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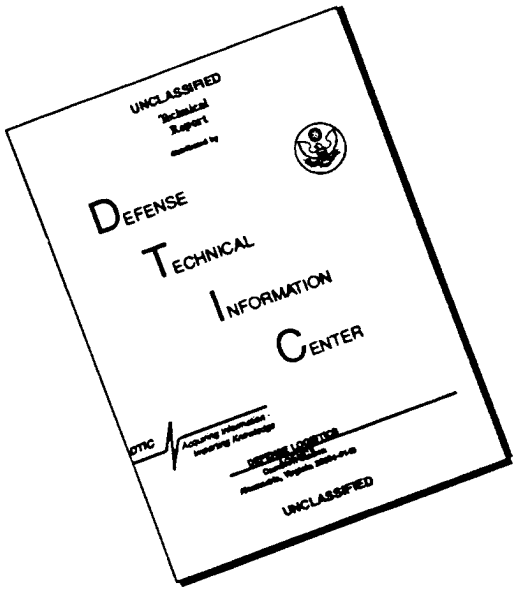
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13. ABSTRACT *(Maximum 200 words)*

A very successful conference was held , 1995 on Modulators of Immune Responses, July 8-15. Responses to the meeting reviewed in Immunology Today and FINews which have worldwide distribution has been very positive and a lot of interest in this area has been generated. The immune system and alterations to its normal function provides a very sensitive indicator of impending disease or danger to health. We now know that all living organisms have an immune system. The study of the immune system of various species and the effect of environmental contaminants provides alternative test models from which extrapolation can be made to human health. This research area, which is growing in importance, is called Immunotoxicology. This was the subject of this conference and the resulting proceedings. Primitive invertebrates such as earthworms, coral, insects as well as fish, amphibians, marsupials, mice, avian species as well as humans were discussed to be used as models. Scientists came from the international community, from almost every continent. Pollution knows no boundaries. We share the same oceans and the same air so these problems are global.

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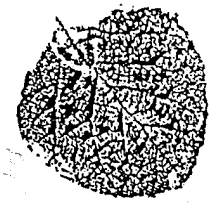
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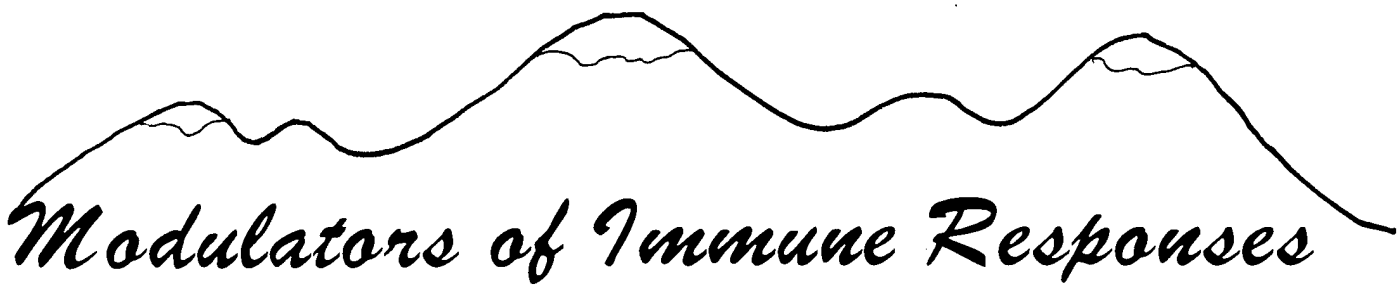
Modulators of Immune Responses

The Evolutionary Trail

Breckenridge Series
Volume 2



Edited by J.S. Stolen, T.C. Fletcher, C.J. Bayne, C.J. Secombes
J.T. Zelikoff, L.E. Twerdok, D.P. Anderson



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Edited by

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Thelma C. Fletcher
Christopher J. Bayne
Christopher J. Secombes

Immunotoxicology
Judith T. Zelikoff
Lorraine E. Twerdok
Douglas P. Anderson

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43 DeNormandie Avenue, Fair Haven, NJ 07704-3303, USA
908/530-3199, Fax: 908/530-5896
E-mail: sosjs@mail.netlabs.net

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Preface

All that's living great and small,
Have immune systems after all.
That after invertebrates evolved,
The lymphoid system to be solved.
That toxicants involve us all.
And uncontrolled all will fall.

The evolutionary perspective in immune regulation and models for immunotoxicology is the subject of this publication.

There is growing realization that the evolution from invertebrate to vertebrate immune systems is essentially the addition of lymphoid components in vertebrate systems. Many researchers are therefore examining immune responsiveness and immune regulation and modulation in some 'non-traditional' or models other than mammals. Many of these models have led to the finding of similar pathways or factors in human systems.

A multitude of immunotoxic compounds exist in the environment affecting all species in the food chain. Species covered in this book range from earthworms to humans. Immunological and host-resistance assays are being used in toxicology safety assessment studies. Immunotoxicology is used in the assessment of ecotoxicity and risk assessment.

The papers in the book were presented at a conference in Breckenridge, Colorado where 100 immunologists from the world community gathered July 8-15,1995. Immune responsiveness was studied in a wide range of species: various aquatic invertebrates, earthworms, insects, marsupials, fish, amphibians, mice, rats, avian species and humans.

This meeting was sponsored by FIN (Fish Immunomodulators Network) and the US Army Medical Research and Materiel Command, Fort Detrick, Frederick, Maryland. Support for this meeting was provided by grant # DAMD17-95-1-5055 of the US Army Medical Research and Materiel Command.

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Joanne Stolen

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Chapter 1

Links Between Alloresponses and their Genetic Background in Colonial Urochordates and Cnidarians: Evidence for the Recognition of "Nonself" as Opposed to "Self"

B. Rinkevich

National Institute of Oceanography
Israel Oceanographic & Limnological Research
P.O. Box 8030, Haifa 31080, Israel

ABSTRACT

Many invertebrates are known to possess highly diverse allorecognition systems which are capable of responding to enormous numbers of "nonself" challenges. The current paradigm for the nature of allorecognition in the invertebrates proposes that, unlike the vertebrates, invertebrate historecognition is based on the concept of the recognition of "self". The present essay concentrates on 3 facets of allorecognition patterns in colonial cnidarians and urochordates: the analysis of transitivity relationships among conspecifics, the capabilities of invertebrates to "individualize" the different types of nonselves and to react differently to different nonself attributes, and the appearance of secondary allospecific responses to prolonged allogeneic challenges. Cumulatively, the results do not meet with the approximation of the paradigm for the recognition of "self" and suggest a complex, but highly regulated panel of effector mechanisms. This strongly suggests that invertebrates may recognize foreign tissues in the context of the "nonself" paradigm of historecognition.

WHY INVERTEBRATES?

It is now above dispute that many invertebrates possess highly diverse allorecognition systems and effector mechanisms which are capable of dealing with enormous numbers of "nonself" challenges (Cooper *et al.*, 1992; Beck *et al.*, 1994; Rinkevich, 1996; and literature therein). Collectively, these historecognition systems have the capacity to identify self cells from nonself entities. Such discrimination can in principle be employed by recognizing either the presence or absence of nonself attributes or by detecting the presence or absence of self molecules (Neigel, 1988). The current guiding paradigm for the nature of invertebrate allorecognition is based on Burnet's (1971) proposal that in contrast to the vertebrates, invertebrate historecognition is based on the self recognition principle. This proposal was constructed on the available information in those days about the nature of colonial hydroid and tunicate allorecognition, where partial sharing of

compatible alleles (Hauenschild, 1954, 1956; Oka and Watanabe, 1957, 1960) was sufficient for fusion between conspecifics.

To further emphasize the validity of alloimmunity in the invertebrates, Hildemann and coworkers (Hildemann *et al.*, 1977, 1979) have suggested three minimal criteria (the existence of antagonistic reactions, the expression of specific activity and the capacity for inducible memory) to be met for a response mechanism to be considered as an immunological system. These criteria have led to a significant number of studies which try to elucidate each one of the above capabilities in a variety of invertebrate phyla and species from the lowest group of multicellular organisms, the sponges, to the highly evolved group of the urochordates (review in Beck *et al.*, 1994; Rinkevich, 1996). Another point of view to the concept of immunity was raised by Janeway (1986), who suggested three other capabilities (the discrimination between self and nonself with a high degree of accuracy, the capability to mount effector responses against nonself molecules, and the capacity for the regulation of these responses) for a biological system to be regarded as an immune system.

Before dealing with the enigmatic nature of invertebrate historecognition, most studies have been engaged with the exceptional precision for discrimination between self and nonself that is expressed in the invertebrates (Grosberg, 1988; Elgar and Crozier, 1989; Weissman *et al.*, 1990; Leddy and Green, 1991). This precision requires: 1. an allorecognition system that is capable of detecting even minute differences between conspecifics, and 2. a genetic repertoire which may directly control the effector mechanisms that are mounted against foreign material (Grosberg, 1988). Although the above criteria seem valid, conceptual difficulties are raised when we consider our recent knowledge of invertebrate immunity. One such example is the results on botryllid ascidian historecognition (Oka and Watanabe, 1957, 1960; Sabbadin, 1962; Scofield *et al.*, 1982). These organisms, when confronted with an alien tissue, do not recognize unshared alleles on their fusibility locus (or the nonself products of these unshared alleles), in the context of the coappearance of shared alleles, and therefore recognize the alien tissue as "self". However, in interacting partners that share no alleles on their fusibility locus, the same alleles that were not recognized before are precisely recognized as "nonself".

The difficulties of formulating the genetics of allorecognition in the invertebrates were discussed elsewhere (Rinkevich, 1996). It is clear that knowing the genetics of invertebrate allorecognition is a key issue for understanding the evolutionary routes of allorecognition in the vertebrates. It will not only result in a unifying concept for immunology but will also answer the main puzzle in vertebrate immunology: All vertebrates reject precisely any transplanted tissue, an adaptation that will never occur in nature (Buss and Green, 1985). Allo- and xenotransplantations in the vertebrates are merely iatrogenic or experimental manipulations that occur in hospitals and research institutions. On the other hand, natural transplantations are very common in the invertebrates, constituting a significant part of their life history portraits (Buss, 1982; Rinkevich and Weissman, 1987a).

Allorecognition phenomena in the invertebrates are not only very precise and specific, but also carry the attribute of high polymorphism (Hildemann *et al.*, 1980; Hildemann, 1981; Taneda *et al.*, 1985; Grosberg, 1988; Leddy and Green, 1991; Rinkevich, 1992, 1993; Sabbadin *et al.*, 1992; Saito *et al.*, 1994; and literature therein). These and other recent studies have revealed many additional new perspectives (Rinkevich, 1996) which may also shed light on the main rationale for the operation

of any immune recognition system: What is the basis for the recognition, is it constructed to distinguish "self" or "nonself"?

It is of great interest that in the vertebrate arena more and more challenges have been proposed to the traditional paradigm that the nonself recognition pattern is the working rationale for the immune system (Burnet, 1971). It has started from the two-signal model (lymphocyte activation vs. anergy, two opposite results which could be elucited from the same antigenic challenge; Bretscher and Cohn, 1970; Bretscher, 1992), and continued with ideas such as the "peptidic self" model (Kourilsky and Claverie, 1986), the "missing self" model (Ljunggren and Kãrre, 1990), and the "high determinant density" idea for alloreactivity (Bevan, 1984) which is somehow on the same line with the "differential affinity" idea (Ohno, 1994). Relevant to the apparent controversy is the theory that the immune system evolved to discriminate "infectious nonself" from "noninfectious self" (Janeway, 1992), the idea that our immune system has elements that recognize "self" and elements for "nonself" recognition (Kãrre, 1992; Versteeg, 1992), the opposing identifications of "self-foreignness" vs. "foreignness" *per se* (Daunter, 1988), the theory that the immune system does not really discriminate self from nonself but "some self" from "some nonself" (Matzinger, 1994), and more.

With regard to the above challenges to the mainstream concept of "nonself recognition" in the vertebrates, and the paradigm for "self recognition" pattern in the invertebrates (Burnet, 1971; Parish, 1977; Stoddart *et al.*, 1985), previous (Neigel, 1988) and recent studies reveal that invertebrates may have developed the capacity of the recognition of "nonself". Results from colonial tunicates and reef corals that support this conclusion are reviewed below.

TRANSITIVITY - THE IMAGE OF ALLORECOGNITION

The analysis of transitivity relationships among conspecifics is performed through allorecognition assays. The simplest transitivity panel involves a combination of 3 organisms (A, B, C) and a relevant specific effector mechanism that has the property of a hierarchial system, or yes/no (fusion/nonfusion) outcome. Transitivity will be established when A = B (compatible) and B = C, then A = C; or when A = B but A ^{1} C (not compatible) then B ^{1} C. A non-transitive character of interactions will be established when A = B and A = C but B ^{1} C. The same rationale holds for the hierarchy in the expression of effector mechanisms, and transitivity will be established when A>B (A dominating B) and B>C, then A>C for transitivity (or linear hierarchy) or A<C for non-transitive relations (circular hierarchy). Generally, nonself recognition may be characterized by non-transitive patterns, while transitivity may be confined to the self recognition framework.

A large number of transitivity tests may not only evaluate partial or full matching paradigms for allorecognition elements (Grosberg *et al.*, 1985; Wulff, 1986), but may also suggest the existence of either a self or nonself recognition pattern (Neigel and Avise, 1983).

However, the ability of transitivity *per se* to discriminate precisely between the self and nonself recognition portrait is not very powerful. It depends on the frequency distribution of histocompatibility alleles, the number of tests of transitivity relevant to the diversity and complexity of the immune entities, the spatial origins of the studied genotypes (Stoddart *et al.*, 1985) and the nature of the recognition elements. An excellent example of the latter point is the fusion-rejection

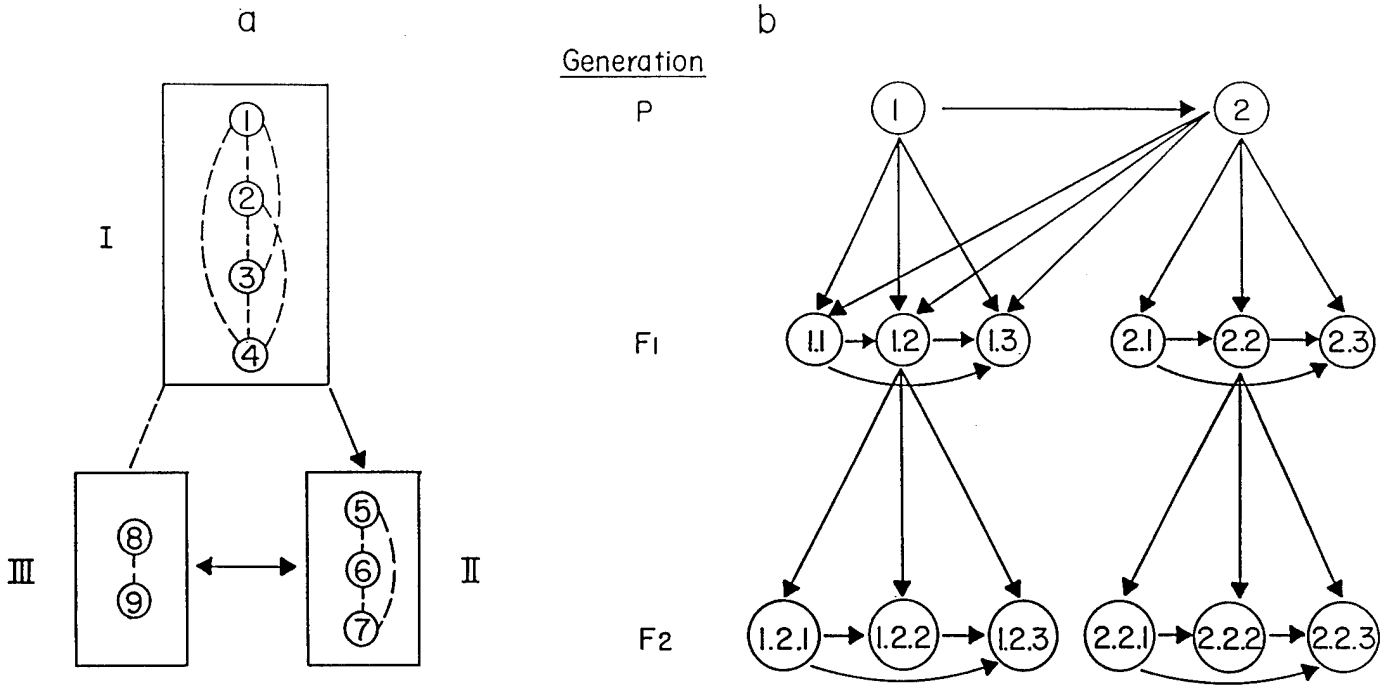


Figure 1. Summary of transivity relationships among cospecifics of the hermatypic coral *Stylophora pistillata* (a) and the compound tunicate *Botryllus schlosseri* (b). A: A network of alloimmune reactions among 9 colonies assigned to 3 histocompatibility groups (I to III). Members of each group are enclosed in a box. Arrows indicate directionality of rejection, superior to the inferior partner. Broken lines - non fusion reaction where colonies first pseudofuse by jamming sutures along contact zones, before tissue-overgrowths are developed (data taken from Chadwick-Furman and Rinkevich, 1994). B. Colony resorption hierachies (arrows) between generations (P, F1, F2) and within a generation in a pedigree of Fu/HC homozygotic (AA line) colonies (data from Rinkevich, 1993; Rinkevich *et al.*, 1993b, 1994b). See text for further details.

phenomenon of botryllid ascidians, where a single, highly polymorphic fusibility/histocompatibility haplotype (Fu/HC; Weissman *et al.*, 1990), with multiple codominantly expressed alleles, precisely controls allorecognition. In this group of colonial tunicates, colonies which share no allele on the Fu/HC locus (e.g. AB vs. CD) will reject each other, whereas colonies sharing one or both alleles (e.g. AB vs. AC, or AB vs. AB, AA vs. AA) will fuse (Oka and Watanabe, 1957, 1960; Sabbadin, 1962; Scofield *et al.*, 1982). Random histocompatibility assays may establish either transitive (e.g. AB = BC = AC and AB = AC) or non-transitive (e.g. AB = BC = CD but AB ¹ CD) interactions. In either case, a self vs. nonself recognition pattern may not be revealed since the minimal outcome for "self recognition" in this system may be established on one positive self-signal, while the "non-self recognition" hypothesis will be based on the threshold for a minimum of two negative nonself signals (see also Neigel, 1988). This rationale of nonself recognition is strikingly compatible with Bevan's (1984) idea for the "high determinant density" requirement for alloreactivity.

Recent transitivity studies on allorecognition of reef corals (Rinkevich *et al.*, 1993a, 1994a; Chadwick-Furman and Rinkevich, 1994; Frank and Rinkevich, 1994) and colonial tunicates (Rinkevich, 1993; Rinkevich *et al.*, 1993b, 1994b) have revealed historecognition patterns, some of which are best explained by the concept of nonself recognition (Figure 1).

As the first example we can use the allorecognition panel depicted for 9 colonies of the hermatypic coral *Stylophora pistillata* (Chadwick-Furman and Rinkevich, 1994; Figure 1a). All possible pairwise colony allorecognition combinations done between these 9 colonies resulted in a network of interactions based on two major responses: 1) nonfusion reactions which continued by the formation of sutures along the contact regions between allogeneic partners, followed by overgrowths; and 2) unilateral rejections. The colonies could be further clustered into 3 distinct allorecognition groups according to the allogeneic responses (Figure 1a). Within each group, colonies overgrew each other in a linear hierarchy pattern. Between groups, they either rejected or overgrew each other: each one of the four Group I colonies engaged in nonfusion-overgrowth interactions with Group III and unilaterally rejected each one of the 3 colonies of Group II. Interactions between Groups II and III were only rejections, and directionality was dependent on the nature of the allogeneic partners. Additionally, out of 400 allogeneic combinations of *S. pistillata*, a real fusion of tissues has not been observed, even between neighboring colonies (Rinkevich and Loya, 1983, 1985; Resing and Ayre, 1985). This complicated pattern of incompatibility in *S. pistillata* cannot be explained by the concept of simple "self recognition", since incompatibility here seems to be coded as a series of discrete alternatives resulting from complex allogeneic genetic elements of the partners involved. Since all allogeneic responses in *S. pistillata* are highly reproducible (Rinkevich and Loya, 1983; Chadwick-Furman and Rinkevich, 1994), the types of the responses are not causative outcomes of external biological or physical parameters.

The second example is the "resorption phenomenon" recorded after the formation of chimeras in *Botryllus schlosseri*, a cosmopolitan compound ascidian (Rinkevich and Weissman, 1987b). After fusion, one partner in the chimera is usually resorbed (morphologically eliminated). The direction of resorption appears to be inherited, as multiple subclones of Colony A always resorb fused subclones from Colony B. The accepted conclusion is that in this group of organisms, fusion occurs between "compatible" partners (sharing at least one allele on their Fu/HC haplotype, which may be regarded as "self" recognition). However, this conclusion is not confirmed here, since the resorption phenomenon that follows fusion may be treated as the second step in a cascade of "non-acceptance" phenomena, leading to the morphological elimination of one genotype. Detailed studies on the genetic background of the resorption phenomenon (Rinkevich, 1993; Rinkevich *et al.*, 1993b, 1994b) have revealed multilevel hierarchies of allorecognition responses, in which the relative state of homozygosity of many allorecognition elements reflects the state of resorption hierarchy. Some aspects of the results are depicted in Figure 1b, where two parent colonies (Colonies 1, 2; both of the AA line on the Fu/HC) are self crossed, giving birth to offspring of generation F1, all AA on the Fu/HC (1.1 to 1.3 and 2.1 to 2.3, respectively; Figure 1b). One of the F1 colonies in each pedigree is self crossed, giving birth to offspring of the F2 generation (1.2.1 to 1.2.3 and 2.2.1 to 2.2.3, respectively; Figure 1b). A typical resorption hierarchy in this scheme (Rinkevich, 1993; Rinkevich *et al.*, 1993b, 1994b, Figure 1b), is manifested as unified directionality from any mother colony (the dominant in the resorption) towards its self crossed offspring (the subordinate), irrespective of the specific generation of a pedigree. Within each generation (P, F1, F2; Figure 1b), a linear hierarchy in the resorption phenomenon is elucidated. Assays between the two pedigrees indicate that a "subordinate" colony of a specific generation (P, Figure 1b) always resorbs self crossed offspring (1.1 to 1.3, Figure 1b) of a "dominant" colony. This polymorphic, ladder-like hierarchial system of histocompatibility loci with codominantly expressed alleles and with directionality in resorption that favors relative heterozygosity of these loci (Rinkevich, 1993) does not meet the approximation of the paradigm for "self recognition". The scenario for selection that resulted in the distinguishing

between clonemates and non-clonemates *per se*, and in the discrimination against the latter, will lead to the loss of necessary polymorphism in the cues used for recognition (Crozier, 1986). This is clearly not the case of *B. schlosseri*, where several hundreds of alleles were suggested to reside in the Fu/HC haplotype alone (Rinkevich *et al.*, 1995).

MANY NONSELVES - AND ONLY ONE SELF

Polymorphism of allorecognition elements *per se* is not favored by selection in models of the within-species recognition type of "self" or "nonself", whereas it could be favored by additional forms of selection associated with the failure to recognize nonself (Neigel, 1988). Therefore, allogeneic assays based on the level of polymorphism cannot solve the puzzle of the "self" vs. "nonself" recognition pattern.

The rationale for allorecognition in a "self recognition" strategy implies that all different types of nonselves will be grouped into one uniform "alien", without individualizing each of these entities. This recognition in principle recognizes either the presence or absence of expected, self molecules. On the other hand, the "nonself recognition" strategy entails the capabilities to individualize the different types of nonselves, and to recognize directly a variety of unexpected, nonself molecules. This type of diversified recognition cannot be experimentally elucidated until the allorecognition elements are studied in detail and until much is known about the nature of the immune system. This is, of course, not the case in any of the studied invertebrates. However, organisms that possess a repertoire of allorecognition effector mechanisms, and express responses against nonself attributes by activating a specific effector mechanism against a specific nonself challenge, may serve as the ultimate model systems for elucidating a pattern of "nonself recognition".

Both colonial cnidarians (Hildemann *et al.*, 1977; Johnston *et al.*, 1981; Neigel and Avise, 1983; Buss and Grosberg, 1990; Salter-Cid and Bigger, 1991; Rinkevich *et al.*, 1993a, 1994a; Chadwick-Furman and Rinkevich, 1994; Frank and Rinkevich, 1994) and colonial botryllid ascidians (Taneda *et al.*, 1985; Rinkevich, 1992; Rinkevich *et al.*, 1994c) have clearly revealed in allogeneic and xenogeneic interactions that individual colonies are able to diversify their responses specifically and repeatedly to different "nonself" challenges, showing the ability to discriminate between different "nonself" attributes.

One representative example is the Red Sea branching coral *Stylophora pistillata*, studied for its historecognition patterns for more than a decade (Rinkevich and Loya, 1983, 1985; Abelson, 1987; Rinkevich and Weissman, 1987a; Rinkevich *et al.*, 1993a; Chadwick-Furman and Rinkevich, 1994). A specific *Stylophora* colony (Figure 2) can reject conspecific A, be rejected by conspecific B, overgrow the tissue of colony C, "pseudofuse" with genotype D, be overgrown by colony E and retreat from conspecific F. When a *Stylophora* colony is engaged in xenogeneic interactions, the colony can reject xenogeneic challenge A, be rejected by species B, reciprocally reject colonies of species C, "stand off" from species D, display allelopathic responses to species E and be overgrown by species F (Figure 2). All the above mentioned allogeneic and xenogeneic responses are repeatable in directionality and are specific to the allogeneic genotype or to the xenogeneic species, featuring a high complexity and a variability in the repertoire of effector mechanisms. This capacity for diversified recognition is also emphasized by Neigel and Avise (1983) who indicated that a single

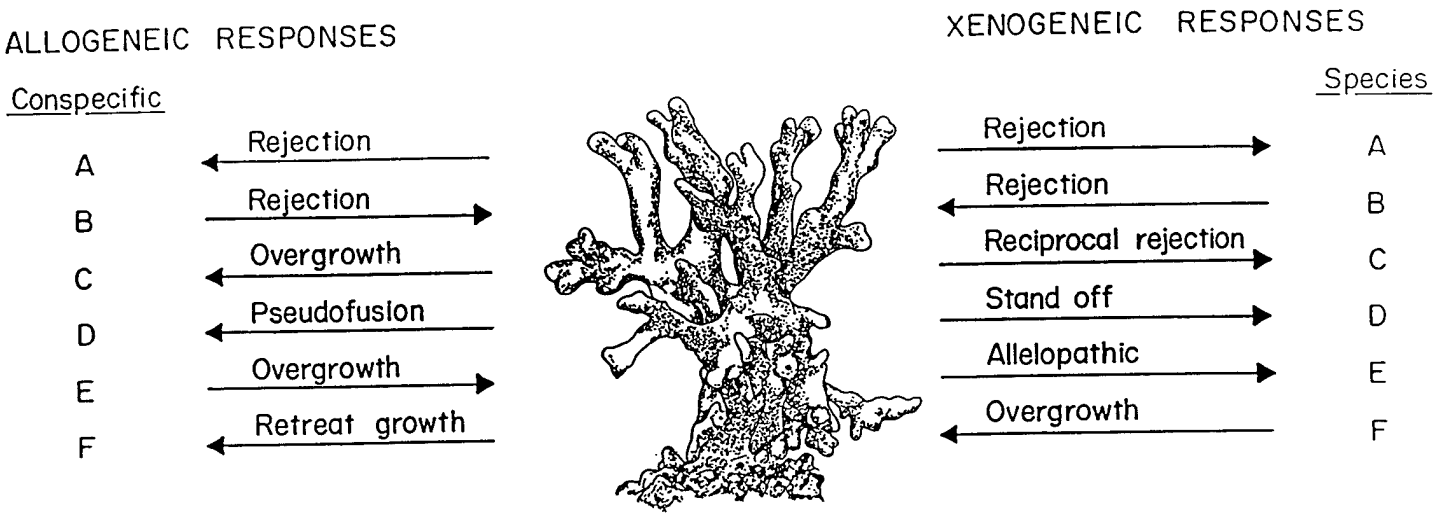


Figure 2. Panel of different historecognition responses in allogeneic and xenogeneic encounters that may potentially be expressed by a single *Stylophora pistillata* colony. Responses can vary by their nature (rejection, overgrowth, pseudofusion, retreat growth, for allogeneic responses; rejection, reciprocal rejections, stand off, allelopathic, overgrowth, for xenogeneic responses) or directionality (such as rejection, overgrowth). See text for further details.

coral colony is not limited to a single mode of interaction in response to allogeneic encounters, and acts respectively to the "property of the system".

One set of experiments for the recognition of "self" vs. "nonself" in the invertebrates was also conducted under *in vivo* conditions (Neigel, 1988). In this study, on *Botryllus schlosseri*, the specificity of cytotoxic activity of blood cells was measured in cases where both chosen genotypes had rejected *in vivo* prior to the *in vivo* experiment as compared to assays done against cells from naive incompatible colonies. This study further demonstrated that blood cells from *Botryllus* are capable of distinguishing among distinct types of nonself allogeneic cells and react differently. *Botryllus* blood cells in this experiment were successfully primed to specific allogeneic attributes.

Both types of results, the *in vivo* results on different specific allo- and xenogeneic responses to different histocompatibility challenges (colonial cnidarians and tunicates) and the *in vivo* specific priming of *Botryllus* blood cells to a given allogeneic challenge, elucidate the ability of invertebrates to respond selectively against different types of nonself. This may suggest that at least some colonial invertebrates may use the "nonself" rationale for the discrimination between self and nonself.

RESPOND - AND RESPOND DIFFERENTLY

Several studies on colonial marine invertebrates have revealed the existence of temporal reversals in the observable outcomes of xenogeneic (Chornesky, 1989) or allogeneic encounters (Chadwick-Furman and Rinkevich, 1994; Frank and Rinkevich, 1994). These reversals were not due to seasonal changes or to other environmental factors; rather, they resulted from inherent differences between the interacting xenogeneic partners (Chornesky, 1989) or between the allogeneic genotypes (Chadwick-Furman and Rinkevich, 1994). However, it is not possible at this stage of knowledge to determine from the above studies whether the results were obtained from exhaustion (on ecological-physiological properties or cellular elements) of the effector mechanisms or from a genuine expression of late xeno- allospecific responses to a specific, chronic historecognition challenge.

However, there are other sets of results which, on the wider scope, indicate that many colonial invertebrates subtly express primary and late, secondary allospecific responses, as a response to a continuous but specific allogeneic stimulus (Chadwick-Furman and Rinkevich, 1994; Frank and Rinkevich, 1994; Rinkevich *et al.*, 1994c). In the above studies, hermatypic corals and hydrocorals not only represent a high variety of allorecognition operations on the morphological level, but also express delayed allogeneic responses such as cytotoxicity, overgrowth, reversal and more. These responses are attributed to the characteristics of selectivity and reproducibility, even on the time scale. The term of "secondary responses" was clearly evaluated in allogeneic responses within the hydrocoral *Millepora dichotoma* (Frank and Rinkevich, 1994).

Relevant to the above discussion on "secondary histocompatibility responses" is the resorption phenomenon in fused botryllid ascidians (Rinkevich and Weissman, 1987a,b, 1992; Weissman *et al.*, 1990). This phenomenon is elicited only after the fusion between "compatible" colonies on the Fu/HC locus and the establishment of the chimera entity. If self vs. nonself recognition could not be judged from the genetics for fusion, the two historecognition levels of fusion and resorption further represent the primary and secondary aspects of this system and indicate the "nonself" nature of allorecognition in botryllid ascidians.

The above three patterns of late responses (reversals in the same effector mechanism, secondary allospecific responses, and acceptance that is followed by non-acceptance response) are fundamental results in many colonial marine invertebrates, including cnidarians and urochordates. These reactions are probably not guided by the failure of the primary responses to detect nonself attributes, but are probably one basic feature of alloimmunity in many groups of invertebrates. The precision that these responses have developed further suggests a complex, but highly regulated panel of effector mechanisms which probably are underlined by a "nonself" recognition capacity.

RECOGNITION OF NONSELF?

Studies in the last few years have clearly demonstrated that invertebrates may possess complex allorecognition systems with a heterogeneous architecture of specialized cells, effector mechanisms and responses (reviewed in Lackie, 1980; Ratcliffe, 1985; Cooper *et al.*, 1992; Beck *et al.*, 1994; Rinkevich, 1996). Although different expressions of alloimmunity may change from one taxa to another, many aspects are ubiquitous, even across phylum boundaries. One such point is the question of the nature of recognition. Is it based on the recognition of "self" or "nonself"?

The present essay has concentrated on three facets of allorecognition: transitivity as an image for allorecognition, the capacity to recognize a variety of nonselves differently and to react accordingly, and the expression of late/secondary responses to chronic allospecific challenges. Cumulatively, these characteristics of the responses recorded in colonial cnidarians and urochordates strongly suggest that many invertebrates recognize foreign tissues in the context of the "nonself" paradigm.

Some of the allospecific responses elicited by the above invertebrates may be explained on the theoretical level, as having been influenced by an "allogeneic threshold". This model characterizes cases where graft acceptance could occur between histoincompatible conspecifics (as fusion between Fu/HC compatible Botryllus colonies) or when disparity between conspecifics is not wide enough for the expression of one rapid acute effector mechanism, and the need for additional alloimmune responses is revealed. This is similar to the notion that the alloreactive target determinant in the vertebrates is a sequence, which is expressed at higher density on the antigen-presenting cells (Bevan, 1984). Even such a relative disparity phenomenon for alloreactivity fits the concept of "nonself" better than the recognition of "self".

We are still in a state in which the genetic frameworks of allorecognitions in the invertebrates and their rationales are explored by searching for the products of allorecognition genes, the effector mechanisms. Nothing is yet known about the genes themselves or the molecular patterns of the cascades of events following self-nonself recognition. It is therefore not easy to determine from the assays on the morphological-cellular levels the molecular nature of allorecognition in these organisms. However, the accepted paradigm for invertebrate historecognition as was suggested by Burnet (1971) is critically challenged by the results presented here. The mechanisms underlying historecognition responses in colonial cnidarians and urochordates are less relevant in the view of recognition of "self" than the alternate operational property of the recognition of "nonself".

The results discussed here further suggest that any simple model for self-nonself discrimination in invertebrates (such as Parish, 1977) is inappropriate to the characteristics of allorecognition in corals and tunicates. It is also probable that the invertebrate allorecognition systems employ both the recognition of the presence of unexpected, nonself molecules and the absence of expected, self molecules. This is also the characteristic of the vertebrate immune systems where B and T cells, for example, use the first strategy whereas NK cells use the second option (see also Janeway, 1992; Kãrre, 1992).

If the argument made here is correct, we have to confront different experimental manipulations which will take this question away from the plausible description of events made on the morpho-

logical level. Not all the theories discussed here may be true, but they may be very useful in stimulating studies that target the question of self-nonself recognition in invertebrates.

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Chapter 2

Antibacterial Defence in Anthozoans, with Special Reference to the Sea Anemone, *Actinia equina*

Danielle M. C. Hutton and Valerie J. Smith*

School of Biological and Medical Sciences
Gatty Marine Laboratory
University of St. Andrews
Fife, KY16 8LB, Scotland.

*author to whom correspondence and reprint requests should be addressed.

ABSTRACT

Recent concern about the effects of anthropogenic disturbance on anthozoans, particularly on the health of corals and their associated marine communities, has stimulated interest in the immunological strategies used by anthozoans to prevent or overcome microbial attack. However, as anthozoans lack a circulatory system and true blood cells, most research has tended to focus on the responses of various scleractinians and gorgonians to foreign tissue grafts. While this work has established that anthozoans are capable of discriminatory allorecognition at the tissue level, it tells us little about the cellular and biochemical strategies used by anthozoans for antimicrobial defence. A number of authors have noted that wounds heal rapidly in anthozoans and that graft sites seldom show signs of infection after surgery. A few papers have further reported antibacterial activity in whole body homogenates of gorgonians and horny corals, but none to date have identified the cell type(s) responsible or offered detailed information about the biochemical character of the active agents involved. Recently, to investigate antibacterial activity by the amoebocytes of anthozoans *in vitro*, we extracted cells from the mesenteric filaments of the sea anemone, *Actinia equina*, and examined their ability to phagocytose and kill Gram positive and Gram negative bacteria ***in vitro***. Our observations confirm that these cells actively phagocytose bacteria and have potent bactericidal properties *in vitro*. Antibacterial activity appears to be due to both the production of superoxide anions and other reactive oxygen species (ROS), through a respiratory burst and the release of soluble antibiotic factors. Preliminary characterization of these factors are described.

INTRODUCTION

The Cnidaria is a large, ancient and diverse phylum, comprising some 9,400 extant species of hydroids, scyphozoans and anthozoans. The group has a wide geographical distribution, with each class well represented in most of the major habitats of the marine environment. Fossil records show that the group evolved in the Precambrian, some 600 million years ago, probably from a bilateral, flagellated planula ancestor (Willmer, 1991). The phylum appears to have arisen separately

from the monociliate ancestors which gave rise to the coelomate line and has produced no further major descendants (Willmer, 1991). Thus, from the standpoint of immune phylogeny, they are interesting group as they would seem to occupy a unique position in animal evolution.

Approximately 68% of all cnidarian species are members of the class Anthozoa (sea anemones, gorgonians, corals and their close relatives). The class is of little direct commercial interest but includes the scleractinian corals, a group of organisms which form the basis of highly productive ecosystems in oligotrophic tropical waters. Anthozoans are therefore of enormous ecological significance, and through tourism to areas in the vicinity of coral reefs, have come to represent an important source of income to local communities in tropical regions.

In recent years, growing awareness about the long term effects of marine pollution and global warming on fragile marine habitats has fuelled concern about the effects of anthropogenic disturbance on the health of coral reefs and other marine ecosystems (Grigg and Dollar, 1990). In particular, disquiet has been expressed about the relationship, if any, between environmental change, coral bleaching, disease and reef erosion. Accordingly, interest has been stimulated in the ways anthozoans, especially scleractinian corals, overcome infection by opportunistic or pathogenic micro-organisms, and how tissue defence, and hence disease susceptibility, might be influenced by external conditions. However, because the restricted distribution of reef corals and the small size of the constituent polyps confounds direct analyses of the cellular and biochemical events underlying disease resistance in these animals, information needs to be obtained from other anthozoan species more amenable to experimental study.

In common with all cnidarians, anthozoans have two body layers, the endoderm and epiderm, which sandwich the mesoglea. Importantly, as acoelomate invertebrates, anthozoans lack a circulatory system and true blood cells; a feature which has complicated investigations into the host defence reactions of these animals at the cellular level.

This paper reviews the information available on the immune strategies shown by anthozoans, and reports recent findings on the antimicrobial defences of the sea anemone, *Actinia equina*. We further highlight areas in need of further research in order to improve our understanding of disease resistance in corals and anthozoans in general.

ANTHOZOAN DISEASES

Anthozoans have been reported to harbour a number of parasites, although the full effects of these pathogens on their host has not always been made clear (Philips 1973; Lauckner; 1980b; Stock, 1988). As might be expected, most observations have been made on scleractinian corals and, the most significant pathological condition known in these animals to date is bleaching. It occurs when the symbiotic zooxanthellae are expelled from the host cells, giving the coral a blanched appearance. It is probably not a recent phenomenon, but has become more persistent and extensive in the last four or five years, affecting large areas of reefs in the Caribbean and Indopacific (Glynn, 1993). It is believed to be linked to environmental stress, especially elevated water temperature, increased ultra-violet radiation or reduced salinity (Glynn,1993; Brown and Ogden, 1993). However, few

details are available about how these factors alter the relationship between the zooxanthellae and their coral host.

Two other diseases known in corals are Blackband disease and Whiteband disease. Blackband disease is caused by the cyanobacterium, *Phormidium corallyticum,* and is characterized by a thin black mat of fine filaments which separates bare coral skeleton from living tissue (Antonius, 1988). It infects through wounds and spreads rapidly; the filaments progressing as much as a few millimeters per day (Antonius, 1988). Whiteband disease is the term applied to the sloughing off of tissue from the exoskeleton, starting at the base of the colony and proceeding to the tips. It may affect many species of coral and has been suggested to represent the end stage of different diseases or physical damage (Sparks, 1993). Unlike bleaching, there appears to be no correlation between these diseases and adverse environmental factors (Peters, 1984).

Finally, tumors have been described in gorgonians and scleractinian corals but, again, the etiology of these lesions and role of the environment in the onset and/or development of tumor growth are unknown (Sparks, 1993).

NON-SELF RECOGNITION

The absence of blood as a defined tissue in anthozoans has led many workers to study self/non-self discrimination phenomena by examining the ability of these invertebrates to respond to foreign tissue grafts *in vivo*. Accordingly, many papers have appeared over the last two decades describing the range and character of discriminatory tissue responses exhibited (e.g. Theodor, 1970; Hildemann *et al.*, 1975a,b; 1977; Bigger and Runyan, 1979; Grosberg, 1988; Bigger, 1988; Leddy and Green, 1991; Rinkevich *et al.*, 1994; Jokiel and Bigger, 1994). This work has shown that anthozoans are capable of complex recognition although the nature of the response varies according to the degree of histocompatibilty between the recipient and the grafted tissue. Autografts or isografts (within colony grafts), which are compatible with 'self', nearly always fuse with the recipient, whereas xenografts, which are incompatible with the host, are rejected (Theodor, 1970; Hildemann *et al.*, 1975a,b; 1977; Bigger and Runyan, 1979; Grosberg, 1988; Bigger, 1988; Jokiel and Bigger, 1994; Rinkevich *et al.*, 1994). Allografts may be either rejected outright or display a range of 'non-fusion' reactions, non-transitive overgrowth or reversals, but never undergo fusion with the host (Theodor, 1970; Bigger and Runyan, 1979; Chadwick-Furman and Rinkevich, 1994; Jokiel and Bigger, 1994; Rinkevich *et al.*, 1994).

Little is known about the cellular events associated with the killing of foreign cells by anthozoans, although it is widely believed to be achieved through cytotoxic reactions by the surviving host. For example, Salter-Cid and Bigger (1991) have reported necrosis at graft interfaces in the gorgonian, *Swiftia exserta,* indicative of cytotoxic events. Likewise, Rinkevich *et al.* (1994) and Chadwick-Furman and Rinkevich (1994) have described discolouration and necrosis at allografts in the hard corals, *Acropora hemprichii* and *Stylophora pistillata,* respectively. Salter-Cid and Bigger (1991) have suggested that tissue damage and destruction, at least in gorgonians, is mediated by direct cell to cell contact or, alternatively, the release of locally-active diffusible cytotoxic molecules. As yet the ability of anthozoan cells to mount cytotoxic reactions, either directly or indirectly, *in vitro* has

not been demonstrated and no cytolytic factors from anthozoans have been isolated, purified or characterized.

The hierarchical character of the recognition responses in anthozoans indicates that anthozoans must possess a repertoire of genetic histocompatibility determinants unique to separate clones, colonies or individuals (Salter-Cid and Bigger, 1991; Rinkevich *et al.*, 1994; Chadwick-Furman and Rinkevich, 1994). The extent to which organisms share common determinants presumably influences the outcome of the challenge. At present, few details are available about the genetics governing histocompatibility in anthozons, but the reports published so far clearly demonstrate that anthozoans exhibit recognition and foreign tissue destruction reactions without the aid of a specialized, blood-based reticuloendothelial network.

WOUND REPAIR AND SURFACE DISINFECTION

Despite the absence of blood and a hemal clotting system to plug wounds and prevent the entry of micro-organisms, anthozoans appear able to effect rapid repair of injured tissues (Young, 1974). Anecdotal information also indicates that they seldom exhibit signs of infection in the tissues after surgery or tissue grafting (Patterson and Landolt, 1979; Bigger and Hildemann, 1982; Bigger and Olano, 1994). With the sea anemone, *Actinia equina*, we have noticed that incisions made into the body wall heal within 48 hr at 20 °C and remain free of infection, even when the injured animals are incubated in non-sterile conditions in natural seawater.

The mechanisms underlying wound healing and tissue disinfection in anthozoans are very poorly understood. Work by Young (1974) and Patterson and Landolt (1979) has implicated the mesogleal connective tissue, and especially the amoebocytes contained within this matrix, in tissue regeneration. Certainly, the mesoglea in anthozoans is populated by a number of amoebocyte-type cells (Patterson and Landolt, 1979; Van der Vyver, 1981; Bigger and Hildemann, 1982), and in sea anemones these cells appear to rapidly infiltrate wounds and secrete fibres, possibly from granules within the cytoplasm, to replace those lost in the damaged or excised mesoglea (Young, 1974; Patterson and Landolt, 1979). The newly-formed matrix is then finally overgrown by already differentiated cells migrating from the ectoderm (Young, 1974). As yet, the extent to which the mesogleal amoebocytes further aid in wound healing by removing or inactivating microbial invaders *in vivo* has not been resolved.

Some surface antisepsis may be achieved through the cleansing and bacteriostatic effects of mucus. In many animals, mucus is known to help maintain disinfection of the respiratory, genital or gastro-intestinal membranes, and it is well established that anthozoans produce copious amounts of mucus especially after mechanical irritation or other forms of disturbance (Lewis, 1973; Krupp, 1984). Mucus in hard and soft corals is generally rich in protein and/or carbohydrate polymers, with a particular abundance of dicarboxylic amino acids and phospholipids (Daumas *et al.*, 1981; Krupp, 1981; Meikle *et al.*, 1988; Drollet *et al.*, 1993). Variations in the composition, structure and antigenicity of mucus produced by different anthozoan species has led to the suggestion that it may mediate self/nonself allorecognition between individuals (Lubbock, 1979). Certainly, mucus provides a slippery, unpalatable outer coat to ward off predators and protect against desiccation (Denny, 1989), and it may play some role in interspecific competition. But it is also possible that it serves

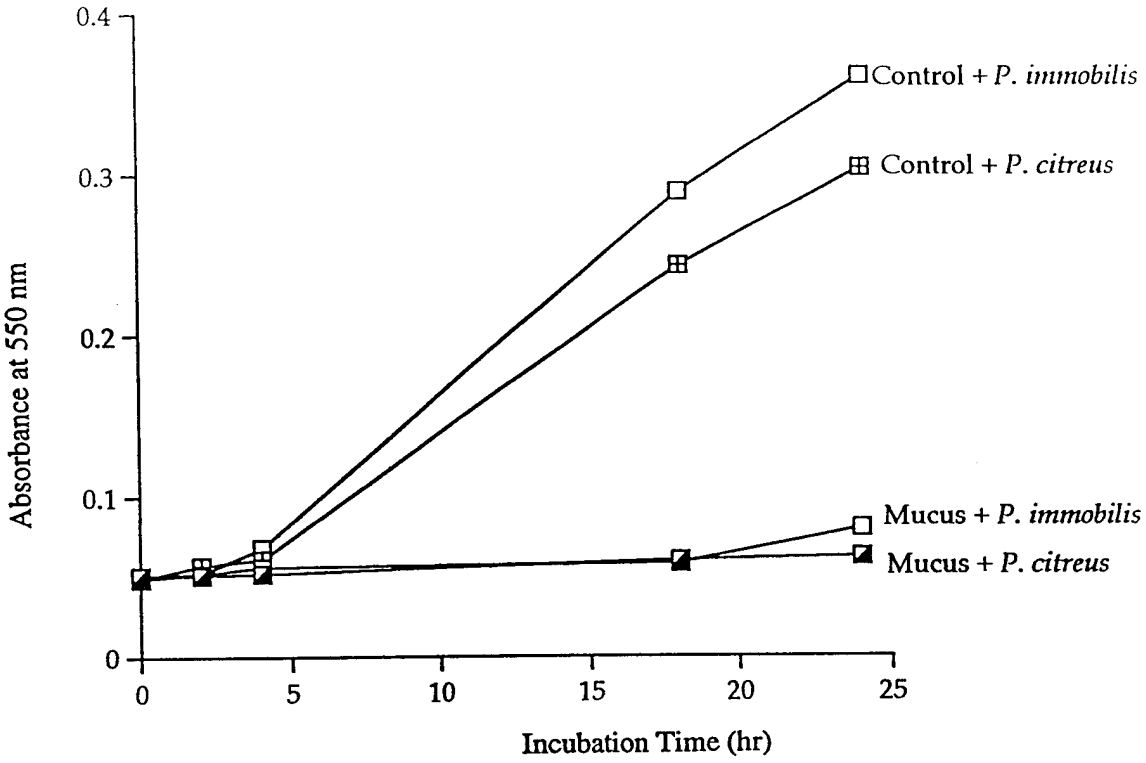


Figure 1. The antibacterial effect of mucus from the sea anemone, *Anemonia viridis* against the marine Gram negative bacterium, *Psychrobacter immobilis,* or the Gram positive bacterium, *Plancoccocus citreus, in vitro.* Mucus was collected from individual animals into a sterile 15 mL plastic centrifuge tube after gently stroking the exterior of the anemone with sterile blunt instrument. Ninety microlitres of the mucus (protein content per well 100 µg) were incubated at 20°C with 10 µL of washed bacteria (2×10^6/ mL) in wells of a sterile 96 well micro-titre plate. For controls, the bacteria were similarly incubated but in 90 µL of Marine saline (MS) (Smith and Peddie, 1992). Bacterial growth was monitored by reading the absorbance at 570 nm at intervals over 24 hr. Inhibition of bacterial growth by the mucus is indicated by the failure of the absorbance in the experimental wells to rise exponentially as in the controls. The data shown are the results of a representative experiment with one animal. The assay was repeated three times with similar trends seen on each occasion.

as a means of controlling epibiosis and tissue disinfection. Indeed, mucus from the sea anemone, *Metridium senile*, have been found to contain hemagglutinins and to exhibit antibacterial properties *in vitro* (Astley and Ratcliffe, 1989). We have similarly observed that mucus from the anemone, *Anemonia viridis,* inhibits the growth of Gram positive and Gram negative bacteria *in vitro* (Figure 1). So far, the identity and origin of the bioactive agent(s) involved are unknown and further work is necessary to establish whether they are present constitutively within the mucus or are released from the tissues after appropriate stimulation.

AMOEBOCYTES

Comparatively few descriptions of the mesogleal cells in anthozoans have been published. Reviewing the literature in 1981, Van der Vyver highlighted two principal amoebocytes in anthozoans; undifferentiated RNA-rich 'interstitial' cells and glycocytes. The interstitial cells were suggested to constitute a self-proliferating cell line which serves to replace worn out cnidocytes, gland cells and other cell types, while the glycocytes were described as true wandering mesogleal cells which contain variable amounts of glycogen (Van der Vyver, 1981). By contrast, Patterson and Landolt (1979) observed only one amoebocyte type, referred to as 'phagocytes', in the anemone, *Anthopleura elegantissima*. These cells were seen in tissue sections to contain numerous eosinophilic or neutrophilic granules but not to be actively mitotic or to enclose ingested particles (Patterson and Landolt, 1979).

One reason for the paucity of information on the mesogleal amoebocytes of anthozoans is the difficulty of obtaining adequate numbers for *in vitro* observation and experimental manipulation. In *A. elegantissima*, for example, the number of cells has been estimated from histological sections to be ca 5 x 10^3 mm^{-2} (Patterson and Landolt, 1979). Recent work in our laboratory, however, has revealed that small (0.5 to 1 mL) volumes of fluid can be drained from the mesoglea of *A. equina*

Table 1.
Characteristics of the mesogleal amoebocytes from the sea anemone, *Actinia equina*.

Characteristic	Hyaline cells[1]	Granular cells[1]
Proportion of total cell population	ca. 65%	ca. 35%
Presence of granules[2]	No	Yes
Adherence to glass[2]	Yes	Yes
Spreading on glass[3]	Yes	No
Phagocytosis of bacteria *in vitro*[4]	Yes	No

[1] The cells were extracted from individual anemones by making a small incision into the ectoderm with a sterile scalpel and allowing the mesogleal fluid to drain into a sterile centrifuge tube.

[2] Differential cell counts were made on mesogleal fluid samples on clean pyrogen-free glass slides examined under phase contrast optics of a light microscope.

[3] Cell spreading was tested by incubating 100 µL volumes of mesogleal fluid on clean, pyrogen-free glass coverslips for 15 min at 15°C and examining under phase contrast optics of a light microscope.

[4] Phagocytosis was examined on washed amoebocyte monolayers using the procedures described by Smith and Ratcliffe (1978) for crustacean hemocytes. Briefly, 100 µL of amoebocytes were added to clean, pyrogen-free glass coverslips, incubated for 15 min at 15°C, washed gently with 2 x 1 mL volumes of sterile MS and challenged with 100 µL of a washed suspension of *Psychrobacter immobilis*, (5 x 10^6/ mL). After 45 min at 15 °C, the cells were washed with 2 x 1 mL volumes of MS to remove loosely adherent bacteria, and scrutinized under phase contrast optics of a light microscope. Intracellular bacteria were distinguished from extracellular forms according to the criteria given in Smith and Ratcliffe (1978).

after surface wounding with a sterile scalpel (Hutton and Smith, unpublished observations). This fluid contains a population of amoebocytes of approximately 2-5 x 10^3/ mL , composed of two main types; the agranular hyaline cells, which represent around 65% of the total, and the granular cells which make up the remaining 35% (Table 1).

The precise number of mesogleal amoebocytes in *A. equina,* and the ratio of hyaline to granular cells, may vary considerably from animal to animal, but for most individuals it generally remains constant during prolonged (ca 4-5 week) aquarium culture (unpublished). We have further noticed that the cell count increases two fold at 48-72 hr after wounding, but reverts back to baseline levels after 96 hr (unpublished). Similar observations have been recorded by Patterson and Landolt (1979) for *A. elegantissima.* In this animal, the number of amoebocytes was seen in histological sections of wound sites to rise from ca 5, 000 to 10,000 mm^2 at 48-72 hr after injury and to return to normal after 120 hr (Patterson and Landolt, 1979). The speed of the cellular response to wounding in anthozoans indicates that the mesogleal amoebocytes are replenished by mobilization of cells rather than by proliferation within the mesoglea itself. This observation supports the hypothesis of Young (1974) that reservoirs of mesogleal amoebocytes occur in the body of anthozoans. Her study of wound responses in *Calliactis parasitica* failed to reveal increased mitosis in the mesoglea after experimental injury and showed that the early stages of tissue regeneration after injury are unaffected by treatment of the animals with colchicine or paradichlorobenzene. Young (1974) considered that a likely source of the mesogleal cells is the endodermal mesenteries, as cells resembling mesogleal amoebocytes are present in the endoderm of *C. parasitica,* and while mitosis occurs in this tissue in both healthy and wounded animals, there is no increase in cell division in the endoderm following tissue damage.

To find a possible source of mesogleal-type defence cells in anthozoans for *in vitro* study, we undertook an examination of the morphological and functional capabilities of the mesenteric filament cells in *A. equina.* Our findings show that mesenteric filaments in *A. equina* do indeed contain hyaline and granular cells, similar to those present in the mesoglea. Moreover, the two cell types are present in approximately the same proportions as in the mesogleal cells and display similar properties in vitro to those cells obtained directly from the mesoglea. We thus believe that the mesenteric filaments of anthozoans constitute a reservoir of amoebocytes and represent a good source of cells for experimental study.

PHAGOCYTOSIS

There have been no detailed investigations of phagocytosis by the mesogleal cells in anthozoans, but by using amoebocytes harvested from the mesenteries of *A. equina,* we have been able to confirm that the cells are able to phagocytose both Gram negative and Gram positive bacteria *in vitro.* The avidity of bacterial uptake by the cells *in vitro* compares well with equivalent values obtained for the blood cells of coelomate invertebrates by other workers (Table 2). In addition, the mesenteric filament amoebocytes in *A. equina* have been found to reduce ferricytochrome c following incubation with phorbol myristate acetate (PMA) or lipopolysaccharide (LPS) *in vitro* (Figure 2), again in a manner akin to that shown by the phagocytes of other animals (Adema *et al.,* 1991; Bell and Smith, 1993), indicating that anthozoan amoebocytes might possess the oxidative machinery used by many species to kill or inactivate ingested micro-organisms.

Table 2.
Comparison of the phagocytic uptake of *Psychrobacter immobilis* by the amoebocytes of *A. equina* with the blood cells of other marine invertebrates *in vitro*

Species	Group	Incubation time	°C	% uptake	Reference
Actinia equina	Cnidaria	45 min	15°	45	Hutton and Smith, 1995
Arenicola marina	Annelida	3 hr	15°	3-5	Fitzgerald and Ratcliffe, 1982
Carcinus maenas	Crustacea	3 hr	15°	15	Smith and Ratcliffe, 1978
Asterias rubens	Echinodermata	3 hr	15°	42	Smith and Saunders, unpubl.
Ciona intestinalis	Ascidiacea	3 hr	15°	40	Smith and Peddie, 1992

In each case, using the Gram negative marine bacterium, *Psychrobacter immobilis*, as test particle. uptake was assessed by scrutiny of monolayer cultures on glass coverslips under phase contrast optics of a light microscope.

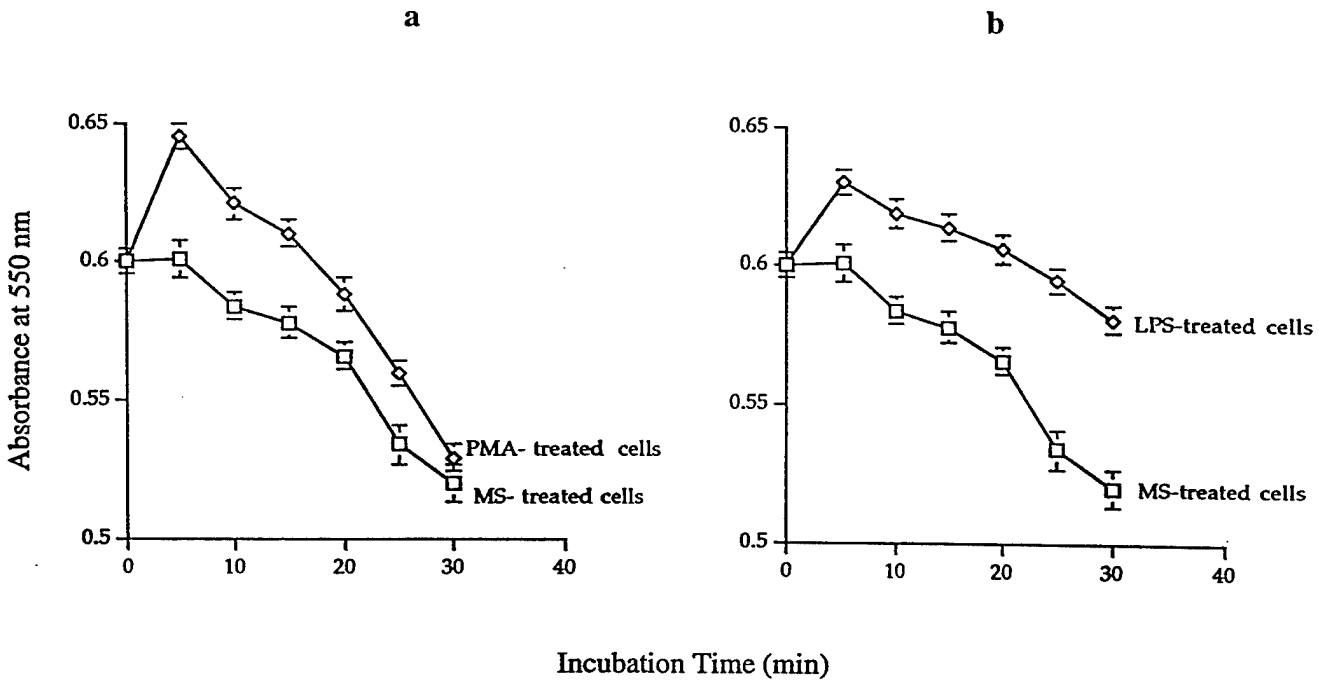


Figure 2. Reduction of ferricytochrome c by amoebocytes isolated from the mesenteric filaments of *A. equina in vitro*. The method used was as described in Smith and Hutton (1995). Briefly, 50 µL of amoebocytes (5.2×10^4/mL) in MS were incubated with 25 µL of ferricytochrome c (stock concentration 160 µM) and 25 µL of elicitor. For controls, the elicitor was replaced with buffer. The absorbance of the experimental and controls were then measured at 550 nm at 5 min intervals over 30 min. Figure 2a shows the change in absorbance using lipopolysaccharide (LPS) (1µg/mL final concentration) as elicitor while Figure 2b shows the response when phorbol myristate acetate (PMA) (8 µg/mL final concentration) is used as elicitor. With LPS, 25 µL of catalase (8 µg final concentration) was included in the reaction mixture to prevent re-oxidation of ferricytochrome c by hydrogen peroxide. Values given are means ± SEM (n = 5).

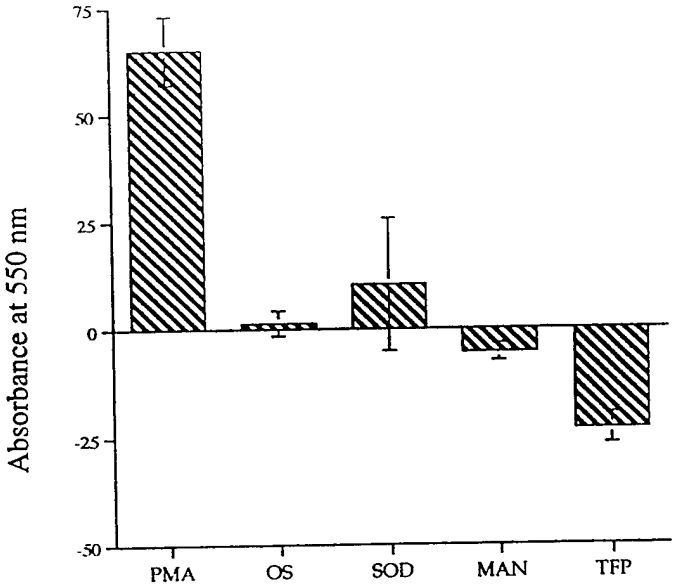


Figure 3. Effect of ROS scavengers or respiratory burst inhibitors on the reduction of ferricytochrome c by PMA -treated amoebocytes from the mesenteric filaments of *A. equina in vitro.* The procedure used was as described in Smith and Hutton (1995). Briefly, 50 µL of amoebocytes (5.2 x 10^4/ mL) in MS were incubated with 25 µL of ferricytochrome c (stock concentration 160 µM), 25 µL of PMA (8 µg/mL final concentration) and 25 µL of either buffer (the positive control), or superoxide dismutase (SOD), mannitol (MAN), or trifluoroperazine (TFP) at final concentrations of 1200 units. mL, 40 mM and 10 µM respectively (the experimentals). For the negative control, PMA and inhibitor were replaced with MS. The absorbance of the experimental and control wells was measured at 550nm at 5 min intervals over 30 min. Values given are changes in absorbance at 550nm after 5 min ± SEM (n = 5).

Confirmation that the mesenteric filament cells in *A. equina* are able to produce superoxide anions and other reactive oxygen species through an NADPH oxidase mediated respiratory burst is provided by the finding that inclusion of trifluoroperazine, mannitol or superoxide dismutase (agents which are known inhibit the action of NADPH oxidase or scavenge superoxide and other reactive oxygen species) in the reaction mixtures reduces the response of the amoebocytes to PMA (Figure 3). As yet, we have no information about the presence of opsonins in anthozoans and no direct evidence that *in vivo* the mesenteric filament cells assist in the sequestration and destruction of infective agents in the tissues. Notwithstanding this, our observation of respiratory burst-type activity by these cells shows that this process has a long evolutionary history and is not confined to the phagocytes of animals with true blood or hemal systems.

ANTIBACTERIAL ACTIVITY

For all animals, one of the most important aspects of host defence is the ability to kill or impair the growth of opportunistic or pathogenic micro-organisms, and often distinct antimicrobial compounds are produced by the tissues to effect antisepsis. In vertebrates, this is achieved by specific antibodies, certain T cell products and a variety of non-specific serum or mucosal components (Levitz *et al.*, 1995). Invertebrates, which do not express immunoglobulins and T lymphocytes, tend to rely instead on the synthesis of a wide range of lytic enzymes and other antibiotic proteins or peptides (Boman, 1991).

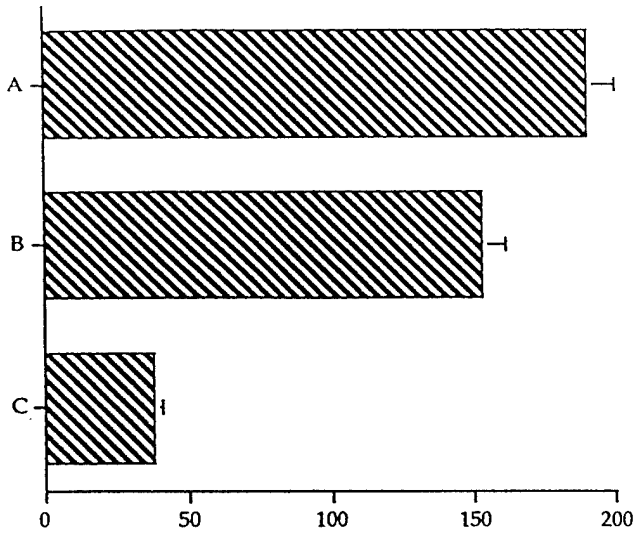


Figure 4. Antibacterial activity by viable amoebocytes isolated from the mesenteric filaments of *A. equina in vitro*. The following cultures were set up in wells of micro-titer plates: (A) 50 µL of a washed suspension of the Gram negative marine bacterium, *P. immobilis* (5 x 10^6/ mL) plus 50 µL of marine saline (MS). (B) 50 µL of amoebocytes (5.2 x 10^4/ mL) plus 50 µL of washed *P. immobilis* (5 x 10^6/ mL) and 50 µL of trifluoroperazine (TFP) (10 µM final concentration). Preliminary eperiments confirmed that at this concentration, TFP was not toxic to the amoebocytes; (C) 50 µL of amoebocytes plus 50 µL of *P. immobilis* (5 x 10^6/ mL) and 50 µL of MS. All wells were incubated for 3 hr at 20 °C. The amoebocytes were then lysed with 150 µL of 0.2% Tween 20 in MS, and 150 µL of sterile marine broth (Difco, Detroit, Michigan) was added to each well to promote the growth of surviving bacteria. The absorbance of each well was read at 570 nm (=T_0) and again after a further 18 hr incubation at 20°C (= T_{18}). Antibacterial activity is expressed as the growth index (SI). This is defined for each well as A_{570} at T_{18} divided by A_{570} at T_0, multiplied by 100. By this notation, values greater than 100 represent bacterial growth (ie no bactericidal effects), whereas those below 100 represent bacterial killing. Values given are means ± SEM (n = 5).

Apart from analyses of mucus, a small number of studies have considered the bactericidal properties of anthozoan tissues. These have been mainly conducted using gorgonians or corals, and have considered only whole body extracts, but they show that anthozoans contain factors able to inactivate or kill both Gram positive and Gram negative bacteria *in vitro* (Burkholder and Burkholder, 1958; Rinehart *et al.*, 1981; Kim, 1994). Few authors have attempted to purify or characterize the active factors involved, little is known about the precise location of these agents in the tissues, their distribution within the group or the extent of their contribution to host defence.

Recently, we have investigated the antibacterial properties of the mesenteric filament amoebocytes from *A. equina in vitro*. We tested first the bactericidal effects of viable cells against the Gram negative marine bacterium, *Psychrobacter immobilis,* and, second, the antibacterial properties of amoebocyte lysate supernatants. We found that growth of this bacterium was significantly impaired in the presence of the viable cells but not in the presence of buffer only (Figure 4). Inclusion of

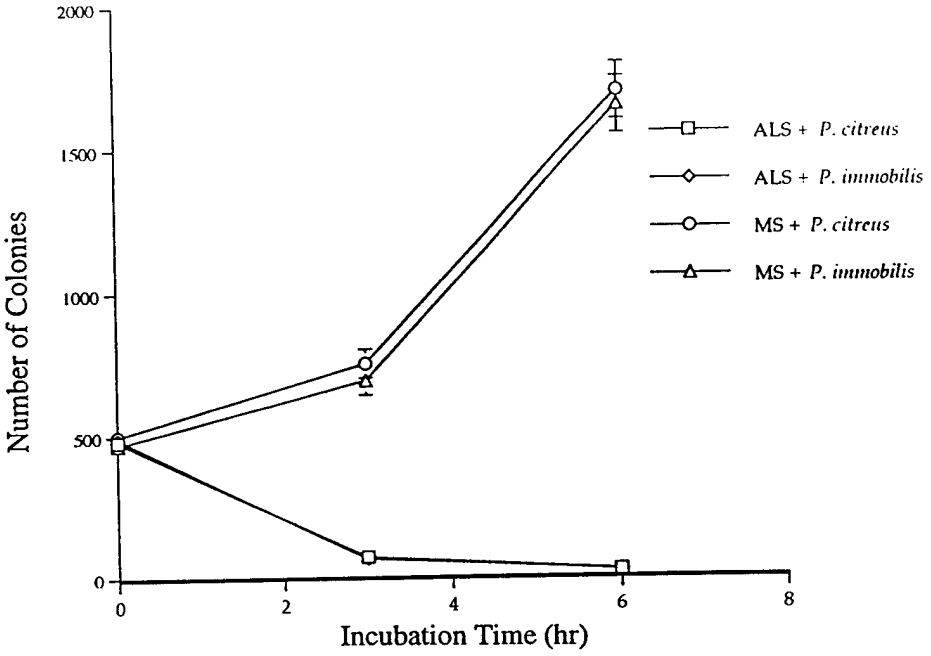


Figure 5. Antibacterial effect of amoebocyte lysate supernatants (ALS) from *A. equina* against the marine Gram negative and Gram positive bacteria, *P. immobilis.* and *P. citreus,* respectively. The method used was as described in Smith *et al.* (1995). Briefly, 180 µl amoebocyte lysate supernatant (ALS) or MS were incubated with 20 µL of washed bacteria (2 x 10^5/ mL) at 20°C for 3 and 6 hr. Aliquots were removed and plated on marine agar and incubated for a further 24 hr at 20°C. The colonies were then counted. Values given are means ± SEM (n = 5).

trifluoroperazine (an inhibitor of NADPH oxidase) in the cell:bacteria mixtures, was observed to impair the antibacterial response of the amoebocytes (Figure 4), showing that this phenomenon must be due, at least in part, to reactive oxygen species generated by the phagocytes through a respiratory burst. With the lysate supernatants of the mesenteric amoebocytes, growth of a wide range of bacterial strains was also significantly reduced (Figure 5).

Preliminary characterization of the active factor(s) involved has further established that activity in *A. equina* amoebocytes is freeze (-20°C, 1 month) and heat (100°C, 10 min.) stable, independent of divalent cations and not due to direct lysis of the bacterial cell wall. Gel filtration of the lysate supernatant on Sephadex G-75 produces four peaks of activity against the Gram positive bacterium, *Planococcus citreus,* corresponding to proteins of molecular weight ca 67 kDa, 13.7 kDa, 7kDa and <kDa (Figure 6). The presence of the small <kDa factor in *A. equina* amoebocytes is of especial interest because, whilst low molecular weight proteins with antibacterial effects have been described for a number of animals (chiefly insects, ascidians, horseshoe crabs, amphibians and mammals) (Nakamura *et al.*, 1988; Azumi *et al.*, 1990; Hoffmann and Hetru, 1992; Ganz and Lehrer, 1994; Cociancich *et al.*,. 1994; Kreil, 1994), none have been previously reported for acoelomates. As yet we do not know whether or not the factor in *A. equina* has any functional or molecular similarities to the low molecular antibiotic proteins in other animal phyla, is effective against Gram negative as well as Gram positive bacteria, and occurs in both granular and hyaline amoebocytes.

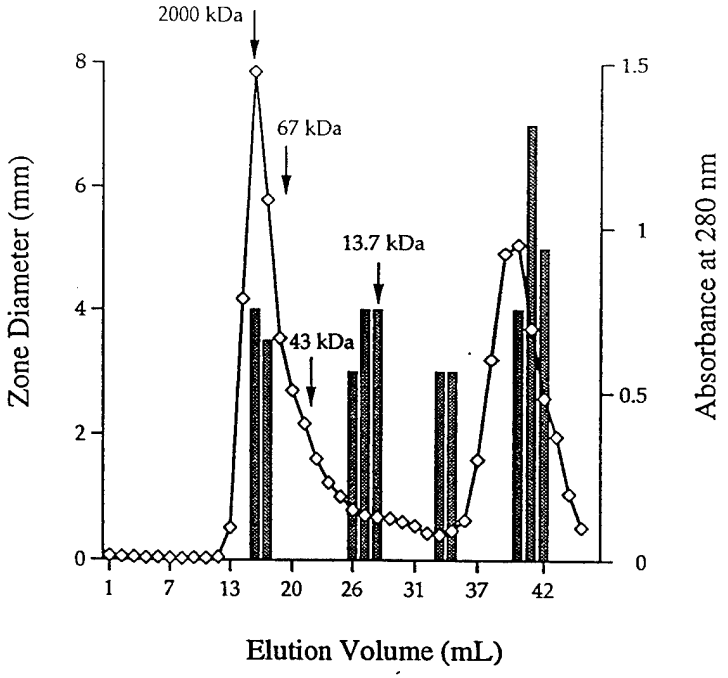


Figure 6. Fractionation of the antibacterial proteins in the amoebocytes of *A. equina* by gel filtration on Sephadex G-75. One millilitre of ALS, prepared in MS (Smith and Hutton 1995) at 2 mg protein/ mL, was applied to a 40 cm column (internal diameter 1 cm) pre-equilibriated with 0.1 M ammonium acetate, pH 6.5 and calibrated with an *in vitro* gel filtration calibration kit for low molecular weight proteins (Pharmacia LKB, Uppsala, Sweden). The proteins were eluted with 0.1 M ammonium acetate, pH 6.5. at a flow rate of 5 mL/ hr. Fractions of 1.2 mL were collected and the absorbancies measured at 280 nm. Each fraction was then freeze dried and reconstituted in 20 µL of sterile distilled water. Antibacterial activity was assessed by the radial diffusion technique (Lehrer *et al.*, 1991) by adding 5 µL volumes to 2 mm diameter wells cut in an 25 mL agarose lawn of *P. citreus* in a 10 cm square petri-dish. The lawns were incubated for 18 hr at 20°C and examined for the appearance of clear zones. Antibacterial vigour was recorded as the diameter of each zone. Arrows indicate molecular weight standards.

CONCLUSIONS AND FUTURE DIRECTIONS

This brief survey of past and present work on the immune responses of anthozoans shows that despite the absence of blood and specialized defence cells, these animals are able to mount highly efficient recognition reactions at the tissue level and to effect antimicrobial protection through a variety of cellular and non-cellular mechanisms. In particular, important roles seem to be played by the amoebocytes that populate both the mesenteric filaments and the mesoglea, and include several of the classical inflammatory responses (amoebocyte infiltration, phagocytosis, ROS production, the synthesis of antimicrobial proteins and, possibly also, cytotoxicity) which are shown by the leukocytes of coelomate animals.

Much still remains to be learned about the biochemical basis for recognition and antibacterial defence in anthozoans, especially if we are to find sensitive and reliable markers for monitoring anthozoan, particularly coral health. For example, the nature of the factor(s) responsible for foreign

tissue destruction in gorgonians warrants detailed analysis, as does the extent to which these agent(s) participate in the killing of parasites. Likewise, with solitary species, the biochemical character of the low molecular weight antibacterial protein(s) needs to be ascertained and their relationship to known antibiotic proteins in other groups clarified. Studies of this type would not only open the way to understanding how the primordial immune systems of coelomates arose but also would enable us to develop a battery of molecular probes which could be used to examine the complex interaction between environment and defence capability in ecologically important members of the group. Other challenging questions that are worthy of consideration include: What are the signalling events associated with bacterial uptake, ROS production and release of microbicidal compounds by the cells? How are these cellular responses regulated *in vivo*, and to what extent do the bioactive factors involved represent unique inventions within the Cnidaria?

Acknowledgment

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Key words: amoebocytes, phagocytosis, superoxide ions, anthozoan, *Actinia,* mesoglea, antibacterial defence.

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Chapter 3

Induction and Regulation of Immune Reactions in an Insect *(Galleria mellonella)*

Andreas Wiesner

Institute of Zoology
Free University Berlin
Königin-Luise-Str. 1-3
D-14195 Berlin, Germany

ABSTRACT

Insects possess effective cellular and humoral mechanisms for the defense against potential pathogens invading the body cavity. The cellular reactions comprise phagocytosis and encapsulation of foreign materials by the hemocytes. The humoral reaction is characterized by a temporary increase of antimicrobial activity in cell free hemolymph. These effector mechanisms have been studied rather extensively. In contrast, little is known about the molecules involved in induction and regulation of defense.

In the studies reviewed here, the humoral and cellular defense reactions of the insect *Galleria mellonella* were induced by synthetic material of non-microbial origin, by hemolymph molecules and by supernatants from phagocytically active hemocytes. The experiments demonstrate the existence of immune activating hemolymph factors and the central role of hemocytes in producing immune regulating factors. As a preliminary result two immune stimulating factors could be identified: Apolipophorin-III of *G. mellonella* as an inducer of the antibacterial humoral immune response *in vivo,* while a <3 kDa factor - presumably released by phagocytosing plasmatocytes - activates the phagocytic reactions of cells from the same hemocyte type *in vitro.*

Introduction

Cellular as well as humoral components belong to the insect immune system. The cellular defense reactions by the insect blood cells, the hemocytes, comprise phagocytosis and encapsulation of invaders, the humoral defense is characterized by a temporary increase of antimicrobial activity in cell free hemolymph. The microscopical appearance of the cellular defense reactions has already been described in detail more than 15 years ago (Ratcliffe and Rowley, 1979), but the regulatory events are only poorly understood up to now (Ratcliffe, 1993a). The humoral response follows the cellular response because time is needed for the synthesis of antibacterial hemolymph proteins (Boman *et al.,* 1981; Hoffmann *et al.,* 1981) which are mainly synthesized by the insect fat body (Faye and Wyatt, 1980). Intensive research on these proteins began in the early 1970s (Boman, *et*

al., 1972), since then a large number of the responsible proteins and some of the corresponding genes have been identified and sequenced (Faye and Hultmark, 1993; Cociancich *et al.,* 1994).

Unlike vertebrates, the immune system in insects seems to lack components such as immunoglobulins, complement cascade and long term immunity (Götz and Boman, 1985). Nevertheless, recent molecular biology studies revealed some parallels between the immune systems of insects and vertebrates (reviewed in Faye and Hultmark, 1993; and Cociancich *et al.,* 1994). For instance, the insect antibacterial peptide cecropin shows similarities with peptidenamed porcine from the pig intestine in regard to its amino acid sequence. Another insect immune protein, the hemolin, belongs to the immunoglobulin superfamily. Furthermore, certain sequences and the activation mechanism of a number of promotor or enhancer regions from immune related genes in insects and mammals are similar.

The absence of immunoglobulins and long term immunity is no hindrance to the extreme efficiency of the insect defense. An example that may help to illustrate this: Experimental induction of insect immune reactions are normally done by intrahemocoelic injections of bacterial suspensions. In our laboratory we inject $1x10^5$ living non-pathogenic bacteria (*Enterobacter cloacae*) suspended in 10µL solution into the hemocoel of a 300 mg *Galleria mellonella* larva. The same dose calculated for a vertebrate with a body weight of 80kg would result in an intravenous injection of 2.7×10^{10} bacteria suspended in an injection volume of 2.7 L saline. Our insects survive such a treatment without any problems, a vertebrate would surely die simply from immediate break down of its physiological balance.

As mentioned above, the effector system of the insect immune system is relatively well examined. In contrast, our knowledge about its induction and regulation is scarce. A number of putative factors influencing insect cellular defense reactions have already been described. A so called "hemokinin" (Cherbas, 1973) was found in *Hyalophora cecropia,* an "encapsulation promoting factor" in *Heliothis virescens* (Davies *et al.,* 1988), a "plasmatocyte depletion factor" (Chain and Anderson, 1982 and 1983) as well as a "phagocytosis stimulating factor" (Mohrig *et al.,* 1979) in *Galleria mellonella.* Unfortunately none of these factors has been further characterized. But in a number of other studies on insects and crustaceans; specific binding proteins for microbial molecules and the subsequent onset of immune related mechanisms have been demonstrated in greater detail. In the crayfish *Pacifastacus leniusculus* the specific association of fungal ß-1,3-glucan with its binding protein is followed by a complex reaction comprising activation of the prophenoloxidase activating system and of a cell adhesion factor (called 76 kDa protein) as well as adhesion, spreading and degranulation of hemocytes (Söderhäll, 1992; Söderhäll *et al.,* 1994). Another ß-1,3-glucan recognition protein from *Bombyx mori* triggers the phenoloxidase activity in this insect (Ochiai and Ashida, 1988). Furthermore, in the same experimental animal; binding of bacterial lipopolysaccharide (LPS) by insect lipophorin seems to be coupled with the termination of cecropin mRNA synthesis (Kato *et al.,* 1994ab). Binding of LPS sugar residues to the immune protein hemolin seems also to be an important step in the induction of the immune genes from the insect *Hyalophora cecropia* (Faye and Hultmark, 1993). The putative role of agglutinins or more generally lectins in the induction process is reviewed by Natori (1990) and Vasta *et al,* (1994).

From the examples cited previously, it can be concluded that there exist specific mechanisms for recognition of foreigness and induction of immunity. In addition, insect immune reactions can also

be provoked by materials and solutions of non-microbial origin. Injection of synthetic material such as latex beads into the hemocoel of insects can be followed by typical "anti-microbial" reactions. These comprise phagocytosis and encapsulation by hemocytes (Götz, 1986; Lackie, 1988), protective immunity against bacterial challenge and induction of strong antibacterial activity in cell free hemolymph (see the following review). Even the injection of sterile saline alone is followed by a distinct humoral response. For *Hyalophora cecropia* it was shown that the induced protein- and RNA-pattern is qualitatively the same after injection of saline as after injection of bacteria (Boman and Steiner, 1981; Boman *et al.*, 1981).

The induction of immune reactions in the insect *Galleria mellonella* without involvement of microbial substances and even without any foreign target was the goal of the studies summarized in the following review. Defense reactions were induced by synthetic materials, by transfer of hemolymph components or by hemocyte released molecules (Figure 1). The ability of these inducers to activate the antibacterial humoral response *in vivo* and to enhance the phagocytic reactions of isolated hemocytes *in vitro* was investigated. The reviewed results will give evidence for the central

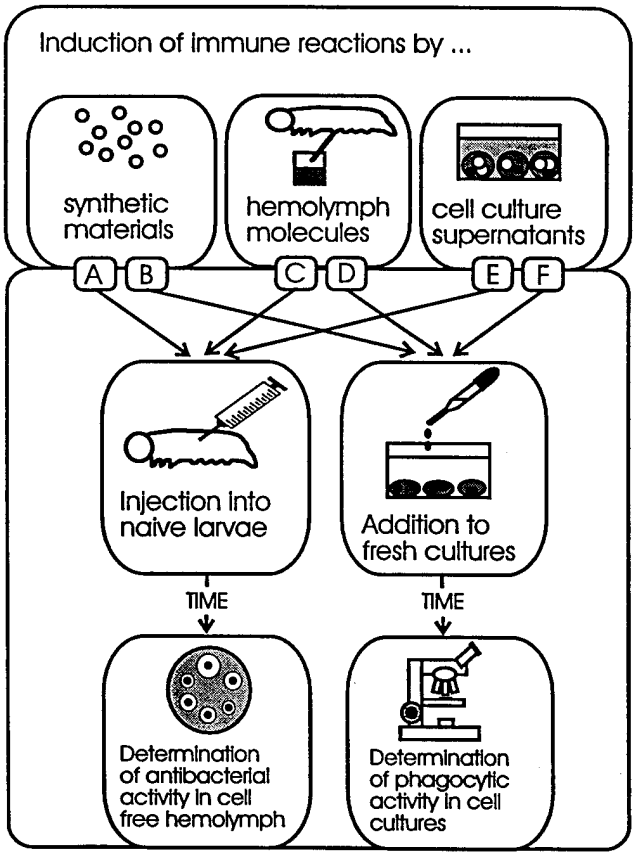


Figure 1. The onset of an antibacterial humoral response *in vivo* as well as phagocytic reactions by isolated hemocytes *in vitro* can be induced by synthetic material such as silica beads (A, B), by hemolymph molecules (C, D) and by cell culture supernatants from hemocytes heavily phagocytosing synthetic beads (E, F). Antibacterial activity in cell free hemolymph is measurable by inhibition or lytic zone assays. Phagocytic reactions of recipient hemocyte cultures can be quantified with the help of a fluorescence assay using yeast cells as targets.

role of hemocytes in the induction of immunity. The general suitability of such studies as a tool for learning more about the recognition of foreigness and to discover new immune regulating factors will be demonstrated.

REVIEW

Basic Assay Methods

In the experiments described below mainly two kinds of assays for the detection of defense reactions were employed. The first is instrumental in determining the onset of a humoral response by measuring the antibacterial activity of hemolymph samples. The second enables the phagocytic reactions of isolated hemocytes *in vitro* to be quantified.

For the determination of antibacterial activity in cell free hemolymph the approved "agar plate methods" were used. An inhibition zone assay with a lipopolysaccharide-defective, streptomycin- and ampicillin-resistant mutant of *Escherichia coli* K12 strain D31 (Boman *et al.*, 1974) as indicator bacterium served for detection of activity against Gram negative bacteria (Faye and Wyatt, 1980). Lysozyme activity was measured with a lytic zone assay according to Mohrig and Messner (1968) using freeze dried *Micrococcus luteus* cell walls. Previously the only enzyme in *G. mellonella* responsible for lytic activity against *M.luteus* was found to be a lysozyme (Powning and Davidson, 1973; Hoffmann *et al.*, 1981) of the chicken type (Jollès *et al.*, 1979; Powning and Davidson, 1976). For standardization, gentamycin was used against *E. coli* and hen egg white lysozyme against *M. luteus* (Wiesner, 1992).

The development of a reliable method for the quantification of phagocytic reactions in *in vitro*-cultures of hemocytes required more effort. At first, the phagocytically active hemocytes must be isolated from the other hemocytes. In *Galleria mellonella* the so called plasmatocytes represent the main phagocytic cell type (Rowley and Ratcliffe, 1981). The most widely used method for the separation of arthropod hemocytes is density gradient centrifugation in Percoll^{TM} (Söderhäll and Smith, 1983; Anggraeni and Ratcliffe, 1991). A disadvantage of this method is the time-consuming diligence necessary in the handling of isolated cell fractions. Cells must be washed several times after separation to remove the Percoll^{TM}. Otherwise precipitation of the gradient medium will create considerable problems during the short cultivation of the isolated cell monolayers (Ratcliffe *et al.*, 1986; Ratcliffe, 1993b). For this reason we adapted a less complicated nylon wool separation method originally developed for the separation of vertebrate blood cells (Greenwalt *et al.*, 1962; Eisen *et al.*, 1972; Julius *et al.*, 1973) for our insect hemocytes. With this method plasmatocytes can be enriched from 52% to 93% and used for phagocytosis experiments immediately after isolation (Wiesner and Götz, 1993). For the quantification of particle uptake by phagocytosing plasmatocytes a fluorescence assay originally developed for vertebrate cells (Hed, 1986) was employed in our laboratory (Rohloff *et al.*, 1994). FITC-labelled yeast cells are added to monolayers consisting of isolated plasmatocytes. After an incubation allowing time for the phagocytosis of yeast cells by the plasmatocytes, trypan blue solution is added. As a consequence, only the ingested yeast cells remain fluorescent, non-ingested yeast cells are quenched by trypan blue. Phagocytic activity can then easily be determined by counting the shining yeast cells under the microscope. Other groups working with

phagocytosing arthropod hemocytes likewise had good experiences with this assay (Thörnqvist *et al.*, 1994; Scapigliati and Mazzini, 1994).

Induction by Synthetic Materials (Figure. 1, A and B)

An important prerequisite for the transfer studies described below was the selection of suitable synthetic material for the induction of defense reactions. For this purpose *Galleria mellonella* larvae were injected with different kinds of provocators such as latex beads, silica beads or ionexchanger beads. Subsequently, the onset of an antibacterial humoral response in the treated animals was recorded. From these experiments it could be concluded that general physicochemical surface properties of the foreign targets greatly influence their inducing capacity. Hydrophilic or positively charged materials revealed to be stronger inducers than hydrophobic or negatively charged ones (Wiesner, 1992; Wiesner and Götz, 1993). In addition, covalent coupling of defined molecules onto latex beads which are normally weak inducers can also enhance the stimulation by the foreign material. This was shown for a number of proteins such as fibronectin or poly-lysine but also for mixtures like bovine serum albumin or hemolymph molecules (Wiesner, 1992).

The same beads which were strong inducers of humoral responses were also potent inducers of the cellular defense reactions *in vivo* and *in vitro*. Hydrophilic silica beads may serve as an example. The intrahemocoelic injection of these beads is followed by a dose dependent increase of antibacterial activity in *Galleria mellonella* larvae (Figure 2). The same beads are also potent activators of the cellular reactions *in vivo* and *in vitro*. More than 63% of the hemocytes in plasmatocyte

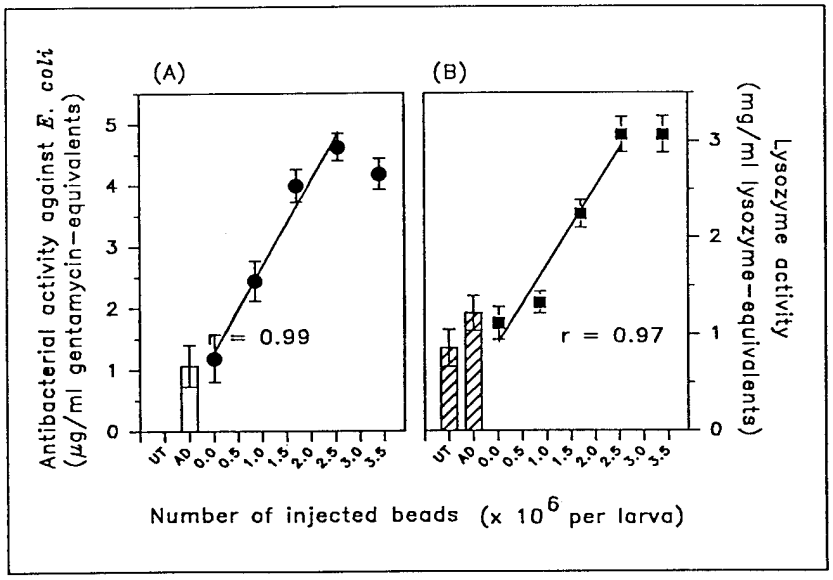


Figure 2. Antibacterial activity against *E. coli* (A) and lysozyme activity against *M. luteus* (B) in cell-free hemolymph of *G. mellonella* larvae 18 hr after an intrahemocoelic injection of hydrophilic silica beads suspended in water at different dosages (10 µL injection volume). The induced antibacterial activities showed a clear dose dependence for dosages between 0.02 and 2.55 x 10^6 beads per larva (each weighing 240-310 mg). UT, untreated larvae; AD, water injected larvae; r, correlation coefficient of a linear regression. Data are given as means ± standard errors from at least 6 larvae, respectively.

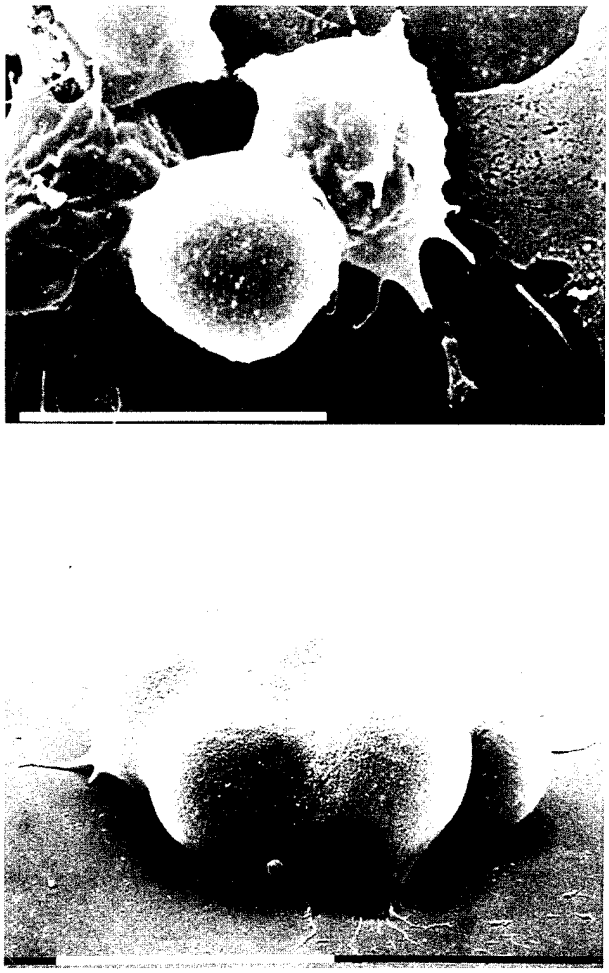


Figure 3. SEM pictures of cellular defenses of *G. mellonella* hemocytes against hydrophilic silica beads *in vitro.* A few minutes after first contact with the beads the isolated plasmatocytes began the phagocytic reaction immediately by enveloping the targets with their membranes (A). Two hours later most of the hemocytes are completely filled with beads (B). Scale bars = 10µm.

monolayers phagocytosed these beads, cells were often filled with beads close to capacity (Figure 3). In contrast, hydrophobic beads did not provoke any noteworthy response (Wiesner and Götz, 1993).

Induction by Hemolymph Molecules

In vivo-in vivo-transfer (Figure 1, C)

Hemolymph transfer studies were conducted in order to prove the existence of immune stimulating factors in hemolymph. In the first experiments on this topic, the donors were preactivated by injection of latex beads. Donor hemolymph was withdrawn later, at various time intervals, and injected onto non-pretreated larvae. The determination of the antibacterial activity in recepient larvae one day after transfer showed clearly that the inducing capacity of the donor hemolymph was

enhanced by preinjection. When cell free hemolymph was transfered this enhancement was restricted to the first 30 minutes after preinjection of the donors with latex beads. At the same time the number of free floating hemocytes in donor hemolymph was greatly reduced because of their engagement in phagocytosis and encapsulation of the latex beads. The hypothesis that the hemocytes contain immune stimulating factors which are released during these defense reactions was further supported by the fact that the enhanced induction capacity of donor hemolymph lasted longer (up to 3 hours after preinjection with latex beads) if hemolymph lysate instead of cell free hemolymph was transfered (Wiesner, 1991).

In further studies methodical progress allowed to prepare strong inducing donor hemolymph without time consuming preinjection of the donor larvae. In these experiments it was shown that 18 hours after transfer of sterile filtrated HLS (hemolymph lysate supernatant) the antibacterial activity in recipient larvae was as high as after injection of high doses of bacteria. The induced antibacterial activity revealed to be dependent of the transfered hemolymph volume (Figure 4). In addition, cell free donor hemolymph proved again to have lower inducing capacities as hemolymph lysate supernatant. This was further support for the hypothetical existence of hemocyte derived inducing factors (Wiesner, 1993).

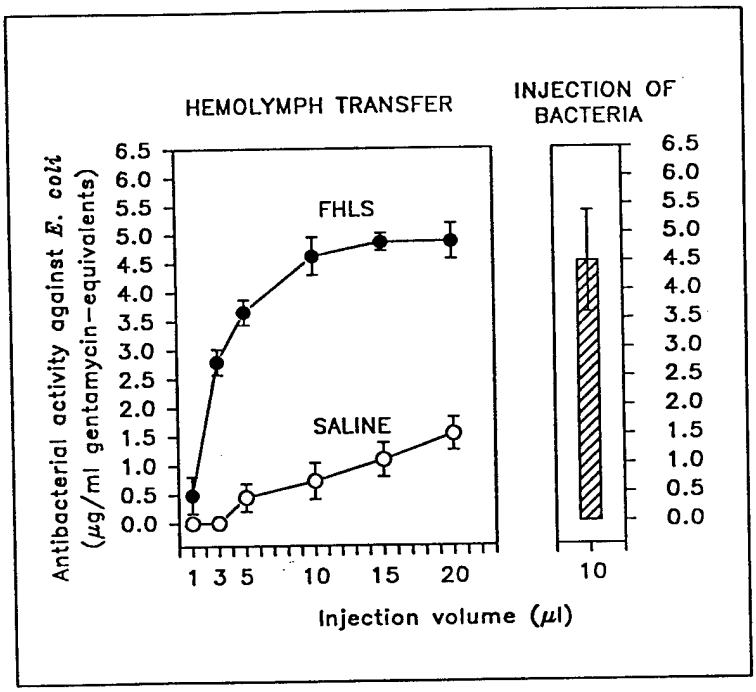


Figure 4: Antibacterial activity against *E. coli* in cell free hemolymph of *G. mellonella* larvae (each weighing 240-310 mg) 18 h after intrahemocoelic injection of 0.2µm-filtered hemolymph lysate supernatant (FHLS). The provoked antibacterial activity depends clearly on the injection volume. On the right-hand side, antibacterial activity after injection of 1 x 10^5 living *Enterobacter cloacae* bacteria is shown for comparison. Data are given as means ± standard errors from at least 8 larvae, respectively.

The consequent continuation with these transfer studies lead recently to the detection of a hemolymph molecule with the ability to induce strong antibacterial activities in recipient larvae. This molecule was purified and identified as apolipophorin III from *Galleria mellonella*. Publishing of these studies is currently under way (Wiesner *et al.*, submitted 1996a).

In vivo-in vitro-transfer (Figure 1, D)

After establishing the methods for isolation and cultivation of plasmatocytes and for the determination of their phagocytic activity (see "basic methods") we started a first attempt to identify phagocytosis stimulating hemolymph molecules. In these experiments, eluates from the nylon wool columns which are used for isolation of plasmatocytes were fractionated by ultrafiltration through membranes with different cut-offs. The resulting fractions - each containing a roughly defined range of molecular sizes - were tested for their ability to stimulate the phagocytosis of yeast cells by freshly prepared plasmatocytes *in vitro* (Rohloff *et. al.*, 1994). The preliminary result of this investigation was the detection of a phagocytosis stimulating activity in the fraction containing molecules >100 kDa (Table 1).

Table 1.
Stimulation of *in vitro* phagocytosis by >100 kDa hemolymph molecules

Addition of hemolymph fractions molecular masses (kDa) approximations	Percentage of phagocytically active plasmatocytes (mean ± SE, n=6)
>100 kDa	32.17 ± 2.13 (***)
100 - 30 kDa	6.65 ± 0.56
30 - 10 kDa	8.27 ± 1.03
10 - 3 kDa	11.72 ± 2.07
Control	14.12 ± 1.68

The addition of >100 kDa hemolymph molecules to monolayers of isolated plasmatocytes results in a significantly enhanced phagocytic reaction of these hemocytes against yeast cells. Addition of the other molecule fractions did not result in an increase compared to the control (addition of cell culture medium only).
(***) = significantly different from the control ($P < 0.001$).

Induction by Cell Culture Supernatants

In vitro-in vivo-transfer (Figure 1, E)

From the experiments cited above we have learned that synthetic inducers as well as hemolymph components are able to provoke defense reactions. In addition, the results support the idea that defending hemocytes are the source of factors which stimulate the onset of a humoral immune response *in vivo*. In order to prove this hypothesis, supernatants from heavily phagocytosing plasmatocytes were injected into previously untreated larvae. 18 hours after injection the onset of a humoral response was monitored by measuring the antibacterial activity in cell free hemolymph

Table 2.
Induction of the humoral response *in vivo* by injection of supernatants (0.2 µm-filtrated) from phagocytosing plasmatocyte monolayers into *G. mellonella* larvae

Injection of 0.2µm-filtrated supernatant from:	Antibacterial activity in hemolymph of injected larvae, 18 hours after treatment (µg/mL gentamycin-equivalents)
Plasmatocytes + Beads + Medium	2.28 ± 0.24 (***)
Plasmatocytes + Medium	1.37 ± 0.23
Beads + Medium	0.79 ± 0.28
Medium only	0.63 ± 0.31
Untreated larvae	0.00 ± 0.00

An intrahemocoelic injection of supernatants from plasmatocytes active in phagocytosis of silica beads is followed by a significant increase of antibacterial activity in cell free hemolymph of injected larvae in comparison to controls (medium only). The activities in larvae which received supernatants from non-phagocytosing plasmatocytes are only slightly enhanced. Antibacterial activity was determined with an inhibition zone assay against *E. coli*.
Data are given as means ± standard errors from at least 8 larvae, respectively.
(***) = significantly different from the control ($P < 0.001$).

of injected larvae. A significantly enhanced antibacterial activity could only be detected in those larvae which were injected with supernatants from phagocytosing hemocytes. Injection of supernatant from non active plasmatocytes resulted in a minor increase in comparison to controls (Table 2). These results give evidence for the existence of hemocyte derived factors capable of provoking the production of antibacterial molecules in intact larvae (Wiesner and Götz, 1993).

In vitro - in vitro - transfer (Figure 1, F)

If hemocytes are the source of factors which induce the humoral response then one can presume that there also should be information pathways between defending hemocytes. Evidence for the existence of such a pathway was given by the fact that the transfer of supernatants from phagocytosing plasmatocyte cultures to freshly prepared ones increases the phagocytic activity in the recipient cultures (Table 3). In the studies dealing with this topic, the donor cultures were activated by the addition of sterile silica beads. The determination of phagocytic activity in recipient cultures was done by the FITC-yeast assay described above. Recently we have characterized the responsible factor to be a very small (<3 kDa), heat-sensitive and hydrophobic molecule. Complete results and methodical details will be reported soon (Wiesner *et al.*, 1996b).

Table 3.
Stimulation of *in vitro* phagocytosis by addition of supernatants (<3kDa fractions) from phagocytosing plasmatocyte monolayers ("donor cells") to freshly prepared monolayers ("recipient cells")

Donor cells are active in phagocytosis	Percentage of phagocytically active recipient cells
Yes	12.34 ± 1.33 (***)
No	5.47 ± 0.68
Control	5.75 ± 0.70

The phagocytic activity of freshly prepared plasmatocyte monolayers can be significantly enhanced by the addition of supernatants (<3 kDa fraction was used in this experiment) from other plasmatocyte monolayers which are active in phagocytosis of silica beads. Addition of supernatants from non-phagocytosing donor cells has no effect in comparison to the control (addition of cell culture medium only).
Data are given as means ± standard errors from 8 experiments, respectively.
(***) = significantly different from the control (*P <0.001)*.

DISCUSSION

The following conclusions have been drawn from the results reviewed:

- Insect immune sytems can be activated by sterile and synthetic material without involvement of microbial molecules. General physicochemical properties of foreign materials greatly influence their inducing capacity. Hydrophilic and positively charged inducers are stronger stimulators than hydrophobic and negatively charged ones.

- Humoral as well as cellular defense reactions can be provoked by hemolymph molecules alone without a foreign target. The hemolymph molecule apolipophorin-III was shown to be an inducer of the humoral response *in vivo*. Another >100 kDa hemolymph factor not further characterized stimulates phagocytosis *in vitro*.

- Hemocytes are a source of inducible immune stimulating factors which can activate the humoral response *in vivo* as well as phagocytic defense *in vitro*. The characterization of the inducible <3 kDa phagocytosis stimulating factor is under investigation, its autocrine like mode of action has already been proved.

The success of the studies described here depended mainly on the use of synthetic inducers instead of microbial ones. As a side effect, the search for good synthetic inducers provided additional informations about the foreign components necessary for the induction of insect immune responses. The results give further evidence for the importance of general physicochemical surface properties of foreign targets for their ability to evoke immune related reactions. Earlier studies by other reseachers have shown that surface charge and hydrophobicity of foreign material have a great

influence on cellular defense by insect hemocytes (reviewed in Lackie, 1988). For detailed discussions of this topic see Wiesner, 1992; Wiesner and Götz, 1993.

Hemolymph transfer studies similar to those presented in this review were also conducted by other researchers. The goal of these earlier studies however, was to substantiate the idea of a putative passive immunization by transferring antibacterial molecules (Stephens, 1963; Mohrig and Messner, 1968). Subsequent transfer studies (De Verno *et al.,* 1983) failed also to give proof for the existence of immune stimulating hemolymph molecules, because the donors were preactivated with bacterial lipopolysaccharides (LPS). As a consequence the results allow the speculation that the LPS itself and not hemolymph molecules were responsible for the immune stimulating activity on recipients (Dunn, 1986). In the experiments presented in this review the avoidance of microbial inducers helped to give better proof for the existence of signaling hemolymph molecules. The identification of apolipophorin-III (apoLP-III) as an inducer of humoral immune reactions was surprising. From investigations on the apoLP-III of *Manduca sexta* and of *Locusta migratoria* the main role of this molecule seems to be the stabilization of low density lipophorins during the transport of lipids between the fat body and the flight muscles (Blacklock and Ryan, 1994). Other reports about the action of lipophorins in insect physiology could probably help to determine connections to insect immunity. For example, the inhibition of hemocyte adhesion by lipophorins (Coodin and Caveney, 1992) and the role of lipophorins in detoxification of bacterial toxins (Kato *et al.,* 1994b) have been described. Further experiments are needed to interpret the possible role of apoLP-III in induction of immunity. The as yet not completely characterized >100 kDa hemolymph factor and its phagocytosis-stimulating activity is surely another part of the complex regulating system. Further studies about the molecule responsible and its' mode of action are currently under way.

The use of cell culture supernatants for the induction of immune reactions helped to discover the existence of a hemocyte derived <3 kDa phagocytosis stimulating factor. Whereas the biological effects are already evaluated in detail, the chemical nature of this molecule must be more closely investigated. It would then be possible to decide if this factor is also responsible for the induction of humoral response after injection of activated supernatants into intact larvae. The existence of hemocyte-derived immune stimulating factors was earlier postulated by others (Boman and Hultmark, 1987; Lackie, 1988; Trenczek and Faye, 1988) because of the obvious importance of these cells in the defense. Hemocytes are in the first line of defense and in direct contact with the foreign invader. Furthermore they are responsible for the rapid sealing of cuticular wounds. Only the hemocytes are therefore able to release signal molecules from which other cells and organs inside the insect body can receive information about the extent of wounds and the number of invaders. The number of reacting hemocytes, the amount of released factors and the increase in cellular and humoral response could be coupled in an effective manner. The results reviewed here support this hypothesis (Figure 5).

The experimental models described have proved to be useful tools in investigating the factors responsible for triggering the defense system. The aim of future experiments will be to continue to detect and isolate immune regulating factors from insects.

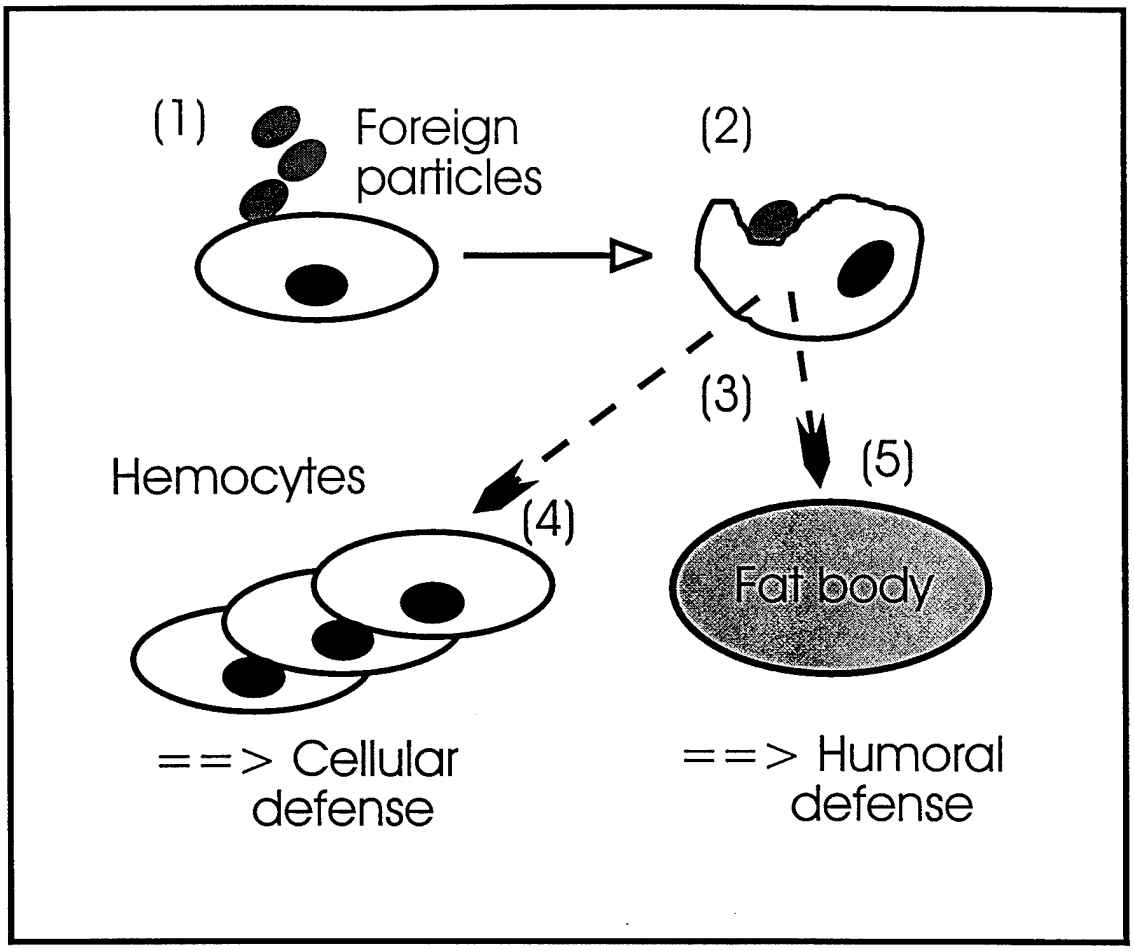


Figure 5. The reviewed results allow the construction of the following model: After recognition of foreigness (1) the phagocytosing hemocytes (2) release immune activating factors (3) which recruit other hemocytes for the defense (4) and trigger the synthesis of antmicrobial proteins by the insect fat body (5).

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Chapter 4

Opioid, Opiate Interaction with Cytokines in Invertebrates: Presence and Significance

George B. Stefano and Thomas K. Hughes[1]

Multidisciplinary Center for the Study of Aging
Old Westbury Neuroscience Research Institute,
State University of New York
College at Old Westbury
Old Westbury, NY 11568

[1]Department of Microbiology
University Medical Branch at Galveston
University of Texas
Galveston, Texas 77550

INTRODUCTION

It is evident that intercellular communication is mediated primarily by chemical signal molecules regardless of the physiological system. During the course of evolution, organisms in which this form of communication developed appear to have increased their chances of survival and thus passed this trait on to their descendents. A plausible explanation for the emergence /dominance of this mechanism of communication can be based on its inherent level of sophistication, as noted by not only synaptic molecules that can enter into intercellular communication, but hormonal and immune ones as well. A further advantage of this method is that it is not limited by spatial contact requirements. Mechanisms which employ direct contact, by their nature, require a great deal of contact space, whereas the only requirement of chemical communication is scaled down space for receptors. This also allows for a greater diversity of the signal molecules and their corresponding receptors. The end result of such chemical communication mechanisms would be a higher degree of sophistication and detailed information transfer, which allows for a greater number of behavioral characteristics to enhance an organism's chance for survival in a changing environment. If indeed this system was favored, it can be predicted that the organisms accumulating the greatest diversity of cellular communication would eventually begin to control their internal as well as external environment.

Molecular evolution, in part, concerns itself with determining the genetic basis of natural selection. Changes in the genetic code, if favorable, will make an organism better prepared to cope with its environment. Other phenomena which are equally important are the mechanism of simultaneous expression of the signal molecule and the receptor in different cell types involved in chemical signaling. It is known that the same signal system can be used in different or the same ways in

different phyla. What causes the precise expression of both complementary systems? This dual expression certainly suggests the existence of a functional interaction of these two "separate" aspects of the same signal system. It would be interesting to speculate that since both "up" and "down" regulation of a receptor population can be regulated by the concentration of the signal molecule, the signal molecule itself can induce the presence of its receptor in a distant cell.

Given the above incorporated strategies for organism survival, one could predict that once this is achieved it would tend to be conserved. The presence of biologically active peptides in invertebrates, which are comparable to those of vertebrates has been known for a considerable period of time (see Leung and Stefano, 1987). The recent upsurge of interest in the diverse roles and modes of operation of these molecules has sparked a search for their evolutionary history. While several reports on the occurrence of endogenous opioids in submammalian vertebrates have become available, comparable data in invertebrates are still emerging. They consist of the demonstration in certain invertebrate ganglia of either opioid peptides or their specific receptor sites (review-Leung and Stefano, 1987).

Regarding the immune system, there is ample evidence documenting the presence and significance of opioid peptides and opiate alkaloids in the immune system (see Stefano and Scharrer, 1994). The same is true for the presence of cytokines and other immunoactive signal molecules in nervous tissue. This chapter focuses on information gained in invertebrates demonstrating that these signal molecules, some mammalian-like (opioid and cytokine), are present and are important in comparative immunology and neuroimmunology.

OPIOID AND OPIATE SIGNAL MOLECULES

Met-enkephalin, Leu-enkephalin and Met-enkephalin-Arg^6-Phe^7 were isolated and identified in the nervous system of *Mytilus edulis* along with opioid receptors and found to be identical to those signal molecules found in man (see Leung and Stefano, 1987). The demonstration of Met-enkephalin-like material in the hemolymph of *M. edulis* was carried out recently by high pressure liquid chromatography (HPLC) and radioimmunoassay (RIA) (Stefano *et al.*, 1989a,b). Morphine-like and codeine-like substances also were demonstrated in the pedal ganglia, hemolymph and mantle tissues of the mollusc *M. edulis* (Stefano *et al.*, 1993). It was further demonstrated that immunocytes of *Mytilus* do not contain opiate alkaloids, at least at the level of the detection and identification. The pharmacological activities of the endogenous morphine-like material resemble those of authentic morphine (Stefano *et al.*, 1993). Morphine and the immunoreactive material corresponding to morphine counteracted, in a dose-dependent manner, the stimulatory effect of TNF-α or IL-1α on human monocytes and *Mytilus* immunocytes. The immunosuppressive effect of this opiate material expresses itself by lowering chemotactic activity, cellular velocity and adherence as well as by making active immunocytes inactive (rounded; Stefano *et al.*, 1993). Codeine mimics the activity of authentic morphine, but only at much higher concentrations. These pharmacological effects of morphine on immunocytes are consistent with those actions attributed to opiates reported in the literature (see Stefano and Scharrer, 1994). Indeed, it has been surmised that morphinergic transmission may regulate the downregulation of immune activation (see Stefano *et al.*, 1993; Stefano and Scharrer, 1994).

CYTOKINES

We have found that *Mytilus edulis* hemocytes and pedal ganglia both produce and respond to immunoreactive (ir) cytokines. Specifically, we have shown that ir-interleukins-1 and -6 (IL-1, -6) and tumor necrosis factor-α (TNF-α) can be demonstrated in this invertebrate (Hughes *et al.* 1990; Hughes *et al.* 1991a). The studies have been performed utilizing reagents and antibodies directed to the human counterpart since none are available for the invertebrate. Thus, a question arises as to specificity of detection and subsequently of action of these "ir-cytokines". For example, could the results that we have seen utilizing a human based enzyme-linked immunosorbent assay (ELISA) be due to the presence of a protein in hemolymph or tissues of *Mytilus edulis* that non-specifically bind human immunoglobulin as has been seen in a different system (Hahn *et al.*, in press). This would result in false positive results. Our control studies indicate that in our model system it is likely not the case. For example, utilizing ELISAs for interferon-γ and IL-8, we have obtained negative results indicating that an immunoglobulin binding substance is not attaching to the ELISA's solid phase anti-human antibodies (data not shown). Additionally, and perhaps more importantly, we have found homologies at the nucleic acid level between invertebrate and human

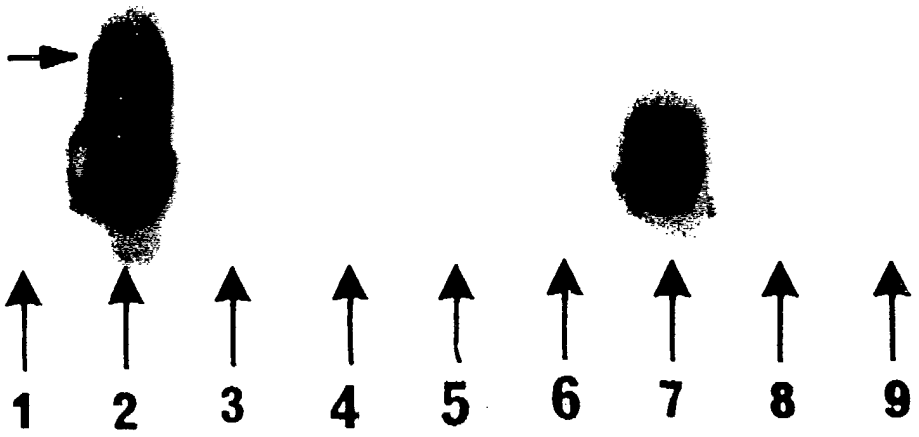


Figure 1. Southern analysis of RT-PCR amplified *Mytilus edulis* RNA for expression of IL-1β related genes.
Total RNA from *Mytilus edulis* and human lymphocytes were reverse transcribed and PCR amplified for IL-1β. Lanes 1-5 contain amplified human RNA, lanes 6-9 contain amplified *Mytilus edulis* RNA. With the exception of lanes 2 and 7, all other lanes are specificity controls. Lane 2 shows a strong positive signal for IL-1β at the expected 802 bp fragment (arrow). There is in addition a strong signal from a positively hybridizing small fragment. In lane 7, containing *Mytilus edulis* RNA, there is positive amplification and hybridization for IL-1β, but co-migrating at the small fragment size.

IL-1β and TNF-α using reverse transcriptase coupled polymerase chain reaction and Southern analysis or Northern analysis, respectively (see Figures 1 and 2). Thus, we feel that our studies provide a strong rationale for further study of these substances in invertebrates as they have been studied in humans, both in the immune and nervous systems.

Given the general lack of conclusive studies to date concerning the presence of cytokines in invertebrates, we thought it important to review our reports concerning the identification of cytokine-like material in these animals. This in turn, would enhance the reliability of our observations and those of others involved with documenting the interaction between cytokines and opioid and opiate signal molecules.

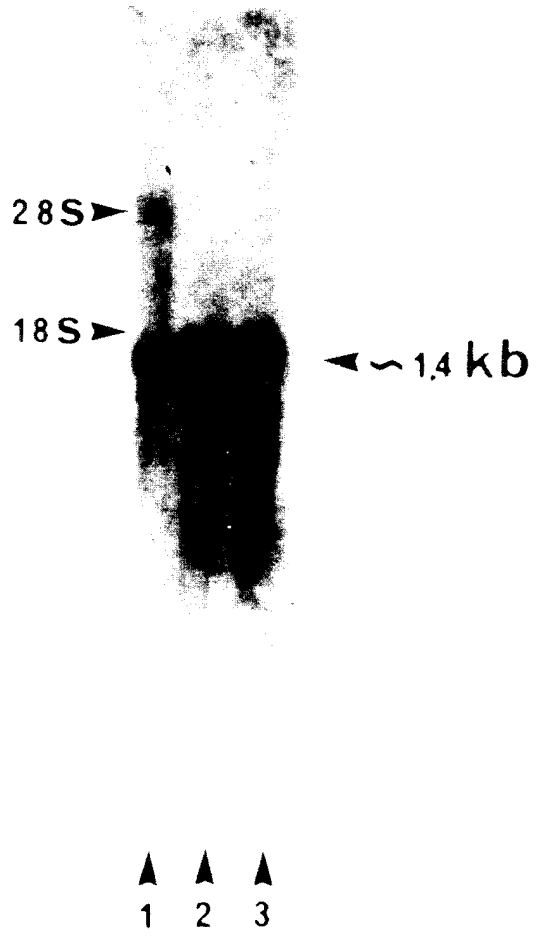


Figure 2. Northern analysis of *Mytilus edulis* immunocyte RNA for expression of TNF-α related genes. To detect the possible existence of a *Mytilus edulis* gene related to mammalian TNF-α, RNA was isolated from immunocytes and probed with a cDNA probe for human TNF-α. Conditions were slightly relaxed (1X SSC/1% SDS @ 65° for 15 min. x 2) to allow for cross hybridization. Lane 1 is control human total RNA from lymphocytes. Lane 2 is RNA isolated from immunocytes in which *Mytilus edulis* was subjected to hypoxic stress. Lane 3 represents LPS stimulated immunocytes. There is a strong signal migrating in all samples at the expected ~1.4 kb position.

CYTOKINES: NEUROTRANSMITTERS/NEUROMODULATORS

This subtitle implies that if cytokines can influence select neurophysiological activities, immunocytes can communicate with neurons (Stefano and Scharrer, 1994). Mammalian cytokines have been shown to alter highly specific neurophysiological activities in invertebrates (Sawada *et al.*, 1991a,b; Stefano, 1992; Szûs *et al.*, 1992a,b). The effect of IL-1 was studied on the voltage activated ion currents of the identified central neurons of *Helix pomatia* L. by use of two microelectrode voltage clamps. The voltage activated inward current (I_{Ca}) was decreased, whereas the outward current ($I_{net\ K}$) was increased by IL-1. IL-1 affects both the transient and the delayed rectifying potassium currents. The IL-1 modulatory effect on the voltage activated ion current is voltage and dose dependent. The threshold concentration for IL-1 was 2 U/mL. The proposed modulatory effect of IL-1 appears to have more than one site of action on the neuron membrane ion channels. Rabbit anti-human IL-1 polyclonal antiserum eliminated the IL-1 effects on the voltage activated inward and outward currents (Szûcs *et al.*, 1992b). Clearly, as recent data indicate, the role of neuropeptides has been broadened to include immunoregulatory mechanisms. These immunoregulatory molecules can alter specific neuronal ionophoric domains. Thus, the signal molecules used for autoimmunoregulation appear to have the potential for neuroimmune communication in invertebrates as well.

OPIOID-CYTOKINE LINK

Opioid induction of an IL 1-like substance in *M. edulis* pedal ganglia and immunocytes has been demonstrated (Hughes *et al.*, 1990, 1991a,b; Stefano *et al.*, 1991). Recombinant human IL-1 can induce the formation of a TNF-like substance as well as initiate specific immunocyte conformational changes that are interpreted as activation in immunocytes (Hughes *et al.*, 1990). Both the immune and nervous systems of *Mytilus* contain an IL-1-like molecule (Stefano *et al.*, 1991; Paemen *et al.*, 1992). In nervous tissue it is apparently localized in microglial cells (Paemen *et al.*, 1992). Opioid challenge can induce the formation of an endogenous IL-1-type molecule that stimulates immunocytes, as does authentic IL-1 (Stefano *et al.*, 1991b; Paemen *et al.*, 1992). This immunocyte stimulation can be blocked by specific IL-1 antibody. DAMA induced stimulation of the production of an IL-1-like substance is shared by both the immune and nervous systems. In *Mytilus*, recombinant human IL-6, although not activating cells directly, potentiated IL-1 activation of immunocytes (Hughes *et al.*, 1991c). Furthermore, an irIL-6 appears to be present in *M. edulis* and the insect *Leucophaea* hemolymph (0.82 ng/mL; Hughes *et al.*, 1991c). It was also found that irIL-6 is produced in pedal ganglia in response to the pharmacological challenge by the Met-enkephalin analogue DAMA. These data also imply that immune signal molecules may have functions that transcend immunomodulation.

Conservation of Signal Systems

Why should these signaling molecules and their apparent systems/mechanisms be retained relatively intact during the course of evolution? In order to answer this question, we must briefly review some basic principles of intercellular signaling. It should also be noted at this time that the same may apply to intracellular signal systems. Major requirements of a compound to be established as a signaling molecule, be it as a neurotransmitter, hormone or cytokine, are (1) presence of the molecule

in a particular cell, (2) its release from that cell upon appropriate stimulation, (3) high affinity, stereospecific binding to a receptor on the target cell, (4) a specific, physiological effect of the molecule on the effector cell, (5) a specific inactivation mechanism and (6) lastly its expressed genetic message.

In peptidergic signal systems, these characteristics are directly gene determined. The enzymes that synthesize and process such signal molecules must be present in their cells of origin and the information to produce these enzymes resides, obviously, in the DNA of the cell. This is also true of the stereoselective receptor molecules found on the target cell as well as all stereoselective components of a given intercellular communication system. The entire sequence of events, from synthesis of the signal molecule to its inactivation, is based on sequential stereospecific events, including in another cell, receptor recognition. Therefore, the components of the system had to evolve simultaneously in order for the system to be operational. Compatible structural conformations had to be found in the synthesizing enzymes, the signal molecule, the receptor molecule, and the inactivation enzymes. Such conformational "matching" of molecules within each signal system is difficult and time consuming, on an evolutionary level, to achieve. In addition, to be operational within an organism, all the components had to be expressed simultaneously. Thus evolutionary changes had to occur on the corresponding genes of the components within a particular signal system if large-scale changes were to take place. The conformational complexity and rigidity of the "match" among the sequential components for a given signal system would thus seem to exert a determining influence during evolution to maintain the conformational integrity of the signal system given the degree of difficulty in obtaining it originally (Stefano 1986,1991). Thus, "ancient" communication systems, e.g., opioid, opiate and cytokine, would tend to remain relatively intact in increasingly complex animal phyla, especially the structure or conformation of the bioactive portions of the molecules themselves.

This principle of conservation does not preclude events that may lead to an old signal system being used in a new functional capacity. In summary, the determining force during evolution which appears to maintain signal systems may well be the number of highly precise stereospecific conformational matching events associated with intercellular communication mechanisms. The list of "mammalian type" signaling molecules in "simpler" organisms is steadily growing (see Stefano, 1982, 1991,1992; Scharrer, 1991). Taken together, the evidence indicates that signal effector and receptor communication mechanisms may be present in unicellular organisms. This in turn suggests that the origin of signal systems may have occurred during prokaryotic development. Indeed, many of these systems may have started out as intracellular communication mechanisms. Thus, the term neuropeptides, may be totally erroneous, even in vertebrates since many serve and are found in locations other than neural tissues, i.e. immune.

The conservative characteristic of signal system evolution also can be noted in the basic structure of neurons and of nervous systems in general. All neurons can generate action potentials and then propagate them to a point where they are coupled to a secretion event. Nervous systems in total are built around three aspects of function: sensory, integrative and motor. Endocrine systems also have similar characteristics. If anything, there is a basic theme in all systems that is modified to be distinctive.

In summary, the present data indicates that invertebrate organisms have many signal systems in common with vertebrates. In this regard, many of these systems have therefore, been developed in invertebrates. If we consider the fact that all organisms age, these systems merely delay the onset of aging until DNA is passed on. Thus, even the strategy of "longevity" would have to be the same in all organisms. The extent to which these similarities manifest themselves may be related to the organisms life span, i.e., rapid. These signal system commonalities may be maintained between organisms by conformational matching which becomes a determining force and not a selective force in establishing a basic signal system that can be enriched as time goes on.

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Chapter 5

A Lectin from the Tunicate *Clavelina picta* is a Homologue of the Mannose-Binding Proteins from Vertebrates

Gerardo R. Vasta[1], Michael S. Quesenberry and Hafiz Ahmed

Center of Marine Biotechnology
University of Maryland Biotechnology Institute
701 E. Pratt Street, Baltimore, Maryland 21202, USA

[1] Corresponding Author: Gerardo R. Vasta

ABSTRACT

In vertebrates, some C-type lectins such as the plasma mannose-binding proteins, are directly involved in attacking putative microbial pathogens both by opsonization and by complement fixation, in those innate defense mechanisms that are considered part of the acute-phase response to infection. We have purified and biochemically characterized several lectins from plasma of the tunicate *Clavelina picta*. Among those, a homodimeric Ca^{2+}-dependent, L-fucose (Fuc) -binding lectin exhibits a carbohydrate recognition domain that partially shares structural (and functional) motifs with the serum mannose-binding protein (MBP) from rat. Among those motifs are the cysteines that participate in the formation of disulfide bonds and the tri-peptide GluProAsn considered to participate in Ca^{2+}-coordination and in the determination of mannose (Man) specificity in the MBPs. This diagnostic tripeptide GluProAsn has not been found in any of the other invertebrate C-type lectins described so far. The apparent differences in specificity between the tunicate lectin and the MBPs can be explained by the fact that both Man and Fuc display equatorial hydroxyls on contiguous carbon atoms of the pyranoside ring, which are the structural determinants recognized by the lectins' CRDs. Mannose-binding proteins, together with pentraxins such as the C-reactive proteins and amyloid P, and clotting factors such as the proclotting factor VIIIc, very likely represent the most ancient form of non-self recognition/defense function that has evolved, through the lineages that gave origin to the chordates, into the vertebrate acute phase response.

INTRODUCTION

Despite the fact that invertebrate lectins were proposed many years ago as mediators of invertebrate recognition/defense mechanisms (Vasta and Marchalonis, 1983), only in the past few years has a central role in invertebrate immunity has become widely recognized. In vertebrate species, lectins directly participate not only in recognition of foreign substances but also display effector functions as opsonins, complement activating factors, or toxins. In addition to directly

recognizing non-self substances, vertebrate lectins also participate indirectly in inflammation and immunity as lymphocyte homing receptors, neutrophil and platelet adhesion molecules or inducing cell activation. Several of these recognition and regulatory functions carried out by C-type lectins and pentraxins are part of the acute phase response to infection, which is probably the most ancient form of non-self recognition/defense (Vasta *et al.*, 1994). Carbohydrate-binding molecules may represent products of very diverse evolutionary histories. Nevertheless, shared structural motifs that have emerged from the analysis of the carbohydrate binding domains (Drickamer, 1988) suggest that regions of their amino acid sequence relevant to their biological roles as recognition and effector factors have been conserved in evolution. The C- and S-type structural motifs in the carbohydrate recognition domains (CRDs) represent well characterized examples of conserved residues that are critical to both structure and function. It is also noteworthy that domains homologous to those present in members of the immunoglobulin superfamily are found in vertebrate and invertebrate lectins (Suzuki *et al.*, 1990; Powell and Varki, 1994; Vasta *et al.*, umpublished). The development of an immunoglobulin-mediated immune response endowed with memory, however, constitutes a relatively late acquisition in the evolution of chordates. Nevertheless, severe immunodeficiencies in childhood originated by defective point mutations of the Man-binding receptor clearly illustrate the critical role still played by lectins in the mammalian internal defense mechanisms against microbial pathogens (Super *et al.*, 1989).

ANIMAL LECTINS MAY DIRECTLY OR INDIRECTLY MEDIATE DEFENSE MECHANISMS

In the past few years it has become evident that in animals biological processes encompassing cell-cell or cell-extracellular matrix interactions, glycoprotein trafficking, fertilization, transmembrane signal transduction and inflammation are at least in part mediated by lectins. Lectins are present in body fluids or at the cell surface, as integral or adsorbed components, and can recognize endogenous or exogenous ligands. Unusual carbohydrate moieties present on tumors or cells infected by pathogens (virus or bacteria) may constitute modified endogenous structures that are recognized as non-self by lectins. In addition to acting directly as non-self recognition molecules in the acute phase response of vertebrates, some humoral lectins such as the MBPs, conglutinin, lung surfactant apo-protein and pentraxins, function as effector factors by promoting phagocytosis and activating the complement system (Drickamer and Taylor, 1993; Tennent and Pepys, 1994). Other lectins, such as the NK cell receptors, selectins, and macrophage and lymphocyte receptors have been proposed to indirectly participate in defense mechanisms by mediating the recognition of pathogen-infected or tumor cells, or the targeting of cells or substances to the appropriate compartments at the time of stress or challenge. Finally, although there is still little evidence about their biological role, structural similarities with molecules that are involved in immune mechanisms or location on surfaces of phagocytic cell populations, suggest that some I-type lectins such as CD22, and galectins such as the Mac2, may be directly or indirectly involved in recognition of non-self determinants in pathogens or tumor cells.

In general, soluble or membrane animal lectins are oligomers of equal or distinct peptide subunits that can be covalently or non-covalently bound to each other. Like most type II transmembrane proteins, the subunits of integral membrane lectins are characterized by the presence of a short transmembrane hydrophobic domain that anchors the protein to the lipid bilayer. The carboxyl-terminal domain of variable length, extracellularly disposed, carries the CRD and putative glycosyla-

tion sites, whereas the intracellular amino-terminal segment may exhibit putative phosphorylation sites. In the less frequent Type I transmembrane lectin subunits, the overall orientation of the polypeptide chain is reversed, with the carboxyl-terminal region of the molecule constituting the cytoplasmic domain. Soluble lectin subunits differ from integral membrane lectins in that hydrophobic residues are distributed throughout the polypeptide sequence without forming a transmembrane domain. The carboxyl-terminal region usually contains the CRD whereas remaining portions of the molecule may exhibit domains with variable structure. Based on amino acid sequence similarities, particularly in the carbohydrate binding site, overall domain organization, and properties such as divalent cation dependence and requirement of free thiols, Drickamer (1988) identified two major groups of animal lectins: the C- and S-types. In the following years, as more amino acid and nucleotide sequences, and three-dimensional structures become available, animal lectins have been further classified in a number of groups and sub-groups that reveal not only the existence of families or at least some structural (and possibly functional) patterns but also that very distinct, and probably unrelated, groups of carbohydrate-binding molecules are included under the term "lectin" (Drickamer, 1988; Caron *et al.*, 1990; Vasta, 1990, 1992; Harrison, 1991; Kornfeld, 1992; Hirabayashi and Kasai, 1993; Drickamer and Taylor, 1993; Barondes *et al.*, 1994b; Powell and Varki, 1995; Vasta and Ahmed, 1995). These additional lectin groups or families include the P-type, I-type, pentraxins and the heparin-binding. We describe below those lectin groups that are directly or indirectly involved in internal defense mechanisms.

The identification of the C-type lectin family is based on a sequence motif conserved in an approximately 120 amino acid Ca^{2+}-dependent carbohydrate-recognition domain (CRD) (Drickamer, 1988). The sequence motif comprises a set of invariant and highly conserved amino acids (approximately 15% of the CRD), including those cysteine residues involved in disulfide bonds that are required to remain intact for binding activity. The set of amino acids defining the motif may vary somewhat depending on the set of CRDs which are chosen for alignment, and subgroups of CRDs with greater or less similarity can be identified, as was done to identify sequences associated with Man- vs. galactose (Gal) -preferring CRDs (Drickamer, 1992) The CRDs are most frequently contained within larger polypeptides comprising mosaic proteins which include multiple domains. Examples include soluble lectins directly involved in defense mechanisms, such as the serum MBPs and the Man receptor from macrophages, and other soluble or integral membrane lectins, such as the liver asialoglycoprotein receptors, the selectins and the lymphocyte receptor for the Fc portion of IgE (Drickamer, 1993) as discussed in further detail below.

A unique feature of the I-type lectins, a recently identified family (Reviewed by Powell and Varki, 1995), resides in their membership in the immunoglobulin superfamily exhibiting a characteristic V1-C2n domain structure (Crocker *et al.*, 1994). All members of this group, that includes CD22 and sialoadhesin, are transmembrane proteins, some with large cytosolic domains having multiple phosphorylation sites, and several extracellular domains. CD22, a cell surface phosphoglycoprotein present on resting mature B cells, has seven extracellular domains with the binding region in the first two domains (Engel *et al.*, 1995) and recognizes sialylated moieties (Powell *et al.*, 1993). This receptor would facilitate antigen-dependent B cell triggering by association with the B cell antigen receptor and with cytoplasmic tyrosine kinases (Leprince *et al.*, 1993), but it may also recognize ligands on activated lymphocytes, monocytes and endothelial cells promoting intercellular adhesion (Engel *et al.*, 1993).

Vertebrate pentraxins, such as C-reactive protein (CRP) and serum amyloid P (SAP), exhibit lectin-like properties, i.e. binding to carbohydrates and related structures, divalent cation dependence, biological functions and overall molecular structure (Baltz *et al.*, 1982; Hind *et al.*, 1985; Kilpatrik and Volanakis, 1985; Tennent and Pepys, 1994) and have been incorporated as an additional group of animal lectins (Vasta, 1990; Drickamer and Taylor, 1993; Powell and Varki, 1995). Like members of the C-type family, some pentraxins behave as acute phase reactants, rapidly increasing their plasma concentration up to 1000-fold or more in response to stress, injury or infection (Marchalonis and Edelman, 1968; Baltz *et al.*, 1982; Liu *et al.*, 1982). Pentraxins constitute a highly conserved family and distant species exhibit a considerable level of structural similarity (Baltz *et al.*, 1982). CRPs share common features independently of the source species, such as the size of the subunits (20,000-30,000) and the presence of Ca^{2+}-dependent phosphocholine- (PC) binding sites. Pentraxins share some properties with lectins: In addition to the PC-binding properties, CRP binds and precipitates galactans (Baltz *et al.*, 1982), fungal extracts (Baldo *et al.*, 1977), and carageenan gums (Liu *et al.*, 1982) in a Ca^{2+}-dependent manner. SAP binds the pyruvate acetal of Gal and has been considered a vertebrate serum lectin that modulates immune responses (Linn *et al.*, 1984).Some invertebrate lectins are homologues of the vertebrate pentraxins (Vasta, 1990). The best characterized example is limulin, the sialic acid-binding lectin from the American horseshoe crab *Limulus polyphemus* (Nguyen *et al.*, 1986). The three subunits *of L. polyphemus* lectin show homology (approximately 25%) to human and rabbit CRP, SAP, and hamster female protein, all mammalian pentraxins that show extensive homology to each other. DCL-I, a lectin isolated from the tunicate *Didemnum candidum* (Vasta *et al.*, 1986a, 1986b; Vasta and Marchalonis, 1986), may be structurally related to a mammalian CRP. The *L. polyphemus* CRP has been proposed to participate, together with an α-macroglobulin-like protein also present in plasma, in a lytic system (Armstrong *et al.*, 1993) that may be involved in internal defense, and that may differ substantially from the mammalian complement system to which it may be equivalent from a functional standpoint.

Cytokines such as IL-Ia, IL-2 and TNF exhibit lectin properties (Muchmore and Decker 1987; Sherblom *et al.*, 1988,1989) but have not been classified within the major lectin categories indicated above. IL-2, a cytokine that binds with high affinity to glycoproteins, such as uromodulin, and to yeast mannans, shares 27% of the primary structure of the CRD with the MBPs (Sherblom *et al.*, 1989). IL-2 is specifically inhibited by diacetyl chitobiose and high Man glycopeptides, and recognizes the core structure of N-linked oligosaccharides and together with IL-1a (Muchmore and Decker, 1987) and TNF (Sherblom *et al.*, 1988), that have been shown to exhibit carbohydrate binding activity towards N-linked oligosaccharides, should also be considered mammalian lectins involved in internal defense (Sherblom *et al.*, 1989).

Galectins (Barondes *et al.*, 1994a) [Soluble ß-galactoside-binding lectins (Barondes, 1984),"S-type lectins" (Drickamer, 1988) or "galaptins" (Harrison and Chesterton, 1980)], bind ß-galactosyl residues and require a reducing environment, but not Ca^{2+} or other divalent cations, for binding activity. Their location is mostly intracellular, in the cytoplasmic compartment. These lectins exhibit considerable similarities in the primary structure and exhibit a pattern of conserved amino acid residues in the CRDs (Drickamer, 1988). The conserved residues are different from the C-type lectins and do not include cysteines (Liao *et al.*, 1994). Some galectins, although not transmembrane proteins, may be present on the cell surface and have been postulated to mediate cell-cell or cell-intercellular matrix interactions in developmental processes (Mecham *et al.*, 1989; Woo *et al.*,

1990; Zhou and Cummings, 1990; Cooper *et al.*, 1991; Harrison and Wilson, 1992). In tumor cells, galectins may be involved in cell adhesion and metastasis. However, the Mac-2 antigen, one of the best characterized S-type lectins and recently included in the galectin-3 group (Barondes *et al.*, 1994a), was found on the surface of murine macrophages (Cherayil *et al.*, 1989) and proposed to be involved in cell adhesion, inflammation and metastasis (Barondes *et al.*, 1994b). The Mac-2/εBP was shown to enhance IL-1 production by monocytes suggesting a role of this lectin in potentiating activities of inflammatory cells and thereby amplifying inflammatory responses (Jeng *et al.*, 1994).

VERTEBRATE C-TYPE LECTINS PARTICIPATE IN INTERNAL DEFENSE FUNCTIONS

C-type lectins may exhibit little or no homology at all in domains other than the carbohydrate recognition site. These additional domains may consist of (a) fibrillar collagen-like structures that may activate complement, (b) structures similar to those in the core protein of proteoglycans, that interact with glycosaminoglycans, (c) domains similar to epidermal growth factors or (d) structures similar to those proteins that bind RNA (Drickamer, 1988) Those domains confer specific functional properties to each particular lectin such as hydrophobic domains that anchor the protein to the plasma membrane, complement binding sites, areas for covalent binding of glycosaminoglycans, etc., resulting in structural and functional mosaic or chimeric molecules (Drickamer, 1991a, 1993).

C-type lectins with similar domain organization can be collectively subclassified into separate groups based on mosaic structures held in common and their gene organization (Drickamer, 1993; Bezouska *et al.*, 1991). Group I includes the cartilage, fibroblast and brain proteoglycan core proteins, aggrecan, versican and neurocan respectively, with specificities for Gal and Fuc. Group II comprises integral membrane proteins such as the hepatic asialoglycoprotein receptors, the Kupffer cell receptor and the IgE-Fc receptor, CD23 (Drickamer and Taylor, 1993). These lectins are specific for Gal/Fuc, Gal/N-acetylgalactosamine (GalNAc) or GlcNAc residues. Most of the CRD coding regions characterized for lectins in these first two groups contain introns. Group III includes the so called "collectins", lectins that are constituted by a CRD connected to collagen-like sequences and here are included the MBPs from serum and liver, the pulmonary surfactant protein SP-A and the bovine conglutinin. Sugar specificities in members of this group include Man, Fuc, GlcNAc and Gal. Group IV is constituted by the membrane cell adhesion molecules known as "selectins", previously known as "LEC-CAMs" (lectin-epidermal growth factor-complement homology-containing cell adhesion molecules), mosaic molecules specific for sialic acids and Fuc, among which Mel-14 and LSM-1 (lymphocyte homing receptors or L-selectins), GMP-140 (Platelet granule membrane protein or P-selectin) and ELAM-1(Endothelial cell-leukocyte adhesion molecule or E-selectin) are the best characterized. Group VII includes 'isolated' CRDs such as those from pancreatic stone protein. The final group termed 'phagocytosis receptor' has as its single known representative the Man-macrophage receptor, which is unique for containing multiple tandem repeats of CRDs within a single membrane-tethered polypeptide. A brief description of those examples that may be directly or indirectly involved in defense mechanisms follows.

Soluble C-type lectins that exhibit collagenous regions linked to the C-terminal sugar binding domain, that are found in body fluids of mammals have been recently classified as "collectins" (Holmskov *et al.*, 1993) and include the MBP (Drickamer *et al.*, 1986; Drickamer, 1988) and conglutinin (Friis-Christiansen *et al.*, 1990) from serum, and the pulmonary surfactant (Lu *et al.*, 1992). The serum MBP, an acute phase response reactant (Ezekowitz *et al.*, 1988) synthesized in the liver, is the best characterized member of this group. These lectins are multimeric proteins (~350 kDa) with a subunit size of about 32 kDa and their function is related to internal defense against viruses (Ezekowitz *et al.*, 1988; Anders *et al.*, 1990) and bacteria (Kawasaki *et al.*, 1989). They not only bind to Man but other monosaccharides as well including Fuc, ManNAc, GlcNAc, glucose (Glc), Gal and others (Holmskov *et al.*, 1993). The gene structure of its subunit indicates that it is a mosaic polypeptide constituted by a signal peptide plus the domains described below, and the presence of stress-response promoter regions suggest that the serum levels are increased upon contact with pathogens (Taylor *et al.*, 1989).

Macrophages and hepatic endothelial cells, also exhibit membrane Man-binding receptors of the C-type that can directly recognize carbohydrate moieties present on viral, bacterial, fungal or protozoan pathogens and mediate their phagocytosis (Ezekowitz *et al.*, 1991; Chakraborty and Das, 1988) through exposed Man, Fuc and GlcNAc residues that are not normally present in host tissues or soluble glycoconjugates (Stahl, 1990). Therefore, they play a role in internal defense. In addition to binding exogenous glycoconjugates, these receptors can bind endogenous ligands such as lysosomal enzymes (Stahl and Schlesinger, 1980) and tissue plasminogen activator (Otter *et al.*, 1991) released during the acute phase response. Like the selectins, the Man receptor is a type I transmembrane protein (Taylor *et al.*, 1990) but the presence of multiple CRDs in a single polypeptide subunit represent a unique feature within the C-type lectins, since the "cluster effect"(Lee *et al.*, 1992) may be achieved without their association as oligomers. In addition to eight CRDs, the amino-terminal portion of the polypeptide contains a cysteine-rich domain and a fibronectin type II repeat of unknown function, but not required for internalization of glycans (Taylor *et al.*, 1992; Taylor and Drickamer, 1993). Another membrane lectin receptor, possibly with analogous functions in non-self recognition, has been identified in placental tissues (Curtis *et al.*, 1992). However, unlike the macrophage Man receptor, this lectin is a type II transmembrane protein. This receptor mediates, *in vitro*, the internalization of HIV through binding to Man present on the envelope glycoprotein gp120, possibly reflecting its biological role (Curtis *et al.*, 1992).

Lymphocytes may also express various surface carbohydrate-binding receptors with C-type CRDs for endogenous ligands. Some receptors are type II transmembrane proteins and are similar to the ones described for phagocytic cells, although their function(s), also related to internal defense mechanisms, may be considerably different: CD23 is a low affinity and Ca^{2+}-dependent IgE receptor on B-cells (Ludin *et al.*, 1987). The ligand on the IgE is likely on the Fc portion, but its nature remains unclear (Bettler *et al.*, 1992; Vercelli *et al.*, 1989; Richards and Katz, 1990). A second ligand for CD23 has been identified as CD21 (Aubry *et al.*, 1992; Pochon *et al.*, 1992) and the interaction would be mediated by Fuc 1-phosphate residues on the latter and complexed IgE (Gordon, 1994).

Natural killer cells recognize their targets by mechanisms that are different from those including rearrangement and T-cell receptors (Shinkai *et al.*, 1992). Type II receptors on natural killer cells such as CD69 (Ziegler *et al.*, 1994), that have been proposed to mediate target cell recognition

functions, are encoded by multigene families and may be also expressed on thymocytes and T-cells. In natural killer cells, these receptors have distal extracytoplasmic C-type, Ca^{2+}-dependent, CRDs and include the genetically linked Ly-49 (mouse) (Yokoyama *et al.*, 1989; Wong *et al.*, 1991; Brennan *et al.*, 1994), NKR-P1 (mouse, rat, and human) (Yokoyama *et al.*, 1991) and NKG2 (human) (Houchins *et al.*, 1991) multigene families. Within each multigene family, members exhibit high similarity throughout the primary structure but only residues present in the CRD (about 25% identity) are shared between families (Wong *et al.*, 1991; Houchins *et al.*, 1991; Yokoyama *et al.*, 1991). In the mouse, Ly-49 (Brennan *et al.*, 1995) represents a multigene family constituted by at least eight distinct genes and members such as Ly-49A and Ly-49C can be expressed by distinct natural killer cell subsets. Target cells are recognized through surface class I major histocompatibility complex antigens (Brennan *et al.*, 1994). The inhibitory properties of sulfated glycans for Ly-49C-mediated cell adhesion suggest that recognition of target cells by this natural killer cell receptor is mediated by protein-carbohydrate interactions.

Selectins (Bevilacqua 1993) are expressed on the surface of both lymphocytes and circulating phagocytic cells, such as neutrophils and monocytes, together with platelets and endothelial cells. The endogenous cell surface ligands recognized, such as the sialyl Lewis X and related structures, contain either sialic acids or sulfate groups, and Fuc (Aruffo *et al.*, 1991; Lasky 1992, 1995; Yuen *et al.*, 1992). Selectins are type I transmembrane proteins with C-type, Ca^{2+}-dependent, CRDs located on the extracytoplasmic amino-terminus of the polypeptide (Kansas *et al.*, 1991), that include additional regions such as epidermal growth factor-like and complement-binding domains. These receptors mediate the rather weak initial phase of adhesion between T-cells, neutrophils, monocytes and platelets and endothelia during inflammatory processes (Lasky 1992). The T-cell homing receptor or L-selectin is involved in the settlement of these cells in peripheral lymph nodes. P-selectins (formerly known as GMP140) are expressed on the surface of platelets and mediate their binding to endothelial cells. Likewise, the endothelial cells express similar receptors, E- and P-selectins, that interact with circulating phagocytic cells in vascular spaces, such as monocytes and neutrophils, facilitating their adhesion and subsequent diapedesis during inflammation (Lasky 1992, 1995; Bevilaqua, 1993).

MANNOSE-BINDING PROTEINS: THE STRUCTURAL BASIS OF THEIR CARBOHYDRATE-BINDING SPECIFICITY

C-type lectins include a subcategory termed 'collectins', exemplified by MBPs. The term collectin arises from the presence of a collagen-like domain and a lectin domain within the same polypeptide. The collectin domain organization observed to date includes an N-terminal cysteine-rich domain, a collagen-like domain, a "neck" or spacer region, and a C-terminal CRD (Drickamer *et al.*, 1986). The homology with complement component C1q found within the collagen-like domain suggests the structural basis of the complement-fixing and opsonic activities found in the molecule. The trimeric assemblage could be understood to derive from the triple-helical associations commonly found among collagen polypeptides. Two different types of molecular organization have been observed among the collectins under electron microscopy (Lu *et al.*, 1990). Among the MBPs and pulmonary surfactant SP-A, the collagen-like portions are bundled together with the CRDs oriented in the same general direction to form a 'bouquet' structure. Conglutinin and surfactant SP-D, in contrast, are gathered at the extreme ends of the molecules associated as oligomers, rather

oligomers, rather than at the collagen-like domains, with the CRDs oriented away from one another. The latter effect causes the higher-order assembly of trimers to appear as a cruciform.

Lectins consisting of associated polypeptides each with a single CRD, as in the case of collectins and asialoglycoprotein receptors, or those that display multiple CRDs in a single polypeptide, such as in the macrophage Man receptor, exhibit increased affinity for ligands exhibiting multiple sugar residues (Lee *et al.*, 1984). This "cluster effect" was demonstrated using multivalent synthetic ligands containing terminal Gal or GalNAc (Lee *et al.*, 1989). This phenomenon was confirmed by demonstrating that isolated CRDs obtained by proteolytic cleavage of the receptor, exhibit reduced affinity for a multivalent ligand (Loeb and Drickamer 1988). However, both valency and geometry of the receptors and their carbohydrate ligands should be important determinants of binding affinity (Taylor *et al.*, 1992; Taylor and Drickamer 1993). From a biological standpoint, it has been proposed that the different forms of clustered CRDs as seen in monovalent or multivalent C-type lectins allows the selection of different types of ligand (Drickamer and Taylor 1993). Clustering of CRDs by association of monovalent subunits such as in asialoglycoprotein receptors may result in increased affinity and optimal binding to the multivalent carbohydrate ligands exposed on desialylated complex oligosaccharides from the plasma glycoproteins cleared by these receptors (Drickamer and Taylor 1993). A linear distribution of CRDs in the macrophage Man receptor would provide the optimal geometry and therefore increased affinity for the diverse linear carbohydrate structures present in microbial surfaces. The presence of multiple CRDs in the Man receptor may provide the flexibility needed to interact with this diversity of foreign ligands (Drickamer and Taylor 1993).

Crystal structures of the CRD portion of the rat serum MBP in both liganded and unliganded form have been reported (Weis *et al.*, 1991, 1992). There is little or no conformational change in the protein observed in the transition from unoccupied to occupied Man-binding site. In fact, the contact between protein and carbohydrate is rather limited, with major interactions between the sugar ring hydroxyls and one of the bound calcium ions occurring in the form of hydrogen-bonding interactions. The calcium changes from 7-coordinate in the unbound form to 8-coordinate in the sugar-binding complex, including displacement of a water molecule forming one of the unliganded hydrogen bonds. Residues relevant to sugar recognition are naturally quite limited. The ability to identity of these amino acids is suggested by the successful alteration of sugar-recognition preference through changing the tripeptide sequence EPN (or GluProAsn in the three-letter amino acid code) in the MBP to QPD (GlnProAsp) in order to obtain Gal-binding preference in the resulting mutant (see below). Furthermore, the amino acids involved in Ca^{2+}-recognition are identified in the crystal structure. There are nine residues either D, N, or E, which contribute interactions to chelate the two Ca^{2+} ions. Only three of the chelating residues are in positions of sequence identity conserved in the C-type CRD motif.

Drickamer has applied the crystal structure-derived information to re-examine the set of amino acids conserved among CRDs with similar monosaccharide specificities, to identify those which are in the best contact position to influence binding (Drickamer, 1992). In this way a limited set of residues was identified to be 'switched' from the sequence EPN associated with Man-binding CRDs to the QPD sequence associated with CRDs having Gal-binding activity. This approach successfully altered the monosaccharide preference of a QPD mutant of the rat serum MBP in the predicted manner. Thus, the contribution of these amino acids to substrate selectivity has been confirmed for one CRD in the set to which the EPN tripeptide common to Man-preferring CRDs is found. Some

limitations of sugar-interactions of this tripeptide are suggested by the observation that the resulting QPD mutant has only a weak affinity for Gal. In fact, the significance of additional contributions is suggested by subsequent studies which improve the strength of the Gal interaction in the mutant by engineering more hydrophobic side chains into the region of the binding pocket (Iobst and Drickamer, 1994). It is important to note that the latter substitutions are adjacent to the critical tripeptide. Overall, this set of experiments confirms the influence of a tripeptide sequence within the circa 120 amino acid-sized CRD in determination of the monosaccharide recognition properties of C-type lectins. This achievement is all the more remarkable in consideration of the limited protein contact made by the sugar ring as seen in the crystal structure, which is perched atop one of the calcium ions, helping to coordinate the calcium through hydrogen bonding interactions with the 3- and 4- 'H's. The importance of the calcium-liganding side chains, as was also indicated in mutagenesis studies (Quesenberry and Drickamer, 1992), is therefore visually evidenced and emphasized in the ternary complex of lectin, calcium, and ligand.

C-TYPE LECTINS IN INVERTEBRATES: HUMORAL AND HEMOCYTE-ASSOCIATED LECTINS FROM THE TUNICATE *CLAVELINA PICTA*

Although the great majority of invertebrate lectins identified so far cannot be placed yet in any of the major lectin groups established for vertebrates lectins, a few *bona fide* members of the C-type lectins, galectins and pentraxins have been identified within the invertebrate taxa. In addition, some protochordate lectins show substantial cross-reactivity with antibodies to immunoglobulin polypeptide chains from elasmobranchs (Vasta *et al.*, 1984) suggesting the possibility that they may be related to immunoglobulin superfamily members, such as I-type lectins.

A number of invertebrate lectins that show significant homology to membrane or soluble C-type lectins from vertebrates have been isolated and characterized. This group includes lectins from the flesh fly *Sarcophaga peregrina* larva (Takahashi *et al.*, 1985), the moth *Bombyx mori* (Kotani *et al.*, 1995) the acorn barnacle *Megabalanus rosa* (Muramoto *et al.*, 1985; Muramoto and Kamiya 1990), the sea urchin *Anthocidaris crassispina* (Giga *et al.*, 1987), the sea cucumber *Stichopus japonicus* (Himeshima *et al.*, 1994) and the tunicates *Polyandrocarpa misakiensis* (Suzuki *et al.*, 1990) and *C. picta* (Vasta *et al.*, 1996). Like most C-type lectins, these proteins share a carboxyl-terminal domain that contains the CRD with a highly conserved set of residues that include cystein and tryptophan. In echinoidin, a lectin from coelomic fluid of the sea urchin *A. crassispina*, the carboxyl-terminal half is similar to the MBPs A and C from rat (35% and 32% identity respectively), the chicken hepatic lectin and the rat asialoglycoprotein receptor (Giga *et al.*, 1987). This would suggest that the carboxyl terminal region of the molecule containing the carbohydrate-binding site is an early development in the evolution of animal lectins (Giga *et al.*, 1987). It also shows homology to the central portion of the lectin from the fly *S. peregrina* (28% identity) (Takahashi *et al.*, 1985) which in turn shows 18% identity with the rat MBPs (Drickamer *et al.*, 1986). The Gal-binding lectin from another echinoderm, the sea cucumber *S. japonicus* is 28.6% identical to echinonectin and exhibits a CRD as well as the Ca2+ binding domain on the carboxyl-terminal region of the polypeptide subunit (Himeshima *et al.*, 1994).

We have isolated four distinct Ca^{2+}-dependent lectins from plasma and hemocytes of the colonial tunicate *C. picta* and designated as CPL-I-IV (Vasta *et al.*, unpublished). Some of these lectins

appear to be mosaic molecules: In addition to the carbohydrate binding site, the multimeric lectin CPL-I exhibits a stretch of amino acid sequence that shows high homology to human proclotting Factor VIIIc, an acute phase reactant synthesized by the liver (Vasta and Pohl unpublished). The lectin CPL-III, a C-type lectin that binds preferentially Fuc but also sulfated glycans and PC (Vasta *et al.*, unpublished) is a covalently-bound homodimer of subunits of approximately 32 kDa. Within the available sequence of CPL-III, we can identify residues which form part of the conserved sequence motif common to all C-type CRDs (Figure 1). Furthermore, the tripeptide sequence EPN, the key primary structural basis for the expected Man/Fuc-binding preference of this is found in CPL III. Restricting the sequence comparison to invertebrate C-type lectins (Figure1), we can examine the distribution of this sugar-preference tripeptide in this collection of CRDs. CPL III is the only CRD in this set which contains the EPN sequence in the relevant position. Only one other CRD has a tripeptide at this position identifiable with either the Gal- or Man-type CRDs, which is BRA-2. BRA-2 contains the sequence QPD, which is associated with those CRDs which prefer Gal. The other CRDs have tripeptide sequences in the critical position which do not match either set.

Examination of CPL III available CRD sequence shows the positioning of three of four critical C (Cys) residues which form a so-far invariant part of the C-type CRD motif (Figure 1, bold). The C residues in CPL III can be seen to have reasonable spacing and positioning relative to the other identifiable residues in the motif. The fourth C should not be present within the length of polypeptide for which sequence data are presently available, and it is therefore reassuring that we do not find that residue in this stretch of polypeptide. It is anticipated that the full-length sequence, when available, will confirm the presence and positioning of the critical fourth C. The three-dimensional structure of rat serum MBP shows the role of this set of two C residues to be to link structural units within the domain through disulfide bonds (Weis *et al.*, 1991). The inner disulfide pair is formed between what corresponds to the first and second C of the available CPL III sequence. In the MBP structure this disulfide bond occurs at the ends of a contiguous stretch of polypeptide forming antiparallel beta sheets. If we presume the presence of an additional C at the N-terminus of CPL III in the proper position, this would be predicted to form a second 'outer' disulfide bond by analogy with the MBP structure. The term outer disulfide bond in this sense does not refer to the three-dimensional structure, but rather means that the residues linked are further apart in the primary sequence of the molecule. In fact, the outer disulfide bond connects the N-terminal a helix and the C-terminal β sheet in the three-dimensional structure of the MBP CRD. So the relative positioning of these C residues is no doubt crucial to maintaining the basic C-type CRD polypeptide fold, and within the known CPL III structure, these residues are present with the required relative positioning (Figure 1).

Among the other residues conservation can be both by identity and type. For the known CPL III sequence, there are nine out of a possible eleven conserved amino acid identities. Of the missing two, one is a E which contacts calcium ion '1' in the crystal structure (numbering based on the structure). The other is the residue N which comes in the WND sequence that comes in the stretch between the inner Cys pair. This is known to contact calcium ion '2' in the MBP structure, which is the calcium in contact with the sugar ring. It can be speculated that other residues within the sequence evolved to make these contacts. This point remains to be investigated by establishing the crystal structure of the CPL III CRD. It is possible to step through (from N- to C- terminus)the nine residues whose identities are conserved in CPL III, to compare the disposition of the MBP

counterparts, as was done above in discussion of the C residues found in CPL III. The first of these is G (Gly) (Figure 1, bold), which in MBP is solvent-exposed. Next in CPL III is W(Trp), the counterpart of which is packed within the hydrophobic core in the MBP structure, against Pro (the next in the CPL III sequence) and W (also found in CPL III). Finally, E (Glu) and D (Asp) residues (Figure 1, bold) correspond to identical amino acids within MBP that contribute interactions with calcium ions '1' and '2'.

```
CPL III          DGMQLGA---FQHWKGY EPN GVGND------GQDCVMAALRDIAAGYETGFWLDSNCGSRA--FYVC
MBP-A            TGGRLT----YSNWKKD EPN DHG-----S--GEDCVTIVDN--------GLWNDISCQASH--TAVC
HFCER            DGSHVD----YSNWAPG EPT SRSQ-------DEDCVMMRGS--------GRWNDAFCDRKLG-AWVC
RASGPR           DGTDYETG--FKNWRPE QPD DWYGHGLGG--GEDCAHFTDD--------GRWNDDVCQRPY--RWVC
MLHR             VGTNKTLTKEAENWGAG EPN NKKS-------KEDCVEIYIKRERDS---GKWNDDACHKRK--AALC
RKCR             DGTPFDYVQSRRFWRKG QPD NWRHGNGE---REDCVHLQ----------RMWNDMACGTAY--NWVC
RPG              DGHSLQ----FEKWRPN QPD NFFAT------GEDCVVMIWHER------GEWNDVPCNYQL--PFTC
HPSAP            DGTPVN----YTNWYRG EPA GRG--------KEQCVEMYTD--------GQWNDRNCLYSR--LTIC
CHL              DGTDTRSS--FTFWKEG EPN NRGF-------NEDCAHVWTS--------GQWNDVYCTYEC--YYVC
RSL              DRSCTD----YLTWDKN QPD HYQN-------KEFCVELVSLTGY-----RLWNDQVCESKD--AFLC

                                   E N       S          N
mannose-type                       Q D W GH  G          D
galactose-type

TUN              DGVSLPTD--SDLWSPN EPS NPQS-------WQLCVQIWSKY-------NLLDDVGCGGAR--RVIC
FLY              PGQAFS----FAYWSEN NPD NYKH-------QEHCVHIWDTKPL-----YQWNDNDCNVKM--GYIC
BRA-2            DDSHSS----HRNWYAT QPD DES---------ELCVLIKEDQY------RQWHDYNCNDRY--NFVC
BRA-3            NGEATD----FTYWSSN NPN NWEN--------QDCGVVNYDTVT-----GQWDDDDCNKNR--NFLC
SUL              DGSPND----FTAWVGS NPD NYGS-------GEDCTQMVMGAG------LNWIDLPCSSTRHY-LIC
SCL              DGSYYD----YQNWCGN DPN AHPDGYGAFSGGSYCN------------GQWVDVHTFTNDQFPFKC
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Figure 1. Comparison of the amino acid sequence of *Clavelina picta* lectin III (CPL-III) with those of mammalian C-type CRDs. HFCER, human lymphocyte IgE receptor (Kikutani *et al.*, 1986); RASGPR, rat asialo glycoprotein receptor-1 (Drickamer *et al.*, 1984); MLHR, mouse lymphocyte homing receptor (Lasky *et al.*, 1989); RKCR, rat Kupffer cell receptor (Hoyle and Hill 1988); RPG, rat proteoglycan core protein (Doege *et al.*, 1986); HPSAP, human pulmonary surfactant apoprotein (Floros et al. 1986); MBP-A, rat mannose-binding protein (Drickamer *et al.*, 1986); CHL, chicken hepatic lectin (Drickamer 1981, 1992); RSL, rattle snake lectin (Hirabayashi *et al.*, 1991); mannose- and galactose-type residues (Drickamer, 1991b); TUN, the tunicate (*Polyandrocarpa misakiensis*) lectin (Suzuki *et al.*, 1990); FLY, the flesh fly (*Sarcophaga peregrina*) lectin (Takahashi *et al.*, 1985); BRA-2 and BRA-3, the acorn barnacle (*Megabalanus rosa*) lectins (Muramoto and Kamiya 1990); SUL, the sea urchin (*Anthocidaris crassispina*) lectin (Giga *et al.*, 1987); SCL, sea cucumber lectin (Himeshima *et al.*, 1994).

Two other positions are similarly correctly filled in CPL III, which can be either aliphatic or aromatic in C-type CRDs. Three positions N-terminal to the first conserved W is a F, and two positions N-terminal to the final conserved C is a Y(Tyr). Both of these meet the criteria for conservation of type of residue found at this position. There is only one position where this is not the case in CPL III. Two positions to the C-terminus of the conserved P falls a G where a side chain capable of

forming a calcium '1' ligand should occur. This `omission' in the CPL III sequence is the second lack of calcium '1' ligand. It is more difficult to envision evolution of the molecule to fulfill two such functional switches, although not impossible. However, it may be more reasonable to infer from the present data that CPL III may lack the calcium `1' binding site, as do selectins. This awaits experimental investigation.

CONCLUSIONS

Mammalian collectins such as the MBPs and conglutinin from serum and the pulmonary surfactant are soluble C-type lectins with collagenous regions linked to a C-terminal sugar binding domain, directly involved in non-self recognition (Drickamer *et al.*, 1986; Drickamer 1988; Friis-Christiansen *et al.*, 1990; Holmskov *et al.*, 1993; Lu *et al.*, 1992). The serum MBPs, acute phase response reactants synthesized in the liver, play a critical role in the internal defense against viruses and bacteria (Ezekowitz *et al.*, 1988; Anders *et al.*, 1990; Kawasaki *et al.*, 1989). They not only bind to Man but other monosaccharides as well, including Fuc, ManNAc, GlcNAc, Glc, Gal and others usually not present or exposed on cell surfaces of the mammalian host (Holmskov *et al.*, 1993). Invertebrates lack the antibody-mediated immunity of vertebrates, characterized by humoral and cell associated immunoglobulins, B and T cells and immune memory. Therefore, humoral and cell-associated lectins and some members of the immunoglobulin-superfamily, such as hemolin, have been proposed as the recognition factors that mediate most invertebrate internal defense mechanisms (Vasta 1991). Like their vertebrate lectin counterparts, lectins from invertebrate species can function not only as recognition but also as effector factors: certain lectins display antibacterial activity (Saito *et al.*, 1995) or promote melanization (Chen *et al.*, 1995). *Bona fide* homologues of C-type lectins and CRP have been described now in invertebrate species (Takahashi *et al.*, 1985; Muramoto *et al.*, 1985; Muramoto and Kamiya 1990; Giga *et al.*, 1987; Himeshima *et al.*, 1994; Suzuki *et al.*, 1990; Vasta *et al.*, 1996) and it has been proposed that these, together with antibacterial peptides, lysozyme, toxins, protease inhibitors and other effector factors, constitute an ancient but efficient defense system, with some components still operative in mammals (Vasta 1991).

Clavelina picta lectin CPL-III contains a CRD with clear homologies to the rat MBP A and other vertebrate collectins. Apparent differences in specificity can be readily explained by similarities in the orientation of hydroxyls critical for recognition by the lectin: the presence of contiguous equatorial hydroxyls in both Man and Fuc. The MBPs activate complement through the classical pathway and hence is similar in function and structure to the C1q, a constituent of complement that binds to immunocomplexes to initiate the activation of the classical pathway (Ikeda *et al.*, 1987). Hence, in addition to direct opsonic effect (Kuhlman *et al.*, 1989), the MBP produces complement-dependent lysis of bacteria. The presence of effector domains, such as those typical of the MBP complement-binding domains described above, in the protochordate lectin CPL-III remains to be investigated. Lectins from *C. picta* recognize a number of bacterial isolates from the environment and those associated with the colony, but also bind to intact sulfated glycans from the colony tunic (Elola and Vasta unpublished). Based on our experimental evidence, we proposed that *C. picta* lectins participate in non-self recognition and defense by agglutinating and opsonizing potentially pathogenic bacteria from the environment, as well as in self recognition and wound repair by binding to Fuc or L-galactose from the non-reducing terminal residues from the tunic glycan exposed to the

plasma and hemocytes upon damage of the body wall. In this aspect *C. picta* lectins are similar to tetranectin, a mammalian lectin that binds to sulfated glycans and to components of the clotting cascade (Clemensen 1989).

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Abbreviations

Abbreviations used are Gal, D-galactose; Glc, D-glucose; Man, D-mannose; Fuc, L-fucose; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; ManNAc, N-acetyl-D-mannosamine; CRP, C-reactive protein, SAP, serum amyloid protein; MBP, D-mannose-binding protein, CRD, carbohydrate-recognition domain.

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Chapter 6

Circadian Neuroendocrine Regulation of Immune Responses

Nicholas J. Nevid

Department of Molecular and Cellular Physiology
University of Cincinnati
Cincinnati, OH 45267-0576

ABSTRACT

Immune processes are temporally integrated with other physiologic and behavioral processes in vertebrates. Based primarily on studies of scale allograft reactions in gulf killifish (*Fundulus grandis*), a general model is proposed for the circadian neuroendocrine regulation of immune responses in vertebrates. According to this model, daily rhythms of immune activities are driven by a neuroendocrine oscillator that is predominantly entrained by nonphotic environmental stimuli. Overall immune responsiveness is influenced by a change in the phase relationships of two or more circadian neuroendocrine oscillators through temporal synergisms of their neural and hormonal expressions. Daily rhythms of cortisol, growth hormone, and prolactin have prominent mediary roles between these neuroendocrine oscillators and the immune system.

INTRODUCTION

The classic texts of Western and Eastern medicine emphasized the importance of daily and seasonal cycles. Hippocrates began one of his books, *Airs, Waters, Places*, by stating that the first subject a medical student should consider is the effects of the seasons and the differences between them (Chadwick & Mann, 1950). In *The Yellow Emperor's Classic of Internal Medicine* (*Huan Ti Nei Ching*, written about 1000 B.C. or earlier), the initial dialogue between the Yellow Emperor and his minister lists rising and retiring in a regular fashion as a key for increased longevity (Veith, 1966). A day-night pattern for fever was described by Hippocrates in *Epidemics, Book I* (section 24) and daily mood cycles were described for patients with liver, heart, spleen, lung, and kidney diseases in *The Yellow Emperor's Classic of Internal Medicine* (chapter 22). It is curious why these cycles, considered by the early physicians to be of fundamental and obvious importance, have been largely ignored by modern medical science.

Because biologic cycles often correspond with celestial events, it is not surprising that an astronomer, a Frenchman named de Mairan, is credited with the first experiment in chronobiology. Using a type of plant that opens its leaves during the day and folds its leaves at night, de Mairan observed that this phenomenon is just slightly less obvious when the plants are confined in a dark place (Marchant, 1729; reproduced in Moore-Ede *et al.*, 1982, page 6). The apparent ability of plants to

sense day and night without being outside or in the sun was related by de Mairan to the sleeping patterns of bedridden patients who were also secluded from the outside world. Numerous studies have since confirmed that daily rhythms of plants and animals persist under experimental conditions wherein suspected temporal cues have been eliminated. Although organisms might still be able to detect subtle geophysical cues when kept in a laboratory, their rhythms usually deviate from a precise 24-hour period under constant conditions. Thus, if allowed to continue for a sufficient number of days, these free-running rhythms eventually become completely out of phase with the external world and the associated cues that normally entrain them. Biologic rhythms that free-run with periods close to 24 hours are called circadian (Latin; circa=approximately, dies=day) and appear to be an endogenous property in organisms.

Individual cells isolated from vertebrates exhibit circadian rhythms *in vitro*. To benefit multicellular animals, these cellular rhythms must be coordinated within and between tissues. The coordination of daily rhythms in mammals (Moore-Ede, 1983; Minors and Waterhouse, 1986; Rossenwasser and Adler, 1986), as well as in other vertebrates (Takahashi and Menaker, 1979; Ooka-Souda and Kabasawa, 1988; Janik *et al.*, 1990), has been linked to hypothalamic cells, called pacemakers or oscillators. Most models of circadian timing systems in vertebrates propose one or two primary oscillators that govern multiple secondary oscillators. The general types of experiments that have provided evidence for separate oscillators are summarized in Table 1. As developed in the following sections of the present paper, interactions between these neuroendocrine oscillators can influence immune capability in vertebrates.

Circadian timing systems represent an adaptive solution of animals to the world in which they evolved. By anticipating the periodic changes of light, temperature, food availability, and the presence of predators in their environment, animals can adjust their daily activities accordingly (Moore-Ede, 1986). Circadian rhythms also play a fundamental role in the reproductive, metabolic, and behavioral changes of vertebrates in different seasons (Meier, 1984; Meier and Russo, 1985). In addition, animals that migrate require a time-compensated mechanism to orient to the constant movement of the sun or stars. Although the underlying mechanisms that generate circadian rhythms remain unknown, the general properties of biologic clocks are similar from simple to complex organisms.

Table 1.
Evidence for multiple neuroendocrine oscillators.*

Type of Experiment	Observation
I.Comparison among seasons	The phases of daily rhythms change relative to each other.
II. Extended free-running conditions.	Some rhythms eventually uncouple and proceed with slightly different periods.
III.Different types of environmental cues provided at different times of day	Simultaneous entrainment of a rhythm to one cue while another rhythm entrains to a second cue.
IV.Sudden phase-shift of an environmental cue	Rhythms reentrain to the shifted cue at different rates

*Specific examples can be found in the reviews by Moore-Ede *et al.* (1982), Rossenwasser and Adler (1986), and Meier (1993).

Investigations of circadian rhythms in gulf killifish and other vertebrates have led to methods for treating intractable human diseases such as maturity onset diabetes, obesity, and atherosclerosis (Cincotta *et al.*, 1989; Meier *et al.*, 1992; Cincotta *et al.*, 1993). Based on those findings, a series of studies were conducted with gulf killifish to examine whether similar neuroendocrine mechanisms regulate immune functions as well. The following review summarizes the results of those and other studies, and then proposes a general model for the circadian neuroendocrine regulation of immune responses in vertebrates. Because this discussion covers an extremely broad field, readers are referred to reviews of circadian timing systems (Bünning, 1973; Moore-Ede *et al.*, 1982; Rossenwasser and Adler, 1986; Meier, 1993), immunologic rhythms (Haus *et al.*, 1983; Lévi *et al.*, 1991; Goulding and Hall, 1993), and neuroendocrine-immune system interactions (Ader *et al.*, 1991; Goetzl and Sreedharan, 1992) for additional background.

REVIEW

Environmental Coupling of Immune Activity Rhythms

Daily rhythms of immune responses against a variety of antigens have been observed in fish (Nevid and Meier, 1993; Michael and Priscilla, 1994), chickens (Skwarlo-Sonta, 1992; Kondo *et al.*, 1992), and many mammalian species including humans (Haus *et al.*, 1983; Lévi *et al.*, 1991). Taken together, these studies provide evidence that nearly all types of immunity, both innate and acquired, undergo predictable daily variations. Although not required for the expression of immunologic rhythms, the light-dark cycle clearly influences the time of day when these rhythms peak or trough. Rhythms of immune activity readjust after a shift of the light-dark cycle (Hiyashi and Kikuchi, 1985) and many investigators maintain animals on staggered photoperiods for convenience in obtaining measurements that correspond to the late night or early morning (Pownall and Knapp, 1980). Daily rhythms of immune function, however, are generally correlated with the diurnal or nocturnal activity pattern of an animal rather than the presence or absence of light per se (Figure 1).

Compared with the modest number of studies that have examined the adjustment of immunologic rhythms to light-dark cycles, fewer studies have examined the adjustment of immunologic rhythms to nonphotic stimuli. Research efforts focused on photoperiodic regulation have been motivated in part by its relevance to jet-lag and shift-work schedules of humans (Hiyashi and Kikuchi, 1985) and by the rearing practices of poultry production (Kirby and Froman, 1991). Daily rhythms of immune function are nevertheless connected with locomotor activity patterns in animals, and several types of nonphotic environmental stimuli might therefore be expected to exert more influence on the phases of immunologic rhythms. Most animals adjust their daily activity to a time of restricted food availability regardless of when the meal occurs during a 24-hour light-dark cycle (Boujard and Leatherland, 1992; Mistlberger, 1994). In addition, many diurnal animals become nocturnal as a behavioral adaptation to altered ambient temperatures. Animals also adjust their activity patterns to avoid predators.

Recent studies of gulf killifish confirm that nonphotic daily stimuli can determine the time of day when immune function peaks (Figure 2). Melanophore destruction during scale allograft rejection is typically 2-3 times greater during the night than during the day in gulf killifish kept on 12-hour

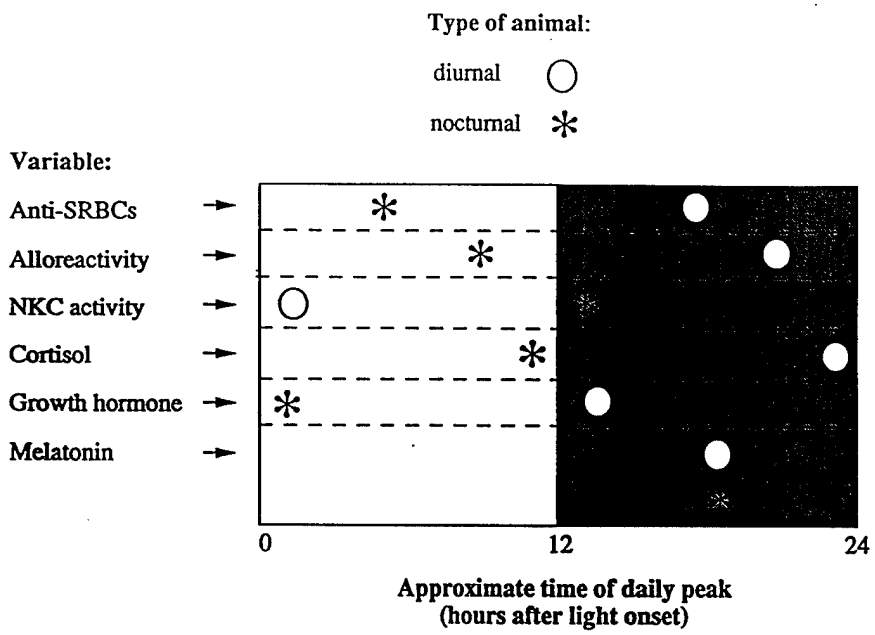


Figure 1. Daily rhythms of immune function are similar among vertebrates when referenced to their daily patterns of locomotor activity instead of the light-dark cycle. Some hormones, like growth hormone and cortisol, are also correlated with locomotor activity, whereas other hormones, such as melatonin, are not. Each point is a rough estimate for illustrative purposes and is based on species that were kept on 12-hour daily photoperiods.

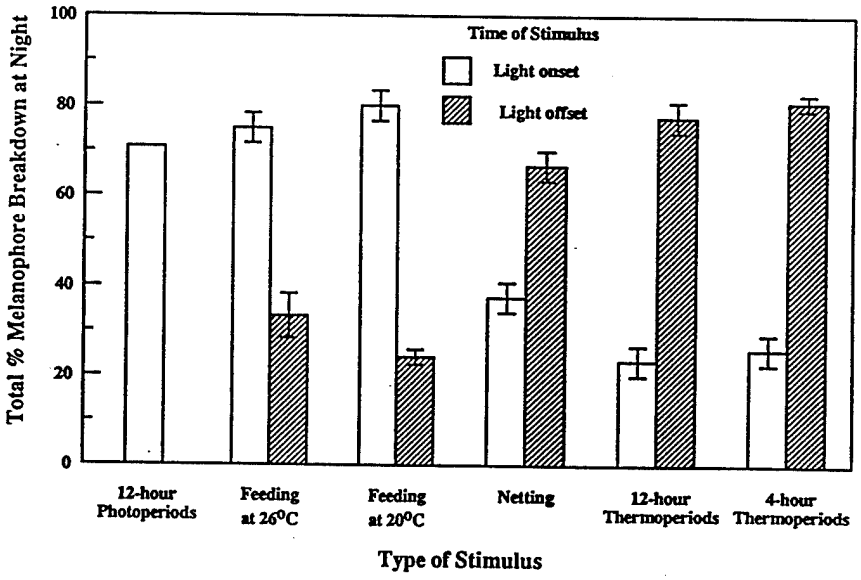


Figure 2. Melanophore breakdown increases each night and decreases each day during scale allograft rejection in gulf killifish maintained under 12-hour daily photoperiods. Nonphotic stimuli applied at specific times of day appear to reverse this daily pattern: melanophore breakdown increases each day (rather than each night) in fish fed a single daily meal at light offset, disturbed daily at light onset, or exposed to a daily increase of temperature from 20°C to 30°C at light onset. Each bar in the graph summarizes the percent of original melanophores (± SEM) that were destroyed during a scotophase over the course of scale allograft rejection (adapted from Nevid and Meier, 1994).

daily photoperiods of about 150 lux (Nevid and Meier, 1993). When gulf killifish are exposed to additional cues at light onset or light offset, however, immunologic rhythmicity does not remain fixed to the light-dark cycle, but instead adjusts relatively quickly to the nonphotic stimulus (Nevid and Meier, 1994). Timed daily feeding, thermoperiods, and disturbances apparently entrain an endogenous oscillation of immune activity in gulf killifish, inasmuch as phase adjustments induced by the nonphotic cues persist for several days after the daily treatments are stopped when gulf killifish are kept under continuous light. Daily rhythms in the number of circulating lymphocytes in mice are also determined by feeding time rather than daily photoperiods (Haus *et al.*, 1983). From these findings, it appears that at least some immunologic rhythms are connected with a neuroendocrine oscillator that is more directly entrained by nonphotic, rather than photic, cues. Because some circadian rhythms preferentially adjust to daily photoperiods and are not reset by nonphotic cues, this oscillator appears distinct from a light-entrained oscillator.

Daily rhythms of the immune activity against sheep red blood cells (SRBCs) in mice readjust at a slower rate than other rhythms following an inversion of the light-dark cycle (Hiyashi and Kikuchi, 1985). Rhythms of immune activity against scale allografts (melanophore destruction) in gulf killifish also appear to readjust relatively slowly to daily photoperiods. When gulf killifish are kept on 12-hour daily photoperiods, netting at light onset for only three days readjusts peak immune activity to the photophase (i.e., 0-12 hours after the netting cue). This altered phase of the immune activity rhythm continues during the photophases for at least three more days after the netting cue is terminated before readjusting to the scotophases (i.e. dark period; Nevid and Meier, 1994). These findings suggest that the phases of at least some immunologic rhythms, which are largely determined by a nonphotically-entrained oscillator, are secondarily influenced by a light-entrained oscillator. When not directly set by nonphotic cues, the oscillator that directly drives rhythms of immune activity adjusts to the light-entrained oscillator.

Such coupling between light-entrained and nonphotically-entrained oscillators may be supported by previously unpublished data from a study of gulf killifish (Figure 3). Immune activity against scale allografts peaks during the photophases when gulf killifish are kept under 12-hour photoperiods and exposed to 30°C during the light and 20°C during the dark (Figure 2; Nevid and Meier, 1994). Daily rhythms of immune activity remain fixed to the scotophases, however, when gulf killifish are exposed to 30°C during the light and 24°C, instead of 20°C, during the dark. Adjustment of this immunologic rhythm may therefore occur abruptly over a relatively small temperature range at night of 20-24°C. This finding emphasizes the functional significance of daily rhythms, since a 24-30°C daily temperature cycle would be expected to have considerable direct effects on the immune activity of an ectotherm.

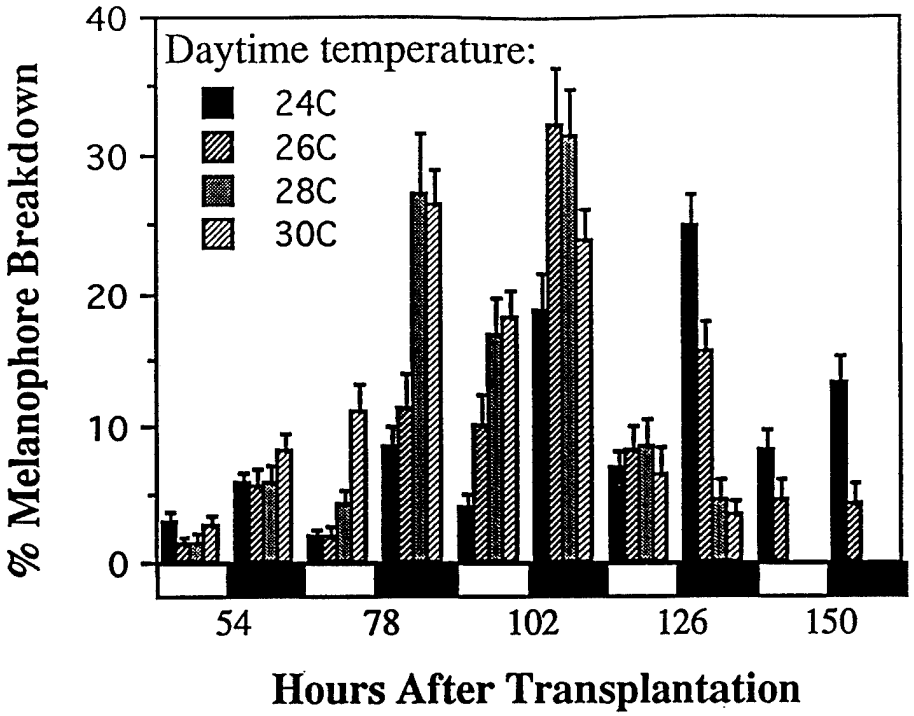


Figure 3. Possible of coupling between a light-entrained oscillator and an oscillator that controls daily rhythms of immune activity. Groups of gulf killifish (6 per group) were maintained on 12-hour daily photperiods, 24°C during the night, and one of four different temperatures during the day for an experiment done in June. Each fish received 6 scale allografts on the day when treatments began. Although scale allografts were rejected faster ($p<0.05$) by fish exposed to 30°C (mean survival time of 5.5±0.1 days; 31 intact grafts) or 28°C (5.6±0.1 days; 26 intact grafts) during the day compared with fish exposed to 26°C (6.8 ±0.3 days; 26 intact grafts) or 24°C (7.2±0.1 days; 34 intact grafts) during the day, all groups exhibited day-night rhythms of immune activity ($p<0.05$) that peaked during the 24°C nights. Because daily thermoperiods of 20°C to 30°C can adjust this daily rhythm (see Figure 2), a threshold for coupling may exist between 20°C and 24°C (materials and methods as in Nevid and Meier, 1994).

Hormonal Coupling of Immune Activity Rhythms

As indicated above, the daily coordination of immunologic rhythms with environmental cycles is mediated by the neuroendocrine system. Since numerous connections exist between the neuroendocrine and immune systems, there may be many neuroendocrine factors that contribute to the daily peaks and troughs of an immune response. The neuroendocrine rhythms that are most important, however, should correlate with to the daily rhythms of an immune response under a variety of environmental conditions and treatments. Although a specific neuroendocrine rhythm that conclusively accounts for an immunologic rhythm is yet to be identified (and may not exist), the hormonal rhythms described below appear closely coupled with at least some immunologic rhythms. The extent to which these neuroendocrine and immune rhythms are internally coupled may reflect their adaptive significance, since environmental conditions or treatments that perturb their temporal relationships usually perturb immune capability as well.

Daily plasma concentrations of glucocorticoids rise just prior to the onset of locomotor activity in most species (Meier, 1975a) and are therefore inversely correlated with the daily rhythms of many immune activities (Lévi *et al.,* 1991). Environmental changes in the onset of light (Hiyashi and Kikuchi, 1985) or the time of food availability (Haus *et al.,* 1983) cause proportionate shifts in both the glucocorticoid and immunologic rhythms. A daily dose of cortisol in small amounts also shifts daily rhythms of immune activity against scale allografts in gulf killifish (Nevid and Meier, 1995a). Fish fed cortisol-treated food at light offset (L:D 12:12) express greater immune activity during the day whereas fish fed cortisol-treated food at light onset, near the anticipated endogenous peak of the steroid, express greater immune activity during the night. These cortisol treatments appear to adjust rather than impose daily rhythms of immune activity in gulf killifish, since the total length of time required to reject scale allografts and the relative differences between day and night immune activities in fish fed cortisol-treated food were equivalent to those of fish fed untreated food. Furthermore, the shifts of immune activity produced by cortisol treatments persist for several days under light-dark cycles after these treatments are stopped (Nevid and Meier, 1995a).

Although glucocorticoids generally inhibit immune function (Dupont, 1988), some immune responses normally peak at a time of day when endogenous levels of this steroid are expected to be high (Lévi *et al.,* 1991). In addition, there is evidence that the inhibitory influences of glucocorticoids do not directly account for the daily rhythms of immune activities that normally peak at opposite times of day from the peak glucocorticoid levels. Corticosterone levels and immune responses against SRBCs in mice readjust at different rates to an inversion of the light-dark cycle, resulting in a few days when elevated corticosterone levels coincide with elevations in the antibody response rhythm (Hiyashi and Kikuchi, 1985). In gulf killifish, immune activity is maximally expressed 0-12 hours after daily handling disturbances (Nevid and Meier, 1994). Although timed daily stress does not appear to entrain daily rhythms of corticosterone in rodents (Ottenweller *et al.,* 1987), such treatments directly cause elevation of plasma corticosterone levels (Kant *et al.,* 1986). Thus, immune activity in gulf killifish can peak when glucocorticoid levels are expected to be high. Stress also elevates growth hormone and prolactin which counteract the suppressive effects of glucocorticoids (Chatterton *et al.,* 1973; Kelly and Dantzer, 1991; Sandi *et al.,* 1992).

Plasma levels of growth hormone in humans rise abruptly about two hours after sleep onset and are extremely low at most other times of day (Weitzman, 1976). Highest growth hormone levels also occur during rest in both nocturnally active rats (Moberg *et al.,* 1975) and diurnally active fish (Leatherland *et al.,* 1974). These temporal patterns make growth hormone an attractive candidate as a factor that drives immunologic rhythms because growth hormone is generally considered to be a positive stimulus for immune function (Gala, 1991; Kelly, 1991) and many immune activities peak while an animal is resting. In support of a direct relationship between growth hormone and immunologic rhythms, timed daily injections of growth hormone drive daily rhythms of immune activity in gulf killifish exposed to continuous light (Nevid and Meier, 1995a); melanophore breakdown in scale allografts is greatest 0-12 hours after each hormone treatment whether it occurs during a subjective morning a evening of continuous light.

It is important to note that growth hormone affects immune activity differently in gulf killifish kept under daily photoperiods compared with continuous light (Nevid and Meier, 1995a). Growth hormone treatments at the offset of 12-hour photoperiods, near the anticipated peak of the endogenous hormone, have no effect on either the phase of the allograft rejection rhythm or the

duration of allograft survival compared with saline-injected controls. In contrast, growth hormone treatments at light onset increase immune activity during the day without apparently diminishing immune activity at night, resulting in significantly shorter allograft survival times compared with saline-injected controls. These observations suggest that a daily rhythm of endogenous growth hormone levels (or of other such stimulants for immune function) might account for the daily rhythm of allograft rejection in untreated gulf killifish, since immune activity does not appear to be refractory to the stimulatory influence of growth hormone during the day.

Although daily rhythms of prolactin have been directly correlated with ornithine decarboxylase activity (a measure of cell proliferation and differentiation) in rat thymocytes (Neidhart, 1989), other findings cast doubt on the role of prolactin as a primary driving stimulus for at least some immunologic rhythms. Daily rhythms of prolactin are highest during sleep in humans (Weitzman, 1976) and can be adjusted by light-dark cycles or ambient temperature in fish (Spieler, 1979), but the relatively modest influences of timed feeding on daily rhythms of prolactin (Bellinger *et al.*, 1975; Spieler *et al.*, 1977) contrast with the abilities of timed feeding to adjust daily rhythms of circulating lymphocytes (Haus *et al.*, 1983) and allograft rejection (Nevid and Meier, 1994) in rodents and fish. Timed daily injections of prolactin influence daily rhythms of the immune response against SRBCs in chickens (Skwarlo-Sonta *et al.*, 1987), but the results of that study provide convincing evidence that the phase of the antibody response rhythm was not directly determined by the timing of prolactin treatments. The more likely role of prolactin, a synergistic factor that has time-dependent effects on immune capability, is discussed below.

Temporal Synergisms and Immune Capability

Daily rhythms of immune activities are correlated with several hormonal rhythms. Under some environmental situations, however, these rhythms appear to become uncoupled with respect to each other in animals. In most cases, such environmental conditions also affect the length of time required by animals to resolve immunologic challenges. In addition, daily rhythms are altered during times of decreased immunocompetence, such as aging (Brock, 1991) and AIDS (Villette *et al.*, 1990; Malone *et al.*, 1992). Changes in immune capability may therefore reflect altered phase relationships between daily rhythms.

Studies of gulf killifish support the idea that interactions between daily rhythms influence immune capability. Daily thermoperiods, single meal feedings, and disturbances have time-dependent influences on the ability of gulf killifish to reject scale allografts (Nevid and Meier, 1995b). Survival times of scale allografts differ by 30% or more when nonphotic stimuli are administered at a most stimulatory time relative to light onset compared with a most inhibitory time. These effects are interpreted as resulting from an interaction between daily rhythms entrained by photic stimuli and other rhythms entrained by nonphotic stimuli. Interactions between these rhythms may stimulate or inhibit immune capability depending on the relationship between their phases.

Interactions between daily rhythms can be understood in terms of stimulus and response rhythms. From this viewpoint, immune responses are greatest when the daily peak of a stimulus rhythm (e.g., an immunostimulatory hormone) coincides with the daily peak of a response rhythm (e.g., receptor numbers on lymphocytes). Other phase relationships between the stimulus and response rhythms suppress immune responses to various degrees. Photoperiodism, for example, involves stimulus

and response rhythms in both plants and animals. As originally put forth by Bünning in 1936, the onset of a photoperiod sets up a rhythm of photosensitivity that peaks at least 12 hours later. Light coincides with this time of high photosensitivity during long daylengths (but not short daylengths), which can elicit photoperiodic effects (Bünning, 1973). It is important to note that photoperiods influence the relationship between stimulus and response rhythms within the organism (i.e., an internal coincidence) and that the coincidence of light with a rhythm of photosensitivity by itself (i.e., an external coincidence) is not sufficient to produce an effect (Meier and Russo, 1985).

A few studies have directly examined photperiodic influences on the immune system. Short photoperiods that decrease testicular weight in hamsters cause an increase in spleen weight (Brainard *et al.,* 1987). In chickens, long photoperiods enhance the proliferative responses of peripheral lymphocytes to Con A (Mashaly *et al.,* 1988), but chickens raised under continuous light produce lower antibody titers against SRBCs and have reduced delayed hypersensitivity responses compared with chickens reared under 12-hour daily photoperiods (Kirby and Froman, 1991). This latter observation may reflect the immunologic consequences of disorganization among daily rhythms, which is likely to occur over time when animals are maintained in an aperiodic environment, and reinforces the concept that photoperiodic influences on the immune system involve more than a coincidence of light with a rhythm of photosensitivity.

Photoperiodic influences depend on the seasonal condition of the animal (Meier, 1993). Many birds, for example, are reproductively, metabolically, and behaviorally (e.g., migratory activities) stimulated in the spring by long daylengths, but not in the late summer or fall when daylengths are as long or longer (Meier and Russo, 1985). In Syrian hamsters, short daylengths inhibit the reproductive system in the fall and early winter and then permit regeneration of the reproductive system when hamsters are kept on these short daylengths for 20 more weeks (Wilson and Meier, 1989). A change in the phase relationships between two circadian oscillators has been proposed as the basis for seasonal conditions in vertebrates (Meier, 1993). One of these oscillators has a serotonergic component and is expressed as a daily rhythm of plasma cortisol, while the other oscillator has a dopaminergic component and is expressed as a daily rhythm of plasma prolactin. The phase relationships between serotonin and dopamine rhythms in the suprachiasmatic nuclei (a primary circadian oscillator) differ in hamsters that are reproductively stimulated or inhibited by short daylengths (Wilson and Meier, 1987). In addition, timed daily injections of 5-hydroxytryptophan (a precursor of serotonin) and L-dihydroxyphenylalanine (a precursor of dopamine), or of cortisol and prolactin, in appropriate phase relationships can produce predictable seasonal conditions in Syrian hamsters, white-throated sparrows, and gulf killifish (Meier and Wilson, 1985).

These mechanisms may relate to the seasonal differences of immune responsiveness that have been observed in many vertebrate species including humans (Haus *et al.,* 1983; Lévi *et al.,* 1991; Zapata *et al.,* 1992). Daily injections of prolactin affect immune responses differently depending on the time of day when this hormone is administered to chickens (Skwarlo-Sonta, 1992) or mice (Cincotta *et al.,* 1995). Immune responses in mice are also influenced differently depending on the time of day when serotonergic or dopaminergic agents are administered, and these agents appear to act indirectly in part through their effects on endogenous prolactin levels (Cincotta *et al.,* 1995). Prolactin receptors are present on many cells of the immune system, including B cells, T cells, and monocytes (Pellegrini *et al.,* 1992; Gagnerault *et al.,* 1993), and may therefore represent targets for an important rhythm that influences immune capability. In addition, prolactin has time-dependent

effects on a variety of physiologic and behavioral conditions in vertebrates (Meier, 1975b), and may therefore serve a more general role in coordinating the immune system with other systems.

Whereas dopaminergic and serotonergic influences on immune function appear to be related with daily rhythms of prolactin (Cincotta *et al.*, 1995), the mechanisms by which other neural activities influence immune function are less clear. Propranolol (a β-adrenergic antagonist) or naloxone (an opioid antagonist) injections at light offset prevent the nighttime peak of immune activity (melanophore destruction) and prolong allograft survival in gulf killifish, whereas injections of the same agents at light onset are ineffective (Nevid and Meier, 1995a). Treatments of gulf killifish with a variety of adrenergic agonists (ephedrine, isoproterenol, or salbutamol) at light onset, however, did not enhance immune activity during the day and had no effect on allograft survival times (Nevid and Meier, unpublished observations). Timed administrations of adrenergic antagonists can also alter immunologic rhythms in mice (Radosevic-Stasic *et al.*, 1987).

DISCUSSION

Based on the available evidence, daily rhythms of immune activity appear to be driven by a neuroendocrine oscillator that is more directly influenced by nonphotic cues compared with photic cues. The phase of this oscillator, however, can also be influenced by a light-entrained oscillator in the absence of appropriate nonphotic cues. This temporal influence between separate neuroendocrine oscillators might account for the slower rates at which immunologic rhythms (expressions of a nonphotically-entrained oscillator) reentrain to light-dark cycles compared with other rhythms (expressions of a light-entrained oscillator). In addition, overall immune capability may be enhanced or inhibited when the phases between the two oscillators, or their neural and hormonal expressions, are altered. MS-222, a general anesthetic, prevents the reentrainment of allograft rejection rhythms by handling disturbances in gulf killifish (Nevid and Meier, 1994). Although the specific actions of MS-222 are not known (Ryan, 1992), similar agents with defined activities would help delineate the neural pathways that couple daily rhythms of immune responses with handling disturbances and perhaps other nonphotic cues.

While basic questions still remain concerning immunologic regulation by circadian mechanisms, the general findings that have been reviewed here are for the most part compatible with models of circadian timing systems that are based on physiologic and behavioral regulation in vertebrates (Moore-Ede *et al.*, 1982; Meier, 1993). The model for circadian neuroendocrine regulation of immune responses diagrammed in Figure 4 represents a composite view of these ideas. The basic components in this model consist of two primary oscillators and three secondary oscillators. Each oscillator can influence the phases of the other oscillators either directly or indirectly through its neural and hormonal outputs. Thus, although inputs from nonphotic stimuli have been assigned to three different oscillators (the secondary oscillators), each of these stimuli has a more direct influence on the phase of daily immune activities (an expression of secondary oscillator Y) compared with inputs from the light-dark cycle (assigned to the primary oscillators). A change in the phase relationships between any two (or more) of the oscillators may alter immune capability through temporal synergisms of their individual circadian expressions.

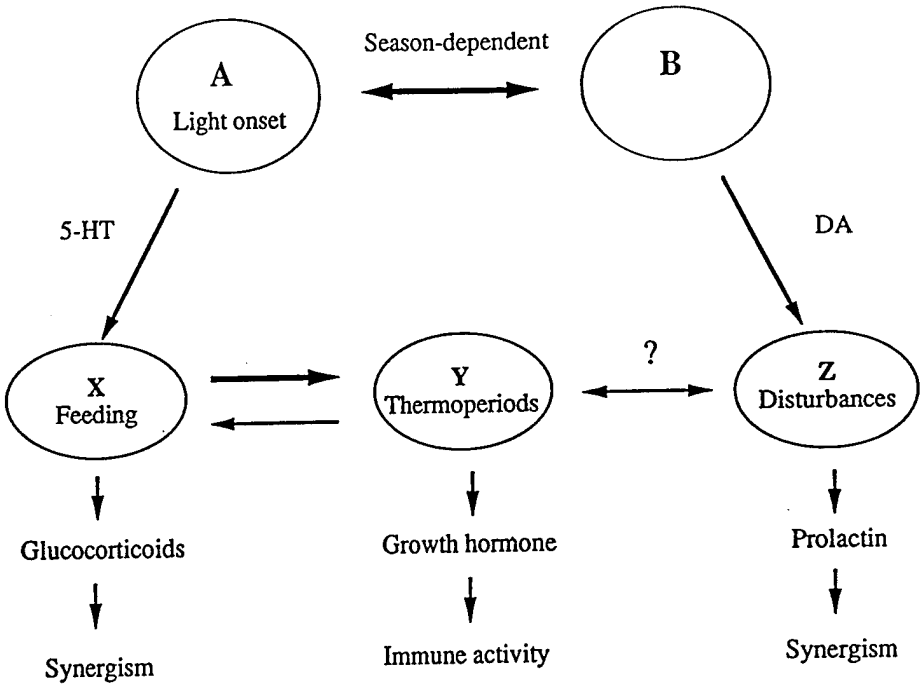


Figure 4. A general model for circadian neuroendocrine regulation of immune responses in vertebrates. Primary oscillator A is entrained by light onset and is coupled with primary oscillator B through a season-dependent mechanism that can be influenced by daylength or ambient temperature. The secondary oscillators, X, Y, and Z, receive temporal information from the light-dark cycle through the primary oscillators, but are more directly influenced by certain types of nonphotic cues (tentatively assigned in the figure). The suspected coupling between Y and Z is based on the observations that growth hormone rhythms adjust to a timed feeding regimen but then readjust back to the original phase under this regimen in rats (Moberg *et al.*, 1975) and the disruption of prolactin rhythms in transgenic mice overexpressing a growth hormone gene (Cecim *et al.*, 1995). Coupling between the other oscillators is based on previous models of circadian timing systems (Moore-Ede *et al.*, 1982; Meier, 1993).

The model of circadian timing systems put forth by Moore-Ede and coworkers (1982), based primarily on studies of humans and primates, fits well with the hormonal and behavioral coupling of daily immune activities in vertebrates. Keeping the original designations of Moore-Ede and coworkers, oscillator Y (a secondary oscillator in Figure 4) controls daily rhythms of locomotor activity, slow-wave sleep, and skin temperature in addition to growth hormone rhythms. Because of the close relationship between locomotor activity patterns and the time of day when immune activities peak or trough (Lévi *et al.*, 1991), this oscillator may directly drive immunologic rhythms in part through its expression of daily growth hormone rhythms. Oscillator X (also a secondary oscillator in Figure 4) controls daily rhythms of REM sleep and core body temperature in addition to cortisol rhythms. Although the daily glucocorticoid rhythms expressed by this oscillator do not appear to account for immunologic rhythms, this oscillator might indirectly influence the phase of immunologic rhythms through its strong temporal influence on oscillator Y. The disproportionate, reciprocal influences between oscillators X and Y proposed by Moore-Ede and coworkers may explain the different effects of cortisol and growth hormone treatments on rhythms of immune activity in gulf killifish kept on light-dark cycles (Nevid and Meier, 1995a).

The other three oscillators depicted in Figure 4 incorporate a model of daily and seasonal regulation in vertebrates put forth by Meier (1984; 1993). Seasonality in vertebrates is thought to have a circadian basis in which the phases of two neuroendocrine oscillators (the primary oscillators A and B in Figure 4) change relative to each other during the year. Primary oscillator A is entrained by light onset and is coupled with primary oscillator B through a season-dependent mechanism that can be influenced by daylength and ambient temperature. Both of the primary oscillators may be located in the suprachiasmatic nuclei where the phase relations of serotonin and dopamine rhythms change in a seasonal manner (Wilson and Meier, 1987). Seasonal changes in serotonergic and dopaminergic activity may in turn alter the phases between daily rhythms of plasma cortisol (an output of secondary oscillator X) and plasma prolactin (an output of secondary oscillator, Z), respectively. Immune responsiveness in mice is influenced differently depending on the time of day when prolactin, serotonergic agents, or dopaminergic agents are administered (Cincotta *et al.*, 1995). Similar treatments in a variety of vertebrate species can alter metabolic, reproductive, and behavioral conditions that are seasonally regulated (Meier, 1984; 1993).

Nonphotic daily stimuli at a specific time of day can have different effects on both the phase of daily immune activity (Figure 2) and the survival times of scale allografts in gulf killifish (Nevid and Meier, 1995b), suggesting that nonphotic cues might directly affect separate neuroendocrine oscillators. Daily rhythms of plasma glucocorticoids are preferentially entrained by timed feeding compared with light-dark cycles (Boulos and Terman, 1980; Boujard and Leatherland, 1992; Mistlberger, 1994). On the other hand, timed feeding appears to shift daily rhythms of growth hormone for several days only (Moberg *et al.*, 1975) and has little effect on prolactin rhythms (Bellinger *et al.*, 1975; Spieler *et al.*, 1977) in animals maintained on light-dark cycles. Thus, food availability may directly influence secondary oscillator X, which regulates cortisol rhythms, and indirectly influence other oscillators. Daily disturbances do not appear to entrain daily rhythms of cortisol (Ottenweller *et al.*, 1987) but might influence secondary oscillator Z based on the release of both prolactin (Kant *et al.*, 1986) and a precursor of dopamine (Kvetnansky *et al.*, 1992) during stress. Daily thermoperiods might influence secondary oscillator Y based on its relation to skin temperature (Moore-Ede *et al.*, 1982) and the temperature threshold for growth hormone secretion in turtles (Licht *et al.*, 1989; Licht *et al.*, 1990). More work concerning the entrainment of circadian rhythms by thermoperiods and disturbances is clearly needed.

Although many of the immune activities discussed in the present paper peak when an animal is resting, there are some types of immune activities that peak at other times of day (Lévi *et al.*, 1991). It is possible that the different phases of these immunologic rhythms are unrelated to daily rhythms of growth hormone, however, it is also possible that growth hormone directly influences some cells and indirectly influences others. This latter explanation appears to account in part for the different phases of protein synthesis rhythms observed throughout the day in different tissues of gulf killifish (Negatu and Meier, 1993). Growth hormone increases plasma levels of insulin-like growth factor 1 (IGF-1) which peak during the photophases in gulf killifish (Wilson *et al.*, 1990). Accordingly, IGF-1 or other cytokines (e.g., interleukins) may link a daily peak of growth hormone at one time of day with immune responses that peak at another time of day.

The relevance of circadian rhythms and the immune system goes beyond the mere cataloguing of biologic rhythmicity. Immune responses of animals are coordinated within a framework of physiologic processes and behavioral activities that fluctuate daily and change seasonally. Altera-

tions in this temporal organization, whether a natural response to environmental change or an abnormality, influence an animal's immune capability. Viewed in the context of daily and seasonal rhythms, there are more similarities than differences when immune regulation is compared among vertebrates. These rhythms are therefore fundamentally important and should be one of the first subjects considered by immunologists.

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Chapter 7

Down-Regulation of Rainbow Trout (*Oncorhynchus mykiss*) Macrophage Activity by Host-Derived Molecules

C.J. Secombes, G.J.E. Sharp[1], S.I. Jang[2], I. Ashton, B. Novoa[3], G.D. Daniels and L.J. Hardie

Department of Zoology
University of Aberdeen
Tillydrone Avenue
Aberdeen AB9 2TN, UK.

[1]Present address:Pharmaceutical and Biological Sciences
Aston University
Birmingham, UK.

[2]Present address: Dept. Aquaculture
College of Fisheries Science
National Fisheries University of Pusan
Republic of Korea.

[3]Present address:Instituto de Investigaciones Marinas
CSIC
Eduardo Cabello
6-36208 Vigo, Spain.

ABSTRACT

A number of factors were investigated for their potential ability to down-regulate rainbow trout (*Oncorhynchus mykiss*) macrophage respiratory burst activity. These included the effects of cortisol and stress on the production of macrophage activating factor (MAF)-containing supernatants from mitogen stimulated head kidney leukocytes, the effect of the eicosanoids 16,16-dimethyl prostaglandin E_2 (diMePGE$_2$) and 13(S)-hydroxy-octadecadienoic acid (13-HODE) on macrophage respiratory burst activity and the effects of the cytokine transforming growth factor β_1 (TGFβ1) on respiratory burst activity. Both addition of cortisol *in vitro* and stress *in vivo* reduced the activity of MAF supernatants, demonstrating for the first time that such factors can interfere with up-regulation of macrophages. However, it is not known whether this phenomenon was due to a reduction in the generation of macrophage activating cytokines or an increase in the production of macrophage deactivating factors such as cytokines or eicosanoids. Both eicosanoids tested

inhibited macrophage respiratory burst activity *in vitro*, in a dose-dependent manner, and diMePGE$_2$ was also able to inhibit the activity of macrophages that had been previously stimulated with MAF-supernatants, tumor necrosis factor α or lipopolysaccharide. Similarly, TGFβ$_1$ was able to inhibit the activity of MAF-treated macrophages, although it had a stimulatory effect on control (untreated) macrophages. The interaction of such suppressive signals and the ability of fish leukocytes to produce macrophage deactivating cytokines are discussed.

INTRODUCTION

Macrophages are key effector cells in innate immune responses against pathogens and tumors, being capable of orchestrating inflammatory events, killing bacteria, parasites and tumor cells, and clearing and inactivating viruses (Lewis and McGee, 1992). In addition, macrophages play an important role in the initiation of specific immune responses, by processing antigens and presenting them to lymphocytes in association with self MHC molecules. Release of cytokines is critical in both functions, especially the release of interleukins (IL) such as IL1, IL6 and IL8, and tumor necrosis factor α (TNFα). It is well known that in order to perform many of these functions macrophages have to be activated (Adams and Hamilton, 1992; Adams, 1994), increasing their competence to effect one or more activities. The regulation of macrophage activation is complex, and it is suggested that multiple states of activation exist controlled by a combination of inductive and suppressive signals (Adams and Hamilton, 1992). Indeed, suppression of macrophages is vital to prevent tissue damage and injury, and inadequate control leads to disease states, as seen in multiple sclerosis and rheumatoid arthritis. Host molecules known to be able to down-regulate macrophage activity include eicosanoids (e.g prostaglandins), hormones (e.g. steroids), neuroendocrine peptides (e.g. catecholamines) and cytokines (e.g. transforming growth factor β, IL4, IL10), some of which may also act in an indirect manner by preventing the release of macrophage activating factors (Sadick, 1992).

As in mammals, fish macrophages are potent effector cells (Secombes and Fletcher, 1992) and appear capable of processing and presenting antigens (Vallejo *et al.*, 1992). They can be activated by a wide range of host- and pathogen-derived molecules (Secombes, 1994), and synergistic interactions between such molecules are known (Hardie *et al.*, 1994; Jang *et al.*, 1995). However, relatively little is known about down-regulation of fish macrophage activity by host-derived molecules. Probably the best studied host molecules capable of suppressing fish macrophage function are the corticosteroids (Barton and Iwama, 1991). Thus, following an episode of stress and release of cortisol, phagocyte responses are generally markedly suppressed (Angelidis *et al.*, 1987; Ainsworth *et al.*, 1991; Thompson *et al.*, 1993). Testosterone has also been shown to be immuno-suppressive in fish (Slater and Schreck, 1993), although little is known about its effects upon phagocyte function. In addition, it has been demonstrated that autonomic neurotransmitters are capable of down-regulating fish macrophage responses, as evidenced by the suppression of respiratory burst activity in the presence of epinephrine or the β-adrenergic agonist isoproterenol (Bayne and Levy, 1991; Flory and Bayne, 1991).

In the present study, a number of potentially suppressive host-derived factors were examined for their direct or indirect effects upon fish macrophage activity. These included determination of the effect of cortisol and stress on the production of macrophage activating factor (MAF) -containing

supernatants from mitogen stimulated leukocytes, and the effect of eicosanoids and the cytokine transforming growth factor β_1 (TGFβ_1) on macrophage respiratory burst activity.

MATERIALS AND METHODS

The effect of cortisol and stress on MAF production

MAF containing supernatants were prepared using rainbow trout *Oncorhynchus mykiss* head kidney leukocytes as described previously (Secombes, 1990). Briefly, head kidney leukocytes were isolated from 300-500 g trout kept at 12-14°C, and stimulated with 10 µg/mL Concanavalin A (Con A, Sigma) and 5 ng/mL phorbol myristate acetate (PMA, Sigma) for 3 hr at 18°C. The cells were then washed x5 to remove the mitogens and cultured for a further 48 hr at 18°C before collection of the supernatants. Supernatants were tested for the presence of MAF by addition to head kidney macrophage monolayers, prepared as described by Secombes (1990). After a 48 hr incubation period, macrophage respiratory burst activity was determined by the reduction of cytochrome c (Sigma)(Secombes, 1990). Cytochrome c solution (2 mg/mL in phenol red-free Hank's balanced salt solution) containing 1 µg/mL PMA to stimulate the respiratory burst, was added to the macrophages and the optical density values at 550 nm recorded for 30 min. Wells containing PMA and 300 iu/mL superoxide dismutase (SOD, Sigma) confirmed the specificity of the reaction and were used as blanks for each treatment.

The effect of cortisol (Sigma) on MAF production was investigated by adding 1, 10 or 100 ng cortisol/mL to the cultures during the pulsing period with Con A/PMA. The cortisol was discarded 3 hr later, during the washing step to remove the Con A and PMA, and the supernatants collected 48 hr later as above. The data obtained after incubation of target macrophages with the supernatants were expressed as a stimulation index, relative to values from macrophages incubated with supernatants from leukocytes not stimulated with mitogens (i.e. a stimulation index above 1 indicated increased activity).

The effect of stress on MAF production was also examined. Trout were exposed to a 2 hr crowding stress, using a protocol shown previously to significantly elevate plasma glucose levels (Thompson *et al.*, 1993). Immediately post-stress, head kidney leukocytes were isolated for the production of MAF-containing supernatants. The data obtained after incubation of target macrophages with supernatants from control (unstressed) and stressed fish were expressed as a stimulation index relative to control macrophage values, as described above.

The effect of eicosanoids and transforming growth factor b on macrophage respiratory burst activity

The relatively stable 16,16-dimethyl prostaglandin E_2 (diMePGE$_2$, Cascade Biochem Ltd) and the 18 carbon fatty acid derivative 13(S)-hydroxy-octadecadienoic acid (13-HODE, Cascade Biochem Ltd) were used to study the effects of eicosanoids on macrophage respiratory burst activity, and represented cyclooxygenase and lipoxygenase products respectively. Each compound was dissolved in ethanol and diluted in tissue culture medium prior to addition to test macrophages to give a constant ethanol concentration of 0.01%. In the case of diMePGE$_2$, two-fold dilutions were tested

from 2.6 μM to 0.08 μM. With 13-HODE, two-fold dilutions were tested from 34 nM to 1 nM. Test macrophages, in 96 well microtiter plates, were incubated with the eicosanoids for 48hr prior to washing and stimulation of respiratory burst activity as described above. The data were expressed as a percent inhibition relative to values from macrophages cultured in the presence of an equivalent concentration of ethanol but no eicosanoid, for the same period of time. In addition, in some cases macrophages were pretreated for 24 hr with either MAF-containing supernatants diluted 1:8, TNFα (British Biotechnology) at 25 units/mL or lipopolysaccharide (LPS, Sigma) at 50μg/mL, prior to exposure to 2.6 μM diMePGE$_2$ for 48 hr. These data were also expressed as a percent inhibition relative to the increase in respiratory burst activity seen with the treated macrophages in the absence of PGE$_2$.

TGFβ is a well known macrophage deactivating factor in mammals (Tsunawaki *et al.*, 1988; Derynck, 1994). To test whether it was also capable of deactivating fish macrophages, natural bovine TGFβ$_1$ (National Institute of Biological Standards, South Mimms) was added to unstimulated macrophages and to macrophages that had been activated previously by a 24hr exposure to MAF-containing supernatants (diluted 1:4), generated by stimulating trout head kidney leukocytes with Con A/PMA as described above. Concentrations of 0.01, 0.1 and 1.0 ng TGFβ$_1$/mL were added to the macrophages for 24hr prior to determination of respiratory burst activity as above. Data from MAF-treated and TGFβ$_1$ exposed macrophages were also expressed as a percent inhibition, relative to the increase in respiratory burst activity seen with MAF treated macrophages in the absence of TGFβ over the response of macrophages treated with control supernatants.

RESULTS

Simultaneous addition of cortisol and mitogens to isolated head kidney leukocytes resulted in supernatants with markedly reduced MAF activity relative to supernatants produced in the absence of cortisol, as evidenced by their ability to increase macrophage respiratory burst activity (Figure 1). There was a dose dependent cortisol effect, with the highest cortisol concentration giving the largest reduction in activity (although this dose effect was not significant overall due to high inter-individual variation). Thus, supernatants generated after incubation of leukocytes with 1 and 10 ng cortisol/mL had approximately 50% less activity than control supernatants, and those generated with 100 ng/mL had 74% less activity than control supernatants. Whilst some carry-over of cortisol into the supernatants was possible, preliminary experiments showed that only the highest dose of cortisol used (100 ng/mL) induced significant ($p<.001$) inhibition of respiratory burst activity after a 48 hr incubation with macrophages. Thus, the low level of residual cortisol potentially present was unlikely to induce the observed reductions in respiratory burst activity.

The effect of a 2 hr crowding stress *in vivo* on the MAF-activity of supernatants from leukocytes stimulated *in vitro* with mitogens was also examined. MAF-activity of the supernatants produced following the crowding stress was markedly decreased (Figure 2). This was most apparent when macrophages were incubated with supernatants diluted 1:4 and 1:8, where activity was some 54% lower than that seen with supernatants from control fish.

Addition of the eicosanoid, diMePGE$_2$, to unstimulated macrophages for 48 hr induced a dose-dependent inhibition ($p<0.01$) of macrophage respiratory burst activity (Figure 3a), with a maximal

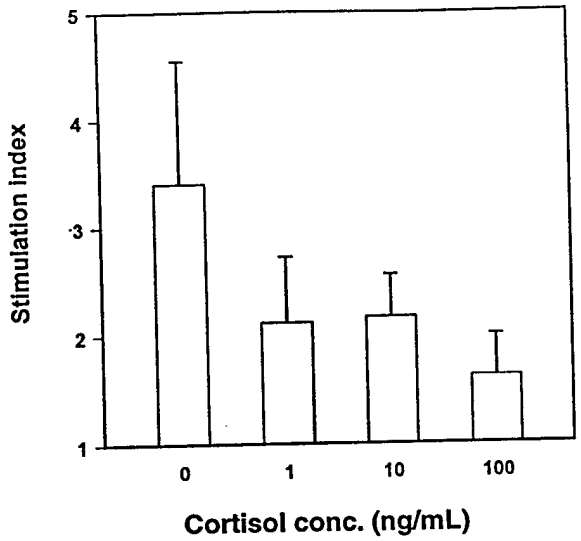


Figure 1. Inhibition of MAF-activity in supernatants (diluted 1:2) from mitogen stimulated head kidney leucocytes cultured in the presence of varying concentrations of cortisol, as assessed by a reduction in their ability to increase respiratory burst activity of target macrophages. Data are presented as mean (+SE) stimulation indices, expressed relative to values from macrophages incubated with supernatants from leukocytes not stimulated with mitogens. N = 3 fish.

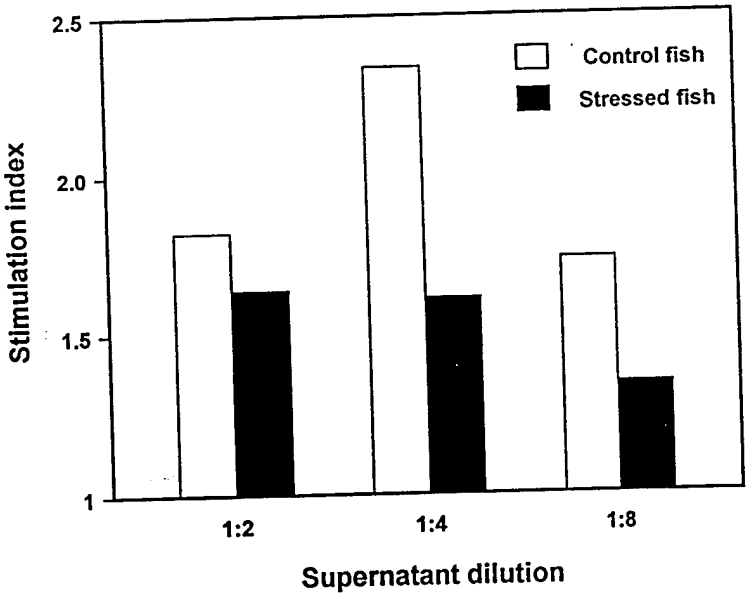


Figure 2. Inhibition of MAF-activity in supernatants from mitogen stimulated head kidney leukocytes obtained from fish given a 2 hr crowding stress, as assessed by a reduction in their ability to increase respiratory burst activity of target macrophages. Data are presented as mean (+SE) stimulation indices, expressed relative to values from macrophages incubated with supernatants from leucocytes not stimulated with mitogens. N = 4 fish.

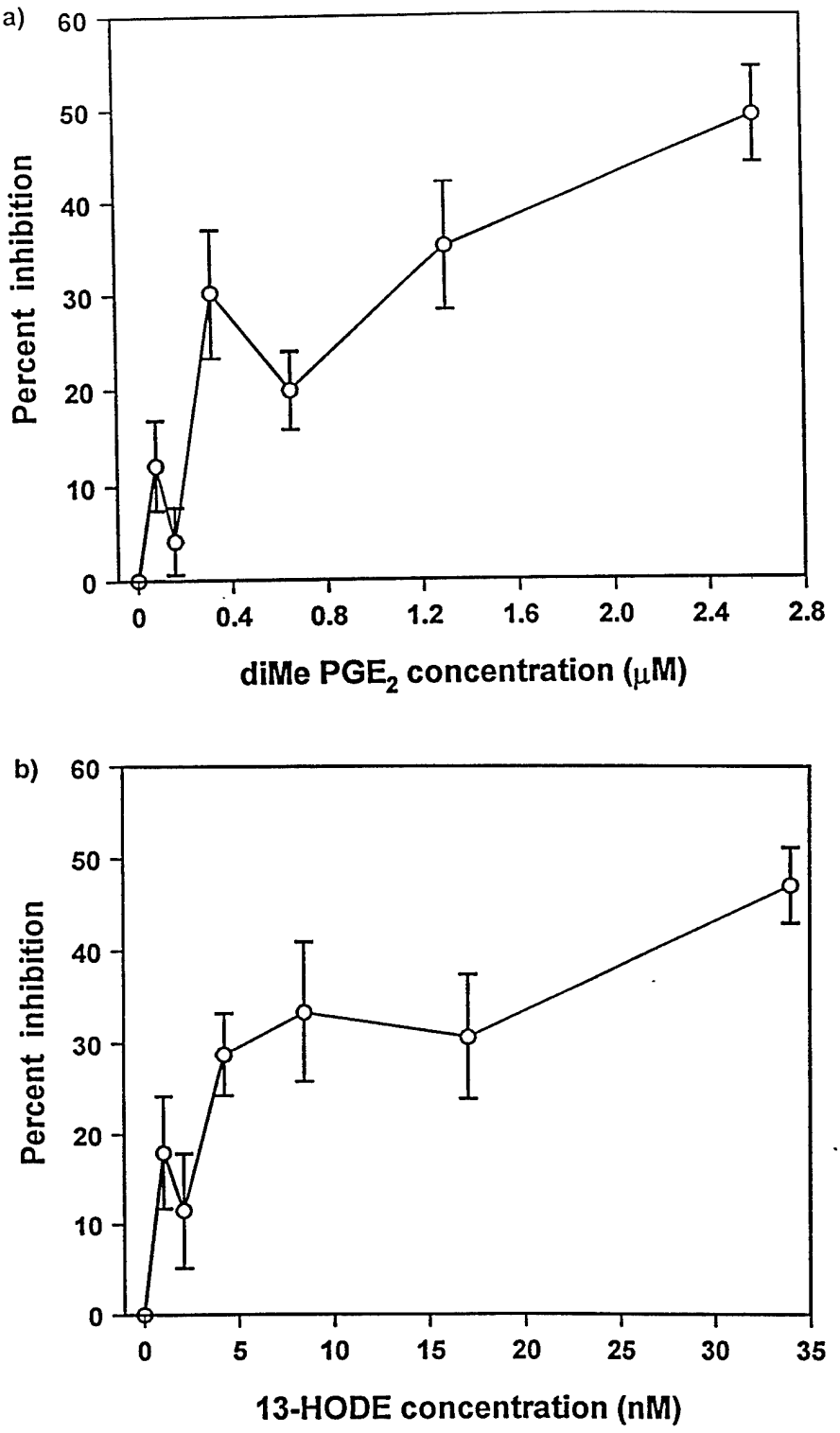


Figure 3. Inhibition of macrophage respiratory burst activity by incubation for 48 hr with varying concentrations of **a)** 16,16-dimethyl prostaglandin E_2 (diMePGE_2) and **b)** 13(S)-hydroxy-octadecadienoic acid (13-HODE). Data are presented as the mean (± SE) percent inhibition relative to valuesfrom macrophages cultured with no eicosanoid for the same period of time. N = 6 fish.

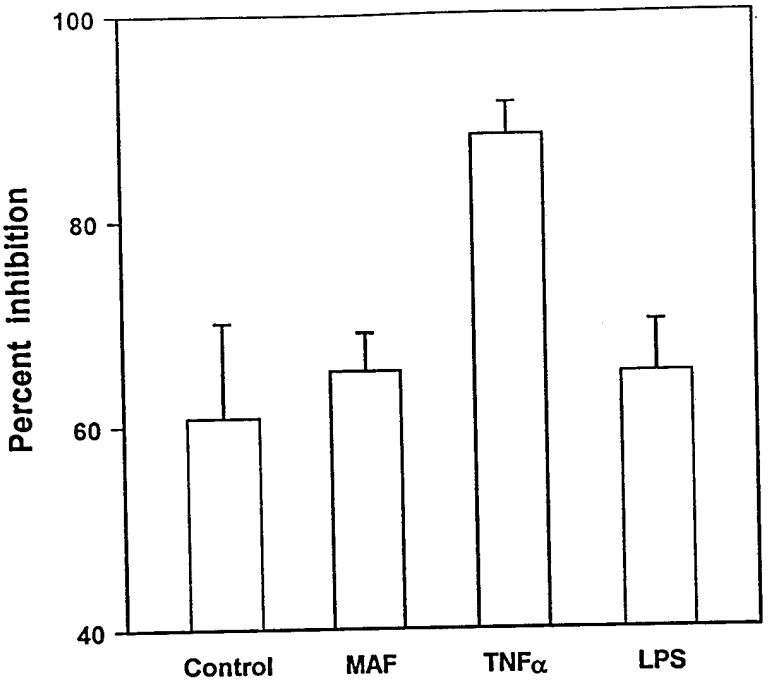


Figure 4. Inhibition of respiratory burst activity in macrophages treated for 24 hr with a MAF-containing supernatant (diluted 1:8), tumor necrosis factor α (25 units/mL, TNFα), lipopolysaccharide (50 µg/mL, LPS) or medium alone (control), followed by an incubation for 48 hr with 2.6 µM 16,16-dimethyl prostaglandin E_2 (diMePGE2). Data are presented as the mean (± SE) percent inhibition relative to the respiratory burst activity seen with respective cultures of macrophages incubated without PGE_2. N = 4 fish.

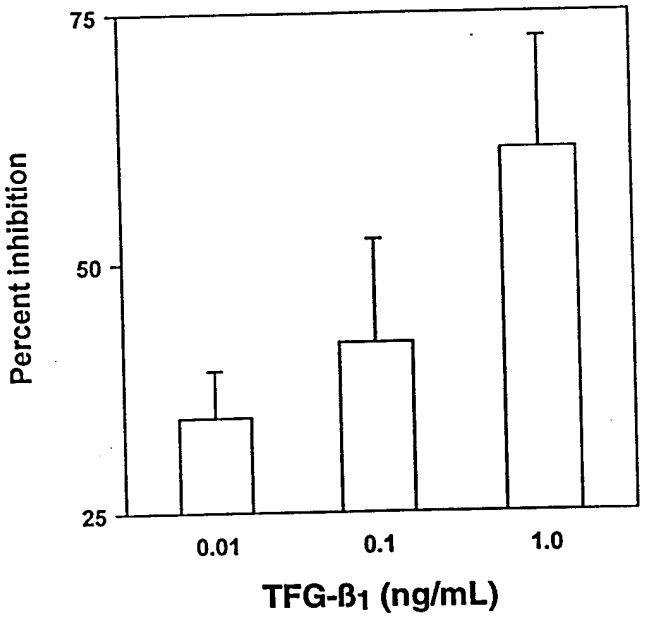


Figure 5. Inhibition of respiratory burst activity in macrophages treated for 24 hr with a MAF-containing supernatant (diluted 1:4), followed by an incubation for 24 hr with varying concentrations of transforming growth factor β1 (TGF β1). Data are expressed as the mean (+ SE) percent inhibition, relative to the increase in respiratory burst activity seen with MAF treated macrophages incubated without TGFβ. N = 4 fish.

inhibition of 50% seen in this experiment using the highest concentration (2.6 μM) tested. However, in other experiments, inhibition as high as 61% was observed using 2.6 μM diMePGE$_2$ (Figure 4). The lowest two concentrations used (0.08 and 0.16 μM) did not induce significant effects. Similarly, addition of 13-HODE significantly inhibited ($p.<0.01$) macrophage respiratory burst activity after a 48 hr incubation (Figure 3b), with a maximal inhibition of 47% seen at the highest concentration (34 nM) tested. As with diMePGE$_2$, the two lowest concentrations tested (1 and 2 nM) did not induce significant effects, although the absolute concentrations used were much lower. Respiratory burst activity of macrophages that had been activated by incubation with MAF-containing supernatants, TNFα or LPS, was also significantly inhibited in the presence of PGE$_2$ (Figure 4), to a similar extent to that seen in unstimulated (control) macrophages.

Lastly, addition of bovine TGFβ$_1$ to unstimulated trout macrophages for 24 hr induced a significant increase ($p.<0.01$) in respiratory burst activity, with maximal activity seen using a concentration of 0.1 ng/mL (data not shown). However, when TGF β1 was added to MAF-treated macrophages it induced significant inhibition ($p<0.05$), relative to macrophages not incubated with TGFβ$_1$, using concentrations of 0.1 and 1 ng/mL (Figure 5). Maximal inhibition of 61% was seen using the highest dose tested and a significant overall dose effect was apparent ($p.<001$).

DISCUSSION

The suppressive effects of cortisol and stress on several aspects of teleost defences have been described previously (Ellsaesser and Clem, 1986,1987; Barton and Iwama, 1991; Thompson *et al.*, 1993), and it is known that stress increases the number of glucocorticoid receptors on fish leukocytes (Maule and Schreck, 1991). Whilst the suppressive effects of stress and cortisol include effects upon respiratory burst activity (Stave and Roberson, 1985; Angelidis et *al.*, 1987), it has not been demonstrated previously that they can also interfere with up-regulation of macrophages. In the present study this was demonstrated indirectly, through effects on the MAF activity of leucocyte supernatants generated in the presence of cortisol or using leukocytes from stressed fish. Whilst the activity of the supernatants was clearly reduced by such treatments, it is not known whether this was due to a reduction in the generation of macrophage activating cytokines or an increase in the production of macrophage deactivating factors such as cytokines or eicosanoids (discussed below). The distinction between such possibilities will only be possible once specific antisera become available to all of the relevant fish molecules. During the generation of the supernatants cortisol was removed since preliminary experiments showed that the highest concentration used (100 ng/mL) induced significant inhibition of macrophage respiratory burst activity in its own right, as expected from previous studies (Stave and Roberson, 1985).

In mammals, two other groups of molecules are also particularly potent macrophage deactivators, eicosanoids such as prostaglandin E$_2$ (PGE$_2$) and cytokines such as transforming growth factor β (TGF β), interleukin (IL) 4 and IL10 (Adams and Hamilton, 1992; Sadick, 1992; Bosco *et al.*, 1995; Wang and Chadee, 1995). In the case of IL4 and IL10, inhibition can also be through an indirect mechanism whereby inhibition of Th1 cells prevents the release of macrophage activating cytokines (Sadick, 1992; Hart *et al.*, 1995). The present study demonstrates that both of these types of molecules are also able to suppress aspects of macrophage activity in fish. With respect to the former (eicosanoids), both cyclooxygenase and lipoxygenase products inhibited macrophage respiratory

burst activity, and PGE_2 was even able to inhibit the activity of macrophages previously activated by incubation with MAF, TNF α or LPS. The inhibitory effect of PGE_2 is known to be mediated by its ability to elevate levels of cAMP, which acts to suppress the activation of Na^+/H^+ exchange by stimulatory molecules (Adams and Hamilton, 1992). In contrast, lipoxygenase products such as leukotrienes and lipoxins typically promote inflammatory events (see Rowley, this issue), and in fish it is known that they are able to promote phagocytosis by macrophages (Knight *et al.*, 1993) and to act as potent chemoattractants for neutrophils (Hunt and Rowley, 1986; Sharp *et al.*, 1992). This is also true for 13-HODE, which in mammals promotes LPS-induced TNF α formation in macrophages (Schade *et al.*, 1993) and can overcome the suppression of the mitogenic response to LPS by splenocytes in the presence of lipoxygenase inhibitors (Elekes *et al.*, 1993). Whilst the highest concentration of 13-HODE used in the present study is some 10-fold lower than the optimal dose used in mammalian studies ($3\text{-}5 \times 10^{-7}$ M), it is within the concentration range that induces these stimulatory effects. Thus, it remains to be determined why fish macrophages are inhibited by incubation with 13-HODE.

Whilst it is clear that macrophage deactivating cytokines exist, it is difficult to categorize a cytokine in such simple terminology. Many of the so-called activating cytokines will down-regulate certain molecules/functions in macrophages, as seen with the down-regulation of the mannose-fucose and transferrin receptors and chemokine production in mammalian monocytes/macrophages by interferon-γ (Auger and Ross, 1992; Adams and Hamilton, 1992; Schnyder-Candrian *et al.*, 1995). Similarly in fish, it is known that at least one function, 5'nucleotidase activity, is decreased in "activated" macrophages (Zelikoff and Enane, 1992; Hepkema and Secombes, 1994). Nevertheless, cytokines that suppress major activities of macrophages, such as microbicidal or tumoricidal activity, are acting as overall inhibitors of macrophage function. Such difficulties in classifying cytokine function were very apparent in the present study, examining the effects of TGF β_1 on trout macrophages. At relatively low concentrations TGF β_1 had a stimulatory effect upon trout macrophages (Jang *et al.*, 1994), but at higher concentrations it down-regulated activated macrophages. Similarly, in mammals whilst TGF β is a potent macrophage deactivator (Tsunawaki *et al.*, 1988; Ding *et al.*, 1990) it can also augment some activities, such as cell migration and angiogenic activity (Wiseman *et al.*, 1988), and is required *in vivo* for optimal Th1 cell development leading to release of IFNγ (Spaccapelo *et al.*, 1995). In accord with this, preliminary studies have shown that addition of TGF β during mitogen-stimulation of trout leukocytes does not inhibit the MAF-activity of harvested supernatants (Hepkema, 1995), previously shown to be dependent upon the presence of fish T cells in the culture (Graham and Secombes, 1990).

Since TGF β is well conserved in mammals (Burt and Law, 1994; Derynck, 1994), probes against conserved motifs have recently been used to isolate a trout TGFβ by PCR from macrophage enriched head kidney leukocytes (Hardie, unpublished data). As in mammals, the translated mature peptide consists of 112 amino acids, with 68% homology to human TGFβ_1, 53% homology to human TGF β_2, 59% homology to human TGF β_3, 62.5% homology to chicken TGF β_4 and 62.5% homology to *Xenopus* TGF β_5. A phylogenetic tree based upon this sequence (Figure 6) clearly reveals that the closest relationship is with *Xenopus* TGF β_5. Thus, not only can trout macrophages respond to mammalian macrophage deactivating cytokines, it is clear that they can also express such molecules.

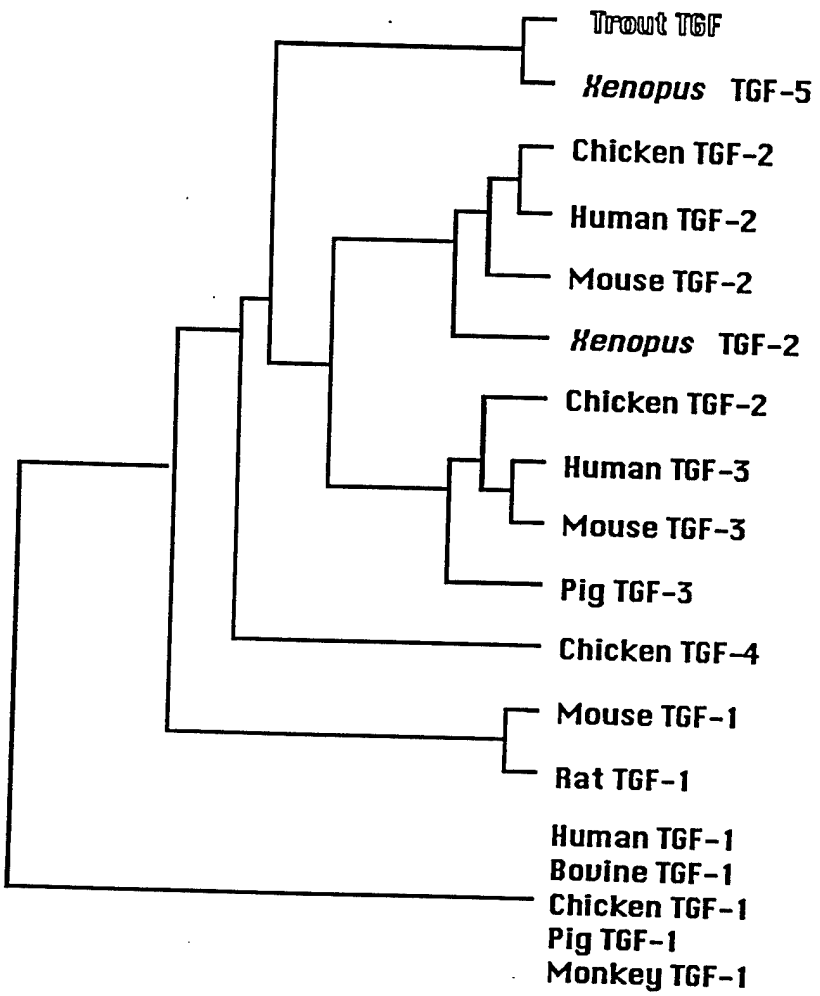


Figure 6. Evolutionary relationships between TGFβ isoforms. Multiple sequence alignment data were used to produce a phylogenetic tree of known TGFβ sequences. The tree was produced using a PHYLIP computer package (Phylogeny Inference Package, version 3.5c) on SEQNET (Daresbury, UK), and bootstrapped 1,000 times.

Finally, while synergistic effects between positive stimuli on fish leukocytes are well known (Hardie *et al.*, 1994; Neumann *et al.*, in press), less is known about interactions between suppressive agents on fish leukocytes. Precedents exist, as with the interaction seen between testosterone and cortisol in decreasing antibody secreting cell number (Slater and Schreck, 1993). Thus, it is possible that there may be additive/synergistic interactions between eicosanoids and cytokines in down-regulating macrophage activity in fish as in mammals (Alleva *et al.*, 1995), as well as potential antagonistic interactions between negative and positive signals (although TNFα, LPS and MAF added individually were unable to prevent the inhibition of respiratory burst activity by PGE_2 in the present study). Interestingly, drug-induced down-regulation of macrophage activity is seen as the goal in some mammalian studies aimed at treating diseases caused by excessive activity of these cells (Cianciolo and Adams, 1994), where the focus of activity is on inhibiting cytokine release and arachidonic acid metabolism.

Acknowledgments

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Chapter 8

The Role of Eicosanoids in Immune Regulation in Fish

Andrew F. Rowley

School of Biological Sciences
University of Wales Swansea
Singleton Park, Swansea, SA2 8PP, U.K.

INTRODUCTION

One of the hallmarks of the mammalian immune system is the array of regulatory mechanisms that integrate the cell types and humoral factors responsible for maintenance of the body free from disease and neoplasia. The interplay between cytokines, eicosanoids and leukocytes, such as macrophages and lymphocytes, exemplifies the complexity of this system. For example, the synthesis/release of cytokines including interleukin-1 (IL-1), IL-2, IL-3, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are all influenced by eicosanoids (Knudsen *et al.*, 1986; Hart *et al.*, 1989; Minakuchi *et al.*, 1990; Stañková *et al.*, 1992; Chan *et al.*, 1993; Daculsi *et al.*, 1993; Li and Fox, 1993) while in turn some of these molecules can stimulate leukocytes to synthesize eicosanoids (Browning and Ribolini, 1987; Sigal *et al.*, 1993). Furthermore, cytokine receptor distribution on leukocytes can also be stimulated or suppressed by eicosanoids (Hancock *et al.*, 1988; Rola-Pleszczynski *et al.*, 1993). Thus, in mammals, eicosanoids can regulate the synthesis and expression of receptors for various cytokines while these cytokines can affect eicosanoid biosynthesis thus providing a feedback loop.

Little is known about the potential role of eicosanoids in the regulation of the piscine immune system, although recent studies have shown that in the rainbow trout, *Oncorhynchus mykiss*, both mitogenic responses to phytohemagglutinin (PHA) and specific antibody production to bacterial antigens are influenced by various eicosanoids (Secombes *et al.*, 1994; Knight and Rowley, 1995). This present review is designed to give a timely overview of these recent findings and suggests future avenues for research in this area.

EICOSANOID GENERATION BY MAMMALIAN AND PISCINE LEUKOCYTES

Before discussing the role of eicosanoids in immune regulation, it is of obvious importance to ascertain which of these compounds are synthesized in fish and therefore of potential importance in this function. This section is not, however, designed to be an exhaustive overview of the biosynthesis and structures of the different types of eicosanoids as these topics have been comprehensively reviewed elsewhere. Hence, the reader is directed to the many excellent reviews of prostaglandin (Smith, 1989), leukotriene (Samuelsson *et al.*, 1987; Ford-Hutchinson *et al.*, 1994), lipoxin (Serhan, 1994) and monohydroxy fatty acid (Spector *et al.*, 1988) biosynthesis for details

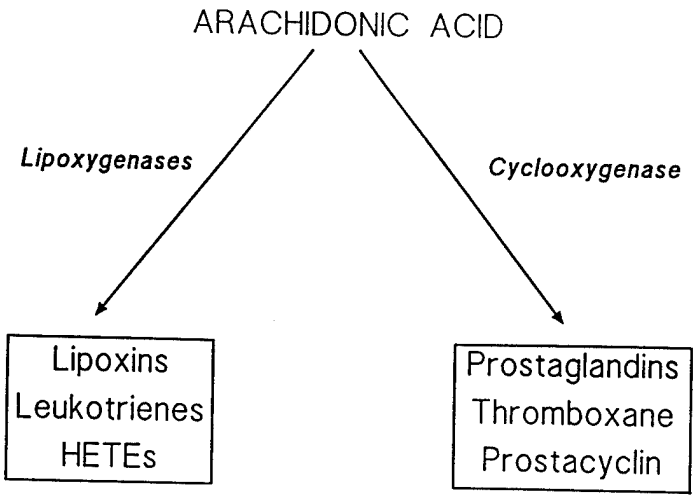


Figure 1. Routes for the biosynthesis of eicosanoids

of the structure and mechanisms of generation of these compounds in mammals. For a more detailed account of eicosanoid generation in fish see Rowley (1991), Serhan (1994) or Rowley *et al.* (1995).

Eicosanoids are principally derived from the C_{20} fatty acid, arachidonic acid (20:4,*n*-6) although in fish where *n*-3 fatty acids often predominate, eicosapentaenoic (20:5,*n*-3) and docosahexaenoic (22:6,*n*-3) acids are also potential substrates for the generation of these compounds (e.g. Henderson and Sargent, 1985; Tocher and Sargent, 1986). There are two main types of eicosanoids, those derived by the action of cyclooxygenase (prostaglandin synthase) termed prostanoids and those produced by the action of lipoxygenases (Figure 1). 5-, 12- and 15-Lipoxygenase activities are involved in the generation of eicosanoids in mammals and are selectively present in a range of leukocyte types including granulocytes (e.g. Turk *et al.*, 1982; McGuire *et al.*, 1985; Rouzer and Samuelsson, 1985; Izumi *et al.*, 1991), mononuclear phagocytes (e.g. Laviolette *et al.*, 1988; Laegreid *et al.*, 1989; Sigal *et al.*, 1993) and lymphocytes (Jakobsson *et al.*, 1992). One of the main lipoxygenase products that has been shown to be involved in immune regulation is leukotriene (LT) B_4 (Claesson *et al.*, 1992), produced by the action of 5-lipoxygenase and LTA_4 hydrolase (Samuelsson *et al.*, 1987). 5-Lipoxygenase has been purified from human granulocytes (Rouzer and Samuelsson, 1985) and found to have a requirement for a number of co-factors including Ca^{2+} ions and an 18 kDa protein termed 5-lipoxygenase activating protein (FLAP) (Dixon *et al.*, 1990; Yamamoto, 1992; Ford-Hutchinson *et al.*, 1994). Although 5-lipoxygenase has not been purified from any fish leukocytes, a FLAP-like protein has been reported in macrophages and peripheral blood leukocytes from the rainbow trout, *O. mykiss* (Rowley *et al.*, 1995). Further lipoxygenase products shown to have immune regulatory activity in mammals are the lipoxins (LX), LXA_4 and LXB_4. For example, LXA_4 and LXB_4 inhibit the cytotoxic activity of human natural killer cells (Ramstedt *et al.*, 1985), although, unlike leukotrienes, less is known about the potential functional importance of lipoxins in immune regulation (Serhan, 1994). Fish leukocytes, like their mammalian

counterparts, have also been found to contain a range of lipoxygenases leading to the biosynthesis of leukotrienes, lipoxins and mono-hydroxy fatty acid derivatives. One of the most carefully studied fish cell types, in terms of eicosanoid generation, is the macrophage. For example, challenge of rainbow trout (*O. mykiss*) macrophages with calcium ionophore, A23187, lipopolysaccharide (LPS) or zymosan causes the synthesis of LTB_4, LXA_4 and 12-hydroxyeicosatetraenoic acid (12-HETE) as well as the equivalent products derived from EPA (LTB_5, LXA_5 and 12-hydroxyeicosapentaenoic acid; 12-HEPE) (Pettitt *et al.*, 1991; Rowley *et al.*, 1994). The nature of the lipoxygenase products generated by other leukocyte types in fish is much less clear, although plaice, *Pleuronectes platessa*, neutrophils synthesize LTB_4/LTB_5 following ionophore challenge (Tocher and Sargent, 1987) and partially purified suspensions of lymphocytes generate 12-HETE and 12-HEPE but not 5-lipoxygenase products, such as LTB_4 (Rowley *et al.*, 1995). Whether fish lymphocytes as a whole do not express 5-lipoxygenase activity is still not proven and requires more sophisticated cell separation techniques to give highly purified (*ca.* minimum 99% pure) lymphocytes than used to date to answer this important question.

Prostaglandin (PG) E_2 has been shown to be a key molecule in immune regulation in mammals (see reviews by Goodwin and Ceuppens, 1983; Phipps *et al.*, 1991). Other prostanoids may also have a similar role but this is far less clear than for PGE_2. Prostaglandins, unlike leukotrienes and lipoxins, are formed by a plethora of mammalian cell types and therefore it is not surprising that these compounds have numerous non-immunological functions including control of water balance in the kidney, hemostasis, gut protection and reproduction (Moore, 1985). Apparently, all mammalian leukocyte types have the capacity to generate PGE_2 although thromboxane generation may be more concentrated in macrophages (e.g. Laegreid *et al.*, 1989), mast cells (Macchia *et al.*, 1995) and platelets (Hamberg *et al.*, 1975). The key enzyme in prostanoid generation is cyclooxygenase (COX) or PGH synthase (PGHS). The constitutive form of the enzyme (COX-1, PGHS-1) is found in the majority of mammalian cell types and its action is inhibited by non-steroidal antiinflammatory drugs including indomethacin, acetylsalicylate and ibuprofen, which have analgesic properties. Recently, a second form of COX was discovered, initially in leukocytes. This form of COX is referred to as inducible COX (COX-2, PGHS-2) and is not found in unstimulated cells. COX-2 can be induced in leukocytes by proinflammatory stimuli including LPS or muramyl dipeptide particularly in the presence of the cytokines IL-1ß, IFN-γ and TNF-α (e.g. Lee *et al.*, 1992; Reddy and Herschman, 1994; Riese *et al.*, 1994). The induction of COX-2 increases the capacity of some cells to generate prostanoids by 342% of the level found in 'resting' cells (Arias-Negrete *et al.*, 1995). COX-2 has more recently been shown to be expressed in a number of other cell types including rheumatoid synovial fibroblasts (Hulkower *et al.*, 1994), peritoneal mesothelial cells (Topley *et al.*, 1994) and human endometrium and decidua (Shaw *et al.*, 1994). As it may be active in disease states, such as rheumatoid arthritis, research has centred on finding selective inhibitors of this enzyme that have no effect on constitutive COX. A number of selective inhibitors have been reported to date including NS-398 (*N*-[2-cyclo-hexyloxy-4-nitrophenyl]methane sulfonamide; Futaki *et al.*, 1994), CGP 28238 (6-[2,4-difluorophenoxy]-5-methyl-sulfonylamino-1-indanone; Klein *et al.*, 1994) and SC-58125. No doubt several new inhibitors of COX-2 will follow and these may find their way into treatment regimes for some inflammatory diseases.

There are many reports of prostaglandin synthesis in fish and, as in mammals, many cell types/tissues express COX activity (Knight *et al.*, 1995). For example, leukocytes of the dogfish, *Scyliorhinus canicula*, have been reported to synthesize PGE_2, PGD_2, $PGF_{2\alpha}$ and thromboxane B_2 (Rowley *et*

al., 1987). In thrombocytes, the platelet equivalent of fish, the principal prostanoid formed is thromboxane B_2 (Lloyd-Evans *et al.*, 1994). Whether fish cells can express two forms of COX activity has yet to be proven. Macrophages from the rainbow trout in culture with bacterial LPS have been shown to have modest (*ca.* 3 fold) increases in PGE_2 generation after 48 hr co-incubation (Rowley *et al.*, 1995). Whether this small increase in PGE_2 is caused by expression of COX-2 or up-regulation of COX-1 is unclear. Furthermore, it is also important to determine if cytokines together with LPS can cause a greater amount of stimulation of PGE_2 generation than LPS alone.

EICOSANOIDS AND IMMUNE REGULATION IN FISH

With a knowledge of the nature by which eicosanoids are generated in fish, it is possible to test whether any of these compounds can influence the behaviour of leukocytes and other cell types in lymphoid organs. Two main experimental approaches have been taken to gain insight into this question and these are discussed in the following two sections of this review.

The *In Vivo* Approach

While this would seem to be a simple approach to determine if eicosanoids are involved in immune regulation it has a number of pitfalls that may jeopardize the validity of the conclusions drawn from such experiments. As already mentioned, a number of selective inhibitors exist that interfere with the synthesis of cyclooxygenase and lipoxygenase products *in vitro* both in mammals and in fish. For example, indomethacin and ibuprofen are relatively 'clean' inhibitors of cyclooxygenase (both COX-1 and COX-2) activity and similarly, nordihydroguaiaretic acid (NDGA) and esculetin inhibit lipoxygenases. When administered to experimental animals their activity may be less specific making it difficult to draw meaningful conclusions. An example of this comes from the work of Rainger *et al.* (1992) who reported that NDGA had a dose dependent inhibitory effect on the generation of antibody in rainbow trout following immunization with *Aeromonas salmonicida*. While part of the explanation of its activity would be expected to reside with its potential inhibition of lipoxygenases, they found that leukocytes taken from fish that had either been injected with NDGA or saline alone showed no significant difference in their capacity to generate lipoxygenase products. This shows that the action of NDGA in these *in vivo* studies may have been via a route independent of eicosanoids and therefore care should be taken in drawing conclusions from such experiments.

An alternative approach is to apply exogenous eicosanoids to the cells of the immune system either by intraperitoneal or intravascular application. Eicosanoids act a 'local' hormones in mammals and their half lives *in vivo* are spectacularly short, ranging from a few seconds in the case of thromboxane, to several minutes for some other eicosanoids. Hence, administration of eicosanoids to experimental animals during immunization trials that may last for days to weeks is unlikely to have any long term effect. Fortuitously, a number of synthetic analogues are available that have all the biological activity of naturally occurring eicosanoids together with markedly increased stability *in vivo* and *in vitro*. These analogues include iloprost and 16,16-dimethyl-PGE_2, prostacyclin and PGE_2 analogues respectively. Intraperitoneal injection of trout with *A. salmonicida* in the presence of 16,16-dimethyl-PGE_2 causes a significant, dose dependent depression in antibody titers compared with controls (Knight and Rowley, 1995). Similarly, fish immunized with sheep erythrocytes

Table 1.
Summary of the main modulatory effects of eicosanoids and inhibitors of their biosynthesis on immune responses in fish

Property	LXA_4	LTB_4	12-HETE	PGE_2	Indomethacin	NDGA	References
PHA-induced mitogenicity *in vitro*	Inhibition	Stimulation	ND*	Inhibition	Stimulation	Inhibition	Secombes *et al.* (1994)
Antibody synthesis (*in vivo*)	ND	ND	ND	Inhibition	Inhibition/stim ulation	Inhibition	Laudan *et al.* (1986), Rainger *et al.* (1992), Knight and Rowley (1995)
Plasma cell generation *in vitro*	-	-	-	Inhibition	Stimulation	-	Knight and Rowley (1995)

*ND = not determined; - no significant effect at the concentrations tested

have a significant reduction in the number of plaque forming cells (PFC) in the spleen in the presence of 16,16-dimethyl-PGE_2 (200 µg/kg body weight). Hence, these and other *in vivo* experiments provide some evidence that PGE_2 is immunosuppressive in fish (Table 1).

The *In Vitro* Approach

This approach has several key advantages for studies aimed at examining how eicosanoids are involved in regulating the immune system. These include the ability to expose cells to selective inhibitors of eicosanoid biosynthesis and simultaneously correlate changes in levels of eicosanoids generated with effects on various immune parameters. Secombes *et al.* (1994) found that PHA-induced mitogenicity of rainbow trout head-kidney leukocytes was influenced by exogenous eicosanoids and by inhibitors of their endogenous generation. Prostaglandins E_2 and E_3 were found to be immunosuppressive while leukotrienes and lipoxins were stimulatory and inhibitory respectively (Table 1). As would be expected, inhibition of prostaglandin synthesis by the exposure of leukocytes to indomethacin resulted in a stimulation of PHA-induced mitogenicity, while NDGA (a lipoxygenase inhibitor) was markedly inhibitory suggesting that the balance of lipoxygenase products are immune-enhancing.

Modulation of the humoral antibody response in *O. mykiss,* as measured by PFC generation *in vitro* in response to sheep erythocytes, is also influenced by the presence of eicosanoids or inhibitors of their biosynthesis (Knight and Rowley, 1995; Table 1). In this study prostaglandins were again found to be immunosuppressive, while other eicosanoids, however, had no significant effect at the concentrations tested (10^{-10} - 10^{-7} M; Figure 2). This potential lack of effect of lipoxygenase products at these concentrations does not rule out the possibility that higher levels may have some significant effect.

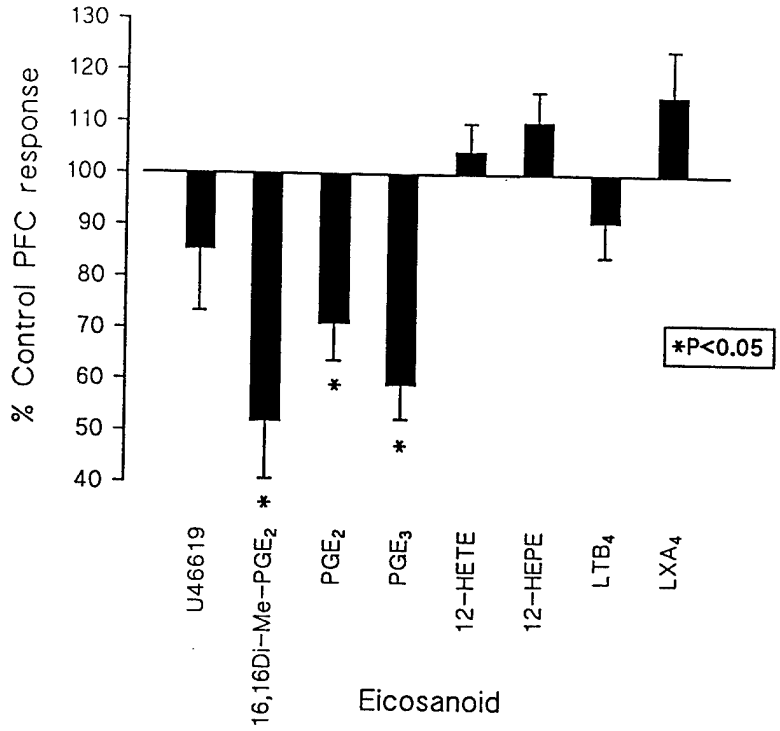


Figure 2. Effect of eicosanoids (all at 10^{-7} M concentration) on the generation of plaque forming cells (PFC) in rainbow trout splenic leukocytes in response to the *in vitro* exposure to sheep erythocytes. Values are expressed as a percentage of the control (no eicosanoid). U-46619 is a stable thromboxane A_2 mimetic. Mean values ± S.D., $n = 5$. Data from Knight and Rowley (1995).

WHAT IS THE MECHANISM OF ACTION OF EICOSANOIDS IN IMMUNE REGULATION IN FISH?

Experiments to date have shown that eicosanoids, in particular PGE_2, have a clear effect on a number of piscine immune responses both *in vivo* and *in vitro* (Table 1). They give little insight, however, into the mechanism of this regulatory activity. Reference to the extensive literature on immune regulation in mammals by eicosanoids suggests that one mechanism of how such effects take place is via cytokines. Modulation of immune reactivity by eicosanoids in mammals is intimately associated with changes in cytokine generation and the distribution of cytokine receptors on leukocytes (Rola-Pleszczynski *et al.*, 1993). Not only can cytokines module eicosanoid generation but the reverse situation is also true leading to a complex interaction between the two classes of molecules (see Figure 3). Other immune modulating molecules are also involved in the interaction between eicosanoids and cytokines including platelet-activating factor (PAF) (see reviews by Braquet and Rola-Pleszczynski, 1987 and Denizot *et al.*, 1994) adding to the complexity of this interaction. Investigations into the potential interplay between eicosanoids and cytokines in fish is hampered by our lack of information on these latter molecules. Few cytokines have been isolated and purified to homogeneity from any fish sources (Secombes, 1994) and the lack of recombinant forms of these molecules will limit what experiments can be performed.

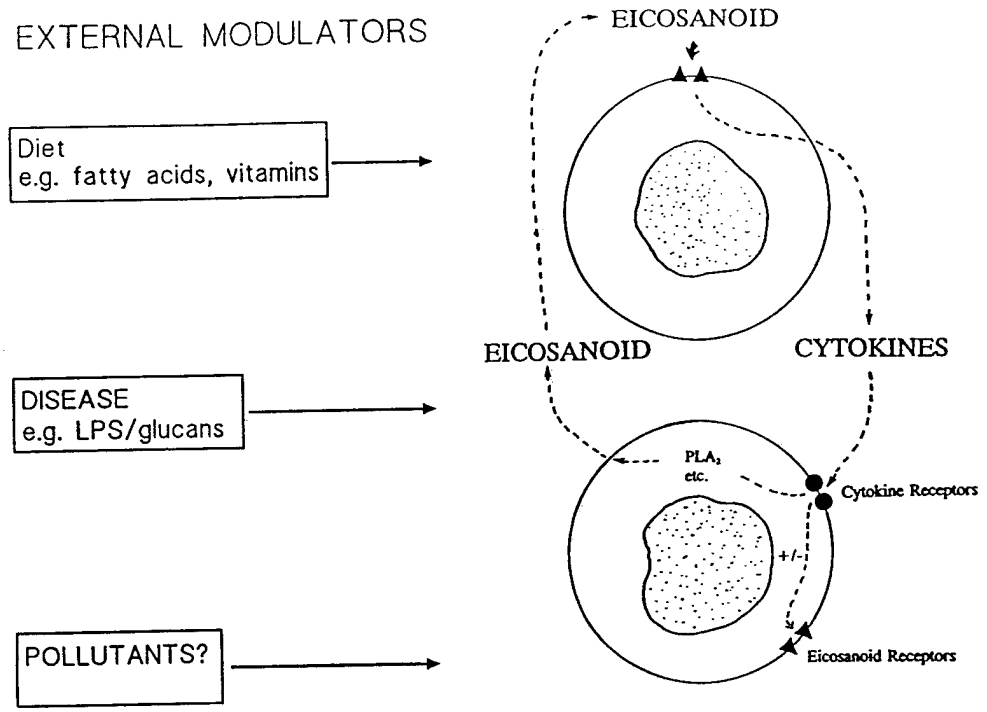


Figure 3. Schematic diagram showing the hypothetical interaction between cytokines and eicosanoids with their target leukocytes and the possible influence of external factors. In this model situation part of the effect of eicosanoids on leukocytes is via cytokine generation and subsequent changes in the expression of eicosanoid receptors on a target cell. Although not shown, eicosanoids could also modify the expression of cytokine receptors hence altering the way in which these cells respond to cytokines released from surrounding cells.

External factors such as diet, infection and pollution probably have profound effects on eicosanoid generation and hence may affect their role in immune regulation (Figure 3). Fatty acids in diets have been shown to cause changes in the profile of eicosanoid generation by fish leukocytes (Bell *et al.*, 1991; Ashton *et al.*, 1994). These changes in turn can affect various inflammatory and immune phenomena (Ashton *et al.*, 1994) due to the role of eicosanoids as mediators of both of these processes. Furthermore, in mammals changes in fatty acids can modulate immune responses by direct mechanisms at the cell membrane independent of eicosanoids (see Yaqoob and Calder, 1993 for review) suggesting that such a process may occur in fish. Erdal *et al.* (1991) have shown that diets rich in *n*-3 fatty acids are immunosuppressive in Atlantic salmon, *Salmo salar*, leading to lower antibody levels although the mechanism (i.e. whether it is independent or dependent on eicosanoids) of this is not fully understood at present. Other dietary factors including vitamins and trace elements may also have indirect effects on eicosanoid generation by altering substrate availability or the behaviour of enzymes involved in eicosanoid biosynthesis. Microbial infections lead to alterations in eicosanoid levels as the agents involved are activators of the synthesis of these factors (Pettitt *et al.*, 1991). Finally, pollutants such as PCBs may also cause changes in eicosanoid generation because of the mechanism by which they interact with phospholipids (e.g. Dzogbefia *et al.*, 1978).

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Chapter 9

Nervous-Endocrine-Immune Interactions in Vertebrates

Barbara Plytycz and Rolf Seljelid[1]

Institute of Zoology
Jagiellonian University
Krakow, Poland

[1]Institute of Medical Biology
University of Tromso, Norway

ABSTRACT

The neuroendocrine and immune systems of vertebrates form a common homeostatic entity reacting in a coordinated way to different disturbing agents (stressors), both of internal and external origin, including pathogenic invasion. This coordination requires different kinds of vertebrate immunocytes which require communication both by contact and via soluble molecules. The necessity for cell-cell contacts involved gradual centralization and compartmentalization of vertebrate lymphoid tissue. A rich vascularization and innervation of lymphoid organs facilitates the communication between the immune, nervous and endocrine cells. All these cells share receptors and some mediatory molecules. For this reason the immune system is affected by both physiological and physical stimuli acting primarily on the nervous system. Moreover, the nervous and endocrine systems are informed about and participate in the ongoing immune response. Throughout our comparative studies on morphology of lymphoid organs and immunocompetent cell activity, as well as our experiments on graft rejection, acute inflammation and bacterial clearance we have also analyzed the effects of neurohormonal factors and/or their agonists/antagonists on certain immune parameters.

PRINCIPLES OF VERTEBRATE IMMUNITY

The immune system of contemporary vertebrates contains two highly efficient, adaptive and specific components (Bayne 1994). First, an ancient mechanism shared by all members of the animal kingdom, based on the functions of multipotent mesenchymal cells. These functions include: phagocytosis, bacteriolytic and bacteriostatic mechanisms, the secretion of enzymes and of various signal molecules (e.g. cytokines, arachidonic acid metabolites) that modify the microenvironment. Second a mechanism evolved in vertebrate ancestors is based on lymphocyte-specific gene rearrangement which creates numerous antigen receptors expressed on individual lymphocytes. Antigen receptors are either components of lymphocyte cell membranes or, as in the case of immunoglobulins, are released into the body fluids. Immunoglobulins bind to specific molecules, marking or

masking the relevant structures that induce or prevent, respectively, further steps in the immune response. These vertebrate-specific mechanisms have both advantages (the memory capability of vertebrate immunity) and disadvantages (predisposition to allergy and autoimmunity).

NEUROENDOCRINE-IMMUNE HOMEOSTATIC SYSTEM

Different kinds of immunocytes require communication both by contact and via soluble molecules. The necessity for cell-cell contacts caused gradual centralization and compartmentalization of vertebrate lymphoid tissues, a rich vascularization and innervation of lymphoid organs, as well as the presence of immunocompetent cells in some endocrine cells (Felten *et al.*, 1992; Zapata, *et al.*, 1982; Zapata, *et al.*, 1981; Zapata and Cooper, 1990; Midonski *et al.*, 1995a). All these cells share receptors and some mediatory molecules (Blalock, 1984; 1992; Blalock and Smith, 1985; Weigent and Blalock, 1987). The immune system is modulated by both psychological and physical stimuli acting on the nervous system. The nervous and endocrine systems interact with an ongoing immune response. It is therefore relevant to examine the immune, nervous and endocrine systems as interdependent components of the homeostatic machinery of the body. Each component of this complex network can be modified and in turn can modify. All components are involved in the maintenance of the homeostatic balance of an organism. This balance can be altered by a variety of environmental or internal stressors. From this perspective, a microbial challenge can be a stressor which in turn evokes a stress response orchestrated by the immune, nervous and endocrine system (Plytycz and Seljelid, 1995 a,b).

We examined this phenomenon using an example of acute inflammation (Plytycz and Seljelid 1995a), extensively studied in the mammalian system (Seljelid 1988; Seljelid and Eskeland 1993; Seljelid and Busund 1994). This response fits the classical description of stress. It involves activation of both the hypothalamo-pituitary-adrenal (HPA) axis and the autonomic nervous system. Non-self macromolecules are stressors which cannot be detected by the nervous system itself but can be recognized by the specialized sensory organ - the immune system (Blalock, 1984). The function of sensory organs is to transform stimuli into signals which allow information to be transmittted to the central nervous system. Microbial macromolecules are recognized and decoded as non-self by leukocytes inducing the release of a battery of mediators with pleiotropic effects like IL-1, IL-6 and TNF. These factors, cytokines and others, initiate the inflammatory response, accompanied by the liver-mediated acute phase resonse (Baumann and Gauldie, 1994) and serve as mediators transferring the information concerning the encounter of foreign material to the central nervous system. Perception of the stressor, both physiological and physical, including microbes, triggers a neuroendocrine cascade which begins by the hypothalmic release of corticotropin releasing factor (CRF) which stimulates corticotropin release from the pituitary, which in turn stimulates glucocorticoid release from the adrenal glands. Adrenal glucocorticoid hormones provide a negative feedback loop by inhibiting CRF, corticotropin and cytokine release. This "classical" HPA axis response is probably the main and certainly the best known feedback mechanism leading to switching off stress responses including immune reactions (Stein and Miller, 1993). There are however several back-up mechanisms and regulatory loops, which as a rule finally terminate the stress response, including the immune reaction. Perception of stress also alerts other nervous and endocrine centers, inducing the involvement of the autonomous nervous system and disperse the endogenous opioid system (Shavit *et al.*, 1985; Sibinga and Goldstein, 1988; Przewlocki, 1993) in the complex homeostatic

reactions. These events are modulated by the moiety of neurochemicals and hormones acting either directly on immunocyte receptors ot indirectly, changing their trafficking patterns and the composition of microenvironments.

EXPERIMENTAL APPROACHES

Studies on the homeostatic system are most advanced in mammalian model species and in humans (e.g. Aarstad *et al.,* 1983, 1991a,b; Boctor *et al.,* 1989; Moynahan *et al.,* 1994; Laudenslager and Fleshner, 1994), in which numerous mediatory molecules and their receptors, shared by the immune and neuroendocrine homeostatic systems are best characterized. However, components of this system are present in representatives of all vertebrate classes (e.g. Cooper, 1984, 1987, 1991, 1992; Maule *et al.,* 1989; Demers and Bayne, 1994). Convenient models for investigation of fundamental aspects of neuro-immune reactions exist in lower vertebrates (Cooper and Walford, 1982; Cooper and Faisal, 1990; Faisal *et al.,* 1989 a,b; Ghoneum *et al.,* 1988; Plytycz, 1994; Saad and Plytycz, 1994), or even in invertebrate species (Franceschi and Ottaviani, 1992; Ottaviani *et al.,* 1991; Stefano *et al.,* 1993).

During our ongoing series of experiments on modulation of immunity by external (environmental) and intrinsic (internal) factors, we have conducted parallel observations and experiments on representatives of mammalian species (mice, rats) and ectothermic animals: amphibians (frogs, toads) and fish (goldfish, salmon). In this review we will discuss some of our results implicating or showing directly the intimate connections between vertebrate immune and neuroedocrine systems, with special emphasis on the advantages of several model animals and experimental protocols.

NEUROHORMONE-DEPENDENT VARIATIONS OF LYMPHOID ORGANS

Lymphoid tissue is strongly dependent on hormonal regulation (Riviere and Cooper 1973; Zapata and Cooper 1990). Lymphoid organs of ectothermic animals undergo pronounced seasonal cyclic changes (Zapata *et al.,* 1992; Cooper *et al.,*1992; Saad and Plytycz, 1994) governed by a pineal gland providing an internal clock (Arendt, 1985; Pierpaolo and Maestroni, 1987; Bartness and Goldman, 1989; Maestroni, 1993; Conti and Maestroni, 1993, Pierpaoli, 1994; Lissoni *et al.,* 1994; Skwarlo-Sonta, 1994). The annual cyclicity may be connected with seasonal fluctuations in levels of several hormones, including corticosteroids (Ghoneum *et al.,* 1986; Saad, 1988; Saad and Plytycz, 1994). The thymus of the common frog, *Rana temporaria,* involutes every winter and recovers every spring, reaching full development with a clear cortico-medullary division every summer (Plytycz and Bigaj, 1983a,b; Bijag and Plytycz, 1984a,b; Plytycz *et al.,* 1991, 1995, 1996a,b; Miodonski *et al.,* 1994; 1995, 1996). The major season-specific morphological features persist in frogs kept in the cold during the summer or warm during the winter. This clearly indicates that the ambient temperature is not a sole or even a major factor which induces these seasonal fluctuations (Bigaj and Plytycz, 1984b). Seasonal cyclic changes of frog thymuses are superimposed on age-related thymic atrophy (Plytycz *et al.,* 1995, 1996a). Thus summer-winter fluctuations are significantly more pronounced in young animals than in senescent frogs. This implies that the same enviromental changes (stressors) induces stronger reactions in young rather than older organisms. Interestingly, Orell and Dwyer (1995) have recently pointed out that aging impairs the capacity of mammals to terminate the stress response and is accompanied by sustained high concentrations of

terminate the stress response and is accompanied by sustained high concentrations of corticosteroids, a possible cause of several age-related changes and diseases including dementia in humans.

In the common frog, the same environmental stimuli influences in various ways, different lymphoid organs of the same individuals. Seasonal changes of jugular bodies and spleens are not so pronounced as those of the thymus and follow different annual patterns which may be connected with organ-specific corticosteroid sensitivity (Plytycz *et al.*, 1993b). Annual cyclic changes, on the other hand, are different in homologous lymphoid organs of different species, e.g. in inguinal bodies of *Bufo bufo* (Plytycz and Szarski, 1987) and *B. Regularis* (Saad *et al.*, 1991).

In addition to reversible, season-specific involution of amphibian lymphoid organs, we have observed their atrophy under laboratory conditions (Plytycz *et al.*, 1986, Dulak and Plytycz, 1989). This was especially striking in malnourished individuals (Plytycz *et al.*, 1993a) but occurred even in those fed *ad libitum* maintaining a constant body weight (Saad and Plytycz, 1994).

COMPENSATORY MECHANISMS IN THE IMMUNE SYSTEM

We assumed that involution of lymphoid organs should be accompanied by impairment of immune reactivity. Surprisingly however, transplantation immunity of the anuran species was unaffected by the season-specific or laboratory-induced atrophy of lymphoid organs. Skin graft survival time was the same in the summer and in the winter (Jozkowicz and Plytycz, 1994). The chronic pattern of allograft rejection was characteristic both for toads kept in the laboratory and for their counterparts living in the wild (Grodzinska and Plytycz, 1990; Goralik *et al.*, 1994). We suggest that even the involuted lymphoid organs possess enough of the functional components and/or can develop some compensatory mechanisms capable of effecting efficient immune responses, at least towards alloantigenic stimulation. In aneurans, significant differences between reactions toward skin allografts and xenografts have been observed (Horton and Manning, 1972; Horton *et al.*, 1992). In our transplantation experiments, malnutrition impaired xenograft, but not allograft rejection (Plytycz *et al.*, 1993a; Jozkowicz and Plytycz, 1994). The viability of allografts was significantly prolonged at low temperatures, while the viability of xenografts was much less temperature-dependent (Jozkowicz and Plytycz, 1994). This indicates that some components of the amphibian immune system, supposedly lymphocytes (Bly and Clem, 1991), are inhibited by low temperature. Xenograft rejection seems to be more dependent on the activity of phagocytic cells, as evidenced by *ex vivo* investigations of graft-infiltrating cells (Jozkowicz, 1995).

Another series of experiments led us to the conclusion that the macrophages can developed compensatory mechanisms allowing them to act efficiently in animals living in the cold (Rozanowska *et al.*, 1992; Jozkowicz *et al.*, 1993b; Plytycz and Jozkowicz, 1994). Fish and amphibian peritoneal macrophages kept overnight at several *in vitro* temperatures (ranging from 1-37°C) were assayed following 3-weeks acclimation. The plastic adherence of macrophages did not vary over the temperature range of 1-22°C. At these temperatures (which are encountered in a natural setting) the general activity of mitochondrial dehydrogenases measured by MTT reduction parallelled increases of *in vitro* temperature. Macrophage endocytic activity assayed by uptake of neutral red and phagocytosis of India ink was strongly dependent on the previous *in vivo* thermal acclaimation. Macrophages from fish or amphibians kept in the cold were significantly more active

at low assay temperatures than cells from their counterparts kept at warm temperatures. This indicates that during prolonged periods of cold or warmth, fundamental functions of macrophages are capable of adapting to thermal changes. This mechanism has enormous adaptive value for ectothermic animals that inhabit temperate climatic zones and ensures effective pathogen clearance during cold periods, compensating for suppressed lymphocyte-mediated immunity (Plytycz and Jozkowicz, 1994).

An acute peritoneal inflammation elicited by sterile irritants is an an attractive model for analyzing temperature-dependence of the immune system in ectothermic animals. Yellow-bellied toads, *B.variegata* injected intraperitoneally with Sephadex G-50 showed the highest numbers of peritoneal cells elicited 2 or 3 days later at the high (22°C) or low (10°C) temperature, respectively. The number and composition of peritoneal cells returned to control levels one week after injecting animals kept at warm temperatures, while it was significantly increased in those maintained in the cold (Jozkowicz *et al.*, 1994c). As revealed by FACScan analysis, a sharp decline in the number of peritoneal cells resulting mainly from *in situ* apoptotic cell death (Jozkowicz *et al.*, 1993b, 1994b,d) which might be induced by elevated levels of corticosteroids. In another series of experiments, we have found that corticosteroid-sensitivity of peritoneal cells is inhibited at low temperatures (Jozkowicz *et al.*, 1993a). Therefore, we can assume and hope to verify that prolongation of acute inflammation in animals kept in the cold may be at least partly related to the low temperature-dependent corticosteroid-resistance of elicited peritoneal cells.

RECEPTOR BINDING STUDIES

Another experimental approach that examines immune-neuroendocrine interactions involves receptor binding. Autoradiography and saturation experiments gave direct evidence for the presence of β-adrenergic and cholinergic muscarinic receptors on fish and amphibian leukocytes (Jozkowicz *et al.*, 1994a, 1995a,b), which are apparently components of leukocyte cell membranes, based on several functional studies (Flory, 1989, 1990; Flory and Bayne, 1991; Bayne and Levy, 1991a,b; Clotheir *et al.*, 1992). In our experiments, *in vitro* incubation of fish or amphibian leukocytes with drugs specific for mammalian receptors modified some functional immune parameters of these cells, e.g. the respiratory burst, phagocytosis or adherence (Jozkowicz, 1994a, Jozkowicz *et al.*, 1995).

In addition to these relatively easy and simple but nonphysiological *in vitro* systems, we have used an *in vivo* treatment of fish or amphibians with different pharmacological ligands that are known to bind specific neurochemical receptors in mammalian systems. For example, propranolol, an agent binding β-adrenergic receptors, has been shown to prolong xenograft, but not allograft viability while atropine, that bind cholinergic muscarinic receptors shortens xenograft rejection from phylogenetically distant species (Jozkowicz *et al.*, 1995b). On the other hand, the ongoing graft rejection modulates the binding of specific ligands by muscarinic cholinergic or β-adrenergic receptors present on the amphibian splenic leukocytes (Jozkowicz *et al.*, 1995).

The results of experiments conducted by E. Cooper's group indicate that endogenous opioids modulate some immune parameters in fish (Cooper and Faisal 1990: Faisal *et al.*, 1989a,b: Ghoneum *et al.*, 1989). Our results show that morphine treatment affects the kinetics of acute inflammation

as well as inhibits bacterial clearance in fish and amphibian lymphoid organs (Gruca *et al.*, 1994; Jozkowicz *et al.*, 1995).

LOOKING FOR A CONVENIENT MODEL SYSTEM

The "playing with receptors" experiments should be conducted by using animals that are carefully matched with respect to age, sex, and general health, which is not easy to achieve in the case of field-collected wild species. The evolutionary immunobiologist working on *in vitro* systems is confronted by problems of possible bacterial contamination from apparently healthy field-collected specimens. We recently discovered that such natural (physiological) splenic bacteraemia is typical for yellow-bellied toads, *Bombina variegata*, that inhabit the bacteria-rich environment,while it occurs randomly in spleens of the fire-bellied toad, *B. Bomnina* and the green frog *Rana esculenta* which inhabits relatively clean habitats. The spleens of yellow-bellied toads kept for prolonged periods in a bacteria-rich muddy water contain numerous viable bacteria, which are completely cleared within one to two days after the toads are moved to containers with clean water (Mika *et al.*, 1995). This could be interpreted to indicate that in this species, the simple environmental change (being a stressing factor) enhances the antibacterial activity of the immune system.This may be connected with the reduced impact of external pathogens which facilitates the successful killing of bacteria by immunocompetent cells. It can also be assumed that a sudden change of environment acts as a stressor which activates the immune system via a neuroendocrine pathway. This intriguing phenomenon is interesting *per se,* but it actually has great adaptive value for animals that inhabit mountain territories where they breed from May until August in small, shallow, muddy, strongly-bacteria-contaminated sites. These conditions lead to bacteraemia, but these bacteria are efficiently cleared following environmental changes connected either with massive rainfalls, cleaning habitable sites, or by massive land migrations forced by drought. This is an example of the beneficial effects of stress in nature.

One of the final goals of our comparative studies is to find the most convenient and suitable species and experimental protocols for further exploration of neural-immune interactions. In conclusion, the neuroendocrine and immune systems of both endothermic and ectothermic vertbrates form a common homeostatic entity reacting in a coordinated way to different stressors, both internal and external in origin including pathogenic invasion.

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Chapter 10

Immune System Development in *Xenopus*

John D. Horton[1], Trudy L. Horton, Pamela Ritchie, Ian Gravenor and Karen Horsham

Department of Biological Sciences
University of Durham
Durham DH1 3LE, U.K.

[1] Author to whom correspondence should be addressed

ABSTRACT

Recently generated anti-*Xenopus* T cell monoclonal antibodies (mAbs) are here used in concert to investigate aspects of T cell development in clawed frogs. The extent to which CD5, CD8 and the 120 kDa XTLA-1 T cell antigen are expressed on thymocytes and splenocytes is followed through larval and early adult life, by employing dual color flow cytometry. Such analysis also confirms the metamorphosis dependence of class II MHC expression by *Xenopus* T cells and indicates that adult T cells express less class II than do adult B cells. Although the pan T cell marker CD5 is not constitutively expressed on *Xenopus* B cells, it can be induced on activated B cells following *in vitro* stimulation of splenocytes with phorbol ester, provided that T cells are present in the culture.

Following larval thymectomy (at 5-6 days) T cell marker expression is frequently abolished from spleen, blood and intestine for at least a year. Together with an absence of allograft rejection responses and T mitogen reactivity displayed by these animals, this suggests there is no major extra-thymic pathway of T cell maturation in this amphibian. Such thymectomized (Tx) *Xenopus* are now being probed for natural killer (NK)-like cells, which may play an important role in the absence of T cells. Recent data reveals that splenocytes from Tx, but not control frogs, can spontaneously lyse B_3B_7 thymus tumour targets (6 hr ^{51}Cr-release experiments), which lack expression of MHC antigens. In contrast MHC-disparate red cell targets, even those coated with *Xenopus* IgY, are not susceptible to splenocyte killing.

INTRODUCTION

The immune system of the clawed frog, *Xenopus,* has been characterized in greater depth than any other ectothermic vertebrate, its free-living embryonic, larval and metamorphic stages of development lending themselves to studies probing ontogenetic immune issues (Du Pasquier *et al.,* 1989; 1995; Horton, 1994; Horton *et al.,* 1995). We here review our recent experiments on *Xenopus* which probe the ontogeny and highlight the thymus dependence of T cell development and which are investigating natural killer cells at this level of evolution.

The first section of the review characterizes ontogeny of cell surface antigen expression on lymphocytes of euthymic frogs, by employing a panel of recently-generated monoclonal antibodies (mAbs) to *Xenopus* T and B cell markers. Special emphasis is given to dual color flow cytometric experiments that provide new insight into differences in T and B cell expression of MHC class II (Du Pasquier and Flajnik, 1990; Flajnik *et al.*, 1990; Rollins-Smith and Blair, 1990) and CD5 (Jurgens *et al.*, 1995) antigens. The second section concentrates on the dramatic immune modulation which occurs following removal of the thymus in the first week of larval life. We reveal through phenotypic and functional studies that current batches of thymectomized (Tx) *Xenopus* display no evidence of an extra-thymic T cell maturational pathway - a pathway recently highlighted by observations on mammals (Lefrancois and Puddington, 1995). Finally, the third section reveals that Tx *Xenopus* possess splenocytes that readily display spontaneous cytotoxicity towards the MHC-deficient *Xenopus* thymus tumour target cells recently described by Robert *et al.* (1994), indicating that NK-like cells may play a crucial role in the defence of these T cell-deficient animals.

REVIEW

Characterization of Cell Surface Antigens on T and B Cells in Euthymic Xenopus.

Ontogeny of T and B cell markers in thymus and spleen.

Figure 1 illustrates mean percentages of thymocytes and splenocytes stained for T and B surface markers as judged by flow cytometry during larval life and in young adults. The studies, to be published in detail elsewhere (Gravenor *et al.*, 1995), employed mAbs against three *Xenopus* T cell specific antigens, XTLA-1, CD5 and CD8. B cells were detected by use of anti-IgM mAbs (see Figure 1 legend for source of mAbs).

For the thymus we demonstrate that T-lineage differentiation is still in its infancy in one week old, stage 48 (Nieuwkoop and Faber, 1967) larvae, there being no detectable $CD8^+$ lymphocytes and only 20% thymocytes express XTLA-1. XTLA-1 differentiation on thymocytes at 7 days (also seen by Nagata, 1986) occurs coincidentally with the initial expression of MHC class II on the thymic epithelium (Du Pasquier and Flajnik, 1990). This indicates a crucial role of MHC proteins in early stages of T cell education in *Xenopus* as occurs in mammals (Kruisbeek, 1993). XTLA-1 expression has developed on virtually all thymocytes by 12 days when some 80% thymocytes are also $CD8^+$. The latter T cell antigen in fact emerges in the 8 day old thymus, as evidenced by our immunohistochemical findings reported elsewhere (Gravenor *et al.*, 1995). Since the anti-CD5 mAb stains thymocytes relatively poorly, the percentages of $CD5^+$ thymocytes during ontogeny may well be an underestimate. IgM^+ B cells were undetectable in the thymus.

In the spleen B cell percentages were shown to gradually decline during ontogeny, whereas T cell percentages increase. Dual color flow cytometry revealed that XTLA-1^+ and $CD8^+$ splenocytes are overlapping, rather than distinct cell populations, both expressing the pan T cell marker CD5 (Figure 6). $CD8^+$ lymphocytes represent about 30-40% of $CD5^+$ T cells in *Xenopus* spleen. Antibodies specific for *Xenopus* CD4 have not yet been generated. Initial ontogenetic studies by Nagata (1986) indicated that T cell differentiation in the *Xenopus* spleen had not begun by stage 50, but could first be identified at stage 52. Although we confirm a low percentage of T cells (both XTLA-1^+ and

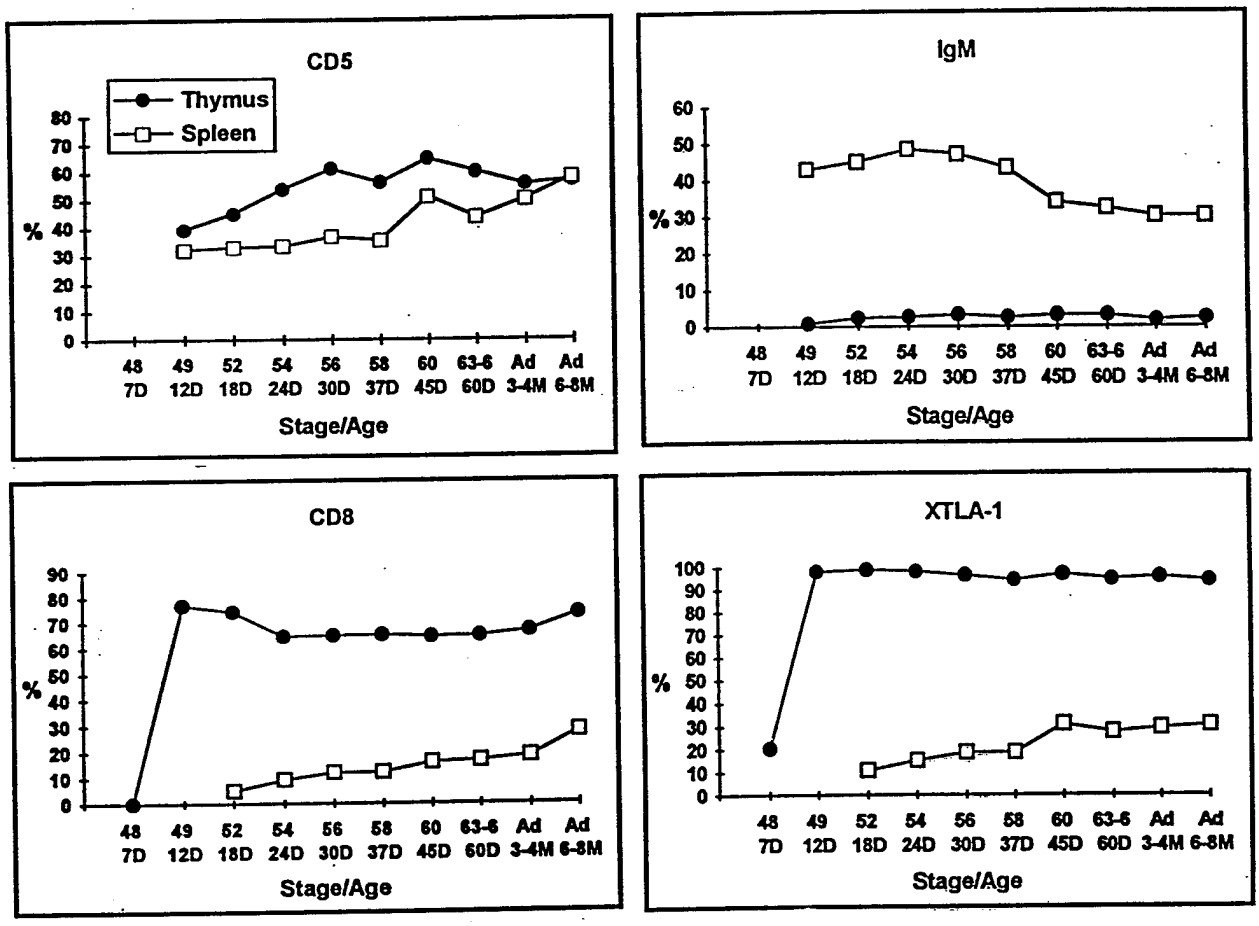


Figure 1. Ontogeny of monoclonal antibody (mAb)-defined T and B cells in thymus and spleen of *Xenopus laevis*. Each data point represents the mean of 2-3 separate flow cytometric experiments (maximum standard deviations for thymocytes = ±10% and for splenocytes = ±7%). Each experiment involved a pool of 10 (stage 63-66) to 20 (stage 48) larval organs and 2-4 adult organs. Murine mAbs used were 2B1 (anti-*Xenopus* CD5 [Jurgens *et al.*, 1995]), AM22 and F17 (anti-*Xenopus* CD8 [Flajnik *et al.*, 1990; Ibrahim *et al.*, 1991]), D8 and 8E4:57 (anti-*Xenopus* IgM [Langeberg *et al.*, 1987; Jurgens *et al.*, 1995]), and XT-1 (specific for a 120kDa *Xenopus* T cell antigen [Nagata, 1988]). mAbs were of IgG isotype, except for anti-CD8 mAbs, which were of IgM isotype. The protocol for flow cytometry is given in the legend to Figure 2.

CD8+) exists in the spleen at stage 52, 30% CD5+ splenocytes are already found in 12 day old larvae (stage 49) and a scattering of $CD8^+$ cells can be visualized immunohistochemically at this time (Gravenor *et al.*, 1995). T cells therefore begin to appear in the spleen within a few days of T-lineage cells first being detected in the thymus.

Differences in class II MHC expression on T and B cells.

Our dual color flow cytometric experiments on metamorphosis-blocked *Xenopus* confirm that class II MHC expression on T cells, but not on B cells, is metamorphosis-dependent (Figure 2), as previously demonstrated by Rollins-Smith and Blair (1990). These authors indirectly showed that adult *Xenopus* T cells were class II^+, on the basis that there appeared only in the adult a major population of IgM^- class II^+ cells. Our dual staining studies with anti-class II and anti-CD5 directly identify that adult splenic T cells express this MHC type. Moreover, co-staining for class II and either IgM or CD5 (see Figures 2 and 3) leads us to suggest that B cells in adult *Xenopus* (until at

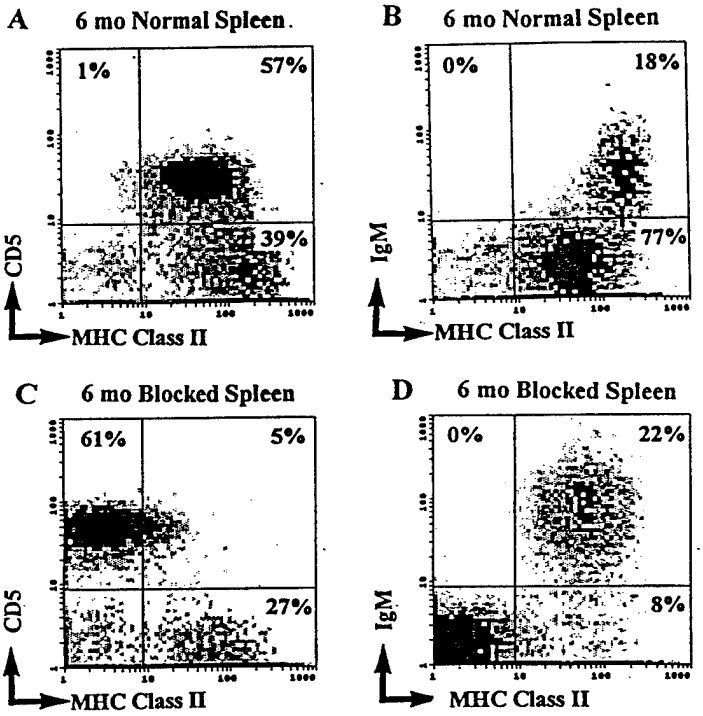


Figure 2. Dual *color* flow cytometric analysis reveals differences in MHC class II expression on splenic T and B cells in normal (A,B) and metamorphosis-inhibited ('blocked') (C,D) 6 month old *X. laevis*. To block metamorphosis sodium perchlorate (1g/litre) was added to aquarial water from larval stage 53. Cells were dual stained for class II MHC (using the AM20 mAb of Flajnik *et al.*, 1990) and either CD5 or IgM expression (to identify T and B cells respectively).

Flow cytometry method. Splenocytes were washed in amphibian strength PBS (APBS with 0.1% NaN_3 and 0.1% BSA) before incubation with the primary mAb at optimal concentration. After washing, cells were incubated with FITC-labelled, rabbit anti-mouse immunoglobulin (DAKO) adsorbed with 5% *Xenopus* serum. Cells were then washed with APBS containing 1% normal mouse serum and counter-stained with a phycoerythrin (PE)-labelled mAb. The control mouse IgG antibody for indirect FITC staining was CT3 (anti-chicken CD3). The PE control was mouse IgG conjugated to PE (DAKO). Markers were set to exclude 98% of cells stained with control reagents from positive analysis. Five to ten thousand lymphocytes were analysed on a Coulter XL or Becton Dickinson Facscan flow cytometer: exclusion of erythrocytes and dead cells being confirmed by propidium iodide staining.

least 1 year of age) express more class II than do T cells. This contrasts with previous findings based on single color FACS analysis that indicated all adult *Xenopus* lymphocytes express equivalent levels of class II molecules (Flajnik *et al.*, 1990). Differences in levels of class II expression by adult *Xenopus* T and B cells could be significant in terms of antigen presentation roles of these two lymphocyte populations.

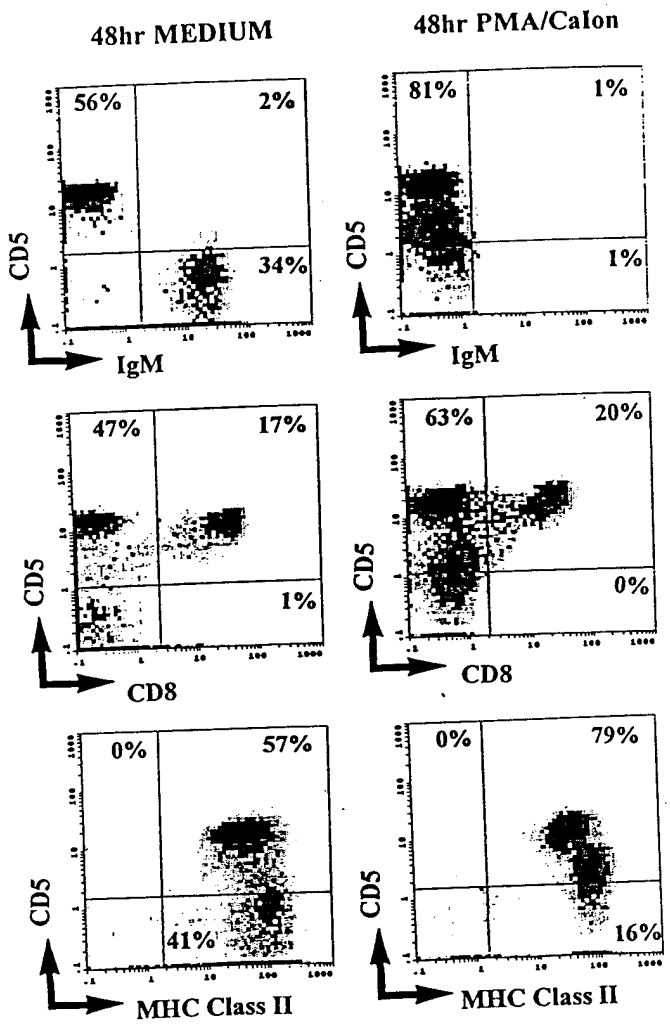


Figure 3. Emergence of CD5 on activated B cells 48 hrs after stimulation of splenocytes with phorbol myristate acetate (PMA) and calcium ionophore A23187 (Ca Ion). Cells assessed for CD5 expression and also for either IgM, CD8 or MHC class II expression using mAbs described in Figure 1. Splenocytes from 1 year old *X. laevis* were prepared in amphibian culture medium (1:1 mixture of L15 and AIM V culture media [Gibco] diluted to amphibian strength, buffered with Hepes and sodium bicarbonate, and supplemented with 5% FCS) and cultured in 24 well plates (Greiner) at 1 x 10^6 leukocytes/ mL/well. Experimental cultures were treated for 18-20 hrs with 10ng/mL PMA (Sigma) and 0.1µg/mL Ca Ion (Sigma), then washed and cultured in fresh medium. Experimental and control (medium alone) cultures were harvested at 48 hrs for dual *color* flow cytometric analysis (see method in Figure 2).

Experimental induction of CD5 expression on *Xenopus* B cells.

Dual color flow cytometry reveals that B lymphocytes in *Xenopus* do not consitutively express CD5, in contrast to the universal expression of this marker by T cells (Gravenor *et al.*, 1995; Jurgens *et al.*, 1995). Lack of significant levels of CD5 is also the situation for rat B cells (Vermeer *et al.*, 1994). In contrast, a minor B cell subset in mice (the B-1a natural antibody-producing population) constitutively express CD5 (Herzenberg and Kantor, 1993) and the majority of rabbit B cells are $CD5^+$ (Raman and Knight, 1992). Since CD5 expression can be induced on human $CD5^-$ B cells following *in vitro* stimulation (Kipps, 1989), we set up experiments to probe whether this was also the case in *Xenopus*.

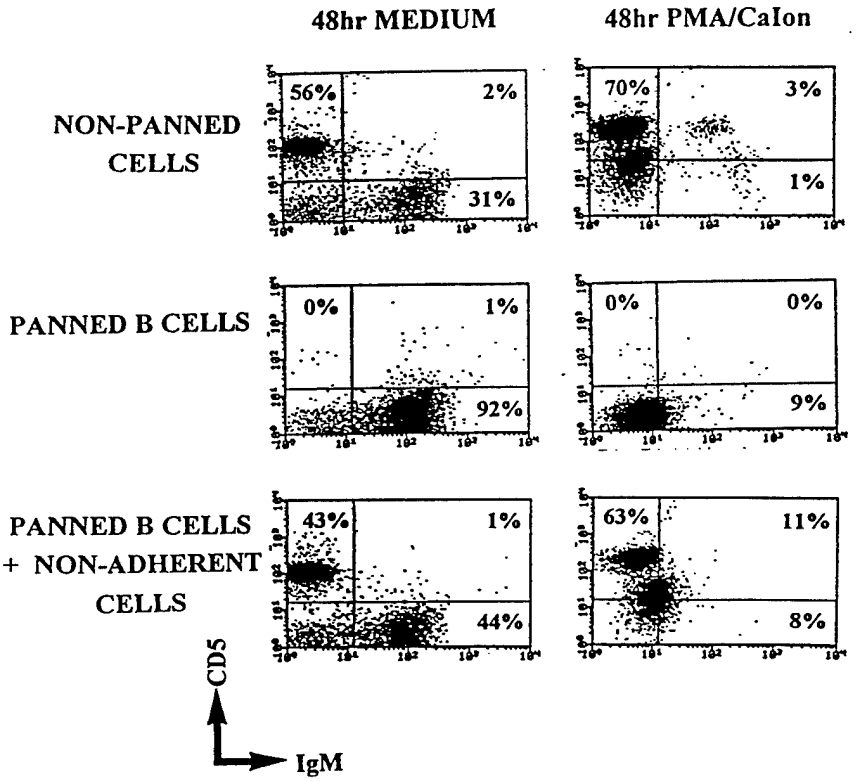


Figure 4. Emergence of CD5 on PMA/Ca Ion-activated B cells requires co-culture with T cells. Splenocytes enriched for T or B cells were prepared by incubating splenocytes from 7 month old *X. laevis* with anti-IgM mAb prior to 'panning' on anti-mouse IgG-coated petri dishes, following the techniques described by Bleicher and Cohen (1981) and Wysocki and Sato (1978). The non-adherent population (removed by gentle washing) comprised approximately 80% T cells, whereas the adherent ('panned') population (removed by vigorous pipetting) contained 90% B cells as judged by flow cytometry. Non-panned splenocytes, the positively-panned B cells alone, and the B cell population plus an equal number of non-adherent (T cell rich) splenocytes, were each cultured at similar leukocyte densities with or without PMA/Ca Ion, as described in Figure 3. After 48 hrs *in vitro*, cells were harvested and dual stained for IgM and CD5 expression and examined by dual *color* flow cytometry.

Figure 3 reveals that after 48 hours of culture in medium alone, cell surface antigenic displays by splenic lymphocytes are directly comparable to the *in vivo* situation. In contrast, following *in vitro* stimulation with the protein kinase C activator phorbol myristate acetate (PMA), together with calcium ionophore (Ca ion), agents known to be profoundly stimulatory for lymphocytes of diverse vertebrates (Rothstein *et al.*, 1986; Lin *et al.*, 1992), CD5 is induced on the surface of a proportion of activated *Xenopus* B cells. By 48 hours after stimulation (even with PMA alone) surface IgM is down-modulated from B cells, but a new focus of cells with less surface CD5 than T cells (Figure 3, top right) is now seen in flow cytometric traces. That this focus of $CD5^{+dull}$ cells represents B cells is suggested by co-staining for CD5 and MHC class II (Figure 3 bottom right), where the cells expressing more class II (that include mainly B cells) represent the $CD5^{+dull}$ population. It is possible that the presence of CD5 on the surface of activated B cells relates to shedding of antigens from stimulated T cells, rather than to induced expression of CD5 by the B cells themselves. However, the failure of the $CD5^{+dull}$ B cell population to 'pick up' any CD8 (Figure 3, middle right) argues against this.

We have now conducted further *in vitro* studies to probe PMA/Ca ion-induced expression of CD5 on *Xenopus* B cells. Figure 4 reveals the outcome of an experiment in which an IgM-panned B cell population (containing 90% IgM^+ cells) from euthymic *Xenopus* spleen is activated (IgM is down-modulated, Figure 4 - middle right), but is **not** induced to express CD5. Co-culture of panned B cells with an equal number of T cell-rich splenocytes restores the capacity of some B cells to be induced to express CD5 following PMA/Ca ion treatment (Figure 4, bottom right). Additionally we and others (Jurgens *et al.*, 1995) have shown that PMA/Ca ion-activated B cells from Tx *Xenopus* exhibit IgM down modulation but **not** expression of CD5 (Figure 5).

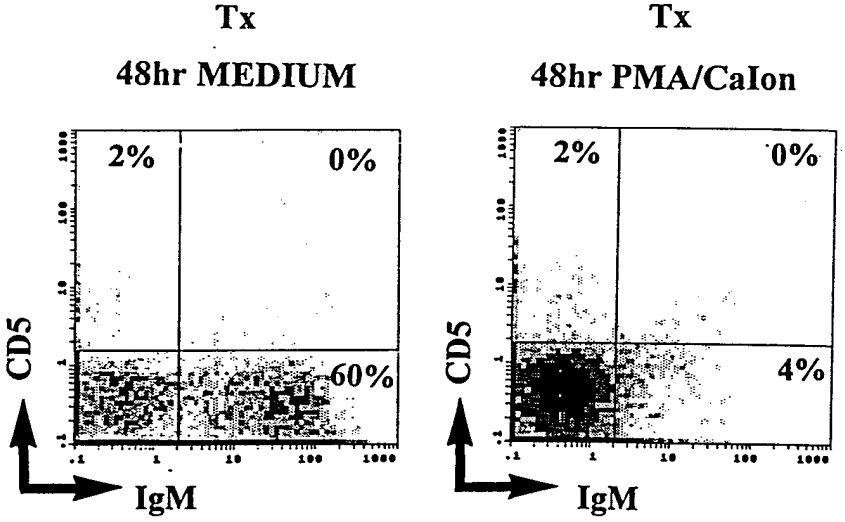


Figure 5. B cells from early-thymectomized (Tx) *Xenopus* fail to express CD5 following PMA/Ca Ion activation. Splenocytes from 6 month old control and 7 day Tx siblings were cultured in medium or PMA/Ca Ion for 48 hrs (see method in Figure 3), stained for IgM and CD5 expression and analysed by dual *color* flow cytometry.

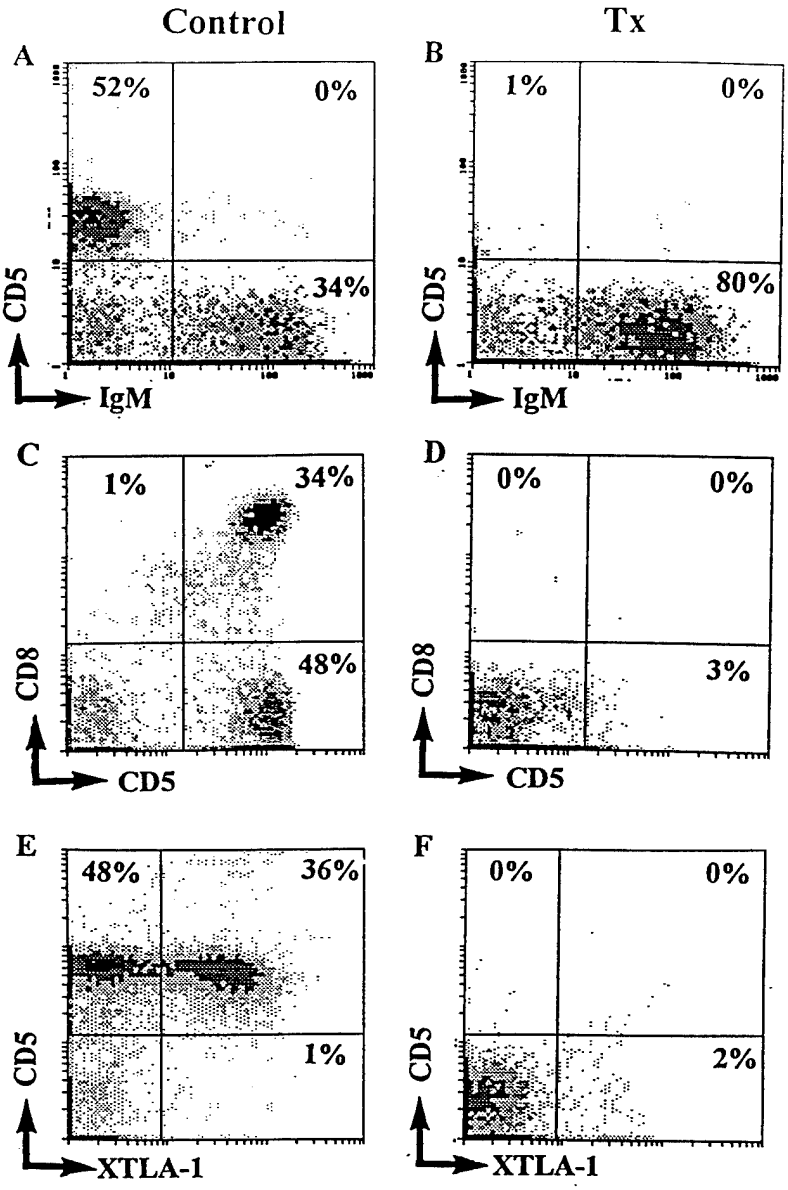


Figure 6. Effect of 5-day thymectomy on lymphocyte surface antigen expression on splenocytes from 3 month old (A/B) and 1 year old (C-F) *X. laevis*. Thymectomy is achieved by microcautery of thymus following the technique of Horton and Manning (1972). Splenocytes stained with combinations of mAbs as shown and analysed by dual color flow cytometry (see legends to Figures. 1&2).

Overall the findings indicate that *Xenopus* B cells require T cells in order to express the CD5 antigen following PMA/Ca ion activation. This contrasts recent studies on human $CD5^-$ B cells which can be activated by PMA *in vitro* to express CD5 in the absence of T cells (Zupo *et al.*, 1994). Clearly further work on induction and immunologic significance of CD5 expression by *Xenopus* B cells is warranted.

EFFECT OF EARLY THYMECTOMY ON T CELL DEVELOPMENT

Lack of T cell marker expression following thymectomy

Our experiments with recent batches of Tx *Xenopus* indicate that removal of the thymus at 5 days eliminates the development of mAb-defined T cells in the *Xenopus* spleen (Figure 6). In young frogs (3 months old) CD5 expression was not evident in the Tx spleen, whereas the percentage of IgM^+ cells increases from 34% to 80% after thymectomy (Figure 6A/B). In one year old euthymic *Xenopus* around 80% $CD5^+$ T cells were maximally found in the spleen, just under half of these co-expressing the XTLA-1 antigen (Figure 6E), a similar percentage co-expressing CD8 (Figure 6C). In spleens of one year old Tx frogs all three T cell markers were undetectable (Figure 6D,F). There was no evidence for emergence of T-like cells in these older Tx animals, contrasting previous flow cytometric analyses (Horton *et al.*, 1995).

Absence of mAb-defined T cell development has been confirmed by immunohistochemical studies and is not confined to the spleen, but is also found in blood and intestine of *Xenopus* thymectomized at 5-7 days of age (Horton *et al.*, 1994; Horton, J.D., Cooper, M.D. *et al.*, in preparation).

Functional studies on Tx *Xenopus*

Earlier work in several laboratories (including our own) indicated that early-Tx *Xenopus* can sometimes display immune responsiveness indicative of T-like cells (e.g. chronic allograft rejection [Horton and Manning, 1972; Nagata and Cohen, 1983] and low level T cell mitogen reactivity [Manning *et al.*, 1976]).

Transplantation studies have recently been carried out on the same batches of control and Tx *X. laevis* used for the cell phenotyping studies quoted above (Figure 7). MHC-disparate skin from *X. laevis/X. muelleri* LM3 clonal *Xenopus* was rejected by 5 control recipients in 16-20 days (Figure 7A/B), whereas LM3 grafts on all 5 Tx recipients remained in perfect condition for 100 days (Figure 7C). Immunohistochemical studies revealed that LM3 grafts on controls became heavily infiltrated by $CD5^+$ and $CD8^+$ lymphocytes, (Figure 7D/E). In contrast, the MHC disparate grafts on Tx animals remained devoid of such cells during a 30 day observation period (Figure 7F).

Lymphocytes removed from additional control and Tx *X. laevis* have also recently been assessed for their ability to respond 3 or 4 days following *in vitro* stimulation with optimal concentration of the T cell mitogen concanavalin A (Con A) or the B cell mitogen *E. coli* lipopolysaccharide (LPS). Con A was highly stimulatory (mean 3HTdR stimulation indices = 15) for splenocytes from 4 control *Xenopus*, whereas all 4 Tx frogs failed to respond (Horton, J.D. Cooper, M.D. *et al.* in preparation). In contrast, splenocytes from Tx animals responded better to LPS than control cells.

Extra-thymic T cell development?

The overall conclusion we now draw from studies carried out over many years on Tx *Xenopus* is that there is no substantial extra-thymic pathway of T cell development at the amphibian level of evolution. Although our studies need support from molecular biological approaches to identify T

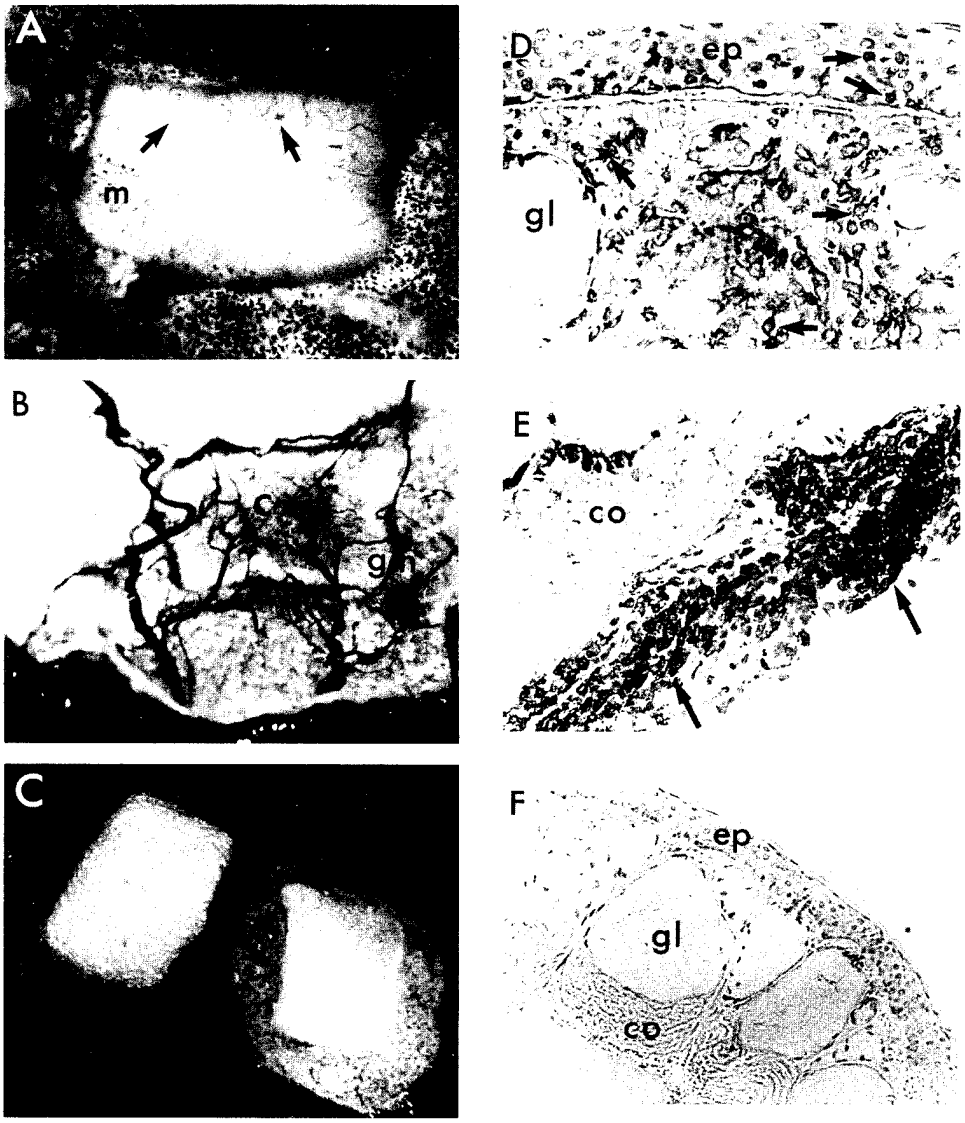


Figure 7. Outcome of transplantation of LM3 skin to 1 year old control and 7-day thymectomized *X. laevis.* **A-C** = Stereomicroscopic views of transplants removed for immunohistochemistry 20 days post-grafting. **7A** (mag x10) The transplant on the control (viewed from above) has been rejected and is now devoid of bright white pigment typical of the ventral skin of the donor but is replete with enlarged and ruptured (arrowed) capillaries. Host melanophores (m) have begun to grow in over graft edges. **7B** (x8) (Same graft viewed from underside) highlights that the vasculature leading to the graft is inflamed, with a dense network of capillaries evident under the center of the graft (c) and at the gratft/host border (g/h). **7C** (x6) Views from above (left) and from the underside (right) of grafts removed from 2 Tx animals reveal the bright white appearance of the healthy LM3 ventral donor skin and no signs of vascular disturbances. **D-F** = Immunoperoxidase-stained cryostat sections (see Gravenor *et al* [1995] for technique) through allografts. **7D** (x200) Graft removed at 10 days from control and stained for $CD5^+$ T cells, which are seen (arrows) in both glandular and epidermal graft regions. **7E** (x200) 20 day graft from control, showing mass of $CD5^+$ T lymphocytes (arrowed) below graft collagen. **7F** (x100) 20 day graft from Tx showing absence of invasive T cells. (ep) graft epidermis; (gl) glandular region; (co) graft collagen.

cell specific molecules, our findings on Tx *Xenopus* do not lend support to the contention that an extrathymic pathway of T cell development has arisen early in phylogeny (Lefrancois, 1991).

SEARCHING FOR NK-LIKE CELLS IN CONTROL AND THYMECTOMIZED XENOPUS SPLEEN

Background

In mammals natural killer (NK) cells are a lymphocyte population that typically display a large granular morphology and are found in various lymphoid tissues, especially the spleen. NK cells display 'natural' or spontaneous cytotoxic activity against certain tumour and virally-infected targets, especially those that are deficient in MHC protein expression (Yokoyama, 1993). Since NK cell activity is high in T cell deficient nude mice (Herberman *et al.*, 1975), we reason that T cell deficient frogs may provide an excellent source of NK-like cells. In general Tx *Xenopus* survive surprisingly well despite absence of T cell development and it may well be that NK-like cells play a crucial role in this situation. Although some in depth studies are characterizing candidate NK cells in chickens (e.g. Kasahara *et al.*, 1993) and have identified NK-like cells in teleost fish (Harris *et al.*, 1991), relatively little (Ghoneum *et al.*, 1990; Watkins *et al.*, 1988) is known about NK cells in amphibians.

Spontaneous cytotoxicity revealed in Tx *Xenopus*

Table 1 reviews the outcome of initial experiments searching for NK-like cell activities in spleens of 9-12 month old control and early-thymectomized *Xenopus*. In these experiments, which

Table 1.
Spontaneous cytotoxicity to allogeneic B_3B_7 thymus tumour cells: studies on control and Tx splenocytes

Incubation time	% Specific ^{51}Cr release* (Mean ±S.D.)	
	Control	Tx
6 hrs	3.3 ± 2.5 (n=6) †	20.5 ± 8.5 (n=9)
21 hrs	11.1 ± 3.5 (n=6)	32.3 ± 6.8 (n=9)

* % specific release = $\frac{\text{Experimental - Minimum}}{\text{Maximum - Minimum}} \times 100$

† splenocytes from 6 control and 9 thymectomized (Tx) *Xenopus laevis* (9-12 months old) cocultured with targets for 6 and 21 hrs, prior to monitoring ^{51}Cr release- see Horton *et al.*, 1989.
Thymectomy performed at 5-7 days of age.
Targets = 1×10^5 ^{51}Cr-labelled B_3B_7 tumour cells. Effector: target cell ratio = 25:1 ± 50:1.

followed the chromium-release protocol described by Horton *et al.* (1989), splenocytes were tested for their ability to spontaneously lyse ^{51}Cr-labelled tumour target cells over a 6 or 21 hour *in vitro* incubation period. The target cells were B3B7 thymus tumour cells derived from a spontaneous thymus lymphoid tumour discovered in *Xenopus* of the *ff* genotype (Du Pasquier and Robert, 1992; Robert *et al.*, 1994). These B3B7 cells fail to express both MHC class Ia and II molecules, but are $CD5^+$ and $CD8^+$ (Robert *et al.*, 1994; our own unpublished findings). Table 1 reveals that spontaneous cytotoxicity after 6 hours of incubation with the B3B7 targets is routinely seen (mean 20% specific Cr release) with thymectomized, but not with splenocyte effectors from controls. After 21 hours, the level of tumour cell killing mediated by Tx splenocytes increases to a mean of around 30%, and a low level of cytotoxicity effected by control cells is suggested. Lack of spleen lymphocyte numbers (especially with Tx animals) precluded testing for spontaneous cytotoxicity at effector: target ratios greater than the range 25:1 to 50:1 used.

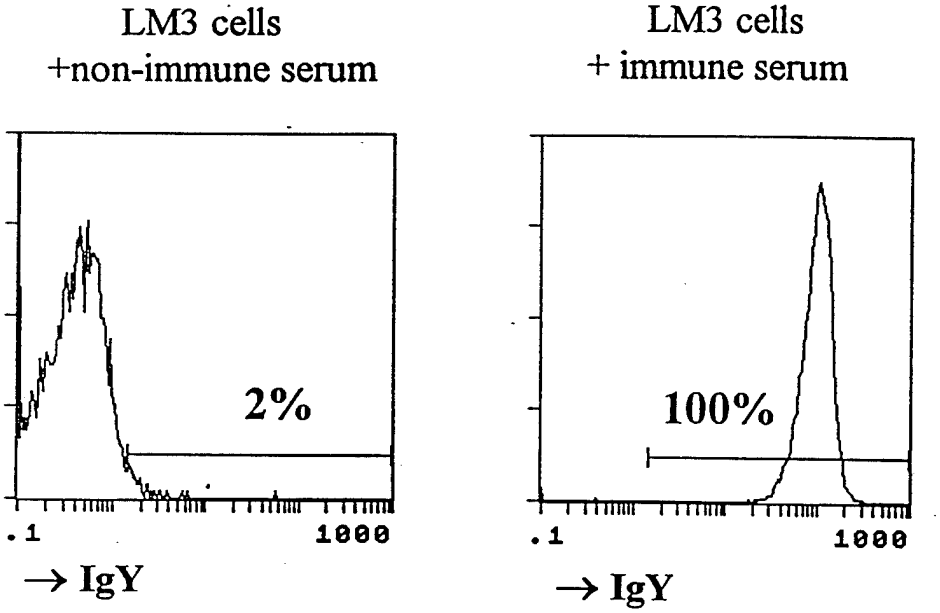


Figure 8. Single *color* flow cytometric histograms identifying IgY-coating of LM3 erythrocytes to be used as targets in ADCC experiments (see Table 2). LM3 peripheral blood was incubated for 30 mins in 1:50 serum from either non-immune or putative anti-LM3 immune *X. laevis* donors. Cells were then incubated with anti-IgY mAb (11D5 from Hsu and Du Pasquier, [1984]) and IgY-coated cells visualised with FITC-labelled anti-mouse Ig (DAKO).

ADCC

We have also tested the ability of *Xenopus* splenocytes to spontaneously lyse other types of ^{51}Cr-labelled targets. Since preliminary evidence (Jurd and Doritis, 1977), involving rabbit antibody-coated chicken erythrocyte targets, had indicated putative antibody-dependent cellular cytotoxicity (ADCC) in *Xenopus* spleen, we have probed for such a killing mechanism using 'more physiologic' (i.e. allogeneic rather than xenogeneic) target molecules. Targets chosen were erythrocytes from LM3 clonal *Xenopus* coated with IgY (IgG-like) alloantibody. The IgY alloantibody was raised by first applying 1st- and 2nd-set LM3 skin grafts to control *X. laevis* and, subsequent to 2nd-set graft rejection, the recipients received two intra-peritoneal injections of LM3 splenocytes and erythrocytes over a 1 month period. IgY-coating of LM3 was confirmed by first incubating the putative alloimmune serum with LM3 RBCs and assaying with an anti-IgY mAb using flow cytometry (Figure 8).

Table 2 reveals there was essentially no spontaneous (6 hour) killing of LM3 erythrocytes, even of the IgY-coated cells, in contrast to the distinct lysis against B_3B_7 targets achieved by splenocytes from the same Tx donors. Although this representative experiment has not identified ADCC in the *Xenopus* spleen, it does serve to highlight that the spontaneous cytotoxicity mediated by Tx splenocytes is limited to only certain appropriate target types (e.g. the MHC-deficient B_3B_7 tumour cells). We have recently found that *Xenopus* splenocytes (from controls and Tx animals) also appear

Table 2.
Failure of control and Tx splenocytes to display significant spontaneous cytotoxicity to IgY antibody-coated allogeneic erythrocytes

Splenocyte source	% specific ^{51}Cr release* - 6 hr assay		
	Target cell type		
	B3B7	LM3 RBCs +non-immune serum	LM3 RBCs +anti-LM3 IgY ab
Control	3	1	6
Tx 1	30	0	6
Tx 2	34	2	7

*% specific release = $\frac{\text{Experimental - Minimum}}{\text{Maximum - Minimum}} \times 100$

Effectors = *X. laevis* splenocytes (1 year old).
Tx = thymectomized on day 7.
Targets = 1 x 10^5 B_3B_7 (E:T ratio 30:1) or 1 x 10^4 LM3 RBCs (E:T ratio 50:1).
Min/Max ^{51}Cr release for B_3B_7 targets = 1294/4483, for LM3 RBCs + non-immune serum = 450/5146, and for LM3 RBCs coated with anti-LM3 IgY ab = 552/5476. See Horton *et al.*, 1989, for chromium-release protocol.

unable to lyse the human erythroleukemic tumour cell line K562, against which human NK cells express strong lytic activity (Andersson *et al.*, 1979).

The Future

In vitro culture of B cell-depleted splenocytes from Tx *Xenopus* with crude supernatants from T cell mitogen-activated cells or purified IL-2-like cytokine (Haynes and Cohen, 1993) is now planned in an attempt to upregulate tumour cell lysis and thereby aid identification of NK-like cells. If mAbs can be generated against *Xenopus* NK-like cell surface antigens, the experience others have had with anti-fish natural cytotoxic cell mAbs (Harris *et al.*, 1991) would indicate that such mAbs may be profitably used to search for evolutionary-conserved NK receptors in diverse vertebrates.

Acknowledgements

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Chapter 11

The Immunobiology of the Marsupial, *Monodelphis domestica*

William H. Stone, Donald A. Bruun, G. Scott Manis, Sarah B. Holste, Eric S. Hoffman, Kay D. Spong and Theresa L. Walunas

Department of Biology
Trinity University,
San Antonio, TX 78212-7200

ABSTRACT

The primary objective of our studies has been to characterize the immune system, at both the cellular and molecular level, of the marsupial, *Monodelphis domestica*, the only laboratory bred marsupial in the USA. The goal of these studies is to shed light on the origin and evolution of the immune system of placental (eutherian) mammals.

Our earlier studies have clearly shown that this marsupial exhibits a typical primary immune response, but an atypical secondary response to particulate antigens such as sheep red blood cells. In addition, *Monodelphis* fails to show a clearly defined isotype switch from IgM to IgG. Also, it fails to exhibit the typical T cell carrier effect. More recent findings demonstrate that *Monodelphis* is unable to mount a strong allogeneic mixed lymphocyte culture response against cells from the stimulating animal, even after the responders have been primed with lymphocytes and/or skin grafts from the stimulating animal. This observation suggests that the T cells of this species are atypical and/or there is little or no polymorphism at the major histocompatibility complex class II locus. In contrast, allogeneic skin grafts are readily rejected, which indicates that there is polymorphism for the class I MHC genes.

Currently, we are using molecular techniques to examine the MHC genes. We have prepared PCR primers from the conserved regions of the class I and class II genes of the Australian marsupial, the red-necked wallaby (*Macropus rufogriseus*). Preliminary data on the orthologous class II gene, amplified from a *Monodelphis* CDNA library, showed an 80% sequence homology to the wallaby gene. Both marsupial genes showed not more than 47% sequence homology to the MHC genes of eutherian mammals. Similar studies are underway to clone and sequence other genes of the immune system, such as the T cell receptor and immunoglobulin genes, in hopes of resolving some of the complexities of the marsupial immune system.

Taken as a whole, our studies suggest that the ontogeny of the immune system of the marsupial, *Monodelphis*, especially the ontogeny of T cells, is different from that of the eutherian mammal. It is interesting to speculate that these differences arose during the more than 130 million years that

separate marsupials from eutherian mammals and whether the absence of a true placenta plays a significant role in these differences.

INTRODUCTION

There is no question that we can gain tremendous insight about the origin and function of the human immune system by tracing its evolutionary history (Travis, 1993). This knowledge of the ontogeny and phylogeny of the immune system may allow us to more efficiently find treatments or cures for many of the immune-related diseases that plague humankind.

Marsupials (Metatheria) separated from eutherian mammals about 130 million years ago (Archer, 1984; Hope *et al.* 1990; Novacek, 1992). Consequently, they can provide an excellent hallmark in reconstructing the immunological evolutionary trail. Marsupials are biologically unique in several aspects which also make them ideal models for studying the immune system. For example, newborn marsupials are still in the fetal stage and thus are ideally suited for experimental situations requiring intervention during early development (VandeBerg, 1990).

Immunological studies of marsupials were initiated over 35 years ago (See Ashman *et al.* 1975, and Rowlands, 1976, for earlier studies, and Jurd, 1994, for more recent studies). However, progress has been very slow, because large numbers of animals suitable for laboratory experimentation have not been available (VandeBerg, 1990). Most of the earlier studies have been done on the North American opossum (*Didelphis virginiana*), and on the Australian quokka (*Setonix brachyurus*). In summary, it was concluded that the functional elements of the immune response in marsupials resembles that found in eutherian mammals (Bryant, 1977), but certain marsupials, especially *Didelphis*, exhibit a somewhat more primitive immune response (Rowlands, 1976). The conclusion that marsupials are "immunological cripples" is not supported by more recent studies, although marked differences in the immune machinery and response is evident between marsupials and eutherian mammals (Infante *et al., 1991).*

There is no longer a shortage of a laboratory-bred marsupial for biomedical research largely because of the efforts of VandeBerg (1990), who has successfully established a prolific colony of the South American opossum, (*Monodelphis domestica*) also known as the grey short-tailed opossum (VandeBerg and Robinson, 1995), hereafter referred to as *Monodelphis* in this paper.

PRESENT STATUS OF THE COLONY OF *MONODELPHIS*

During the past 15 years, tens of thousands of *Monodelphis* have been produced and weaned at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, Texas. In fact, some pedigrees go as far back as 22 generations. In addition, some newly wild-caught animals have been introduced into the colony and have been maintained as a separate population (Population II). Crosses between the 2 populations (Populations I and II) have yielded viable and fertile offspring. At the same time, some partially inbred strains have been produced, with an inbreeding coefficient as high as 0.674 (VandeBerg and Robinson, 1995). The effect of the introduction of these newly-introduced, wild-caught animals on the genetic structure of the breeding colonies has been discussed in detail by van Oorschot *et al.* (1992). Because pedigree records have been diligently

kept on every animal, it has been possible to conduct genetic experiments that would not have been possible otherwise (Stone *et al.* 1987; Sevilir *et al.* 1989; van Oorschot *et al.* 1990, 1992; Infante *et al.* 1991; Chiscano *et al.* 1993; VandeBerg and Robinson, 1996).

LIFE CYCLE AND HUSBANDRY

Monodelphis is an ideal laboratory animal for several reasons (VandeBerg, 1990; VandeBerg and Robinson, 1996). It is docile and can be raised singly in standard plastic mouse cages or, when in mating, in plastic rat cages containing nest boxes. A commercial, high protein, dried fox food (Reproduction Diet, Milk Specialties Products, Inc., New Holstein, WI) and water are supplied *ad libitum*. *Monodelphis* breed continuously throughout the year, and are maintained on a cycle of 14 hr light:10 hr dark conditions. The average litter size is 7 with a range from 2 to 13. Recommendations of the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985) were followed by certified animal caretakers for all work reported from our laboratory.

Animals are generally placed in mating at about 6 months of age. Estrus is induced in the female within 8 days of pairing. The gestation period is approximately 14 days. Unlike other marsupial species, *Monodelphis* does not have a pouch; thus the newborn attach to the mothers' teats and remain there for approximately 2 weeks. The animals are weaned at about 2 months and reach sexual maturity at 5-6 months. The onset of reproductive decline occurs between 18-30 months, and an animal is considered aged after 30 months. Natural, age-related death occurs between 36-42 months.

Many of the studies that use *Monodelphis* require blood samples. We have found that the most efficient and humane technique for obtaining blood samples is by cardiac puncture. Despite the small size of these animals, [year-old males weigh on average 115g and females 70g (Hubbard *et al.* 1991)], they are remarkably tolerant to chronic blood loss (Manis *et al.* 1992; Robinson and VandeBerg, 1994). Before bleeding, the animals are anesthetized by inhalation using methoxyflurane (Metofane, Pitman-Moore, Washington Crossing, NJ) in a large (4 L) beaker equipped with a plastic cover and cotton balls saturated with anesthesia at the base. For bleeding, a 3cc syringe and a 27-gauge, 0.5-inch needle is used. After bleeding, the animals are placed in a box containing a heating pad until they awake from the anesthesia.

ONTOGENY AND PHYLOGENY OF THE THYMUS GLAND

Our studies of the gross anatomy and histology of the thymus gland (Hubbard *et al.* 1991) show that the ontogeny of the *Monodelphis* thymus is similar to that of eutherian mammals. The thymus is present at birth and quite large relative to *Monodelphis* body weight, but no clear-cut cortex or medulla and no Hassall's corpuscles are visible. The thymus remains large until adulthood (6 months), when a well-defined cortex, medulla, and Hassall's corpuscles are clearly visible. At about 6 months of age, it begins to atrophy and fat replaces the cortex and medulla. By about 28 months of age, 50% of the animals have thymuses that are completely atrophied. The thymus is located in the anterior mediastinum, anterior and adjacent to the heart. *Monodelphis*, like the North American opossum, has only a thoracic thymus, whereas with few exceptions (Poskitt *et al.* 1984),

the Australian marsupials possess a cervical thymus in addition to a thoracic thymus. This is of some evolutionary significance, since it supports the view that the North and South American marsupials are more closely related to each other than either is to the Australian species.

HISTOPATHOLOGY OF THE IMMUNE SYSTEM

We have studied the lymphoreticular system through gross dissection, histological examination, and immunohistochemical staining of *Monodelphis* lymphoid tissues (Unpublished). Although the microscopic structural organization of the immune system is similar to humans, differences exist. Plasma cells are present in both lymph nodes and splenic sinuses despite serological studies that have demonstrated a weak secondary humoral response to particulate antigens such as sheep red blood cells (SRBC) (Croix *et al.* 1989). However, there does not appear to be any correlation between intraperitoneal immunization status and activation of germinal centers, which may imply that T cell help is impaired. In general, the splenic sinuses and thymic epithelial complexes are poorly developed, and compartmentalization of the B cell populations is clearly evident using specific isotypic (anti-B and anti-T cell) monoclonal antibodies. Still unexplained is the finding that the neutrophils do not contain the same complement of the hydrolytic enzyme (chloroacetate esterase) that is found in eutherian mammals. It is interesting to note that the lymph nodes are extremely difficult to locate by gross inspection. Those most easily located are in the bowel mesentery. Curiously, there are no differences between immunized and unimmunized animals in nodal size or cellularity. In contrast, the spleen is easily located caudad to the stomach within the left peritoneal cavity. The average weight of the spleen of adults is 265mg (Range 189-330mg), which is 0.3% of the average body weight; this is exceptionally large for such a small animal.

Coutinho *et al.* (1993) studied the organization of the gut cellular immune components in the marsupial, *Didelphis albiventris* (the white belly opossum) using monoclonal and polyclonal antibodies against human lymphocyte specificities. They found MHC class II-like antigens in the Peyer's patches and in cells of the follicular-associated epithelium suggesting that these cells act as antigen-presenting cells for T cells. Also, Jones *et al.* (1993) demonstrated B cells in opossum spleen using a polyclonal anti-human pan B cell antibody.

B AND T LYMPHOCYTES

We were unable to separate B and T cells of *Monodelphis* using rosetting with SRBC (Unpublished). Thus, it is likely that this species, unlike eutherian mammals, lacks the SRBC receptor. However, B and T cells were separated efficiently using nylon wool columns and their proportions were similar to those reported for humans by Greaves *et al.* (1974). In peripheral blood of both sexes, we found 76 ± 3% non-adherent cells, which were shown by monoclonal antibodies directed against T cells (raised in Balb/C mice to PBMCs and thymocytes) and by activity in mixed lymphocyte cultures (MLC) to be T cells. We found 24 ± 3% adherent cells, which were shown to be B cells, because they expressed surface immunoglobulin (Ig). This was demonstrated by treating the adherent population with a rabbit anti-*Monodelphis* Ig serum (prepared in our laboratory) followed by a commercial goat anti-rabbit FITC (fluorescein-isothiocyanate) antiserum. Using a fluorescence microscope, we found that a large majority of the adherent cells were labelled. In

contrast, very few of the non-adherent (T cells) were labelled when they were treated in the same way (Infante *et al.* 1991). We confirmed these results using a FACS^{TM} Analyzer (Becton-Dickinson Co.). We also examined the proportion of B and T cells in spleen and in lymph nodes and found that the proportions were very similar to those found in eutherian mammals.

Attempts to define sub-populations of lymphocytes using a battery of monoclonal antibodies that we produced against peripheral blood mononuclear cells (PBMCs) as well as against thymocytes have not yielded clear-cut results. However, there was suggestive evidence of some B and T lymphocyte sub-populations (Unpublished). This is an area of research which requires further investigation.

IMMUNOGLOBULINS

Purified IgG and IgM, as well as their respective heavy chains, were prepared from adult non-immunized *Monodelphis* by caprylic acid and ammonium sulfate precipitation (McKiney and Parkinson, 1987) and column fractionation (Coligan *et al.* 1994). These preparations were shown to be specific by immunoelectrophoresis and Western blot analysis using anti-Ig sera that we produced in rabbits. We found definite evidence of at least 2 sub-classes of IgG. Evidence for 2 IgG sub-classes in *Monodelphis* has been presented recently by Shearer *et al.* (1996). Interestingly, only 2 IgG sub-classes were detected in *Didelphis* serum (Rowlands and Dudley, 1969; Rowlands, 1970). However, in humans, for example, there are 4 IgG sub-classes, only 2 of which cross the placental barrier (Coffman *et al.* 1993). Since marsupials do not possess a true (eutherian) placenta, perhaps there is no functional need for the 2 classes of IgG that cross the placenta, although Bell (1977) reported that the quokka had 2 types of light chains and 4 Ig sub-classes. In summary, the bulk of the evidence suggests that the Igs of marsupials are not structurally or functionally different from those of eutherian mammals despite the differences in the number of kinds of isotypes.

CYTOKINES AND COMPLEMENT

Brozek and Ley (1991) reported that *Monodelphis* interleukin-1 (IL-1), obtained from lipopolysaccharide-stimulated macrophage and skin cultures, did not stimulate proliferation of mice thymocytes. Also, neither human nor mouse IL-1 stimulated *Monodelphis* thymocytes, and there was no serological cross-reactivity between an anti-human IL-1 antiserum and *Monodelphis* IL-1. Taken as a whole, these results indicate that the IL-1 of *Monodelphis* is different from the IL-1 of humans and mice. These are interesting results, because IL-1 is presumably a highly-conserved molecule throughout the animal kingdom. Further studies, including those at the molecular level, need to be done to explain these inconsistencies. Wilkinson *et al.* (1992b) reported that Concanavalin A (Con A)-stimulated PBMCs of the koala (*Phaseolarctos cinereus*) produced an IL-2-like growth factor. Unfortunately, there are no detailed studies of the cytokines of marsupials. This is another area which would benefit from further study.

Complement has not been studied extensively in marsupials. One study (Wirtz and Westfall, 1967) reported lower activity in marsupials than in guinea pigs with reduced C1 levels. More recently, Koppenheffer, *et al.* (1994) investigated the classical and alternative pathways of *Monodelphis* and

concluded that the complement activation pathways required divalent cations and complement activators similar to those of eutherian mammals.

MATERNAL-FETAL RELATIONSHIP

It is well-known that in eutherian mammals the transfer of passive immunity from mother to offspring occurs transplacentally before birth and/or via the milk after birth. Clearly, *Monodelphis* and other marsupials lacking a true placenta have rather stringent limitations imposed on their ability to acquire passive immunity from the mother. We have concluded that *Monodelphis* newborn obtains its immunity entirely via the mother's milk (Samples, *et al.* 1986). Pregnant females were immunized with SRBC, and anti-SRBC hemolysins were detected in the mother's milk and in saline extracts of the newborn only if the newborn suckled the mother. More recently, Wild (1994) clearly demonstrated that passively acquired antibodies cross the gut of the newborn *Monodelphis* and can be found in the newborn's circulation. However, Deane *et al.* (1990) demonstrated passage of IgG through the yolk sac placenta to the fetus and neonates in the tammar wallaby (*Macropus eugenii*). Thus, while it is generally agreed that the newborn marsupial is unable to synthesize Igs, there does appear to be some variation in the manner in which the newborn marsupial acquires its passive immunity.

We were unable to demonstrate any lymphocytotoxic antibodies in *Monodelphis* mothers (Samples *et al.* 1986). This agrees with the observations of van Oorschot and Cooper (1988), who were unable to detect complement-dependent cytotoxic antibodies in parous tammar wallabies. It seems safe to conclude that there is no trans-yolk sac immunization of the pregnant female by lymphocytes of the fetus in *Monodelphis*. This is in stark contrast to humans, where many lymphocytotoxic antibodies can be harvested from maternal serum (Amos and Kostyn, 1980; Chao, 1993). Perhaps the short period during which the conceptus remains in the uterus is not long enough for fetal-maternal immunization (van Oorschot and Cooper, 1988).

HUMORAL IMMUNE RESPONSE

We developed a micro-hemolytic assay using guinea pig complement to measure the humoral antibody response of *Monodelphis* to SRBC. The primary response was similar to that observed in other mammalian species, but the secondary response was weaker and less persistent than in eutherian mammals. In fact, the shape and amplitude of the secondary response curve was almost identical to that of the primary response (Croix *et al.* 1989). Several repetitions of these experiments have confirmed our original results (Unpublished). Thus, we feel confident in concluding that the secondary response of *Monodelphis* to a particulate immunogen such as SRBC is different from that in eutherian mammals. Earlier studies with particulate immunogens in other marsupials appear to parallel our observations of a somewhat weaker and atypical secondary immune response (Jurd, 1994).

We have been unable to produce precipitating antibodies against serum proteins, such as human Ig (HGG) and albumin (HSA) (Unpublished). Difficulty in producing antibodies in opossums to soluble antigens such as bovine serum albumin and hemocyanin was reported by Taylor and Burrel (1968) and Marx *et al.* (1971). However, using an enzyme-linked immunosorbent assay (ELISA),

we were able to detect antibodies against the protein, keyhole limpet hemocyanin (KLH). But the secondary response to this soluble immunogen was noticeably weaker than normally observed in eutherian mammals (Unpublished). Weak secondary responses to both particulate and soluble immunogens were noted also in other marsupials (Rowlands, 1976; Wilkinson, *et al.* 1992a).

ISOTYPE SWITCH

In view of the relatively weak secondary response exhibited by *Monodelphis* to SRBC, we were not surprised to find that they did not exhibit the typical mammalian isotype switch (Roitt, 1994; Coffman *et al.* 1993) from IgM as the primary response antibody to IgG as the predominant secondary response antibody (Unpublished). We screened *Monodelphis* sera by FACS for IgM and IgG antibodies to SRBC. Serum samples were obtained at weekly intervals during the primary and secondary response. Using rabbit antisera produced in our laboratory and shown by Western blot to be specific for either IgM (μ heavy chain) or IgG (γ heavy chain), we found both IgM and IgG anti-SRBC antibodies during the primary and the secondary response, although it appeared as though the concentration of IgG was slightly higher than the IgM concentration during the secondary response.

We obtained similar results using antisera produced against the soluble antigen, KLH. The kinetic curves that were generated using an ELISA indicated that, in *Monodelphis*, the appearance of IgM and IgG in response to immunization with a soluble immunogen (such as KLH) and adjuvants is indistinguishable with respect to time to first antibody response, as well as to the intensity and duration of response. In addition, the primary and secondary response curves (as against SRBC) were also indistinguishable (Unpublished). Recently, Shearer *et al.* (1995) measured the humoral immune response in *Monodelphis* to soluble immunogens and found, as we did, that both IgM and IgG were detectable in both the primary and the secondary responses. Thus, we conclude that *Monodelphis* exhibits an unusual isotype switch.

T CELL CARRIER EFFECT

One of the hallmarks of the humoral secondary immune response in eutherian mammals is the so-called T cell carrier effect (Katz *et al.* 1970) in which a secondary response to a hapten requires that the carrier molecule used in the booster injection be the same as the one used in the priming injection. We used dinitrophenol (DNP) as the hapten and KLH as the carrier and immunized *Monodelphis* subcutaneously with Freund's complete adjuvant. We measured the immune response by an ELISA assay. The results (unpublished) clearly showed that *Monodelphis* does not exhibit a typical T cell carrier effect. Once again, these provocative results support our hypothesis that *Monodelphis* T cells (T helper cells) do not function in the same manner as eutherian T cells.

CELLULAR IMMUNITY AND THE MHC

The question of whether cellular immunity is well-developed in newborn *Monodelphis* remains unanswered despite the report by Fadem and Hill (1985) and Fadem *et al.* (1988) that newborn *Monodelphis* can tolerate grafts of a mouse melanoma. In our experiments, transplanted Hela cells were rejected by the newborn *Monodelphis* (Unpublished). Further, we could not induce acquired tolerance in *Monodelphis* who were immunized as neonates with either Hela cells or mouse (Balb C) lymphocytes.

The MHC of eutherian mammals (human and mice) have been extensively studied (Klein, 1986; Strominger, 1989). The overall organization of the MHC consists of several linked genes representing 3.5-4.0 million base pairs (bp) in humans. The major function of the MHC molecules is to present processed antigen to T cells. The class I molecules bind peptides from intracellular antigens, whereas Class II molecules bind peptides from extra-cellular antigens. (Cruse and Lewis, 1994).

CLASS I ANTIGENS

We produced 30 lymphocytotoxic antisera by skin grafting and alloimmunization in *Monodelphis*. Of these, 18 appeared to detect from 5-7 class I antigens. Genetic studies suggested that these antigens are controlled by genes at 2 or more autosomal loci (Stone *et al.* 1987). Evidence that these genes are encoding class I antigens of the MHC is supported by the fact that the antisera were produced by tail skin grafting and were directed to antigens on the lymphocyte membrane, and because allografts made between adult individuals matched for these antigens lasted significantly longer than grafts made between adult individuals mismatched for these antigens. Further support came from our molecular studies in which a mouse class I CDNA probe (pH-2IIa) coding for a relatively conserved region, hybridized in Southern blots with 3 or 4 (depending upon stringency) *Monodelphis* DNA fragments obtained by EcoR1 enzyme digestion. These results suggest that the class I genes of Monodelphis and mice share at least 70% sequence similarity.

Earlier, we reported (Infante *et al.* 1991) that allogeneic tail skin transplants were rejected in a manner not unlike those in eutherian mammals. Interestingly, second-set grafts were not rejected as rapidly as expected. (In eutherians, second-set grafts are usually rejected in about half the time it takes for first-set grafts to be rejected). These data add further credence to our hypothesis that the secondary response in *Monodelphis* is quite unlike that of eutherian mammals. More extensive skin graft studies completed recently (unpublished), confirm these earlier results and more precisely identify the chronology of the allograft response. Grafts were made within and between two genetically different populations of *Monodelphis* (See above, "Present Status of the Colony of *Monodelphis*"). The genetic relationships of the donors and recipients were defined in terms of their kinship coefficient (KC). The KC is a measure of genetic relationship between 2 animals and is equivalent to the inbreeding coefficient of actual or hypothetical offspring of the two individuals (Hartl and Clark, 1989). The average time of onset of rejection of first-set grafts was 18.6 ± 6.2 days, and the average time to complete rejection was 30.6 ± 6.4 days. As expected, there was a correlation between the graft response and the KC. In other words, grafts between closely related animals (high KC) were retained longer than those between distantly related (low KC) animals.

CLASS II ANTIGENS AND THE MIXED LYMPHOCYTE RESPONSE

The mixed lymphocyte culture response (MLR) is a measure of T cell function and is highly dependent on polymorphism at the class II loci (Lachmann *et al.* 1993). When lymphocytes (responding cells) are mixed in culture with allogeneic irradiated lymphocytes (stimulating cells), the immunologically competent responding cells will respond to the stimulating cells, as evidenced by the incorporation of tritiated thymidine (^{3}HTdR). Evidence that the responding lymphocytes are indeed capable of responding is usually obtained by treating the cells with a mitogen. We routinely used Con A as the mitogen of choice with *Monodelphis* (Infante, *et al.* 1991).

Assays of nylon-wool-separated lymphocytes showed that the non-adherent cells (T cells) cultured alone or with irradiated adherent cells (B cells) are readily stimulated by Con A, while the adherent population is not. These results suggest that *Monodelphis* T lymphocytes proliferate with mitogens in the absence of accessory cells, in contrast to the response of eutherian lymphocytes (e.g., mouse). Our preliminary results (Unpublished) showed that *Monodelphis* PBMCs proliferated in response to certain bacterial superantigens, suggesting conservation of important structures involved in T cell antigen recognition. Later, Brozek *et al.* (1992) reported that *Monodelphis* lymphocytes were easily stimulated with Con A, phytohemagglutinin (PHA), and pokeweed mitogen (PWM) but, curiously, not with bacterial lipopolysaccharide (LPS). Wilkinson *et al.* (1992b) reported that the optimum responses to mitogens in the koala generally paralleled those required for eutherian mammals.

The most intriguing result in our study of the class II genes in *Monodelphis* was the failure to detect a strong MLR response with allogeneic cells (Infante *et al.* 1991). We are not alone in this finding—Fox, *et al.* (1976) reported a weak or absent MLR response in *Didelphis*. Brozek (Personal Communication), failed to detect a significant MLR response in *Monodelphis* using whole blood, and Wilkinson, *et al.* (1992a) reported the virtual absence of an MLR response in koalas even though the lymphocytes responded strongly to mitogens (Wilkinson *et al.*, 1992b). More recently, Zuccolotto, *et al.* (1995) examined T cell function in the tammar wallaby and found that the MLR response was virtually absent, and Harris (1995) also failed to generate an appreciable MLR response in the Australian opossum (*Trichosurus vulpecula*) despite extensive attempts to optimize the culture conditions.

One possible explanation for the failure to obtain an MLR response in *Monodelphis* is that it could be an artifact of the technique by which the lymphocytes were purified. We ruled out this possibility, since we obtained good mitogen response, but no MLR responses when the cells were prepared by a variety of purification techniques (such as ficoll-hypaque, nylon wool column separation, and differential centrifugation). Even using whole blood, we observed good mitogen stimulation but no MLR response.

Significantly, we found (Unpublished) that the MLR response could not be augmented even after the responding animal had rejected skin grafts and/or had been immunized with lymphocytes from the stimulating animal. Taken together, these results strongly indicate that *Monodelphis* (and several other marsupials) do not exhibit an MLR response either because their T cells are unusual and/or they have little if any polymorphism at the MHC class II loci. Consistent with this latter possibility is the recent report by McKenzie and Cooper (1994) that describes the very low degree

(%) of class II polymorphism in the tammar wallaby based on the absence of variability using restriction fragment length polymorphism (RFLP) at the class II ß-chain encoding loci. These authors suggest that the degree of polymorphism in the MHR class II genes is somewhere between that observed in cheetahs and the lions from the Ngorongoro crater (O'Brien and Evermann, 1988; Parker *et al.* 1991). Recently, Mikko and Andersson (1995) reported a low class II polymorphism in European and North American moose presumably due to a population bottleneck. Whether or not polymorphism exists for the class II genes in *Monodelphis* is currently being tested in our laboratory.

MOLECULAR STUDIES

MHC

Recently, we have turned our attention to molecular studies of the genes of the *Monodelphis* immune system in hopes of resolving some of the complexity of the immune system of this species. We hope to compare directly the DNA sequences of MHC genes of the metatherian and eutherian species, a strategy which will perhaps throw some light on the evolution of the immune system.

A genomic library was prepared from *Monodelphis* liver, and a CDNA library was prepared from the spleen (Sambrook *et al.* 1989). We obtained a number of DNA probes to use in screening these libraries, including a human class I MHC probe (pH-2IIa), which we used in our preliminary studies to detect class I genes (Stone *et al.* 1987), and 2 additional class I probes (UB-1 & UB-2) from the red-necked wallaby, kindly supplied by Dr. Jan Klein. In addition, Dr. Klein supplied us with 2 class II probes (DAB & DBB) from the red-necked wallaby, and Dr. Ellen Kraig provided the human class II (DQß) probe. We used these probes to screen these libraries under standard stringency conditions (Sambrook *et al.* 1989; Ausbel, *et al.* 1989). Initial screening of 10^6 plaques of the genomic library with the DQß probe using a chemiluminescent, non-isotopic technique (Martin *et al.* 1990) gave us a number of weakly positive clones, but none that could be confirmed as truly positive on secondary screening. These negative findings are similar to those of Schneider, *et al.* (1991), who could not obtain a definitive DQß response in the red-necked wallaby.

These preliminary studies indicate that the similarity between the DNA sequence of *Monodelphis* and eutherian mammals (from which our probes were derived) is too low to hybridize to homologous genes. However, we have been able to obtain positive clones using MHC probes from red-necked wallaby. We constructed primers for PCR from known DNA sequences of the Class II genes of the wallaby to amplify the homologous genes in *Monodelphis*. The primers were located in conserved regions of Exons II and III of the wallaby sequence. As expected, we were able to amplify a clone directly from the cDNA library. Using these primers in conjunction with primers flanking the vector's cloning site, we have been able to amplify and sequence an entire *Monodelphis* class II gene. The results show 80% homology with a wallaby gene (Bruun *et al.* 1995; Stone *et al.* 1995). We have also screened plaques from each of the libraries with the class I PCR primers, but these studies are ongoing.

Reports have recently appeared dealing with molecular studies of the MHC of other marsupial species. Schneider *et al.* (1991) isolated class II beta chain-encoding MHC genes in the red-necked wallaby, whose sequences were not orthologous (a gene that has evolved directly from an ancestral locus) to any of the genes in eutherian mammals studied thus far. One of the 3 sequences appeared to be derived from a processed pseudogene. These authors suggested that the class II gene family of eutherians and metatherian mammals evolved from different ancestral genes.

Greville and Houlden (Personal communication) have identified three MHC class IIß loci in koalas. Two expressed loci, referred to as Phci-DAB1 and Phci-DBB1, appear to be orthologous to Maru-DAB1 and Maru-DBB respectively previously identified by Schneider *et al.* (1991). Phci-DAB1 alleles show about 86% homology with Maru-DAB1 (300bp exon II and III) and Phci-DBB1 alleles show about 92% homology with Maru-DBB (200bp exon II and III). From 12 captive bred koalas, they have identified 5 alleles for Phci-DAB1 locus (33% heterozygosity) and 3 alleles for Phci-DBB1 locus (no heterozygosity). The third locus is most likely a processed pseudogene, lacking an intron and containing a stop codon.

Mayer *et al.* (1993) identified 3 different full-length class I MHC sequences in a cDNA prepared from spleen mRNA of the red-necked wallaby. At least 2 loci were inferred, and the sequences suggested that the marsupial class I genes arose from a different ancestral element than the class I genes of eutherians.

A similar study in the tammar wallaby by Slade *et al.* (1994) reported that they had cloned 3 genes related to the human class II DQα genes and the mouse MHC (H-20a) genes. *In situ* hybridization to the tammar wallaby chromosomes mapped these genes to the long arm of chromosome 1. However, in contrast to Schneider *et al.* (1991), these authors did not conclude that the class II gene families of eutherian and metatherian mammals evolved from different ancestral genes.

In view of the findings by McKenzie and Cooper (1994) that there is very low polymorphism at the class II loci in the tammar wallaby, it would be quite interesting to determine if the genetic variation in the class II genes reported by Slade *et al.* (1994) represents a significant class II polymorphism.

Bronwyn (Personal communication) demonstrated that koala MHC class I constitutes a polymorphic, multi-gene family. Six partial exon II and III (MHC) class I sequences were evident from at least 3 loci from a single koala. Polymorphic variation was detected by examination of sequences from a number of individuals. Comparisons revealed approximately 80% homology with sequences from the red-necked wallaby. Phylogenetic analysis demonstrated that all koala sequences are more related to one another than they are to any of the wallaby loci. This indicates that they are probably not orthologous genes. Thus, the koala sequences represent a new class I gene family. According to Greville (as reported by Bronwyn), this contrasts with the orthologous relationship evident between class II sequences from koala and those from red-necked wallaby.

Ig genes

Molecular studies of the *Monodelphis* Igs are in their infancy. Miller (Personal Communication) has been attempting to identify and clone V, D, J, and constant region gene segments from the Ig heavy chain locus (Igh) and the genes which encode proteins known to be important for V(D)J recombination. Toward these goals, Miller and co-workers have amplified, cloned and sequenced a region of the *Monodelphis* recombination activation gene 1 (Rag1) and have identified a candidate clone of the terminal deoxynucleotidyl transferase (TdT) gene. Both of these cloning procedures were performed using degenerate oligonucleotide "cocktails" to amplify a region of genomic DNA by PCR. They initially focused on Rag1 and TdT as interesting markers for developmental stages. In all mammalian species so far studied, Rag1 expression is an obligatory step for V(D)J recombination to occur. They intend to use Rag1 expression as a marker for when lymphocyte ontogeny first reaches the point where V(D)J recombination is initiated. In mice and humans, TdT expression is postnatal, usually detected in the first week of life (Bogue *et al.* 1992). Fetal and neonatal antibodies rarely contain N nucleotide additions. These studies are destined to elucidate the timing of TdT expression in *Monodelphis*.

Miller has shown that the *Monodelphis* Rag1 gene has approximately 90% identity with the human and mice Rag1 genes at the amino acid level. They have also cloned the Rag1 gene from *Didelphis*, but the sequence data have not yet been compared to other marsupial genes.

SUMMARY AND CONCLUSIONS

Research with *Monodelphis* has already contributed to the pool of knowledge regarding the evolution of the immune system. But much more remains to be done. It appears that the immune system of *Monodelphis*, and perhaps other marsupials, has not evolved quite to the level of that of true placental mammals. In many respects, its immune system more closely resembles that of the salamander (Cohen, 1980) than even that of the frog (DuPasquier *et al.* 1989). The molecular studies are likely to help us understand the structural changes that may account for the atypical aspects of the immune system of *Monodelphis* and other marsupials. As stated by Jurd (1994), "One also looks forward to the application of molecular biology and immunogenetic techniques to the elucidation of such features as the MHC map and the nature of the immunoglobulin genes...".

It is hoped that *Monodelphis* will play an increasingly important role in basic and biomedical research of the immune system.

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Chapter 12

Molecular Characterization of an Evolutionarily Conserved Function-Associated Molecule (FAM) on Natural Killer (NK) Cells

David T. Harris, Ph.D. and Reuben Kapur, Ph.D.

Department of Microbiology and Immunology
Bldg. 90-Main Campus
University of Arizona
Tucson, AZ 85721

ABSTRACT

The role of a novel function-associated molecule (FAM) expressed on natural killer (NK) cells and cells displaying NK-like activity has been analyzed and characterized. The FAM was identified using a monoclonal antibody (mAb) isolated by immunizing mice with fish NK cells (termed NCC, natural cytotoxic cells). NCC are capable of recognizing and lysing human and rodent NK-sensitive tumor cell lines. The mAb (termed 5C6) isolated in this fashion reacted with a 43-45 kDa cell surface molecule present on NK cells of fish, mouse, rat and man; indicating the presence of a highly evolutionarily conserved molecule. This FAM was also present on LAK cells and non-MHC-restricted T cells. Two color flow cytometry demonstrated that FAM was unique in relation to all other known NK and T cell molecules. The anti-FAM mAb was capable of inhibiting NK lysis in all species examined. Inhibition of NK function was mediated via an inhibition of conjugate formation (i.e., recognition). When FAM was removed from the surface of NK cells by mAb-mediated modulation, the FAM (-) effector cells were severely impaired in functional capabilities. Further, expression of the anti-FAM mAb on the surface of normally insensitive target cells resulted in lysis of the target cells. Triggering of the FAM by mAb-mediated crosslinking resulted in signal transduction activities as shown by phosphatidylinositol lipid hydrolysis, mobilization of intracellular calcium, expression of activation antigens, and secretion of lymphokines. Protein and molecular characterization of FAM revealed that it was highly homologous to the intracellular intermediate filament protein, vimentin. Treatment of NK cells with anti-sense oligonucleotides directed against the vimentin mRNA inhibited FAM expression as assessed by flow cytometry, with a resultant inhibition of NK cell function. Thus, FAM is a novel molecule that appears to function as a receptor involved in NK cell recognition processes that lead to NK cell activation and triggering of NK cell function.

INTRODUCTION

Natural killer (NK) cells exhibit "natural" cytolytic activity against virus-infected cells and certain tumor cells in a non-major histocompatibility complex (MHC) restricted fashion (Herberman *et al.*, 1975). NK cells are usually defined as CD16+56+3- large granular lymphocytes (LGL) in humans (Herberman and Ortaldo, 1981; Reynolds *et al.*, 1985), and by the expression of the surface marker NK1.1 in the mouse (Koo and Peppard, 1984). The equivalent of mammalian NK cells (termed nonspecific cytotoxic cells, NCC) have been observed in several lower vertebrate species, e.g. teleost fish (Graves *et al.*, 1984; Evans *et al.*, 1984a; Evans *et al.*, 1984b; Evans *et al.*, 1984c), and is consistent with the concept that the NK cell system developed early in the evolution of the immune system. The observation that fish NCC were capable of recognizing and killing the same target cell lines as human NK cells (Evans *et al.*, 1984a; Jaso-Friedman *et al.*, 1988) led us to hypothesize that fish and human NK cells may recognize the same target cell antigens, and may utilize the same or very similar cell surface structures (receptors) for this purpose. Although NK cell antigen receptors capable of recognizing MHC class I antigens have now been identified, these receptors appear to be limited in their expression and distribution, and do not appear to be the only ones involved in NK cell function. These molecules include several molecules present on human NK cells (Ciccone *et al.*, 1992; Lanier *et al.*, 1995), NKR-P1 on rat NK cells (Chambers *et al.*, 1989; Giorda *et al.*, 1990; Giorda and Trucco, 1991), and NK1.1, 5E6 and Ly49 expressed on murine NK cells (Stoneman *et al.*, 1995; Yokoyama, 1995). Due to the abundance of receptor-like molecules in NK cells it is quite probable that many others remain to be identified and characterized.

Previously, using a monoclonal antibody (mAb) directed against a novel function-associated molecule (FAM), we identified an evolutionarily-conserved structure on fish (Evans *et al.*, 1988), rat (Friedman *et al.*, 1992), human (Harris *et al.*, 1991), and mouse NK cells (Kapur *et al.*, 1994). In the teleost fish, expression of FAM is restricted to the natural cytotoxic cells (NCC), which are thought to be equivalent to mammalian NK cells. On murine NK cells, the expression of FAM is restricted to NK cells that express the NK1.1 antigen. In the rat the expression of FAM is limited to cells expressing the NKR-P1 molecule. In the human system, FAM expression is only seen on NK cells and T cells displaying NK-like functions (i.e., non-MHC-restricted T cells). Activation of NK cells to become LAK cells results in higher levels of FAM expression. The results obtained in these studies have indicated that FAM is a novel molecule that possesses all of the characteristics of a receptor molecule. Further, it appears to be intimately involved in the processes of antigen recognition and activation of NK cell function. Thus, this highly evolutionarily conserved molecule may have been one of the first antigen receptors on NK cells as this effector system evolved during the development of the immune system. As FAM appears to be a possible candidate for an antigen receptor, experiments were conducted to analyze and characterize this molecule in an attempt to investigate its role in NK cell function.

MATERIALS AND METHODS

NK Cells. Natural cytotoxic cells (NCC) were obtained from the head kidney of *Ictalurus punctatus* (catfish) and purified as described previously (Evans *et al.*, 1988). C57BL/6 mice were used as a source of murine NK cells and used in experiments at 6 to 10 weeks of age. In some experiments, spleen mononuclear leukocytes were passed over nylon wool columns to remove

macrophages and B cells, and enrich for NK cells. Mouse lymphokine activated killer (LAK) cells were obtained by culturing fresh NWNA cells in complete medium supplemented with 250 U/mL rIL-2 at 2 $X10^6$ cells per mL for a period of at least 5 days. Adherent lymphokine activated killer (ALAK) cells were derived by a procedure described previously (Vujanovic *et al.*, 1988). Rat NK and LAK cells were obtained from spleens of Fischer 344 rats as described (Friedman *et al.*, 1992). Heparinized peripheral blood obtained from young donors (aged 18-30 years) was used as a source of human NK cells. Peripheral blood lymphocytes (PBLs) were obtained by centrifugation of blood over Ficoll-Hypaque gradients and adherence (2X) to plastic plates. Purified NK cells were obtained by first panning these cells over anti-Ig coated Petri dishes to remove B cells, followed by panning over mAb OKT3 coated Petri dishes to remove T cells. NK cells isolated as described were used as a source of fresh, endogenous human NK cells (80-95% CD16+, 5-20% CD3+). Lymphokine-activated killer (LAK) cells were obtained by culturing the above cells for 5 days in RPMI-10% FBS medium containing 1000 units/mL recombinant human IL 2.

Derivation of monoclonal antibody

The anti-FAM mAb 5C6 was derived as described (Evans *et al.*, 1988). Briefly, mice were immunized with purified fish nonspecific cytotoxic (NCC) cells (from *Ictalurus punctatus*), spleen cells were fused with the P3-X63-Ag8.653 myeloma, and wells positive for growth were screened by ELISA and flow cytometry with NCC. The anti-FAM mAb was used either as hybridoma supernatants or was purified by TSD BioServices, a Taconic affiliate (Germantown, NY 12526). The anti-FAM mAb selected for use in the experiments was of the IgM isotype.

Cytotoxicity assays

Effector cells were tested for cytolytic activity in a standard microcytotoxicity assay (Koren *et al.*, 1981). Lytic units at the 20% lysis level (LU20) for 1 X 10^6 effector cells were calculated by computer-assisted regression analysis as described by Pross *et al*, 1981. All effector to target (E/T) ratios were performed in triplicate. Spontaneous ^{51}Cr - release under any of the conditions never exceeded 10%. In experiments in which the effector cells were treated with the mAb, the effector cells were plated in the wells of the microtiter plates and incubated with the mAb for 1 hr at 4°C before the addition of the target cells to the wells. In other experiments, the effector cells were preincubated with the mAb at 4°C for 1 hr in conical tubes, washed and then used in the cytotoxicity assays. The percent inhibition of cytotoxicity was based on comparisons of the LU20.

Redirected lysis

NK and LAK cells were tested for redirected cytolytic activity in a typical microcytoxicity assay using ^{51}Cr-labeled target cells. Assays to measure redirected lysis by the effector cells were performed by using the mAb-secreting hybridoma cells as target cells. The P3 fusion partner used for construction of the hybridomas was used as a control.

Conjugate assays

The effects of the mAb on the ability of the various effector cell populations to form conjugates with target cells was examined as described previously (Harris *et al.*, 1987b). Briefly, equal numbers

of effector cells and target cells (0.5 X 10^6 each) were mixed in 1 mL of RPMI-10%, the mixture was centrifuged for 2 min. at 50 xg and incubated for 15 min. at room temperature. At this time cells were vigorously pipetted (four to five times) to disrupt spontaneous conjugates, an aliquot was placed on a hemocytometer and conjugates were enumerated.

NK cell stimulation with anti-FAM MAB

To measure lymphokine production by NK cells upon anti-FAM mAb stimulation, NK cells were treated with anti-FAM mAb for a period of 24 hr with 10 μg/mL of anti-FAM mAb. Cells were washed with 37°C media, and total cellular RNA was extracted (Nicolaides and Stoeckert, 1990). cDNA was prepared using mouse Moloney leukemia virus reverse transcriptase. Reverse transcriptase-Polymerase chain reaction (RT-PCR) was performed on each of these samples using primers specific for Tumor necrosis factor-α (TNF-α) (Clontech, Palo Alto, CA). The nucleotide sequences of the 5' and the 3'primers are 5'-ATG/AGC/ACA/GAA/AGC/ATG/ATC/CGC/-3' and 5'-CC/ AAA /GTA/ GAC/CTG/CCC/GGA /CTC/-3', respectively. The samples were amplified for 30 cycles, consisting of 94°C for 1 min., 60°C for 2 min., and 72°C for 3 min. In some experiments the supernatants from stimulated NK cells were tested for biological activity of secreted lymphokines using indicator cell lines specific for TNF-α and IL-2. Tumor necrosis factor (TNF) was assessed by lysis of WEHI tumor cells. T cell growth factor (TCGF) activity was measured both by proliferation of CTLL cells as well as by ELISA quantification of IL 2 levels (Genzyme, Boston, MA).

Anti-FAM MAB modulations

Down-modulation of FAM from the surface of NK cells was achieved by treating various effector cell populations (1 x 10^6 cells) with 10 μg/mL of anti-FAM mAb for 30 mins at 4°C. Cells were subsequently cross-linked with a goat anti-mouse IgM mAb and incubated for 18 hrs at 37° C. Cells were washed repeatedly with 37° C media and flow cytometric analysis was performed to determine the loss of FAM expression from the surface of NK cells.

Flow cytometric analysis

Cell surface phenotype and mAb binding were analyzed by flow cytometry with the use of a Becton Dickson FACStar Plus flow cytometer. Controls consisted of fluorescein conjugated goat anti-mouse IgM antibody. Viable cells were gated by a combination of forward light scatter and size. A minimum of 10,000 cells were analyzed for each histogram and the data were collected on a log scale. For the two-color flow cytometric analysis, cells were first stained with anti-FAM mAb and an anti-IgM-FITC second antibody (green fluorescence). The cells were then stained with a PE-conjugated mAbs and then analyzed for simultaneous red and green fluorescence. Propidium iodide staining was also used to identify viable cells. In the experiments 10 μg mAb/1 x 10^6 cells was used for all mAbs (previously determined to be optimal for staining).

Signal Transduction assays

The ability of the mAb-stimulated effector cells to transduce signals was assessed by measurement of phosphatidylinositol lipid hydrolysis and intracellular calcium mobilization as previously described (Harris *et al.*, 1987; Kozumbo *et al.*, 1987).

Northern Blot Analysis

Total cellular RNA was extracted as previously described (Nicolaides & Stoeckert, 1990). Northern analysis was performed as described (Ausubel *et al.*, 1990). Briefly, 25 μg of total RNA was electrophoresed through 1% agarose/formaldehyde gels, transfered to nylon membranes, UV-cross-linked at 0.3 joules/cm^2 using a Bioslinker (Bios Corp., New Haven, CT), and probed with a ^{32}P-labeled 1 Kb cDNA vimentin probe (Oncor, Gaithersburg, MD). Hybridizations and washings were carried out as suggested by the manufacturer.

Polymerase Chain Reaction (PCR) Analysis

Total cellular RNA was isolated as described above. cDNA was synthesized from total RNA using the uni-amp plus kit (Clontech, Palo Alto, CA). PCR was performed on each of these samples using primers specific for the head, core and the tail region of the vimentin gene. Briefly, seven different primers against different regions of the vimentin gene were synthesized. Primer 1 corresponded to the head region of the vimentin gene (5'-CAA / CCG / GAG /CTA / TGT / GAC / CAC /-3'), primer 2 corresponded to the region approximately 40 nucleotides up stream of primer 1 (5'-TGT / CTA / CCA / GGT / CTG /TGT / CCT / -3'), primer 3 corresponded to the head region of the vimentin gene, approximately 200 nucleotides downstream of primer 2 (3'-CTC / TTC / CAT / CTT / GAC / GTC / CTC / -5'), primer 4 corresponded to the conserved core region of the gene, and is approximately 350 nucleotides downstream of the primer 3 (3'- GAC / GTG / CTA / CTT / CTC / TAG / GTC / -5'), primer 5 also corresponded to the conserved core region of the vimentin gene, and is approximately 110 nucleotides downstream of the primer 4 (3'- TTC / TTG /GAG / GTC / CTC / CGG / CTC / -5'), primer 6 also corresponded to the conserved core region of the vimentin gene (5'- AAG / AAC / CTC /CAG / GAG / GCC / GAG / -3'), and primer 7 corresponded to the tail region of the vimentin gene (3'-GAG / TCG / TAG / TGC / TAC / TGG / AAC / -5'). The samples were amplified for 30 cycles, consisting of 94°C for 1 min., 60°C for 2 min., and 72°C for 3 min.

Anti-Sense Oligonucleotide Treatment

Sense and anti-sense treatment of FAM(+) positive NK cells was performed as described earlier (Orbea *et al.*, 1990). Briefly, 21 mer sense and anti-sense oligonucleotides were generated to the core and the head region of the vimentin gene (Macromolecular structures facility, University of Arizona). Sense oligonucleotides used in experiments were of the following sequence: 5'- TGT /CTA /CCA /GGT /CTG /TGT /CCT /-3' (against the head region) and 5'- CTG /CAC /GAT /GAA /GAG /ATC /CAG /-3' (against the core region). Anti-sense oligonucleotides were of the following sequence: 3'-ACA /GAT /GGT /CCA /GAC /ACA /GGA /-5' (against the head region) and 3'-GAC /GTG /CTA /CTT /CTC /TAG /GTC /-5' (against the core region). FAM(+) 5 day LAK and ALAK cell populations were treated with either sense or anti-sense oligonucleotides for a period of 24 hr

at a final oligonucleotide concentration of 50 μM in 96 well plates. The cells then were washed and used either for flow cytometric analysis and cytotoxicity assays. This treatment protocol did not result in significant decreases in cell viability as determined by propidium iodide staining and flow cytometric analysis.

RESULTS

The function-associated molecule, FAM, is an evolutionarily conserved molecule

Experiments were conducted to examine the distribution of FAM in several species having well-characterized NK cells. Two color flow cytometry (FACS) was used for this purpose, analyzing FAM expression in combination with a variety of mAbs directed against T cell, B cell, and macrophage antigens. As shown in the summary presented in Table 1, FAM was detected in each species only on cells known to be NK or NK-like effector cells. It was of interest to note that FAM expression could be detected on T cells displaying NK-like activity. FAM expression was not detected on typical T cells, B cells or macrophages in any species examined. Generally, in each species the anti-FAM mAb reacted with the majority (although not 100%) of the NK cell populations. Thus, FAM was shown to be a novel NK cell-specific molecule which due to its species distribution was evolutionarily conserved.

Table 1
Cellular Distribution of the Function-Associated Molecule in Various Species

Species	NK cells	LAK cells	T cells	Activated T cells	Other cells
Fish	ND	ND	ND	ND	No
Rat	Yes	Yes	No	ND	No
Mouse	Yes	Yes	No	ND	No
Human	Yes	Yes	No	Yes	No

The distribution of FAM was assessed in each of the indicated species by one and two color flow cytometry as described in Materials and Methods. Data are persented as whether FAM was present (Yes) or absent (No) from the indicated lymhocyte populations. ND indicates not done. The results are a summary of all experiments.

The anti FAM mAb inhibits NK cell cytotoxicity and conjugate formation in all species

The role of FAM in NK cell function was first examined by analyzing its role in NK cell mediated cytotoxicity in each of the species (see Table 2). It was observed that the lytic activity of NK cells and LAK cells from each species was inhibited by the addition of the anti-FAM mAb to the cytotoxicity assays. Anti-FAM mAb could be added directly to the assay or the effector cells could be pretreated with the mAb, with equivalent results. The anti-FAM mAb did not react with the target cells used in the assays indicating that its effects were restricted to the effector cells. Analysis of the mechanism of lytic inhibition via single cell assays revealed that the mAb inhibited the formation of conjugates between the effector cells and the target cells, thereby inhibiting recogni-

tion. These results were found regardless of the type of target cells used in each species. Thus, FAM is a functional molecule involved in some fashion in NK cell recognition and function.

Table 2.
Inhibition of Cytotoxicity and Conjugate Formation by anti-FAM mAbs

	Percent Inhibition	
Species	**Cytotoxicity**	**Conjugates**
Fish	82	84
Rat	54	51
Mouse NK	70	75
House LAK	99	78
Human NK	80	71
Human LAK	45	46

The effects of anti-FAM mAb on NK and LAK cell function was assessed by addition of the mAb to cytotoxicity and conjugate assays as described in Materials and Methods. Data are presented as the percent inhibition as compared to control mAbs of the same isotype which also bound to the effector cell poplations. A representative experiment is shown.

Expression of FAM on NK cells is required for efficient NK cell lysis

To further confirm the role of FAM in NK cell lysis, a series of experiments were performed in which the expession of FAM was altered. Modulation of FAM from the surface of NK cells by mAb-mediated capping resulted in the inhibition of NK cell lysis as well as the loss of conjugate formation (see Table 3). These results were observed in both the mouse and human systems. Furthermore, use of target cells expressing the anti-FAM mAb on the cell surface resulted in the lysis of normally insensitive (i.e., not killed) target cells using both mouse and human effector cells

Table 3.
Effect of FAM Modulation of NK Cell Cytotoxicity and Conjugate Formation

Species	Modulation	Cytotoxicity	Conjugates
Mouse	No	140	60
	Yes	30	17
Human	No	79	69
	Yes	14	10

FAM expression was modulated from the cell surface of NK cells by mAb-mediated crosslinking as described in Materials and Methods. Flow cytometry confirmed that effector cells did not express FAM prior to use in functional assays. Modulated cells then were used in cytotoxicity and conjugate assays. The results from the cytotoxicity assays are presented as LU20 (lytic units), while the results from the conjugate assays are presented as percent conjugates observed. A representative experiment is shown.

(see Table 4). Thus, it appeared that FAM expression was required for efficient NK cell lytic function.

Table 4.
Redirected Lysis of Insensitive Target Cells by Anti-FAM mAbs

Species	Anti-FAM hybridoma	P3 fusion partner
Mouse	360	18
Human	360	1

The ability of anti-FAM mAb to mediate redirected lysis was performed as described in Materials and Methods. Target cells used in the experiments were either the anti-FAM hybridoma or the P3 myeloma fusion partner. Data are presented as the LU20 (lytic units). A representative experiment is shown.

FAM is a signal-transducing molecule on NK cells

The above results indicated that FAM had the characteristics of an antigen receptor. In order to further explore this hypothesis, experiments were performed to determine if FAM was capable of activating signal transduction pathways in the effector cells (see Table 5). If a molecule is an antigen receptor, then crosslinking it with either antigen or a mAb directed against it should trigger signal transduction pathways that eventually lead to cellular activation. It was observed that crosslinking of FAM with its mAb resulted in the detection of second messengers, i.e., phosphatidylinositol lipid hydrolysis and intracellular calcium mobilization, indicative of activation of the typical lymphocyte signal transduction pathway (Harris *et al.*, 1987a; Kozumbo *et al.*, 1987). These second messengers (which eventually resulted in activation of protein kinases essential for gene activation) were detected as early as 5 min. following FAM crosslinking. Furthermore, FAM crosslinking resulted in prolonged cellular activation (indicative of gene activation), which 48-72 hr later resulted in the detection of secreted lymphokines (TNF-α and TCGF) and the upregulation of the activation

Table 5.
Signal Transduction Induced by Anti-FAM mAb Crosslinking

Species	PI turnover	Ca^{2+} flux	Activation Ag	Lymphokines
Fish	2X	6X	ND	ND
Rat	2X	2.5X	ND	ND
Mouse	ND	ND	ND	5X
Human	4X	3X	25X	100X

The ability of the FAM to mediate signal transducing activities was assessed as described in Materials and Methods by crosslinking FAM with anti-FAM MAb on NK cells in each of the indicated species. Signal transduction was assessed by measurement of phosphatidylinositol (PI) lipid hydrolysis, mobilization of intracellular calcium (Ca^{2+}), flow cytometric analysis of activation antigen expression and secretion of lymphokines. Data are presented as the relative increase above media stimulated control cells. ND indicates not done. The data are a summary of all experiments.

antigens HLA-DR and the transferrin receptor on the NK cells. Thus, FAM is indeed a receptor-like molecule physically linked to intracellular signal transduction pathways.

Molecular characterization of FAM

In an effort to further characterize this novel molecule, FAM was immunoprecipitated, purified and subjected to limited amino acid sequencing. Three peptides derived from FAM were successfully sequenced. Surprizingly, each of the peptides was found to be identical to the intermediate filament, vimentin (Evans *et al.,* 1993). However, as typical vimentin is expressed only intracelluarly and has a molecular weight of 57 kDa, it was postulated that FAM might be highly homologous but not identical to this protein. To examine this possibility, experiments were performed to attempt to identify the gene encoding FAM (see Table 6). Northern blot analysis using a probe for vimentin revealed only one mRNA transcript for both FAM (+) and FAM (-) cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses using primers specific for defined regions of the vimentin gene also revealed identical products in both FAM (+) and FAM (-) cells. Thus, it was not possible to detect any novel gene product derived from vimentin or related to vimentin in cells expressing FAM, as compared to FAM(-) cells.

Table 6.
Summary of molecular characterization of the FAM on NK cells

Northern Blot	Only one mRNA transcript observed typical of true vimentin
RT-PCR	All PCR products were typical of true vim
AA Sequencing	Peptides were 100% identical to vimentin core region

FAM (+) and FAM (-) cells were molecularly analyzed to identify the gene encoding for FAM. Northern blot analyses, RT-PCR analyses and amino acid sequencing of peptides were performed as described in Materials and Methods. The overall results did not indicate that FAM was significantly different from vimentin. The table presents a summary of the findings.

In a final attempt to resolve the identity of FAM, experiments were performed in which the expression of the gene for vimentin was inhibited through the use of anti-sense oligonucleotides (see Table 7). Treatment of mouse effector cells with such anti-sense oligonucleotides resulted in an almost complete loss of FAM expression on these cells as assessed by FACS. Furthermore, concomitant with the loss of FAM expression there was a significant loss of cytolytic activity due to anti-sense oligonucleotide treatment. Thus, it appeared that FAM indeed was derived in some fashion from the typical vimentin gene.

Table 7.
Effects of Vimentin Anti-Sense Oligonucleotides on NK Cell Function

Effector cells	Anti-sense	FACS analysis	Cytotoxicity
Mouse LAK	No	20%	95
	Yes	1%	5
Mouse ALAK	No	40%	250
	Yes	5%	90

Effector cells were treated with antisense oligonucleotides directed against various regions of the vimentin gene as described in Materials and Methods. The cells then were analyzed by flow cytometry (FACS) for FAM expression and were used in cytotoxicity assays to test for lytic activity. Data are presented as the percent of cells staining positive for FAM expression and as the LU20 (lytic units)observed in the cytotoxicity assays. A representative experiment is shown.

DISCUSSION

The NCC population in teleost fish is thought to be equivalent to mammalian NK cells (Graves *et al.*, 1984; Evans *et al.*, 1984a; Evans *et al.*, 1984b; Evans *et al.*, 1984c). The observation that fish NCC exhibit spontaneous cytotoxicity against a variety of human target cells led us to conclude that fish and human NK cells recognize a common determinant on these target cells. Thus, it was reasonable to assume that fish and human NK cells would utilize similar types of receptors or function-associated molecules. In agreement with this hypothesis, anti-NCC mAbs were generated that not only bound NCC, but also to the majority of freshly isolated peripheral blood human NK cells (but not to T cells, B cells or monocytes) as well as rodent NK cells. The function-associated molecule (FAM) defined by the mAb 5C6 is a novel evolutionarily-conserved, cell surface protein present on fish NCC (Evans *et al.*, 1988), human NK cells (Harris *et al.*, 1991), rat NK cells (Friedman *et al.*, 1992), and mouse NK cells (Kapur *et al.*, 1994), as demonstrated by flow cytometric analysis of NK cells, and by ^{125}I surface labeling and immunoprecipitation experiments. Overall, the data suggests that FAM is involved in a common recognition mechanism utilized by NK and LAK cell populations in each species. However, it is not known if FAM is an antigen receptor.

FAM is intimately involved in NK cell recognition and function as demonstrated by experiments showing that anti-FAM mAb inhibits cytotoxicity and conjugate formation by NK cells. Interestingly, a proportion of LAK cells displayed both the novel mAb-defined structure as well as the CD8 antigen, indicating that some IL 2-activated T cells are also capable of expressing this molecule. In other experiments (Harris *et al.*, 1992b), such T cells were found to be capable of NK-like cytotoxicity and this lytic activity was inhibited by the anti-NCC mAbs. The ability of the mAbs to inhibit lysis of a variety of target cells by NK and LAK cells implied that these effector cells utilized this structure in their interactions with different types of target cells. One could hypothesize either that this molecule is involved in the cytolytic function of NK cells against many different target cells regardless of the target antigen involved, or that the effector cells expressed more than one such structure on their surface and each of these structures reacted with our mAbs. These results

could imply that NK antigen receptors are either broadly antigen-reactive (in the first instance) or are closely related to one another (in the latter instance). In either case, this would imply that our mAbs define a common portion of this structure, similar to anti-framework determinant mAbs. To further analyze the role of FAM in NK cell cytotoxicity, an attempt was made to down-modulate FAM from the surface of the various effector cell populations. As shown by flow cytometry, an almost 100% decrease in the expression of FAM was observed after an overnight modulation with the mAb. Down-modulation of FAM resulted in a significant decrease in cytotoxicity and conjugate formation by the effector cell populations. These results support the hypothesis that FAM is intimately involved in NK cell recognition and triggering of cytolysis. Absolute inhibition of cytotoxicity of target cells was not achieved even upon complete modulatation of FAM from the surface of NK cells. It is possible that either the modulated effector cells still express low levels of FAM that were not detected by flow cytometry and/or the loss of FAM results in the use of other molecules on NK cells involved in cytotoxicity. One can hypothesize that each of these distinct effector cell populations utilizes similar molecules in mediating NK-like cytotoxicity.

Generally, to qualify as an antigen receptor, a molecule must mediate signal transduction functions which result in cellular activation. FAM appears to be a signal-transducing molecule, as anti-FAM mAb treatment of NK cells stimulates lymphokine production (TNF-α and TCGF), mediates redirected lysis of normally insensitive target cells, causes phospholipid hydrolysis and intracellular calcium mobiliziation, and induces the expression of activation antigens (class II MHC molecules and upregulation of transferrin receptors). Normally insensitive target cells are rendered susceptible to lysis by bearing an anti-receptor mAb on its surface. This process bypasses the normal receptor-antigen recognition step while triggering the receptor-bearing effector cell. These observations are characteristic of those seen with mAbs directed against antigen-binding receptors. Also, observations regarding the limited expression of this molecule and its role in NK cell function, along with its evolutionary conservation, would seem to indicate that it is unique in nature.

A partial amino acid sequence of FAM isolated from rat NK cells has been shown to be highly homologous to the core region of the intermediate filament (IF), vimentin. We also have shown that FAM is molecularly related to, if not identical with, vimentin by its cross-reactivity with anti-vimentin mAbs. These anti-vimentin mAbs bind specifically to NK cells in each species and inhibit cytotoxicity and conjugate formation (Harris *et al.,* 1992a). IF are composed of distinct intermediate filament proteins (IFP) that are encoded by a multigene family, the members of which are regulated developmentally in a tissue specific fashion (Lazarides, 1982; Steinert and Roop, 1988). The expression of IFP is predominantly restricted to cells of mesenchymal origin, undifferentiated cells, and malignant cells. Moreover, the expression of vimentin is growth regulated (Ferrari *et al.,* 1986). Vimentin is expressed in all stages of B cell development, but is lost during terminal differentiation of B cells into plasma cells (Dellagi *et al.,* 1983). Previous studies have shown that vimentin is induced in normal resting murine T lymphocytes during the Go to S phase transition by mitogens and growth factors, including Concanavalin A (Kaminska *et al.,* 1990), polyamines (Kaminska *et al.,* 1990), and IL-2 (Podolin and Prystowsky, 1991). These studies suggest vimentin to be a growth responsive gene in T cells. The function of vimentin during growth and differentiation is not clear (Klymkowsky *et al.,* 1989). Vimentin appears to link the nucleus to the plasma membrane (Georgatos and Blobel, 1987), becoming phosphorylated during mitotic reorganization (Chou *et al.,* 1990). Based on these findings it can be postulated that vimentin plays an important role in signal transduction and intracellular transport processes. Although the function

of vimentin in lymphocytes is not understood, vimentin aggregates with lymphocyte antigen receptors (Dellagi and Brouet, 1982) and undergoes extensive cytoplasmic reorganization in B cells after antigen receptor cross-linking (Albrecht *et al.*, 1990). Similar to FAM, vimentin also is highly evolutionarily conserved in its structure, cellular localization, and amino acid sequence (particularly in the core region) (Steinert *et al.*, 1985). Vimentin along with other IFs has a conserved central α-helical rod domain of 311-314 amino acids, which is flanked by end domains of variable size and chemical character (Steinert *et al.*, 1985). Differences in the sizes and properties of IF subunits are due almost entirely to the variability in portions of the end domains, and the basis for the structural uniformity of diverse IF appears to reside in the conserved structure of the rod domains (Steinert *et al.*, 1985). The presence of vimentin either in the cytoplasm or on the cell surface of NK cells has never been reported. It should be noted that the sizes of these proteins (vimentin, 57 kDa and FAM, 42-45 kDa) are quite different. To examine the relationship between FAM and vimentin at the molecular level, northern blot analyses were performed on FAM(+) and FAM(-) cells. An attempt was made to determine if more than one vimentin-like mRNA existed, that might encode for FAM. Using a full-length vimentin cDNA probe, only a single mRNA transcript corresponding to the size of classical vimentin was detected. To further search examine for separate mRNA encoding for FAM and vimentin, PCR analyses were performed using cDNA derived from FAM(+) and FAM(-) cells. In particular, we sought subtle differences in the size of PCR products between the two cell types that may have arisen as a result of alternate splicing of a single mRNA. Primers made against different regions of the vimentin gene were used to amplify various regions of the cDNA from FAM(+) and FAM(-) cells. No differences in the types of PCR products were observed between the two cell types. Finally, to examine the functional relationship of FAM and vimentin, and to determine whether the mRNA encoding these two proteins was identical, an antisense oligonucleotide approach was utilized. This strategy has been used successfully in the past to study specific gene function of perforin (Orbea *et al.*, 1990) and c-myc (Wickstrom *et al.*, 1988). Upon treating FAM(+) cells with antisense oligonucleotides made against the core (i.e., conserved) and the head (i.e., variable) regions of the vimentin gene, greater than 90% loss in the cell surface expression of FAM was observed by flow cytometry with 5 day LAK and ALAK cells. The decrease in the cell surface expression of FAM also resulted in a parallel decrease in the cytotoxicity of the effector cells versus target cells. These results were the most convincing evidence that FAM was a novel form of vimentin. As shown, complete inhibition in the killing of target cells was not achieved upon treating FAM(+) cells with antisense oligonucleotides. It is possible that the loss of FAM results in the utilization or up-regulation of other NK cell function-associated molecules, which may play a role in NK cell cytotoxicity. Based on the partial amino acid sequence and the anti-vimentin mAb cell surface staining of NK cells, as well as northern blot analyses, PCR analyses, and the antisense oligonucleotide treatments, it appears that FAM present on NK cells is a novel form of vimentin. The mechanism by which this "vimentin-like" FAM is expressed on the surface of NK cells is not clear. It is possible that a small undetected transmembrane domain (exon) might be added to the FAM mRNA transcript, which allows it to anchor in the plasma membrane and function as a receptor-like molecule. It is also conceivable that post-translational processing of vimentin in NK cells in some fashion allows the "vimentin-like" FAM to be transported to the cell surface. It should be remembered that the molecular weight of FAM is slightly smaller than that of classical vimentin, possibly indicative of post-translational modification.

In conclusion, using a comparative immunological approach we have identified a novel cell surface molecule present on the majority of human peripheral blood NK cells. This structure is involved

in NK cell function as evidenced by the ability of mAbs directed against it to inhibit NK cell antigen recognition as well as to mediate signal transducing activities (i.e., redirected cytotoxicity, stimulation of lymphokines release, and cellular activation). Although this molecule is reminiscent of T cell antigen receptors in structure, it is only found on cells with NK activity and is highly evolutionarily conserved. This molecule appears to be a candidate NK cell antigen receptor. Experiments conducted at the cellular and molecular levels demonstrate FAM to be identical to (or highly homologous to) the IF, vimentin, and thus represents a novel form of this molecule. It is very interesting that NK cells appear to have modified a normal intracellular structure for use in extracellular recognition processes. FAM, like vimentin, is a highly evolutionarily-conserved molecule and plays an important role in NK cell cytotoxicity. Finally, it was very intriguing that the molecule defined by our mAbs was present on fish, rodent and human NK cells. This finding would seem to imply that the structure appeared very early in the evolution of the immune system and selective pressures have existed to maintain its presence. It is possible that the molecules in each species are not identical, but at least a portion of the complex that is recognized by our mAbs and that is involved in NK cell function is very conserved antigenically. The discovery of this molecule in fish leads us to speculate that nonspecific immunity evolved prior to the development of antigen-specific T cells. This defense system probably utilized very primitive molecules to mediate this activity and may have given rise to the development of the more sophisticated function-associated molecules and receptors found on present day T cells. Overall, these results indicate that FAM is a novel receptor-like molecule that is intimately involved in murine NK cell recognition and function. It is quite possible and should be considered however, that FAM may be an accessory molecule that is tightly associated (physically or otherwise) with other antigen receptors on NK cells. In either case FAM would be intimately involved in antigen recognition by NK cells in a variety of species.

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Chapter 13

Preliminary Characterization of the Antibacterial Proteins in the Blood Cells of the Solitary Ascidian, *Ciona intestinalis*

Cameron Findlay and Valerie J. Smith[1]

School of Biological and Medical Sciences
Gatty Marine Laboratory
University of St Andrews
Fife, Scotland KY16 8LB

[1]Author to whom correspondence should be addressed

INTRODUCTION

Ascidians occupy an important phylogenetic position close to the origin of the vertebrates, and hence are of considerable interest in comparative immunology. Previous work on ascidian antimicrobial defences has shown that phagocytosis is executed by the hyaline amoebocytes and is aided by opsonins released by the morula cells (Smith and Peddie, 1992). It has also been shown that enriched populations of phagocytes produce free oxygen radicals *in vitro* (Bell and Smith, 1994) and that the morula cells migrate to wounds, sites of non-fusion reactions and sites of infection *in vivo* (Sabbadin *et al.*, 1992). Recently, we have shown that the blood cells from the solitary ascidian, *Ciona intestinalis*, display potent antibacterial activity *in vitro*, and that this activity is constitutively present in non-immunized animals (Findlay and Smith, 1995). Activity tends to be stronger against Gram positive than Gram negative bacteria and to reside principally within the morula cells, with some weaker activity also present in the amoebocytes (Findlay and Smith, 1995). Physiochemical characterization of the phenomenon has further established that it is stable following heat (100 °C, 10 min) or freeze (-20°C, 1 month) treatment, requires Ca^{2+}, but not Mg^{2+}, ions for effect and does not entail direct lysis of the bacterial cell wall (Findlay and Smith, 1995). Here we describe preliminary biochemical characterization of the factors responsible and show that there are at least two antibacterial proteins in *C. intestinalis* blood cells. One lies in the molecular weight range of 60-70 kDa and affects both Gram positive and Gram negative organisms. The other lies in the molecular weight range 7-15 kDa and is effective only against Gram positive strains.

MATERIALS AND METHODS

Preparation of blood cell lysates (CLS)

Blood cells were obtained by bleeding individual animals directly from the heart into marine anticoagulant pH 7.0 (0.1 M glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM EDTA, 0.45 M NaCl) as in Smith and Peddie (1992). The blood cells were then pooled from 50 individuals and washed twice (400 xg) in Mg^{2+} free marine saline (MS) (0.5 M NaCl; 20 mM $CaCl_2.6H_2O$; 11 mM KCl; 43 mM Tris; pH 7.4; 940 mosM) before homogenization (30 min, 4 °C) in 0.1 M ammonium acetate, pH 6.5, containing 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma, Poole, Dorset, UK). The homogenate was finally centrifuged at x 48,000 xg for 20 min (4 °C), and the protein concentration in the resulting supernatant, designated CLS, adjusted to 1 mg/ mL.

Antibacterial assays

Antibacterial activity was determined using *Planococcus citreus* (NCIMB 1493) and *Psychrobacter immobilis* (NCIMB 308) as representative Gram positive and Gram negative test strains, respectively. The bacteria were grown to log phase in Marine Broth (Difco, Detroit, Michigan) (18 hr, 20°C), harvested and washed twice (800 xg) in MS before final resuspension in sterile MS at a concentration of $1x10^7$ cells/ mL. One mL of each suspension was mixed with 9 mL of sterile, molten (40 °C) 1 % agarose (Sigma; low endosmotic type) made up in MS and supplemented with 3 % w/v Marine Broth (Difco), and 0.02 % v/v Tween-20 (Sigma), and poured into a sterile 9 cm square petri dish. Two microliter volumes of each CLS fraction were added to 1.5 mm diameter wells cut in the agarose lawn. Controls consisted of 2 µL of 0.1 M ammonium acetate pH 6.5 in place of the CLS The plates were then incubated at 20 °C for 3 hr, overlaid with fresh agarose mixture minus bacteria and re-incubated at 20 °C for a further 18 hr. Antibacterial activity was recorded as the diameter of clear zones formed in the bacterial lawn.

Gel filtration

CLS samples were subjected to gel filtration on a 40 x 1 cm Sephadex G-75 column, pre-equilibriated with 0.1 M ammonium acetate pH 6.5. One mL of CLS was applied to the column and eluted at a flow rate of 5 mL/ hr. Fractions (1 mL) were collected and concentrated by freeze drying and reconstition in 50 µL of distilled water. The column had previously been calibrated using a Pharmacia molecular weight standards (ribonuclease A 13.7 kDa; ovalbumin 43 kDa; bovine serum albumin 67 kDa; and blue dextran 200 kDa).

SDS-PAGE

The composition of the biologically active CLS fractions obtained after gel filtration were examined by SDS-PAGE using the method of Schägger and von Jagow (1987) for the separation of proteins in the range 1-100 kDa. Fractions within the two active peaks (Figure 1), incorporating the 60-70 kDa and 7-13 kDa factor(s), were pooled to make two samples. The non-active fractions between the two active peaks were also pooled and made into one sample. Sigma low molecular weight protein standards, optimised for tricine SDS-PAGE, were used. The gels were stained using Coomassie blue (Sigma).

Protein

Protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

RESULTS

Figure 1 shows the main protein peaks obtained by fractionation of *C. intestinalis* CLS on Sephadex G-75. Parallel measurement of the antibacterial effects of these fractions revealed that there are two zones of activity. One represents proteins in the molecular weight range 60-70 kDa

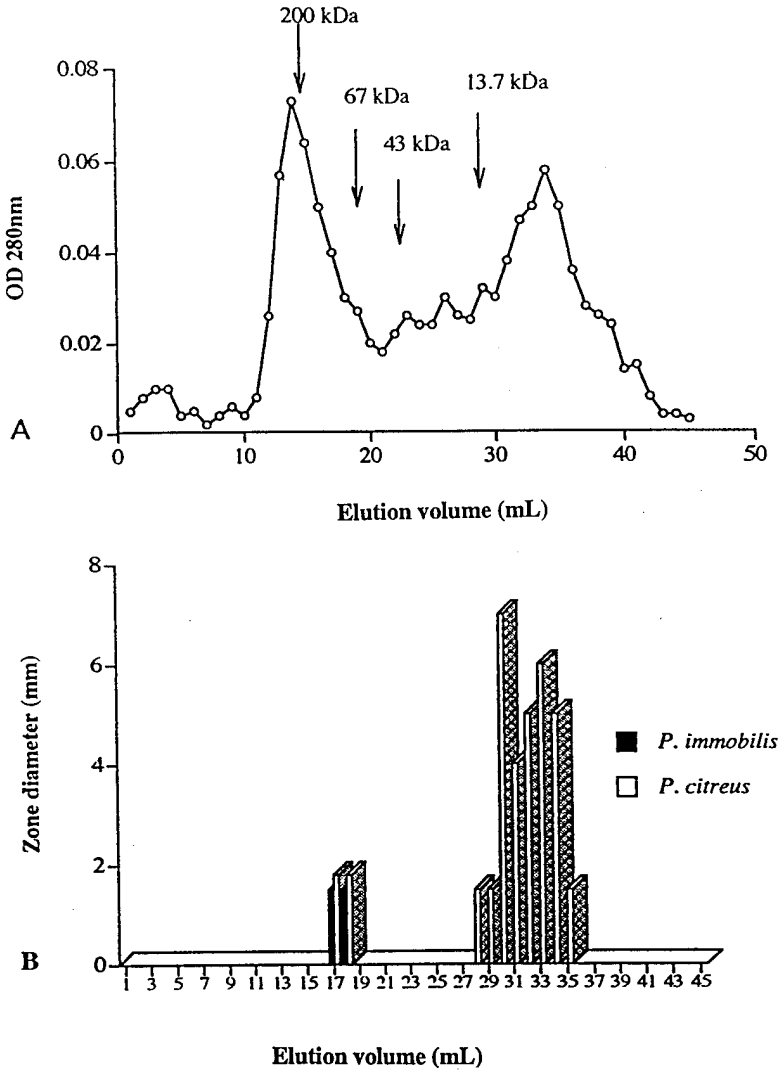


Figure 1.(A) Sephadex G-75 gel filtration of *Ciona intestinalis* blood cell lysate supernatant (CLS). The column was eluted with 0.1 M ammonium acetate pH 6.5 at a flow rate of 5 mL/ hr with 1 mL fractions collected and freeze dried. (B) Fractions were reconstituted in 50 µL distilled water, and 2 µL added to a single well cut in an agarose lawn of *P. citreus* or *P. immobilis*. The plates were then incubated for 18 hr at 20 °C and the diameter of the clear zones measured.

and is active against both *P. citreus* and *P. immobilis* (Figure 1). The other represents proteins in the molecular weight range 7-15kDa and is active only against *P. citreus* (Figure 1). Activity in the 60-70 kDa fraction was substantially weaker than that containing the 7-15 kDa proteins against *P. citreus* as clear zones of 1.5 mm were recorded with the higher molecular weight fraction for both strains of bacteria, whereas clear zones of 7 mm were observed with the lower molecular weight fraction against *P. citreus* (Figure 1). Analysis of the protein composition of the active peaks by SDS-PAGE revealed that there are at least three protein bands within the 7-15 kDa fractions (Figure 2). These represent proteins with molecular masses of 7, 10 and 13 kDa. These three bands were present only in this final peak (lane 4, Figure 2). The antibacterial peak which included the higher molecular weight fractions, active against both Gram positive and Gram negative strains, contained a number of protein bands which were all of higher molecular weight than 16.8 kDa. None of these bands were present in the low molecular weight antibacterial peak, or in the non-antibacterial fractions (Figure 2).

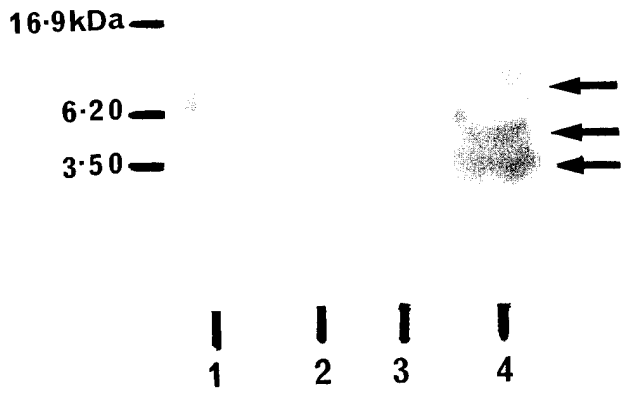


Figure 2. Sephadex G-75 fractions from separated CLS were subjected to SDS-PAGE and stained with Coomassie blue. The arrows indicate three major protein bands present in the low molecular weight antibacterial peak (lane 4). Lane 1: Sigma low molecular weight standards, Lane 2: Elution volume 15-19, Lane 3: Elution volume 20-27, Lane 4: Elution volume 28-35.

DISCUSSION

This preliminary characterization of the antibacterial factors within the blood cells of the solitary ascidian, *C. intestinalis,* by gel filtration shows that there are at least two antibacterial Proteins present within the CLS, which differ in molecular mass, spectra of activity and bactericidal potency *in vitro*. Previously, we have shown that activity in unfractionated CLS operates against a range of Gram positive and Gram negative bacteria (Findlay & Smith, 1995). Here we show that the protein(s) in the higher molecular mass range appear to be active against both of the Gram positive and Gram negative bacteria, but are comparatively weak, whereas those in the lower molecular weight range are effective only against Gram positive forms and are much more potent.

A number of antibacterial proteins with molecular weights within the 50-80 kDa range are known to be present in both vertebrate and invertebrate systems (Elsbach and Weiss, 1993; Vaara, 1992). They include a 50-60 kDa bacterial permeability increasing protein (Elsbach and Weiss, 1993) and 78 kDa lactoferrin (Vaara, 1992). However, both are active only against Gram negative strains. By contrast, the larger molecular weight (60-70 kDa) antibacterial protein(s) in *C. intestinalis* is/are active against both the Gram positive and Gram negative bacteria tested, indicating that it/they might constitute novel type(s) of antibiotic protein(s).

As yet, it is unclear which of the three proteins revealed to be present within the low molecular weight antibacterial fraction of *C. intestinalis* CLS by SDS-PAGE are responsible for activity against *P. citreus*. One well characterized bactericidal protein known to fall within the 7-15 kDa range and to target exclusively Gram positive bacteria is lysozyme. This attacks the 1,4 glycosidic links of the bacterial cell wall and, in invertebrates, has a molecular mass of 13-14 kDa (Boman *et al.,* 1991). However, it is unlikely that lysozyme is one of the factors present in *C. intestinalis* as we have previously shown that the bactericidal activity in this animal is non-lytic and distinct from lysozyme in several respects (Findlay and Smith, 1995). Of the other low molecular weight antibacterial proteins known to occur in invertebrates, most in the 7-15 kDa range (eg dipterin, coleopterin, hymenopterin), affect only Gram negative bacteria (Cociancich *et al.,* 1994). Proteins which target only Gram positive bacteria (eg the defensins) tend to have molecular weights of 3-4 kDa (Cociancich *et al.,* 1994; Lehrer, 1993). Previous work on antimicrobial factors in solitary ascidians has revealed several families of antimicrobial proteins, including the halocyamines (Azumi *et al.,* 1990), the lissoclinotoxins (Litaudon *et al.,* 1994) and the didemnins (Davidson, 1994). All the factors found so far have very small molecular weights (less than 1-2 kDa) and, in general, are more effective against Gram negative organisms than Gram positive. This again indicates that one or more of the factors in *C. intestinalis* might constitute new forms of antibacterial agent(s). Further purification and amino acid sequence analyses of both the high and low molecular weight proteins in *C. intestinalis* are necessary to reveal whether or not these ascidian factors bear any similarity to the antibacterial proteins of other species. We are currently undertaking a more detailed study of *C. intestinalis* CLS by reverse phase HPLC as the first step towards resolving this question.

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Chapter 14

Antibacterial Proteins in the Hemocytes of the Shore Crab, *Carcinus maenas*

Denni Schnapp and Valerie J. Smith*

School of Biological and Medical Sciences
Gatty Marine Laboratory
University of St Andrews, Fife,
KY16 8LB, Scotland

* Author to whom correspondence should be addressed

INTRODUCTION

Much of the research into the immune strategies of crustaceans has been stimulated by the increasing importance of decapods in aquaculture, and over the last 10 years great strides have been made in our understanding of the cellular and humoral defences in these animals. However, despite the progress made in elucidating some of the biochemical events associated with non-self recognition and hemocyte activation (Söderhäll, 1992; Smith and Chisholm, 1992), comparatively little is known about the repertoire, biochemical characteristics and biological importance of microbicidal agents synthesized in crustacean tissues.

Previously the hemocytes of the shore crab, *Carcinus maenas*, have been shown to have potent antimicrobial activity against a range of Gram positive and Gram negative bacteria *in vitro* (Chisholm and Smith, 1992). Activity resides chiefly in the granular cells, is independent of divalent cations and is stable following either heat (100°C, 10 min) or freeze (-20°C, 1 month) treatment (Chisholm and Smith, 1992). Equivalent activity is also exhibited by the hemocytes of a variety of other marine crustaceans (Chisholm and Smith, 1995) and for most species the response does not appear to entail bacterial agglutination, direct lysis of the bacterial cell wall or phenoloxidase activity (Chisholm and Smith, 1992; 1995). The present paper describes some of our preliminary work to identify and characterize the antibacterial factor(s) in crab, *C. maenas* hemocytes.

MATERIALS AND METHODS

Animals and preparation of hemocyte lysate supernatants (HLS)

Specimens of *C. maenas* were collected from St. Andrews Bay and maintained in filtered seawater in a flow-through aquarium at 12-15°C until use. Only healthy adults, showing no wounds or signs of infection were used for experimental purposes. Hemolymph was collected and hemocyte extracts prepared in sterile *Carcinus* saline (CS) (0.38 M NaCl, 13 mM KCl, 20 mM $CaCl_2$, 0.05 M Tris, 26 mM $MgCl_2$, pH 7.4) as described in Chisholm and Smith (1992), except that the hemocytes were homogenized in the presence of 0.01% phenylthiourea (PTU).

The protein content of the sample was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Antibacterial assays

Antibacterial activity was determined using the Gram negative marine bacterium, *Psychrobacter immobilis* (NCIMB 308), as test agent. The bacteria were maintained on slopes of Difco Marine Agar (Difco, Detroit, Michigan) at 4 °C, and prior to assay, were raised to log phase growth in Difco Marine Broth (18 hr, 25 °C). They were then harvested, washed and resuspended in sterile buffer (0.45 M NaCl, 0.05 M Tris; pH 7.2) at a concentration of ca $1x10^6$/ mL

Antibacterial activity in whole HLS or HLS fractions was assessed using a modification of the spread plate assay described in Chisholm and Smith (1992). Briefly, 10 mL of bacterial suspension were incubated in 90 µL of HLS sample or, in the case of controls, 90 µL of sterile CS (the final concentration of bacteria was ca 10^4 mL). After 4 hr, each bacterial suspension was diluted with 900 µL of sterile CS, and 100 mL aliquots were plated in triplicate onto Marine Agar plates. These plates were then incubated for a further 48 hr at 25°C and the number of colony forming units (cfu) determined. Antibacterial activity was calculated as the percentage reduction of cfu in the samples as compared to the saline control.

Gel filtration

A 60 x 0.8 cm column of Sephadex G75 (Pharmacia, Uppsala, Sweden) was equilibrated with 0.1 M ammonium acetate (pH 6.8) and calibrated with dextran blue and molecular weight markers (bovine serum albumin (66.7 kDa), ovalbumin, (43 kDa), chymotrypsinogen A (25 kDa) and RNAse A (13.7 kDa) (all from Pharmacia). One millilitre of HLS (protein content ca 1.5 mg mL^{-1}) was applied to the column and eluted at a flow rate of 6 mL/ hr. Fractions of 1 mL were collected and the absorbance determined at 280 nm. Each fraction was then freeze dried, reconstituted in 100 µL of sterile CS and assayed for antibacterial activity as above.

Electrophoresis and bacterial overlays

SDS PAGE was performed by the method of Schägger and von Jagow (1987). The acrylamide-bis concentrations were 4 % for the stacking gel, 10 % for the spacer gel and 16.5 % for the separating gel. Native PAGE under acidic conditions was carried out following the method of Lehrer *et al.* (1991) using 12% acrylamide for the gels. The gels were stained with Coomassie Blue. Bacterial lawn overlays to identify protein bands with antibacterial activity were also made according to procedures described in Lehrer *et al.* (1991), except that the overlays consisted of double strength Marine Broth (Difco) (74.8 g/ L) in 1% agarose.

RESULTS

Figure 1 shows the gel filtration profile of whole HLS and typical SDS-PAGE profiles of peaks with antibacterial activity. At least three active peaks (A, B and C), corresponding to proteins with molecular weights in the range of 80-90, 35-40 and 14 kDa, respectively, were eluted from the column (Figure 1). SDS-PAGE analysis of peaks A and B showed that the fractions contained a heterogeneous mixture of proteins with molecular weights ranging from 17 kDa to over 67 kDa (Figure 1). Peak C showed some traces of previously eluted proteins and two clear bands representing proteins with molecular weights of ca 11 kDa and 6.5 kDa (Figure 1).

Figure 2 shows native PAGE of whole HLS and the corresponding lawn overlay of *P. immobilis*. The gel shows a number of protein bands, three of which produce clear zones on the bacterial lawn overlay (Figure 2).

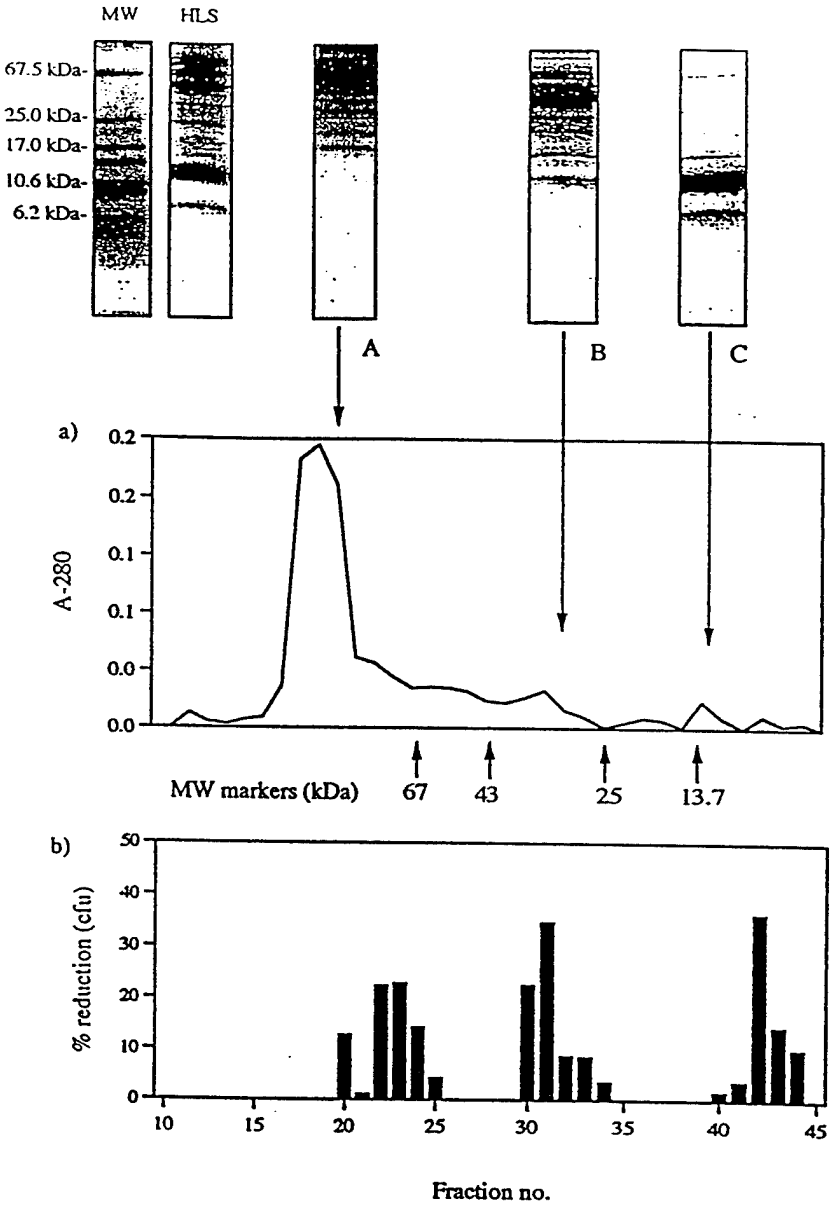


Figure 1. Gel filtration chromatography of whole crab hemocyte lysate supernatant (HLS) and typical SDS PAGE profiles of antibacterial peaks. A 0.9 x 57 cm column of Sephadex G-75 was equilibrated with 0.1M ammonium acetate (pH 6.8). 1 mL HLS (protein content ca 1.5 mg/ mL) was added to the column and eluted at 6 mL/ hr. Fractions of 1 mL were collected and the absorbance read at 280 nm. They were then freeze-dried, reconstituted in 100 mL *Carcinus* saline and assayed for antibacterial activity as described in the text. (a) absorbance at 280 nm; (b) activity profile against *Psychrobacter immobilis*. The inset shows SDS PAGE profiles of antibacterial peaks from a different run, corresponding to the three activity peaks A, B and C.

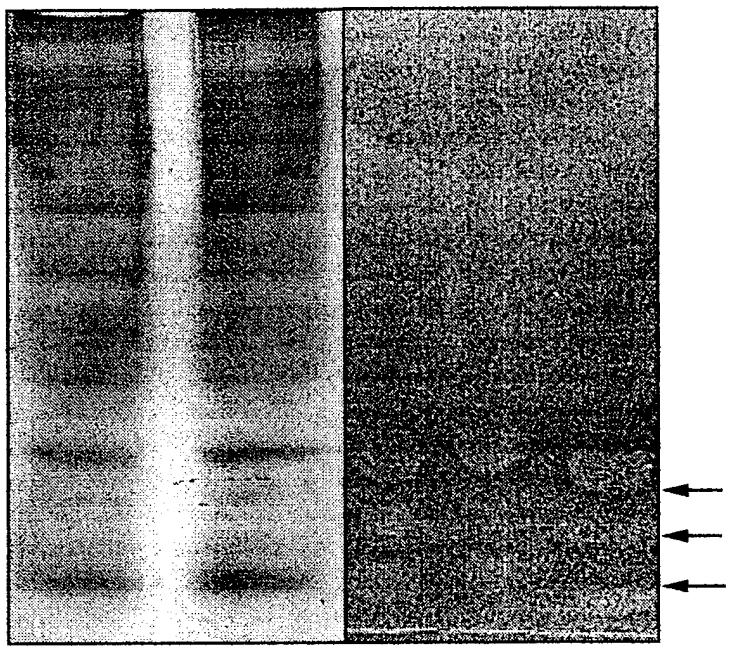


Figure 2. Acid native PAGE of whole crab hemocyte lysate supernatant and overlay of a lawn seeded with *P. immobilis.* The method was modified from the two-layer-method of Lehrer *et al.*, (1991). Briefly, a 1.2 mm thick layer of 10 mM phosphate buffer with 1% agarose was seeded with ca $3x10^6$ bacteria/mL and overlaid with the gel. After 1 hr, the gel was replaced with an overlay consisting of double strength Marine Broth (Difco) plus 1% agarose.The gel used for the overlay is shown on the left (Coomassie Blue stain). Arrows indicate zones of antibacterial activity.

DISCUSSION

This preliminary fractionation of *C. maenas* HLS by gel filtration and PAGE shows that the hemocytes of this crab contain at least three proteins which inhibit the growth of the Gram negative bacterium, *P. immobilis in vitro.* The molecular weights of these proteins lie within the ranges, 80- 90 kDa, 35-40 kDa and, importantly, 6.5 -11 kDa. Low molecular weight antibacterial proteins are known to be key components in the non-specific defenses of both vertebrates and invertebrates and are believed to represent an ancient form of tissue protection against opportunistic microbial exploitation (Boman, 1991). They have been found in insects (Hultmark, 1993; Cociancich *et al.*, 1994), horseshoe crabs (Nakamura *et al.*, 1988), ascidians (Azumi *et al.*, 1990), amphibians (Kreil, 1994) and mammals (Zasloff, 1992; Ganz and Lehrer, 1994; Selsted and Oullette, 1995). None have previously been described for the Crustacea.

Comparison of the antibacterial proteins characterized to date shows that they constitute a heterogeneous collection of molecules which differ in their amino acid content, size, structure, specificity and mode of action (Boman, 1991, 1994). Some, such as the defensins, target mainly Gram positive bacteria (Ganz and Lehrer, 1994), others, such as apidaecin and sarcotoxin are active only against

Gram negatives (Casteels *et al.*, 1989, Natori, 1994), while a number of types, eg the cecropins and bactenecins, have broad spectrum activity *in vitro* (Cociancich *et al.*, 1994)

The data presented in the present report tell us nothing about the identity, structure or range of biological effects of the antibacterial proteins in *C. maenas*, although previous work by Chisholm and Smith (1992) has shown that *C. maenas* HLS has broad spectrum activity against a number of bacterial strains, including Gram positives. *Psychrobacter immobilis* was used as a convenient sentinel test organism in the present study but for related experiments on *C. maenas* HLS, we have used the Gram positive bacteria, *Planococcus citreus* (NCIMB 1493) and *Micrococcus luteus* (NCIMB 376) as test agents (Schnapp and Smith, unpublished). This work has indicated that there are indeed proteins present in *C. maenas* hemocytes, including at least one in the molecular weight range 6-14 kDa, which are active against Gram positive bacteria (Schnapp and Smith, unpublished). Experiments are currently underway to clarify whether or not the response to these micro-organisms is mediated by the same proteins as those which affect *P. immobilis.*

From our estimates of the molecular weights of the active proteins in *C. maenas*, it is unlikely that antibacterial activity in this animal is mediated by proteins related to cecropins or defensins as these agents generally have molecular weights of 2-4 kDa and defensins show weak activity against Gram negative bacteria (Cociancich *et al.*, 1994; Ganz and Lehrer, 1994). Instead, they fall into the range exhibited by bactenecins and the glycine-rich peptides, coleopterin and diptericin, or anti-LPS factor from horseshoe crabs (Cociancich *et al.*, 1994; Iwanaga *et al.*, 1994). The small 6.5 kDa protein, in particular, has a molecular weight close to those of the bactenecins, bac7 and bac5, isolated from bovine neutrophils (Gennaro *et al.*, 1989). At this stage, we cannot make predictions about the character of the antibacterial proteins in *C. maenas* or about their affinity to known antimicrobial proteins in other animal groups.

What is clear from the present study is that these proteins are present in *C. maenas* hemocytes without prior stimulation of the crabs with bacteria, bacterial products or other treatments. This further distinguishes them from the low molecular weight antibacterial peptides in insects, where pre-stimulation by bacteria or wounding is necessary for synthesis and release of the proteins from the fat body (Cociancich *et al.*, 1994). It is possible that induction of antibacterial protein synthesis by wounding or exposure to microbial carbohydrates is unnecessary for those animals which partition their antibacterial armoury within the blood cells, as localized release of the compounds can be achieved through regulated exocytosis. Certainly degranulation by crustacean blood cells seems to be regulated by a complex biochemical pathway in the blood (Söderhäll, 1992). As yet, the relationship between exocytosis and the delivery of antibacterial proteins at sites of infection awaits further investigation.

Acknowledgements

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Chapter 15

Natural and Transgenesis-induced Antimicrobial Activities in Marine Invertebrates

Philippe Roch, Evelyne Bachère, Viviane Boulo, Jean-Paul Cadoret, Florence Hubert, Annie Morvan, Thierry Noël and Wil van der Knaap

Défense et Résistance chez les Invertébrés Marins (DRIM)
Université de Montpellier 2, cc 80
34095 Montpellier Cédex 05, France.

ABSTRACT

In mussel *Mytilus galloprovincialis*, a potent cytotoxic activity directed against eukaryotic cells including protozoa parasites, but not bacteria, was found to be mediated by a high molecular mass multimeric protein of 320 kDa, acting through a polymerization process. Naturally occurring antibacterial small proteins were also evidenced in the hemolymph. In shrimp *Penaeus japonicus*, an α-macroglobulin-like molecule was found to be associated with a cytolytic activity of the plasma. In the oyster *Crassostrea gigas* and *Ostrea edulis*, and in Penaeid shrimps antibacterial activity was found that was directed against various pathogenic and non pathogenic bacteria.

Anti microbial activity of heterologous peptides was found against the oyster parasite *Bonamia ostreae*. Although non-toxic for self hemocytes, tachyplesin, magainin and to a lesser extent cecropin, were able to kill the parasite *in vitro*.

Transformation experiments with reporter genes were developed to establish experimental parameters and the functionality of available promoters. *In vivo*, two mass transformation methods were applied to mussel and oyster eggs: electroporation and high velocity particle bombardment. *In vitro*, the lipofection procedure was used to transfect oyster heart primary cell cultures. Inducibility of an heat-shock protein promoter and temporal expression of mammalian viral promoter were demonstrated in bivalve molluscs.

INTRODUCTION

Bivalve and shrimp aquaculture is threatened by several phenomena that dramatically decrease production. Among these, pathogens are crucial factors acting directly on the cultivated marine invertebrates. To overcome disease problems, better knowledge of the natural defence system of the commercially important molluscs and shrimps is needed. Another approach consists of using already known antimicrobial molecules for transgenesis. These are the strategies developed by the DRIM lab at the University of Montpellier 2 - France, under the sponsorship of the Institut Français

de Recherche et d'Exploitation de la Mer (IFREMER) and the Centre National de la Recherche Scientifique (CNRS).

In addition to cellular immunity, where macrophages play a prominent role, bivalve molluscs possess several types of so-called non-specific humoral defence molecules: agglutinins (Renwrantz and Stahmer, 1983), bactericidins (Mori, *et al.*, 1980), lysosomal enzymes (Cheng, 1983) and lysozyme (Cheng, *et al.*, 1975; Takahashi, *et al.*, 1986). Also, factors related to serine proteases have been demonstrated in oyster hemolymph (Bachère, *et al.*, 1990). Cytolytic activity directed towards vertebrate erythrocytes has been extensively studied in various invertebrate extracts. In the mussel *Mytilus edulis*, an adaptation of the *in vitro* plaque assay was employed to demonstrate the presence of agglutinins and cytolytic molecules released by hemocytes (Leippe and Renwrantz, 1988). In oysters (*Crassostrea virginica*), interactions with parasites have recently been investigated, both in terms of mortality (Burreson, *et al.*, 1994) and of host immune response involving lysozyme (Chu and LaPeyre, 1989), circulating and tissue-infiltrating hemocytes (Ford, *et al.*, 1993), plasma hemagglutinin (LaPeyre *et al.*, 1995), iron-binding proteins (Gauthier and Vasta 1994) and the role of stress proteins (Tirard, *et al.*, 1995).

Anti infectious immunity

Cytotoxic activity of mussel plasma

Mammalian erythrocytes were lysed by incubation in the presence of dialyzed mussel plasma. Differences in the observed percentages of cell death probably reflected differences in cell membrane composition. A tumor cell line from the mouse was also sensitive, as was *Bonamia ostreae*, an intra-hemocytic protozoa parasite of the flat oyster *Ostrea edulis* (Hubert, *et al.*, 1996). In contrast, the viability of the two tested Gram negative bacteria, *Escherichia coli* and *Vibrio alginolyticus*, was not affected.

Mussel cytotoxic activity depends on the concentration of active molecules and on the contact duration between effector molecules and target cells. Completely inactivated by heating at 45°C, the cytotoxic activity was strictly dependent on the pH of the incubation medium with an optimum for pH 8.5. Individual mussels presented enormous differences in their cytotoxic activity ranging from 8 to 81 % but stimulating the activity by various injections restricted such diversity.

Purified in anion exchange chromatography followed by gel filtration, the cytotoxic activity appeared mediated by a high molecular mass protein complex, acting as a polymer of 320 kDa, binding to the target cell surface to produce transmembrane pores. The 320 kDa protein is also a complex containing monomers of 25, 43 and 100 kDa.

Cytolytic activity in shrimp plasma

Several *Penaeus japonicus* plasma components were fractionated by anion exchange chromatography. Using monoclonal antibodies prepared against *P. japonicus* hemocytes and plasma, we located an α-macroglobulin-like (α-m) protein of 170 kDa (Bachère, *et al.*, 1995). Identified by immuno cross reactivity with commercially available antibodies, the shrimp α-m was found associated with

a cytolytic activity. Moreover, the monoclonal antibody used, recognized the crayfish *Pacifastacus leniusculus* 190 kDa α-m.

Antibacterial activity in oysters

In the two species, the European flat oyster *Ostrea edulis* and the Pacific oyster *Crassostrea gigas*, lysates of hemocytes and hemolymph plasma were assayed for antibacterial activity against an array of marine bacteria. These comprised Gram negative bacteria: *Vibrio alginolyticus, V. tubiashi* (both pathogenic to marine invertebrates), *E. coli*, and one Gram positive, *Micrococcus luteus*. Of naive animals, a low proportion showed either bactericidal or bacteriostatic activity, or both. These two activities have different spectra of target species-specificity. Induction was achieved by physical stress and the injection of saline solutions or bacterial suspensions (Hubert, *et al.*, 1996). Such treatments resulted in the induction of anti bacterial activities in virtually all the negative oyster specimens.

The activities in both oyster species are most probably due to peptides. As a first attempt of identification, chromatographic separations were performed with lysates from *O. edulis*: activity was located at the end of the NaCl gradient. The mode of action remains to be investigated, but it is probable that not one, but at least two active molecules are present.

Effect of heterologous anti microbial peptides

Anti-microbial activity of heterologous peptides was tested *in vitro* against several bivalve pathogens, *B. ostrea* and *Perkinsus marinus*, and bacteria belonging to Vibrionaceae family. Tachyplesin, a 17-residue peptide isolated from horseshoe crab hemocytes, was the most toxic and did not impair the bivalve hemocyte viability nor their phagocytic capabilities as demonstrated by respiratory burst activity. Magainins and to a lesser extent cecropins were also found as toxic for oyster pathogens (Morvan, *et al.*, 1994). Alternately, defensin from human neutrophils was not active until a concentration of 500 µg/mL.

Genetic transformation

Two main techniques were used as a first step of transgenesis in marine bivalves : *in vitro* lipofection of primary oyster hart cell culture and *in vivo* high velocity particle bombardment of mussel and oyster zygotes or young embryos. Reporter gene was luciferase under the control of two heterologous promoters : the heat shock protein 70 from *Drosophila* and the cytomegalovirus early promoter. Optimizing the technical parameters such as culture conditions, DNA ratios, limit pressure, heat shock temperature and duration, recovery time, sensitivity, was our first goal. In both cases, transient expression was obtained ranged from 10 to 30 times the background of luminescence.

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Chapter 16

Lucigenin- and Luminol-Dependent Chemiluminescent Measurement of Oxyradical Production in Hemocytes of the Pacific Razor Clam, *Siliqua patula*, and the Oyster, *Crassostrea gigas*

Evan Greger

BIOMED
1720 130th Ave N.E.
Bellevue, WA 98006-2203

Hemocytes of the Pacific razor clam, *Siliqua patula*, and the oyster, *Crassostrea gigas*, produced reactive oxygen intermediates during *in vitro* phagocytosis of zymosan particles, resembling the oxygen-dependent antimicrobial metabolism of activated mammalian polymorphonuclear leukocytes. Light emission during phagocytosis by zymosan-activated hemocytes was enhanced with lucigenin or luminol and measured on a liquid scintillation counter calibrated to detect single photon events (Greger *et al.*, 1995).

The chemiluminescent response of oyster and razor clam hemocytes was compared using probes which enhance light emission of activated blood cells during phagocytosis, the cyclic hydrazide luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Allen and Loose, 1976) and the acridinium salt lucigenin (10,10'0-dimethyl-9,9'-biacridinium dinitrate) (Allen, 1981). Lucigenin enhancement produced measureable CL in both oyster and razor clam hemocytes, similar to human polymorphonuclear leukocytes (Allen, 1981, 1986; Muller-Peddinghaus, 1984; Ward *et al.*, 1990) (Figure 1). Luminol-dependent CL of razor clam hemocytes was barely measurable and did not resemble the comparatively high levels observed in oyster hemocytes, suggesting inter-specific dissimilarity in hemocyte oxidative function. When added to either luminol or lucigenin, oyster hemocytes produced CL without zymosan stimulation. Razor clam hemocytes did not produce comparable autoreactivity in either medium, further implying differences in the hemocyte oxygen-dependent metabolism of the two bivalve species.

Participation of the superoxide anion in lucigenin-dependent CL of razor clam hemocytes was assessed by addition of superoxide dismutase at an interval prior to the CL peak (Webb *et al.*, 1974). Addition of superoxide dismutase produced concentration-dependent reduction in lucigenin-enhanced CL, suggesting that activated razor clam hemocytes produced the superoxide anion (Figure 2). The reaction resembled the lucigenin-enhanced CL of human leukocytes (Webb *et al.*, 1974, Nyberg and Klockars, 1990), as well as the luminol-dependent CL of hemocytes of the scallop,

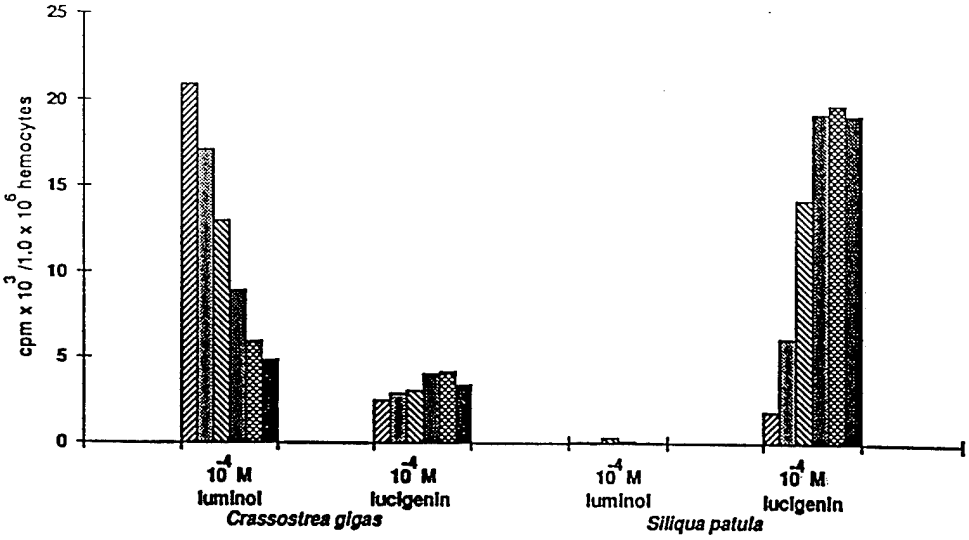


Figure 1. Bivalve species/CL probe comparison experiment. Representative CL response of 1.0 x 10^6 pooled hemocytes in 2 mL serum from the razor clam, *Siliqua patula*, or the oyster, *Crassostrea gigas*, added to a 10-4M concentration of lucigenin or luminol in 2 mL sterile seawater, and activated with zymosan in 2 mL sterile seawater (final concentration 1.33 mg/mL) (n = 13, 19, respectively). Six CL counts at consecutive 8 minute time intervals are shown.

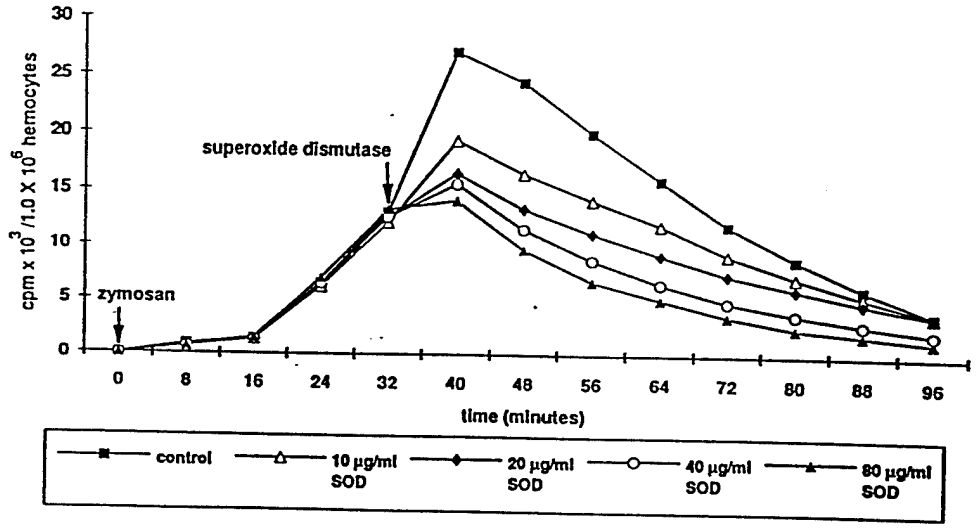


Figure 2. Superoxide dismutase experiment. Representative CL response of 1.0 x 10^6 pooled razor clam hemocytes in 2 mL serum added to a 10-4M concentration of lucigenin in 2 mL sterile seawater, and activated with zymosan in 2 mL sterile seawater (final concentration 1.33 mg/mL) (n = 11). Superoxide dismutase was added to vials at a 32 min. interval prior to the CL peak at final concentrations of 10, 20, 40, and 80 µg/mL. Thirteen CL counts at consecutive 8 min. time intervals are shown.

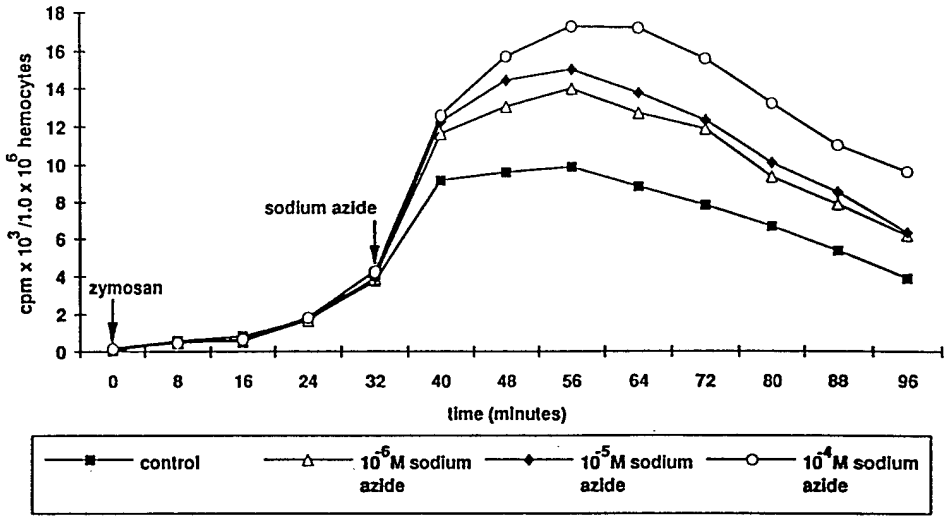


Figure 3. Sodium azide experiment. Representative CL response of 1.0 x 10^6 pooled razor clam hemocytes in 2 mL serum added to a 10-4M concentration of lucigenin in 2 mL sterile seawater, and activated with zymosan in 2 mL sterile seawater (final concentration 1.33 mg/mL) (n = 11). Sodium azide was added to vials at a 32 min. interval prior to the CL peak at final concentrations of O M, 10-4M, 10-5M, and 10-6M concentrations. Thirteen CL counts at consecutive 8 minute time intervals are shown.

Pecten maximus (Le Gall *et al.,* 1991), and the snails *Lymnaea stagnalis*, *Helix aspersa*, and *Achatina achatina* (Andersen and Amirault, 1979; Dikkeboom *et al.,* 1988; Adema *et al.,* 1992). Production of the superoxide anion was also indicated by inhibition by SODof the histochemical reduction of nitroblue tetrazolium observed microscopically on zymosan-activated hemocyte monolayers attached to glass slides.

Myeloperoxidase and catalase activities were evaluated by addition of sodium azide and potassium cyanide (Klebanoff *et al.,* 1971; Nakagawara *et al.,* 1981). Concentration-dependent amplification of razor clam hemocyte lucigenin-dependent CL with exogenous sodium azide, similar to that observed in human macrophages (McCord and Fridovich, 1969; Gyllenhammar, 1987; Jungi and Peterhaus, 1988), suggested oxidative activity somewhat independent from myeloperoxidase (Allen, 1986) (Figure 3). Since potassium cyanide also inhibits myeloperoxidase, similar enhancement of lucigenin-dependent CL with potassium cyanide also implied reduced myeloperoxidase involvement (Nakagawara *et al.,* 1981) (Figure 4). Increased CL with addition of exogenous cyanide and azide resembled the lucigenin-dependent CL of human macrophages (Muller-Peddinghaus, 1984), but contrasted with the inhibition of invertebrate hemocyte luminol-dependent CL (Dikkeboom *et al.,* 1987; Le Gall *et al.,* 1991), suggesting that the probes enhance CL by different mechanisms and detect distinct oxidative events (Allen, 1986). Further evidence for reduced myeloperoxidase activity in razor clam hemocytes was suggested by the lack of luminol-dependent CL, a probe reliant on myeloperoxidase, hydrogen peroxide, and halide (DeChatelet *et al.,* 1982; Seim, 1983).

The lucigenin-dependent CL of activated razor clam hemocytes formed a bell-shaped curve that could be consistently measured above that of control vials containing lucigenin and zymosan and

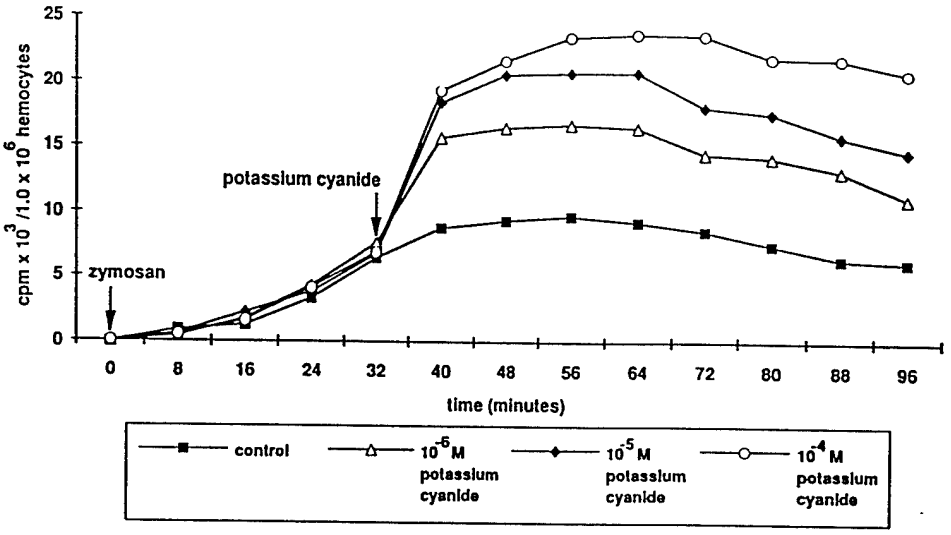


Figure 4. Potassium cyanide experiment. Representative CL response of 1.0 x 10^6 pooled razor clam hemocytes in 2 mL serum added to a 10-^{4}M concentration of lucigenin in 2 mL sterile seawater, and activated with zymosan in 2 mL sterile seawater (final concentration 1.33 mg/mL) (n = 11). Potassium cyanide was added to vials at a 32 min. interval prior to the CL peak at final concentrations of O M, 10^{-4}M, 10^{-5}M, and 10^{-6}M concentrations. Thirteen CL counts at consecutive 8 minute time intervals are shown.

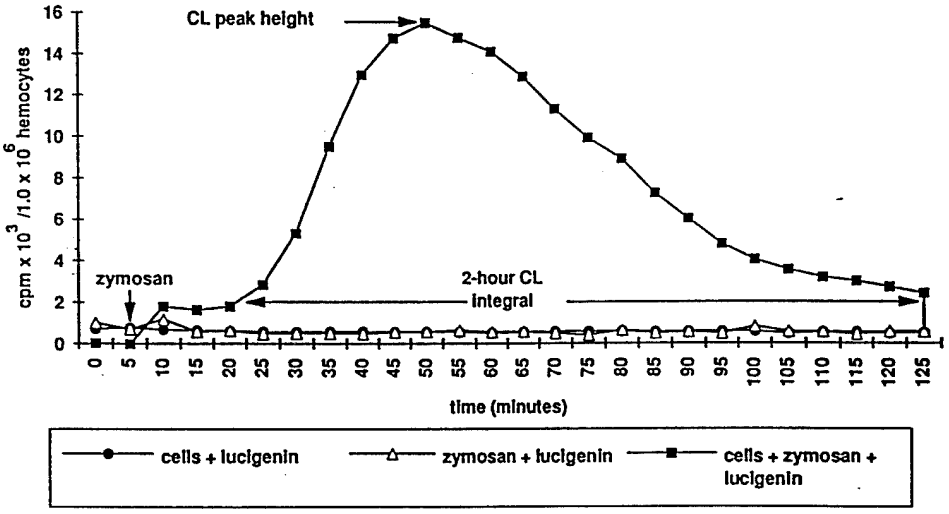


Figure 5. Lucigenin-dependent CL of razor clam hemocytes. Representative CL response of 1.0 x 10^6 razor clam hemocytes (individual clam) in 2 mL serum added to a 10^{-4}M concentration of lucigenin in 2 mL sterile seawater, and activated with zymosan in 2 mL sterile seawater (final concentration 1.33 mg/mL), compared to the CL produced by cells and lucigenin only or zymosan and lucigenin only (n = 139). Twenty-six CL counts at consecutive 5 min. time intervals are shown.

cells and zymosan (Figure 5). Lucigenin produced a delayed time-to-peak CL compared to luminol in oyster hemocytes, allowing for consistent measurement of both CL peak height and integral (Figure 1). A comparative delay in lucigenin-dependent CL peak height and a reduced standard of deviation compared to luminol, consistent with that of human phagocytes, has been used to propose the use of lucigenin in large-scale kinetic experiments (Muller-Peddinghaus, 1984). In addition, lucigenin could be used to measure hemocyte oxidative activity of both bivalve species and was soluble in water, avoiding exposure of hemocytes to dimethyl sulfoxide and other solvents that might affect phagocytosis and/or scavenge oxygen radicals. These factors, along with a specificity mainly for the primary oxygen intermediate, the superoxide anion, suggest that lucigenin may provide a valuable tool for measurement of the blood cell phagocytosis.

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Chapter 17

Effects of Peptidyl Membrane Interactive Molecules™ on *Crassostrea Virginica* Hemocyte Function and *Perkinsus Marinus* Viability

Beverly A. Weeks-Perkins[1], Wayne A. Tompkins[1], Jesse M. Jaynes[2], Suzanne-Kennedy Stoskopf[1], Jia Ma[1], Jay F. Levine[1] and Frank O. Perkins[1]

[1]Department of Microbiology, Pathology and Parasitology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606, U.S.A.

[2]Demeter BioTechnologies, Ltd. Research Triangle Park, NC 27709, U.S.A.

ABSTRACT

We investigated the *in vitro* cytotoxic effects of 4 synthetic lytic peptides termed peptidyl membrane interactive molecules™ (peptidyl MIMs™; modified mellitin and defensins) at concentrations from 0.1 to 200 μM against *Perkinsus marinus*, the histozoic protozoan parasite of the Eastern oyster, *Crassostrea virginica*. Viability of axenically-cultured *P. marinus* was assessed microscopically by trypan blue exclusion and by uptake of the fluorescent dyes acridine orange and ethidium bromide. Following exposure to one of the peptidyl MIMs™ (D1D-2), *P. marinus* viability was reduced by 93-100% at the 100 and 200 μM concentrations. None of the peptidyl MIMs™ at concentrations from 0.1-200 μM demonstrated toxicity for *C. virginica* hemocytes as assessed by viability of the hemocytes and their capabilities to chemotactically respond to and phagocytize *P. marinus*. In fact, lower concentrations of the peptides (0.1 and 10 μM) stimulated chemotactic and phagocytic activity of hemocytes. The most active peptide (D1D-2) at a concentration of 10 μM stimulated chemotaxis from a mean of 60 ± 7% to 89 ± 7% of granulocytes and phagocytosis from a mean of 23 ± 5% to 52 ± 6% of total hemocytes. These results demonstrate that our selected synthetic lytic peptides have substantial protisticidal activity against *P. marinus* and may stimulate hemocyte function *in vitro*.

INTRODUCTION

Perkinsus marinus which kills unacceptably large numbers of the Eastern oyster, *Crassostrea virginica*, along the Gulf of Mexico and Atlantic coast of the United States, is probably the most important pathogen of *C. virginica* and has been the most important factor in causing the catastrophic decline of the commercial oyster industry along the eastern coast of the U.S. (Andrews, 1988).

Recognizing these facts, many proposals have been advanced for controlling the pathogen, all without significant success.

Several distinct and structurally related classes of small (23-39 amino acids) amphipathic cationic lytic peptides exist in a diversity of organisms including insects, amphibians, horseshoe crabs, mice and humans. They comprise melittin (M) (Bowman and Hultmark, 1987), cecropins (C) (Bowman and Hultmark, 1987), magainins (G) (Zasloff, 1987) and defensins (D) (Dimarcq *et al.*, 1990), which exert their host-protective antimicrobial effects by causing severe perturbation of the membranes of target pathogens such as bacteria, fungi, protozoa, tumor cells and viruses. Lytic peptides also exhibit concentration-dependent cell proliferating or antineoplastic effects (Jaynes *et al.*, 1989). Analogues of M and D, termed peptidyl membrane interactive molecules^{TM} (hereafter referred to as peptidyl MIMs^{TM}), were designed and synthesized to maximize antipathogen cytotoxicity and minimize toxicity for normal host cells. These peptides are commercially available from Demeter BioTechnologies, Ltd., Research Triangle Park, NC 27709, USA.

Lytic peptides have been shown to be important in the antimicrobial defense of Metazoa (Kimbrell, 1991), and using this knowledge, workers have been successful in controlling diseases by transgenically altering the host's genome to induce synthesis of effective lytic peptides (Jaynes *et al.*, 1993; Norelli *et al.*, 1994). Therefore, in a search for an answer to the problem caused by *P. marinus*, the present authors are engaged in research to determine which synthetic lytic peptides are the most effective in killing *P. marinus* without compromising the host oyster. Our preliminary results on the *in vitro* effects of selected peptidyl MIMs^{TM} are reported herein.

MATERIALS AND METHODS

Oysters

Adult *Crassostrea virginica* were collected from the James River seed beds and held in a 700 gal. recirculating sea water system with biofiltration at a salinity of 22-24 ppt. and 22-24°C for less than 45 days before hemocytes were obtained. During the holding period, the oysters were fed a suspension of *Thalassiosira weissflogii* at about 6 X 10^5 cells/L every other day. Sea water was prepared using 40 Fathom Marinemix sea salts (Marine Enterprises, Inc., Baltimore, MD).

Perkinsus marinus

Cells of the pathogen were axenically grown in modified Kleinschuster and Swink (1993) medium at 22-24°C and harvested by centrifugation while in exponential or early stationary phase. The cells were then washed in filtered sea water (FSW) at 24 ppt. and resuspended in FSW to yield 2.8 X 10^6 cells in 500 µL. The parasite suspensions were stored on ice for no longer than 1 hr before the toxicity assays were performed.

Lytic Peptides

Four synthetic lytic peptides, modeled after naturally-occurring molecules and termed peptidyl MIMs^{TM}, were provided by Demeter BioTechnologies, Ltd., dissolved in phosphate buffered saline

(PBS) and held at -70°C to provide working stock solutions of 1 mM. Their designations are D1D-2, D2B-15, D4E-1 and D1D-6. Their molecular weights range from 2,000 to 5,000, and they are 17 to 37 amino acids in length.

Assay for Peptidyl MIMs™ Toxicity Against *Perkinsus marinus*

The four peptide solutions were thawed and dilution series using FSW were prepared. For each peptide final concentration (0.1, 1, 10, 100, and 200 µM), *P. marinus* cell suspensions were diluted in FSW and dispensed into microcentrifuge tubes in 36 µL aliquots of ca. 2 X 10^5 cells each. For each dilution, 4 µL of peptidyl MIM™ solution was added to each 36 µL of cell suspension. The same volume of FSW was added as the control. Replicates of 3 for each condition were evaluated. The resultant mixtures were incubated at room temperature for 60 min. before *P. marinus* viability was assessed.

Determination of *P. marinus* Viability

Two viability staining techniques were used. In one, dye exclusion of aqueous trypan blue (9 parts of an aqueous 0.4% stain solution or the same concentration in FSW at 24 ppt. salinity plus 1 part cell suspension in FSW) was noted for the four peptidyl MIMs™ tested. As a confirmation of the efficacy of trypan exclusion in detecting *P. marinus* viability, fluorescent stains were used in an experiment involving the peptidyl MIM™ D2B-15. A solution of acridine orange (0.12 mg/100 mL) and ethidium bromide (2.95 mg/100 mL) (Morvan *et al.*, 1995) in FSW (24 ppt salinity) was used and examined by epifluorescence microscopy using an exciter/barrier filter combination of 450/490 mµ and 520 mµ. Dead cells fluoresced red and live cells fluoresced green. The staining time was 5 min before counting the percentages of viable and dead parasites. Replicates of 3 were averaged for each condition using trypan blue.

Electron Microscopy

Control and D1D-2 peptidyl MIM™-treated parasites were fixed for 45 min. in 2% glutaraldehyde + 1.5% paraformaldehyde in 0.1 M Millonig's phosphate buffer and 2.7% glucose at pH 7.2 and room temperature. After buffer rinses, postfixation was accomplished for 1 hr. in 1% OsO_4 in 0.1M Millonig's phosphate buffer and 2.7% glucose at pH 7.2 and 4°C. Cells were embedded in Spurr's resin, sectioned at 60 nm and stained in Reynold's lead citrate and uranyl acetate.

Effects of Peptidyl MIMs™ on the Viability of Oyster Hemocytes

Hemocytes were obtained from the adductor muscle by puncture using a 23-gauge needle. The hemolymph was drawn into a 5 mL hypodermic syringe containing cold modified Alsever solution (Bachère *et al.*, 1988) so that a three-fold dilution resulted, thus preventing clotting of the hemocytes. Hemolymph from three oysters was pooled, hemocytes were counted using a hemocytometer, concentrated by centrifugation at 500 xg for 5 min and adjusted to a final concentration of 2 X 10^5 hemocytes per 36 µL. Replicates of 3, 36 µL aliquots were placed in microcentrifuge tubes and to each was added 4 µL of the four peptidyl MIMs™ in FSW at concentrations of 0.1, 1, 10, 100 and 200 µM. Controls consisted of 4 µL of FSW, and secondly, 4 µL of non-lytic peptide (α-PI) in FSW. This methodology is a modification of that described by Morvan, *et al.* (1994). Hemocytes

were incubated for one hour and viability was evaluated by trypan blue exclusion for all 4 peptidyl MIMs^{TM} and acridine orange/ethidium bromide staining for peptide D2B-15 as described above.

Effects of a Selected Peptidyl MIM^{TM} on Function of Oyster Hemocytes

Immune function of hemocytes was determined *in vitro* using the following assays after hemocytes had been treated or not treated with the appropriate concentrations (0, 0.1, 1, 10, 100 and 200 µM) of the peptidyl MIM^{TM} D1D-2 in FSW for 60 min. Since hemocytes are known to be the primary defense agents against invading microbes, events which occur during the endocytotic process, i.e., chemotaxis and phagocytosis, were quantitated in control and treated cells. For observing chemotaxis, a modification of the techniques of Weeks *et al.* (1986) and Weeks-Perkins and Ellis (1995) were used. Meronts, merozoites and schizonts of *P. marinus* were placed in the lower compartments of Boyden chambers which consist of two compartments separated by a filter of 5 µm pore size. A control consisted of adding FSW without *P. marinus* cells to the lower chamber. After a 1.5 hr incubation period, chemotactic activity was quantified by counting at least 100 cells on the upper and lower surfaces of the filter being certain to vary the focal plane so that all cells were observed. The ratio of hemocytes on the lower surface to the total number counted represented percent chemotaxis.

The percent phagocytosis was determined by quantifying the number of hemocytes which had internalized *P. marinus* per total number of hemocytes, using a modification of the techniques of Weeks and Warinner (1984), Bodhipaksha and Weeks-Perkins (1994) and Weeks-Perkins *et al.* (1995). The hemocyte suspensions (1 X 10^5 in 100 µL) were allowed to adhere to the bottom of plastic wells in multiwell plates for approximately 30 min. *P. marinus* cells (5 X 10^5 in 100 µL) were then placed in each well containing hemocytes. The plates were incubated in a moist chamber at room temperature for 2 hr. Cells were then stained *in situ* using Diff-Quik solutions and examined on a Zeiss Axiovert inverted microscope using a 40X adjustable, bright field objective or removed from the wells by scraping with rubber policemen and jetting FSW against the bottom of the wells. The dislodged cells were allowed to dry on a microscope slide, stained with Diff-Quik solutions and examined using a 100X bright field objective. Since the counts for dislodged cells yielded lower levels of phagocytosis and not all cells could be dislodged, the percent phagocytosis values reported herein are from the cells counted ***in situ*** in the wells. Cells observed using a 100X objective could be seen in greater detail thus permitting a better assessment as to whether cells of *P. marinus* had been internalized, but the technique was inferior due to the problem with dislodging the most firmly attached cells and possibly a problem with disruption of some of the cells. Use of the 40X objective presented the problem of reduced resolution; however, where a judgement had to be made as to whether a *P. marinus* cell had been internalized or was lying on or under a hemocyte, the parasite cell was considered to be outside the hemocyte. Therefore, the counts should be an underestimation rather than an overestimation. In addition, no attempt was made to evaluate phagocytosis in clumped hemocytes. For each control and concentration of D1D-2 at least one hundred phagocytes were counted.

Each of the assays involving viabilities of *P. marinus* and hemocytes as well as chemotaxis and phagocytosis of hemocytes was conducted on 4 separate occasions.

Statistics

The statistical analysis of data was performed using analysis of variance for determination of significant differences in values for control and treated cells. The least significant difference method of multiple comparisons was used to identify significant differences among treatment groups (Dowdy and Wearden, 1991). Statistical significance was set at ≤ 0.05.

RESULTS

Effects of Peptidyl MIMs^{TM} on *Perkinsus marinus* and Hemocytes of *Crassostrea virginica*

Four peptidyl MIMs^{TM} at concentrations of 0.1, 1, 10, 100 and 200 µM were tested for their cytotoxicity toward *P. marinus* (Table 1). The most active peptide (D1D-2) was cytotoxic for *P. marinus* with 97 ± 3% killing at 100µM and 100% at 200 µM. The other 3 peptides at 100µM concentration resulted in 65 ± 3, 51 ± 1 and 40 ± 6% killing. None of the peptidyl MIMs^{TM} were cytotoxic for hemocytes at any concentration tested (Table 1).

Table 1.
Effects of P-MIMs^{TM} on *Perkinsus marinus* and *Crassostrea virginica* Hemocytes

Target Cell	Peptide	Concentration (µM)					
		0	0.1	1	10	100	200
		% Killed					
P. marinus	D1D-2	4±2	37±4	50±3	61±5	97±3	100
	D2B-15	3±2	12±2	39±3	53±2	65±3	69±3
	D4E-1	8±2	8±4	5±3	20±3	51±1	61±3
	D1D-6	6±4	1±1	3±2	10±4	40±6	46±5
Hemocytes	D1D-2	3±2	3±3	3±3	5±4	5±3	4±2
	D2B-15	6±2	6±2	8±2	5±3	6±3	7±2
	D4E-1	6±3	9±3	6±2	7±3	5±1	7±3
	D1D-6	7±2	7±3	5±1	7±2	7±1	6±1

Effect of a Selected Peptidyl MIM^{TM} Against *P. marinus*

The most active peptidyl MIM (D1D-2) was cytotoxic for *P. marinus* in a dose-dependent manner (Figure 1). When 2 X 10^5 parasites were treated with 0.1 µM, the parasite viability was reduced by 37%. Mid-range concentrations of 1 and 10µM killed, respectively, 50 ± 3 and 61 ± 5% of the parasites for the same exposure duration. At the highest concentrations (100 and 200µM), extensive lysis of parasites was visible at the light microscope level and only 3 ± 3% and 0%, respectively, were viable after 1 hour.

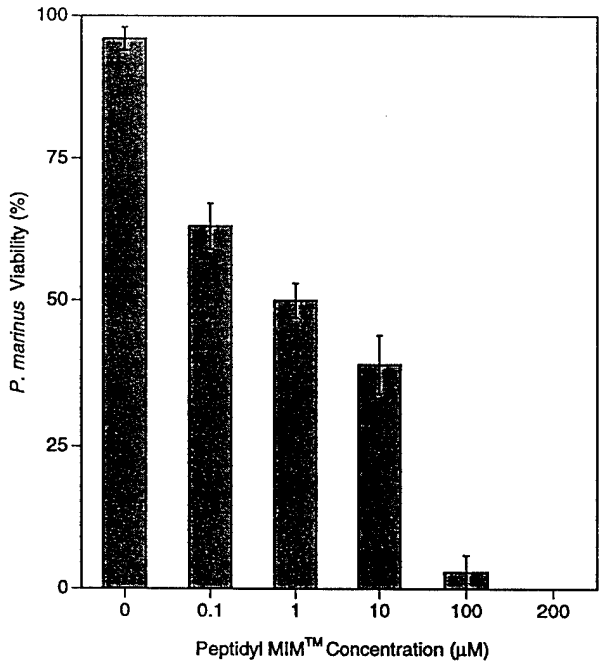


Figure 1. Effect of the peptidyl MIM™ D1D-2 on the viability of *P. marinus*. Results are the mean and standard deviation (SD) of 3 replicate samples for each concentration in 4 separate experiments. At least 100 viable parasites (unstained in the trypan blue procedure and green fluorescing in the acridine orange procedure) were counted in a volume corresponding to parasites in the control sample. Relative viability= number of live treated *P. marinus*/total viable and dead cells.

Ultrastructure of Control and Peptidyl MIMTM (D1D-2)-Treated *P. marinus* and Hemocytes

The fine structure of control cells was the same as has been previously reported (Perkins, 1969) (Figure 2A) with the exception that some meronts and merozoites did not form cell walls. Those cells which had cell walls and which were treated with D1D-2 at 100 μM were disrupted to the point at which only vesicles and non-membranous cellular debris were visible within the intact cell walls (Figure 2B). Meront, merozoite and schizont cell walls were retained. Remnants of those cells which had not formed cell walls before treatment were visible as clusters of non-membranous cell debris which resembled the non-membranous cell debris found in the cell "ghosts" which had formed walls before treatment. Figures 2A and 2B are representative of typical conditions for untreated cells and cells which were pretreated with 100 μM D1D-2.

The ultrastructure of D1D-2-treated and untreated hemocytes was indistinguishable (micrographs not included).

Effect of Peptidyl MIM™ on Hemocyte Chemotaxis

The most active peptide (D1D-2) at concentrations of 1 and 10 μM significantly ($p \leq 0.05$) enhanced chemotactic responses of hemocytes as compared to the control and those treated with 0.1, 100 and 200 μM peptide (Figure. 3). In the control, where there were no *P. marinus* cells added to the lower chamber, negligible numbers of hemocytes were observed on the lower surface of the filter.

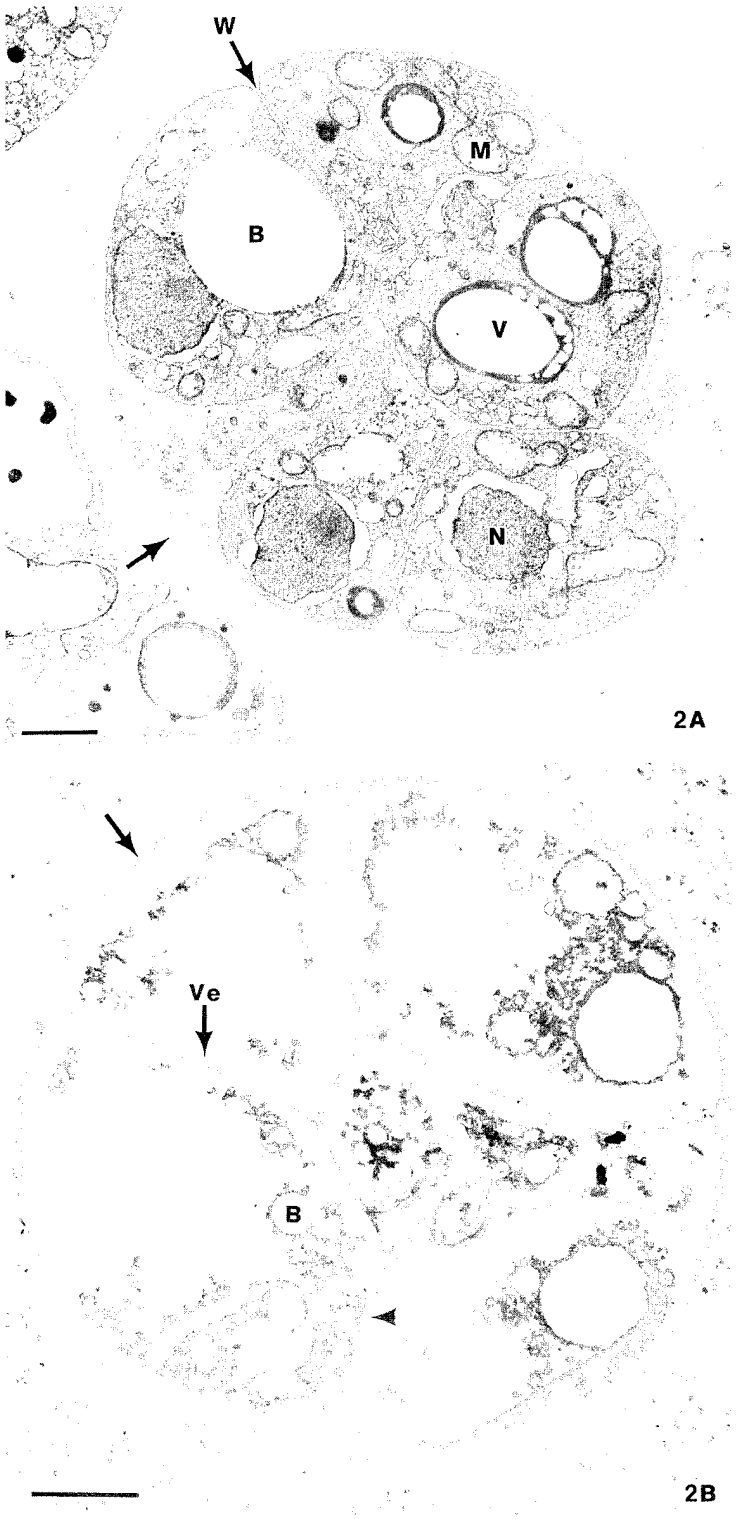


Figure 2. A. Schizont of *Perkinsus marinus* incubated in filtered sea water (FSW) for 1 hr prior to fixation. Portions of 6 merozoites are visible in the section plane. Nucleus (N); lipoid body (B); vacuoplast material (V); mitochondrion (M); forming cell wall of merozoite (W); schizont cell wall (arrow). Bar= 1µm. **B.** Schizont of *P. marinus* incubated in 100 µM of peptidyl MIM D1D-2 for 1 hr. in FSW. The schizont (arrow) and merozoite (arrow head) cell walls are the only identifiable cellular elements which remain. Membrane bound vesicles (Ve); lipoid body (B). Bar = 1µm.

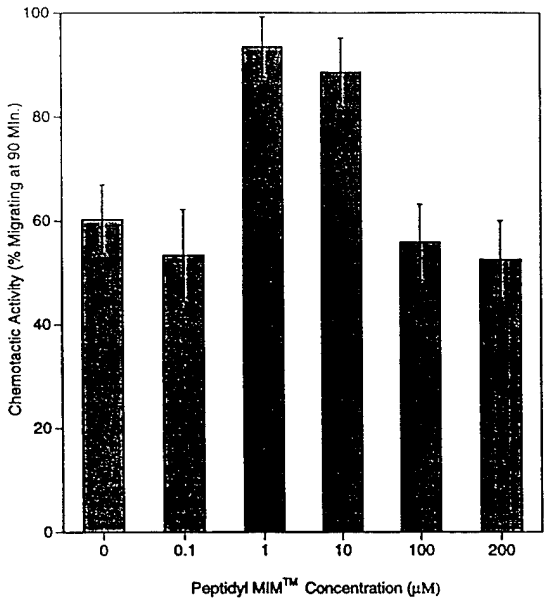


Figure 3. Chemotactic responses to *P. marinus* by *C. virginica* hemocytes treated with peptidyl MIM™ D1D-2 at 0 to 200 µM for one hour. Results are the mean and SD of 3 replicates expressed as percentage of cells migrating in 4 separate experiments.

Effect of Peptidyl MIM™ on Hemocyte Phagocytosis

Peptide D1D-2 at concentrations of 1 and 10 µM significantly ($p \leq 0.05$) stimulated hemocyte phagocytosis as compared to phagocytosis by hemocytes exposed to 0, 0.1, 100 and 200 µM D1D-2 (Figure 4).

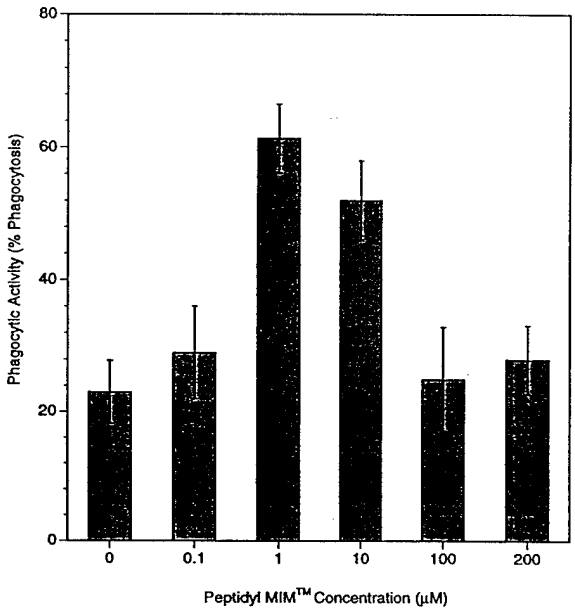


Figure 4. Phagocytosis of *P. marinus* by *C. virginica* hemocytes treated with peptidyl MIM™ (D1D-2 at 0 to 200 µM) for one hour. Results are the mean and SD of 3 replicates expressed as percentage of cells migrating in 4 separate experiments.

DISCUSSION

The effects of 4 peptidyl-membrane interactive molecules™ (peptidyl MIMs™) were investigated *in vitro* against the histozoic protist *Perkinsus marinus* and the host cells in which it proliferates, the hemocytes of the Eastern oyster, *Crassostrea virginica*. Two of these peptides were synthetic analogues of the naturally occurring lytic peptides, defensins, which have been extracted from phagocytes of a variety of mammalian species and from hemocytes of several species of insects. They have been reported to have activity against bacteria, fungi, tumor cells and viruses (Kimbrell, 1991; Ma *et al.*, 1996). The other two peptidyl MIMs™ were synthetic peptides of the melittin class which were originally extracted from bee venom and have been shown to be cytotoxic for bacteria (Wade *et al.*, 1992).

A number of studies have detailed the effects of various types of lytic peptides and their synthetic analogues against pathogenic protists. These include *Blastocystis hominis, Entamoeba histolytica, Trypanosoma cruzi* (Huang *et al.*, 1990; Jaynes *et al.*, 1988), *Plasmodium cynomolgi* (Gwadz *et al.*, 1989), *P. falciparum* (Jaynes *et al.*, 1988), *Acanthamoeba castellanii* (Feldman *et al.*, 1991), *Acanthamoeba polyphage* (Schuster and Jacob, 1992) and *Bonamia ostreae* (Morvan *et al.*, 1994). However, none of these were in the mellitin or defensin class of lytic peptides or analogs of such lytic peptides.

By determining the viability of parasites treated *in vitro* with 4 peptidyl MIMs™, we demonstrated the cytotoxicity of these antimicrobial peptides against the apicomplexan protist *P. marinus*. The decrease in viability was greatest for the D1D-2 peptide and ranged between 94 and 100% for a one hour treatment with the two highest concentrations tested. Further *in vitro* studies using the other 3 peptides were not conducted since they were less toxic for *P. marinus*. Electron microscopy of D1D-2 peptidyl MIM™-treated *P. marinus* showed complete lysis of the cells leaving only cell walls, small vesicles, lipoid droplets and unidentifiable cell debris.

Since *C. virginica* hemocytes are host cells for *P. marinus*, the effects of peptidyl MIMs™ on hemocyte viability and function were investigated. The presence of *P. marinus* in oyster hemocytes is a result of phagocytosis, there being no mechanism on the part of the pathogen to actively penetrate host cells. Once internalized, some killing of *P. marinus* may occur, but obviously not at a level which is always adequate to protect the host, as evidenced by the virulence of the pathogen. La Peyre (1993) reported a reduction of 30% in numbers of merozoites and meronts in the presence of *C. virginica* hemocytes *in vitro*. Whether this level of killing is a reflection of the levels attained under natural conditions remains to be determined.

Chemotaxis and phagocytosis have been shown to be reliable indicators for determining the *in vitro* effects of pollutants on hemocyte function (Bodhipaksha, 1993; Bodhipaksha and Weeks-Perkins, 1994; Weeks-Perkins *et al.*, 1995). Therefore, these assays were used to evaluate the effect of a selected peptidyl MIM™ on hemocytes which function as host cells and as immune cells. No impairment of hemocyte viability was observed and treated hemocytes were capable of chemotactic and phagocytic responses. In fact, one peptidyl MIM™ at intermediate concentrations stimulated these two functions. In addition, there were no discernable ultrastructural alterations in the

hemocytes. Therefore, this peptidyl MIM™ appears to be harmless and perhaps beneficial to *C. virginica* hemocytes. The mechanism of action for hemocyte stimulation is unknown.

Our observations on the cytotoxicity of peptidyl MIMs™ against *P. marinus* and the lack of damage to host cells is consistent with the results obtained by Jaynes *et al.* (1988) in their *in vitro* studies of lytic peptide activities against *Plasmodium falciparum* in human erythrocytes and *Trypanosoma cruzi* in Vero cells, and those of Morvan *et al.* (1994) in their *in vitro* investigations of *Ostrea edulis* parasitized by *Bonamia ostreae.*

Lytic peptides are remarkably toxic to microbes and non-toxic to cells of the Metazoa and higher plants. An exception is noted in mammalian cells. If they are neoplastic or the cytoskeleton of the cells is disrupted by agents such as cytochalasin D or colchicine (Jaynes, 1990), then they are not resistant to lytic peptides suggesting that integrity of the cytoskeleton is crucial.

Also of interest are the phylogenetic implications. The difference in susceptibility of the protist, *P. marinus*, as opposed to the metazoan species, *C. virginica,* underlines the fundamental differences which exist between the Animalia and the Protista, differences which have long been recognized by morphologists and now are supported by molecular biologists (Patterson and Sogin, 1992). The differences in the present context appear to lie at the level of the plasmalemma. As the primary structure of peptidyl MIMs™ is an amphipathic α–helix with hydrophobic amino acid side chains on one face of the α-helix and hydrophilic amino acid side chains on the other, it is proposed that these peptides disrupt susceptibile cell membranes by pore formation and subsequent lysis due to loss of osmotic integrity (Christensen *et al.,* 1988; Jaynes, 1990). This is supported by transmission electron microscopic examination showing openings in cell membranes treated with peptidyl MIMs™ and other lytic peptides (Christensen *et al.,* 1988; Jaynes, unpublished data; Morvan *et al.,* 1995).

Techniques of transgenics offer the greatest promise for practical application of these findings. Genes which code for synthesis of selected peptidyl MIMs™ can be formulated and transgenically inserted into the genome of oysters. If appropriately expressed, this would provide a strain of *P. marinus*-resistant oysters. Jaynes *et al.* (1993) and Norelli *et al.* (1994) have already been successful in transgenically inserting genes for the synthesis of lytic peptides into two plant species, resulting in the host acquiring resistance to a pathogen to which it was previously susceptible.

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Chapter 18

In Vitro Modulating Effects of Monomer and Dimerized Lysozyme on Polymorphonuclear (PMN) and Mononuclear (MN) Cell Activity

A.K. Siwicki, P. Klein[1] , W. Kiczka[1], M. Morand[2] and M. Studnicka[3]

Fish Pathology and Immunology Research Laboratory IFI
Zabieniec near Warsaw
05-500 Piaseczno, Poland

[1]Nika Health Products
Princeton, New Jersey, USA

[2]Laboratoire Veterinaire Departemental Conseil General du Jura
39016 Lons le Saunier, France

[3]Department Biology and Toxicology of Catholic University
Lublin, Poland

INTRODUCTION

Lysozyme is an enzyme with bacteriolytic properties and is ubiquitous in its distribution among living organisms. It especially attacks structures containing muramic acid, and has also been reported to have antiviral, antibacterial and anti-inflammatory properties. In nature, lysozyme is found only as a monomer (Osserman *et al.*, 1974). Under natural conditions, a number of substances achieve their full effectiveness only as a dimer or polymers. Therefore, a question arose whether greater activity can be generated through lysozyme dimerization. Nika Health Products Princeton, NJ, USA prepared the dimer of lysozyme for experimental studies and verified the accuracy of this hypothesis. Lysozyme dimer turned out to be significantly less toxic than its monomer, and experimental studies showed that dimerized lysozyme activates immunocompetent cells, induces phagocytosis, stimulates alpha-interferon synthesis and modulates TNF (tumor necrosis factor) generation (Kiczka, 1994). Since it prevents excess TNF generation, the lysozyme dimer also precludes all the negative effects associated with excess TNF levels. While it modulates TNF generation, lysozyme dimer it does not completely eliminate TNF which, in small amounts, is indispensable in fighting the results of infection (Beutler, 1992; Kiczka, 1994). Toxicological tests carried out on animals, revealed moderate toxicity of high doses of this preparation. The cytotoxic effect was recorded at concentration over 500 µg/mL in the V 79 cells line. Concentration up to 1000 µg/mL did not show any toxic effect on cells of the Vero, HeLa, MRC5 and MDCH type (Klein and Kiczka, 1994). The experimental study in animals showed that lysozyme dimer activates the phagocytic activity of blood leukocytes (Kiczka *et al.*, 1994; Pomorski *et al.*, 1994).

The use of immunostimulants to activate the nonspecific defence mechanisms and prevent diseases in fish culture is an important development (Anderson 1992, Siwicki *et al.*, 1994). Several drugs and chemicals have been shown to be effective in stimulating immunocompetent cells and defense mechanisms in fish, including levamisole (Siwicki 1989, Siwicki *et al.*, 1990), glucans (Yano *et al.*, 1989; Robertsen *et al.*, 1990), animal extracts (Jeney and Anderson 1993; Siwicki *et al.*, 1994), and other products (Anderson 1992). The effect of immunostimulants on the defence mechanisms must beverified by *in vitro* and *in vivo* studies. The most important part of an *in vitro* study is the influence of potential immunomodulators on the two major functions of the immune system in fish, 1. the specific mechanism of lymphocyte proliferation and antibody secreting cells and 2. the nonspecific mechanisms, phagocytosis and metabolism of phagocytic cells.

In the present *in vitro* study, the influence of monomer (Sigma, St Louis, MO, USA) and dimerized lysozyme (KLP-602, Lydium KLP) on rainbow trout (*Oncorhynchus mykiss*) lymphoid and myeloid cells were examined by using a respiratory burst activity assay of neutrophils and macrophages stimulated by PMA, a MTT assay for proliferation of lymphocytes and ELISPOT assay for total and specific antibody secreting cells (ASC) stimulated by *Yersinia ruckeri* O-antigen.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss*) weighing 200-250 g were purchased from a local fish farm. Fish were maintained in 500 L tanks and held in concrete receways fed with spring water (12 ± 1° C). The fish were fed a commercial pelletized dry feed.

Immunostimulants and antigen

In this *in vitro* study, lysozyme monomer from chicken egg white (L 6876; approx. 95% protein and 50,000 units/mg protein) was obtained from Sigma (USA) and two forms of dimerized lysozyme were obtained from Nika Health Products (USA):

- Lydium-KLP (Nika) commercial products for veterinary use, manufactured to the specifications of Nika Health Products by G. Streul and Co.AG, Uznach, Switzerland. Lydium-KLP includes, as the active ingredient, natural, highly purified dimer of lysozyme extracted from hen egg's ovalbumin (active ingredient - 5 mg, solvent - 10 mL, preservative).

- KLP-602 highly purified, lyophilized dimer of lysozyme extracted from hen egg's ovalbumin (powder without solvent and preservative, used for preparation of Lydium-KLP (Nika).

Experimental design

Four experiments were conducted analyzing the effects of doses of lysozyme monomer and dimer on the macrophages and neutrophils respiratory burst activity, proliferation of lymphocytes and the numbers of antibody secreting cells. In each experiment, similar concentration of monomer and dimerized lysozyme at 0.1, 1.0, 5.0, 10, 50 and 100 μg/mL of medium were used.

Before dissection, fish were anesthetized in Propiscin (IFI, Poland) and bled from the caudal vein in order to reduce the blood volume in the pronephros. Blood, pronephros and spleen from 10 fish were removed and single cell suspensions obtained by teasing the tissues in HBSS medium through a nylon mesh. The cells from pronephros and spleen were washed in heparinized Hank's balanced salt solution (HBSS) and isolated on Percoll gradient (Pharmacia, Uppsala, Sweden). Blood cells were purified on Gradisol G (Polfa) or Lymphoprep (Nicomed, Oslo, Norway) gradients.

Respiratory burst activity assay

The modification of Secombes (1990) technique was used for study the respiratory burst activity of neutrophils and macrophages stimulated by Phorbol myristate acetate (PMA, Sigma, stock solution: 50 µg/mL in ethanol, kept frozen at -20°C). The neutrophils were isolated from blood and macrophages from head kidney. One hundred µL of cells suspension ($1x10^6$/well in RPMI-1640) are added to 96-well culture plates (Costar, USA) and incubated with 100 µL of different concentrations of monomer or dimer lysozyme in RPMI-1640 (Roswell Park Memorial Institute-1640) for 2 hr at room temperature. After incubation non-adherent cells were removed by washing in RPMI 1640. The medium was replaced by 0.1 mL of prewarmed RPMI, and 0.1 mL of NBT (Sigma) at 2 mg/mL in RPMI with or without PMA, to give final concentrations of 10 µg/mL PMA and 1 mg/ml NBT. Replicate wells for subsequent assessment of cell numbers were treated similarly but without the addition of NBT or PMA. The plates were incubated 30 min at room temperature. The supernatant NBT solution was removed from the wells and the wells washed throughly with methanol. The wells were then allowed to air dry. The insoluble blue formazan was solubilized by adding first 120 µL/well 2M KOH, and then 140 µL/well dimethyl sulphoxide (DMSO, Sigma). The contents of the wells were then mixed immediately with an automatic pipette to complete solubilization and give a final solution with an intense blue color. The OD_{620} of this solution was read on a micro-reader, using as a blank a well without cells which was incubated with NBT solution, and subjected to the same fixing, washing and solubilization steps; this remained colorless.

Lymphocyte proliferation

Isolated lymphocytes were suspended at $5x10^6$ cells/mL in RPMI 1640 containing 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomicin, 0.02 mM 2-mercaptoethanol, 1% Hepes buffer and 10% rainbow trout plasma decomplemented. One hundred µL of cell suspension was distributed in each of the 96 well (Costar, Cambridge, MA, USA) and 100 µL of monomer or dimerized lysozyme (Lydium-KLP, KLP-602) solution at differents concentrations (2X) was added per well (in triplicate) or 10 µg/mL of Concanavalin A (ConA) or medium for control wells. The cells were cultured for 3 days at 22°C an incubator without CO_2.

Lymphocyte proliferation was determined by MTT colorimetric assay method according to Carmichael *et al.*, (1987) with the modification of authors. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma M2128) was dissolved in PBS at 5 mg/mL and filter sterilized. Fifty µL of MTT solution was added to all wells and plates were incubated at 24°C for 4 hr. After centrifugation of the microplates media was removed and 100 µL of isopropanol or DMSO (Sigma) was added to wells and mixed thoroughly to dissolve the dark blue crystals. After a 5 minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a micro-reader,

using a test wavelength of 620-630 nm. Plates were normally read within 5 minutes of addition of isopropanol or DMSO.

Total and specific ASC assay

A new method for *in vitro* immunization and culture of rainbow trout spleen sections and ELISPOT assay for the quantification of antibody secreting cells have been used (Siwicki and Dunier, 1993). In each experiment, spleen from 18 healthy rainbow trout were sectioned and placed in a 35 mm sterile well (six wells, Costar, Cambridge, MA, USA) containing 10 ml RPMI 1640 (Sigma) with 2% fetal calf serum (FCS, Gibco) and 0.1, 1.0, 5.0, 10, 50 or 100 µg/mL dimerized lysozyme KLP-602 or Lydium-KLP. The spleen section were injected with 10 µg doses of *Yersinia ruckeri* O-antigen and incubated at 17°C. Media was changed every other day by taking off 5 mL of old media and replacing 5 mL of new media with the appropriate levels of monomer and dimerized lysozyme Lydium-KLP or KLP-602. Spleen sections were sampled on day 9 after lysozyme monomer or dimer and antigen exposure. Single cell suspensions were obtained by teasing the tissues in medium through a steel mesh and were purified on a 61% Percoll gradient (Pharmacia). Counting living cells from spleen was done with trypan blue, using a hemacytometer after washing 3 times in RPMI-1640.

Non-specific ELISPOT assay

Multiscreen-HA 96-well filtration plates of cellulose esters (0.45 µm, Milipore) were coated with 100 µL of monoclonal anti-trout Ig (1-14 from DeLuca) diluted 1:100 and incubated overnight at 4°C. After washing 3 times with PBS, the plates were incubated with 200 µL RPMI-1640 + 5% FCS for 1 hr at 37°C to block the sites not bound by antigen. After removal of the blocking medium, 100 µL of the lymphocyte suspension was added to each well ($1x10^6$ cells/ well). The cells were incubated 6 hr at room temperature. After incubation, the plates were washed three times in PBS and eight times in PBS + 0.05 Tween 20, then 100 µL peroxidase labeled goat anti-trout Ig (Kirkegaard and Perry Lab, Gaithersburg, MD), diluted 1:2000 in PBS-Tween + 1% FCS, was added to each well and incubated overnight at 4°C. After three washing in PBS, 100 µL TMB Membrane Enhancer diluted 1:10 in the peroxidase substrate and the B peroxidase solution (Kirkegaard and Perry Lab.) was added to each well. The plates were incubated for 15 min before washing in tap water and drying. The blue spots were counted using a light microscope and results expressed as spot-forming cells per 10^6 spleen leukocytes. The assays were done in quadruplicate for each spleen.

Specific ELISPOT assay

The multiscreen-HA plates were coated with 100 µL of $1x10^8$ *Yersinia ruckeri* bacteria (Yersivax, Rhone-Merieux IFFA Laboratory, Lyon, France) in PBS and incubated overnight at 4°C, 24 hr before testing the cells . After three washings in PBS the previously described methodology for the non-specific ELISPOT assay was applied.

Statistical analyses

Statistical analyses were performed by the Student *t*-test. Differences on means were considered statistically significant at P.05.

RESULTS

The analysis of the effects of differents doses of monomer or dimer (Lydium-KLP) lysozyme on neutrophil and macrophage metabolism was done by examining respiratory burst activity. With the dimer there was significant increase compared to the control, but this was not the case when the 50 µg/mL and 100 µg/mL doses of lysozyme monomer were used (Figures 1 and 2). The 50 µg/mL and 100 µg/mL levels of lysozyme monomer suppressed the respiratory burst activity of neutrophils and macrophages. In all concentrations used between 0.1 µg/mL to 100 µg/mL lysozyme dimer significant ($P<0.05$) increased the respiratory burst activity of neutrophils and macrophages occurred when compared to monomer and control (Figures 1 and 2).

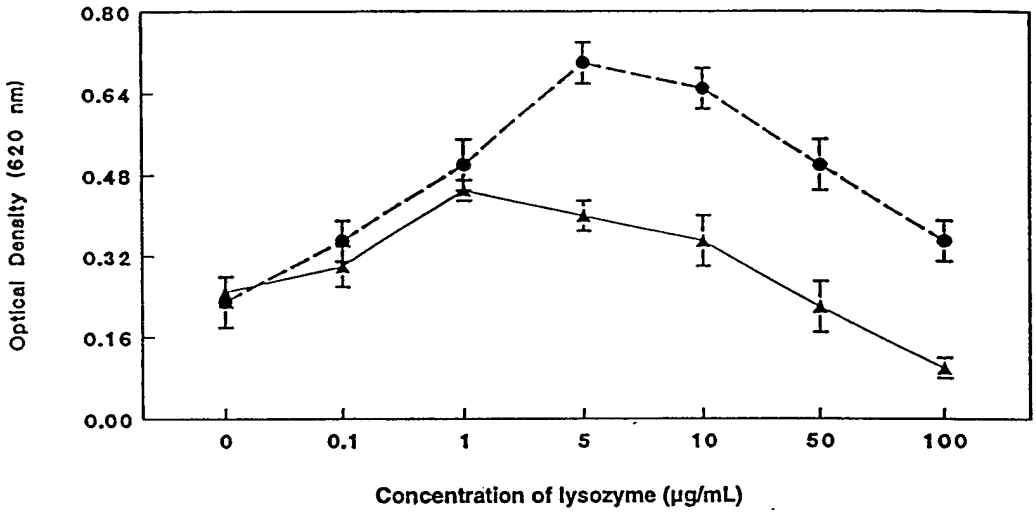


Figure 1. *In vitro* influence of different doses of lysozyme monomer (Sigma L 6876) and dimer (Lydium-KLP) on rainbow trout respiratory burst activity of blood neutrophils stimulated by PMA (Mean ± SE, n=10). ● Dimer Lydium-KLP, ▲ Monomer L6876

The lymphocyte proliferation analysis by MTT assay indicated a similar pattern. The results showed that monomer and dimer of lysozyme stimulated lymphocyte proliferation, but this was not the case when the 50 µg/mL and 100 µg/mL lysozyme monomer were used (Figure 3). This dose significantly decreased the lymphocyte proliferation as compared to the lysozyme dimers ($P<0.05$). In all concentrations tested (0.1 µg/mL to 100 µg/mL) a significant increase in lymphocyte proliferation occurred when compared to the lysozyme monomer. At 10 µg/mL, proliferation was similar to the effect of the mitogen Concanavalin A (Figure 3).

The respiratory burst activity of neutrophils and macrophages stimulated by PMA after incubation with different concentrations of two dimerized lysozyme (KLP-602 and Lydium-KLP) are shown in Figures 4 and 5. The analysis of the effects of differents doses of two dimers of lysozyme on respiratory burst activity of neutrophils and macrophages showed that there was a significant increase when the KLP-602 and Lydium-KLP were used at doses of 0.1 µg/mL to 100 µg/mL. At

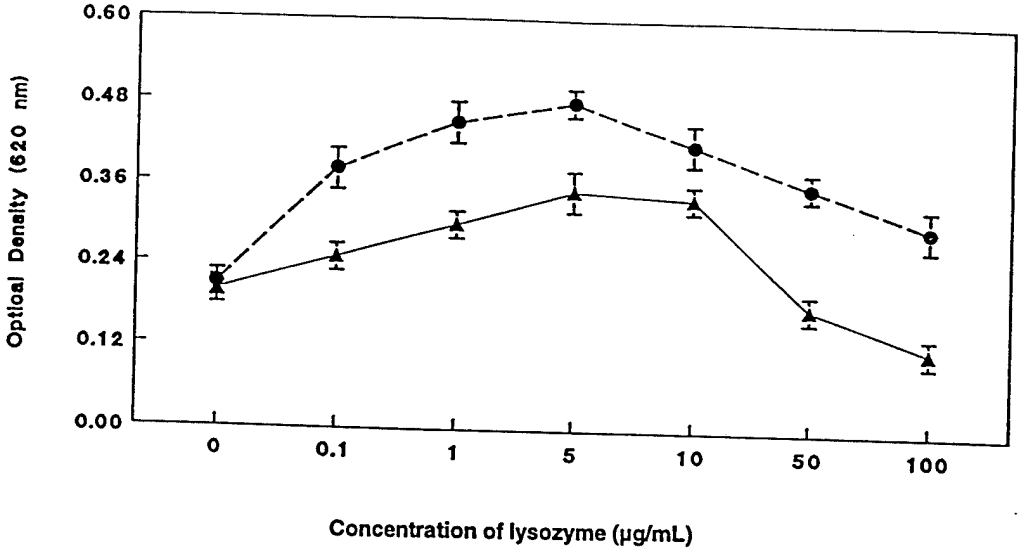


Figure 2. *In vitro* influence of different doses of lysozyme monomer (Sigma L 6876) and dimer (Lydium-KLP) on rainbow trout respiratory burst activity of head kidney macrophages stimulated by PMA (Mean ± SE, n=10). ● Dimer Lydium-KLP, ▲ Monomer L6876

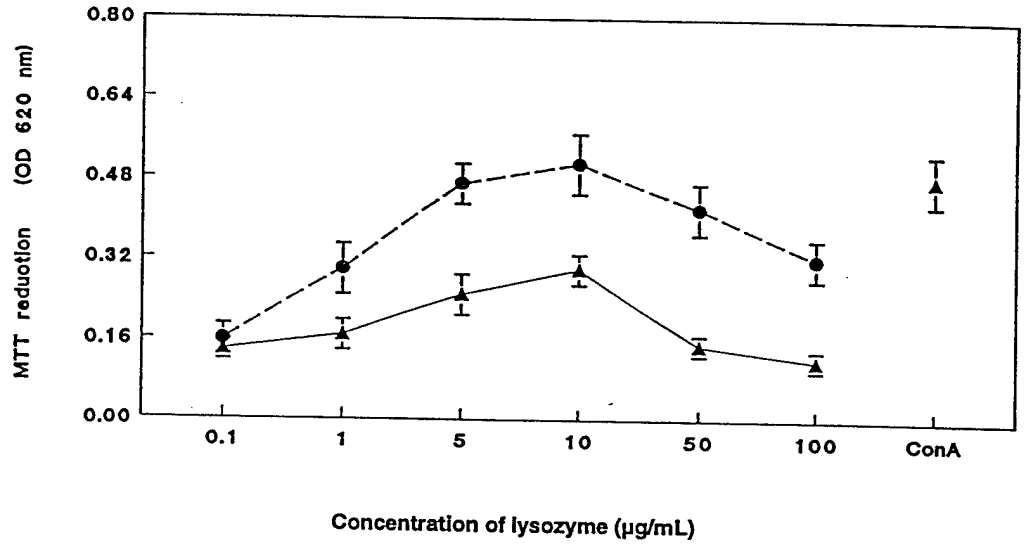


Figure 3. *In vitro* influence of different doses of lysozyme monomer (Sigma L 6876) and dimer (Lydium-KLP) and ConA on rainbow trout proliferation of head kidney lymphocytes by MTT assay (Mean ± SE, n=10). ● Dimer Lydium-KLP, ▲ Monomer L6876

concentrations of 1 µg/mL to 100 µg/mL of KLP-602, a statistically significantly higher stimulatory effect ($P<0.05$) was observed, compared to the similar concentrations of Lydium-KLP.

The lymphocyte proliferation analysis by MTT assay indicated a similar pattern. In all experimental concentrations of two dimers of lysozyme (KLP-602 and Lydium-KLP) statistically significant ($P<0.05$) stimulatory effects were observed, compared to the control (Figure 6). The lymphocyte

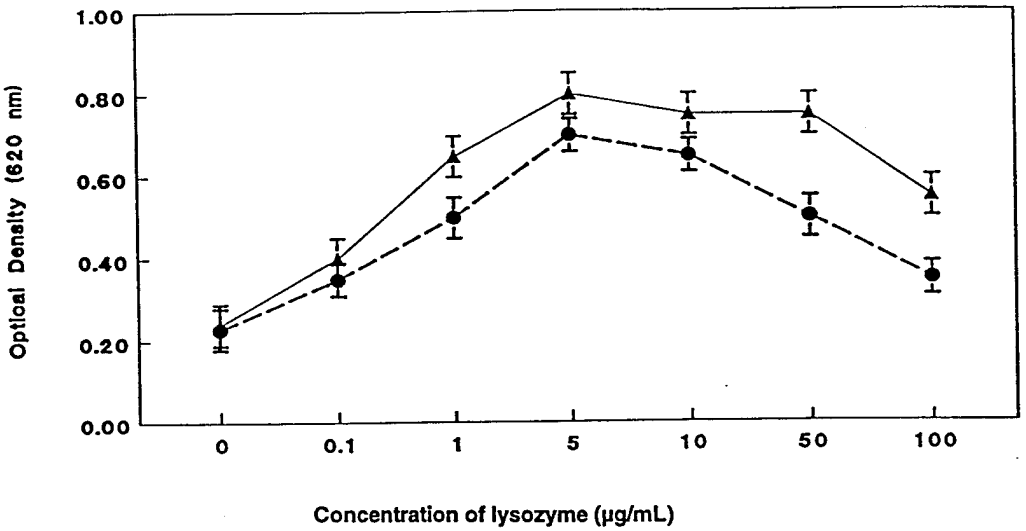


Figure 4. *In vitro* influence of different doses of dimerized lysozyme: KLP-602 and Lydium-KLP on rainbow trout respiratory burst activity of blood neutrophils stimulated by PMA (Mean ± SE, n=10). ● Dimer Lydium-KLP, ▲ Dimer KLP-602

proliferation with KLP-602 was higher when compared to the similar concentrations of Lydium-KLP. The two concentrations 5 µg/mL and 10 µg/mL of KLP-602 had a statistically significant higher stimulatory effect ($P<0.05$), compared to Concanavalin A (Figure 6).

The effects of dimerized lysozyme on the total and specific immune response by enumeration of antibody secreting cells (ELISPOT assay) are summarized in Figures 7 and 8. In all concentrations of two dimers of lysozyme (KLP-602 and Lydium-KLP), total and specific antibody secreting cells significantly ($P<0.05$) increased, compared to lysozyme-free immunized spleen sections of fish. In all experimental doses, significantly higher immunostimulatory effects of KLP-602 on the total and specific antibody secreting cells were observed as compared to similar doses of Lydium-KLP.

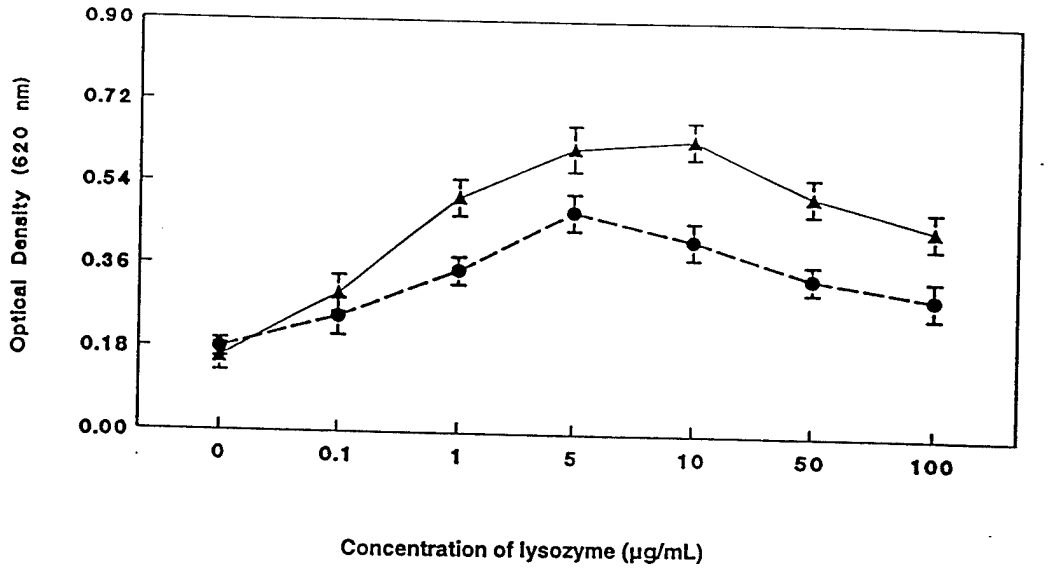


Figure 5. *In vitro* influence of different doses of dimerized lysozyme: KLP-602 and Lydium-KLP on rainbow trout respiratory burst activity of head kidney macrophages stimulated by PMA (Mean ± SE, n=10). ● Dimer Lydium-KLP, ▲ Dimer KLP-602

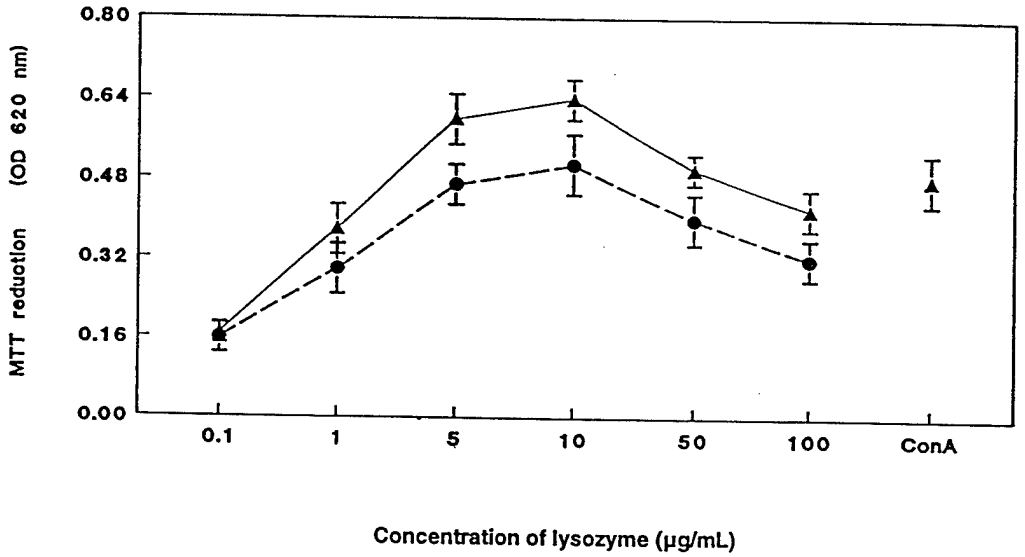


Figure 6. *In vitro* influence of different doses of dimerized lysozyme: KLP-602 and Lydium-KLP and ConA on rainbow trout proliferation of head kidney lymphocytes by MTT assay (Mean ± SE, n=10). ● Dimer Lydium-KLP, ▲ Dimer KLP-602

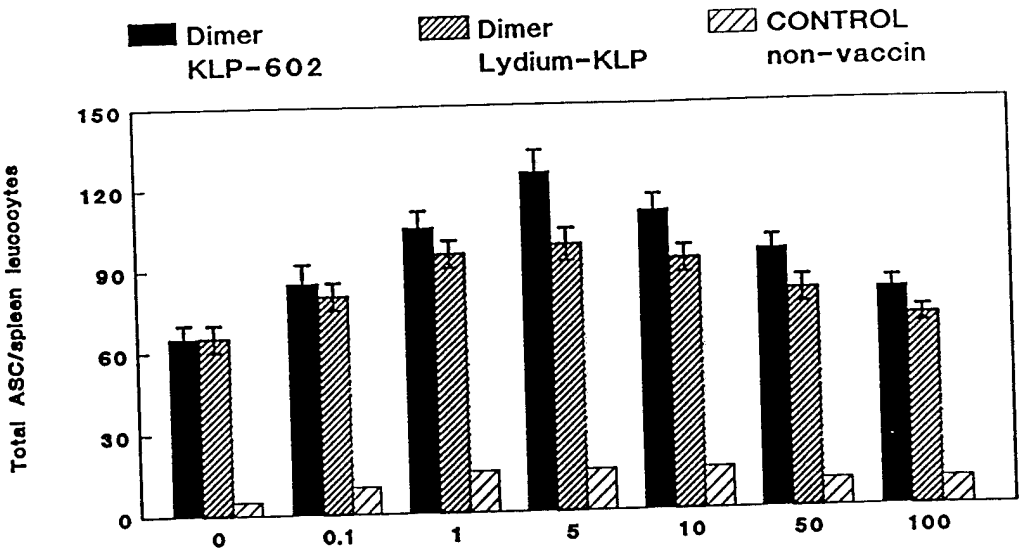


Figure 7. *In vitro* effect of different doses of dimerized lysozyme: KLP-602 and Lydium-KLP on the numbers of total ASC by ELISPOT assay from spleens of rainbow trout injected with 10 *µg Yersinia ruckeri* O-antigen or non immunized (non-vaccine). Mean + SE, n=10.

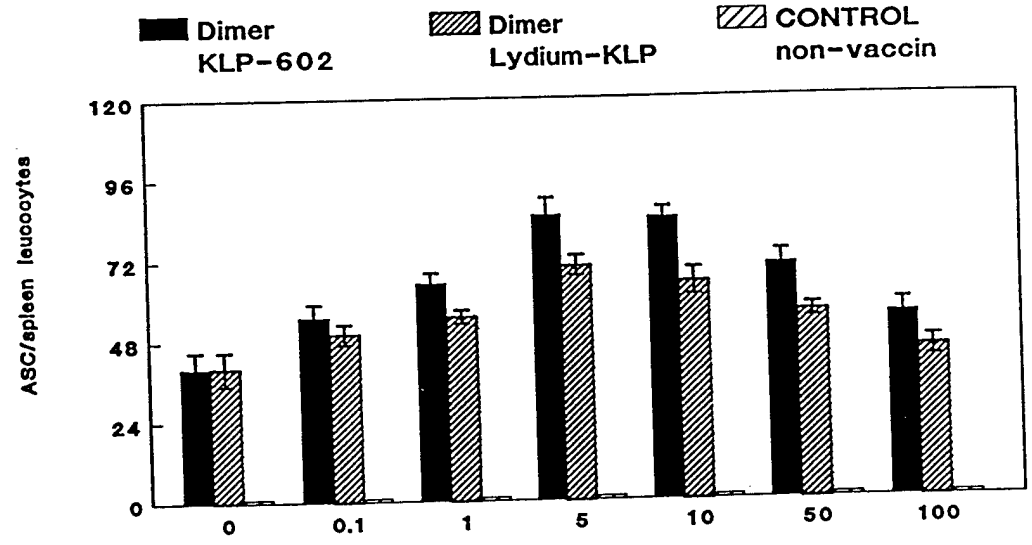


Figure 8. *In vitro* effect of different doses of dimerized lysozyme: KLP-602 and Lydium-KLP on the numbers of specific ASC by ELISPOT assay from spleens of rainbow trout injected with 10 *µg Yersinia ruckeri* O-antigen or non- immunized (Mean + SE, n=10).

DISCUSSION

The development of the *in vitro* assays to study influence of immunomodulators on the immunocompetence cells is an effective way to do preliminary screening on candidate drugs or biological response modifiers before doing more extensive *in vivo* experiments. Presently there are many chemicals being proposed in medical and veterinary sciences for use in the treatment and prevention of diseases. Information from *in vitro* assays on the influence of different doses of immunomodulators are very important for the more expensive field tests *in vivo*.

Lysozyme monomer and dimers (KLP-602, Lydium-KLP) were tested for the first time as an immunomodulator *in vitro* on the phagocytic cells (neutrophils and macrophages) and cell-mediated immunity in fish.

In the present study we observed a immunostimulatory effects of monomer and two dimers (KLP-602 and Lydium-KLP) of lysozyme *in vitro* on the respiratory burst activity of neutrophils and macrophages, lymphocyte proliferation and antibody secreting cells. The results showed that dimerized lysozyme (KLP-602 and Lydium-KLP) are more active on two major functions of the immune system, *ie* the specific mechanisms of lymphocyte proliferation and antibody secreting cells, and the nonspecific mechanism, phagocytosis of neutrophils and macrophages. For both functions lysozyme monomer followed a dose-effect, acting as a suppressor at high concentrations and as immunostimulator at low concentrations. Compared to the lysozyme monomer, for both functions dimerized lysozyme had a higher immunostimulatory effect. The results suggests that dimer of lysozyme is more effective and less toxic on polymorphonuclear and mononuclear cells. Similar immunostimulatory effects of dimerized lysozyme have been observed in humans and animals (Kiczka, 1994). The studies conducted so far show that it induces increased activity of phagocytizing cells.

Under natural conditions, a number of substances (IgM, IL-5, TNF-α) achieve their full effectiveness only as dimer or polymers (Beutler, 1992). In nature, lysozyme is found as monomer. Therefore, a question arose whether higher activity can be generated through lysozyme dimerization. The presented studies verified the hypothesis and suggested that dimerized lysozyme has more immunomodulatory influence on immunocompetent cells *in vitro*.

The *in vitro* results showed that dimerized lysozyme may be utilized for the enhancement of the immune functions. The application of KLP-602 and Lydium-KLP for stimulating or modulating the cellular and humoral defence mechanisms and for protection against diseases in fish is of increasing interest to veterinary medicine.

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Chapter 19

The Effects of Stress and Cortisol on Phagocyte Function in Juvenile Salmonids

James R. Pegg and George K. Iwama

Department of Animal Science
University of British Columbia
Suite 208, Main Mall
Vancouver, B.C. Canada V6T 1Z4

INTRODUCTION

Stress is experienced by fish both in aquaculture (eg. handling, crowding) and in the wild (eg. pollution). The physiological effects of stress in fish have been well studied (see Barton and Iwama, 1991). While the stress response is adaptive, there may be some negative effects, especially in the case of severe or prolonged stress. Stress has been shown to have negative effects on some components of the immune system (Maule *et al.,* 1989; Mazur and Iwama, 1993) and to result in decreased disease resistance (Maule *et al.,* 1989). Phagocytes are a very important part of the immune system, and therefore disease resistance, in fish. They play a role in both the non-specific immune system by engulfing and destroying microorganisms, as well as play a role in the specific immune system through antigen presentation and cytokine production (Abbas *et al.*, 1991). This study examines whether confinement stress, and the stress and saltwater adapting hormone cortisol, affect phagocyte function in juvenile salmonids.

MATERIALS AND METHODS

Phagocyte Isolation

Phagocytes were isolated according to the methods of Secombes (1990). Anterior kidneys were aseptically removed and placed in Leibovitz medium (L-15) supplemented with 10 u/mL heparin and 100 u/mL penicillin/streptomycin and a cell suspension was created by gently teasing the tissue through nylon mesh (50-100 μm) or by gently drawing the tissue in and out of a 1cc plastic syringe (cortisol injection experiment). The cell suspension was enriched for phagocytes by centrifuging on a 34/51% discontinuous Percoll gradient for 20 min. at 400 xg and seeded into microplates or chamber slides (cortisol injection experiment) at 10^6 cells per well. The cells were further enriched for phagocytes by allowing them to adhere for 30 min. before performing functional assays.

Assays of Phagocyte Function

A phagocytosis assay was performed in which the medium was replaced with L-15 containing Congo red stained yeast in a microplate adaptation of a method described by Seeley *et al.*, (1990). The phagocytes were incubated with the yeast suspension for 2 hr after which time the unphagocytized yeast were washed away with phosphate buffered saline and the adherent phagocytes were solubilized overnight at 37° C in 1.5 g/L trypsin. The red stained yeast released from the phagocytes were quantified at 525 nm in a microplate reader against trypsin blanks and standard solutions. Superoxide production was measured by nitro blue tetrazolium (NBT) reduction without stimulation according to the method of Secombes (1990). The phagocytes were incubated in 1mg/mL NBT in L-15 for 2 hr and then fixed in methanol, washed in 70% methanol, air dried, and dissolved in 2M KOH and dimethylsulfoxide (DMSO). The resulting turquoise/blue colour was measured with KOH/DMSO blanks and NBT standards at 620 nm in a microplate reader. Adherent cell protein was determined using a bicinchoninic acid protein assay (Smith *et al.*, 1985) and used to standardize the results of the phagocytosis and NBT reduction assays. All three assays were performed in duplicate or triplicate wells for each fish sampled.

A slide assay of phagocytosis was used in the cortisol injection experiment in which the adherence and phagocytosis steps were performed at the same time. After washing away the nonadherent cells and nonphagocytized yeast, the slide was fixed and stained with Diff-Quik, and the percentage of cells containing yeast was counted under a microscope.

Confinement Stress

In order to determine whether stress could affect phagocyte function, groups of five juvenile chinook salmon, *Oncorhynchus tshawytscha*, were placed in 10 L of water in white 25 L buckets at room temperature with aeration. This was a severe combined stressor of confinement, temperature, and water quality. Four fish were sampled at 2 hr, 1,3,5 days and compared to control fish from 170 l tanks. Plasma cortisol, glucose, and lysozyme concentration were measured at 2 hr and 3 days.

Cortisol Injection

Juvenile coho salmon, *O. kitsutch*, (average 22g) were maintained in Cultus Lake (British Columbia, Canada) water on a commercial salmon diet. Fish were injected with Prednisolone (a cortisol analog; 20 (µg/g fish weight) or saline (controls) into the dorsal sinus and kept in 75 L aquariums. Four fish from each group were sampled regularly for 2 weeks.

Cortisol Implantation

Juvenile chinook salmon (average 30g) were maintained in dechlorinated Vancouver City water on a commercial salmon diet. Cortisol (hydrocortisone, Sigma) was dissolved in a mixture of 50% coconut oil and 50% vegetable oil, at two concentrations and injected into the peritoneal cavity to give final doses of 50 and 100 µg/g fish weight (Specker *et al.*, 1994). Control fish were injected with implants without cortisol. Fish were kept in 70 L tanks and sampled regularly for one week.

In vitro Cortisol Incubation

Phagocytes were isolated from juvenile chinook salmon and allowed to adhere to microtitre plates as described above. Half of the wells from each fish were incubated with media containing 5x10-7M cortisol (hydrocortisone 21-hemisuccinate, Sigma). Phagocytes were incubated with cortisol for 4, 8, 24, and 48 hr at the end of which time phagocytosis, superoxide, and protein assays were performed. At 4, 8, and 24 hr four fish were used with duplicate wells for each assay, and at 48 hr two fish were used with four replicate wells per assay.

Blood sampling

Fish were killed with an overdose of MS222 and blood was taken from the caudal vein immediately using a heparinized syringe. Blood samples were centrifuged to separate the plasma which was stored at -50°C. Plasma cortisol concentration was determined with a radioimmune assay (coat-a-count, Diagnostic Products Corporation), and plasma glucose concentration was determined with a microplate assay using the Trinder method (Sigma). Lysozyme was measured using the lysoplate method (Osserman and Lawlor 1966, modification by Lie *et al.* 1986).

Statistical analysis

For the confinement stress experiment Student's t-tests were used to compare means for each day. Two way analysis of variance was used for the cortisol implantation and *in vitro* cortisol experiments and on arcsin square root transformed proportion data for the cortisol injection experiment. Student-Newman-Keuls tests were used to determine group differences. Significance level for all experiments was $p<0.05$ and all data is presented as means ± standard errors.

RESULTS

Confinement stress decreased phagocytosis ($p=0.027$) and increased superoxide production ($p=0.002$) after 3 days, however, there was no significant differences at the other sampling times (Figure 1). No difference was observed in plasma lysozyme concentration (Table 1). Plasma cortisol

Table 1.
The effects of confinement stress on plasma cortisol, glucose, and lysozyme concentrations

Factor	2 hr control[1]	2 hr stress	3 days control	3 days stress
cortisol(ng/mL)	25.5±13.2	240.1±21.2[2]	8.3±3.6	48.6±10.7[2]
glucose(mg/dL)	59.0±5.7	103.2±10.6[2]	70.1±10.7	76.2±10.6
lysozyme(u/mL)	162.0±23.2	106.4±12.5	202.3±78.8	184.8±40.9

[1]mean values ± standard error, n=4
[2]significantly different from control at $p<0.05$

concentration was increased at 2 hr and 3 days while plasma glucose concentration was increased at 2 hr but not at 3 days (Table 1).

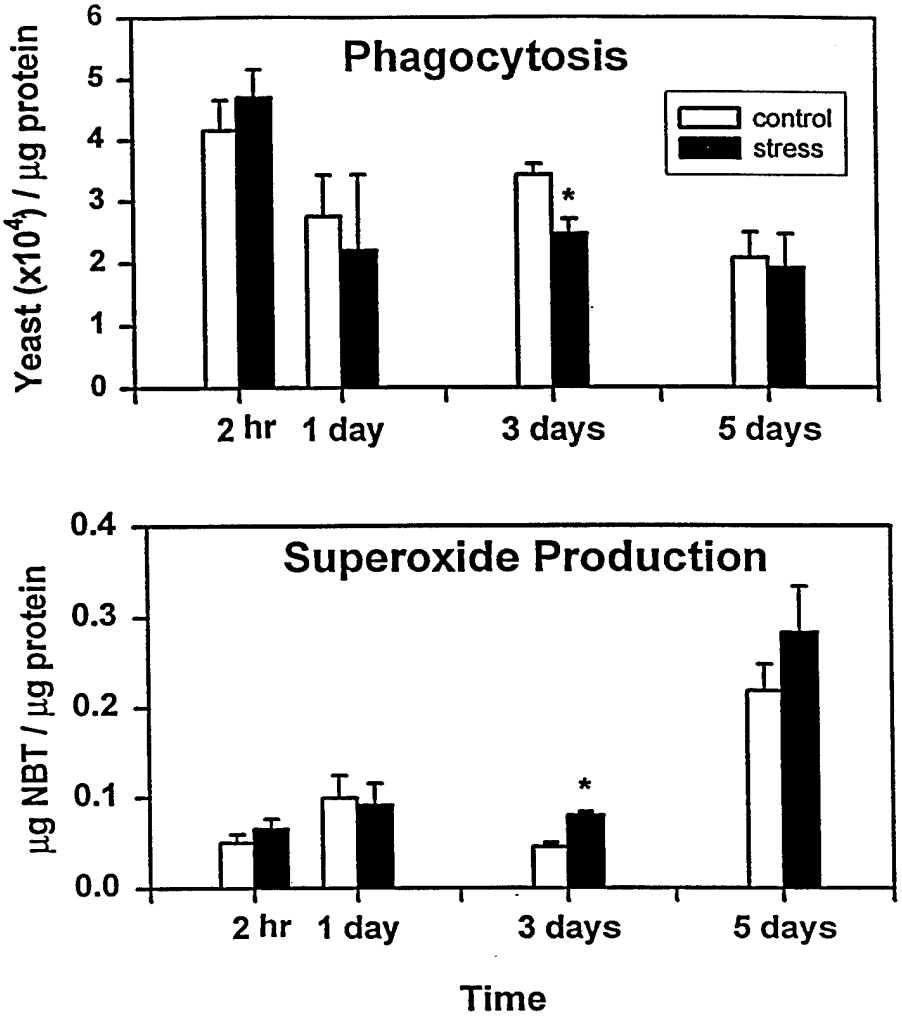


Figure 1. The effects of confinement stress on phagocytosis and superoxide production of microplate adherent phagocytes from juvenile chinook salmon, *Oncorhynchus tshawytscha*, (n=4). Data presented as means ± S.E. * means significantly different from controls at $p<0.05$.

On days 4,7, and 14 cortisol injected fish showed significantly lower phagocytic activity ($p=0.0001$) (Figure 2A). Cortisol implantation, however, caused an increase in phagocytosis over controls at both doses (Figure 2B) ($p=0.0008$), but the two doses were not different from each other. Cortisol implantation did not significantly affect superoxide production or plasma lysozyme concentration (data not presented).

Cortisol had no effect on phagocyte function in vitro (data not presented). Cortisol implantation raised plasma cortisol concentration for the duration of the experiment to between 507 and 395

ng/mL for the 100 (µg/g dose and between 431 and 173 ng/mL for the 50 (µg/g dose as compared to between 56 and 15 ng/mL for sham implanted fish.

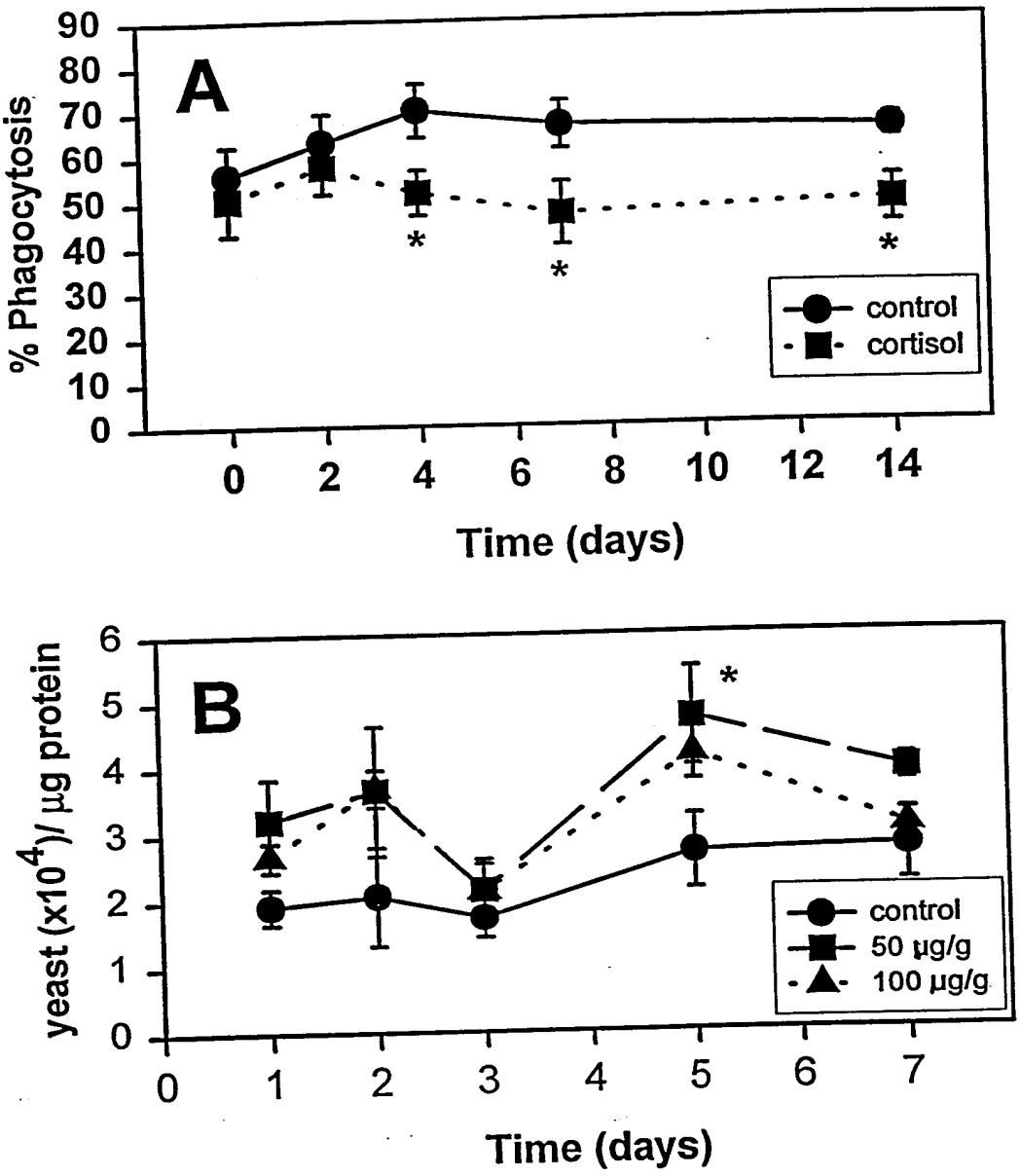


Figure 2. A. The effects of cortisol injection (prednisolone acetate 20 µg/g in the dorsal sinus) on phagocytosis in juvenile coho salmon, *Oncorhynchus kitsutch*. B. The effects of two doses of cortisol implants (50 and 100µg/g) on phagocytosis in juvenile chinook salmon, *O. tshawytscha*. Phagocytosis was significantly higher in the cortisol implanted fish ($p=0.0008$), however, the two doses were not different from each other. Data presented as means ± S.E. * means significantly different from controls at $p<0.05$.

DISCUSSION

The two major findings from this study are: a) confinement stress had both an inhibitory effect on phagocytosis and a stimulatory effect on superoxide production after three days; b) when injected into juvenile coho salmon cortisol had the opposite effect on phagocytosis as compared to when it was implanted into juvenile chinook salmon.

Decreases in immune function due to stress and the stress hormone cortisol has been reported by several other authors (see Narnaware *et al.*, 1994; Maule *et al.*, 1989; Tripp *et al.*, 1987), and is often considered a general consequence of stress (Barton and Iwama, 1991). However, stimulation of immune function due to stress has also been observed such as a transient increase in the number of antibody producing cells in the anterior kidney 24 hr following stress (Maule *et al.*, 1989). Stress may stimulate some aspects of the immune system while suppressing others and these effects change over time. It is possible that although phagocytosis is decreased after stress, superoxide production or killing ability is enhanced at the same time. For practical reasons, experiments usually concentrate on one aspect of immune function so that suppression and stimulation of different aspects of immune function are not usually observed in the same experiment. These results emphasize the need to measure a variety of indicators of immune function since suppression of some components of the immune system may be compensated for by stimulation of other components.

There are many possible reasons for the observed difference between the effects of cortisol injection and cortisol implantation including: the methods used to measure phagocytosis; the dose and type of cortisol (prednisolone acetate vs. hydrocortisone); species related differences. The two phagocytosis assays used actually measure phagocytosis slightly differently. The slide assay measures the proportion of adherent phagocytes that are actively engulfing yeast while the microplate assay measures the number of yeast taken up by a population of adherent phagocytes so that although the proportion of phagocytes that are actively engulfing yeast cells is lower, those phagocytes may be engulfing more yeast per phagocyte so that more yeast cells are engulfed overall. This emphasizes the problems with interpreting and extrapolating the results of one assay of immune function to immune competence and disease resistance.

The lack of effects of cortisol *in vitro* indicate that the effects of cortisol on phagocyte function are probably not direct. A similar lack of response to physiological levels of cortisol *in vitro* has been reported in rainbow trout, *O. mykiss*, (Narnaware *et al.*, 1994).

The results presented here indicate that stress and cortisol do have an effect on phagocyte function. However, this may not be due to simple suppression of phagocytosis. Other aspects of phagocyte function such as superoxide production may be enhanced to compensate for decreases in phagocytosis. More information is needed, and work is in progress to clarify the biological relevance of these changes in phagocyte function through disease challenges of stressed fish.

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Chapter 20

Skin Component May Protect Fishes from Sunburn and Fungal Infection Resulting from Exposure to Ultraviolet-B Radiation

David L. Fabacher and Edward E. Little

National Biological Service
Midwest Science Center
4200 New Haven Road
Columbia, Missouri 65201

ABSTRACT

Elevated levels of ultraviolet-B (UVB) radiation, as a consequence of stratospheric ozone depletion, may cause harmful effects in freshwater fishes. To determine the extent and significance of solar UVB effects on aquatic organisms, we investigated the effects of simulated solar UVB radiation on rainbow trout (*Oncorhynchus mykiss*), Apache trout (*Oncorhynchus apache*), Lahontan cutthroat trout (*Oncorhynchus clarki henshawi*), and razorback suckers (*Xyrauchen texanus*). In our solar simulator, fishes received daily 5-hr exposures to UVB (290-320 nm) during a 16-hr photoperiod. Lahontan cutthroat trout and rainbow trout had significant sunburn on day 3 and significant fungal infection on day 6 of exposure to an irradiance of UVB that simulated ambient mid-latitude summer irradiance. When methanol extracts of dorsal skin from unexposed fishes were scanned in a spectrophotometer we observed a large absorbance peak in the UVB wavelength range. The peak area was calculated and the relative amount of this unidentified component was estimated in the dorsal skin of each fish species. Apache trout and razorback suckers had significantly larger amounts of this component than Lahontan cutthroat trout and rainbow trout and did not develop sunburn or fungal infection. These findings indicate that this component may be photoprotective and act as a natural sunscreen.

INTRODUCTION

Increases in UVB at the earth's surface have resulted from stratospheric ozone depletion (Kerr and McElroy, 1993). Aquatic organisms are at risk when UVB penetrates the water column (Smith and Baker, 1979; Smith *et al.*, 1992; Gleason and Wellington, 1993; Hader, 1993; Siebeck *et al.* 1994; Williamson, 1995) causing sunburn in some fishes (DeLong *et al.*, 1958; Dunbar, 1959; Allison, 1960; Bullock and Roberts, 1981; Bullock, 1982; Bullock *et al.*, 1983; Bullock and Coutts, 1985; Bullock, 1988; Fabacher *et al.*, 1994; Little and Fabacher, 1994; Ramos *et al.*, 1994). We compare and discuss the incidence of sunburn and fungal infection in four species of freshwater fishes exposed to simulated solar UVB. We also describe a skin component that may protect some

of these fishes from sunburn and fungal infection resulting from exposure to simulated solar UVB radiation.

METHODS

The solar simulator (0.61 m wide by 1.83 m long) contained ten 160-watt cool white lamps, four 160-watt UVB313 lamps, eight 160-watt UVA365 lamps, two 20-watt cool white lamps, two 20-watt SF20 sun lamps, and eight 75-watt halogen incandescent flood lamps.

UVB lamps were controlled by a recycling 24-hour timer that operated for 5 hours to simulate a total solar daily dose. Cool white and UVA fluorescent lamps were controlled by a second timer that operated for a 16-hour period simulating a midsummer photoperiod. The simulator was suspended over a water bath of similar dimensions and was enclosed with reflective specular aluminum to contain the radiation in the exposure area. Output of the simulator was calibrated as previously described (Fabacher *et al.*, 1994; Little and Fabacher, 1994).

Juvenile fishes (60-75 days post-hatch; mean length 4.8 cm; mean weight 0.95 g) were exposed in 2-L glass 15 x 15 x 23 cm tall airlift chambers (Cleveland *et al.*, 1991) which received a 0.6 L/min flow. The chambers were set in the water bath under the solar simulator. Water quality and temperature (18°C) in the water bath was the same as used in culture. Control conditions, which provided a minimal UVB irradiance of 4.3 $\mu W/cm^2$ and a dose of 0.08 J/cm^2/day, were created by covering the top and bottom of each exposure chamber with 0.76 mm polycarbonate, and then covering the sides with 0.13 mm mylar. The simulated solar UVB irradiance of 190 $\mu W/cm^2$ and a dose of 3.42 J/cm^2/day was generated by covering the top of each exposure chamber with 0.13 mm thick cellulose acetate. Lahontan cutthroat trout, rainbow trout, and Apache trout were exposed for 7 days; razorback suckers for 21 days.

Fish were stocked in groups of 5 per chamber. Three replicate groups per treatment were randomly distributed under the solar simulator, and the entire experiment was repeated. Values obtained from replicates among treatments were pooled to generate a sample size of six per treatment. Fish were examined daily for sunburn, infection, and mortality, and were fed 24-hour old *Artemia sp.* and salmon starter several hours prior to and at the conclusion of each exposure. The effect of treatment level and species on the response of fishes was evaluated using analysis of variance techniques performed with the Statistical Analysis System (SAS, 1989). Cumulative percentages of sunburn and fungal infection were arcsine transformed and analyzed using a randomized block model with a repeated measures ANOVA to account for effects between experimental trials (Snedecor and Cochran, 1980). Mean values were compared using Fisher's protected LSD test ($p \leq 0.05$).

In addition to the exposures, each of five unexposed fish of each species was killed by freezing. A fish was held between a forceps on a watch glass under a binocular dissecting microscope, skin behind the head and opercula was punctured with a pointed forceps, and a small microscissors was used to cut a section of skin from the dorsal surface of the fish. This section of skin encompassed the area from just behind the head to the dorsal fin and just above the lateral lines and was peeled off the underlying musculature. Each skin section was weighed to the nearest milligram, extracted with 100% methanol, and refrigerated. Chilled methanol extracts were scanned in a Beckman 5230

UV/vis recording spectrophotometer. The approximate absorption maximum (λmax) of a skin component was calculated from the recording on the chart paper. Peak area was calculated using the formula 1/2 baseline x height to give a semiquantitative estimate of the amount of skin component. The results were expressed as area units/milligram wet weight of tissue. Statistical significance ($p \leq 0.05$) of the amount of component was determined by t-test and Duncan's multiple range test (Snedecor and Cochran, 1980).

RESULTS

The simulated solar ultraviolet (UV) irradiance produced by the simulator was similar to solar irradiance measured in Columbia, Missouri, on June 21, 1993, at an altitude of 271 m and a latitude of 38.5° N (Figure 1).

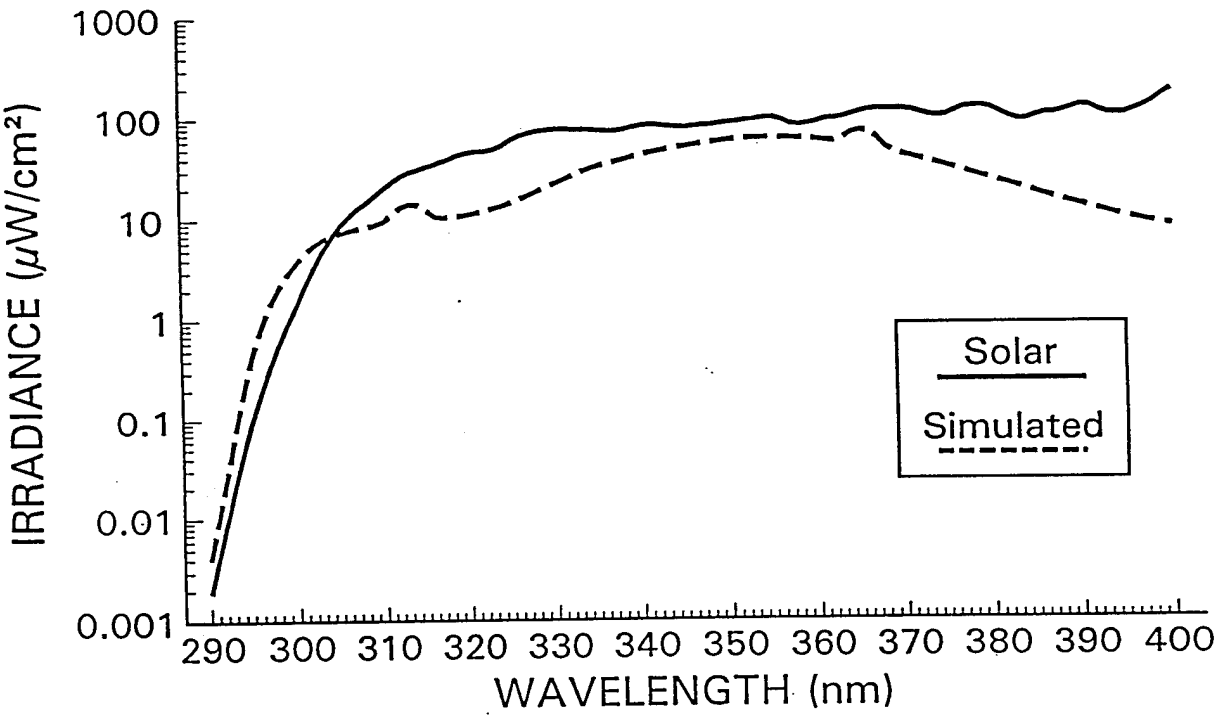


Figure 1. Spectral composition of simulated solar exposure irradiance and solar UV irradiance measured on June 21, 1993 at 38.5°N latitude (modified from Little and Fabacher, 1994).

In order to provide an indication of the wavelength composition and sunburning potential of the simulated solar UV irradiance, we compared the weighted irradiance values for simulated solar and solar UV using the Diffey action spectra (McKinlay and Diffey, 1987) for human erythema (sunburn). Since shorter wavelengths induce erythema more readily than longer wavelengths, this weighting provides a sunburning dose relative to the spectral wavelength composition of the exposure. The Diffey erythemal weighted irradiance reflects the total of the weighted irradiances

for all wavelengths necessary to produce erythema in humans. The Diffey erythemal weighted irradiance was 3.256 x 10^{-5}W/cm^2 for sunlight and 3.021 x 10^{-5}W/cm^2 for the simulated solar irradiance. Thus, the sunburning potential of the simulated solar irradiance was similar to that of ambient solar exposure on June 21, 1993, in Columbia, Missouri.

The presence of sunburn was a qualitative observation which first appeared as a characteristic darkening of the skin on the dorsal surface of the fish between the head and caudal fin and ventrally to about the lateral lines (Fabacher *et al.* 1994; Little and Fabacher, 1994). The largest area of sunburn usually occurred just posterior to the head and anterior to the dorsal fin. After day 2 of the 7-d exposure, incidence of sunburn increased significantly for Lahontan cutthroat trout and rainbow trout exposed to simulated solar UVB (Table 1).

Table 1.
Percent sunburn among fishes during a 7-day exposure to ultraviolet-B radiation

Day of exposure	Lahontan cutthroat trout		rainbow trout	
	control[a] (%)	treated[b] (%)	control[a] (%)	treated[b] (%)
1	0	0	0	0
2	0	16.7[7.4][c]	0	3.3[6.0][c]
3	0	53.3[14.8][c,d]	0	26.6[11.1][c,d]
4	0	73.3[11.0][c,d]	0	33.3[25.2][c,d]
5	0	80.0[8.0][c,d]	0	53.3[11.4]c,d
6	0	86.6[5.1][c,d]	0	83.3[3.7][c,d]
7	6.6 [5.1][c]	90.0[5.5][c,d]	0	93.3[4.7][c,d]

[a]Minimal UVB irradiance of 4.3 μW/cm^2.
[b]Solar simulated irradiance of 190 μW/cm^2.
[c]Mean[SE], n=6.
[d]Significantly different ($p \leq 0.05$) from control.

In addition to sunburn, Lahontan cutthroat trout and rainbow trout also developed fungal infection (Table 2). Fungal infection, observed to be *Saprolegnia* sp. (Fabacher *et al.*, 1994), appeared as mycelia on the darkened areas of the dorsal skin and on the dorsal fin. Later, patches of fungus covered the darkened areas and the dorsal fin giving those areas a white to gray colored appearance. Fungal infection appeared on day 4 and increased significantly from control after day 5 for Lahontan cutthroat trout. Fungal infection was significantly different from control beginning on day 6 for rainbow trout. Apache trout did not develop sunburn or fungal infection within 7 days of exposure. Razorback suckers did not develop sunburn or fungal infection within 21 days of exposure.

In methanol extracts of the dorsal skin from unexposed Lahontan cutthroat trout, rainbow trout, Apache trout, and razorback suckers we observed an unknown skin component (Fabacher and Little, 1995) with an absorption maximum around 292 nm (Figure 2).

Table 2.
Percent fungal infection among fishes during a 7-day exposure to ultraviolet-B radiation

Day of exposure	Lahontan cutthroat trout		rainbow trout	
	control[a] (%)	treated[b] (%)	control[a] (%)	treatedb (%)
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	0	13.3[9.4][c]	0	0
5	0	23.3[13.3][c]	0	0
6	0	46.6[9.4][c,d]	0	20.0[8.1][c,d]
7	0	60.0[8.1][c,d]	0	30.0[7.5][c,d]

[a]Minimal UVB irradiance of 4.3 μW/cm^2.
[b]Solar simulated irradiance of 190 μW/cm^2.
[c]Mean[SE], n=6.
[d]Significantly different ($p \leq 0.05$) from control.

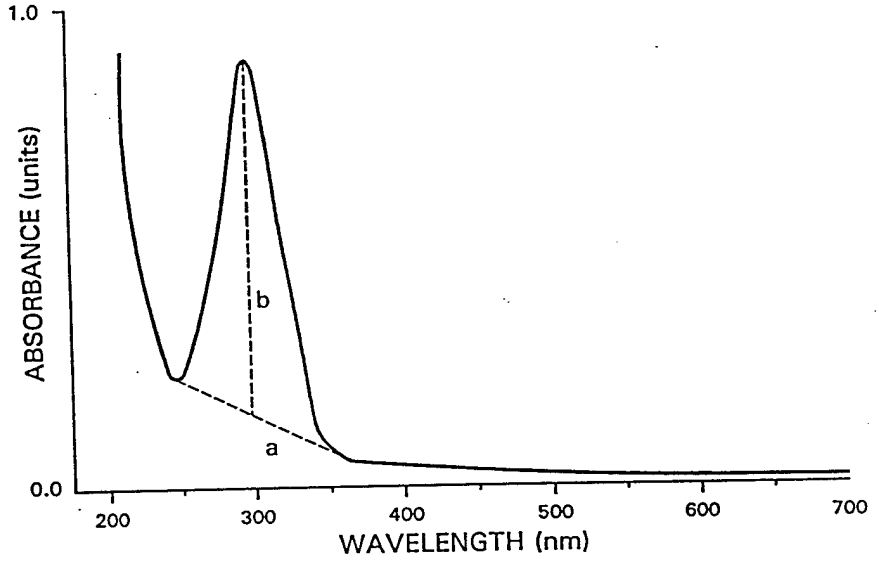


Figure 2. A representative UV-visible light absorption spectrum of dorsal skin methanol extract from a razorback sucker (modified from Fabacher and Little, 1995). Dotted lines are baseline (a) and height (b) of peak.

Apache trout and razorback suckers, which did not develop sunburn or fungal infection, had significantly larger amounts of this component than Lahontan cutthroat trout and rainbow trout (Table 3).

Table 3.
Amount of component in skin of unexposed fishes and day when UVB-exposed fishes were observed with significant sunburn and fungal infection

Species	Amount of component*	Day to sunburn	Day to fungal infection
Lahontan cutthroat trout	23.9[2.8][a+]	3	6
Rainbow trout	23.9[1.2][a]	3	6
Apache trout	49.6[7.8][b]	>7	>7
Razorback suckers	101.2[3.8][c]	>21	>21

*Values are mean[SE] area units/milligram wet weight of tissue for five fish of each species.
+Means with the same letter are not significantly different.

DISCUSSION

We observed a darkening of the dorsal skin within 48 hours of exposure to UVB radiation among Lahontan cutthroat trout and rainbow trout. Widespread dispersion of melanosomes occurred in the dermis of Atlantic salmon (*Salmo salar*) that were thought to have been exposed to direct solar ultraviolet radiation (McArdle and Bullock, 1987). Darkening of the dorsal skin of fishes exposed to simulated solar UVB may have resulted from melanosome dispersion and yielded the first grossly observable symptoms of sunburn.

The appearance of sunburn and subsequent fungal infection we observed are consistent with observations of other investigators. Bell and Hoar (1950) observed changes in pigmentation and fungal infection in irradiated coho salmon (*Oncorhynchus kisutch*) fry and goldfish (*Carassius auratus*). Sunburn and fungal infection were observed in chinook salmon (*Oncorhynchus tshawytscha*) exposed to sunlight (DeLong *et al.*, 1958).

In our study, fungal infection may have resulted from cell necrosis within the sunburned skin (Bullock, 1982), as well as suppression of the immune system by UVB. A wide range of environmental stress factors can depress the immune system in fishes, including chemical contaminants, drugs, and X-radiation (Zeeman and Brindley, 1981; Anderson *et al.*, 1984). Fishes may be more susceptible to infection by pathogens after the immune system has been depressed (Sindermann 1979; Weeks *et al.*, 1986; Anderson, 1990).

We did not observe darkening of the dorsal skin and fungal infection in UVB-exposed Apache trout and razorback suckers. Among Lahontan cutthroat trout and rainbow trout, not all fish in the same exposure chamber experienced sunburn and subsequent fungal infection. Similar to our observations, Bullock (1988) observed considerable variability in response of individual rainbow trout to intense simulated solar radiation and suggested that the ability of some fishes to tolerate UV radiation

may result from elevated levels of a photoprotective factor of genetic origin. The unknown skin component we observed may function as the photoprotective factor suggested by Bullock (1988).

The spectral characteristics of the unknown skin component we observed are similar to mycosporine-like amino acid compounds (MAAs) extracted from a variety of marine organisms, including eggs and eyes of fishes (Chioccara *et al.*, 1980; Dunlap *et al.*, 1989; Karentz *et al.*, 1991; Karentz, 1994). When methanol extracts of tissues containing these low molecular weight, polar MAAs are scanned in a spectrophotometer, absorption maxima can be observed from 310-360 nm. Thus, MAAs may offer organisms protection against UV radiation that occurs around those wavelengths. Even though the λ max of around 292 nm we observed in fish dorsal skin extracts is lower than that normally observed for MAAs, the unknown we observed may be related to MAAs. Preliminary results with high pressure liquid chromatography indicate that the unknown component we observed is a low molecular weight, polar, single compound.

Fish skin is more susceptible than human skin to sunburn damage when exposed to UVB because fish skin lacks a keratinized outer layer, has dividing cells in all layers of the epidermis, and normally does not contain protective epidermal melanin-containing cells (Bullock *et al.*, 1978; Bullock, 1982). The unknown component we observed is probably produced in epidermal cells and concentrated in the mucus. Once in the mucus it could effectively absorb, and thus block, UVB radiation occurring around 292 nm from reaching critical macromolecules in cells of the epidermal and dermal layers of the skin.

In conclusion, we observed an unidentified component in the dorsal skin of unexposed fishes. There appeared to be direct relationship between the amount of this component and the day when UVB-exposed fishes were observed with significant sunburn and fungal infection. This component may protect natural populations of fishes from sunburn and fungal infection resulting from exposure to UVB. The degree of photoprotection would probably depend on the amount of component present as well as on the UVB irradiance. We plan to identify this component, measure the levels of this component in UVB-exposed fishes, and determine if this component can be used as a bioindicator to identify UVB-sensitive fish species.

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Chapter 21

Effect of α-Tocopherol and n-3 HUFA Deficient Diets on Blood Cells, Selected Immune Parameters and Body Composition of Gilthead Seabream (*Sparus aurata*)

D. Montero, LL.Tort[1], M.S. Izquierdo[2], J. Socorro, L. Robaina[2], J.M. Vergara[2] and H. Fernández-Palacios

Instituto Canario de Ciencias Marinas, Gobierno de Canarias
Aptdo. 56, 35200 Telde
Las Palmas, Canary Islands, Spain

[1] Universitat Autonoma de Barcelona
Departament de Biologia Cellular i de Fisiologia
Unitat de Fisiologia Animal de Ciences
Edifici C 08193 Bellaterra, Barcelona, Spain

[2] Universidad de Las Palmas de Gran Canaria
Facultad de Ciencias del Mar
Departamento de Biología, edificio de Ciencias Básicas
Campus Universitario de Tafira 35017
Las Palmas de Gran Canaria. Canary Islands, Spain

ABSTRACT

Fish fed α-tocopherol depleted diets are reported to show a markedly reduced immunocompetence. Besides, essential fatty acids (EFA) deficient diets also cause disturbances in the physiology and biology of farmed fish. A feeding experiment was conducted to study some effects caused in gilthead sea bream (*Sparus aurata*) juveniles by EFA deficient diets and non supplemented α-tocopherol diets.

Juveniles of 55g were fed on diets containing three different supplementation levels of α-tocopherol (0, 50 and 150 mg α-tocopherol/Kg diet) plus an extra treatment consisting on 150 mg α-tocopherol/kg diet and deficient on n-3 HUFA, EFA for marine fish. Biochemical composition of liver and muscle were studied, and some hematological parameters (Hematocrit, hemoglobin, red blood cell count, white blood cell count, MCHC, MCV and MCH) as well as NBT index and alternative serum complement pathway (ACH_{50}) were measured. In addition, histological modifications were also studied.

Red blood cell fragility of the fish fed non-suplemented α-tocopherol diet was significantly higher than the others. Some significant differences were found between diets in some blood cells parameters. Histological differences such as dilated glomerular capillares and reduced space of Bowman' capsule were found in the kidney of fish fed on n-3 HUFA deficient diets. Pancreatic damages were also found in fish fed the α-tocopherol deficicent diets. On the other hand, fish fed the deficient diets showed a lower NBT index and depleted ACH50 when compared with fish fed control diet.

These results suggest that gilthead seabream fed diets with marginal deficiences in nutrients such as α-tocopherol and n-3 HUFA, essential components of the cell membrane, may be exposed to a reduction in their stress resistance potential. Further experiments are conducted to clarify this fact.

INTRODUCTION

Infectious diseases are a major cause of economic losses in intensive fish culture. One of the factors which has been directly correlated with disease outbreak is the immunosupresive effect of diets with deficiency in certain essential nutrients (Blazer, 1982; Blazer *et al.* 1989; Blazer, 1992; Landolt, 1989; Lall and Olivier, 1993). Such effect has been particularly noticed with deficiences in certain vitamins and highly unsaturated fatty acids of the linolenic (n-3 HUFA) family, which fish can not synthesize de novo.

Vitamin E is known to be directly involved in the inmunological processes of freshwater fish. Deficiencies in α-tocopherol have been reported to modify hemagglutination and specific immune responses to sheep red blood cells and *Yersina ruckeri* in rainbow trout (Blazer and Wolke, 1984), to produce a decrease in macrophage function (Blazer, 1982), and alterations in serum complement activity in Atlantic salmon (Hardie *et al.,* 1990). The quality of dietary polyunsaturated fatty acids also affects the physiological condition of freshwater fish (Watanabe, 1982) and influences the modulation of the immune system and macrophage function. Dietary lipids influence the fatty acid composition of membrane phospholipids, alter the physical properties of cell-membrane, the activity of membrane-associated receptors and the production of eicosanoids (Sheldon and Blazer, 1991).

However, little is known on the effect of vitamin E and n-3 HUFA deficiencies in marine fish species. Thus, the present work focuses in the gilthead seabream (*Sparus aurata*), which is a relevant fish species within Mediterranean aquaculture. The effects of α-tocopherol and n-3 HUFA deficiencies were studied on selected immune parameters such as the white blood cell (WBC) number and the number of each WBC type, the respiratory burts activity of circulating neutrophils and the activity of the alternative complement pathway. At the same time a number of analysis from the same fish were performed, including hematological parameters as well as the biochemical composition of liver and muscle. Histological effects on hepatopancreas, spleen and kidney were also studied.

MATERIAL AND METHOD

Fish and diets

Seventy two gilthead seabream (*Sparus aurata*) juveniles of 55 g mean weight, were randomly distributed in 12 circular, 100 L flow-through, fibre-glass tanks (6 fish/tank). Each tank received seawater at a flow rate of 0.5 L/ min, and was provided with aeration and natural light cycle.

The composition of the experimental diets is showed in Table 1. The diets were prepared with the same proximal composition and containing respectively 150 mg α-tocopherol/kg diet (Control diet), 50 mg α-tocopherol/kg diet and 0 mg α-tocopherol/kg diet. A fourth diet contained also 150 mg α-tocopherol/kg deficient on n-3 HUFA. Each diet was fed twice a day at a feeding rate of 2.5% fish body weight per day, 6 days per week for 9 weeks. Body weight was measured at the beginning and at the end of the experiment.

Table 1.
Ingredients of the experimental diets

	Diet No.			
	1	2	3	4
Sardine meal(S.M.)	64.02	64.02	64.02	16.00
Oil extracted S.M	-	-	-	42.94
Sardine oil	1.83	1.83	1.83	-
Beef tallow	3.40	3.40	3.40	10.31
Starch	12.00	12.00	12.00	12.00
Dextrin	4.00	4.00	4.00	4.00
α-Cellulose	10.04	10.05	10.05	10.04
CMC (1)	0.50	0.50	0.50	0.50
Vitamin mix (2)	2.00	2.00	2.00	2.00
Mineral mix (3)	1.30	1.30	1.30	1.30
Choline Chloride	0.90	0.90	0.90	0.90
α-tocopherol (mg/kg diet)	150	50	0	150

(1) Carboximethilcellulose

(2) (mg/kg diet): Vit A: 25; Vit D3: 5; Vit K: 20; Vit B12: 0.5; Vit H: 1; Folic acid: 10; Vit B6: 40; Vit B1: 40; Vit B_2: 50; Pantothenic acid: 117; Nicotinic acid: 200; Myo-inositol: 2000; Ascorbic acid: 5,000; Ethoxiquin: 100.

(3) (g/kg diet): $(H_2PO_4)Ca$: 1.605; $CaCO_3$: 4.0; $FeSO_4 \cdot 7H_2O$: 1.5; $MgSO_4 \cdot 7H_2O$: 1.605; K_2HPO_4: 2.8; $Na_2PO_4 \cdot H_2O$: 1; $Al(SO_4)_3 \cdot 6H_2O$: 0.02; $ZnSO_4 \cdot 7H_2O$: 0.24; $CuSO_4 \cdot 5H_2O$: 0.12; KI: 0.02; $CoSO_4 \cdot 7H_2O$: 0.08; $MnSO_4 \cdot H_2O$: 0.08.

Biochemical analysis

Proximal and fatty acid composition of the experimental diets were measured. Liver and muscle lipid and fatty acid contents of fish fed different diets were also determined.

Crude protein was determined with Kjeldahl method. Lipids were extracted as described by Folch *et al.* (1957). Fatty acids were obtained by transmethylation as described by Christie (1982) and identified using gas chromatography (Izquierdo *et al.*, 1990).

Hematology

Fish were anesthetized with chlorobutanol (1,1,1 trichloro-2-methyl-2-propanol). The anesthetic was added to the tanks to avoid the net capture effects on hematological parameters (Pearson and Stevens, 1991). Fish handling time was less than 2 min per fish to minimize stress effects on hematological parameters. Blood was obtained by caudal sinus puncture with 1 mL plastic syringe. Then, it was immediately transferred to an eppendorf tube coated with litium heparin as anticoagulant.

Each fish was bled immediately after the first blood extraction. Blood samples were transferred to an eppendorf tube without anticoagulant and allowed to clot at 4°C for 2 hr. The serum was separated by centrifugation at 3000 rpm for 10 min, and stored at -80°C for serum complement activity determination.

Hematocrit (Packed Cell Volume -PCV-) was measured by microcentrifugation (3,000 rpm, 10 min). Blood hemoglobin content (g/dL) and red blood cells number (RBC) were obtained using a hematological counter Sysmex 800. Mean cellular hemoglobin concentration (MCHC), mean cellular volume (MCV) and mean cellular hemoglobin (MCH) were calculated as described by Fletcher (1975).

Erythrocyte fragility was studied using the method described by Draper and Csallany (1969) and modified by Wilson *et al.* (1984).

Immune parameters

Fifty µL of blood were fixed in 2.5% glutaraldehide, buffered with Tris 1mM, and diluted in Cortland saline. This suspension was used for counting the lymphocyte number in a Neubauer hemocytometer. Smears were prepared from each fish to study the leukocrit, using the stain method of Quick Panoptic (Química clínica aplicada S.A., Tarragona, Spain). The reaction between nitroblue tetrazolium (NBT) and oxygen radicals from circulating neutrophils activity was measured spectrophotometrically as described by Siwicki *et al.* (1993).

Serum ACH_{50} was measured as described by Sunyer and Tort (1995). Rabbit red blood cells ($2.5x10^{-8}$ cells/mL) were used as mammal erythrocyte suspension.

Histological studies

At the end of the experiment, spleen, kidney and hepatopancreas from 2 fish from each tank were fixed in 10% neutral-buffered formalin. Samples were stained with hematoxylin-eosin (HandE) and periodic acid-Schiff-hematoxylin (PAS-H) for histological examination (Martoja and Martoja-Pierson, 1970).

Statistical analysis

All the data were subjected to one-way analysis of variance (ANOVA), and differences between means compared by Tukey test at a 95% interval of confidence ($P<0.05$) (Sokal and Rolf, 1979).

RESULTS AND DISCUSSION

Diet composition analysis

Uniform levels of total lipids, protein and moisture were obtained for the different diets. Fatty acids composition of the experimental diets shows that the beef tallow diet had a lower content of n-3 HUFA than the other diets and can be considered as deficient in these fatty acids for this species (Table 2). Ibeas *et al.,* (1994) showed that levels up to 0.9% of n-3 HUFA are deficient for this species.

Table 2
Approximate composition and some fatty acids of experimental diets (% dry weight)

	Diet No.			
	1	2	3	4
Crude Protein	50.92	50.78	51.16	49.52
Moisture	10.80	10.02	11.65	11.57
Crude Lipids	12.27	11.60	11.63	12.98
n-3 HUFA	2.04	1.98	1.78	0.52
20:4n-3	0.06	0.05	0.05	0.01
20:5n-3	1.06	0.99	0.22	0.22
22:5n-3	0.04	0.12	0.03	0.03
22:6n-3	0.88	0.82	0.75	0.26

Fish growth

No mortalities were observed during the experiment. As described for other species like red seabream (Yone and Fujii, 1975), low n-3 HUFA dietary levels significant reduced ($P<0.05$) gilthead seabream growth (Figure 1). On the contrary, dietary α-tocopherol levels did not affect gilthead seabream growth as occurs in red seabream (Sakaguchi and Hamaguchi, 1979) and sea bass (Stephan *et al.*, 1993).

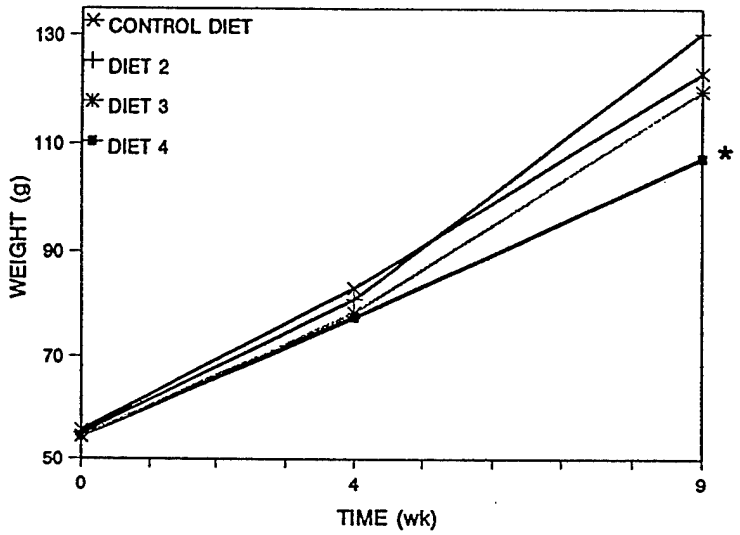


Figure 1. Fish growth during the feeding period. * Denotes significant diferences ($P<0.05$)

Muscle and liver composition

Liver lipid contents of fish fed any of the deficient diets were significantly ($P<0.05$) higher than the control, denoting an advanced grade of deficiency in those fish (Table 3). Muscle water content showed a trend to increase with the reduction of the dietary α-tocopherol or n-3 HUFA levels. This two characteristic sympthoms of deficiency (Castell *et al.*, 1972; Takeuchi *et al.*, 1979) have been also observed in gilthead seabream fingerlings (Kalogeropoulos *et al.*, 1992) and juveniles (Ibeas *et al.*, 1994) fed EFA deficient diets. Either dietary lipids lacking EFA or containing oxidation products have been shown to affect body composition (Watanabe, 1982). But crude lipids of livers from fish fed diet 2 with only 50 mg/kg of α-tocopherol showed the highest levels. This effect seems to be due to the relation of dietary α-tocopherol with the fat metabolism as the lipid composition of this diet (Table 2) did not indicated a significant oxidation.

Figures 2 and 3 illustrate the levels of some fatty acids in the total lipids of fish liver and muscle. There was a slight reduction in the levels of eicosapentaenoic (EPA:20:5n-3) and docosahexaenoic (DHA:22:6n-3) acids in the livers of fish fed the EFA deficient diet (Diet 4) (Figure 2a). Accordingly, the n-3 HUFA content and the relative proportion of n-3 fatty acids with respect to n-6 in liver of fish fed diet 4 was significantly ($P<0.05$) lower than in the other groups, denoting an EFA deficiency (Figure 2b). Accumulation of crude lipids in the liver due to impairment of lipoprotein biosynthesis (Fukuzawa *et al.*, 1971) together with the low n-3 HUFA levels in diet 4 caused a

TABLE 3
Liver and muscle lipid content of the fish fed the experimental diets (% dry weight)*

	Diet No.			
	1	2	3	4
LIVER				
Crude Lipids	23.74a	32.89b	27.72c	26.88c
Moisture	68.37a	64.14b	67.41ab	69.06a
MUSCLE				
Crude Lipids	9.46a	13.55a	12.44a	10.00
Moisture	73.39a	74.11a	74.73a	74.80a

* Values with different letter within a line are significantly different (P<0.05); n=18.

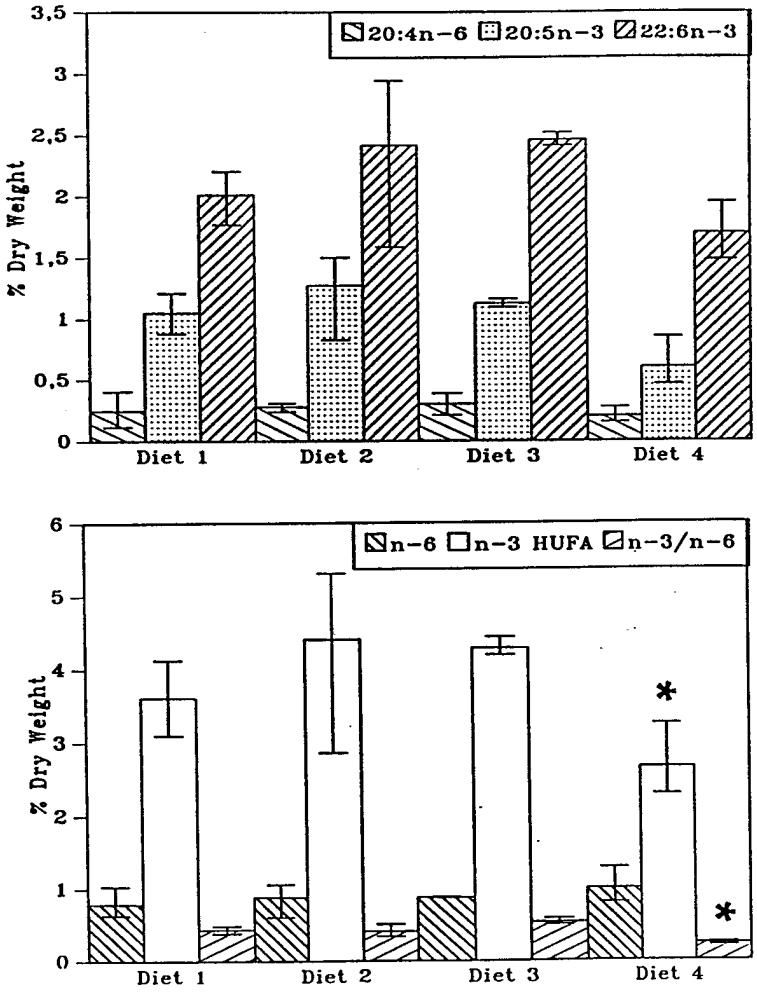


Figure 2. Some fatty acid contents of liver lipids. * Denotes significant differences (P<0.05)

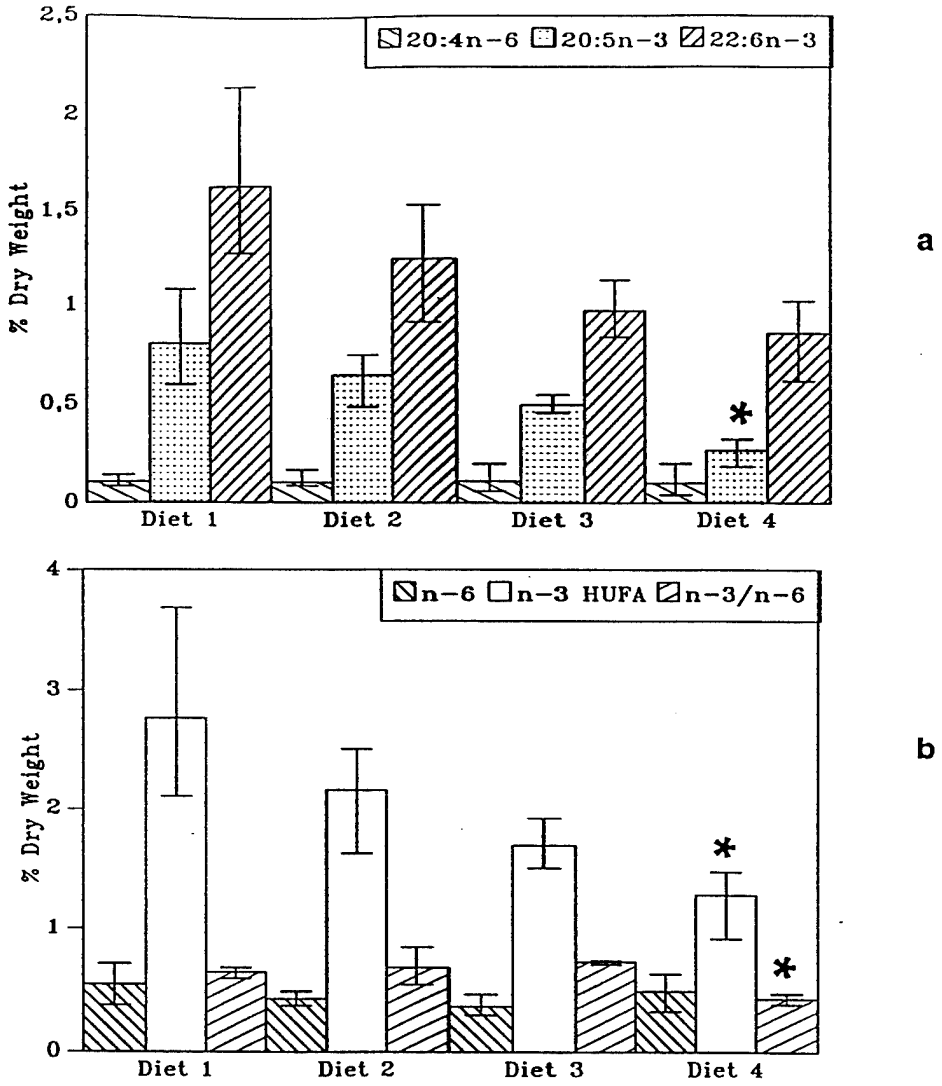


Figure 3. Some fatty acid contents of muscle lipids. * Denotes significant diferences (P<0.05)

significantly (P<0.05) lower n-3 HUFA contents in fish muscle (Figure 3b), another sympthom of EFA deficiency. Thus, EPA contents of muscle were significantly lower in fish fed diet 4.(Figure 3a), but those of DHA were not significantly lower. This fact suggests the preferential conservation of DHA for growth and maintenance of cell structures and membranes and denotes the importance of DHA (Izquierdo *et al.*, 1989; Watanabe *et al.*, 1989), while EPA is utilized as an energy source. The EPA, DHA and n-3 HUFA contents of muscle from fish fed the other diets showed a reduction trend with the reduction of the dietary α-tocopherol levels (Figure 3), reflecting the diet composition.

On the other hand, levels of araquidonic acid did not shown significant differences (P<0.05) neither in muscle nor in liver of fish fed the experimental diets, suggesting a trend towards conservation of this fatty acid as the n-3 HUFA acid decreased. Araquidonic acid is the main precursor of eicosanoids

in mammals using both lipooxygenase and cyclooxygenase pathways. Similar mechanisms are involved in the eicosanoid production involved in the response of fish macrophages, including the use of the same enzymatic pathways in eicosanoids production (Sheldon and Blazer, 1991). Some of the eicosanoids from cycloxygenase pathway, such as some prostaglandins, have immunosupresive effects, while eicosanoids from lipooxygenase pathway such as leukotriens have immunostimulatory effects. The maintenance of immunological status requires a balance between immunosupresive and immunostimulating eicosanoids.

n-3 HUFA are reported to be precursors of certain eicosanoids in fish (Blazer, 1991). In mammals, dietary supplementation of n-3 HUFA involves inhibition of prostaglandin synthesis using two pathways: a) There is an competitive inhibition for desaturases between fatty acids 18: 2n-6, 18: 1n-9 and 18: 3n-3, producing a depletion in the synthesis of araquidonic when dietary n-3 HUFA is increased. b) N-3 HUFA competitively inhibit the araquidonic acid oxygenation by cyclooxygenase (Hwang, 1989). The former pathway is unlikely to occur in marine fish, due to the very low or lack of $\Delta 6$ desaturase activity (Mourente and Tocher, 1993). However, recent studies (Bell *et al.*, 1994) indicate that arachidonic acid is the preferred precursor for eicosanoid synthesis in fish and a competitive inhibition by EPA at the cyclooxygenase active site. In the present study, the significant reduction in the EPA levels in fish tissues together with the conservation of the arachidonic acid modified the ratio between these two fatty acids, possibly leading to an imbalance in the production of eicosanoids. Thus, the reduction of EPA, due to the low levels in the diet together with its preferential use as energy, may have caused an increase in eicosanoids from the cycloxygenase pathway derived from arachidonic acid.

Hematology

The α-tocopherol dietary levels did not affect PCV in gilthead seabream (Table 4), as shown for other fish species like rainbow trout (Moccia *et al.*, 1984), channel catfish (Wilson *et al.*, 1984; Gatlin *et al.*, 1986) or Atlantic salmon (Raynard *et al.*, 1991). N-3 HUFA dietary levels did not significantly affect PCV as described for rainbow trout (Greene and Selivonchick, 1990).

There were no significant differences ($P>0.05$) for MCHC, meaning that erythrocyte swelling caused by handling stress was not produced (Soivio *et al.*, 1981). Blood hemoglobin levels and RBC count were not affected by α-tocopherol dietary levels (Table 4), as described for rainbow trout (Moccia *et al.*, 1984) or for channel catfish (Wilson *et al.*, 1984). N-3 HUFA deficient diets did not significantly affect either blood hemoglobin or RBC count in gilthead seabream. However, both values were slightly higher values in fish fed on n-3 HUFA deficient diet, as described for rainbow trout by Greene and Selivonchick (1990).

Significant differences ($P<0.05$) were found in the results from erythrocyte fragility test between the group fed on non supplemented α-tocopherol diet and the control group. These differences suggest oxydative damages in the RBC membranes of fish fed α-tocopherol deficient diets. No significant difference ($P>0.05$) on erythrocyte fragility was found between fish fed the control diet and those fed the n-3 HUFA deficient diet, but values of this parameter for the latter doubled those of the former, suggesting some relation between the EPA deficiency and the erythrocyte fragility (Table 4).

TABLE 4
Effect of different diets on some hematological and immunological parameters*

	Diet No			
	1	2	3	4
PCV (%)	37.82a	35.15a	33.44a	35.75a
Hemoglobin (g/dL)	9.70ab	9.73ab	9.18a	11.29b
MCHC (g/dL)	26.02a	28.25a	27.93a	32.03a
MCV (fL)	129.27a	128.49a	124.42a	105.52a
MCH (Pg)	32.39a	34.67a	33.75a	31.42a
RBC ($x10^6/mm^3$)	3.00ab	2.86a	2.75a	3.60b
Erythrocyte fragility (%)	3.91a	3.34a	15.09b	10.38ab
WBC ($x10^3/mm^3$)	41.22a	38.31a	40.93a	33.40a
Neutrophils ($x10^2$)	30.19a	29.35a	31.86a	23.71a
Monocytes ($x10^2$)	6.77a	6.61a	8.93a	5.60a
NBT Index	0.83a	0.79a	0.25b	0.45b
ACH50 ($U\ mL^{-1}$)	169.17a	-	90.58b	88.57b

* Values with different letter within a line are significantly different ($P<0.05$); n=18.
Abbreviations:MCHC: Mean cellular hemoglobin concentration; MCV: Mean cellular volume; MCH: Mean Cellular hemoglobin. RBC: Red blood cell; WBC: White blood cell; PCV: Packed cell volume; ACH50: 50% hemolysis effected by the alternative complement pathway; NBT: Nitroblue tetrazolium.

Dietary levels of α-tocopherol significantly ($P<0.05$) affected the erythrocyte fragility in *Sparus aurata,* as described for other species such as channel catfish (Wilson *et al.,* 1984) or rainbow trout (Cowey *et al.,* 1983; Boggio *et al.,* 1985). On the other hand, fish fed on n-3 HUFA deficient diet showed higher but not significantly different erythrocyte fragility when compared with the control diet. This is in agreement with the results decribed by Kiron *et al.* (1994) for rainbow trout fed on different dietary fatty acid status. The increase of erythrocyte fragility in the n-3 HUFA deficient group is in agreement with the results obtained by Erdal *et al.* (1991) for Atlantic salmon, who found a positive correlation between the cell membrane strength and the increase of dietary n-3 HUFA.

A great amount of smudged or disintegrated erythrocytes was observed in the smears from fish fed on unsupplemented α-tocopherol diet, as well as in those coming from n-3 HUFA deficient diet. This is in accordance with Moccia *et al.* (1984) who described this effect for rainbow trout fed on diets with different levels of oxidized fish oil, vitamin E and ethoxiquin. These authors found smudged erythrocytes in fish fed all diets, but these cells were more abundant in fish fed non supplemented vitamin E and/or non supplement ethoxiquin diets with oxidized fish oil.

Histology

No relevant differences were found in spleen or liver from gilthead seabream fed the experimental diets. A longer experimental period may be needed to confirm the histological signs of nutritional deficiences found in other fish species (such as migrated nuclei hepatocytes or liver vacuolization).

Alterations of the kidney glomerules were found in those fish fed on the n-3 HUFA deficient diet. These alterations were present in approximately 50% of the fish, and absent in fish fed the other diets. These alterations consisted in dilated glomerular capillaries and reduced space of Bowman' capsule, as described for channel catfish fed on low PUFA level diets without ethoxiquin and α-tocopherol (Lovell *et al.,* 1984). Moreover, some cloudy swelling degeneration of the renal tubules were found in the kidney of these fish.

On the other hand, approximately 80% of fish fed on non supplemented α-tocopherol diets showed alterations in pancreas cells, consisting in cellular degeneration and some points of necrosis (Figure 4), suggesting cell membrane and intracellular organelles membrane damage probably due to the vitamin E deficiency. Raynard *et al.* (1991) found pancreas disease in atlantic salmon fed vitamin E deficient diets. The fish susceptibility to pancreatic disease is associated with a great oxidative stress due to deficience in vitamin E in conjuntion with increased unsaturation index of the diet. In our experiment, dietary vitamin E had a greater effect on the incidence of the pancreas damage than the dietary lipids as described for Atlantic salmon (Raynard *et al.,* 1991).

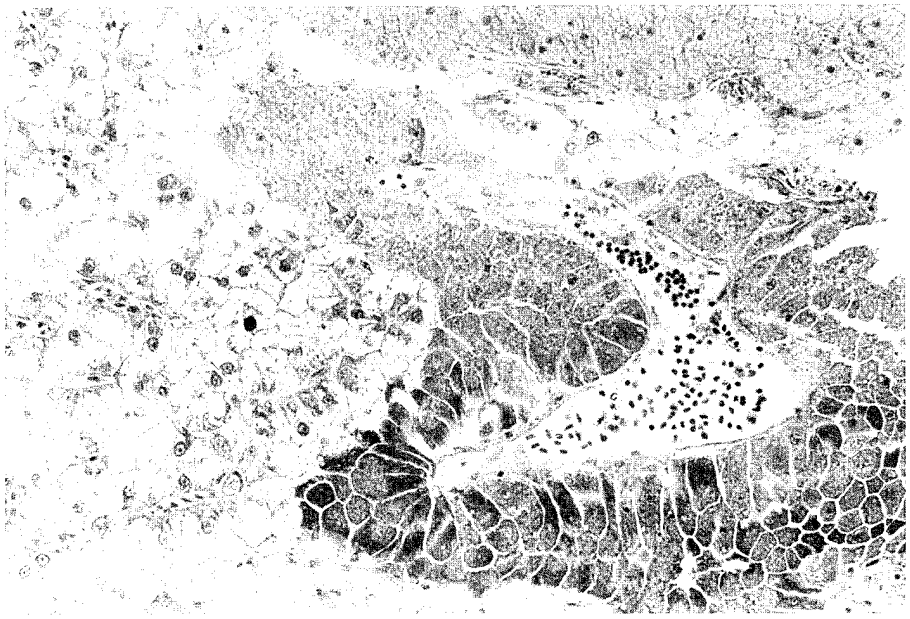


Figure 4. Alterations in pancreas cells consisting of cellular degeneration and some points of necrosis from fish fed the non-supplemented α-tocopherol diet.

Immune parameters

There were no significant differences ($P>0.05$) for lymphocyte, neutrophil or monocyte count in gilthead seabream fed different treatments. Similar results were described for Atlantic salmon fed different α-tocopherol levels (Hardie *et al.*, 1990). However, we found lower ($P<0.05$) respiratory burst activity of circulating neutrophils of fish fed both deficient diets, measured by NBT reduction and expressed as an NBT index (Table 4). Both deficiences caused this activity depletion, suggesting oxidative damage in the cell membrane due to the oxygen radicals produced from neutrophils. In previous work (Anderson and Siwicki, 1994), it has been suggested that reduced NBT activity may be caused by different factors, such as environmental stress and toxicants, including dietary vitamin deficiences.

Table 4 shows the hemolytic activity driven by the alternative complement pathway (ACP) obtained for different treatments. Fish from the control group showed a higher degree of hemolytic activity ($P<0.05$) when compared with fish fed on deficient diets. Obach *et al.* (1993) found higher complement activity in fish supplemented with vitamin E. Hardie *et al.* (1990) found a marked reduction in the total complement activity in serum from fish fed on vitamin E deficient diets. These authors suggested that this decrease in complement activity is not simply a reflection of a general decrease in all serum proteins, but an specific effect on the complement proteins. Our results agree with these previous results, but in addition, they also show that the reduction is significant in alternative pathway activity in both n-3 HUFA and α-tocopherol-deficient diets. Several hypothesis can explain this reduction of activity, since it has been shown that changes in membrane composition can regulate the ACP, and malnutrition conditions generates less complement proteins (Mold *et al.*, 1989). However, further studies are needed in this area as no definitive answers have been demonstrated at present.

CONCLUSIONS

Both α-tocopherol and n-3 HUFA deficiences produced a series of remarkable effects. Alterations in the cell membrane composition due to the n-3 HUFA dietary deficiences could cause erythrocyte fragility and depleted neutrophil activity. Higher incidence of these problems together with pancreatic damages in the vitamin E deficiencies suggested an added effect by alteration of cell membrane oxidative status. The fact that both deficient diets caused a reduction in the oxidative activity of neutrophils and a reduction of the hemolytic capacity of ACP, indicates that these nutritional deficiences significantly decreased the immunocompetence in gilthead seabream. Thus, gilthead seabream fed diets with marginal deficiences in α-tocopherol and n-3 HUFA, may be exposed to a reduction in their stress resistance potential. Further stress experiments are currently under way in order to verify this.

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Abbreviations:EFA: Esential fatty acid; n-3 HUFA: Highly unsaturated fatty acid of the linolenic family. EPA: Eicosapentaenoic acid (20:5n-3); DHA: Docosahexaenoic acid (22:6n-3); PUFA: Polyunsaturated fatty acid; MCHC: Mean cellular hemoglobin concentration; MCV: Mean cellular volume; MCH: Mean Cellular hemoglobin. RBC: Red blood cell; WBC: White blood cell; PCV: Packed cell volume; ACP: Alternative complement pathway; ACH50: 50% hemolysis effected by the alternative complement pathway; NBT: Nitroblue tetrazolium.

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Chapter 22

An *In Vitro* Culture System that Supports Long-Term Production of Functional Myeloid Cells from Rainbow Trout Spleen and Pronephros

Rosemarie C. Ganassin, John L. Brubacher and Niels C. Bols

Department of Biology
University of Waterloo
Waterloo, ON N2L 3G1, Canada

Correspondence: Niels C. Bols

ABSTRACT

Long-term hemopoietic cultures, similar to those produced from mammalian bone marrow, have been generated from rainbow trout pronephros and spleen. The trout organs are dissociated, and placed in culture flasks, leading to the formation of a complex stromal layer on the culture surface. In time, small, round cells appear on top of the stromal layer, proliferate, and are released into the medium as mature progeny cells. Cultures that produce these progeny cells develop only after specific dissociation procedures and in medium supplemented with a specific amount and type of serum. Spleen cultures must be dissociated with collagenase and the medium must be supplemented with a high (30%) concentration of fetal bovine serum (FBS). Nonhemopoietic cultures resulted after explant outgrowth or dissociation with trypsin and in medium with low FBS concentrations or a horse serum supplement. These cultures contained adherent cells, including macrophages and fibroblasts, and could be passaged to form cell lines, but did not develop a non-adherent population. Kidney hemopoietic cultures resulted after dissociation by either mechanical means or collagenase digestion, and supplemented with 10, 20 or 30% FBS. Spleen cultures produce macrophages and a unique cellular product with the characteristic morphology and motility of mammalian dendritic cells. The progeny cells produced by the pronephros include macrophages and neutrophils, and these progeny cells adhere differentially to extracellular matrix proteins. This adhesion is enhanced by exposure to the phorbol ester, phorbol -12-myristate 13-acetate (PMA).

INTRODUCTION

Long-term bone marrow cultures (LTBMC) (Allen *et al.*, 1990; Dexter *et al.*, 1977; Spooncer *et al.*, 1993) have been valuable tools for studying the process of hemopoiesis in mammals. Recently, long-term cultures have been developed from the two major hemopoietic tissues of the rainbow trout, *Oncorhynchus mykiss*, the pronephros (Diago *et al.*, 1993) and the spleen (Ganassin and Bols, 1996). In these cultures, a multilayered stroma forms on the tissue culture surface.

Cellular products develop in association with the stromal layer, and are released into the culture medium as mature cells. The cells produced by this culture system are difficult to identify definitively because of the scarcity of monoclonal antibodies to fish leukocyte surface markers. However, the major products of cultures of rainbow trout spleen include macrophages and cells with the characteristic morphology and motility of mammalian dendritic cells (Ganassin and Bols, 1996), and the major products of kidney cultures have been described as heterophils (Diago *et al.*, 1993).

Both kidney and spleen cultures, like long term bone marrow cultures, are dependent upon supplementation of the culture medium with a high concentration of fetal bovine serum. The method of organ dissociation is also important to successful hemopoiesis, particularly from spleen tissue. Hemopoietic cultures from rainbow trout spleen are only established when the tissue is digested at 10°C for 12-18 hours with collagenase, and not when the tissue is disrupted using mechanical means, such as mincing, or forcing the tissue through a screen or a syringe. Digestion with other enzymes such as trypsin results in cultures that cannot sustain hemopoiesis. Rainbow trout kidney cultures, on the other hand, can be established successfully by either mechanical dissociation (Diago *et al.*, 1993), or by enzymatic digestion.

Both kidney and spleen hemopoietic cultures will be useful in studies of the factors controlling production of immune system cells, and will provide a convenient source of cells for studies of their functions. For example, preliminary experiments indicate that the kidney culture products, which are primarily neutrophils, adhere differentially to various extracellular matrix (EM) proteins. This adhesion is enhanced by exposure to the phorbol ester, PMA.

MATERIALS AND METHODS

Preparation of kidney and spleen cell cultures

Cultures were initiated from rainbow trout spleen as previously described (Ganassin and Bols, 1995). Kidney cultures were prepared using the same protocol. Briefly, rainbow trout weighing 100- 250 grams were killed by cerebral fracture and their blood was removed by caudal puncture to reduce the blood volume in the kidney. A ventral incision was made, and the viscera were removed to expose the kidney. The connective tissue was removed, and the pronephric tissue was scraped out with a sterile scoop and placed in petri dish with 5 mL of sterile phosphate buffered saline supplemented with the antibiotics Gentamicin, (50 µg/mL) and Fungizone, (100 µg/mL) (Canadian Life Technologies, Burlington, ON). The tissue was teased apart with a sterile scalpel and scissors. At this point, two different dissociation methods were used. In the first instance, the tissue was forced through a 40-mesh screen of a tissue dissociation kit (Sigma). Alternately, an equal volume of collagenase A (Boehringer Mannheim, Dorval, Que) was added to the kidney fragments, which were then incubated for 12 hr at 10 °C. After either dissociation step, the resulting suspension of cells and fragments was centrifuged in a tabletop centrifuge for 5 minutes at 1,000 rpm (HN-SII, International Equipment Co., Needham Heights, MA). The cell pellet was resuspended in the growth medium, Leibovitz's L-15, supplemented with 10, 20 or 30% fetal bovine serum (FBS) (catalog number 200-6140), or with 30% horse serum (HS) (catalog number 16050) from Canadian Life Technologies (Burlington, ON).

The cell suspensions in different growth media were added to 12.5 cm^2 flasks (Falcon, Oxnard, CA), and incubated at 22°C. The tissue from each organ was distributed into three flasks. After two weeks, the medium was completely removed, the surface of the flask was rinsed with medium, and fresh medium containing penicillin (100 I.U. /ml) and streptomycin (100 µg/mL) was added. At this point the only cells that were present were those attached to the surface of the culture flask. Cultures were maintained at 22°C, and fed by complete changes of medium until the appearance of hemopoietic foci. At this time, cells were harvested from the cultures weekly by removing half of the culture medium containing non-adherent cells, and replacing it with fresh medium.

Morphology and cytochemistry

A Nikon Diaphot inverted microscope with phase optics was used to observe and photograph living cultures in flasks. Non-adherent cells were harvested from a culture flask and a cytocentrifuge (Shandon Cytospin) was used at 500 rpm for 10 min. to deposit the cells on to microscope slides. The slides were fixed for 5 min. in methanol, and were then stained with Wright's and Giemsa stains (Sigma) in distilled water, 1:3:50 for 20 min., to distinguish general morphology. Positive staining for the leukocyte enzyme myeloperoxidase is a characteristic of rainbow trout neutrophils (Zelikoff and Enane, 1991). Myeloperoxidase activity was demonstrated using diaminobenzidine (DAB), with a modification of Sigma procedure 390. DAB was used as a substrate rather than p-Phenylenediamine diHCl and catechol, because staining results with the latter were unclear. Cytocentrifuged slides were fixed for 30 sec. at room temperature in fixative solution consisting of 5 mL of 37% paraformaldehyde mixed with 45 mL of 95% ethanol. Following fixation, they were washed in gently running tap water and air dried in the dark for 10 min. Fast DAB tablets (Sigma) were used to prepare the staining solution, and washed, fixed slides were incubated for 30 min. in the dark in a 37°C waterbath. The slides were rinsed in tap water, allowed to air dry and counterstained with Acid Hematoxylin Solution (Sigma catalog No. 285-2) for 10 min.

Transmission electron microscopy

Non-adherent cells were harvested from kidney cultures and collected by centrifugation. Cells were fixed for 10 min. in modified Karnovsky's fixative, pH 6.8, rinsed gently 3 times with 0.1 M sodium cacodylate, and post-fixed in osmium tetroxide. Cells were dehydrated in ascending ethanol concentrations, taken to acetone, then infiltrated and embedded in Epon:Araldite. Ultrathin sections were cut on a microtome, stained with lead citrate and uranyl acetate, and observed with a Phillips transmission electron microscope.

Cell Adhesion Assay

Wells of 48-well tissue culture plates (Costar, Cambridge, MA) were coated with laminin, fibronectin, collagen type I, or collagen type IV (Sigma). Collagen I and collagen IV were dissolved in 0.1 M and 0.25% (v/v) acetic acid respectively. Both were sterilized by underlying with chloroform (Sigma , 1991), and diluted to working concentrations in Dulbecco's PBS. Laminin and fibronectin were purchased as sterile solutions and diluted further in Dulbecco's PBS. Wells were coated with 200 µL of each extracellular matrix protein, at final concentrations indicated in figure legends, by overnight incubation.

Following the overnight incubation, wells were washed twice with PBS, and 100 µL of L-15, with or without 100 ng/mL phorbol -12-myristate 13-acetate (Sigma), was added to each well. Cultured kidney cells were prepared by collecting non-adherent cells by centrifugation, and resuspending in L-15 without serum to a concentration of 10^6 cells/mL. 100 µL of this suspension was added to each well, and to uncoated wells, for a final concentration of 10^5 cells per well, and 50 ng/mL of PMA in phorbol treated wells (Rüegg *et al.*, 1992).

To quantify the degree of cell attachment, the fluorescent dye, 5-(6)-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) (Molecular Probes) was used. Although CFDA-AM has been shown to have a detrimental effect on cell attachment (De Clerk *et al.*, 1994), in our assay it is not present during the period of cell attachment. After a 10 hr incubation, medium containing unattached cells was gently decanted from the culture vessels, and 150 µL of Dulbecco's PBS containing CFDA-AM at a concentration of 4µM was added to each well. The dye-containing buffer was aspirated out of each well after 30 min., and 250 µL of Dulbecco's PBS was added. The plate was scanned using the CytoFluor 2350 (Millipore), a cytofluorometer, with filters 485/530, using area scan at a sensitivity setting of 4.

RESULTS

Development of hemopoietic cultures

In both kidney and spleen cultures, collagenase dissociation led to hemopoietic cultures with a complex stroma that released cells into the medium. Hemopoietic spleen cultures could only be established with collagenase digestion, and not with trypsin or mechanical dissociation (Ganassin and Bols, 1996, summarized in Figure 1). Kidney tissue produced hemopoietic cultures when dissociated by either mechanical or enzymatic means. While spleen cultures absolutely required a supplement of 30% FBS to establish hemopoiesis, kidney cultures produced progeny cells when supplemented with 10 or 20% FBS, although the number of cells produced was greater with a 30% supplement (data not shown).

At the time of the first complete medium change, two weeks after culture initiation, only cells adherent to the tissue culture surface were present. In spleen cultures, the establishment of a complex stromal layer on the culture surface was followed by the appearance of small foci of round, phase bright cells (Figure 2b), which proliferated into large masses and produced non-adherent cells that were released into the culture medium. The time period from the initiation of the culture and the appearance of non-adherent cells ranged from 4 weeks to 6 months.

Kidney cultures developed in a different manner than those of the spleen (Figure 2). The stromal layer did not cover as much of the culture surface as the stromal layer of spleen cultures. The presence of numerous melanomacrophages gave a black appearance to the culture surface. Progeny cells did not develop in discrete foci, as was seen in spleen cultures, but rather over the entire surface of the stromal layer, and were much more numerous. The kidney cultures produced progeny cells as early as 2 weeks following culture initiation, and when supplemented with 30% FBS, maintained production for a minimum of 2 months. In cultures supplemented with a lower FBS concentration, hemopoiesis was not as prolonged, and the stromal layer declined and began to peel off of the surface

after 2 months. In cultures supplemented with horse serum, a stromal layer did not develop, and non-adherent cells were not produced.

Generation of cell lines

As described previously (Ganassin and Bols, 1996), and summarized in Figure 1, the techniques for producing hemopoietic cultures have led to the generation of numerous cell lines from rainbow trout spleen and kidney. These cell lines have been maintained and cryopreserved as described by Bols and Lee (1994), and are awaiting further characterization.

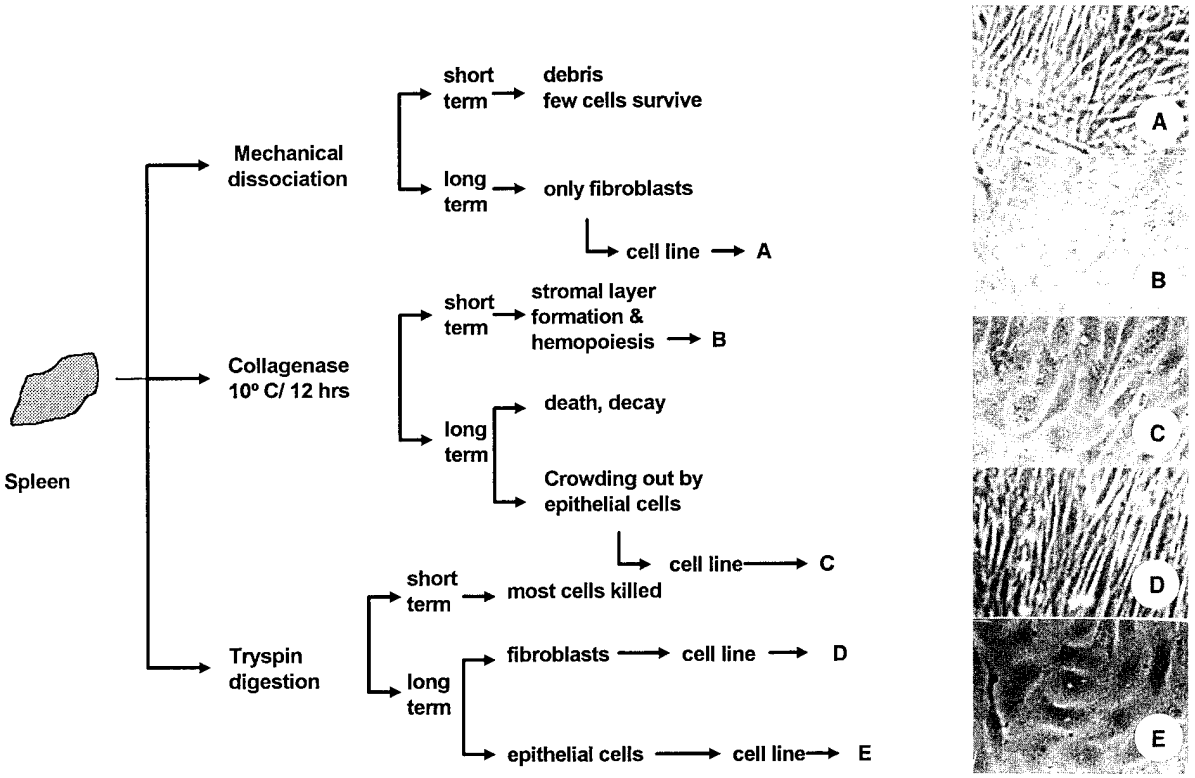


Figure 1. Effect of dissociation protocol on spleen culture development. Only specific dissociation protocols, as summarized in this chart, lead to hemopoietic cell cultures of rainbow trout spleen. Mechanical dissociation, including mincing tissue, or forcing it through a fine screen or a syringe, results in the death of most cell types. Only fibroblast-like cells survive, which can be passaged to form cell lines (A). Collagenase digestion, for 12 hrs at 10°C, leads to the formation of a stromal layer and the proliferation of hemopoietic cells (B). These cultures ultimately either die, or are crowded out by a cell type with epithelial morphology (C), which can also be passaged to form cell lines. Digestion of the tissue with trypsin results in the survival of either fibroblast-like (D) or epithelial-like (E) cells, and these will also form cell lines.

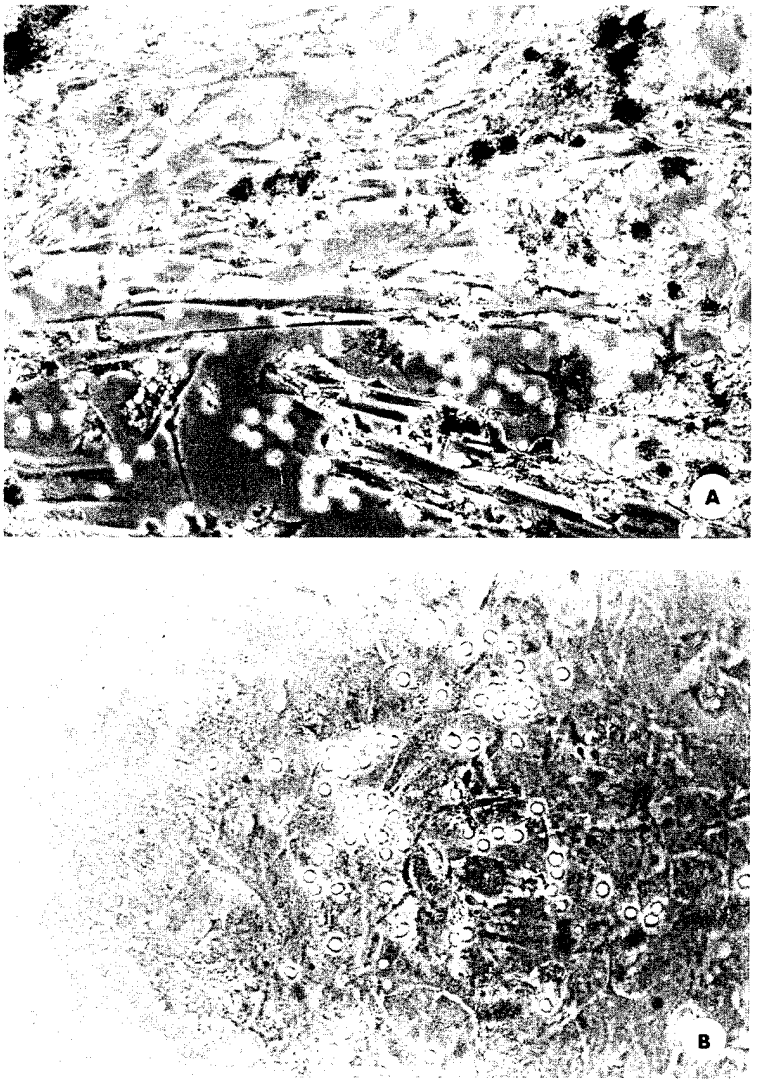


Figure 2. Spleen and kidney hemopoietic cultures differ in character. The production of progeny cells (the small, round, phase-bright cells evident in these micrographs), occurs in discrete foci associated with the complex stromal layer in spleen hemopoietic cultures (B). In kidney cultures (A), the stromal layer is not as extensive and contains numerous melanomacrophages (black pigmented cells). Progeny cells develop all over the stromal layer, and are not concentrated in foci.

Products of hemopoietic cultures

The progeny cells of spleen cultures have been previously described (Ganassin and Bols, 1996). The products of kidney cultures include neutrophils, which are myeloperoxidase positive cells (Figure 3c) , approximately 60 μm in diameter, often with lobed nuclei (Figure 3a). Another identifiable product is the monocyte or macrophage, with a diameter greater than 100 μm, an oval shaped nucleus, numerous lysosomes and residual bodies (Figure 3b), and negative staining for myeloperoxidase. Other cell types are not easily identifiable, and may be earlier developmental stages of neutrophils or macrophages.

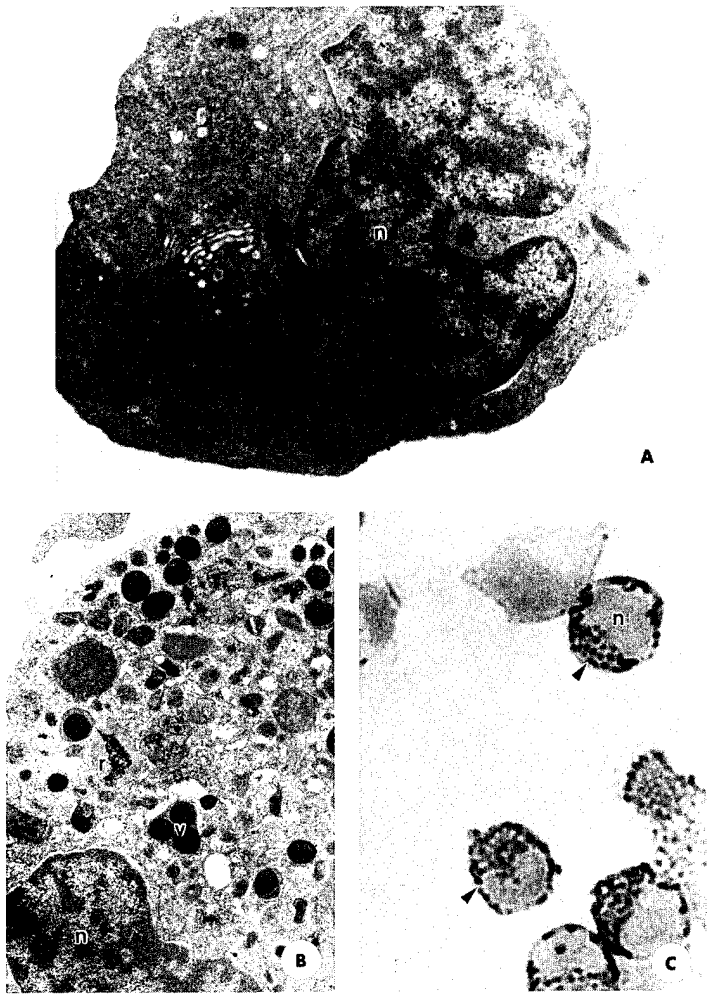


Figure 3. Characterization of the products of kidney hemopoietic cultures. The major products of these cultures are neutrophils (A), which have large multi-lobed nuclei (n) occupying approximately half of the cell area and a granular cytoplasm (TEM, mag. 22,500 x , actual cell size 60 mm diameter). Fewer macrophages (B) are formed, and are easily distinguished by their larger size (TEM, mag. 13,200 x, actual cell size, 110 mm diameter). The bean-shaped nucleus (n) occupies much less of the cell area, and there are numerous phagocytic vesicles (v) , residual bodies (r), and other inclusions. In cytocentrifuge preparations stained for the enzyme myeloperoxidase (C), most of the cells show dark granulation (arrowheads), indicating a positive reaction typical of rainbow trout neutrophils.

Adhesion of kidney culture products

Unstimulated kidney culture products adhere to uncoated plastic and to surfaces coated with fibronectin to the same degree (Figure 4). Adhesion to laminin is approximately one half of adhesion to uncoated plastic, while adhesion to either collagen I or collagen IV is very limited.

When the phorbol ester PMA is included in the attachment medium, attachment to collagen I or collagen IV coated surfaces is relatively unaffected; however, the cells attach to one another and form large aggregates (Figure 5b). Attachment to uncoated surfaces is enhanced slightly by

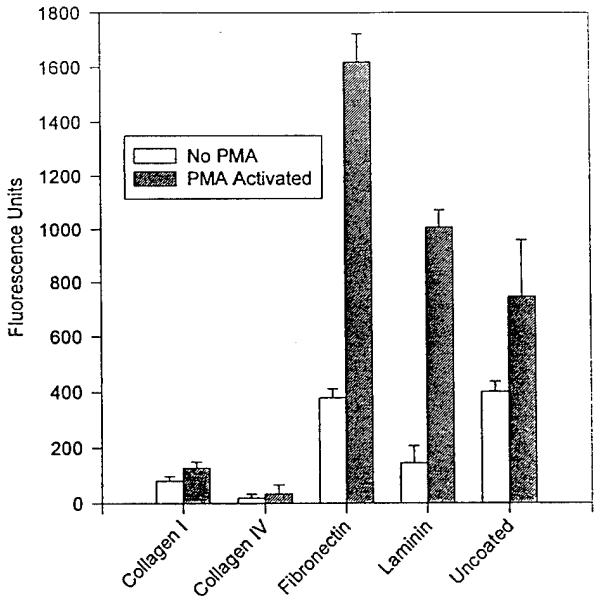


Figure 4. Adhesion assay of kidney culture products (primarily neutrophils). Wells of a 48 well plate (Costar) were coated with the extracellular matrix proteins collagen I or IV (10 mg/well), fibronectin (5 mg/well), or laminin (2 mg/well). Adhesion was measured after 10 hrs. Cells were added in medium without a serum supplement, and with or without the phorbol ester PMA, and allowed to adhere for 10 hrs. After 10 hours, non-adherent cells were removed, and 150 mL of 4 mM CFDA-AM (carboxyfluorescein diacetate acetoxy methyl ester, Molecular Probes) was added to each well. After 30 min. incubation, the solution was aspirated and replaced by Dulbecco's PBS, and the plate was scanned using a CytoFluor 2350 microfluorometer (Millipore), using filters 485/530, sensitivity 4. Results shown are of a single representative experiment, with error bars representing the standard deviation of triplicate wells.

exposure to PMA. PMA dramatically enhances the attachment of kidney culture products to surfaces coated with fibronectin and laminin. The adherence of PMA stimulated kidney cells to fibronectin is four times that of the unstimulated controls, while adherence to laminin is increased five fold. Cells attach strongly and spread in fibronectin coated wells (Figure 5d), but are weakly adherent and spread less in wells coated with laminin (Figure 5f).

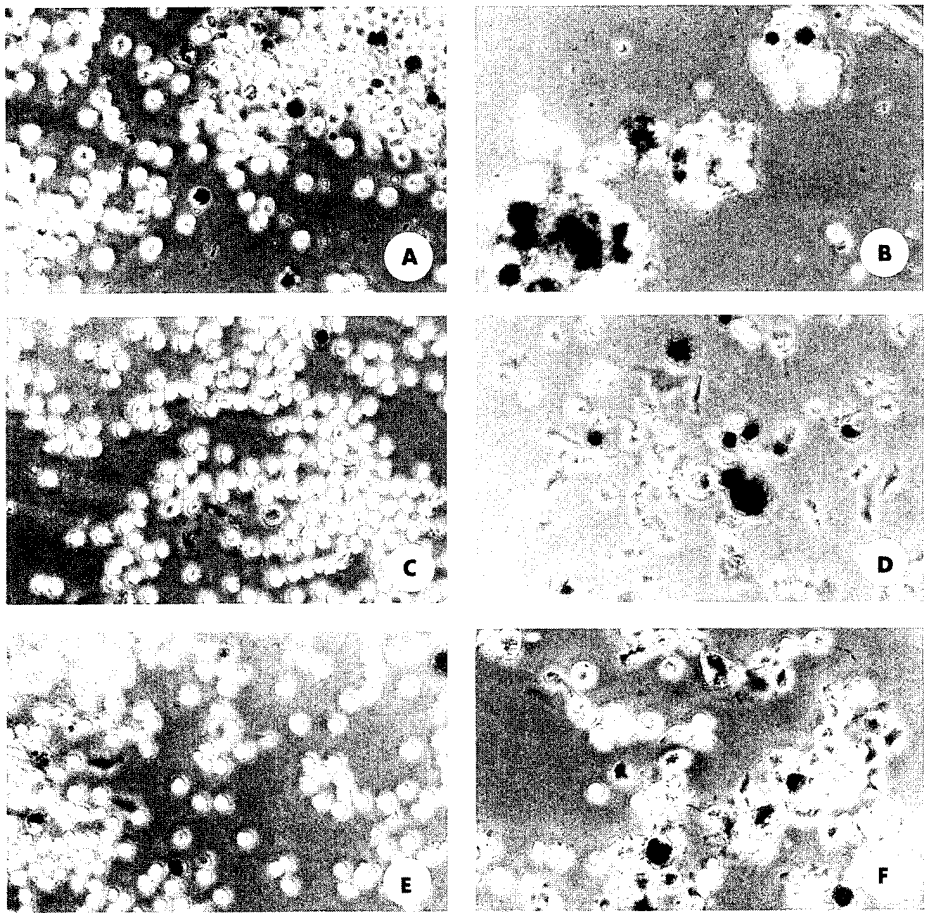


Figure 5. Appearance of cells adhering to extracellular matrix proteins. Without PMA in the attachment medium, cells did not attach appreciably to surfaces coated with collagen I (A), but attached to surfaces coated with fibronectin (C), or laminin (E). When PMA, 50 ng/mL, was added to the attachment medium, cells plated in wells coated with collagen I (shown) or IV (not shown, but similar) formed large aggregates, not attached to the culture surface (B) (this photograph is taken in the plane of the floating cells). In wells coated with fibronectin (D), attachment was greatly enhanced and the cells rapidly spread. In laminin coated wells (F), attachment was much greater than in uncoated, but cells attached tenuously and did not spread appreciably. Phase contrast, Mag. 200x, photographed 1 hr after plating.

DISCUSSION

Hemopoietic cell cultures have been established from the two major hemopoietic organs of the adult rainbow trout, the anterior kidney (pronephros) and the spleen. Cultures from these organs initiated and maintained under the same conditions differ in several respects, including the requirement for a specific dissociation protocol and serum supplement, the time course of culture development, the extent of stromal layer formation, and the progeny cells produced.

Kidney hemopoietic cultures can be established after dissociation of the tissue by mechanical means, or by enzymatic digestion, unlike those of spleen, which are only successful when initiated by collagenase digestion (Ganassin and Bols, 1996). In spleen cultures, digestion by collagenase is essential to the establishment of a complex stromal layer. In mammalian long-term bone marrow culture (LTBMC), the stromal layer provides cell-cell contacts and some of the diffusible regulatory molecules that stimulate blood cell formation and maturation. Other diffusible factors are provided by a high serum concentration supplementing the growth medium (Spooncer *et al.*, 1993). The long period of time between culture initiation and the production of progeny cells in rainbow trout spleen cultures suggests that the stromal layer may produce some critical factor(s) that must accumulate to high enough levels to support hemopoiesis. Kidney cultures develop in a much shorter time than those of the spleen, and do not appear to require the establishment of an extensive stromal layer prior to cell production. Kidney cultures begin to produce non-adherent progeny cells very soon after culture initiation, which suggests that the factors required differ from those of the spleen, or are required in smaller amounts and are supplied adequately by the fetal bovine serum supplementing the culture medium.

Spleen progeny cells develop in discrete foci, and may require specific cell-cell contacts in addition to soluble factors, as has been observed in LTBMC (Allen *et al.*, 1990). The associations between developing kidney cells and the stromal layer are less obvious.

The progeny cells produced by spleen cultures, as described previously (Ganassin and Bols, 1996), include monocytes, macrophages, and dendritic-like cells. The kidney cultures also produce monocytes and macrophages, but their major product appears to be neutrophils, as judged by their morphology, and positive myeloperoxidase staining. This division may reflect the *in vivo* situation.

Kidney cultures produce many more progeny cells than those of spleen. This is also true *in vivo*. The rainbow trout kidney is considered the primary hemopoietic organ, analogous to mammalian bone marrow. Histologically, far more blood cell production is observed there than in the spleen (Yasutake and Wales, 1983). The spleen has been considered analogous to the mammalian lymph node (Anderson, 1986), which functions primarily in the response to antigens and has a secondary role in blood cell production.

The culture conditions previously described for pronephric cultures (Diago *et al.*, 1993), produced only small numbers of hemopoietic cells. When the pronephros is cultured using the same method and culture medium described for spleen cultures (Ganassin and Bols, 1996), the cultures reliably produce large numbers of neutrophils for a period of months. The cultures provide a convenient source of cells for further experimentation.

Preliminary experiments of the adhesive properties of the kidney culture products demonstrate the potential usefulness of these cultures. In the inflammatory response, the migration of leukocytes from the blood or tissues to the site of infection or tissue damage involves interactions with extracellular matrix proteins, such as laminin, fibronectin and collagen (Snyderman and Goetzl, 1981).

Our data shows that PMA enhances the attachment of the kidney culture products to laminin and fibronectin, and promotes the aggregation of the cells in the presence of collagen I or IV. The phorbol ester PMA is a potent activator of protein kinase C (PKC), its specific cellular receptor (Blumberg, 1988), and mimics the effects of physiological activators (Rosales and Juliano, 1995). Leukocytes of mammals respond to phorbol esters by increased cell-to-substratum or cell-cell adhesion, either due to increased expression of specific receptors on the cell surface, as has been reported in human neutrophils (Yoon *et al.*, 1987) or by the phosphorylation of proteins that are involved in adherence to extracellular matrix proteins (Pontremoli, 1987). The mechanism of the response of fish neutrophils to PMA requires further investigation.

Hemopoietic spleen and kidney cultures have many potential uses. First, they provide a model system for the study of the hemopoietic process similar to mammalian long-term bone marrow culture. LTBMC have allowed the study of numerous questions that are difficult or impossible to interpret in the more complex *in vivo* situation, including the identification and isolation of many of the known cytokines. The factors affecting rainbow trout blood cell production can now be examined and the identification and isolation of novel fish cytokines from these cultures is possible. Secondly, these cultures provide a ready source of immune system cells for further studies. The dendritic-like cells produced by the spleen cultures will be useful in studies of antigen presentation, and of cellular motility. The kidney neutrophils can be used to help to elucidate the process of inflammation in fish.

Finally, the culture conditions result in the production of numerous cell lines, which are potentially useful in such areas as fish virology, toxicology, cell biology, and fish physiology (Bols and Lee, 1991,1994).

Abbreviations: fetal bovine serum (FBS), phosphate buffered saline (PBS), long-term bone marrow cultures (LTBMC), phorbol-12-myristate 13-acetate (PMA), extra-cellular matrix (EM), 5-(6)-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM)

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Chapter 23

Neurotransmitter and Opioid Modulation of the Amphibian Transplantation Immunity

Szczepan Jozefowski[1,2], Alicja Jozkowicz[1], Lucyna Antkiewicz-Michaluk[2], Barbara Plytycz[1,3], Rolf Seljelid[3]

[1]Institute of Zoology
Jagiellonian University
Krakow, Poland

[2]Institute of Pharmacology
Polish Academy of Sciences
Krakow, Poland

[3]Institute of Medical Biology
University of Tromsø
Tromsø, Norway

ABSTRACT

The experiments were performed on the involvement of the autonomous nervous system in the modulation of the amphibian transplantation immunity. The presence of adrenergic and cholinergic muscarinic receptors on the amphibian leukocytes was demonstrated using radioligand binding assays. *In vivo* administration of muscarinic (atropine) and ß-adrenergic (propranolol) antagonists affected the rate of xenograft but not allograft rejection in froglets of *R.esculenta*. Morphine administration had no effect on the fates of both allografts and xenografts in *R.esculenta* and *R.temporaria*. The ongoing xenograft but not allograft rejection in *B.bufo* was accompanied by the increased specific binding of β-adrenergic radioligands to whole blood cells. In the case of xenograft rejection, the increased specific binding of muscarinic radioligands was detected as well. It seems that autonomic nervous system participates significantly in the modulation of immunity to xenogeneic stimuli.

INTRODUCTION

Studies in the field of neural modulation of the immune system are most advanced in mammalian model species and in humans since in these organisms numerous mediatory molecules and their receptors, shared by the immune and neuroendocrine systems, are best characterized (Hu, *et al.*, 1991; Kavelaars, *et al.*, 1990; Shadiack, *et al.*, 1993). However, one should keep in mind that very convenient models for the investigation of fundamental aspects of neuroendocrine-immune interactions can be found among "lower" vertebrates or even invertebrate species.

We will focus here on investigations of modulation of the amphibian transplantation immunity by neurotransmitters and neuropeptides.

MATERIALS AND METHODS

Animals

The adult common toads *Bufo bufo*, fire-bellied toads *Bombina bombina*, froglets of the green frog *Rana esculenta*, and froglets and adult individuals of the common frog *Rana temporaria* were field collected. In the laboratory they were kept in moist terraria with fresh plants, grasses and leaves which were changed twice a week. The animals were fed with earthworms, fruit flies and snails *ad libitum*. The experiments were conducted after at least 2 weeks' acclimated to laboratory conditions.

Skin grafts

The frogs and fire-bellied toads *B. bombina* were anaesthetized in 0.1 % MS-222 (Sandoz); the common toads *B. bufo* were etherized. Squares of dorsal skin (approximately 4 mm^2 and 25 mm^2 in the case of young and adult individuals, respectively) were transplanted to the backs of the recipients. The grafts were not secured. Three-day isolation of recipients prevented loss of the grafts. The grafts were checked under a stereomicroscope every second day. Graft rejection was considered complete when all the melanophores of the graft were destroyed. Each froglet received 3 skin grafts: allograft, xenograft from a froglet of *R. temporaria*, and xenograft from adult *B. bombina*. The froglets of *R. temporaria* were allografted and xenografted with the skin from a froglet of *R. esculenta* and adult *B. bombina*. Each adult toad *B. bufo* received only one graft: either autograft or allograft or xenograft from an adult *R. temporaria*.

Drug administration

Grafted individuals of *R.esculenta* were injected with 100 µL of either dl-propranolol hydrochloride (Sigma) (0.01 mg/g body weight), atropine sulphate (Sigma) (0.67 mg/g b.w.), morphine sulphate (Polfa) (10 mg/kg b.w.) or saline into the dorsal lymph sac every second day, at 4-7 P.M. Grafted froglets of *R. temporaria* were similarly injected with either morphine (10 mg/kg b.w.) or saline.

Isolation of peritoneal leukocytes

Adult male *Rana temporaria* and *Bufo bufo* were injected intraperitonealy with Sephadex G-50 (Pharmacia, Fine Chemicals), (0.5 mL, 3%) two days prior to cell harvesting. Sephadex-elicited peritoneal leukocytes were harvested by peritoneal lavage with HBSS (220 mOsm, pH 7.5) without divalent cations, containing 10 U/ml heparine. Cells were washed twice with HBSS by centrifugation at 400 xg for 10 min and resuspended at 6.7 x 10^6 cells/mL in fresh buffer.

Blood cells

Peripheral blood was collected in HBSS with heparin (final concentration 10 U/mL) from the allo-and xenografted common toads killed by decapitation at the rejection phase. Blood from the

autografted animals killed at the same time served as a control. Following centrifugation at 400 xg for 10 min, blood cells were resuspended in fresh HBSS with heparin (10 U/ml) at 6.7 x 10^6 cells/mL.

ß-adrenoreceptor assay

The ß-adrenoreceptors were defined as [^{3}H]-CGP 12177 (Amersham, specific activity 49 Ci/mmol) binding sites. Peritoneal cells (1x10^6) were incubated with increasing concentrations of radioligands without (total binding) or with (nonspecific binding) unlabelled dl-propranolol hydrochloride (Sigma) (final concentration 100 mmol/L). The incubation was carried out in duplicate in a shaking water-bath at 25° C for 50 min. Addition of the radioligand initiated the incubation, which was terminated by vacuum-assisted filtration of the incubation mixture through GF/C Whatman fiber-glass filters. The filters were then rinsed twice with 5 mL of ice-cold incubation buffer and placed in plastic scintillation minivials. The samples were assayed for radioactivity in a Beckmann LS 3801 scintillation spectrometer at 50% efficiency. The protein content was analyzed according to the Lowry' (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Specific binding was defined as the difference between total and non-specific binding, and was expressed in fmol/mg protein. The results were evaluated by Scatchard analysis for assessment of Bmax and Kd values.

Muscarinic receptor assay

The cholinergic muscarinic receptors were defined as [^{3}H]-QNB (Amersham, specific activity 49 Ci/mmol) binding sites. Peritoneal cells (10^6/mL) were incubated with increasing concentrations of radioligands without (total binding) or with (nonspecific binding) unlabelled atropine sulphate (Sigma) (final concentration 1 mmol/L). The incubation was carried out in duplicate in a shaking water-bath at 25°C for 60 min. The futher experimental procedure and data analysis were the same as for β-adrenergic receptor assay.

Radioligand binding by toad blood cell suspension in HBSS was determined in triplicates using a single radioligand concentration (4.2 nM QNB and 6 nM CGP) with or without appropriate receptor blockers.

Statistical analysis

For comparison of samples, t-test (Statgraphics, version 2.6) was used. Values were considered significantly different at $p<0.05$.

RESULTS

Figures 1 and 2 show that the radioligand binding to peritoneal leukocytes of toads and frogs was saturable and specific; both radio ligands were displaced from the binding sites by an exess of corresponding unlabelled antagonists. The low Kd values obtained from the Scatchard plots (inserts) indicate that in both species radioligands bind to leukocytes with a high affinity.

Figure 3 shows radioligand binding to blood cells of common toads *B.bufo* bearing either skin autografts, or allografts or xenografts during the rejection phase. Binding to both adrenergic and

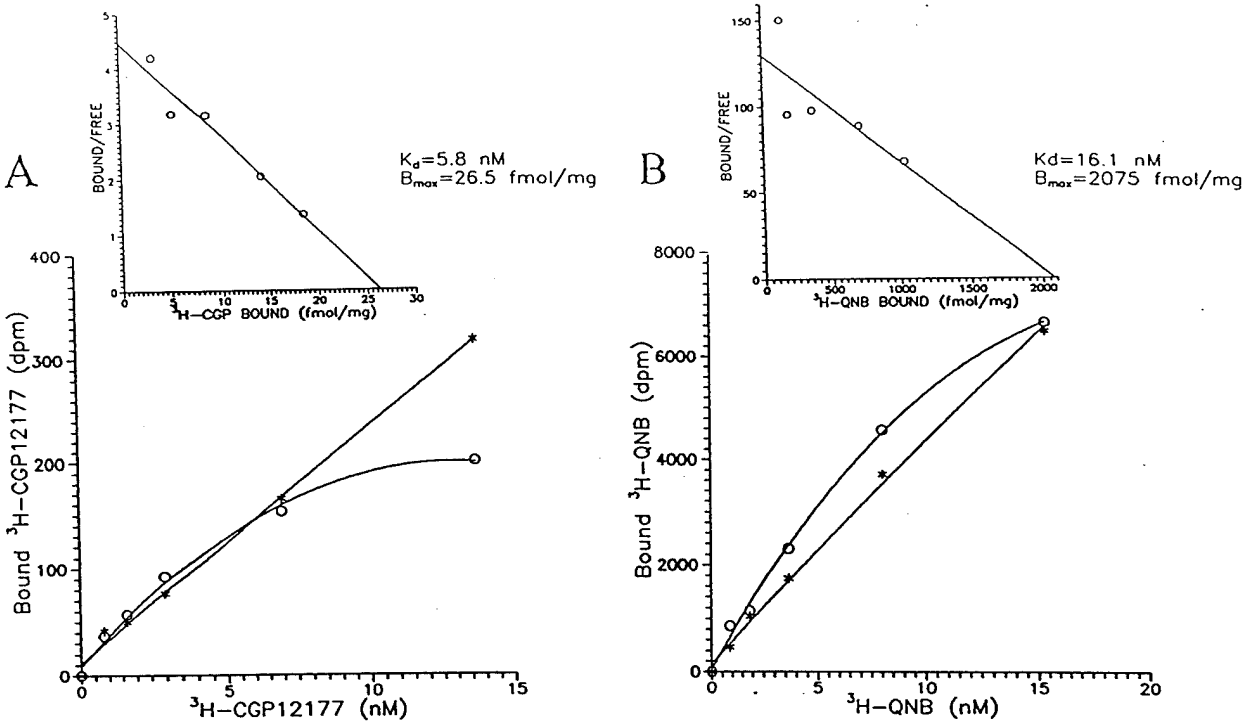


Figure 1. Saturation curves and Scatchard plots (inserts) of [^{3}H]-CGP 12177 (A) and [^{3}H]-QNB (B) biding to common toad (*B. bufo*) peritoneal leukocytes. (o) specific binding, (*) nonspecific binding. Each figure shows the results of one out of tree (CGP) or two (QNB) representative experiments, each point being the average of duplicate.

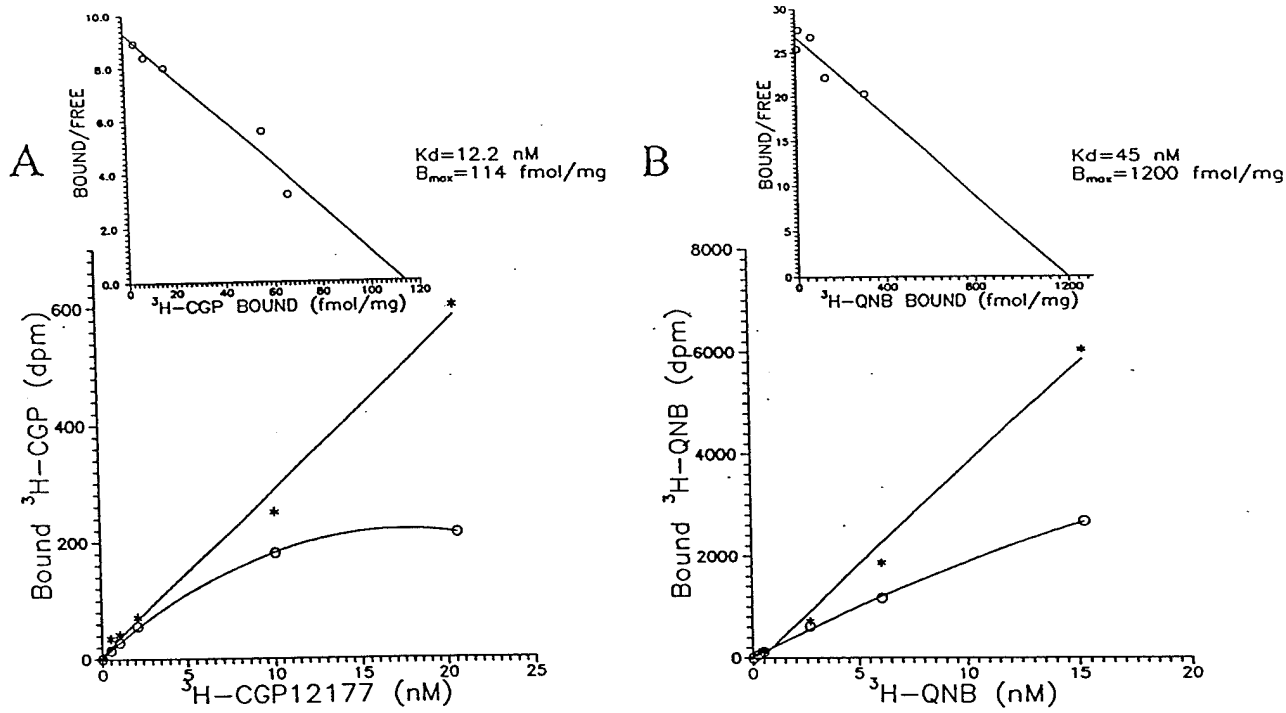


Figure 2. Saturation curves and Scatchard plots (inserts) of [^{3}H]-CGP 12177 (A) and [^{3}H]-QNB (B) binding to common frog (*.R. temporaria*) peritoneal leukocytes. (o) specific binding, (*) nonspecific binding. Each figure shows the results of one out of two representative experiments, each point being the average of duplicates.

muscarinic receptors was significantly increased in xenografted animals (Figure 3a,b). Binding of adrenergic receptors was not affected in hosts of allografts (Figure 3b).

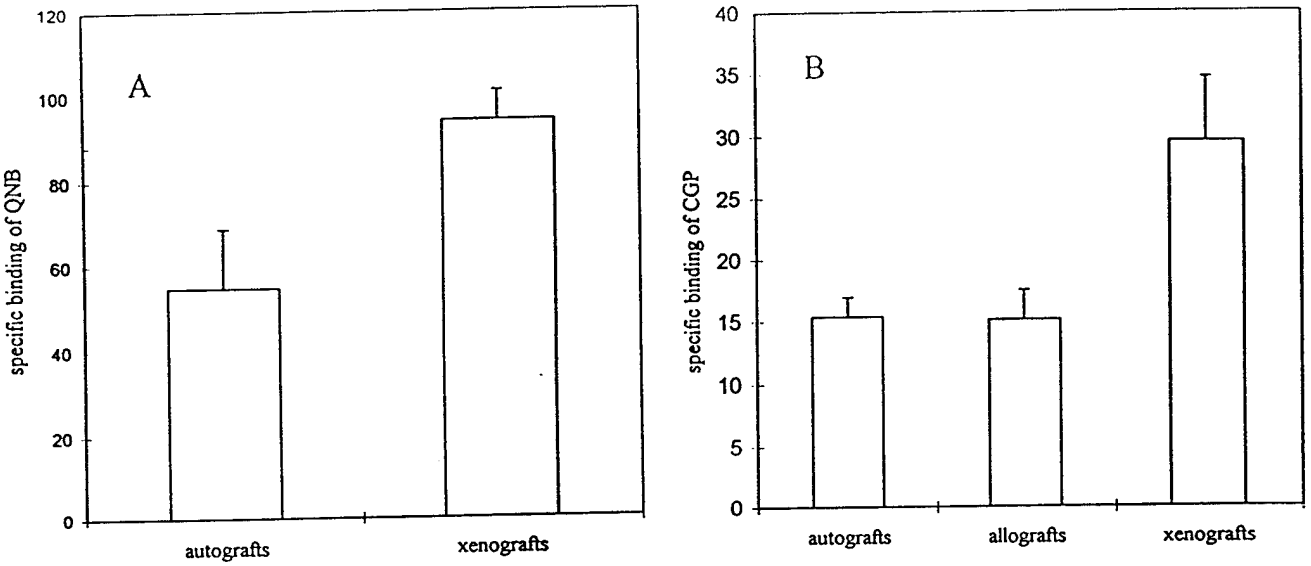


Figure 3. The specific binding of 4.2 nM of tritiated QNB (A) and 6 nM of tritiated CGP (B) to blood cells of the common toad *B.bufo* at the rejection phase of allo- or xenografts and to blood cells of their autografted counterparts. Each value is the mean ± SE from 6-8 animals.

Figure 4 illustrates mean survival times of skin allografts and xenografts in the froglets of *R. esculenta* injected every second day with saline (control animals), β-adrenergic (propranolol), or muscarinic (atropine) antagonists. Propranolol significantly prolonged the survival time of xenografts from both *R. temporaria* and *B. bombina* while allograft rejection was not significantly affected. Atropine significantly accelerated rejection of skin xenografts from *B. bombina*, while survival of xenografts from *R. temporaria* and allografts was not significantly affected.

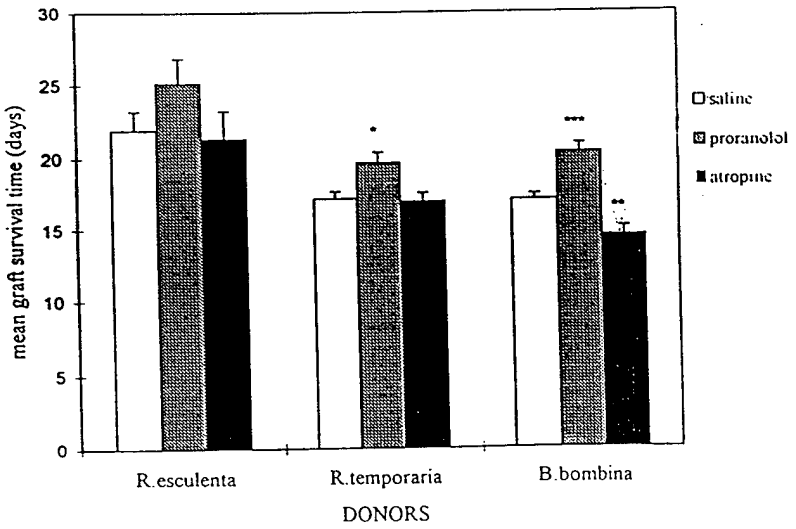


Figure 4. Effects of chronic treatment with beta-adrenergic and muscarinic antagonists on the mean survival times of skin allo- and xenografts in the green frog *R.esculenta*. Each value is the mean ± SE from 14-17 animals. Asterisks indicate values statistically different from those of the saline treated group: * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$.

Figure 5 presents mean survival times of skin allografts and xenografts in froglets of (A) *R. esculenta* and *(B) R. temporaria* injected every second day with saline (control group) or morphine. Chronic morphine treatment had no effect on graft viability.

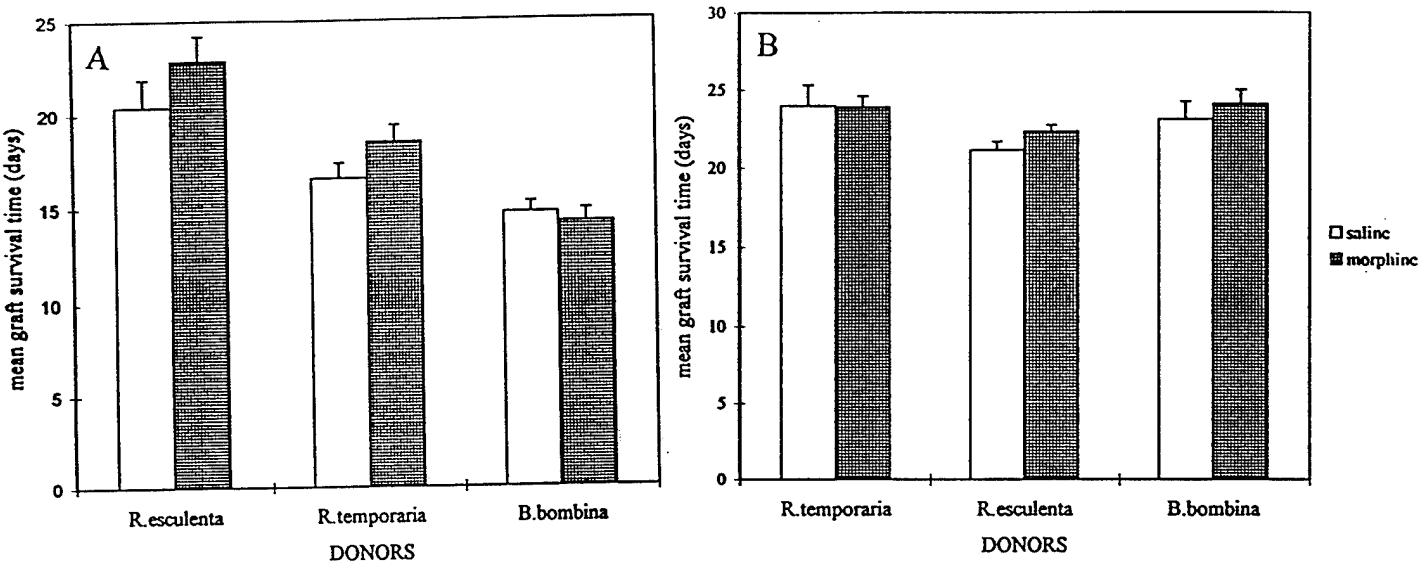


Figure 5. Effects of chronic treatment with morphine on the mean survival time of skin allo- and xenografts in the green frog (A) *R.esculenta* and the common frog (B) *R.temporaria*. Each value is the mean ± SE from 14-17 animals.

DISCUSSION

Saturation experiments analyzed according to the Scatchard method indicate the expression of cholinergic muscarinic and adrenergic receptors on both toad and frog peritoneal leukocytes (Figures 1, 2). Recently we demonstrated the expression of both types of receptors in goldfish head kidney leukocytes (Jozefowski, *et al.*, 1996). Their presence on the mammalian leukocytes has been already well documented by different groups of investigators (Atweh, *et al.*, 1984; Lopker, *et al.*, 1980; Abrass, *et al.*, 1985; Khan, *et al.*, 1986).

Radioligand binding to blood cells of the common toad *B. bufo* during the rejection phase of allo- or xenografts was compared to that in autografted animals. Binding to adrenergic receptors was not changed in the hosts of allografts (Figure 3b). We cannot exclude, however, that different cell populations were affected in opposing ways. Binding to both adrenergic and muscarinic receptors was significantly increased in xenografted animals (Figure 3a,b). We should stress that whole blood was used for this investigations and that the composition of blood leukocytes was not determined. Thus the relative contribution of particular cell types in the receptor binding experiments is unknown. The increased level of radioactivity bound may result from the increased number of receptor-rich cells and/or increased receptor expression on particular cell populations. The former phenomenon may be related to leukocyte proliferation and/or redistribution, while the latter may

be evoked by multiple neurohormonal changes during the ongoing immune response. Therefore we can only speculate on the mechanisms underlying up-regulation of radioligand binding to receptors during xenograft rejection.

Increased binding to both adrenergic and muscarinic receptors during xenograft rejection corresponds with the results on effects of drug administration on the fates of skin grafts: only the fates of xenografts were affected by chronic administration of propranolol or atropine.

Drug affect transplantation immunity of *R. esculenta* in various ways. Propranolol significantly prolonged the survival time of xenografts from both *R. temporaria* and *B. bombina*, while allograft rejection was not significantly affected. Atropine significantly accelerated rejection of skin xenografts from donors of the phylogenetically distant species *B. bombina*, while survival of xenografts from *R. temporaria* and allografts was not significantly affected. Morphine treatment did not affect the fates of grafts in both *R. esculenta* or *R.temporaria* hosts.

It seems that autonomic neurotransmitters principally affect immunity towards xenogenic stimuli. Blocking of muscarinic receptors increased the speed of the immune reaction. One might speculate that acetylcholine acting via muscarinic receptors would delay immunity towards xenogeneic stimuli. In contrast blocking of β-adrenergic receptors caused retarded the immune reaction. Noradrenaline, acting on ß-adrenergic receptors, appears to have acccelerated reactions to xenogeneic stimuli. It seems that the two parts of the autonomic nervous system have opposite effects on immunity to xenografts .

Early thymectomy impairs allograft more profoundly than xenograft rejection in *Xenopus laevis*. In this species allografts are heavily infiltrated by lymphocytes, while the antibody-mediated response predominates in xenograft rejection (Horton, *et al.,* 1992). Lowering of ambient temperature prolongs allograft viability more than xenograft viability (Tahan and Jurd, 1978; Jozkowicz & Plytycz, 1994). Host malnutrition prolonged xenograft but not allograft viability (Plytycz, *et al.,* 1993). Oxygen radicals and nitric oxide are more involved in xenograft than allograft response. (Jozkowicz, 1996). This indicates that xenograft rejection depends mainly on innate immunity and on the antibody-mediated response. These mechanisms seem susceptible to autonomic modulation than those involved in allograft rejection.

Adrenergic stimulation of both antibody production and NK activity has been demonstrated previously by chemical sympathectomy (Ackerman, *et al.,* 1991) and by infusion of adrenergic agonists (Murray, *et al.,* 1992; Fujiwara & Orita, 1987). the immunological effects of stress-induced activation of the sympathetic nervous system blocked by peripheral ß-adrenoreceptor antagonists (Benshop, *et al.,* 1994; Irvin, *et al.,* 1990; Croiset, *et al.,* 1990; Fujiwara & Orita, 1987) also imply adrenergic involvement. Acute sympathetic stimulation preferentially increases the number of circulating lymphocytes from populations expressing a higher level of ß-adrenergic receptors, e.g. B lymphocytes and NK cells (Landmann, 1992; van Tits, *et al.,* 1990). Concanavalin A-induced *in vitro* activation of porcine (Westly & Kelley, 1987) and murine (Radojcic, *et al.,* 1991) splenocytes leads to increased expression of ß-adrenoceptors.

Activation of the immune system induces stress-like neuroendocrine changes, including activation of the hypothalamus-pituitary-adrenal axis (Chuluyan, *et al.,* 1992; Carlson, *et al.,* 1981) leads to

an increased level of glycocorticosteroids, which are known ß-adrenoceptor up-regulators (Reid, *et al.*, 1992). It has been shown that hydrocortisone treatment increases the number of muscarinic receptor sites on rat thymocytes (Maslinski, *et al.*, 1988).

Hodgson *et al.* (1979) demonstrated the considerable variability of the adrenergic system within urodele and anuran amphibians. Among amphibians, the interactions between the adrenergic system and immune responses were most extensively studied in *Xenopus laevis* (Clothier, *et al.*, 1992). Chemical sympatectomy by 6-hydroxydopamine prior to immunization decreased thymus-dependent but increased thymus-independent antibody responses in *Xenopus laevis*. Noradrenaline administration either increased or decreased antibody production depending on time of administration. The authors suggested that noradrenaline does not act directly on B cells but up-regulates T cell functions by affecting their maturation (Clothier, *et al.*, 1992). Experiments on immune-neuroendocrine interactions in amphibians conducted so far show enormous complexity in the underlying mechanisms, needing further elucidation.

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Chapter 24

Effects of Morphine on Acute Peritoneal Inflammation and Bacterial Clearance in Fish, Amphibians and Mice

Piotr Gruca[1,3], Alicja Jozkowicz[1], Magdalena Chadzinska[1], Joanna Mika[1,2], Szczepan Jozefowski[2], Barbara Przewlocka[2], Ryszard Przewlocki[2], Barbara Plytycz[1,3], Rolf Seljelid[3]

[1]Institute of Zoology
Jagiellonian University
Krakow, Poland

[2]Institute of Pharmacology
Polish Academy of Sciences
Krakow, Poland

[3]Institute of Medical Biology
University of Tromso, Norway

ABSTRACT

The effects of exogenous opioids, morphine and naltrexone, on the acute peritoneal inflammation and/or bacterial clearance have been examined in representatives of phylogenetically distant vertebrate species: fish (goldfish, *Carassius auratus* and salmon, *Salmo salar*), amphibians (yellow-bellied toads *Bombina variegata*, green frogs *Rana esculenta* and common frogs *R.temporaria*) and mammals (Swiss mice). Looking for a convenient model system we checked the number and activity of peritoneal leukocytes retrieved 2 days after i.p. injection of a sterile irritant (thioglicollate) with and without morphine; with or without pretreatment with naltrexone. Goldfish and salmons injected with a single dose of morphine had significantly lower number of peritoneal leukocytes than their saline-injected counterparts. The effect of morphine was completely anatagonized by pretreatment of goldfish with naltrexone. The number of elicited peritoneal cells was unaffected by an acute morphine treatment in *B.variegata* and *R. esculenta.* An increased number of elicited peritoneal cells ($p=0.064$) was induced by chronic morphine administration to *R.temporaria* froglets. Swiss mice had lowered number of peritoneal cells following a single dose of morphine. Morphine treatment caused the enhanced bacterial growth in the lymphoid organs of some animals with a moderate natural bacteriemia.

INTRODUCTION

An acute inflammation may be looked upon as a stress reaction with the evoking factor considered to be a stressor (Plytycz and Seljelid, 1995). Indeed, macrophages stimulated by microbial molecules or products of tissue injury release an array of "alarm" cytokines with pleiotropic effects (Seljelid and Eskeland, 1993). They are recognized both by other immunocytes leading to further steps of inflammation (Seljelid and Busund, 1994) as well as by the nervous and endocrine cells activating the hypothalamic-pituitary-adrenal "stress" axis as well as autonomous nervous system and endogenous opioids (Khansari *et al.*, 1990, Przewlocki, 1993). Immunocytes possess receptors for neurotransmitters and opioids and can synthesize some of these substances themselves (Blalock *et al.*,1985, Kavelaars *et al.*, 1990, Blalock, 1994). Well-orchestrated action of all components of the immune, nervous,and endocrine origin leads finaly to the removal of inflammatory stimuli and tissue clearance.

The details of intercellular communication during these reactions are studied mainly in the mammalian model species. However, all vertebrates possess all components of the hypothalamic-pituitary-adrenal axis, well developed autonomous nervous system, and, probably, at least some components of the opioid system (Faisal *et al.*, 1989, Clothier *et al.*, 1992, Saad and Plytycz, 1994). Among the so-called "lower" vertebrates we may very well be able to find the most convenient model organism(s) for studies on acute inflammation and its dependence on the nervous and endocrine factors.

The aim of this investigation was to study the effects of exogenous opioids injection on the elicited peritoneal inflammation and/or clearance of naturally occurring bacterial contaminations in representatives of mammals (mice), amphibians (toads, frogs), and fish (goldfish and salmon).

MATERIALS AND METHODS

Animals

Goldfish *Carassius auratus* (19 -27 g body weight) and salmons *Salmo salar* (35-60 g b.w.) were obtained from fish farms in Poland and Norway, respectively and kept at 22°C or 10°C, respectively. Amphibians: yellow-bellied toads *Bombina variegata* (5-7 g b.w.), froglets of the green frog *Rana esculenta* and common frog *Rana temporaria* (1.8 ± 3 g b.w.) were field-collected in Poland and kept in aquaria at 22°C. Swiss mice (17-39 g b.w.) were reared at the Institute of Pharmacology of the Polish Academy of Sciences.

Experimental protocols

Morphine hydrochloride (Nycomed) or sulphate (Polfa), or naltrexone hydrochloride (Sigma) were dissolved in physiological saline adjusted to a species-specific osmolarity. Injections were intraperitoneal (i.p.) or into the anuran dorsal lymph sac (l.s.).

Chronically treated froglets of *R. esculenta* and *R.temporaria* received l.s. morphine injections (10 mg/kg b.w.) every second day for 30 days at 4-7 PM.

Animals subjected to an acute morphine treatment received single i.p. injection of morphine (20 mg/kg b.w., and 10 or 20 mg/kg in the case of *Rana esculenta*) with or without pretreatment (20 min earlier) with i.p. (fish, mice) or l.s (amphibians) injection of a single dose of naltrexone (1 mg/kg b.w.). Control animals were injected with a respective volumes of physiological saline.

Acute inflammation

For a model of an acute inflammation, we used peritoneal inflammation elicited by a sterile irritant, thioglycollate medium (Oxoid Limited, London). Animals were injected intraperitoneally with thioglycollate medium (TG) with morphine (10 or 20 mg/kg b.w.) or with saline. Some of them were i.p. or l.s. injected 20 min earlier with naltrexone (1 mg/kg) or saline. Two days after injection the peritoneal leukocytes were collected in PBS adjusted to species-specific osmolarity supplemented with heparin (10 U/mL), counted in haemocytometer, and incubated in vitro for analysis of their activity.

Cell suspensions

Amphibian spleens and goldfish head kidneys were aseptically isolated, teased into cell suspensions, and incubated in 2 mL of PBS adjusted to goldfish, salmon or amphibian osmolarity (260 mOsm, 360 mOsm or 220 mOsm, respectively). Samples of supernatant fluid were used for the detection of live bacteria.

Detection of live bacteria

The number of bacteria living in organs (fish head kidneys and anuran spleens) were estimated by MTT reduction assay as described previously (Mika *et al.*, 1996). Five 10 µL samples of supernatant fluid from each organ was transferred to 96-well flat-bottomed microtiter plates filled with a 90 µL/well bacterial broth (Wytwornia Surowic i Szczepionek, Warszawa) and incubated overnight at room temperature 22°C. Wells with bacterial broth, supplemented with 10 µL of the sterile water served as a control of sterility. After overnight incubation the MTT reduction assay was performed. This assay developed by Mosmann (1983) relies on the conversion of the yellow tetrazolium salt (MTT) by dehydrogenases of viable bacteria to blue formazan. From MTT (Sigma) stock solution (5 mg/mL), 10 µL was added per each 100 µL well (final concentration 500 µg/mL) and plates were incubated further for 30 min. The optical density (O.D.) of the blue dye was determined spectrophotometrically on a Uniscan II at 570 nm 30 minutes after stopping the reaction with 50 µL of extraction buffer (Hansen *et al.*, 1989, slightly modified by Plytycz *et al.*, 1993).

Statistical analysis

The results were analysed using Statgraphics version 2.6 computer program. Samples were compared using Student`s t-test. Differences between mean values at $p<0.05$ were considered to be significant.

RESULTS

Figure 1 shows that the number of goldfish *C.auratus* peritoneal leukocytes was lower in the morphine-treated than in the control group. This effect was prevented by pretreatment of the animal with naltrexone although naltrexone itself had no significant effect on the peritoneal cell number (Figure 1a). The significant lowering of the number of TG-elicited peritoneal cells was also observed in salmon *S.salar* (Figure 1b).

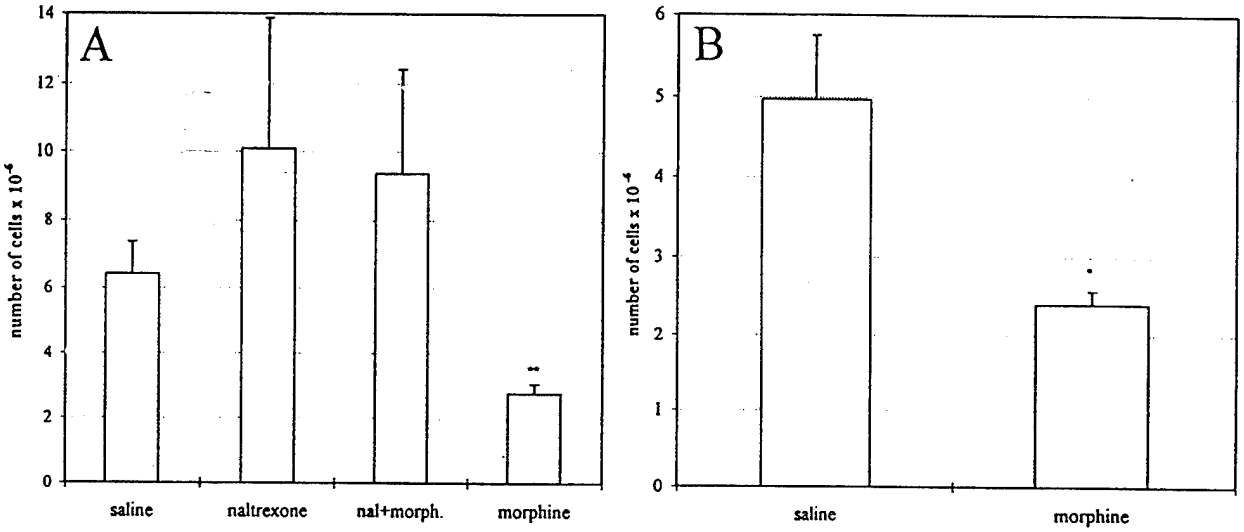


Figure 1. The effect of an acute opioid treatment on the number of thioglycollate-elicited peritoneal cells of fish: a) goldfish *C. auratus*; b) salmon *S.salar*. Asterisk indicates values statistically different from those of control group: * - $p<0.05$, ** - $p<0.01$.

An acute morphine/naltrexone treatment did not affect the number of elicited peritoneal cells of anurans, *B.variegata* and *R. esculenta* (Figure 2), while a chronic morphine treatment (every second day for one month) suggested some enhancement of number of the peritoneal leukocytes counted 2 days after irritant injection of *R.temporaria* and *R. esculenta* froglets ($p=0.064$ and $p=0.23$, respectively) (Figure 3).

Figure 4 shows the number of murine peritoneal cells which was significantly decreased in animals treated with morphine.

Figure 5 shows the effect of an acute dose of morphine on the occurrence of live bacteria in the head kidneys of goldfish. The results of three separate experiments demonstrate that morphine may exert a very profound enhancing effect, a moderate one, or no effect on head kidney bacterial contamination. Morphine induced enhanced bacteriemia in fish is demonstrated in the first and second experiment, while no such effect was visible in third experiment. Saline-injected control fish from

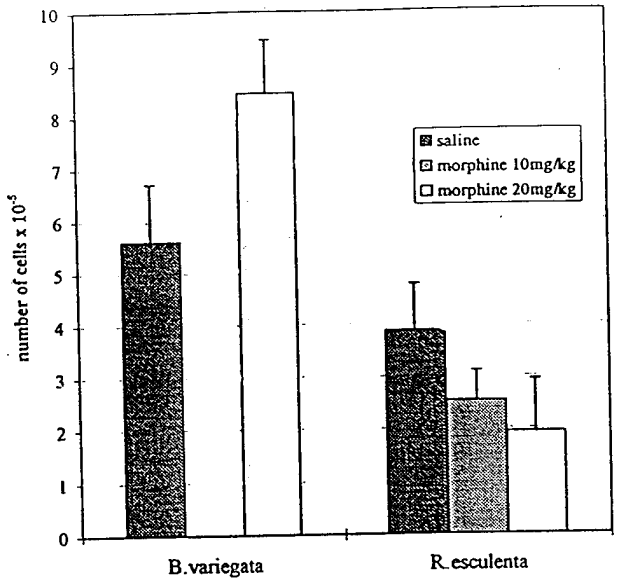


Figure 2. The effect of an acute opioid treatment on the number of thioglycollate-elicited peritoneal leukocytes in amphibians: yellow-bellied toads *B. variegata* and green frogs *R. esculenta*.

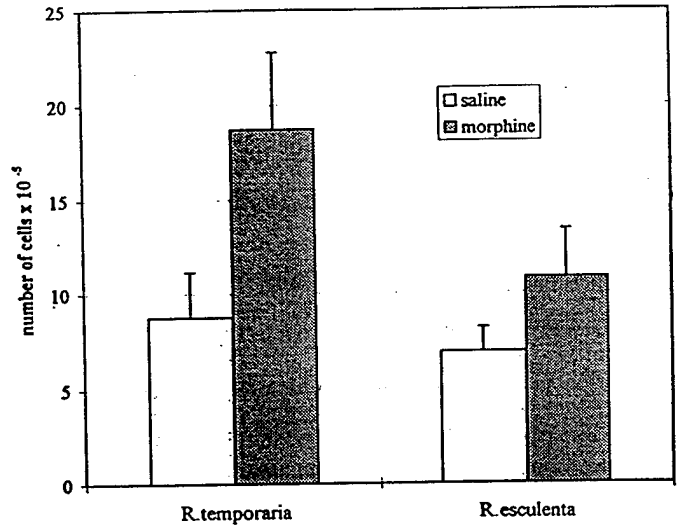


Figure 3. The effect of a chronic opioid treatment on the number of thioglycollate-elicited peritoneal leukocytes in amphibians: common frogs *R. temporaria*, and green frogs *R.esculenta*.

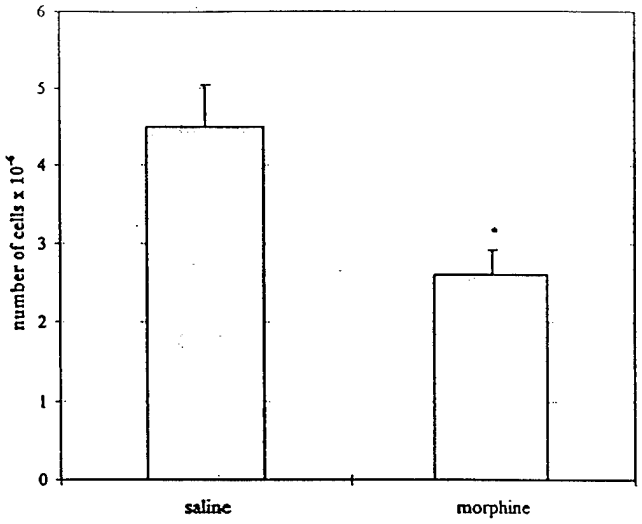


Figure 4. The effect of an acute morphine treatment on the number of thioglycollate-elicited peritoneal cells of Swiss mice. Asterisk indicates values statistically different from those of control value: * - $p<0.05$.

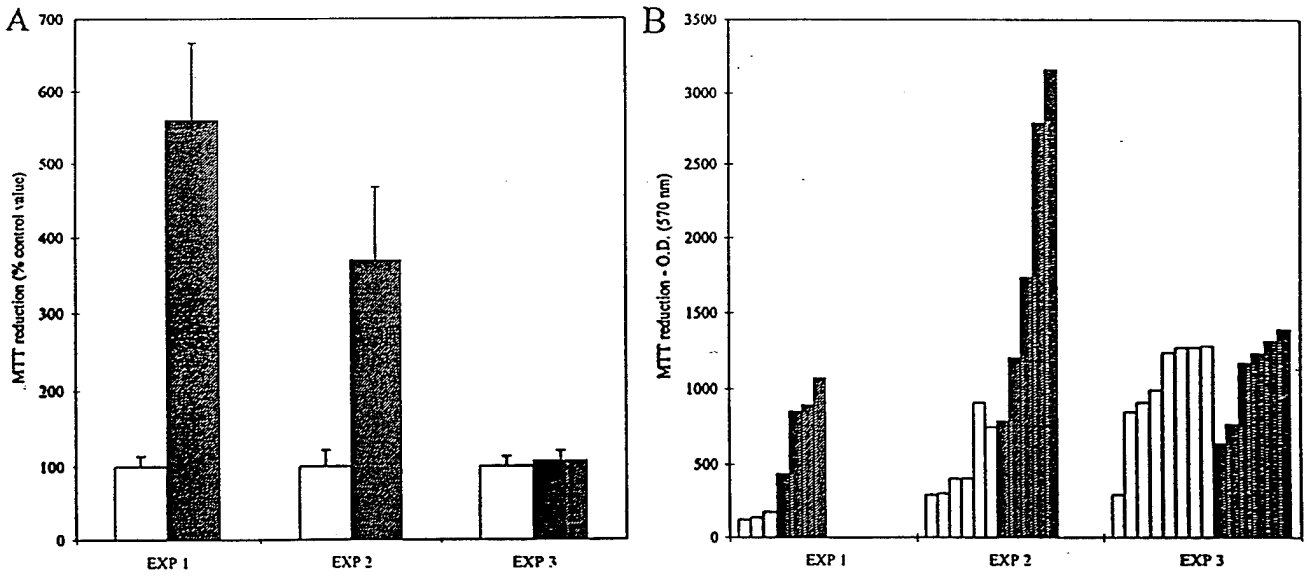


Figure 5. The effect of an acute morphine treatment on the survival of bacteria in the head kidney of goldfish estimated by MTT reduction assay. a) Percentage of the control values from saline-injected fish. Each value is the mean ± SE from 3-8 individuals; b) The results from individual organs.
❑ saline, ■ morphine

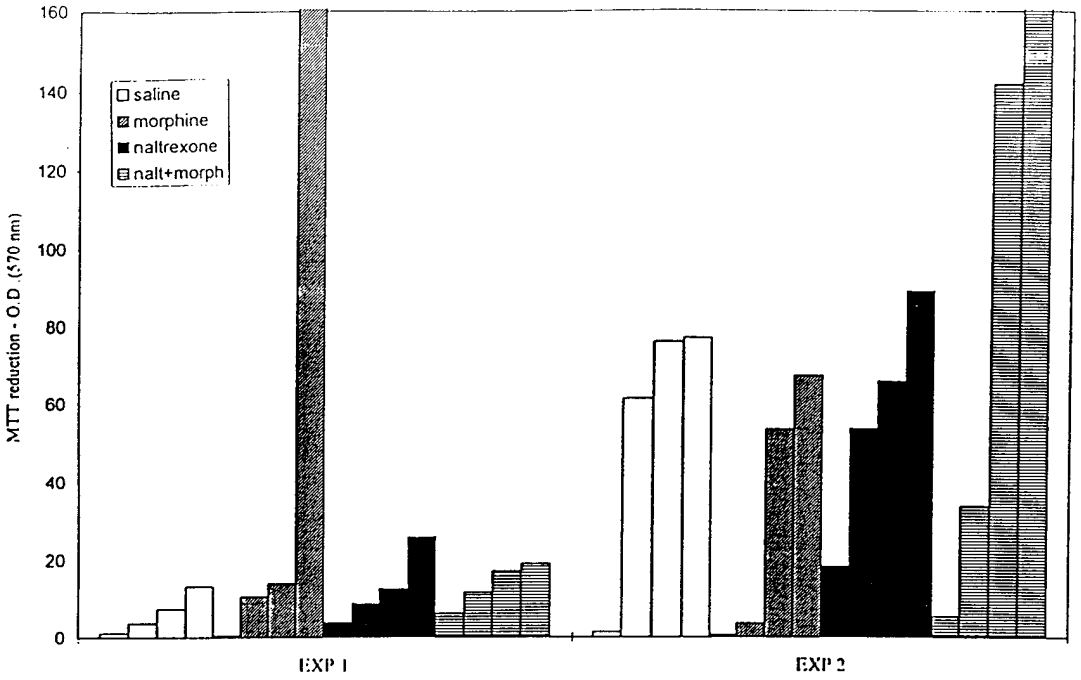


Figure 6. The effect of an acute opioid treatment on the survival of bacteria in the spleen of the yellow-bellied toad *B.variegata* estimated by MTT reduction assay. Results from individual organs.

the first experiment were relatively bacteria-free in comparison to control animals from experiments 2 and 3 (Figure 5b).

Figure 6 shows the effect of an acute dose of morphine and/or naltrexone on the occurrence of live bacteria in the spleens of yellow-bellied toads. In the first experimental series all but one animal was apparently bacteria-free while in the second experimental series, the majority of them were bacteria-rich. The opioid treatment had no effect in both experimental series.

DISCUSSION

The effects of morphine and/or naltrexone on the number of the elicited peritoneal leukocytes retrieved 2 days after intraperitoneal injection of the sterile inflammatory agents, thioglycollate, into the peritoneal cavities of representatives phylogenetically distant vertebrates: fish, amphibians, and mammals. When compared to control animals injected with irritants only, the number of elicited leukocytes was significantly lower in acute morphine-treated goldfish, salmon, and mice; unaffected in similarly treated toads and frogs, and higher (P=0.064) in chronically morphine-treated froglets of *R.temporaria*. It does not necessarily mean that there are three different species-specific patterns in response to the morphine treatment. This part of our ongoing experiments is focused on one time point only, namely the second day after injection. Our preliminary results suggest, that morphine treatment may cause different effects at various time points, e.g. we recorded increased cell numbers

24 hours after morphine-thioglycollate treatment of mice and goldfish, while on day 3 after injection the inhibitory effect of morphine seems to be even stronger than on day 2. Therefore among the possible explanations of the effects, it seems reasonable to consider the probable influence of morphine on kinetics of thioglycollate-elicited acute peritoneal inflammation.

We have observed the profound effect of ambient temperature on kinetics of an acute peritoneal inflammation in yellow-bellied toads *B.variegata* (Jozkowicz *et al.*, 1994) and on activities of fish and amphibian peritoneal macrophages assayed at different *in vitro* temperatures following *in vivo* acclimation to cold or to warmth (Plytycz and Jozkowicz, 1994). The number of elicited peritoneal leukocytes increases rapidly at both high and low temperature. However at room temperature (22°C) it returns to the control level within one week, while it persists over control for more than two weeks at 10°C. The temperature-related variability on the cell number is connected with cell composition and activity (Jozkowicz *et al.*, 1994). Studies on the cellular composition (using FACScan analysis) and *in vitro* activities of leukocytes collected from peritoneal cavities of opioid-treated animals are in progress.

Morphine, a well-known agonist of the opioid receptors, can affect several immune parameters (for review see Peterson *et al.*, 1993). In a classical system, effects of morphine is prevented by blocking of opioid receptors with naloxone or naltrexone (Adler *et al.*, 1993). In our experiments this classical pattern was detected only in the case of goldfish.

In mammals morphine reduces resistance to infections (Tubaro *et al.*, 1983). We have shown (Mika *et al.*, 1996) that the field-collected animals often bear live bacteria in lymphoid organs. In this study we compared the presence of live bacteria in fish head kindeys and anuran spleens in the control and morphine-treated animals. Figure 5 shows that an acute dose of morphine under certain conditions can have profound effects on preexisting bacterial flora in lymphoid organs of the investigated animals. Morphine can exacerbate infections in apparently healthy goldfish with a low preexisting bacteriemia, while opioids do not change bacteriemia in the heavily-infected fish and toads and they had no effect on the bacteria-poor specimens of *B.variegata*.

In conclusion, exogenous opioids may have a modulatory effects on the immune processes in vertebrates. Among ectothermic animals we find a model species, e.g. goldfish, for studies of mechanisms of the opioid-dependent immunomodulation.

Acknowledgements

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Chapter 25

Profiling Immunotoxicology: Past, Present, and Future

Loren D. Koller, DVM, PhD

College of Veterinary Medicine
Oregon State University
Corvallis, Oregon 97331

ABSTRACT

Immunotoxicology has experienced considerable growth and expansion since its inception in the early 1970's. During the 1970's and early 1980's, immunotoxic chemicals were identified, immunoassays were developed and refined, mechanisms of action investigated, and the immune system identified as the "target organ" for selected chemicals. Initial studies were conducted in the mouse while the rat was being pursued as an appropriate animal model. The impact of toxic substances on the human immune system was virtually unknown in the early era of immunotoxicology but has gained substantial momentum during the past ten years. *in vitro* techniques were initially used to better understand mechanisms of action but have recently been expanded to screen chemicals and evaluate human cells and tissues for the immunomodulating effects of xenobiotics. More recently, it has been proposed that biologic markers of exposure, effect, and susceptibility be identified for immunotoxicology. These immune markers could have application in quantitative human health risk assessment. Immunotoxicology is presently a widely diversified discipline that transcends several fields. The future of immunotoxicology will become more complex which will require continued training of new scientists and acquiring the necessary information to bridge the chasm between basic and applied research in order that immunotoxicology data can be appropriately utilized for human health risk assessment activities.

INTRODUCTION

Immunotoxicology originated in the early 1970's when immunologists and/or toxicologists began investigating the immunotoxic potential of prominent environmental chemicals. Some of the investigators who published in this area in the 1970's and remained active as the discipline progressed are recognized in the following references (Gainer, 1972; Vos and DeRoij, 1972; Koller, 1973; Street and Sharma, 1975; Levy *et al.*, Munson, 1975; Exon *et al.*, 1976; Loose and DiLuzo, 1976; Faith and Moore, 1977; Kerkvliet and Kimeldorf, 1977; Luster *et al.*, 1978; Silkworth and Loose, 1978; Thomas and Hinsdill, 1978; Lawrence *et al.*, 1978; Dean *et al.*, 1979, Hinton *et al.*, 1979). From these and other early studies it became apparent that chemicals known to be ubiquitous in the environment could compromise immunity in animals. Thus, the stage was set for a new discipline, presently known as immunotoxicology. The adverse immune effects exerted by chemicals were confirmed when exposure to these agents resulted in increased susceptibility of the host to infectious agents. These initial discoveries provided the foundation for a series of events

which today have established immunotoxicology as a prominent discipline that is shared by immunopharmacology.

After it was recognized that chemicals could modulate immunity, conventional immunoassays were adapted to assess chemical-induced immune dysfunction. These studies generally included evaluation of the humoral- and cell-mediated immune responses as well as macrophage activity. In addition, animals exposed to chemicals for prolonged periods were frequently challenged with an LD_{50} dose of an infectious agent to assess for natural resistance to infectious disease.

During 1970's and early 1980's, a number of immunoassays were developed and adapted to test for immunotoxicity of chemical agents. Some of those procedures included responses to mitogens, antibody plaque-forming cells (PFC), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), delayed-type hypersensitivity (DTH), mixed lymphocyte reaction, lymphocyte cytotoxicity, helper T/suppressor T cell ratios, NK cell cytotoxicity, bone marrow progenitor cells differentials, cytokine activity, and several host-resistant assays. The discovery of monoclonal antibodies stimulated renewed interest in flow cytometry and immunocytochemistry. Many of those procedures have been validated as acceptable assays to assess chemical-induced immune dysfunction.

Immunotoxicology gained credibility when it was confirmed that some chemicals such as toxaphene (Allen *et al.,* 1982), pentachlorophenol (Kerkvliet *et al.,* 1982), lead and polychlorinated biphenyls (Koller *et al.,* 1983) produced immunosuppression at dosages lower than those that altered other known and commonly used toxicological indices. This feature revealed that the immune system was indeed a sensitive indicator to detect perturbation by chemicals and that the ensuing immunosuppression could not only render an animal susceptible to infectious agents, but could also potentially cause an increased risk of cancer. There is substantial evidence that immunocompromised individuals are highly susceptible to some forms of cancer. This is exemplified by Kaposis sarcoma, which occurs in patients with Acquired Immune Deficiency Syndrome, AIDS; (Safai *et al.,* 1985).

Most chemicals target a specific organ within the body. Toxicologists, pathologists, and clinicians have used this characteristic to diagnose, treat, and ascertain the biological mechanism by which a chemical produces toxicosis. Thus, the symptoms and pathology associated with a chemical that is primarily hepatotoxic are expressed in those parameters used to detect injury to the liver. The immune system, on the other hand, is distributed throughout the body in cells, tissues, organs, and the circulating blood and lymph. Therefore, different components of the immune system can be exposed to the parent chemical and/or its metabolite(s) systemically at different sites throughout the body. This factor in itself acknowledges the immune system as a highly sensitive organ for detecting toxicosis. Compromised immune function could be life-threatening, as are many other manifestations of toxicosis.

The impact of toxic substances on human immune function was virtually unknown in the 1970's and early 1980's. There was, however, documented evidence that exposure to chemicals such as the polychlorinated and polybrominated biphenyls and lead suppressed immune responses in man (Bekesi *et al.,* 1978; Sachs, 1978; Bekesi:*et al.,* 1983, Lui and Wong, 1984; Ewers *et al.,* 1984). The continued development of new clinical immunological technology to assess immune function

in man has facilitated accumulation of data to evaluate chemical-induced immune dysfunction in humans.

Mechanisms OF Immunotoxicology

Immunotoxicology is the study of injury to the immune system that can result from occupational, inadvertent, or therapeutic exposure to a variety of environmental chemicals or biologic materials (National Research Council, 1992). The founding years of the discipline were devoted primarily to identifying immunotoxic chemicals and to developing a battery of sensitive, quantitative immunoassays. However, much of the research during the past several years has concentrated on investigating the mechanisms by which immunotoxic chemicals compromise immune function.

Because optimum performance of the immune system is dependent upon a cascade of immune events, disruption of any one of the components of this circuit can alter the immune response. Interference in the early stages of the immune sequence would be suspected to result in a generalized effect on immune function. However, occasionally a chemical will elicit a more selective response within this chain of events. This can be attributed to the chemical exerting a more specific effect directly upon an immunocyte or upon a particular subset of cells. Thus, the immunomodulating response may range from highly selective to generalized. These features account, at least in part, for the lack of a typical linear dose response normally observed in other standard toxicological procedures.

The mechanisms of immunity are extremely complex. A complete immunological paradigm would include assessment of several compartments of the immune response which are inclusive of humoral and cell-mediated immunity, macrophage activity, NK cell cytotoxicity, cytokine production/activity, and other active components of the immune system. Chemicals can compromise one or several reactive sites within the immune network. The actual mechanistic effects, however, may occur by altering internal cell structures, membranes, surface antigens, and/or a variety of receptors. Chemicals may alter the composition of these structures, bind to or block their activity, or interfere with numerous putative non-immune regulators required for activation, differentiation, proliferation, and normal development of immune responsiveness.

Assessment OF Immunotoxicity - Biologic Markers

A myriad of immunoassays were initially developed to assess the integrity of the immune system both *in vivo* and *in vitro*. Most immunotoxicologists agreed that a panel of immunoassays should include procedures to assess humoral immunity, cell-mediated immunity, macrophage function, pathotoxicologic examination of lymphoid tissues, and host resistance to an infectious and/or oncogenic agent. This panel was later expanded to include NK cell cytotoxicity and production/activity of numerous regulatory cytokines.

Several laboratories organized a multitiered approach to evaluate the immunotoxic potential of xenobiotics. The initial tier generally included nonspecific but quantitative procedures to assess the major compartments of the immune system. Other tiers were designed to specifically evaluate compounds identified as immunotoxic in tier one in greater detail by investigating compartments

of the immune response which affected by the test chemical. Thus, a comprehensive immune profile could be characterized for each positive agent tested.

The National Academy of Science, National Research Council, formed a Subcommittee in 1989 to review immunotoxicology. The result of that subcommittee's effort was preparation of a document, "Biologic Markers in Immunotoxicology" that was published by the National Academy Press in 1992. The text includes immune-mediated disease (hypersensitivity), autoimmune disease, biologic markers of immunosuppression, animal models, methods of detecting immunotoxicants, biologic markers for immunotoxicity of humans, applications in epidemiology, and areas of controversy. It was determined that immunotoxicology has rapidly expanded from fundamental to applied research with considerable interest in its application in developing health standards and permissible levels for human exposure to xenobiotics. It was also pointed out that the interaction between the nervous, endocrine, and immune systems makes it virtually impossible to duplicate these systems *in vitro*, and thus, intact animal systems are essential to assess the immunotoxic potential of xenobiotics for human health risks.

The review focused on biologic markers of immunotoxicology. Biologic markers are indicators of events in biological systems, i.e., variations in the number, structure, or function of cellular or biological components. A biologic marker of exposure is the ability to measure a chemical in a biologic specimen; ie., urine, blood, tissue, organs, hair, etc. Biologic markers of effect quantitatively assess cellular or biochemical alterations within an organism that can be associated with changes in the status of health. Immunological markers of effect can be any detectable change within the immune system and changes in other tissues resulting from immune-mediated dysfunction. A biologic marker of susceptibility is an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic; i.e., differences in individuals over time or between individuals and populations. Biomarkers of immunity must be able to recognize exposure, predict species susceptibility to exposure, and/or identify biologic effects or disease as a result of exposure to a xenobiotic. Methods to measure biological markers must be sensitive and specific to have value in predicting health risks and pending disease.

The subcommittee concluded that several assays have been validated in animals to detect immunomodulation by xenobiotics. Further, a large body of immunotoxicological information has been catalogued for many chemicals using animal models. On the other hand, a paucity of immunotoxicological data is available in humans who have been exposed to environmental substances. Nevertheless, a battery of tests currently exist to assess, to a degree, immune competence in humans who have been exposed to known or suspected immunotoxicants. Finally, development of biological markers as surrogates for human disease will better characterize the immunotoxic potential of xenobiotics in humans.

MODELS OF IMMUNOTOXICITY

Unlike most target organs that are confined anatomically within a host, the immune system is dispersed throughout the body and can be exposed to xenobiotics at many locations and during various stages of metabolism. The immune system promises to be one of the most sensitive biological systems for detecting adverse reactions resulting from exposure to drugs and toxic

substances. The difficulty in testing for chemically-induced immune dysfunction in the past has limited the knowledge available as to the immunotoxic potential of a multitude of environmental pollutants. Nevertheless, a wealth of information is available from animal studies to suggest that many chemicals found in the environment could be putative immunotoxins for humans.

Advances in biotechnology and animal models have improved the process of interpreting and extrapolating data from laboratory animals to man. Many of the immune procedures utilized for immunotoxicology are reproducible, quantitative, and highly sensitive in detecting chemical-induced immune dysregulation. It is universally accepted that the immune systems of some animals and man are comparable; animal models are available to objectively assess immune dysfunction, positive immunosuppressants such as cyclophosphamide and corticosteroids are used t; validate assays, and that data obtained in animals have been validated in humans. Although the principles and phenomena in humans and animals are basically similar and comparable, it is recognized that variations in these responses can occur between various species.

Animal models have been used successfully during this century to develop methods of diagnosis, prevention, and treatment of human disease. Two laboratory animals, the mouse (Buck *et al.*, 1985, Vos *et al.*, 1994) and the rat (Exon *et al.*, 1984, 1985, 1990, Vos *et al.*, 1994) have been proposed as animal models for assessing xenobiotic-induced immunomodulation. The primary advantages of the mouse are that the immune system has been described in great detail and many inbred stains are available. Multiple immunoassays can be performed using a single mouse, but the small size of the animal limits the number of tests and amounts of sample that can be collected per animal.

The rat model can be used to evaluate multiple, concomitant immune compartments within an individual animal (Exon *et al.*, 1984, 1985, 1990). The multiple parameters of immunity assessed in each rat include humoral immunity (ELISA), cell-mediated immunity (DTH), NK cell cytotoxicity, and the production of three potent regulatory immunocytokines: macrophage-derived IL1, PGE2, and lymphocyte-derived IL2. The multi-assay single-animal approach represents an economical, versatile, sensitive, and relatively comprehensive paradigm for assessing the immunotoxicological properties of xenobiotics. This model minimizes animal-to-animal and day-to-day variation, reduces experimental and comparative error, is sensitive to detect immunosuppression as well as immunoenhancement and can simultaneously test the major types of immune responses, immunocyte populations, and immunoregulatory pathways. Another advantage of utilizing the rat is that almost all pharmacological/toxicological data are amassed for this species, permitting comparison of systems to ascertain the relative sensitivity of different body functions to the test agent.

In vitro IMMUNOTOXICOLOGY

In vitro technology has been used for decades to better understand "mechanisms of action". Renewed interest in the application of *in vitro* technology has occurred in concert with the "animal rights" movement and by scientists who are interested in reducing animal numbers that have been used conventionally in drug experiments. Although there are biologic complexities that basically make it impossible to duplicate *in vivo* immune responses *in vitro*, *in vitro* studies may be used to initially "screen" xenobiotics for immunotoxic effects in addition to being extremely useful in

dissecting and manipulating immune responses that occur *in vivo*. Furthermore human cells and tissues can be obtained, cultured, and evaluated directly (*in vivo* exposure) or indirectly (*in vivo* exposure/*in vitro* testing) for the immunomodulating effects of xenobiotics. These exposures could occur voluntarily, occupationally, environmentally, or accidentally. *in vitro* toxicology is advancing at a rapid pace.

It is not uncommon to apply *in vitro* immunoassays upon cells and tissues that have been exposed to a xenobiotic *in vivo*. Thus, the xenobiotic has been "systemically processed" whereby it has been "naturally" absorbed, distributed, metabolized (if required) and excreted. During this process, the encompassing components of the immune system, if involved, have been activated to proceed through a complex chain of events. Many of these reactions can be measured at various points by *in vitro* technology.

Removal of tissue and cells for *in vitro* exposure has limitations. The chemical has not been "processed" *in vivo*, the cascade of immune events can not be duplicated *in vitro*, nor are the interactions between the immune, endocrine, and central nervous systems in place. Thus, a given drug/chemical must be administered to an intact host and properly "processed" to ultimately obtain an accurate immunotoxic profile for that drug/chemical. Measuring immune function is essential and critical in order to interpret and relate data from animals to humans and when correlating immune competence to disease resistance. During this process, however, it must be kept in mind that many immune procedures are available that do not merit the criteria to be classified as functional; i.e, represent some type of action that correlates with innate or naturally acquired disease resistance.

HUMAN IMMUNOTOXICOLOGY

Several procedures are currently available to assess humoral and cellular as well as nonspecific immunity in humans (NRC, 1992; Shearer *et al.*, 1994). In the 1970's and early 1980's, very few studies concerning the immuntoxicity of xenobiotics for humans were reported. This was in part due to little interest in the subject, the lack of appropriate non-invasive immune procedures coupled with inability to accurately evaluate results, and the question of how to interpret "positive" results. However, in the past 10 years, there has been a proliferation of human investigations and case reports appearing in the literature. Many of these studies have supported animal data while others have raised questions. In addition to studies that have included subjects exposed to xenobiotics either occupationally, environmentally, or by accident, data is being accumulated on human cells and tissues that are exposed to chemicals *in vitro* and are compared with animal data. Nevertheless, there remains a need to design well-delineated human epidemiological studies, either cross sectional, retrospective, or prospective, to better understand the immunotoxic potential of xenobiotics, either actively or passively, in humans.

RISK ASSESSMENT

Quantitative health assessments include hazard identification, exposure-dose-response assessment, exposure assessment, and risk characterization. This information forms the basis of risk assessment, risk management, and risk characterization. There are gaps or deficiencies in many data end points, many methods and models have not been validated, uncertainly factors are often

applied universally "across the board", variability is frequently not considered in calculating individual and population risk, and chemical mixtures and/or exposure via multiple pathways needs to be addressed. Application of credible scientific principles and statistical methods are essential ingredients for developing meaningful estimates of likely human health effects. Appropriate dose/response relationships, extrapolation of dose, estimation of exposure, assessment of safety/risk, *in vivo* exposures simulating human exposure, use of validated *in vivo/in vitro* assays, using "functional and/or diagnostic" sensitive and predictive measures, determining "what is an adverse health effect", and establishing the "no observable adverse effect level" (or benchmark dose) in the spirit of good laboratory practices are a few of the many parameters that are desired (required) to develop satisfactory assessments of risk associated with human exposure to xenobiotics.

Immunotoxicology has been identified to have a role in the application of risk assessment (Dean *et al.*, 1994, Luster *et al.*, 1994b). Utilizing immunotoxicity data as a part of evaluating drugs, chemicals and biologics for human risk assessment has recently come to the fore front of science. For example, the Environmental Protection Agency (EPA) and Agency for Toxic Substances and Disease Registry (ATSDR) have used immunotoxicity data in establishing "reference doses" and "minimum risk levels", respectively. Immunotoxicology presently meets several of the criteria required for optimum risk assessment analysis while future research will no doubt improve the application of immunotoxicology for human health risk assessment. Nevertheless, immunotoxicology could selectively be the most sensitive endpoint for a given chemical thereby qualifying as the adverse health effect chosen for risk assessment purposes. Considerable interest will be devoted to this aspect of immunotoxicology in the immediate future.

CURRENT AND FUTURE RESEARCH

Research in immunotoxicology progressed steadily during the 1970's and early 1980's. With more scientists entering the field during the past decade, coupled with advancement in technology, the discipline is rapidly expanding. A cross-section of topics presented at the 1995 Society of Toxicology meeting represents a spectrum of diverse research activities presently being pursued by a cadrae of scientists.

One symposium focused on "Second Messengers" discussing signal transduction, redox regulation, calcium homeostasis and adenylate cyclase. A second session was devoted to interactions between immune and non-immune cells such as glial cells, keratinocytes, hepatocytes, and endothelial cells. A platform season was devoted to mechanisms of immunotoxicity discussing such topics as nitric acid and nitrate production, reactive oxygen, nitrogen species, and apoptosis. A poster discussion session focused on cytokines in inflammatory and immunotoxic responses. This session included topics on cytokine toxicity and gene expression, chemotactic factors, tumor necrosis factor, DNA binding, interleukins 2 and 6, and interferon. Poster sessions were titled "Models and Mechanisms of Immunotoxicology" and "Hazard Identification and Immunomodulation". Topics presented were aquatic models, biomarkers in fish, flow cytometry, mouse intranasal models, systemic anaphylaxis model, interlaboratory validation of an ELISA, DNA signal amplifications, SCID mouse models, the local lymph node assay, interleukin 4 and interferon, eosinophils and platelets, humoral immune response, Kupffer cells, contact hypersensitivity, host resistance, cytotoxicity,

developing immune system, receptors, neuro-endocrine factors, natural killer cells, cytokine mRNA, growth factors, adjuvants, thymocyte abnormalities, immunophenotyping, anaphylaxis, and precursor cells.

These diverse research activities are representative of studies that are contributing to the future of immunotoxicology. New methods of technology will be developed and adapted, additional assays validated, mechanisms of action pursued, *in vitro* procedures developed and compared to *in vivo* responses, models (animal, immune reactions, etc) refined and developed, human data obtained to support/dispute animal data, identification of biologic markers of exposure, effect, and susceptibility, and interactions between cells and chemicals. Considerable attention will be devoted to adapting immunotoxicology for human risk assessment and identifying those xenobiotics in which the immune system is the primary target organ. Current interest in exposure to chemical mixtures, sensitivity of the young (developing immune system) and the effect of other confounding environmental factors (pathogens, etc) will carry over into immunotoxicology. Transgenic animals, computerized image analysis, and polymerase chain reaction as well as other emerging technology have promise in advancing our knowledge of cellular and molecular biology in immunotoxicology. Further, in order to bridge the gap between basic (mechanistic) and applied (risk assessment) research, measuring "immune function" is essential and critical in order to interpret and translate data from animals and/or *in vitro* studies to humans and to ascertain the relationship between immune competence and disease resistance. One must recognize that some procedures do not meet the criteria to be classified as functional; i.e, represent some type of "action" that correlates with innate or naturally acquired disease resistance. Finally, training of new scientists, promotion of multidiscipline research, and organizing immunotoxicological response teams to gather epidemiological data from human exposures will assure advancement of the state of immunotoxicology.

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Chapter 26

Approaches to Immunotoxicology Testing: A Brief Overview

Peter T. Thomas and Michael I. Luster[1]

Head, Immunotoxicology
Life Sciences Dept.
IIT Research Institute, Chicago, IL

[1]Environmental Immunology and Neurobiology Section
National Institutes of Environmental Health Sciences
Research Triangle Park, NC

ABSTRACT

Over the last 20 years, evidence has accumulated demonstrating that the immune system is a sensitive target organ for toxicity resulting from exposure to drugs, or chemicals of environmental concern. This evidence prompted the Government to initiate an effort lead by the National Toxicology Program to develop and validate a panel of tests in rodents designed to screen compounds for potential immunotoxicity. Over the past decade, this testing approach has produced a database which exceeds 50 compounds. Furthermore, this protocol is proving to be a scientifically valid approach for predicting immunotoxicity.

REVIEW

The immune system comprises a complex set of cellular and biochemical components which serve to recognize and protect the body against foreign materials. It is unique in that it must perform this task without responding adversely to self. The distinction between self and non-self is accomplished by means of an elaborate recognition system depending on specific receptor molecules associated with the surface of all cells in the body; including those of the immune system. Immunotoxicology is a relatively new science concerned with understanding the potential deleterious effects of chemical xenobiotics on the immune system. Potential adverse effects can range from immunosuppression (leading to an increased risk of infection and tumor growth) to immunoenhancement (an increased risk of allergy and autoimmunity).

There is considerable evidence from laboratory animal studies that drug or chemical exposure produces specific and injurious effects on the immune system (Murray and Thomas, 1992). In addition to concerns about inadvertent chemically-induced modification of the immune system, the pharmaceutical and biotechnology industries are placing increased emphasis on the development of selective modulators of immune function for the treatment of disease. Consequently, there has

been an increase in immunologic data submitted in support of Investigational New Drug (IND) applications to the Food and Drug Administration (FDA).

Methods for assessing potential immune suppression in experimental animals may be classified into three general categories. These methods are commonly used in conjunction with subacute (14-28 day) or subchronic (90 day) exposure to the test material. One set of methods focuses on evaluating toxicologic pathology parameters associated with the immune system including body and lymphoid organ weights, CBC and differential counts as well as histopathology. A second category of methods utilize cell culture techniques to evaluate functional competence of immune cells. The third category of tests evaluate the immune system in total by evaluating the host response to infectious or tumor challenge. Due to the redundancy in the immune system, animal models of host resistance are particularly relevant for immunologic risk and safety assessment.

The growing body of evidence that the immune system was an increasingly important target organ for toxicity prompted the National Toxicology Program (NTP) to conduct an interlaboratory validation in which this laboratory participated. Initiated in the early 1980s, the goal of this exercise was to study a battery of immunological screening tests to supplement the routine carcinogenicity and toxicity testing conducted by the NTP. Due to the wide variety of tests that could be utilized and the potentially large number of immunotoxic compounds, an initial tier approach for immunotoxicity assessment (Table 1) was developed (Luster, *et al.*, 1988). For the most part, the assays resemble those used clinically for diagnosis of immunodeficiency disease. Further validation was required, however, to determine whether or not those tests ultimately selected for a screening panel were sensitive and comprehensive enough to detect the more subtle immunologic alterations that could occur after chemical exposure. Recently, an interlaboratory validation effort using similar tests has been performed in the rat model (White, *et al.*, 1994).

Since the most relevant end point for immune dysfunction is altered host resistance (e.g., to bacteria, viruses, parasites, or tumor cells), emphasis was placed on developing sensitive infectivity models for detecting altered host resistance and correlating immune aberrations with specific host resistance assays. Many different host resistance models were evaluated during development and validation of this testing scheme. Initial criteria for consideration included (1) relevance to human disease, (2) knowledge of pathogenesis or mechanisms of protection, (3) availability of a quantifiable end point, and (4) biohazard safety.

The assays in the initial first tier were selected because they are relatively sensitive, reproducible, and measure defined aspects of the immune response. For example, after experimental immunization, the antibody plaque-forming cell (PFC) assay measures the production of specific antibody by quantitating the numbers of antibody-producing cells in the spleen. The antigen used is sheep erythrocytes, which induce an immune response through cooperation with different accessory cells in addition to B-cells. T-lymphocytes and macrophages, in addition to playing a key role in antigen recognition, produce soluble factors required in maturation of B-cells to antigen-producing plasma cells. On the other hand, the mixed lymphocyte culture (MLR) response is a widely used *in vitro* clinical test to measure cell-mediated immunity (CMI). The MLR evaluates the same mediators that are involved in graft versus host disease and graft rejection in humans.

The second tier of assays in this original protocol represent a more comprehensive evaluation of immunity. The rationale for proceeding with these more mechanistically-based tests includes: evidence of immune effects in the absence of significant toxicity, evidence that these changes occur at relevant dose levels and, ideally, an observable dose response. In the case of pharmaceuticals, these criteria must be interpreted in the context of the anticipated therapeutic dose. With respect to chemicals of occupational or environmental significance, these would relate to potential human exposure levels. The *in vitro* assays proposed for the second tier probe more specific effector cell functions. By contrast, the *in vivo* host resistance models evaluate the overall state of the immune system. The choice of host resistance model to use is dependent upon several factors including the particular exposure route for the compound and/or immunological profiles already known.

At the present time, the database generated from studies utilizing assays in this tier encompasses over 50 compounds. The data indicate that the performance of only 2-3 immune tests are sufficient to predict potential immunotoxicity in the mouse model. As a result, the Tier utilized by the NTP was recently revised to reflect this (Table 1). The highest association with immunotoxicity was noted when either the antibody PFC assay or lymphocyte surface marker analyses were performed (Luster *et al.*, 1992). While compounds that are known or suspected carcinogens were often immunotoxic, over 100 compounds generally regarded as safe and widely used as food additives were not positive in this tier as expected (Gaworski *et al.*, 1994). Details concerning the development, validation and utility of this testing protocol are described in detail elsewhere (Luster *et al.*, 1988, 1992, 1993).

Certain limitations are inherent in any screening approach. Since animals are usually evaluated at a single time point, there is no opportunity to determine persistence or recovery of effects. Furthermore, examination of the relationship between antigenic or infectious agent challenge and chemical exposure is not possible. Nevertheless, the panel provides a fairly in-depth evaluation for potential immunotoxic compounds that can provide valuable information for risk assessment. The schemes outlined in Table 1 represents one approach to evaluate xenobiotics that have unknown potential for immunotoxicity. When developing pharmaceutical safety assessment protocols, additional information is usually available concerning the pharmacodynamics, pharmacokinetics, and mechanisms of action of the drug candidate. In such instances, a tier testing scheme employing standardized tests such as the one described above may be inappropriate.

In addition to approaches developed by the NTP, other useful protocols for assessment of immunotoxicity have been proposed. These protocols, in some cases, utilize the rat model (Van Loveren and Vos, 1989). From a regulatory perspective, various governmental agencies are requiring such data or are in the process of determining whether immunotoxicological end points should be included in the routine preclinical toxicology testing requirements under Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Toxic Substances Control Act (TSCA) or as part of an FDA Investigational New Drug submission (Sjoblad, 1988; Hoyle and Cooper, 1990; Hinton, *et al.*, 1992).

Table 1
Immunotoxicology Testing Panels

Original	Revised
TIER I Hematology Weights - Body, spleen, thymus, kidney, liver Cellularity - Spleen, bone marrow Histology of lymphoid organ IgM antibody plaque-forming cells (PFCs) Lymphocyte blastogenesis T cell mitogens (PHA, Con A) T cell(mixed leukocyte response MLR) B cell (lipopolysaccharide, LPS) Natural killer (NK) cell activity	**TIER I** Hematology Weights - Body, spleen, thymus, kidney, liver Cellularity - Spleen, bone marrow Histology of lymphoid organ IgM antibody plaque-forming cells (PFCs) Quantitation of splenic B and T lymphocytes (surface markers)
TIER II Quantitation of splenic B and T lymphocytes (surface markers) Enumeration of IgG antibody PFC response Cytotoxic T lymphocyte (CTL) cytolysis or delayed hypersensitivity response (DHR) Cytotoxic T lymphocyte (CTL) cytolysis or delayed Host resistance Syngeneic tumor cells PYB6 sarcoma (tumor incidence) B16F10 melanoma (lung burden) Bacterial models *Listeria monocytogenes* (morbidity) *Streptococcus species* (morbidity) Viral models Influenza (morbidity) Parasite models *Plasmodium yoelii* (parasitemia)	**TIER II** Natural killer (NK) cell activity Enumeration of IgG antibody PFC response Mixed leukocyte response (MLR) Host resistance models Reticoloendothelial System (RES) Clearance

SUMMARY

Immunotoxicology is a relatively new science that can be defined as the study of the consequences of exposure to drugs, chemicals or environmental toxicants on the structure and function of the immune system. The need to design environmental and drug safety studies to evaluate potential toxicity to the immune system has prompted the development of several comprehensive testing approaches. Once such approach developed and validated by the National Toxicology Program has accumulated a large drug and chemical database and is proving to be a scientifically valid protocol for predicting immunotoxicity.

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Chapter 27

Comparative Immunotoxicology and Risk Assessment

Dr. Maurice Zeeman

U.S. Environmental Protection Agency
Office of Pollution Prevention & Toxics (7403)
401 M St., S.W.
Washington, D.C. 20460

ABSTRACT

In 1983, the U.S. National Research Council (NRC) proposed a framework for risk assessment and recommended that U.S. federal agencies develop guidelines on how risk assessment is done. The U.S. Environmental Protection Agency (EPA) has been involved in the development and publication of specific risk assessment guidelines since the mid-1980's. The focus of those guidelines has primarily been on specific human health issues, such as cancer or developmental effects, or on other aspects of risk assessment, such as exposure assessment.

Non-cancer risk assessment endpoints, such as immunotoxicity, appear to be becoming more important in the assessment of chemicals. U.S. federal agencies, international organizations, and others have been developing various forms of background guidance documents on immunotoxicology test methods and on the use of such immunotoxicity results for chemical risk assessment purposes.

The importance of immunotoxicity as a potential non-cancer endpoint for assessing the risks of chemicals or other stressors to humans and other organisms in the environment will increase as reliable and meaningful immunotoxicity test methodologies and immunotoxicology risk assessment guidelines are developed. The development and use of a standardized approach to comparative immunotoxicology and the test species selected could allow for a more accurate and meaningful determination of the relative sensitivity of immunotoxicity endpoints versus the other toxicity endpoints used in risk assessment. Dioxin (2,3,7,8-TCDD) is one recent example where immunotoxicity may be a more sensitive endpoint than cancer (carcinogenicity). Therefore, immunotoxicity could play an important role in the risk assessment of this environmental contaminant and perhaps become more significant in the evaluation of other chemicals undergoing risk assessment.

INTRODUCTION

In the past, the risk assessment of chemicals for human health purposes often focused primarily upon one test endpoint, cancer. The results of carcinogenicity testing in only a few rodent species, along with some conservative assumptions, were used to extrapolate such test results to the one species of interest, humans. Non-cancer endpoints, such as immunotoxicity, neurotoxicity and, developmental toxicity often seemed to end up not being very critical to the risk assessment of chemicals to humans.

However, the significance of and the sensitivity seen in the intimate and complex feed-back relationships between the immune and the neuroendocrine systems is starting to be appreciated (Blalock, 1994; Fuchs and Sanders, 1994). The impacts of some common chemical contaminants in the environment on these two different sets of systems have resulted in recent calls for using a more comparative toxicology approach to adequately assess the risks of chemicals (Colburn and Clements, 1992).

A more thorough comparative toxicology approach should help us in appreciating the significance of "The Wildlife/Human Connection" in the assessment of the long-term risks of synthetic chemicals to the endocrine and to the immune systems of humans and other organisms (Colborn, 1994). That this comparative toxicology approach is important to wildife and that we should pay attention to it is further evidenced by a recent report that persistant chemical contaminants found in fish in the Baltic Sea suppress the immune systems of seals in this area, and that this immunosuppressive mechanism could be readily related to the recent mass mortalities of seals and other marine mammals caused by viral infections (Ross *et al.*, 1995).

Out of all of this, the importance of immunotoxicity data in the risk assessment of chemicals is actively evolving. This appears to be strenghtened by a recent report that an immunotoxicological endpoint may be more sensitive than a cancer endpoint for dioxin (Birnbaum, 1994). Therefore, immunotoxicology could end up as a critical concern in the risk assessment of dioxin and also perhaps for other chemicals undergoing risk assessment.

Immunotoxicology does not seem to have been generally acknowledged as a distinct sub-discipline of toxicology until about the mid 1970s to early 1980s (Vos, 1977; Sharma, 1981). The adverse effects of chemotherapeutic agents and environmental contaminants on the immune systems of humans and their mammalian surrogates was the primary developmental focus of this field and, for the most part, remains so up to the present (Burleson *et al.*, 1995; Dean *et al.*, 1994; Descotes, 1988, 1993; Luster *et al.*, 1988, 1992, 1993; Schuurman *et al.*, 1994; Selgrade *et al.*, 1995).

Many vertebrate and invertebrate organisms in the environment had also gradually been shown to have immune structures and functions that were somewhat similar (or analogous) to those of their mammalian counterparts (Cooper, 1976; Marchalonis, 1977). In fact, a new international journal, i.e., *Developmental and Comparative Immunology*, was started in 1977 (Cooper, 1987) to address the substantial scientific interest being directed towards comparing the immune systems and responses of other organisms to those in the species most typically studied, i.e., humans and rodents.

Substantial data on the immunotoxicity of drugs, pesticides, environmental contaminants, radiation, heavy metals, and other chemicals have been collected and reviewed in mammals and in other vertebrates such as fish and birds (Anderson and Zeeman, 1995; Dean *et al.*, 1985, 1994; Descotes, 1988; Dunier and Siwicki, 1993; Dunier, 1994; Fairbrother, 1994; Luster *et al.*, 1992, 1993; Porter *et al.*, 1984; Sharma, 1981; Sharma and Zeeman, 1980; Vos, 1977; Zeeman and Brindley, 1981; Zelikoff, 1993, 1994). More limited chemical immunotoxicity data have also been collected for invertebrates in the environment, such as worms, molluscs, etc. (Anderson, 1994; Goven *et al.*, 1994).

The practical utility of mammalian immunotoxicology data for chemical risk assessment purposes is currently being actively explored by industry, academics, and some regulatory agencies worldwide (Basketter *et al.*, 1995; de Waal *et al.*, 1995; FDA, 1993; Hinton, 1992, 1995; ILSI, 1995; Luster *et al.*, 1988, 1992, 1993; Neumann, 1996; NRC, 1992; OTA, 1991; Schuurman *et al.*, 1994; Selgrade *et al.*, 1995 a,b; Sjoblad, 1988; Wartenberg *et al.*, 1993; WHO, 1996). As a result, a few recent background or summary documents have been made available that give some guidance on possible immunotoxicity test methods and/or on the use of appropriate immunotoxicology results in chemical risk assessment (Table 1).

Table 1
Recent Immunotoxicology Summary or Background Guidance Documents

Title	Source*	Year
Identifying & Controlling Immunotoxic Substances	OTA	1991
Biologic Markers in Immunotoxicology	NRC	1992
Risk Assessment in Immunotoxicology I	NIEHS	1992
Risk Assessment in Immunotoxicology II	NIEHS	1993
Immunotoxicity Studies (draft)	FDA	1993
Immunotoxicity Testing and Risk Assessment	ILSI	1995
Principles & Methods for Assessing Direct Immunotoxicity Associated with Exposure to Chemicals	WHO	1996

* See references for complete citations (NIEHS, see Luster *et al.* 1992 & 1993)

RISK ASSESSMENT

In 1983, the National Research Council (NRC) of the U.S. National Academy of Sciences (NAS) published the NRC "Red Book" entitled *Risk Assessment in the Federal Government: Managing the Process* (NRC, 1983). Their definition of risk assessment was "the characterization of the potential adverse health effects of human exposures to environmental hazards." The NRC also identified the following basic steps of a human health risk assessment: hazard identification, dose-response assessment, exposure assessment, and risk characterization. This general framework of four steps for human health risk assessment was intended to facilitate the development of uniform technical guidelines.

Risk Assessment Guidelines

Development of formal risk assessment guidelines by the EPA had its origins in the NRC "Red Book" recommendations that U.S. federal agencies develop "inference guidelines" to describe how its risk assessments are done (Norton *et al.*, 1995). The EPA's risk assessment guidelines are not intended to be specific test methods, or step-by-step rules, instead they are to represent general scientific principles and approaches to assist the EPA in conducting its own risk assessments.

The EPA's risk assessment guidelines are developed by numerous agency senior scientists. Historically, the EPA's guideline activities have focused on human health risk assessment, as was evident when the first set of five risk assessment guidelines were published in 1986 (Table 2). Some of the risk assessment guidelines have been revised since then and some additional proposed guidelines (such as ones on neurotoxicity, reproductive toxicity, and ecological risk assessment) are in various stages of review by scientific peers and by the public. Such guidelines are intended to assist the agency in its risk assessment process.

Table 2
Original EPA Risk Assessment Guidelines

Guidelines for Carcinogen Risk Assessment (1986)
Guidelines for Mutagenicity Risk Assessment (1986)
Guidelines for the Health Risk Assessment of Chemical Mixtures (1986)
Guidelines for the Health Assessment of Suspect Developmental Toxicants (1986, revised 1991)
Guidelines for Estimating Exposures (1986, revised 1992)

The Risk Assessment Process

The process for assessing the risk of a chemical or other stressor (e.g., radiation) requires certain basic pieces of information, such as the exposures to and the hazards of the chemical or other stressor. Some measurement or estimation of the degree of exposure to the organisms of concern should be made. If there are no exposures to a chemical stressor, then a quantitative measure of risk will probably not be able to be estimated.

Also the hazard of a chemical, its inherent toxicity to test organisms, must be determined. Highly hazardous chemicals are those that cause adverse effects in certain organisms or organ systems at very low test concentrations or exposures. A chemical may cause significant adverse effects in one test species (such as frank lethality) or in an organ system endpoint (such as liver cancer or immunopathology in the spleen and lymph nodes), and may not cause these adverse effects in another test species or on other target organ system endpoint(s). The type and degree of responses that are seen will depend on several factors, such as differences in species sensitivity, the exposures (dose), relative organ sensitivity, sex (gender), age, etc.

The risk characterization phase of the risk assessment process takes the chemical exposure estimates and contrasts these with the various hazard estimates to determine the possibility and degree of harm to the organisms of concern. The simple way to make this comparison is via the quotient method,

where dose levels causing little or no hazards are adjusted for experimentaluncertainties (see below) and then contrasted with the predicted levels of exposure to see if there appears to be any risk of adverse effects. Simply put, if the exposure predicted or measured exceeds the dose shown to cause specific hazardous effects in the lab, the possibility for seeing such impacts in real circumstances must be considered to be relatively high.

Uncertainty (or "safety") factors are typically applied to the hazard endpoints to account for experimental uncertainties and provide some conservatism to these predictions of hazard. Uncertainty factors of 10 or less are commonly used to take into account some uncertainties such as intra- and interspecies extrapolations, inadequate test durations, extrapolating from the lowest-observed-adverse-effect-level (LOAEL/LOEL) to a no-observed-adverse-effect-level (NOAEL/NOEL), etc. (Beck *et al.*, 1994; Dourson and Lu, 1995).

When actual or reasonably accurate estimations of exposure are unknown (an all too common an occurrence), the general approach has been to define the dose level(s) of potential concern in the following manner. The dose levels where the most sensitive and/or meaningful (non-cancer) toxicity endpoints occur are adjusted downwards by the use of various uncertainty factors (such as the above). These adjustments are made to define dose levels above which there may be a concern and below which there is some conservative margin that the risk of such toxicity effects are not likely to occur.

Risk Assessment Endpoint Estimators

The above such procedures have been used to calculate quantitative estimates of the acceptable oral doses/exposure of the chemical to humans, such as the acceptable daily intake (ADI), and an EPA variant of the ADI called the reference dose (RfD). The RfD is an important estimate that is commonly provided in the regulatory assessment of the non-cancer risk of many chemicals (Beck *et al.*, 1994).

The ADI is "the daily intake of a chemical which, during a lifetime, appears to be without appreciable risk on the basis of all the known information at the time" (Dourson and Lu, 1995). The RfD is more specific, and is "an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure (via ingestion) to the human population (including sensitive subgroups) that is likely to be without appreciable risk of deleterious effects during a lifetime" (Barnes and Dourson, 1988; Dourson and Lu, 1995).

Simplistically, the RfD is calculated using the most significant and sensitive toxicity endpoint, to which uncertainty factors are applied. "The RfD is calculated from a NOAEL for a critical effect, which is then divided by uncertainty factors (UFs)..." (Barnes *et al.*, 1995). [For the sake of simplicity, other issues in calculating an RfD, such as modifying factors, are not presented here].

$$RfD = NOAEL/UFs$$

Similarly, a Reference Concentration (RfC) could be estimated for a chemical entering via the inhalation route of exposure. This versatile method of assessing chemical risk (by setting no risk

levels) has also been applied to different species, and therefore is very useful in comparative toxicology.

Analogous risk assessment approaches have also been used in some parts of the EPA in the determination of the environmental (ecological) risk of industrial chemicals to organisms in the environment, and in the setting of chemical water quality criteria to protect wildlife in the Great Lakes (EPA, 1995; Zeeman and Gilford, 1993; Zeeman, 1995). Basically, the hazards are estimated and uncertainty factors are used (smaller in magnitude as the toxicity data base increases) to set levels of chemical exposure in the environment that are below regulatory levels of concern (hopefully these are below the levels that could cause adverse environmental impacts).

An important goal for immunotoxicologists is that meaningful immunotoxicity results be used more often as key endpoints in the risk assessment estimations of chemicals. To be used more often means that the immunotoxicity test results must be both very sensitive and yet also be recognized as a critical endpoint of concern, i.e., be one of the most significant health or environmental (ecological) endpoints of concern.

That will require that generally acceptable and reliable standard immunotoxicology test methods be developed and, for comparative purposes, certain environmentally relevant species (that are probably also of significant commercial importance) be emphasized in testing.

COMPARATIVE IMMUNOTOXICOLOGY

Primarily because of potential concerns for human health, the immunotoxicity of chemicals to mammals has been an experimental concern over the last 20 or so years. Immunotoxicity test methods and test results have only relatively recently become the subject of regulatory concern during the actual risk assessment of chemicals (Basketter *et al.*, 1995; Birnbaum, 1994; FDA, 1993; Hinton, 1992, 1995; ILSI, 1995; Luster *et al.*, 1992, 1993; Sjoblad, 1988; WHO, 1996). This regulatory interest could increase as a limited number of test methods are agreed upon as critical and as more refined risk assessment guidelines for immunotoxicology are developed.

From a comparative species point of view, scientific interest in the immune systems of vertebrates and invertebrates in the environment has been longstanding (Cooper, 1976; Marchalonis, 1977). The testing of the immunotoxicity of chemicals to a wide variety of organisms, especially vertebrates in the environment, has also been the subject of experimentation over the last 20 or so years (Anderson and Zeeman, 1995; Fairbrother, 1994; Porter *et al.*, 1984; Sharma and Zeeman, 1980; Weeks *et al.*, 1992; Wester *et al.*, 1994; Zeeman and Brindley, 1981).

The natural scientific interest in the diverse immune responses of a wide variety of species in the environment and in the numerous methods that can potentially be used for detecting the impacts of toxic chemicals on such immune responses needs to be contrasted with the relative value of such data for quantitative risk assessment purposes.

As is seen in the above section on Risk Assessment, data from toxicity tests are used in certain ways to assess the risk of test chemicals. The most compelling cases for action will come from test results

that are closely related to the endpoints that are considered the most relevant to important species and are, therefore, significant for risk assessment. For example, a test finding adverse impacts on the growth, development, and/or survival of the young at specific doses of a chemical will probably be considered to be more significant than one showing, at the same dose levels, some effects on the number or types of white blood cells. This means that only the most meaningful and significant immunotoxicology endpoints will need to be determined for risk assessment purposes because only these will be the primary ones focused upon in the risk assessment process.

Research in mammalian immunotoxicology is now actively taking this approach into consideration. For example, Luster *et al.* (1992) analyzed the results of over 50 selected compounds tested by the National Toxicology Program. They showed that the results of certain tests (alone and in specific combinations) taken from their screening battery of immunological tests (Luster *et al.*, 1988) appeared to be more predictive of actual immunotoxic impacts and were probably much more meaningful than others, e.g., the splenic plaque (antibody) forming cell response and the natural killer cell activity response tends to be a very predictive combination. From this study, and their follow-on one examining at host resistance tests, Luster *et al.* (1993) stated that "as few as two or three immune parameters were needed to predict immunotoxicants in mice."

The choice(s) of mammalian species to be used for immunotoxicity risk assessment purposes appears to be the subject of some discussion, e.g., the mouse versus the rat. Leaving such matters to the human health specialists, comparative immunotoxicologists need to seriously consider focusing their attention to the use of a very limited number of representative species. There is a compelling reason for this. The public will really only care about (support and fund research on) the environmental species that they can relate to and that mean something to them. Therefore, working with species that are interesting, but not especially significant to the public, can serve to dilute needed efforts. This lack of focus on only a few important species could probably divert attention from the hoped for increasing relevance and utility of comparative immunotoxicology.

To enchance the practical utility of immunotoxicity in the risk assessments of chemicals, comparative immunotoxicologists may need to start focusing more of their attention upon just a few representative species. The chicken, trout (and/or salmon), and perhaps oysters (and/or clams), could serve to represent birds, fish, and invertebrates in the environment. Recognizing that such choices are limiting, and that the end results are not truly representative of the enormous species diversity in the environment (especially where extrapolations from a few species will need to serve for thousands of species, e.g., trout for all fish), such difficult decisions still need to be made. This could help avoid the marginalization of research in comparative immunotoxicology and be an impetus in its use in the risk assessment of chemicals.

The reason for the choice of the aforementioned species center on their commercial importance. This leads to several very important considerations. People typically care about what they eat, they want it to be nutritious, and they do not want their food to be contaminated or harmful to them or their children. This concern is often translated into active political and even governmental regulatory actions and protections.

The public spends a great deal of money on their food and this results in large agricultural and aquacultural interests that are willing and even eager to attempt to grow and husband such species.

This often leads to intensive animal culture, where infectious disease is typically a major issue, and where host immunology can be a critical protective factor.

The need to grow and husband such food species means that a great deal may already have been learned about them, i.e., existing databases. However, much still remains to be learned about their biology so that their culture can be improved (and they can also become a more suitable "laboratory" species). Some of the industry money will go to their own and to academic research facilities to learn about the culture, biology, and disease resistance of such species. Also federal agencies and labs often perform or support such research in academic arenas using public monies.

To summarize, the public thinks these species are important and will financially support research, political and government actions, and the large industrial interests dealing with them. There are often large research databases on many of these species and they have already generally been selected to be good species to be studied (cultured) under laboratory circumstances. Even so, much more needs to be learned about their immune responses and how these are impacted by their culture methods (foods, intensive culture, disease resistance factors, normal biological rhythms, etc.) and the chemicals that are often used in their culture (antibiotics, hormones, etc.) or that they are exposed to via their environment (pesticides, PCBs, PAHs and other industrial chemicals, etc.).

DISCUSSION

The science of immunotoxicology resulted from advances in, and a melding of, both immunology and toxicology. Similarly, the impetus for the active development of the field of comparative immunotoxicology comes from advances in both comparative immunology and environmental toxicology. As these various disciplines seek to receive continued funding, and to become more pertinent to the processes used for the risk assessment of chemicals, a focus different from the past typical basic-research type agendas of these disciplines will probably need to be considered.

The risk assessment process typically requires specific toxicity data endpoints and centers on their reliability and significance. These toxicity endpoints need to be generally agreed upon as significant, as they can often end up as being critical in risk assessment decision-making. Like Luster *et al.* (1992, 1993), the comparative immunotoxicologists in the field need to critically examine the significance of their endpoints being tested or proposed as useful for the risk assessment of chemicals.

Typical significant risk assessment endpoints for humans tend to focus upon adverse organ or tissue effects, with results such as carcinogenicity, reproductive toxicity, neurotoxicity, etc. Endpoints used for environmental (ecological) risk assessment often focus upon adverse effects (such as death, decrements in growth and development, and decrements in reproduction) that are expected to have impacts at the population level, not just the individual level (Zeeman and Gilford, 1993; Norton *et al.*, 1995; Zeeman, 1995).

Therefore, while immunotoxicologists interested in human effects and risk assessment can afford the luxury of focusing only on individual level effects, comparative immunotoxicologists interested in environmental (ecological) risk assessment must consider focusing more upon immune effects

that are likely to also have significance at the population level. In addition, as described in detail above, only a limited number of commercial species are likely to be recognized and considered truly important enough by most people to be suitable in many environmental chemical risk assessments.

The natural academic interest in determining the diverse immune capabilities (and the sources of immune variability) of a wide variety of environmental species is, of course, necessary and commendable. However, the significance of such results may be very difficult to support in the practical chemical risk assessment context discussed above. For example, while the author has a long-standing interest in the significance of biological rhythms upon fish immune systems (Zeeman, 1986), perhaps this needs only to be determined using the culture methods of the most important commercial species.

Irregardless of such possible limitations, comparative immunotoxicology is a discipline that will remain active on many fronts. Both immunology and toxicology appear to be fields that are exploding with new research results. Consider the growing significance of the research in the endocrine disruptor field. Then there are the discoveries of the complex interactions between the immune and neuroendocrine systems. Finally there is the probable impact of comparative immunotoxicology on risk assessment and vice versa. For the foreseeable future, there will be no lack for stimulating things to do or possibilities to consider.

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Chapter 28

Invertebrates, Ectotherms, Immunotoxicology: Extrapolation to Human Health

Edwin L. Cooper and Nicolo Parrinello[1]

Laboratory of Comparative Immunology
Department of Neurobiology
School of Medicine
University of California, Los Angeles
Los Angeles, CA 90024

[1]Laboratory of Marine Immunobiology
Zoology Institute
University of Palermo
via Archirafi 18
Palermo, 90123, Italy

ABSTRACT

We recognize that increasing concentrations of xenobiotics in the environment, industry, food and water supplies, have become a problem of significant proportions. Today, the danger is greater than ever before. Many environmental pollutants are toxic to animals and to humans, resulting in overt clinical disease and occasionally death. For example, mercury and lead are non-essential elements for living organisms, however, their ability to produce disease and even death after acute or chronic exposure is well documented. Mercury and lead are apparently immunosuppressive and the damage may occur to a particular cell (B, T, or macrophage) or it may involve more than one cell which regulates the proliferation and differentiation of other cells responsible for a normal functioning immune system. This paper will be concerned with a focus on the comparative aspects of immunotoxicology. In so doing, points will be raised offering the advantages of using invertebrates, fish, amphibians and reptiles as animal models for at least three main reasons. First, they are highly relevant in that they are members of the biosphere and any alterations of their immune systems shoudl be understood. Second, they are important to elements of the food chain and are therefore good candidates for vectors of disease. Third, if we look for the need to use certain correct models to ascertain the effects of immunotoxicants, it is not always a mammal (and certainly not humans for legal and ethical reasons) that is the appropriate model. It is therefore at this juncture that an appropriate animal model be chosen. Finally, by viewing the effects of xenobiotics as potentially deleterious to an essential system of all organisms as one survival strategy, the comparative approach to the immune system, takes on an evolutionary flavor that should be of deep concern for us all. For the biological consequences of such pollution (as a source of stress) may range beyond that which is immediately apparent.

INTRODUCTION

What can history tell us about the use of animal models?

Animals have been used as models for centuries to predict the risks chemicals and environmental factors have on human health. Much of what is recorded in early history about toxicological testing indicates that humans were the test subjects, surely long before the stringent rules of today. The earliest clear description of the use of animals in the scientific study of the effects of environmental agents appears to be by Priestley (1792) in his study of gases. The first systematic use of animals for the screening of a wide variety of agents was published by Orfila (1818), and was described by Dubois and Geiling (1959) in their historical review. This work consisted of dosing test animals with known quantities of agents (poisons or drugs), and included the careful recording of the resulting clinical signs and gross necropsy observations. This paper will present four models—earthworms, tunicates, fish and amphibians, demonstrating damage to the immune system by experimentally induced changes in the environment. In the invertebrates, the examples may be termed purely immunotoxic whereas the damage caused by other environmental variables, such as temperature and aggressive behavior are somewhat peripheral. Alternative species have been given considerable attention as has been pointed out by Chengelis (1992).

A modern view of using diverse animal species

Present-day toxicologists adopt a variety of techniques from other scientific areas and develop new skills unique to the questions asked and the studies being pursued. "The use of animals as predictive models in experimental biology in general, and in toxicology and pharmacology in particular, continues to cause more than a little controversy. On the one hand, animal models, that include invertebrates and ectotherms, have provided the essential building blocks that have permitted the explosive growth of understanding in these fields, with numerous benefits to both humans and other animal species. At the same time, the benefits of such use, balanced against costs, in terms of animal lives and potential suffering and discomfort, have been subject to an increasing level of questioning" (Di Carlo and Oehme 1992). This caution may be due to the difficulty of extrapolating results directly to humans.

With respect to earthworms, the advantages far outweigh the disadvantages although supporting this assertion is surely controversial. For example, cost advantages are clear: (100 earthworms = $2.00; 100 mice = $125-$175). There is no need for elaborate vivarium space; the 48 hour contact test requires little capital investment other than a dedicated under-the counter refrigerator set at 15-20°C; standard lethality test, at 48 hours is in marked contrast to 7-14 days for mice. There are the disadvantages however which include: limited endpoints other than death or abnormalities (Stenerson, 1979;1984; Drewes, *et al.,* 1984) and the test does not yield much qualitative information. There may also be some institutional bias. Because the test is not cutting edge technology (e.g. no tissue culture) and uses a non-mammalian model, it has been easy to dismiss the utility of this system. These interpretations need to be reevaluated because there are new ways to culture earthworm cells to identify surface markers and to develop short term assay systems (Vetvicka, *et al.,* 1994).

REVIEW OF IMMUNE SYSTEMS

The Invertebrates

Clearly invertebrates possess elements of non-specific and specific immune responses (Figure 1.) although this assumption has been subjected to intense scrutiny with advocates and protagonists on both sides (Beck *et al.*, 1994; Brehelin 1986; Cooper *et al.*, 1987; Cooper, 1992; Cooper 1994; Cooper and Nisbet-Brown, 1993; Cooper *et al.*, 1992; Ganz and Lehrer, 1994, Garside and Mowat, 1995; Greenberg, 1987; Hoffman, 1995; Hoffman *et al.*, 1994; Hultmark, 1993; Humphreys, 1994; Janeway, 1992; Marchalonis and Schluter, 1990; Mitchell, 1984; Quintans, 1994; Ratcliffe, 1981a; Smith, 1992; Stewart, 1992; Tauber, 1994; Vetvicka, *et al.*, 1994). A commonly held belief concerning the immune system is that its progressive phylogenetic development paralleled the appearance of immunoglobulins. Thus, the entire animal kingdom can be divided according to the narrow view that invertebrates that produce only non-specific reactions and vertebrates that show specific responses. This view also implies that specificity must only accompany antibody synthesis. In the case of cell-mediated immunity, however, invertebrates have been shown to possess specificity and memory (Cooper *et al.*, 1992).

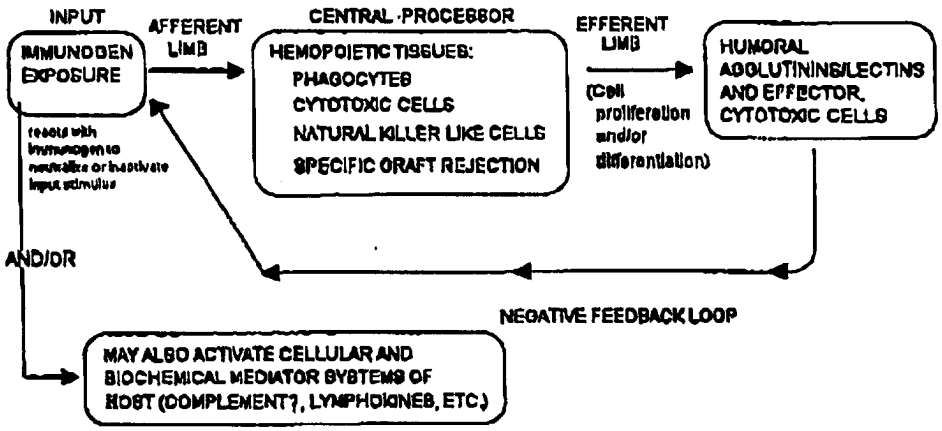


Figure 1. A model of the competent immune system of invertebrates depicting normal interrelations of the major components. Although created primarily for mammals, the general events are still applicable (adapted from Dean, *et al.*, 1994).

Despite the conceptual difficulty in considering immunoglobulins and specificity, it is acceptable to divide the animal kingdom into invertebrates with humoral immunity and cell-mediated immunity and vertebrates with specific T-cell and B-cell immunities (Cooper, 1982). With respect to humoral immunity in invertebrates, it may be compared with discussions on antibodies in vertebrates for several reasons. First, it is not the intention, however, to equate humoral immunity in invertebrates point for point with immunoglobulin or antibody synthesis in vertebrates. Second, invertebrate immunity and antibody synthesis in vertebrates share one common feature, both are produced or initiated in response to an encounter with a soluble or particulate antigen. After synthesis occurs,

foreign material or antigen, are either sequestered or eliminated. Third, the other feature they share in common is that substances are synthesized by both, these being antibodies, agglutinins, lysins, etc. (Cooper, 1982, Cooper *et al.,* 1992; Ratcliffe, 1981b). Available evidence from living relatives of extinct fossils has been examined to set the stage for an in-depth understanding of the immune system and how it could be expected to be compromised by environmental variables, in hopes that students might be moved to analyze the immunotoxicity of exotic animals, uniting immunotoxicology with the broad discipline of biology (Cooper, 1976; Cooper, 1982; Cooper and van Muiswinkel, 1982; Cooper *et al.*, 1985; Stolen *et al.*, 1986).

Immunosuppression by Xenobiotics in Invertebrates

PCBs and earthworm immunity

There is a growing wealth of studies indicating that invertebrate immune systems may be affected by xenobiotics (Figure 2). For example, both humoral and cellular immunodefense responses of the earthworm have been analyzed with respect to PCBs (Eyambe, *et. al.* 1991; Cooper and Roch, 1992; Fitzpatrick, *et al.,* 1990; Rodriguez, *et al.,* 1989; Roch and Cooper, 1991; Chen, *et al.,* 1992; Fitzpatrick, *et al.,, 1992).* More recently in other parallel studies *Eisenia fetida andrei, Eisenia hortensis,* and *Lumbricus terrestris,* have been compared after exposure to the PCB Aroclor 1254 (Ville, *et al.,* 1995). Responses mediated by free factors, detected by *in vitro* assays for lysozyme, hemolysis, and proteases, were increased in both *Eisenia.* Antibacterial activity directed against pathogenic bacteria was increased in *E.f. andrei.* The resistance of *L. terrestris* against nonpathogenic bacteria was decreased. Nonspecific cellular functions, including phagocytosis and those related to wound healing, decreased dramatically in all earthworms.

Presented here is a summary of the modulation of cellular- and molecular-related immunodefense activities in earthworms due to PCB exposure. First, three cell types are involved, i.e. macrophages, leukocytes, and chloragocytes. PCBs did not decrease the *in vivo* sensitivity to pathogenic bacterial infection as measured by LD50, nor the humoral antibacterial responses as measured *in vitro.* As a consequence, this chemical pollutant did not exert any negative effect on antibacterial coelomic fluid proteins nor on the chloragocytes involved in their synthesis. Second, cell-free-related activities, such as lysozyme and protease, were increased. Third, the cellular arm as affected in that PCBs exerted a strong inhibitory effect on macrophage-mediated functions as reflected by the results of assays designed to measure wound healing and phagocytosis. This decrease was evident as soon as the earthworms, especially *E. hortensis*, was in contact with PCB'sas low as 1 $\mu g/cm^2$. Moreover, this concentration induced no gross morphological nor behavioral modifications of the earthworm. Recently, spontaneous cytotoxicity, which resembles natural killer cell activity, has been shown to be suppressed in co-cultures of allogeneic coelomocytes from *L. terrestris* (Suzuki and Cooper, 1995, in press). The macrophage-mediated functions, quantified by phagocytosis of yeasts, represents a simple, rapid, and sensitive assay, one that can be applied to both earthworm genera from soils (*L. terrestris*) and from manure (*Eisenia*). This assay provides an indicator of the potentially deleterious effects of soil contamination, at least by PCBs, using the immunodefense system as the yardstick (Ville *et al.*, 1995).

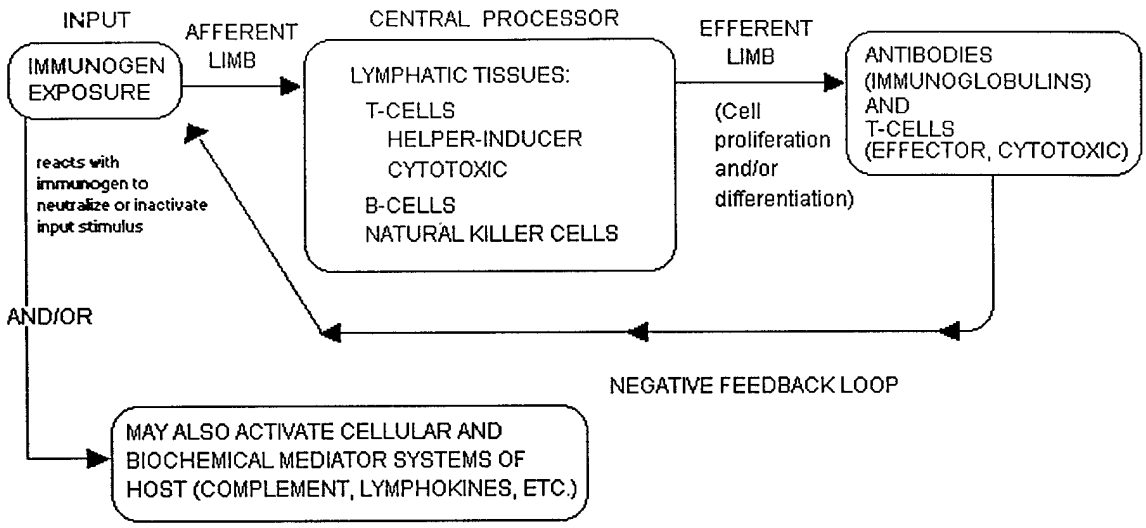


Figure 2. A model of the competent immune system of most invertebrates depicting sites of potential effects on the major components by toxic factors. Although created primarily for mammals, including humans, the general events are still applicable (adapted from Dean, *et al.*, 1994).

TBT affects phagocytosis in tunicates

Organotin compounds have been used in marine anti-fouling paints as biocides. Although much research has been done on the effects of organotins on rats (Funashi *et al.*, 1980; Krajnc *et al.*, 1984; Mushak *et al.*, 1982; Pieters *et al.*, 1989; Snoeij *et al.*, 1988; Vos *et al.*, 1984; Vos *et al.*, 1985), very little is known of its effects on invertebrates. Because tunicates are vulnerable to these xenobiotics in their natural habitats, we used *Ciona intestinalis*, to establish an *in vitro* assay for phagocytosis of yeast by hemocytes after exposure to different concentrations (0.0015, 0.015, 0.15, 1.5 μM) of four organotin compounds: tributyltin (TBT), triphenyltin (TPT), diphenyltin (DPT), and dibutyltin (DBT). To evaluate phagocytic activity, we used a method based on fluorescence excitation of yeasts pretreated with eosin-Y. The percentage of phagocytosis decreased from control 45±3.49 to 22.4±5.14 at 1.5 μM of TBT ($p<0.001$), and it was significantly reduced in the presence of the ionophore A23187. TPT, DPT, and DBT did not show significant effects on phagocytosis. These results indicated that for future analysis, tunicates could potentially seve as excellent models for dissecting events such as phagocytosis that are associated with xenobiotic-induced immunosuppression (Cooper *et al.*, 1995).

Heavy metals

It is now generally recognized that the increasing concentration of metals in the general environment, industry and particularly in water supplies, has become a problem of significant proportions (Nicoll, 1987; Goyer, 1986; Hayes, 1983). The biological consequences of such pollution may range beyond that which is immediately apparent (Ludwiski, 1987). Today, the danger is greater than ever before. Many environmental pollutants are toxic to animals and human beings, resulting in overt clinical

disease and occasionally death (Hayes, 1983). Mercury and lead are non-essential elements for living organisms, however, their ability to produce disease and even death after acute or chronic exposure has been extensively reported (Hillam, 1986; Eisler, 1988). It has been well documented that, mercury and lead are immunosuppressive (Blakely, 1980) and the effects may be associated with a particular cell (B, T or macrophage) or may involve more than one cell which regulates the proliferation and differentiation of other cells responsible for a normal functioning immune system (Killer, 1973). The immunological and histopathological effects of mercury and lead on lymphoid tissues (spleen, lymph nodes and thymus) of mice as well as histopathological, biochemical changes in kidney and liver in animals exposed to different metals have been recorded (Suda, 1986; Alonso, 1988). Sites of potential effects on immunological components in ectotherms have been speculated. However, such a focus of work has not yet been reported to my knowledge with respect to ectotherms and certainly not to invertebrates which lack in many instances defined areas that house leukocytes.

Hussein (1989), has demonstrated that chronic exposure to lead or mercury in albino mice causes clear signs of immunosuppression (unpublished). Cellular and humoral responses are decreased by prolonged exposure to mercury or lead. Spleen cells of mice exposed to mercury had significantly reduced conjugate formation against YAC-1 target cells. The level of suppression was dose dependent, with maximal decreases obtained at 10 ppm of methyl mercury after two weeks of exposure. This suggests a direct inhibitory effect of mercury on conjugate formation. Exposure of mice to lead acetate levels between 14-140 ppm also resulted in a reduction in conjugate formation. However, acute exposure (different single exposures) of lead acetate resulted in increases in conjugate formation.

Antibody production by single cells was evaluated by the hemolytic plaque method. Antibody production was observed to be significantly less when mice were treated with organic or inorganic mercury. Maximal reduction in antibody production was again reached after two weeks at 10 ppm. B-cell or plasma cell synthesis of IgM may be affected by mercury exposure. Chronic doses of lead acetate also caused decreases in antibody production, but acute doses caused increases in antibody production (Hussein, 1989).

what are the clinical implications for immunotoxicology?

According to Newcombe *et al.* (1992), "Clinical immunotoxicology emphasizes the detection and expression of alterations in the human immune system caused by toxic substances. Understanding such immune dysfunctions requires a knowledge of the organization, cellular expression, and regulation of the human immune system. Furthermore, the immune system has a unique role in the assessment of toxicity since xenobiotic metabolism can occur via pathways of its cellular components and products of immune cell activity may alter the structure and effects of xenobiotics presented to these cells. Xenobiotic-induced interruptions or inappropriate modifications of the essential cell-to-cell communications circuitry of the immune system by foreign substances may result in immunotoxic responses. A variety of vectors (antibodies, viruses, cytokines) provoke immunopathologic responses and provide excellent models for the characterization and elucidation of the mechanisms by which immunotoxic agents result in immune dysfunction. These models also emphasize the potential role of xenobiotics in the dissection of interactions within the immune system. There is an expansion in information available to scientists,

industrialists, government agencies, and the public at large in their recognition and publication of the effects of toxic substances, thus making it possible to dissect the mechanisms of immunotoxic substances and the diversity of clinical expressions xenobiotics may have for the human population.

Can we offer some criticism with respect to animal models?

One should not discount the use of animal models since as pointed out by Gad (1992 a, b): 1) animals can serve as accurate predictive models of toxicity in humans (or other species); 2) the selection of an appropriate species to use is key to accurate prediction in humans; 3) understanding the strengths and weaknesses of any particular model is essential to understanding the relevance of specific target organ toxicities to what would be expected in humans. Although direct transfer of effects from animals to humans is controversial, as our understanding of molecular biology advances and we learn more about the similarities of structure and function of higher organisms at the molecular level, the more it becomes clear that the mechanisms of chemical toxicity are largely identical in humans and animals, (Zbinden, 1987).

The following is a criticism offered by Newcombe *et al.,* 1992, with respect to exotic species: "Although we recognize the importance of animal models, there is a delicate balance that must be achieved between making what may sometimes seem inaccurate predictions that may be associated with extrapolations from these models to humans and the inherent problems associated with the role of low dose exposures, chronic immunosuppression, and cancer. Once some of these parameters become more clear and we recognize host defense dysfunctions, leading to a full understanding of pathogenesis, can we hope to intercede and control at least some of these disorders". "One new, but not necessarily unique approach is the design of new programs that will attract young investigators with new and ever expanding technologies, ideas and training. Their challenge would be to not be timid in taking positions in industry, in government and in those that unite all three of these facets of society". Invariably once a field such as immunotoxicology advances because of new information, newer techniques (*in vitro* assays; see Whittaker and Faustman, 1994), there is merging and expansion until new fields appear on the horizon. Such is the case now for ecotoxicology (see Walker *et al.,* 1991), which is the study of the harmful effects of chemicals upon ecosystems.

Perspectives and Future Directions: The Challenge of Comparative Immunotoxicology and Extrapolation to Human Health

Understanding the toxic potential and mechanisms of environmental xenobiotics is essential in assessing risks to public and environmental health. We can then make appropriate legislative and regulatory decisions to protect both. Numerous methods have been developed for screening chemicals and studying their modes of action on a variety of acute toxic endpoints, and subchronic-chronic processes such as carcinogenicity. Toxic effects on the immune system have been shown and scientists have become aware of the broad spectrum of xenobiotics that alter immune function (Vos, 1977; Sharma, 1981; Dean, *et al.,* 1985). The immune system is a potential target organ system for studying the toxicology of chemical exposure (Dean, *et al.,* 1986).

Much is known about the molecular and cellular biology of the immune system, making it especially well-suited for studying mechanisms of toxicity. Cells of the immune system have the following characteristics that make them appropriate and sensitive for studying chemically-induced cellular toxicity: (1) capacity to proliferate rapidly following activation with antigen or non-specific stimuli; (2) gene products that can be used as markers of maturation, and (3) potential to undergo terminal differentiation that results in production of measurable humoral mediators (e.g. antibodies and cytokines in mammals and, agglutinins in earthworms) or providing effector functions (e.g. tumor cell killing, Luster, *et al.,* 1988). Since assessment of function and enumeration of immune cells following exposure to xenobiotics require small volumes of blood or lymphoid tissue, observations in experimental animals can be confirmed (i.e. validated) in humans using immune cells collected by such methods (Dean, *et al.,* 1986). Insight into alteration of immunocompetent cells by xenobiotics is essential since they are required for host resistance (Luster, *et al.,* 1988).

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Chapter 29

Interspecies Immunotoxicity: Relative Importance of Acquired and Innate Immune Functions

Stephen B. Pruett[1], Deborah Keil[1], Michael Ensley[2] and Robert W. Luebke[2]

[1]Department of Biological Sciences
[2]College of Business and Industry
Mississippi State University
Mississippi State, MS 39762, USA

[2]Environemental Research Center
USEPA, Research Triangle Park, NC 27711, USA

ABSTRACT

Rodents are the most commonly used species in immunotoxicology studies, and their immune systems are comparable to the human immune system in most respects. However, several pitfalls and practical problems remain in extrapolating rodent data to humans. For example, recent evidence indicates reactive nitrogen intermediates constitute an important anti-microbial mechanism of rodent macrophages, but this does not seem to be generally true for human macrophages. In addition, some drugs and chemicals are immunotoxic because they induce stress responses in the host. Mouse and human lymphocytes respond differently in some respects to glucocorticoids, one of the key immunosuppressive stress mediators. Such differences complicate extrapolation of data from rodents to humans. Lack of sufficient data regarding the quantitative relationships between easily measured immunological functions and host resistance also impedes extrapolation. For example, many immunological measurements can be made for humans using a blood sample, but the impact on host resistance to infection or cancer of particular decrements in particular immune functions is not known. Using dexamethasone as a prototypical immunotoxicant, we developed multivariate statistical models in mice that predict host resistance on the basis of immunological functions. For some host resistance models the results were not surprising, but for others it seems that innate immune mechanisms play a larger role than anticipated in host resistance. Strategies can be conceived to use these models to estimate the increased susceptibility to infectious disease or cancer anticipated in persons with particular decrements in immunological functions. However, caveats such as differences between rodents and humans in metabolism of immunotoxicants and in target sites in the immune system must be considered in evaluating the validity of such extrapolations.

INTRODUCTION

Bridging the gaps between basic immunology, toxicology, and risk assessment is a major challenge in immunotoxicology. Immunotoxicologists have used rodent models to achieve considerable success in identifying immunotoxicants (Luster, *et al.*, 1993), determining their target site(s) and mechanism(s) of action in the immune system (Burns and Munson, 1993; Cao, *et al.*, 1990; Davila, *et al.*, 1994; De Krey, *et al.*, 1993), and evaluating their effects on resistance to selected microbial pathogens or cancer cells (Luebke, *et al.*, 1984; Selgrade, *et al.*, 1992). However, it is still not possible to quantitatively predict the effects of suppression of individual immune functions or sets of immune functions on host resistance to infection or cancer. Until such predictions are possible in the most widely used experimental animals (rodents), the meaning of suppressed immunological parameters in humans will remain unclear. However, even if such predictions become possible in rodents, interspecies differences in the effects of immunotoxicants in mammals (Smialowicz, *et al.*, 1994) will complicate attempts to extrapolate from animal models to humans. In the present review, selected data from this laboratory will be used to illustrate one approach that may lead to statistical models to predict host resistance on the basis of changes in various immunological parameters. Results summarized in this review indicate the status of innate immune parameters may be particularly important in predicting host resistance. One of these parameters, production of reactive nitrogen intermediates by macrophages is reviewed as an example of differences in innate immune mechanisms in humans and rodents. Such differences, as well as differences in the absorption, distribution, metabolism, and excretion of toxicants in various mammalian species will need to be addressed when using predictive models developed in rodents for risk assessment in humans.

QUANTITATIVE MODELS FOR PREDICTING HOST RESISTANCE ON THE BASIS OF IMMUNOLOGICAL PARAMETERS IN MICE

Luster and colleagues analyzed the correlation between a number of individual immunological parameters and host resistance to infectious agents or cancer cells in mice (Luster *et al.*, 1993). Several expected correlations between immunological functions and host resistance were demonstrated. In addition, some sets of three immunological parameters correlated well with the overall status of a compound as an immunotoxicant. However, no immunological parameter was reliable in quantitatively predicting host resistance (Luster *et al.*, 1993). In addition, substantial suppression of one or more immunological parameters by an immunotoxicant does not always decrease resistance to infectious agents or tumor cells (Holsapple, *et al.*, 1988; LeVier, *et al.*, 1994; Luster *et al.*, 1993). Such observations are interpreted by some investigators as an indication of an "immunological reserve" capacity. However, host resistance can be influenced by the challenge dose of the infectious agent (Luster *et al.*, 1993). In addition, an immunotoxicant may suppress resistance to one pathogen, but not others (Luster *et al.*, 1993). Because of this complexity, the existence of an immunological reserve capacity would be very difficult to prove. However, it is clear that individual immunological parameters are generally more sensitive to suppression by immunotoxicants than are more holistic measures of immune function, host resistance assays (Luster *et al.*, 1993).

Redundancy, overlap, and compensatory mechanisms are becoming increasingly evident in the immune system, and these properties may further complicate efforts to predict host resistance on the basis of individual immunological parameters. For example, several apparently conclusive lines of evidence caused most immunologists to accept that Interleukin-2 is essential for the development of cytotoxic T lymphocyte responses. However, the construction of transgenic IL-2 knockout mice demonstrated only minor defects in CTL development and anti-viral immunity in the complete absence of IL-2 *in vivo* (Kundig, *et al.*, 1993). Subsequent studies have revealed that IL-15 might function as a substitute for IL-2 under some circumstances (Ihle, 1995). A similar partial redundancy has been reported for IL-4 and IL-13 (de Vries and Zurawski, 1995). Different cell types may also share some key functions (e.g., production of Interferon-gamma by NK cells or Th cells or phagocytosis and killing of microbes by both neutrophils and macrophages). Hence, it is not surprising that important immunological parameters can sometimes be substantially suppressed with little impact on host resistance, if one or more redundant systems is left intact. In addition, it should be noted that some immunotoxicants suppress some immunological parameters, but enhance others. For example, morphine suppresses NK cell activity, lymphocyte numbers, and macrophage function, but it substantially increases neutrophil numbers and also increases host resistance (LeVier *et al.*, 1994).

Because of the complexity of the immune system and its dependence on multiple (often interacting) mechanisms for host resistance, it is not surprising that single immunological parameters fail to quantitatively predict host resistance (Luster *et al.*, 1993). Multivariate statistical methods, such as those used by ecologists and social scientists, are designed to model and predict quantitative relationships in complex, interactive systems. These methods can be visualized as regression models which describe the relationships between multiple independent variables and a dependent variable by using a multi-dimensional response surface, instead of the single line used in simple regression techniques (Hair, *et al.*, 1995). Unfortunately, these methods were developed with the assumption that all variables can be measured for each individual. For a number of theoretical and practical reasons, it is not possible to do this in immunotoxicology studies. In particular, measurement of host resistance, innate immunity, and acquired immunity in the same animals is not possible, because the immunizations or microbial challenges required affect other immunological parameters. Thus, it was necessary to validate the use of multivariate methods with multiple sets of mice exposed to the same dosages of the same immunotoxicant. Dexamethasone was selected as a prototypical immunotoxicant for use in these studies. Several sets of mice included a vehicle control group and four groups given a daily dose of dexamethasone (subcutaneously) at 0.3, 1, 10, or 30 mg/kg/day. Some sets were used to evaluate resistance to B16F10 tumor cells, *Listeria monocytogenes*, *Streptococcus agalactiae*, or *Trichinella spiralis*, and other sets were used to evaluate various innate and acquired immune functions or parameters. The impact of randomly pairing data from different mice (within dosages groups) on the outcome of multivariate analyses was investigated using a data set for which all variables were analyzed for each mouse. These data were then randomized within each dosage group, and the multivariate analyses obtained using the two data sets were compared. Factor analysis and subsequent multiple regression using the original data set and the randomized data set produced similar models. Thus, multivariate methods seem appropriate for immunotoxicology data (Keil, *et al.*, 1995). However, work is continuing to more precisely determine the reliability of such results.

Several multivariate models have been developed in which approximately 35 immunological parameters (independent variables) were used to predict host resistance in dexamethasone-treated mice. These models account for up to 65% of the variance in host resistance, and indicate that four factors (comprising a total of 24 variables) are important in predicting host resistance to B16F10 tumor cells (Keil *et al.*, 1995). An important aspect of these models is the use of factor analysis to group variables with similar dose-response relationships and to eliminate collinearity. The factor scores which are derived from each group of variables are then analyzed by multiple regression or logistic regression. Assessment of the influence of random data pairing from different groups of mice on these models is continuing, but results to date suggest that these methods can be used with immunotoxicology data and may provide a more holistic method for modeling and predicting host resistance than simple regression techniques.

An interesting feature which emerged from these models was the importance of some innate immunological parameters as compared to acquired, specific immune parameters. For example, the number of blood neutrophils was an important factor in resistance to *Listeria monocytogenes.* Dexamethasone caused a substantial (~4-fold) dose-dependent increase in blood neutrophil numbers, and significantly decreased resistance to *Listeria monocytogenes* (as indicated by lethality) only at the highest dosage (30 mg/kg/day). However, antibody production, NK cell activity, and the number of CD4+ CD8- and CD4-CD8+ T cells in the spleen were significantly suppressed at much lower dosages (0.3-10 mg/kg/day). Until recently, the latter parameters (along with macrophage function) were thought to be the key determinants of resistance to *Listeria monocytogenes* (Baldridge, *et al.*, 1990; Czuprynski, *et al.*, 1989; Dunn and North, 1991). However, recent studies demonstrate that neutrophils play a key role in resistance to *Listeria monocytogenes* in mice (Czuprynski, *et al.*, 1994). Thus, the conclusions of our study are understandable because increased numbers of neutrophils may compensate for loss of other important immunological functions that are typically involved in resistance to *Listeria monocytogenes.* This illustrates the importance of evaluating most of the major aspects of immunity and using statistical methods that consider all these immunological variables together in predicting host resistance.

INTERSPECIES VARIATIONS IN THE IMMUNE SYSTEM: IMPLICATIONS FOR RODENT-TO-HUMAN EXTRAPOLATION

Substantial differences between rats and mice have been reported with regard to the effects of some immunotoxicants (Smialowicz *et al.*, 1994). Undoubtedly, there are many such differences among mammalian species. The most obvious explanation would be differences in absorption, distribution, metabolism, and excretion. However, differences in the immune system among species might also be involved by providing different targets for immunotoxicants. In addition, a particular immunological function may be more important in some species than in others. Such issues must be considered in extrapolating from rodent (or other) models to humans.

High output production of reactive nitrogen intermediates (RNI) by macrophages provides an excellent illustration of an apparently substantial difference between the rodent and human immune systems. It is now well-established that high output RNI production by the inducible nitric oxide synthase (iNOS) of activated macrophages is an important anti-microbial mechanism against many (Denis, 1994), but not all (Higginbotham, *et al.*, 1992; Higginbotham and Pruett, 1994; Leenen, *et*

al., 1994) microbial pathogens in mice. Rat macrophages often produce greater quantities of RNI than mouse macrophages, suggesting an equally important anti-microbial role for RNI in rats. However, human macrophages derived from peripheral blood monocytes as well as alveolar macrophages do not seem to produce anti-microbial levels of RNI in response to a variety of stimuli, including Interferon-g and bacterial lipopolysaccharide (LPS) (Cameron, *et al.*, 1990; Murray and Teitelbaum, 1992; Padgett and Pruett, 1992). Although human macrophages contain an iNOS gene, there are apparently only a few stimuli that induce high level RNI production. For example, live *Mycobacterium avium* induce the production of high concentrations (20 μM) of nitrite (a stable end product of RNI, frequently used as an indicator of RNI production), but other Mycobacteria species do not (Dumarey, *et al.*, 1994). In addition, nitrite production has been used in most studies as an indicator of RNI production. It is possible that the predominant form of RNI produced by human macrophages is not nitric oxide and does not readily form nitrite, or that nitric oxide is produced but rapidly scavenged (Denis, 1994). In any case, it is clear that human and rodent macrophages differ at least in the stimuli required to induce high output RNI production and/or in the nature of the RNI produced. The data are consistent with an even more striking species difference, in which RNI constitute an important anti-microbial mechanism in rodents, but not in humans. Substantive differences between humans and some species/strains of rodents have also been reported in the complement system (Ong and Mattes, 1989), ratios of neutrophils to lymphocytes in the blood (Hollinger, 1995), and undoubtedly in other important immunological parameters. The impact of these differences on rodent-to-human extrapolation in immunotoxicology has received little attention, and there are important immunotoxicants which affect some of these parameters (LeVier *et al.*, 1994; White, *et al.*, 1986).

QUANTITATIVE MODELING AND EXTRAPOLATION FROM RODENTS TO HUMANS: CHALLENGES FOR THE FUTURE

It seems feasible to construct multivariate statistical models that predict changes in host resistance on the basis of changes in immunological parameters caused by a particular immunotoxicant (Keil *et al.*, 1995). However, it is unlikely that a single model will predict changes in host resistance on the basis of changes in immunological parameters for all immunotoxicants. The patterns of immunological changes induced by immunotoxicants are so diverse that it is unlikely a single model could describe all possibilities (Luster *et al.*, 1993). However, it is possible that a few comprehensive models might adequately predict host resistance for groups of immunotoxicants which share similar patterns of immunological effects. Immunotoxicants could be grouped on the basis of a relatively small number of immunological parameters using cluster analysis (Hair *et al.*, 1995), and the suitability of a limited number of comprehensive models for predicting host resistance within clusters could be evaluated. If successful, this approach would permit prediction of quantitative relationships between easily measured immunological parameters and host resistance in rodents.

A number of issues must be addressed if quantitative statistical models derived from rodent studies are to be used in predicting the impact of altered immunological functions on host resistance in humans. Although most of the immunological parameters used to develop mouse models can also be measured for humans, the source of lymphocytes in mouse studies is usually spleen, whereas peripheral blood lymphocytes are generally used in human studies. Although initial efforts to evaluate this issue indicate similar effects of at least some immunotoxicants on spleen and blood

lymphocytes in rats (White, *et al.*, 1991), this issue will need further evaluation. In addition, immunotoxicants which affect immunological parameters that differ markedly in humans and rodents will require special attention and perhaps modification of the rodent models to account for estimated greater or lesser importance of a particular parameter in host resistance in humans as compared to rodents. Incorporation of toxicokinetic data to account for differences in absorption, disposition, metabolism, and excretion would be ideal, but will often be impossible due to lack of human toxicokinetic data for many potential immunotoxicants. Ultimately, an intelligent system (using pattern recognition and/or artificial intelligence) might be the only practical approach by which to incorporate all such adjustments and to develop a comprehensive predictive structure that will "learn" from each new data set added to it. Such techniques are already used in ecology and evolution research (Edwards and Morse, 1995; Mangel, 1990), and evaluation of their feasibility in immunotoxicology and ecotoxicology studies seems warranted.

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Chapter 30

Comparative Sensitivity of Different Species to Environmental Chemical-Induced Immunotoxicity

Joseph G. Vos[1,2], Nanne Bloksma[2], Albert D.M.E. Osterhaus[3,4], Henk Van Loveren[1] and Piet W. Wester[1]

[1]National Institute of Public Health and the Environment
P.O. Box 1
3720 BA Bilthoven, The Netherlands

[2]Faculty of Veterinary Medicine
Utrecht University

[3]Seal Rehabilitation and Research Centre
Pieterburen

[4] Institute of Virology
Erasmus University, Rotterdam

ABSTRACT

When adequate human data are not available, and human risk assessment has to be based on toxicity studies in laboratory animal species, interspecies differences in toxicity becomes a major issue. The same holds true for assessing the risk of wildlife populations to become adversely affected by environmental chemicals, when data in the species at risk are not available. The use of toxicity data obtained in laboratory animal species for risk characterization for man or wildlife poses difficulties when 1) species differ qualitatively in target organ of toxicity (hazard identification), 2) interspecies variation in sensitivity to the contaminant is substantial (dose-response assessment), or 3) levels of contaminants are unknown (exposure assessment). This pertains to the assessment of adverse effects in general as well as immunotoxicity.

Examples of environmental contaminants for which data in man and in wildlife populations are necessary for an adequate understanding of the immunotoxic risks at current levels of environmental pollution include: polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs), organotin compounds, and hexachlorobenzene. PCBs, PCDFs and PCDDs induce thymic atrophy and immune suppression in all species investigated, but there are extremely large interspecies differences in toxicity. This hampers risk assessment for man and wildlife (*e.g.* marine mammals occupying a high trophic level). Results of recent studies are discussed in which i) the toxicity of TCDD for the human thymus is investigated following transplantation of fetal human thymic tissue in severe combined immunodeficient (SCID) mice; and ii) immune effects of ambient

levels of immunotoxic contaminants are investigated in a semi-field study with harbour seals fed herring from the contaminated Baltic Sea. In contrast to the PCBs and related compounds, the thymotoxicity of organotin compounds is species specific. Rats are very sensitive and host resistance is strongly impaired. However, thymus atrophy does not occur in mice, guinea pigs or Japanese quail following oral organotin exposure. Studies performed in related fish species with the anti-fouling agent TBTO show thymotoxicity in the guppy (*Poecilia reticulata*) and no effect on the thymus of medaka (*Oryzias latipes*). Finally, hexachlorobenzene is an immunomodulating compound that appears immunosuppressive in the mouse, but immunostimulating in the rat resulting in elevated (auto)antibody levels. The possible role of autoimmunity in clinical symptoms of patients of an HCB poisoning is discussed.

The results indicate that for certain chemicals interspecies differences in immunotoxicity are found, although among higher vertebrates the immune system appears highly conserved, and immune components and their interactions are extremely similar between mice, rats and humans. As the purpose of risk assessment is to make inferences as to the potential risk to human health, the process will benefit from human data, either by testing in humans themselves, or using human material (*in vitro*), or special constructs (*e.g.* xenografts, transgenic animals). For risk assessment at the ecosystem level, semi-field studies are an adequate strategy.

INTRODUCTION

Risk assessment is the process of analyzing relevant toxicological effects, dose-response and exposure data of a particular agent in an attempt to establish qualitative and quantitative estimates of adverse outcomes, including immunotoxicity. It comprises four steps: hazard identification; dose-response assessment; exposure assessment; and risk characterization (National Research Council, 1983). Since the purpose of risk assessment is to make inferences about potential risk to human health, the most appropriate data to be used are those derived from human studies. However, adequate human data are seldom available and the vast majority of risk assessment must be based upon animal studies. Likewise, for risk assessment at the ecosystem level, data in wildlife populations to environmental chemicals are seldom available and risk assessment has therefore, also to rely on laboratory animal studies.

The use of toxicity data obtained in laboratory animal species for risk characterization of man or wildlife poses difficulties when: 1) species differ qualitatively in target organ of toxicity, 2) interspecies variation in sensitivity to the contaminant is substantial, or 3) levels of contaminants are unknown.

Examples of environmental contaminants for which data in man or in wildlife populations are necessary for an adequate understanding of the immunotoxic risks posed by current levels of environmental pollution are discussed. They include: polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs), organotin compounds and hexachlorobenzene (HCB).

REVIEW

PCBs, PCDFs and PCDDs

These environmental pollutants elicit a broad spectrum of toxicological effects that are dose-dependent and species- and target organ-specific. The 2,3,7,8-substituted PCDDs and PCDFs and some non-ortho and mono-ortho substituted ("dioxin-like") PCBs can be clearly distinguished from the di-, tri-and tetra-ortho substituted ("non-dioxin-like") PCBs. Most of the toxic effects of the "dioxin-like" compounds, including the immunotoxic effects, are thought to be initiated by binding to the aryl hydrocarbon (Ah) or TCDD receptor. A risk assessment method for complex mixtures is based on this common mechanism of action and the observed additivity of effects from *in vivo* and *in vitro* studies. Each congener is assigned a potency relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the toxic equivalence factor (TEF). Based on these individual TEF values and congener concentrations the total amount of TCDD toxic equivalents (TEQs) in a sample can be calculated (Safe, 1990).

Numerous studies to examine the toxicity of TCDD have been carried out in laboratory animals. These studies have shown that TCDD causes a wide variety of toxic effects, with a remarkable interspecies variation both in target organs and toxicity (e.g. LD-50 values). Despite this variation, TCDD at sublethal doses causes thymic atrophy and immunotoxicity in all species investigated (Vos, *et al.*, 1991; Holsapple, *et al.*, 1991). The atrophy of the thymus is histologically characterized by reduced cellularity of the thymic cortex. Upon functional assessment, particularly thymus-dependent immune responses appear impaired, leading to a decreased host response to various infectious agents.

The extremely large interspecies differences in toxicity hamper risk assessment for man and wildlife (*e.g.* marine mammals occupying a high trophic level). Results of recent studies will be discussed in which: i) the toxicity of TCDD for the human thymus is investigated following transplantation of fetal human thymic tissue in severe combined immunodeficient (SCID) mice; and ii) immune effects of ambient levels of immunotoxic contaminants are investigated in a semi-field study with harbour seals fed herring from the contaminated Baltic Sea.

Immunotoxicity in Man

Immune suppression was not only seen in laboratory animals but also in humans inadvertently exposed to PCBs, PCDFs or PCDDs; the developing immune system being very sensitive (Vos, *et al.*, 1991; Birnbaum, 1995). Unequivocal immune alterations have been observed in Taiwanese residents following consumption of rice oil contaminated with PCBs and PCDFs. Exposure to these compounds caused acneiform skin lesions, pigmentation of skin and nails, liver damage and abnormal immune function. Serum IgM and IgA concentrations and the percentage of T lymphocytes in the peripheral blood were decreased (Chang, *et al.*, 1981). The cell-mediated immune system was investigated by delayed-type hypersensitivity responses. The percentage of patients showing a positive skin test to streptokinase and streptodornase was significantly lower as compared to controls (Chang, *et al.*, 1982). This suppression of cell-mediated immunity was reproduced in a follow-up study by tuberculin skin testing (Wu, *et al.*, 1984). A disease similar to the Yu-Cheng

poisoning in Taiwan occurred in Japan in 1968, the so-called Yusho disease. Yusho patients frequently suffered from respiratory infections. Serum IgA and IgM levels had considerably decreased during 2 years after the onset of the poisoning, but returned to normal in most cases. Respiratory symptoms persisted for longer time periods (Shigematsu, *et al.*, 1978). There also appears to be an association between elevated exposure to PCBs, PCDDs and PCDFs and immune effects in infants from arctic Quebec (Dewailly, *et al.*, 1992; 1993). A 20-fold higher incidence of infectious disease (e.g., meningitis, measles and otitis) has been reported in the first year of life of Inuit children exposed to high levels of organochlorines as compared to children living in southern Quebec. Inuit infants had some primary immune dysfunctions as measured by a low immunization 'take rate'.

From these investigations in man it can be concluded that PCBs and related compounds cause imune alterations, particularly of the thymus-dependent immunity. The findings in man correlate in qualitative terms with the findings in experimental animals, thus illustrating the relevance of studies in laboratory animals. However, as exposure data on these mixtures of contaminants are virtually lacking for those individuals in which immune parameters were investigated, and because of the remarkable interspecies variation in toxicity, assessment of the risk of effects of these chemicals on the immune system of man is not possible in quantitative terms. A model in which human thymic tissue is transplanted in experimental animals may provide an opportunity to study the sensitivity of the human thymus to these compounds. For this reason, severe combined immunodeficient (SCID) mice were used.

SCID mice have an autosomal recessive defect that impairs the rearrangement of antigen receptor genes in both T and B lymphocyte progenitors (Schuler, *et al.*, 1986), and as a result they lack functional T and B cells. SCID mice engrafted with human fetal thymus and liver tissue fragments under the kidney capsule (SCID-hu mice) have been shown to sustain human T cell differentiation in the human thymus grafts for at least 6 months (Namikawa, *et al.*, 1990). The grafts display a normal thymic architecture and function, and have shown their usefulness in the study of the human immune system and disorders thereof, e.g. alterations induced by human immunodeficiency virus (McCune, 1991). The model also provides the opportunity to examine the sensitivity of the human thymus to thymotoxic chemicals like TCDD (De Heer *et al.*, 1995b). In this study Wistar rats and SCID-hu mice were exposed once to doses up to 25 µg TCDD/kg body weight. The relative size of the cortex, evaluated on day 4 after treatment, was decreased both in rat thymus and the grafted human thymus at the same dose levels. TCDD tissue concentrations in the normal rat thymus and the grafted human thymus were similar. The study indicates that the human thymus and the Wistar rat thymus display a similar sensitivity to TCDD.

Chemical Contaminants and Seal Immunocompetence

Marine mammals are of special interest as top-predators in a marine environment in which persistent lipophilic compounds such as PCBs, PCDFs and PCDDs accumulate (Luckas, *et al.*, 1990). Mass mortalities of seals, such as the 1988 phocine distemper virus epizootic in Europe that resulted in the death of approximately 20,000 seals (Osterhaus, *et al.*, 1988), led to speculation that environmental pollution via comprome of the immune system, had rendered these animals more susceptible to virus infection.

To investigate the hypothesis that pollution of the marine environment had impaired the immunocompetence of seals, a 2.5-year experiment under semi-field conditions was carried out in which two groups of approximately 14-month-old harbour seals were fed herring from either the relatively uncontaminated Atlantic Ocean or the contaminated Baltic Sea (De Swart, *et al.*, 1994). The diets were analysed for the content of organochlorines particularly of compounds that act through the Ah-receptor since these were previously found to cause immunotoxicity in laboratory animals. Daily intakes of 2,3,7,8-TCDD Toxic Equivalents (TEQs) for PCBs, PCDFs and PCDDs were 288 and 29 ng TEQ/day for seals fed Baltic Sea and Atlantic Ocean herring, respectively (De Swart, *et al.*, 1994). Vitamin A levels were used as a "biomarker" of organochlorine exposure, since results of an earlier, similar semi-field study had shown an inverse correlation between organochlorine exposure and vitamin A levels (Reijnders, 1986; Brouwer, *et al.*, 1989).

Because of the absence of specific immunological reagents for seals, immunological assays in the recent immunotoxicity experiment were limited to a general immune function screen, including white blood cell counts, mitogen- and antigen-induced proliferative responses of peripheral blood mononuclear cells (PBMC), natural killer (NK) cell activity, serum antibody responses following primary immunization with antigens and delayed-type hypersensitivity (DTH) reactions. In the 2.5-year study, T cell mitogen-induced lymphocyte proliferation and NK cell activity were consistently reduced in the Baltic group as compared to the Atlantic group (De Swart, *et al.*, 1994; Ross, *et al.*, 1996). Also, DTH reactions and serum antibody titers raised to immunization with ovalbumin were significantly lower in the former group (Ross, *et al.*,1995; De Swart, 1995). Results are summarized in Table 1.

Table 1.
Differences in Immunological Parameters in Seals Fed Contaminated Herring from the Baltic Sea as Compared to Seals Fed Relatively Uncontaminated Herring from the Atlantic Ocean

Parameter	Effect[1]
NK cell activity PBMC	decrease
T cell mitogen-induced response PBMC	decrease
B cell mitogen-induced response PBMC	no effect
MLR-induced response PBMC	decrease
Antigen-induced response PBMC	decrease
Delayed-type hypersensitivity response	decrease
Specific serum antibody responses	no effect/decrease
ex vivo/in vitro immunoglobulin production	*no effect*
lymphocyte counts in peripheral blood	*no effect*
neutrophil counts in peripheral blood	increase

[1]significant effect over time in the seals fed Baltic herring as compared to the seals fed Atlantic herring.

As NK cells play an important role in the first line of defence against viruses and as T lymphocytes play a major role in the clearance of virus infections, it was concluded that the recent virus-induced mass mortalities in seals may well have been exacerbated by immunotoxic contaminants. Analysis of blubber samples collected by biopsies indicated that seals consuming herring from the Baltic Sea had PCB concentrations roughly three times higher than seals consuming Atlantic Ocean herring; the contribution of PCBs to the total TEQ values amounted to 94 %, while PCDDs and PCDFs contributed only 6 % (Ross, *et al.*, 1995). These data suggest that the PCBs are largely responsible for the observed effects.

The results of this semi-field study have direct relevance, since three seal species (harbor, ringed and grey) currently inhabit the Baltic Sea. Moreover, the Baltic Sea herring which was shown to cause the impairment of immune function in the captive seals was intended for human consumption, which raises concerns about the potential for adverse immunological effects in certain human consumer groups.

Organotin Compounds

Organotin compounds are used in a variety of applications. The major uses are as stabilizers of plastics, as biocides and as catalytic agents in industrial processes. The first indications of potential immunotoxicity of certain organotin compouds came from toxicity studies with triphenyltin acetate and triphenyltin hydroxide (Verschuuren, *et al.*,1966). A selective action of dialkyltin compounds on the immune system was first described by Seinen and Willems (1976). The organotin-induced thymic atrophy is determined by the lenghth of the alkyl chain, probably related to the water-lipid partition of the homologues. Among the various di- and trisubstituted organotins studied, di-*n*-octyltindichloride (DOTC), di-*n*-butyltindichloride (DBTC), bis(tri-*n*-butyltin)oxide (tributyltin oxide, TBTO), and tri-*n*-butyltin chloride (TBTC) have been shown to be markedly thymotoxic and immunosuppressive in the rat (Penninks, *et al.*, 1990). DOTC-induced thymic atrophy in the rat was shown to be associated with a suppression of T cell function as evidenced by reduced delayed-type hypersensitivity (DTH) response, delayed allograft rejection, suppressed T lymphocyte proliferation and reduced thymus-dependent antibody responses.

Overall, similar findings were obtained with TBTO: suppression of DTH reactions, T lymphocyte proliferation, and T-dependent antibody responses. In addition, TBTO treatment led to decreased resistance to infection with *Listeria monocytogenes, Trichinella spiralis* (Vos, *et al.*, 1984) and with rat cytomegalovirus (Garssen *et al.*, 1995). Long-term low level oral exposure did not affect DTH reactions but suppressed natural killer (NK) activity and resistance to infection. Even when long-term exposure was started only at an age of 12 months, both the *Trichinella spiralis* and *Listeria monocytogenes* infection models showed immunotoxicity of TBTO (Vos, *et al.*, 1990).

Although the di-*n*-butyl-, di-*n*-octyl- and tri-*n*-butyltins are considered prototype immunotoxicants in the rat, no selective lymphoid atrophy occurred in mice, guinea pigs and Japanese quail following oral exposure (Seinen, *et al.*, 1977). However, thymic atrophy was produced in mice following intravenous administration of DBTC or DOTC, suggesting that interspecies differences in susceptibility after oral administration of these compounds may be due to differences in toxicokinetics. Due to the antibacterial activity of dialkyltins, guinea pigs die from disturbed caecal bacterial fermentation. A recent study indicates that the human thymus may be sensitive to organotins:



Figure 1. Low power microphotograph of a control (left) and a TBTO-exposed guppy (right) showing the localization of the paired thymus (arrows). Note the very strong thymic atrophy in the exposed animal (HE, x 25).

fermentation. A recent study indicates that the human thymus may be sensitive to organotins: SCID-hu mice exposed to DBTC showed reduction in the relative size of the cortex of the human thymus transplant, similar to the effect observed in the rat (De Heer *et al.*, 1995a). Species diferences were also noted in fish. DBTC and TBTO (a compound of paramount importance for the aquatic environment because of its use as an anti-fouling agent) induced thymic atrophy in the guppy (Figure 1) but, surprisingly, the medaka showed no such effect (Wester *et al.*,1990). Currently under investigation is whether TBTO is immunotoxic to the flounder (*Platichthys flesus*), a flatfish species common to coastal areas, estuaries and large rivers in the Netherlands and therefore, particularly exposed to pollution. Field studies and studies with flounder kept under semi-field (mesocosm) conditions indicate a causal link between water/sediment contamination, liver tumor occurrence and lymphocystis virus infection resulting in wart-like lesions in the skin (Vethaak, 1993). The role of immunotoxicants including TBTO in these diseases will be investigated in a study with flounder kept under laboratory conditions (Grinwis *et al.*, 1995).

HCB-Induced Immunotoxicity and Autoimmunity

HCB is a highly persistent environmental chemical that has been used in the past as a fungicide. Presently, emission in the environment may occur as a result of the use of HCB as a chemical intermediate or as a byproduct in chemical processes. It is an immunomodulating compound, with immunosuppressive effects in mice and immunostimulatory effects in rats (Vos, 1986).

HCB reduced in BALB/c mice the cell-mediated immunity and the humoral immunity (primary and secondary antibody responses to sheep red blood cells), as well as the resistance to protozoan

infections (*Leishmania* and *Plasmodium berghei*) and a number of tumor cell lines (BALB/c and DBA/2 mice). Moreover, it increased the lethal action of endotoxin (Loose, *et al.*, 1977; 1978). HCB also diminished graft-versus-host activity (Silkworth and Loose, 1981), and the cytotoxic, phagocytic and microbicidal activity of alveolar, splenic and peritoneal macrophages (Loose *et al.*, 1981). The susceptibility to HCB is greater following pre- or perinatal administration (Barnett, *et al.*, 1987).

In rats, prominent changes following dietary exposure include elevated IgM levels and an increase in the weights of the spleen and lymph nodes. Histopathologically, the spleen shows hyperplasia of B-lymphocytes in the marginal zone and follicles, while lymph nodes show an increase in proportions of high endothelial venules, indicative of activation. High endothelial-like venules are induced in the lung, as are accumulations of macrophages. Functional tests revealed an increase in cell-mediated immunity, as measured by DTH reactions, a notable increase in primary and secondary antibody response to tetanus toxoid, and decreased NK activity in the lung (Vos, *et al.*, 1979a; 1979b; Van Loveren, *et al.*, 1990). Stimulation of humoral and cell-mediated immunity occurred at dietary levels as low as 4 mg/kg following pre- and postnatal exposure; at such a dose conventional parameters of hepatotoxicity were unaltered (Vos, *et al.*, 1983). Therefore, the developing immune system of the rat seems particularly vulnerable to the immunotoxic action of HCB.

More recent studies indicate that HCB might cause autoimmune-like effects in the rat. Wistar rats treated with HCB had elevated levels of IgM, but not IgG, against the autoantigens single-stranded DNA, native DNA, rat IgG (representing rheumatoid factor), and bromelain-treated mouse erythrocytes (that expose phosphatidylcholine as a major autoantigen) were elevated. It has been suggested that HCB activates a recently described B cell subset committed to the production of these autoantibodies (Schielen, *et al.*, 1993). The role of these autoantibodies is still a matter of controversy. Increased levels have been associated with various systemic autoimmune diseases, but a protective role of these autoantibodies against development of autoimmune disease has been postulated as well. Interesting in this respect are the observations that HCB had opposite effects in two different models of autoimmune disease in the Lewis rat. HCB treatment severely potentiates allergic encephalitis elicited by immunization with myelin in complete Freund's adjuvant, while it strongly inhibits the development of arthritic lesions elicited by complete Freund's adjuvant (Van Loveren, *et al.*, 1990).

The chemical HCB has been the subject of intense research following the accidental poisoning in Turkey from 1955 to 1959: it has been estimated that 4000 people developed porphyria due to the ingestion of wheat that had been treated with the fungicide HCB (Cam, 1960). Clinically, patients developed skin lesions that have been attributed to the the toxicity of photochemically activated cutaneous porphyrins. In a follow-up study of 204 patients, 20-30 years later, dermatological and other abnormalities, such as painless arthritis, still persisted (Cripps, *et al.*, 1984). Regarding the mechanism of HCB-induced hepatic porphyria, an oxidative metabolite and not the parent compound has been found to be responsible for the porphyrinogenic action. In rats treated with the combination of HCB and triacetyloleandomycin (TAO, a selective inhibitor of cytochrome P450IIIA), porphyria was greatly reduced (Van Ommen, *et al.*, 1989; Den Besten, *et al.*, 1993). Remarkably, combined treatment with HCB and TAO did not substantially affect the incidence and severity of skin lesions. In addition, TAO did not influence the immunomodulatory effects of HCB,

Table 2
Role of Metabolism in the Target Organ Toxicity of Hexachlorobenzene in the Rat

Treatment	Hepatic porphyria	Immune effects	Skin lesions
HCB	+	+	+
HCB + TAO[1]	-	+	+

[1]Triacetyloleandomycin, a selective inhibitor of P450IIIA
After: Van Ommen, *et al.*(1989), Den Besten, *et al.*(1993), Schielen, *et al.*(1993; 1995).

including the formation of autoantibodies. From these findings it has been suggested that an immunological component, at least in part underlies the HCB-induced skin lesions in the rat and possibly in man (Schielen, *et al.*, 1995) (Table 2). Similarly, an autoimmune etiology is conceivable as to other symptoms in HCB-poisoned patients in Turkey such as enlarged thyroid and painless arthritis (Cripps, *et al.*, 1984). This certainly needs future research.

CONCLUSIONS

The use of toxicity data obtained in laboratory animal species for risk characterization of man or wildlife poses difficulties when 1) species differ qualitatively in the target organ of toxicity, 2) interspecies variation in sensitivity to the contaminant is substantial, or 3) levels of contaminants are unknown. Examples of environmental contaminants for which data in laboratory animals show qualitative or quantitative interspecies differences are PCBs, PCDFs and PCDDs, organotin compounds, and HCB. For these chemicals, toxicity data in man and in wildlife populations are necessary for an adequate understanding of the immunomodulating risks.

PCBs, PCDFs and PCDDs. Data on the sensitivity of the human immune system, necessary for the process of risk characterization, were obtained in the SCID-hu and SCID-ra models. Results of the study indicate that the human thymus and the Wistar rat thymus display a similar sensitivity to TCDD. In a study carried out under semi-field conditions, seals fed fish from an organochlorine-contaminated environment have suppressed immune function, as measured by NK cell activity, and T cell functions. Seals inhabiting heavily polluted coastlines may therefore be more susceptible to infectious diseases, and environmental contaminants may have played a role in the 1988 phocine distemper virus epizootic among harbor seals in Europe.

Organotins: the thymotoxicity of organotin compounds is species specific. Rats are very sensitive and host resistance is strongly impaired. However, thymus atrophy does not occur in mice, guinea pigs or Japanese quail following oral organotin exposure. An indication for the sensitivity of the human thymus comes from a recent study in which SCID-hu mice were exposed to DOTC. Studies performed in fish with the anti-fouling agent TBTO show thymotoxicity in the guppy and no effect on the thymus in the medaka, indicating the importance of further investigations.

sensitive. HCB exposure of Lewis rats can modulate models of autoimmune disease. Studies also show that rats treated with HCB have elevated autoantibody levels. These findings and the association in rats of skin lesions with immune effects and less so with accumulation of porphyrins, make an autoimmune etiology conceivable for the disease symptoms that have persisted for 20-30 years in HCB-poisoned patients in Turkey.

For certain chemicals, interspecies differences in immunomodulation are found, although among higher vertebrates the immune system appears highly conserved, and immune components and their interactions are extremely similar between mice, rats and humans. As the purpose of risk assessment is to make inferences about potential risk to human health, the process will benefit from human data, either by testing in man itself, or using human material (*in vitro*), or special constructs (*e.g.* xenografts, transgenic animals). For risk assessment at the ecosystem level laboratory studies with the species at risk or semi-field studies are an adequate strategy.

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Chapter 31

Approaches to Interspecies Extrapolation: Use of Human Clinical and Laboratory Mouse Data from Ozone and Ultraviolet Radiation Immunotoxicity Studies to Generate Prototype Parallelogram and Ladder Models

MaryJane K. Selgrade, Ph.D.

Experimental Toxicology Division
National Health and Environmental Effects Research Laboratory
United States Environmental Protection Agency
Research Triangle Park, NC 27711

ABSTRACT

One of the most problematic issues in risk assessment is extrapolation from animal data to human health effects. Parallelograms have often been used to make qualitative comparisons between effects in laboratory rodents and humans. (In such models different species are represented by the vertical sides and *in vitro* vs *in vivo* or immune function vs host resistance are represented by the horizontal sides of the parallelogram. Each corner represents a measurable parameter). These models also address problems associated with extrapolation from *in vitro* data to *in vivo* effects and extrapolation across levels of biologic organization from effects at the cellular level (e.g., immune function) to effects at the organism or population level (e.g., disease susceptibility). By stacking parallelograms to make ladders both *in vitro* to *in vivo* and level of organization issues can be considered. Parallelograms and ladders are currently being used to develop more quantitative models. Interspecies comparisons are ideally made when data from similarly designed, controlled experiments are available for both species. For humans, such data are rare. For two agents, ozone (O_3), which suppresses alveolar macrophage (AM) phagocytosis, and ultraviolet radiation (UVR) which suppresses contact sensitivity, there are data from controlled human studies that are similar to data generated in rodent studies. Results of experiments in which AM from humans and mice were exposed *in vitro* to 0.8 ppm O_3 indicated the sensitivity of AM from the two species was the same. Similarly, when effects of *in vivo* O_3 exposure on AM phagocytosis were compared following dosimetric adjustments, the sensitivity of the two species was essentially identical. Suppression of AM function shows good correlation with enhanced susceptibility to bacterial pneumonia in mice and rats. Likewise, sensitivity of mice and humans to UVR-induced suppression of contact sensitivity is very similar in mice and humans; suppression of this response in mice results in enhanced susceptibility to mycobacterial infections. These studies suggest that data obtained in mice predict effects in humans, and that human studies may be useful in developing more

quantitative risk assessment models that can then be applied to situations where human data is not available.

INTRODUCTION

One of the most problematic issues in risk assessment is extrapolation from animal data to human health effects. It is rare to have dose-response data on the toxicity of a compound in humans. Hence, it is necessary to rely on animal data in establishing safe exposure levels or in determining risks associated with various levels of exposure. Fortunately, the immune system has been highly conserved across species such that the organs and cells of the immune system in humans, mice, and rats are very similar (Du Pasquier, 1989). Therefore, it is reasonable to test for potentially adverse effects on the human immune system using laboratory rodents. Also, in the few instances where it has been possible to compare the effects of an immunotoxic agent in humans and laboratory rodents, results have been comparable (Selgrade *et al.*, 1995a). This paper reviews human clinical and laboratory rodent data for two immunotoxic agents: (1)ozone (O_3), which suppresses alveolar macrophage (AM) phagocytosis, and (2) ultraviolet radiation (UVR), which suppresses contact hypersensitivity (CHS). Qualitative comparisons between the two species are made, and approaches are suggested for using the data to develop more quantitative models that might then be applied to situations where human clinical data is not available.

Parallelograms have often been used to make comparisons between effects in laboratory rodents and humans (Figure 1). In these models different species are represented by the vertical sides. In addition to interspecies extrapolation (moving horizontally across the diagram), moving vertically

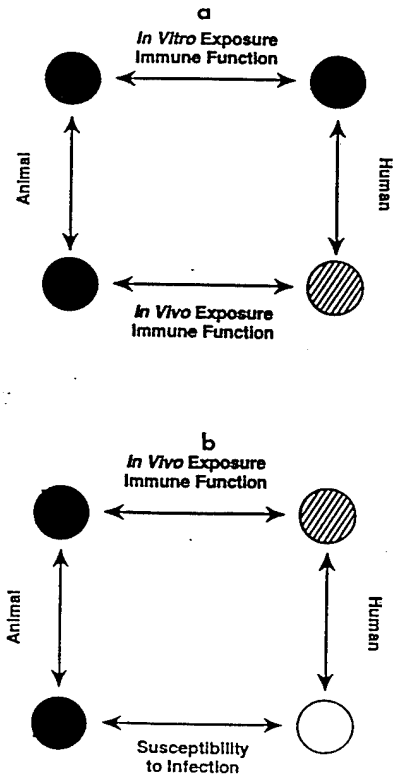


Figure 1. Parallelogram models. (a) Model of the relationship between suppression of human and rodent immune cell function following *in vitro* and *in vivo* exposure to a toxicant. (b) Model of the relationship between suppression of immune function and susceptibility to infection in humans and laboratory rodents following toxicant exposure. ● =- data which can be readily obtained experimentally; Ø = data that can sometimes be obtained in human clinical studies and sometimes must be extrapolated from other points on the parallelogram; O = data that cannot ordinarily be obtained experimentally and must be extrapolated based on other points on the parallelogram.

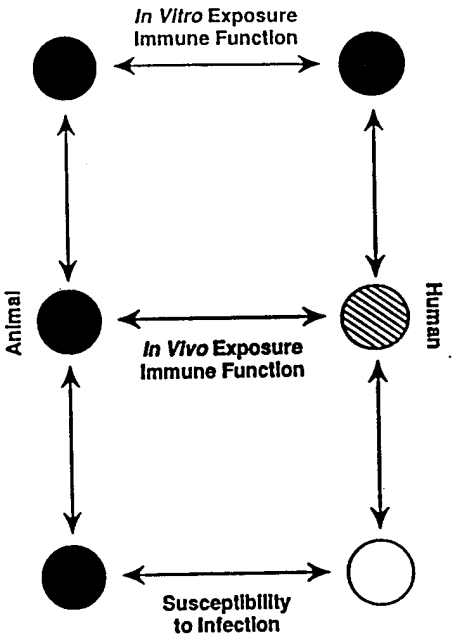


Figure 2. Ladder model designed to use both *in vitro* and *in vivo* data. ● = data that can be readily obtained experimentally; Ø = data that can sometimes be obtained in human clinical studies and sometimes must be extrapolated from other points on the ladder; O = data that cannot ordinarily be obtained experimentally and must be extrapolated based on other points on the ladder.

down the models addresses problems associated with extrapolation from *in vitro* data to *in vivo* effects and extrapolation across levels of biologic organization from effects at the cellular level (e.g., immune function) to effects at the organism or population level (e.g., disease susceptibility). Each corner represents a measurable parameter. The models can be expanded by stacking parallelograms to make ladders (Figure 2). To date this approach has only been used to make qualitative comparisons; however, more quantitative comparisons could be made by developing dose-response curves for two or more corners of the model, obtaining a function (or equation) to describe these dose responses, and then making quantitative comparisons from one corner to another. Although thay have been developed to make interspecies comparisons between laboratory rodents and humans, these same approaches could also be applied to other types of interspecies comparisons, including alternate species mention in other chapters in this volume. Although the primary focus has been on health risk assessment, these models might also be useful for certain types of ecologic risk assessments. Also, the problems that these models address are not unique to immunotoxicity but are common to many other toxicity endpoints. Hence this approach might have wider applications. Immunotoxicity data from human clinical and rodent studies of O_3 and UVR provide ideal data sets to begin developing such models.

OZONE

A number of air pollutants enhance susceptibility of mice to challenge with *Streptococcus zooepidemicus*, a relatively avirulent group C *Streptococcus* (reviewed by Selgrade and Gilmour, 1994). In these experiments mice are exposed to pollutant or filtered air for 3 hr. Both treatment groups are then put in the same chamber and challenged with an aerosol of *Streptococcus zooepidemicus*, which results in the deposition of between 200-4000 bacteria (Miller *et al.*, 1978) in the lung (not an overwhelming dose). Subsequently, mortality over 20 days is measured as an indicator of disease susceptibility. Few if any air controls die, however significant increases in mortality have been observed at O_3 levels as low as 0.1 ppm (Coffin and Gardner, 1972; Ehrlich *et*

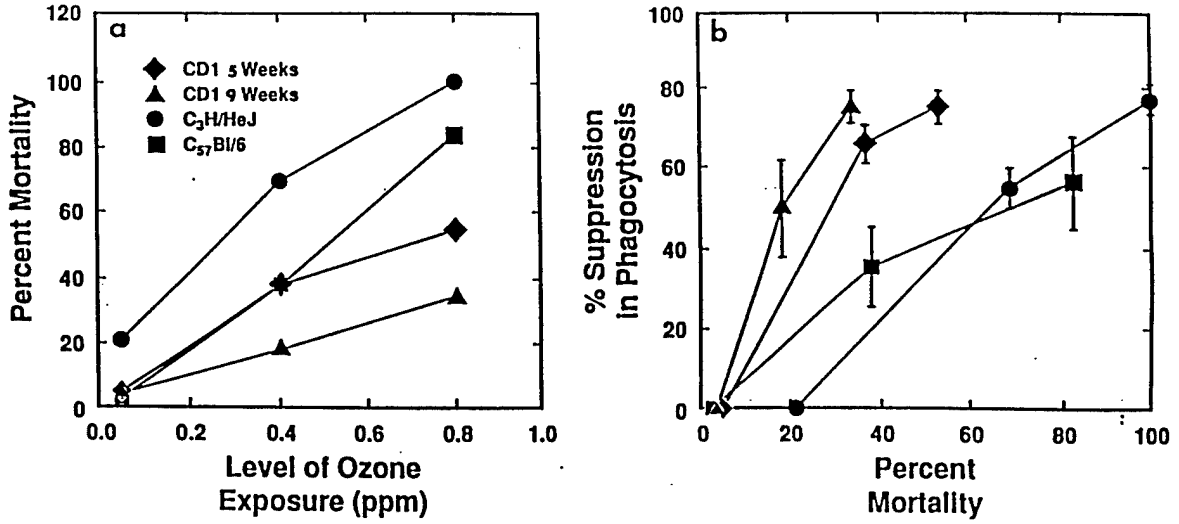


Figure 3. Relationship between suppression of alveolar macrophage phagocytosis and O_3-enhanced mortality due to infection. The strain of mouse that is most sensitive to O_3-enhanced infection (a) is the strain that can tolerate the least suppression of AM phagocytosis (b) and the strain that is the most resistant to O_3-enhanced infection (a) can tolerate the greatest suppression of AM phagocytosis (b) suggesting that toxicant effects on AM phagocytosis should be predictive of enhanced susceptibility to bacterial infection.

al., 1977). It should be noted that the National Ambient Air Quality Standard for O_3 is 0.125 ppm. In air controls the bacteria are cleared from the lung by 24 hr postexposure; delayed clearance and then replication of bacteria are observed in mice exposed to O_3. In the classic experiment using 5 wk old, CD-1 mice, greater than 60% mortality was observed following exposure to 0.8 ppm O_3 (Gilmour *et al.*, 1993a). Different ages and strains of mice have different susceptibilities to O_3-enhanced infection (Gilmour *et al.*, 1993a,b) In order of susceptibility 5-wk-old C3H/HeJ mice are most susceptible, followed by 5-wk-old C57BL/6, 5-wk-old CD-1 mice, and finally, the most resistant, 9-wk-old CD-1 mice (Figure 3). When AM cells from the different mouse strains were obtained from broncho-alveolar lavage fluid and assessed *in vitro* for phagocytic activity using uptake of fluorescein-labeled latex beads, C3H/HeJ mice had lower baseline AM phagocytic activity in the air-exposed mice. It is therefore not surprising that the phagocytic activity following O_3 exposure of these mice was lower than that for other mouse strains (Gilmour *et al.*, 1993b). When the percent suppression of phagocytosis for the different ages and strains of mice with different susceptibilities to O_3-enhanced infection were plotted against percent mortality (Figure 3), the strains that were most resistant to ozone could tolerate the greatest decrease in phagocytosis; whereas, the most susceptible strain could tolerate the least suppression of phagocytosis. In fact, in the most susceptible strain some deaths occurred in air controls with 0% suppression of phagocytosis. Certainly these data are consistent with the hypothesis that effects of O_3 on this infectivity model are primarily due to effects on AM phagocytosis. Similar strain differences have

been observed with NO_2 and phosgene enhancement of Streptococcal infection (unpublished data), suggesting that the macrophage is also an important target for other pollutants. Hence, AM phagocytic function should be predictive of enhanced susceptibility to infection and can be used as the parameter of interest for the top left hand corner of the parallelogram in Figure 1b when susceptibility to *Streptococcus zooepidemicus* is used as the parameter of interest for the lower left hand corner.

Recently, it has been demonstrated that the *Streptococcus zooepidemicus* infectivity model can be applied to rats (Gilmour and Selgrade, 1993). Exposure to O_3 suppressed AM phagocytic function and delayed clearance of bacteria from the lung. However, rats did not die as a result of the infection, but eventually cleared the bacteria from their lungs. Recovery from infection in the rats appeared to be due to a more rapid influx of neutrophils (PMNs) in the rat as compared to the mouse. Recovery of PMNs from lavage fluid peaked at 24 hrs as compared to 2-4 days in mice. Rat PMNs may also be more efficient at killing bacteria than mouse PMNs. Although rats do not die, they do experience delayed clearance of bacteria and an inflammatory response in the lung that does not occur with bacteria or O_3 (at the exposure levels used in these studies) alone. These effects then become the parameters of interest for disease susceptibility in the lower left hand corner of the parallelogram for rat vs human comparisons. Suppression of AM phagocytosis remains the parameter of interest for the upper left hand corner. Enhanced susceptibility of the rat to *Streptococcus* has also been demonstrated following acute and chronic phosgene exposure (Yang *et al.*, 1995; Selgrade *et al.*, 1996).

Finally, suppression of AM phagocytic activity has been demonstrated in human subjects following O_3 exposure (Devlin *et al.*, 1991). Hence there are data to apply to the upper right hand corner of the parallelogram. To date, effects of O_3 on human AM phagocytosis have only been tested using one exposure regimen, 0.08 ppm for 6.6 hr while undergoing intermittent moderate exercise. Table 1 shows a comparison of mouse and human AM sensitivity to *in vivo* O_3 exposure. Dosimetric differences between mice and humans have been reported by Slade et al. (personal communication) based on a comparison of ^{18}O deposition in humans and mice exposed to O_3 labeled with this stable isotope. When one makes dosimetric adjustments based on this data and assumes that the response of AM to O_3 is linear over some portion of the dose-response curve, the sensitivity of human and mouse AM to *in vivo* O_3 exposure appears to be similar (Selgrade *et al.*, 1995a). With this limited amount of data qualitative comparisons can be made between the top corners of the parallelogram in Figure 1b. Currently, efforts are under way to develop dose-response curves for these two corners so that a more quantitative relationship can be established.

It is rare to be able to obtain data from controlled human studies following *in vivo* exposure to toxicants. O_3 is an exception. It is, however, possible to obtain AM from humans and expose them to toxicants *in vitro*. When human and mouse AM obtained from broncho-alveolar lavage were exposed to 0.8 ppm O_3 for 3 hr, the percent suppression in phagocytic activity was 29 and 21%, respectively. The difference between human and mouse AMs was not statistically significant (Selgrade *et al.*, 1995a). Again, efforts are underway to obtain a dose-response for these endpoints which are represented by the two upper corners of the parallelogram model in Figure 1a and the ladder model in Figure 2. The hypothesis is that quantitative relationships can be developed using O_3 data in these models and that such information will then be useful in interpreting data for more toxic air pollutants, such as phosgene, where human *in vivo* data is not available.

Table 1.
Effect of *In Vivo* Ozone Exposure on Alveolar Macrophage Phagocytosis

Treatment	Mice	Humans
Air	330.6 (10.4)[a] n=4	714.9 (46.1) n=10
Ozone[b]	194.0 (19.7)[c] n=4	539.2 (22.3)[c] n=10
% suppression	42%	25%
% suppression corrected[d] for dosimetric difference	28%	25%

[a]Mean (standard error) of the phagocytic index i.e., the number of fluorescent particles ingested per 100 macrophages.
[b]Mice were exposed to 0.8 ppm for 3 hr; humans were exposed to 0.08 ppm for 6.6 hr while undergoing intermittent exercise.
[c]Significantly different from air control ($p<0.05$; Student's T test)
[d]Based on studies using ^{18}O, alveolar macrophages of mice exposed to 0.8 ppm ozone for 3 hrs receive roughly 1.5 times more ozone than those of humans exposed to 0.08 ppm ozone for 6.6 hrs while exercising moderately. (Selgrade *et al.*, 1995 for more details).

The current data base certainly suggests that humans exposed to O_3 are at increased risk of bacterial infection. The consequences of that risk depend on exposure to an infectious agent at the time AM macrophage function is compromised, the virulence and dose of that agent, and how rapidly secondary lines of defense such as PMNs are mobilized.

ULTRAVIOLET RADIATION (UVR)

Human clinical and laboratory rodent studies of UVR-induced immune suppression provide another opportunity to make direct comparisons between human and rodent responses. A large body of research in mice has shown that exposure to UVR suppresses both contact and delayed-type hypersensitivity (CHS and DTH) responses in mice as the result from of cytokines released from keratinocytes following UV-exposure (reviewed by Rivas and Ullrich, 1994; Chapman *et al.*, 1995). These cytokines alter antigen presentation such that Th1-type responses are suppressed.

When mice were exposed to UVR at one site and injected 3 days later at a distant site with *Mycobacterium bovis* BCG (the vaccine strain for tuberculosis), the DTH response to BCG was suppressed and the total number of bacteria in the spleen and lymph node were increased in a dose responsive manner (Jeevan and Kripke, 1990). These data can be applied to the two left hand corners of the parallelogram in Figure 1b. The lowest exposure dose that caused significant suppression of the DTH response was 1.4 kJ/m^2 whearas the lowest dose at which significant increases in bacteria were observed was 0.7 kJ/m^2. Hence, the sensitivity of the immune function test and the host resistance model were similar. It should be noted that the dose of UVR that produces minimal erythema in BALB/c mice is 2.2 kJ/m^2. Enhanced susceptibility of mice to *Mycobacterium lepraemurium* (an infection which resembles human leprosy) has also been demonstrated following UVR exposure (Jeevan *et al.*, 1992), suggesting that the course of chronic as well as acute infections may be altered by UVR.

Table 2
Comparison of Ultraviolet Radiation Doses That Cause 50% Suppression of Contact Sensitivity in Mice and Humans

Mice[a]	Human[b]		
Sensitivity of Phenotype	mJ/cm^2	Skin Type	mJ/cm^2
high (C57BL)	70-230	fair	90
intermediate (C3H)	470-690	intermediate	140
low (BALB/c)	960-1230	dark	150

[a]Data from Noonan and Hoffman, 1994
[b]Data from Oberhelman *et al.*, 1994; Cooper personal communication

UVR also suppresses the contact hypersensitivity response to haptens such as dinitrofluorobenzene (DNFB), a response that is similar although not identical to the DTH response. The UVR dose response associated with suppression of CHS in mice is the same when UVR exposure occurs at the site of DNFB sensitization or at a distant site (Noonan and De Fabo, 1990). However, effects at a distant site require a 3-day delay between exposure and sensitization; whereas, effects at the same site can be observed immediately. Also, different strains of mice have different sensitivities to the immunosuppressive effects of UVR (Noonan and Hoffman, 1994).

Although there have not been controlled human studies on the effects of UVR on the DTH response, there are data on effects of UVR on CHS responses to DNCB (Cooper *et al.*, 1992; Yoshikawa *et al.*, 1990), including dose-response data (Table 2). If CHS responses in mice and humans are used to describe the top corners of the parallelogram in figure 1b, the response of humans and mice appear to be very similar (Table 2). In fact the intraspecies variability among strains of mice appears to be greater than the interspecies variability between humans and mice.

Finally, just as mice exhibit phenotypic differences with respect to UVR-induced immune suppression, UVR-resistant and susceptible phenotypes (unrelated to pigmentation) have been described for the human population (Yoshikawa *et al.*, 1990). The susceptible phenotype occurred in approximately 60% of normal healthy volunteers but occurred in 92% of skin cancer patients (Yoshikawa *et al.*, 1990). This data suggests that the UVR susceptible phenotype is a risk factor for skin cancer and provides qualitative information for the bottom right hand corner of the parallelogram in Figure 1b.

CONCLUSIONS

The studies described here demonstrate that the effects of O_3 and UVR on immune responses in mice and humans are similar. Hence, for these two agents, effects in the mouse were good predictors of human effects. Complementary human and rodent studies such as these may be used in constructing more quantitative risk assessment models based on a parallelogram approach. These models may then be helpful in interpreting rodent and *in vitro* data for agents for which human clinical data is not available. These models could certainly be extended to disease endpoints and immune function endpoints other than those specifically described in this paper. They also could be applied to other types of interspecies comparisons.

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Chapter 32

Chemical Toxicity and Host Defense in Invertebrates: an Earthworm Model for Immunotoxicology

A.J. Goven, L.C. Fitzpatrick and B.J. Venables[1]

Department of Biological Sciences
University of North Texas
Denton, Texas 76203

[1]TRAC Laboratories
113 Cedar Street
Denton, Texas 76201

ABSTRACT

Assays using immune function in vertebrates have been used to assess sublethal toxic potential and modes of action of chemicals and environmental pollutants. Some of these assays are based on immune functions that are phylogenetically conserved throughout the animal kingdom. Herein, we suggest that several of these functions found in invertebrates are likely candidates for use as biomarkers to evaluate toxic potential of chemicals and environmental pollutants. We discuss immunoassays developed with earthworm, *Lumbricus terrestris*, coelomocytes (immune cells) in support of using invertebrates for cost-effectively screening chemicals and assessing toxic potential of environmental contaminants.

INTRODUCTION

Understanding toxic potential and mechanisms of action of environmental contaminants is fundamental for assessing risk to public and environmental health. Although methods have been developed for screening chemicals and studying their modes of action on a variety of acute toxic endpoints and subchronic-chronic processes, only relatively recently have scientists become aware of the broad spectrum of chemicals that alter immune function and of the immune system's potential for use in assessing sublethal toxicity of exposure to chemicals (Dean, *et al.*, 1986). Since there is considerable information on the immune system, immune responses are especially well suited for comparative analyses which emphasize mechanisms of chemical toxicity. Additionally, since immune responses are important in host defense, their modulation may result in increased infections that could influence survival of individuals and populations. Subcellular, cellular and organismal immunological indicators of exposure to or effects of chemicals, especially those that can be demonstrated at exposure levels shown to be nontoxic by traditional toxicity evaluation, can be used for risk assessment tools. Such immunological indicators fall within the category of

sensitive sublethal measurement endpoints (Suter, 1989) defined as biomarkers (Weeks, *et al.*, 1992).

Although immune system complexity has increased during animal evolution, certain aspects of immunity have been conserved phylogenetically; immunocytes in one form or another can be found in phyla above Porifera. Existing basic and comparative information on immune systems of invertebrates suggest that invertebrates are useful for developing immune based biomarkers to assess toxic potential of environmentally relevant chemicals (see Beck, Habicht, Cooper and Marchalonis, 1994). Invertebrates, which often dominate animal biomass, energy flow pathways and nutrient cycling within ecosystems represent an ecologically important route of environmental contaminants to vertebrate species. Alterations in invertebrate immune function may serve as valuable early warning symptoms of contaminants which threaten survival of individuals or populations and ecosystem health. Among invertebrates, earthworms possess a number of attributes which make them an especially appropriate choice for development of immune-based biomarkers for investigation of effects of chemicals on host immune defense systems.

REVIEW

Rational for the Earthworm Model for Terrestrial Toxicology

Physico-chemical complexity of parent materials and dispersion heterogeneity of contaminants pose notable challenges for toxicological risk and clean-up assessments of terrestrial hazardous waste sites (HWS) as well as accidental contamination events in terrestrial ecosystems. Dispersion heterogeneity often increases the number and bulk of samples to be handled. Physico-chemical heterogeneity of solid matrix samples requires special mixing techniques to assure consistent exposure for test organisms. Specialized techniques are necessary for fractionation and chemical analyses of solid matrices. And, direct exposure to parent materials requires using non-traditional and ecologically relevant test organisms.

In 1988 we began to address these challenges to terrestrial toxicology by developing a model immunoassay system using earthworms, *Lumbricus terrestris,* as non-vertebrate surrogates for assessing immunotoxic potential of chemicals. Development of earthworm immune-based biomarkers was based on a need for rapid, sensitive, cost-effective and socially non-controversial surrogate immunoassay protocols for use as an adjunct or complement to existing protocols with mammals. Such an assay system would be used to screen chemicals to determine if further tests using mammals are required.

Earthworms were selected for several reasons: (1) Their immune functions appear to be sufficiently complex for use in screening chemicals for immunotoxic potential (Chen, *et al.*, 1991; Goven, *et al.*, 1993, 1994; Suzuki, *et al.*, 1995; Cooper, 1974, 1976; Hostetter and Cooper, 1974; Cooper and Stein, 1981); (2) being virtually ubiquitous and ecologically important soil organisms, they are valuable in situ sentinels for use in assessing environmental contamination; (3) earthworm behavior and morphology enable their direct exposure to complex environmental mixtures and matrices of pollutants (Reineke, 1992); (4) earthworms are easy and inexpensive to maintain for immunological

experiments; (5) their basic biology is well known; and (6) they are currently used in standardized acute toxicity protocols for laboratory and in situ bioassays (Greig-Smith, *et al.*, 1992).

Selection of Immunoassays

Criteria we used to select immunoassays were that they had to: (1) Be coelomocyte-based; (2) measure earthworm immune functions analogous to those in vertebrates; (3) predict secondary immunodeficiency effects such as reduced host resistance; and (4) have low inherent variability. Coelomocytes, earthworm immunocytes, are easily obtained in large numbers through a non-invasive extrusion technique we developed (Eyambe, *et al.*, 1991). Immunoassays must be analogous to those in vertebrates for use in predicting potential chemical effects on vertebrate immune responses. Immunoassays diagnostic or predictive of secondary immunodeficiency effects, such as reduced host resistance to disease following chemical exposure, are valuable because they measure functional immunologic responses. As such they may be useful in public health risk assessment. Biomarkers or measurement endpoints should have low variability in response to chemicals but, consistency in response to non-chemical influences (i.e. habitat changes) (Suter, 1989) to achieve maximum signal-to-noise ratio (chemical response: natural variability). Ideally, biomarkers should measure something of ecological significance, be easily and unambiguously quantifiable, biologically appropriate for expected exposure routes and indicative of specific classes of chemicals (Suter, 1989).

Herein we report on immunomodulatory effects of chemicals on earthworms, *L. terrestris*, as indicated by immunoassays measuring: (1) Innate immunity as lysozyme activity; (2) immunopathology as total coelomocyte counts (TCC), differential coelomocyte counts (DCC) and coelomocyte viability; and (3) nonspecific immunity (NSI) as phagocytosis and nitroblue tetrazolium (NBT) dye reduction, which indicates a cell's ability to oxidatively kill phygocytosed microorganisms. We limit this review to immunoassays which satisfy criteria noted above.

Assessment of Immunotoxic Effects of Chemicals on Earthworm Host Defense

Innate immunity: Effect of Cu++ on lysozyme activity

Lysozyme, is an enzyme capable of bactericidal activity via action on peptidoglycan of Gram-positive bacterial cell walls and functions as a component of an organism's innate or natural antimicrobial defense mechanism (Salton, 1975). We have observed lysozyme activity in earthworm coelomic fluid and coelomocyte extracts, and have demonstrated that substrate specificity and temperature influence is similar to that found in mammalian serum and leukocyte extracts (Goven, *et al.*, 1994). Correlation between increasing lysozyme activity in coelomocyte extracts and increasing coelomocyte numbers suggests that lysozyme is produced by and released from coelomocytes (Goven, *et al.*, 1994). It is believed that serum lysozyme activity in mammals results from lysozymes being released by neutrophils within the vasculature (Zucker, *et al.*, 1970). Lysozyme activity is a primitive innate immune defense mechanism associated with the granulocyte, monocyte-macrophage system of mammals, and with coelomocytes in earthworms (Zucker, *et al.*, 1970; Goven, *et al.*, 1994). Reduced lysozyme activity in earthworms after chemical exposure should enable prediction of chemical immunotoxicity in vertebrates.

We evaluated earthworm lysozyme activity as an assay for chemical immunotoxicity using Cu^{++} (as $CuSO_4$), which is known to inhibit vertebrate lysozyme activity (Feeney, *et al.,* 1956). Earthworms exposed on filter paper (FP) to sublethal concentrations of Cu^{++} (LC_{50} = 2.58 µg Cu^{++}/cm^2FP) at 10°C for 5 days showed significantly reduced lysozyme activity in both coelomic fluid and coelomocyte extracts (Goven, *et al.,* 1994). Coelomic fluid lysozyme activity decreased to 40 and 50% of controls after exposure to 0.5 and 1.0 µg/cm^2 Cu^{++}, respectively. Lysozyme activity of coelomocyte extracts decreased to 54% of controls after exposure to 1.0 µg/cm^2 Cu^{++}. Although exposure to 0.5 µg/cm^2 Cu^{++} did not significantly reduce lysozyme activity of cell extracts, exposure reduced enzyme activity to 72% of controls. Body-burden Cu^{++} concentration was 28.5 and 73.1 µg/g dry mass in earthworms exposed to 0.5 and 1.0 µg/cm^2 of Cu^{++}, respectively, indicating a dose response relationship between Cu^{++} and reduction in lysozyme activity.

It is likely that Cu^{++} interacts with or binds to lysozyme in a manner adversely affecting the functional conformation of the enzyme leading to attenuation or inactivation. Metal ions, including Cu^{++}, tend to bind to basic proteins such as lysozyme (Jolles and Jolles, 1984) leading to chemical reactions that catalyze hydrolysis of peptide bonds or disulfide linkages, or breakage of hydrogen bonds causing structural changes in enzymes (Feeney, *et al.,* 1956).

Reduced lysozyme activity suggests immunosuppression that could reduce resistance to bacterial challenge. The earthworm lysozyme activity assay appears to be sufficiently sensitive for measuring sublethal effects of chemicals on an important innate immune function.

Cytological biomarkers: Coelomocyte morphology and effects of PCB on TCC, DCC and coelomocyte viability

Analysis of cellular light scatter properties (forward angle light scatter; integrated 90° light scatter) using flow cytometry indicates that *L. terrestris* possesses three subpopulations of coelomocytes as defined by size and granularity (Figure 1). These subpopulations can be morphologically characterized as : Small agranular; large granular; and large agranular. Coelomocytes exhibit light scatter properties that are similiar to murine peritoneal lymphocytes, granulocytes and monocytes, respectively. Similarities to murine peritoneal phagocytic cells (monocytes and neutrophils) strengthens our contention that phagocytosis by earthworm coelomocytes has potential as a broadly applicable measurement endpoint for assessing non-specific immunotoxicity risks to higher wildlife, including mammals. Identification and density gradient separations of phagocytic coelomocytes should enable direct comparison with similarly enriched murine phagocytic cells, allowing establishment of earthworm: mouse dose equivalence response profiles for various classes of chemicals and their mixtures. Such profiles, established *in vitro* and *in vivo* would be invaluable in assessing immunotoxic risks to mammals.

Cytological parameters of TCC, DCC and coelomocyte viability represent a logical set of easily measured, sensitive and stable biomarkers for assessing acute and chronic immunotoxicity. Chemical effects on these basic cytological parameters indicate effects on both the overall health of an earthworm, and potential problems in NSI and specific-immunity as a complete blood count would in mammals.

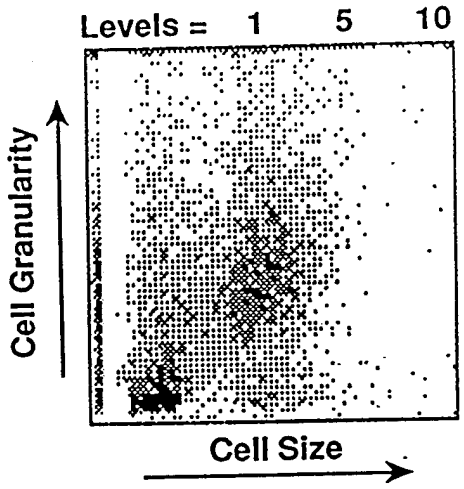


Figure 1. Coelomocytes extruded from unexposed control earthworms (*Lumbricus terrestris*)analyzed by flow cytometry. A dual parameter dot plot of cell size (forward angle light scatter) vs cell granularity (integrated 90° light scatter) illustrates the representative cell populations recovered from the coelomic cavity.

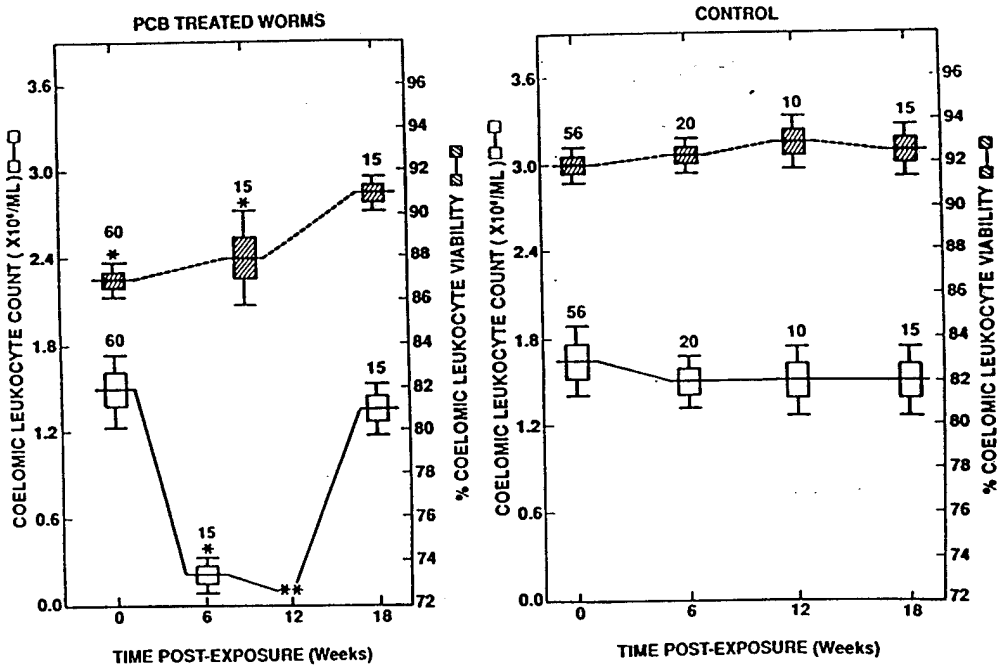


Figure 2. Effects of 5-day PCB (Aroclor 1254) filter paper exposure (10 µg/cm^2) on total numbers (open rectangles) and viability (shaded rectangles) of coelomocytes collected from *Lumbricus terrestris.* All exposed and unexposed earthworms were extruded at 0 hours, and 6, 12 and 18 weeks postexposure. Horizontal line is the mean,rectangles are standard error and vertical lines represent 95% confidence interval. Sample sizes are indicated by numbers above assay points. Asterisks indicate means significantly different from controls. Double asterisks at 12 weeks indicate too few cells were extruded to obtain reliable data.

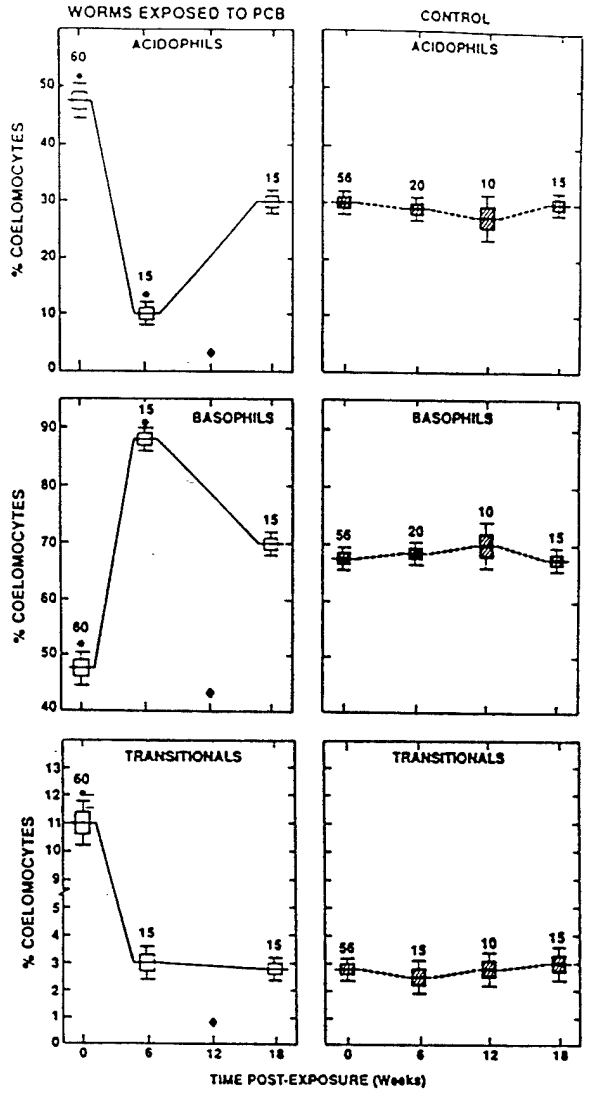


Figure 3. Comparison of mean acidophil, basophil and transitional coelomocyte percentages between earthworms (*Lumbricus terrestris)* exposed for five days to 10 µg/cm^2 PCB (Aroclor 1254) on filter paper (open rectangles) and controls exposed to saline (shaded rectangles). Coelomocytes were collected by extrusion at 0 hrs, and 6, 12 and 18 weeks postexposure. Asterisks indicate significant difference from controls and diamond at 12 weeks depuration indicates too few cells were extruded to obtain reliable data. Symbols same as Figure 2. Sample sizes are indicated by numbers above assay points.

Much of our cytological work has been conducted following earthworm exposure to Aroclor 1254 (Goven, *et al.,* 1993), a commercial mixture of polychlorinated biphenyls (PCB) with known vertebrate immunotoxic effects (Lee and Chang, 1985). For laboratory exposure we used a 5-day filter paper (FP) contact exposure to 10 µg PCB/cm^2. This represents a sublethal exposure well below the LC_{50} (300 µg/cm^2 FP) and results in a whole body tissue concentration of approximately 100-200 µg/g dry mass (LD50 = 1140 µg/g dry mass). PCB exposure results in both acute and chronic effects on coelomocytes.

Acute PCB effects on cytological parameters were evident on coelomocytes collected immediately (0 hr depuration) after exposure. Significant differences in viability (Figure 2) between coelomocytes from exposed and unexposed earthworms suggest that PCB was somewhat toxic to coelomocytes. Although TCC (Figure 2) were not immediately (0 hr depuration) affected by PCB exposure, DCC (Figure 3) showed a striking decrease in percentage of basophils, a cell responsible for HMI, and a concomitant increase in acidophils, a phagocytic cell. DCC for controls agree with reports that basophils, acidophils and transitionals represent 60, 30 and 3% of the total coelomocyte population for normal earthworms (Cooper and Stein, 1981).

Our extrusion technique (Eyambe, *et al.,* 1991) for obtaining coelomocytes enables sequential collections from individual earthworms at 6-week intervals. These sequentially collected coelomocytes have normal cytological parameters (TCC, DCC, viability) (Figures 2, 3) (Eyambe, *et al.,* 1991). The technique works well in studying effects of chronic exposures on regeneration of immunocompetent coelomocytes. Chronic PCB effects were measured by changes in the ability of earthworms to repopulate their coelomic cavity with immunocompetent coelomocytes as determined by TCC (Figure 2) on coelomocytes collected by secondary extrusion at 6, 12 and 18 weeks post exposure when body burden concentrations of PCB were 41.0, 30.2 and 15.7 µg PCB/g dry mass, respectively. There were significant differences in TCC between exposed and unexposed earthworms at 6 weeks. At 12 weeks, exposed earthworms extruded too few cells to obtain accurate cell counts. By 18 weeks, when PCB tissue concentration was lowest (15.7 µg/g dry mass), TCC returned to levels of controls, indicating a repopulation of coelomic cavity. Viability (Figure 2) and DCC (Figure 3) of coelomocytes collected from exposed earthworms followed a similar pattern: Abnormal cytological patterns at 6 weeks; too few cells to assay at 12 weeks; and return to normal parameters at 18 weeks postexposure.

Because PCB-exposed earthworms were unable to extrude normal numbers of immunocompetent coelomocytes until 18 weeks postexposure, when tissue concentration of PCB was lowest, we suggest that PCB affected coelomopoietic tissues. Similarly, mammals show a decreased number of circulating leukocytes after PCB exposure (Carter and Clancy, 1980; Fishbein, 1974).

NON-SPECIFIC IMMUNITY

Assessment of chemical effects on phylogenetically conserved immune responses such as immunocyte spreading (activation), phagocytosis and bacteria killing should allow for predicting xenobiotic effects on homologous vertebrate non-specific responses.

Effect of PCB and Cu^{++} on coelomocyte phagocytosis

As with other biomarkers, much of our work on phagocytosis has been conducted using coelomocytes extruded from earthworms after a 5-day filter paper exposure to sublethal concentrations of PCB (10 µg/cm^{2}FP) or Cu^{++} (1.0 µg/cm^2 FP) (Goven, *et al.,* 1994; Rodriguez-Grau, *et al.,* 1989).

Effects of whole worm exposure to Cu^{++} or PCB on phagocytosis were determined using flow cytometry. For identification of phagocytic coelomocytes, exposed and unexposed earthworms were injected intracoelomically with 5 X 10^7 fluorescent latex beads (2.22 µm diameter) in a total volume of 0.2 mL Lumbricus balanced salt solutions (Rodriguez-Grau, *et al.,* 1989) divided equally among five injection sites Following fluorescent bead injection earthworms were placed in moist containers and incubated at 10°C, in the dark, for 3 hr. Coelomocytes were collected immediately after incubation and fluorescent latex bead ingestion was determined using a Coulter EPICS V flow cytometer. For analysis, the single 5 watt argon laser set at 488 nm and the output adjusted to 400 mW. Extruded non-phagocytized latex beads were excluded by setting appropriate sample and gating parameters prior to data collection. Coelomocytes from exposed and unexposed earthworms were examined and divided into two groups: (1) Those ingesting at least one bead, i.e. all phagocytosing cells; and (2) those ingesting three or more. The former provides an index of the

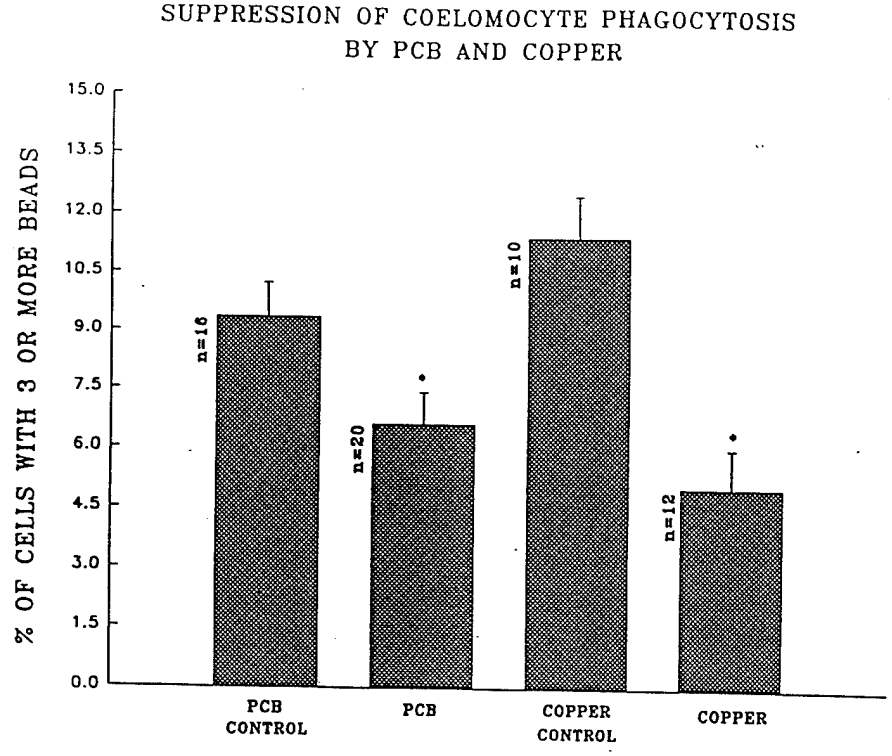


Figure 4. Suppression of phagocytic activity in earthworm (*Lumbricus terrestris*) coelomocytes after whole worms were exposed via 5-day filter paper contact to sublethal concentrations of Cu^{++} (as $CuSO_4$, 1.0 $\mu g/cm^2$) or PCB (Aroclor 1254, 10.0 $\mu g/cm^2$). Expressed as percentage, vertical lines represent standard errors, n represents sample size.

ability to ingest while the latter represents phagocytic efficiency, an index of protective immune function.

Coelomocytes from both Cu^{++} and PCB exposed earthworms had significantly lower phagocytosis than controls (Figure 4). Additionally, both Cu^{++} and PCB exposure significantly lowered coelomocyte ingestion of three or more fluorescent beads compared to coelomocytes from unexposed controls (Figure 5). Phagocytosis, a well-documented nonspecific immune function of coelomocytes, is the most primitive of the protective response in animals. Suppression of the phagocytosis in coelomocytes exposed to PCB may be homologous to PCB suppression of mammalian cell phagocytosis. (Thomas and Hinsdill, 1978; Street and Sharma, 1975).

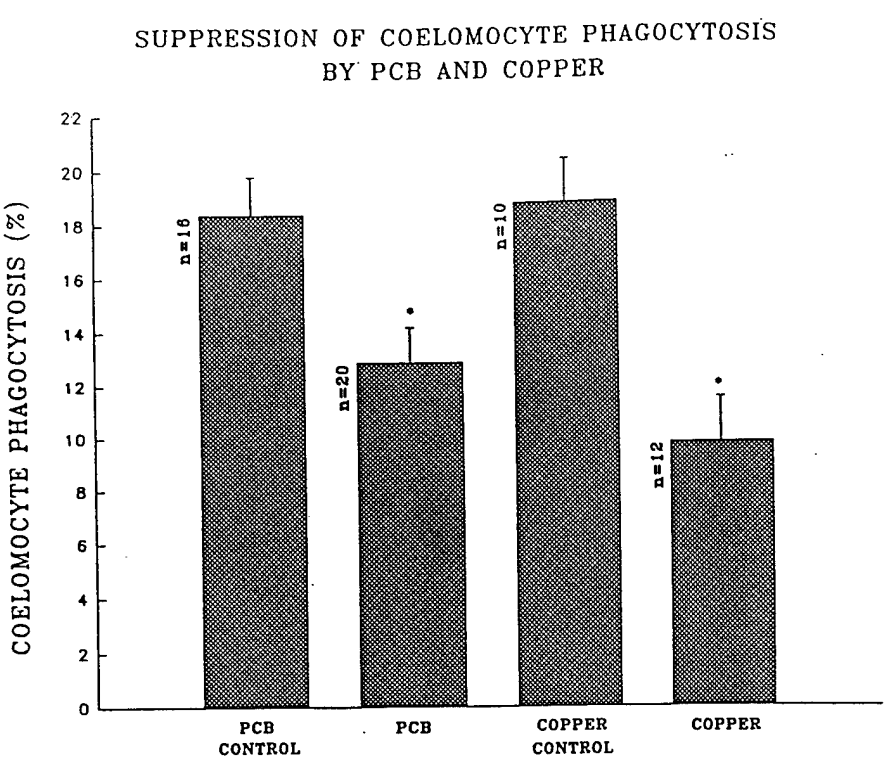


Figure 5. Suppression of phagocytic efficiency, measured by ingestion of three or more beads, of earthworm (*Lumbricus terrestris*) coelomocytes after whole worms were exposed via 5-day filter paper contact to sublethal concentrations of Cu^{++} (as $CuSO_4$, 1.0 $\mu g/cm^2$) or PCB (Aroclor 1254, 10.0/cm^2). Expressed as percentage, vertical lines represent standard errors, n represents sample size.

Effect of refuse-derived fuel-fly ash (RDFF) on NBT dye reduction

The major roles of phagocytes are ingestion and killing of microorganisms, principally by the oxygen-dependent "respiratory burst" involving super oxide anion and hydrogen peroxide (H_2O_2) production (Drutz and Mills, 1984). Activation of oxygen metabolism of phagocytosis is a useful marker of phagocytic ingestion and an important process related to killing of ingested microbes (Braunde, 1981).

The NBT dye reduction assay has been used to evaluate the ability of phagocytes to catabolize and kill phagocytosed bacteria by the "respiratory burst" (Braunde, 1981). This colorimetric assay, which indirectly measures intracellular production, was developed to detect metabolic defects associated with chronic granulomatous disease in humans. The NBT assay complements our phagocytic assay. It identifies xenobiotics that interfere with intracellular oxidative bactericidal activity of phagocytic cells but, do not affect either their stimulation or phagocytosis or both. We have demonstrated that earthworm coelomocytes have the ability to reduce NBT dye in a nearly linear fashion over incubation times, as expected for leukocytes collected from mice and humans (Chen, *et al.*, 1991). Thus, it appears that the responsible cellular mechanisms are broadly conserved phylogenetically.

Sensitivity of the earthworm NBT dye reduction assay to chemical exposure was determined using coelomocytes harvested from earthworms exposed for five days to sublethal RDFF: Commercial

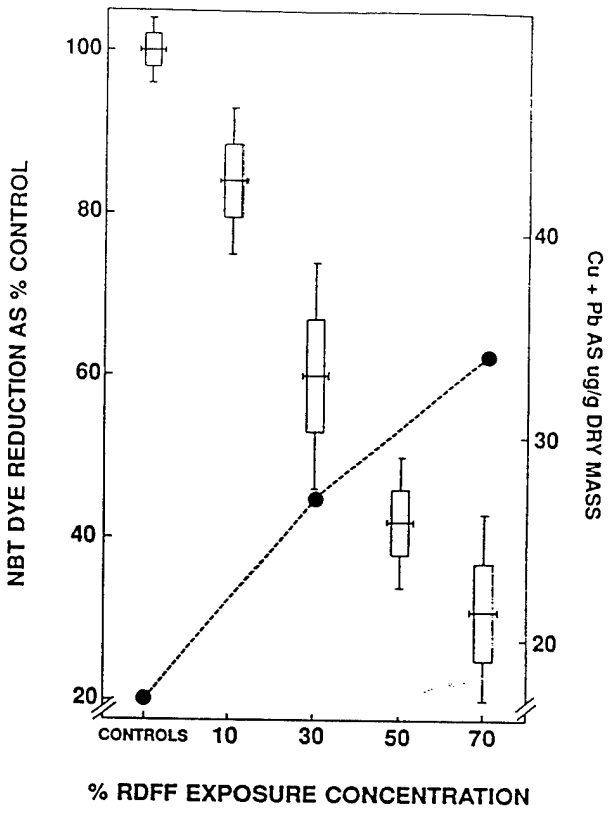


Figure 6. NBT dye reduction after 60 minutes incubation by coelomocytes from earthworms (*Lumbricus terrestris*) exposed for five days to commercial soil (controls) and refuse-derived fuel fly ash (RDFF) mixtures with commercial soil of 10, 30,50, and 70%. Expressed as percent of control $OD_{515\ nm}$. Symbols same as those in Figures 2. Data based on pooled coelomocyte samples from six different groups of five earthworms for each exposure concentration. Dashed line represents corresponding tissue levels of metals (Cu + Pb).

soil mixtures of 10:90, 30:70; 50:50 and 70:30, by dry mass (Chen, *et al.*, 1991). Earthworms were exposed in 1-L glass jars with metal caps within an environmental chamber at 10°C without light. Five earthworms were housed in each jar, which contained 150 g dry mass of the RDFF: Commercial soil mixture hydrated with 10 mL deionized water. Controls were exposed similarly,but to 150 g of soil only. Concentrations of principal RDFF heavy metals (Cd, Cu, Cr, Zn, Ni, and Pb) were determined for parent material and acid-digested tissues in selected earthworms by atomic absorption spectrometry.

NBT reduction by coelomocytes was influenced significantly by RDFF exposure concentration (Chen, *et al.*, 1991). Expressed as percent of controls, NBT reduction was inversely related RDFF concentrations (Figure 6). Dye reduction by coelomocytes from 30, 50, and 70% RDFF exposure groups was significantly lower by 40, 59, and 64%, respectively, than that of controls. Suppression of NBT dye reduction exceeded the 25% value used in clinical medicine to define immune suppression in humans (Maderazo and Ward, 1980).

Concentrations of Zn, Pb, Cu, Cr, and Ni, and Cd were 2,342, 610, 470, 104, 49 and 20 mg/kg dry mass, respectively, in undiluted RDFF. Metals were analyzed in control earthworms and those exposed to 30 and 70% RDFF concentrations -extremes of the range, where there were significant effects on NBT reduction. Tissue concentrations of Cu and Pb were significantly higher than those

of the controls. The other metals were not significantly different between controls and exposed earthworms.

Because NBT reduction in phagocytic cells occurs by a chemical reaction between the dye and , and is produced by the one-electron reduction of 0_2 (a reaction catalyzed by NADPH oxidase), suppression of dye reduction suggests that heavy metals (known enzyme toxicants) interfered in the pathway leading to formation. Both Cu and Pb are known to affect glucose-6-phosphate dehydrogenase (G-6-PD) (Vallee and Ulmer, 1972), the enzyme in the hexose monophosphate shunt involved in conversion of $NADP^+$ to NADPH, which is the reducing agent in production (Absolom, 1986). Additionally, Pb has been linked to G-6-PD deficiency in mammals (Stokinger, 1981) and suppresses resistance to bacterial infection in mice (Hemphill and Kaeberle, 1971). The latter accords with increased susceptibility to infection found in children poisoned by Pb (Stokinger, 1981) and our preliminary data suggesting decreased resistance to infection with *Aeromonas hydrophilia* in the manure worm, *Eisenia foetida*, after 5-day exposure to RDFF concentrations of 30, 50, and 70%.

Suppression of NBT dye reduction occurred at tissue concentrations of Cu and Pb similar to those in some natural populations (roadside, mining sites). The assay also showed toxicity of Pb and/or Cu at exposure levels below those affecting growth and reproduction, and causing mortality (Ma, 1982). Thus, the earthworm NBT assay appears sufficiently sensitive for measuring a sublethal effect of heavy metals on an important nonspecific immune function of cells (the ability to resist infection by killing microorganisms oxidatively) common to a wide diversity of animals, including important wildlife, at realistic environmental concentrations and below those reported to produce other forms of toxicity.

Application of nonspecific immunoassays to hazardous waste site (HWS) soils

We have performed a preliminary assessment of sublethal toxicity of soils from a Superfund HWS using nonspecific immunoassays on *L. terrestris* exposed in situ to soils having different contamination levels (Venables, *et al.,* 1992). The HWS was used to mix and batch agrochemicals including herbicides and pesticides for resale. Earthworm mortality was highly correlated to tissue and soil concentrations of chlordane and DDT (Callahan and Linder, 1992). Earthworms were categorized as having been exposed in situ to high, intermediate or low contamination levels according to in situ morbidity and mortality data (Greene, *et al.,* 1989). We also examined immunologic effects in *L. terrestris* exposed in our laboratories to the highly contaminated HWS soil diluted with artificial soil to 5% (Venables, *et al.,* 1992).

Coelomocyte spreading, phagocytosis of antigenic rabbit red blood cells and reduction of NBT dye were used as assays for non-specific immune function (Table 1). Spreading indicates membrane activation, leading to phagocytosis or enhanced ingestion of particulate antigens. Reduction in any of these responses would indicate a suppression in NSI. Coelomocytes from earthworms exposed in situ for 48 hours to HWS soils of low, intermediate and high levels of contamination demonstrated suppressed coelomocyte activation (spreading) and ability to reduce NBT dye at all three exposure levels while phagocytosis was reduced at intermediate and high levels. There was an apparent dose response which correlated with the exposure level. All three NSI functions were suppressed in

Table 1
Effects of *in situ* exposure to hazardous waste site (HWS) soil of low (L), intermediate (I) and high (H) levels of contamination,and laboratory exposure to 5% dilution of HWS soil on coelomocyte activation (spreading), phagocytosis and nitroblue tetrazoliuim (NBT) dye reduction. Expressed as percent normal unexposed controls.

Immune Parameter	*In situ* Exposure			Laboratory Exposure
	L	I	H	5% HWS Soil
Spreading	76	22	45	67
Phagocytosis	100	44	13	28
NBT	67	43	13	45

coelomocytes from earthworms exposed in the laboratory for 5 days to a 5% dilution of HWS soil. Results indicate a good correspondence between field and laboratory exposure.

DISCUSSION

Our work with earthworms suggests that invertebrates have considerably greater potential for use in immunotoxicology than generally realized. They are sufficiently complex with immune processes that are broadly conserved phylogenetically for use as surrogates for vertebrates in studying the immunotoxic effects of environmental pollutants. Among invertebrates, earthworms possess a number of attributes which make them an appropriate choice for investigation of effects of chemicals on host immune defense systems and development of immune-based biomarkers, especially for terrestrial toxicology. Several earthworm coelomocyte-based biomarkers are sensitive indicators of sublethal immunotoxicity of single chemicals and complex mixtures. A comprehensive suite of biomarker measurement endpoints with invertebrates should provide for cost-effective screening of chemicals, and risk assessment of environmental pollutants and HWS soils.

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Chapter 33

Expression and Modulation of Immunological Activities by Tunicate Hemocytes

N. Parrinello, V. Arizza, M. Cammarata and D.M. Parrinello

Laboratory of Marine Immunobiology
University of Palermo
Institute of Zoology
Italy

*Correspondence should be addressed to:Prof. Nicolò Parrinello
Istituto di Zoologia
Via Archirafi, 18
90123 Palermo, Italy

INTRODUCTION

Tunicates are filter-feeding marine invertebrates, considered to be the most primitive members of Chordata which exhibit features characteristic of the vertebrates (Berril, 1955): (1) pharyngeal region divided in a branchial basket and alimentary endostyle which has been related to thyroid gland; (2) the larval form which presents a notochord and a dorsal neural tube. Recent studies of the molecular phylogeny of the animal kingdom by comparison of sequences of 5S rRNA (Hori and Osawa, 1987) and 18S rRNA (Field *et al.*, 1988; Wada and Satoh, 1994), as well as studies of the structure of genes for muscle actin (Kusakabe *et al.*, 1992) support this view. Accordingly, tunicates are excellent models of chordate evolution and for dissecting events associated with regulation of immune response and immunesuppression after exposure to xenobiotic.

In spite of numerous studies on ascidian immunological reactivity, little is known about hemocyt-differentiation and function. In the present report, some of their activities related to immune functions have been discussed, and differentiation pathways suggested.

ASCIDIAN HEMOCYTES

Stem-cells (lymphocyte-like), hyaline amoebocytes, granular amoebocytes, signet-ring cells, compartment cells, morula cells are hemocytes usually found in the hemolymph of the ascidians (for reviews see: Rowley, *et al.*, 1984; Wright, 1981; De Leo, 1992). Moreover, hemocytes may be typical of certain species: hemocytes with acidic vacuole (*Ciona intestinalis, Phallusia mamillata*), two types of compartment cells in *Phallusia mamillata* (Cammarata, *et al.*, 1993), univacuolar refractile granulocytes (*Ciona intestinalis*), two types of morula cells in *Ciona intestinalis* (Par-

rinello, *et al.,* 1995). There are considerable differences in the number of circulating hemocytes between species, individuals, body size, season, and characteristic hemocytes have been found.

IN VIVO IMMUNE REACTIONS

1) Phagocytosis, and opsonization by circulating hemocytes and humoral factors are components of natural immunity (Wright, 1974; Wright and Cooper, 1975).

2) In solitary ascidians, tunic graft rejection was characterized by specific immune recognition and hemocyte cytocidal activity, restricted by cellular polymorphic histocompatibility antigens, and provided with immunological memory (Reddy, *et al.,* 1975; Raftos, *et al.,* 1987a,b; 1988; Raftos and Briscoe, 1990; Raftos, 1991). Genes which express a considerable allogeneic polymorphism are also responsible of fast reactions between allogeneic and xenogeneic *in vitro* combinations of hemocytes, which results in mutual death of apposing cells (Fuke, 1980; Fuke and Nakamura, 1985).

3) In colonial ascidians, nonfusion reactions represent histocompatibility discrimination, controlled by a defined gene locus (Fu/HC) with 100 codominantly expressed alleles (for review see: Watanabe and Taneda, 1982, Scofield and Nagashima, 1983)).

4) Tunic inflammatory cell-mediated reactions against foreign materials include phagocytosis, degranulation, tissue injury and encapsulation, and suggest release of cytokines, proliferation of stem cells, chemotaxis (Parrinello, 1981; Parrinello, *et al.,* 1977, 1984; Parrinello and Patricolo, 1984; Parrinello, *et al.*, 1990).

HEMOCYTES INVOLVED IN IMMUNE FUNCTIONS

1) Stem cells (LLCs; solitary ascidians), proliferate in first-set and second-set tunic allografts (Raftos, 1991; Raftos and Cooper, 1991; Raftos *et al.*, 1987b) and infiltrate the tunic inflamed by injection of foreign materials (Parrinello and Patricolo, 1984).

2) Granular amoebocytes of differently sized granules, "macrophages", univacuolar granulocytes, signet-ring cells, probably originated from stem cells or intermediates, degranulate in the inflammed tunic (Parrinello *et al.*, 1984; Parrinello *et al.*, 1990). The inflammatory reaction can be extremely destructive for surrounding tissues.

3) "Morula cells" are conspicuous participants in tunic allograft rejection lesions, tunic inflammatory reactions and nonfusion reactions of colonial species (De Leo *et al.*, in prep.; Raftos *et al.*, 1987b; Scofield and Nagashima, 1983; Sabbadin *et al.*, 1992).

4) "Vacuolated cells" and "granular amoebocytes" undergo mutual lysis following an allogeneic *in vitro* "contact reaction" (Fuke, 1980).

***In vitro* assays of hemocyte immunological activities.**

Fractionation of hemocytes by density-gradient centrifugation was used to examine the functions of the various types of hemocytes (Parrinello and Cammarata, 1995). The hemolymph, obtained using a sterile syringe containing cold anticoagulant, was centrifuged onto a Percoll discontinuous gradient. Bands of cells were collected by gentle aspiration and washed in sterile artificial sea water. Cell viability was estimated by the eosin-y exclusion test, and cell types were identified.

Species differences in separated hemocyte populations as well as typical hemocyte types have been found (reviewed in: Parrinello and Cammarata, 1995), e.g. "univacuolar refractile granulocytes" appear to be specific of *Ciona intestinalis*. Moreover, hemocytes identical by their morphology can show density difference in their inclusions: *Phallusia mamillata* pigment cells were separated in the bands B1 (26%) and B2 (26%), granular amoebocytes in B1 (45%) and B2 (48%), univacuolar signet-ring cells in B6 (97%) and B7 (98%). These differences could depend on the differentiation pathway of the vacuolar material which may in turn be related to hemocyte functional stages as well as to the biology of the species.

Stem cells

This cell type has been named lymphocyte-like (LLC) on the basis of its morphology (Figure 1 A). These hemocytes appear to circulate in the vascular system and continuously replace aging differentiated cells (Sabbadin and Zaniolo, 1979; Rinkevich and Weissman, 1987; Ermark, 1975, 1982). *In vivo*, the pharynx is a major hematopoietic organ in which stem cells undergo proliferation and differentiation to yield a variety of morphologically distinct hemocyte types (Ermak, 1975, 1982). *In vitro*, pharyngeal explants remain viable for up 70 days when cultured in a compatible medium (Raftos, *et al.*, 1990). Cell proliferation is maintained in these cultures for extended periods. Recombinant human interleukin-2 and phytohemagglutinin-P stimulate stem cells of *Styela clava* to proliferate (Raftos *et al.*, 1991).

The proliferation of tunicate stem cells, in the pharynx or other visceral tissues, contributes to hematopoiesis as well as to immunological reactivity. The high level of proliferative activity evident among pharyngeal cultures in the absence of IL-2 reflects this hematopoietic activity. On the other hand, the proliferation was induced by allogeneic stimuli (Raftos and Cooper, 1991) and stem cells were associated with the recognition of allogeneic tissue (Raftos, *et al.*, 1987b) indicating that they have immunological function and may act as immunocompetent cells.

Proliferative activity was also evident among the circulating hemocytes. Rinkevich and Rabinowitz (1993) demonstrated that *Botryllus schlosseri* hemocytes may proliferate *in vitro* conditions, although differentiation of these cells into hemocyte types was not achieved. Cells were not markedly responsive to lectins, but mixed interleukin solution (IL-1, IL-2) had some proliferative effects. In addition, *Botryllus* heterologous plasma supplementation resulted in agglutination of the most hemocytes and, thereafter, in cell proliferation.

The physiological activities of endogenous interleukin-like fractions in tunicates are conjectural. The evidence suggests that one role may be to regulate cell proliferation. However, the physiological

relevance of such modulation is unclear in that tunicate cytokines apparently are constitutively present in the hemolymph.

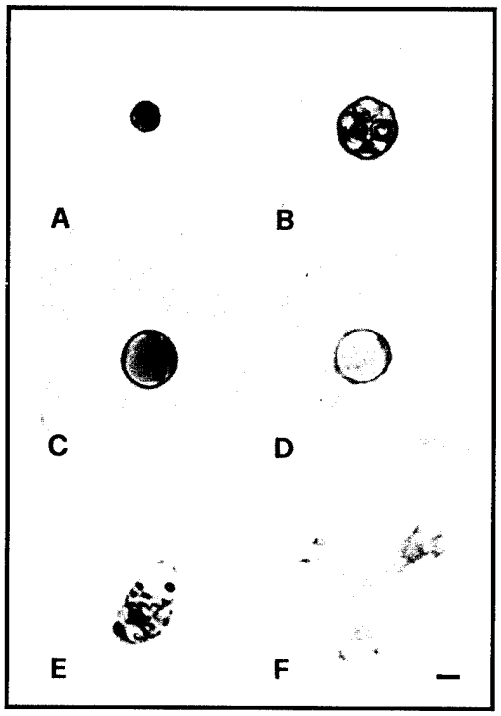


Figure 1. *Ciona intestinalis* hemocytes from a Percoll density gradient.
(A) Stem cell;
(B) morula cell;
(C) univacuolar refractile granulocyte;
(D) univacuolar granulocyte (signet-ring cell);
(E) granular amoebocytes;
(F) hyaline amoebocytes.
Pigment cells and intermediate stages of morula cells are not shown. Bar 4 μm.

Cytotoxic cells

Styela plicata

Because of the well known immunopotentiality of *S. plicata* hemocytes in allograft rejection (Raftos *et al.*, 1987a,b; Raftos, 1991), we examined the cytotoxic activity of the hemocytes after density gradient separation (Parrinello *et al.*, 1996.). Raftos *et al.* (1987b) reported that the frequency of "lymphocyte-like cells" and "morula cells" increased during tunic tissue allograft reactions both in first-set and second-set immune responses. Morula cells are usually berry-like by light microscopy with large vacuoles containing homogeneous masses of strongly-dense material (Figure 1B; Figure 2), they have been reported to be mainly involved in nonfusion reactions between allogeneic colonies of *Botryllus* and probably partecipate in effector reactions (Saito and Watanabe, 1982; Scofield and Nagashima, 1983; Sabbadin *et al.* 1992). Scofield and Nagashima (1983) speculated that morula cells are involved in biochemical pathways which can generate cytotoxic iodide and hydroxyl radicals. Also the association between proPO system contained in this hemocyte type and cytotoxicity has not been demonstrated. However, "morula cells" when associated with degeneration of the implanted foreign tissue, could release cytotoxic molecules as a consequence of the

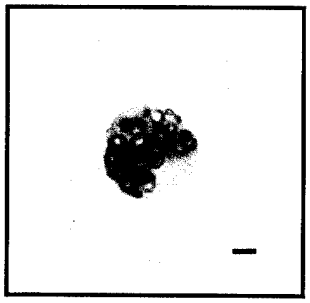


Figure 2. Cytochemical staining with L-DOPA which shows phenoloxidase activity in morula cells of *Styela plicata*. Bar 2.5 μm.

activated eumelanotic reaction mechanisms presumably generated by the proPO-system during immune reaction (Parrinello, 1995). Phenoloxidase, a bifunctional copper containing redox enzyme, catalizes the orthohydroxylation of monophenol (i.e. tyrosine) forming o-diphenol, and then the dehydrogenation of the diphenol into a quinone which may be highly toxic (see review: Nappi and Vass, 1993). The phenoloxidase activity was measured, according to Söderhäll and Hall (1984), in the hemocyte lysate supernatant (Arizza, *et al.* 1995). After a Percoll density gradient separation of the hemolymph only the hemocytes from band 2 contained PO activity which was significantly enhanced by trypsin and chymotrypsin pretreatment and preincubation with microbial lipopolysaccharides. Soybean trypsin inhibitor, copper chelants tropolone and phenylthiourea were specific inhibitors. Microscopic observations of hemocytes from this band showed that it was enriched in "morula cells" (55% cells) and amoebocytes (31% cells). Cytochemical staining with L-DOPA of separated hemocytes in smears confirmed the presence of the phenoloxidase in the globular granules of *Styela plicata* morula cells (Figure 2). PO activity of this cell type was reported for other ascidian species (Jackson, *et al.*, 1993). The hemocytes from the Band 2 also showed cytotoxic activity (in preparation) against K-562 tumour cells (chromium release assay) and rabbit erythrocytes (haemoglobin release assay).

When assayed by a plaque forming cell micromethod with rabbit erythrocytes, both the hemocyte types produced plaques of lysis. The plaques induced by morula cells appeared to be smaller in diameter than those by amoebocytes.

Although supporting evidence is requested, it can be assumed that the high frequency of "morula cells" in *S. plicata* allograft tissue rejection (Raftos, *et al.*, 1987b), could be related to the degenerating process of grafted tissue. A such relationship may also be proposed for colonial ascidian nonfusion reaction.

Ciona intestinalis

In *C. intestinalis* tunic allograft rejection (Reddy, *et al.*, 1975) and inflammatory reaction (Parrinello, 1981; Parrinello and Patricolo, 1984; Parrinello, *et al.*, 1977, 1984a,b), cytotoxic responses and tissue degeneration were produced. To identify the cytotoxic cells, the hemolymph was separated in a Percoll density gradient and an *in vitro* assay was performed using erythrocytes as targets (Parrinello, *et al.*, 1995). In Figure 1 only density-gradient separated hemocytes involved in immune functions are shown. The hemocytes of the Band 5 lysed rabbit erythrocytes. This activity was Ca2+-dependent and could be inhibited by sphingomyelin (25 μg/mL). The Band 5 predominantly contained morula cells (52%; Figure 1B) and "univacuolar refractile granulocytes" (URGs, 40%; Figure 1C). The functional relationship between hemolysis degree (%) and frequency of specific cell types enriched by density gradient, was examined by the logistic regression model (Homser and Lemeshow, 1989). Only the amounts of URGs were in a statistical close relationship (goodness of fit, R= 0.88) with anti-rabbit erythrocyte lytic activity whereas morula cells could not be related (R = 0.058) to the hemolysis. In addition, the URGs were plaque forming cells (Parrinello *et al.*, 1996) and their hemocyte lysate supernatant contains the cytotoxic factors against rabbit erythrocytes and tumour K-562 cell line (paper in prep.). Cytochemical staining of separated hemocyte smears with L-DOPA showed an evident PO reaction of the large granules content, inhibition with tropolone and phenylthiourea supported the specificity of PO reaction.

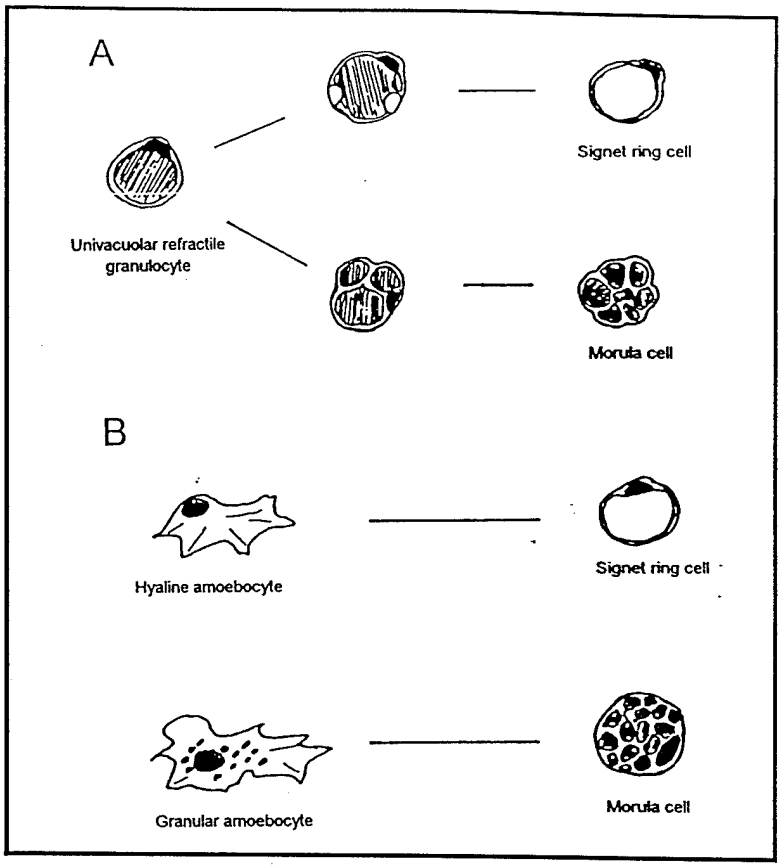


Figure 3. Differentiation pathways of "signet-ring cell" and "morula cell". (A) From the univacuolar refractile granulocyte of *Ciona intestinalis*. (B) From the hyaline or granular amoebocytes of *Botryllus schlosseri* (Ballarin *et al.* 1993, 1994).

Since, non phagocytic hyaline amoebocytes (B1, B2) and phagocytic amoebocytes (B2, B3), reported by Peddie and Smith (1993) to have anti-tumour activity, failed to show anti-rabbit erythrocyte cytotoxicity, two mechanisms could exist in *Ciona*.

The hemocyte type "univacuolar refractile granulocyte" we defined on the basis of the vacuolar content, has been previously described by Rowley (1981) as "compartment cell". However, such a terminology increases confusion in hemocyte classification because it has also been used to indicate a multivacuolated hemocyte of other ascidians (see: De Leo, 1992). According to Rowley (1981), URGs and morula cells apparently differed from each other only in terms of their degree of vacuolization, and could be stages of a single cell differentiation pathway due to the compartmentalization of the large univacuolar refractile inclusion (Figure 3A). Since our results (Parrinello, *et al.*, 1996) showed that *in vitro* cytotoxic activity against erythrocytes is a property of URGs, this activity appears to be expressed at an early stage of morula cell differentiation pathway.

A second differentiation pathway from URGs was suggested by inflammatory reactions induced in the tunic of *C. intestinalis*. We reported (Parrinello, *et al.*, 1984; Parrinello and Patricolo, 1984; De Leo, *et al.*, 1992) that numerous URGs and signet-ring-like cells (Figure 1D) infiltrated the inflamed tissue. Light and TEM observations (De Leo, *et al.*, 1992), showed that URGs presented changes in the structural organization of the refractile material which, in a spot-like fashion (Figure 3A), became less dense and similar to that contained in the vacuole of the signet-ring cells. These intermediate forms were also observed in the density-gradient enriched populations separated from the hemolymph (in preparation).

The available data suggest that univacuolar signet-ring-like cells as well as morula cells could originate from URGs which express a direct cytotoxicity, probably activated by *in vitro* conditions, or could differentiate into morula cell or signet-ring cell (Figure 3A) depending on the stimuli.

A lectin releasing cell

Lectins are sugar-binding proteins, humoral and cell-bound, that have been found in the hemolymph of the invertebrates and have been proposed as partecipants in immune recognition mechanisms (for reviews see: Olafsen, 1986; Vasta, 1991; Parrinello, 1991). Hemocytes can contain and also secrete lectins. Although supporting evidence has been reported for only a few species, it has been assumed that these molecules are involved in ascidian cell-mediated defense reactions.

We reported that ascidian hemocytes contain surface lectins (Parrinello and Arizza, 1988, 1989; Cammarata, *et al.,* 1993), moreover we showed, for the first time in tunicates, that the hemocytes from *Phallusia mamillata*, known as compartment cells, contain and release *in vitro* lactose-specific lectins (Cammarata *et al.*, 1993; Arizza *et al.*, 1993; Figure 4).

Short-term cell microcultures were performed in a medium based on ionic and osmotic analysis of the hemolymph, enriched with M199 and L-glutamine. Hemocytes separated from each band on density gradient were cultured into wells of a sterile 24-well, flat-bottomed culture plate at 15°C. The hemagglutinating activity of the cell-free supernatants was estimated after 3-24 hours with rabbit erythrocytes.

The examination of the differential distribution of hemocytes among the fractionated bands of cells (B1-B8) showed that the supernatant from Band 4 hemocytes in microcultures possessed the highest hemagglutination titer (128) than those of Band 3 and 5 (4-8). The released lectins were lactose-specific as shown by inhibition experiments. Microscopical observations and differential counts showed that Band 4 was prevalently enriched in "compartment cells" (98%), whereas 15% were contained in the cell population of Band 3, and 31% in Band 5. This hemocyte type, spherical in shape, is characterized by angular vacuoles in variable numbers distributed regularly at the periphery of the cytoplasm (Figure 4A). Linear regression equation, calculated by comparing hemagglutinating activity of supernatants and the percentage of compartment cells present in the cultures, expresses the relationship ($P < 0.001$; $r = 0.98$) between numbers of compartment cells and hemagglutinating titer.

Released lectins were lactose-specific, and possess the same molecular and immunochemical properties that characterize those purified from the hemocyte homogenate which were also found on the surface of compartment cells (Figure 4B).

A

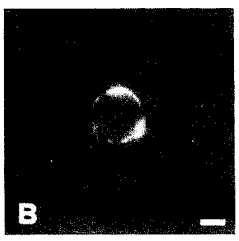


Figure 4. Immunocytochemical (anti-lectin purified primary antibodies) localization of lectins in *Phallusia mamillata* compartment cells. (A) Staining of the globular vacuoles (peroxidase conjugated secondary antibodies). (B) Evidence of surface lectins (rhodamine conjugated secondary antibodies). Bars 4 µm.

Phagocytes

Phagocytic activity is a basic strategy of innate immunity, being a function of both hemocytes and hemolymph plasma. Several ascidian hemocytes have been reported to be phagocytes. *In vivo* studies showed that small particulate foreign bodies injected into the tunic or vascular system of *Halocynthia aurantum* (Smith, 1970) and *Ciona intestinalis* (Wright, 1981) were phagocytosed by hyaline amoebocytes. Carmine particles were identified in the cytoplasm of amoebocytes, and tryphan blue was found in the cytoplasm and vacuoles of signet ring cells after intracardial injections into *Molgula manhattensis* (Anderson, 1971). Fuke (1979) reported phagocytic activity in *Halocynthia roretzi* of the several hemocyte types. Sawada *et al.* (1991) postulated that the phagocytes of this species consist of small granular amoebocytes and Ohtake *et al.* (1994) then identified them by *in vitro* experiments and TEM examination.

Phagocytosis in *C. intestinalis* was also investigated using mixed and separated populations of hemocytes (Smith and Peddie, 1992; Rowley, 1981, 1982). Only the granular (Figure 1E) and hyaline (Figure 1F) vacuolar amoebocytes were seen to ingest bacteria and erythrocytes *in vitro*. We performed phagocytosis assay in a glass slide/coverslip-chamber with o-fluorescein-conjugated yeast cells (0.125%) as target (Parrinello and Cammarata, 1995). After 60-90 min stirring, dye quenching solution was added and hemocyte-containing fluorescent yeast cells were evaluated by UV-light microscopy. In unseparated hemocyte preparations, about 45% cells were found to be phagocytes.

Hyaline amoebocytes, macrophage-like cells, and signet-ring cells are the cell types involved in phagocytosis of *Botryllus schlosseri*. Ballarin *et al.* (1994) suggested that macrophage-like cells and signet-ring cells represent cell types which are engaged in processing engulfed materials and which have their precursors in hyaline amoebocytes. These phagocytes after particle ingestion, withdraw their cytoplasm projections and change their shape from flat and fusiform to spherical. This view is indirectly supported by *in vivo* time course studies with carmine particles (Ballarin, *et al.*, 1994).

MODULATORS OF LYTIC AND PHAGOCYTIC ACTIVITY

Hemolymph factors, hemocyte lysate supernatant and xenobiotic substances can modulate in various ways, immune functions of ascidian hemocytes assayed *in vitro*.Under certain experimental conditions, although media isomotic with hemolymph was used, the possibility exists that manipulations of the hemolymph affected the expression and modulation of cell mechanisms.

Cytotoxicity

To examine the role of hemolymph components in modulation of *Ciona intestinalis* hemocyte cytotoxic activity, cell-free hemolymph was added in the reaction mixture. Preliminary results showed that diluted (50 µg/mL protein) hemolymph caused significant inhibition of anti-RE cytotoxicity respectively by URGs (84%) and URGs-lysate (74%) (Figure 5; paper in prep.). Probably a negative regulation on the cytotoxic mechanism could be exerted by humoral compo-

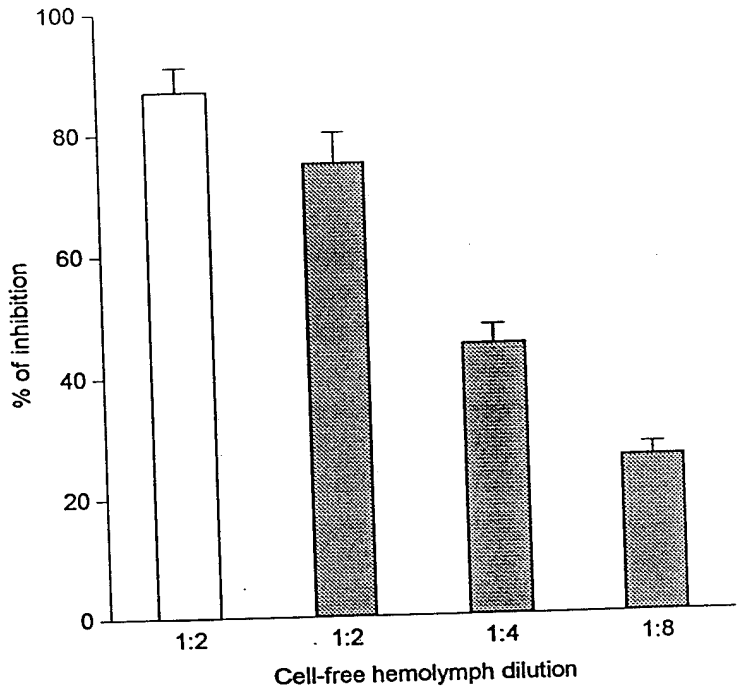


Figure 5. Inhibitory effect of homologous cell-free hemolymph (50 µg/mL protein) versus anti-rabbit erythrocytes cytotoxic activity of *Ciona intestinalis* hemocytes □ or hemocyte lysate supernatant ■ (200 µg/mL protein) from density separated hemocytes B5 band.

nents of the hemolymph. We do not know the nature of the hemolymphatic modulators. However, when these factors are absent, the hemocytes become active in some of their functions.

Phagocytosis

In clearing and eliminating foreign materials, plasma factors can enhance the phagocytic mechanism. In *Halocynthia roretzi*, within 20 min incubation 71% of small granulocytes phagocytosed sheep erythrocytes in the hemolymph, while only 39% phagocytosed the erythrocytes in artificial sea water. Latex beads were readily ingested in every medium (Ohtake, *et al.*, 1994). Factors that enhance phagocytosis of yeast and sheep erythrocytes are present in the plasma of *Botryllus schlosseri* (Ballarin, *et al.*, 1994).

Tunicates possess cytophilic molecules which are functionally homologous to interleukin-1 and regulate cellular activities during inflammation (Beck, *et al.*, 1989). Interleukin-1-like molecules have been isolated (approx. 20 Kd) from the hemolymph of *Styela clava* (*tun*IL-1) and assayed for IL-1 activities in a mouse thymocyte proliferation assay (Beck, *et al.*, 1993). Two isoelectric forms of *tun*IL-1 (α and β) were identified, and both enhanced phagocytosis of yeast and formalinized-sheep erythrocytes by acting as an opsonin. In addition, *tun*IL-1 directly activated tunicate phagocytes. The enhanced ingestion was distinct from opsonization and resulted from a general activation of phagocytic cells. In fact, although latex beads cannot be opsonized, phagocytic activity toward both yeast and latex beads was enhanced when *tun*IL-1 was incubated directly with amoebocytes.

In *Ciona intestinalis* phagocytosis of bacteria was not affected by plasma factors but was enhanced by a lysate supernatant made from enriched population of morula cells which probably cooperate *in vivo* with amoebocytes in an opsonic phenomenon during host defense responses (Smith and Peddie, 1992).

Recently we reported the inhibitory effect of tributyltin (TBT) on phagocytes from *C. intestinalis* assayed *in vitro* (Cooper, *et al.*, 1995). The hemocytes were exposed at different concentrations (0.04-40 ppb) of four organotin compounds (TBT, tryphenyltin, diphenyltin, dibutyltin, used in marine anti-fouling paints as biocides), and their mortality was low (<5%). The percentage of phagocytosis decreased from 46.6 (controls, unexposed) to 22.0 ($p < 0.001$) at 40 ppb of TBT, whereas the other compounds did not exerted significant effects on phagocytosis at each of the concentrations that were used.

DISCUSSION

Although, further study is needed to understand differentiation pathways and functions of ascidian hemocytes, information has been obtained by research on their immunological activities. Some hemocyte types (stem cells, hyaline amoebocytes, granular amoebocytes, morula cells, signet-ring cells) are present in all the examined ascidian species and some of them express similar activities. Stem cells appear to be immunocompetent in the sense of their immunoreactivity and proliferation which also include specific recognition and adaptive responses. It is generally established that hemocytes and immunocytes originate from stem cells in hemopoietic tissue and in circulating hemolymph to replace aging differentiated cells or to react against foreign stimuli.

However, the examination of hemocyte morphology and some immunological activities suggests differences among ascidian species. In some cases, hemocytes may represent intermediate cells which differentiate into immunocytes showing different activities, e.g. in *Ciona intestinalis* hyaline amoebocytes (non phagocytic), hyaline vacuolar amoebocytes and granular amoebocytes (phagocytes; Rowley 1982; Smith and Peddie, 1992) have been considered (Rowley, 1982) to be part of the same developmental series. Although functional relationships of separated hemocyte populations need to be confirmed, it is interesting to note that (Peddie and Smith, 1993) hyaline amoebocytes (non phagocytic) and phagocytic amoebocytes appear to be cytotoxic against mammalian tumour cell lines.

Hemocytes typical of some ascidian species, could be related to the biology of the species and represent early stages of differentiation pathways involved in some immunological responses (e.g. phagocytic appearance of signet-ring cells in *Botryllus*; possible differentiation of signet-ring cells or morula cells from univacuolar refractile granulocytes in *C. intestinalis*). In *Ciona*, URGs are cytotoxic against erythrocyte targets, contain PO, and could differentiate morula cells which are not cytotoxic and contain both phenoloxidase and opsonins (Smith and Peddie, 1992). On the other hand, *Phallusia* compartment cells, which could be included in the differentiation pathway leading to morula cells, contained and released lectins whereas morula cells did not. It is tempting to speculate that, in *Ciona intestinalis*, a differentiation pathway could start from a typical hemocyte, the univacuolar refractile granulocyte, which compartmentalizes to form a morula cell (Figure 3A) or undergoes vacuolar content changes developing univacuolar signet-ring-like cell. Although we

do not have evidence on the origin of URG, the possibility exists that this cell type originates from stem cells.

Another tentative differentiation pathway includes hyaline amoebocytes, macrophage-like cells, and signet-ring cells which are involved in phagocytosis in *Botryllus schlosseri* (Ballarin *et al.*, 1994; Figure 3B). This agrees with the results of a histoenzymatic analysis that showed hydrolytic enzymes within these cell types (Ballarin *et al.* 1993) and also suggested that morula cells originate from granular amoebocytes because both are positive for arylsulfatase, peroxidase and phenoloxidase. Thus, it is noteworthy that, in different ascidian species, a same hemocyte type (i.e. morula cell) seems to originate from different early hemocyte stages and differentiation pathways.

Further studies on functional relationships of the hemocyte types, as well as approaches to identify cell surface antigenic markers could help to distinguish the existing cell types and differentiation pathways.

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Chapter 34

Signal Transduction in Teleost Lymphocytes as a Target for the Action of Environmental Metals

Karen G. Burnett, Kent C. MacDougal, and Patricia Mericko[1]

Marine Biomedical and Environmental Sciences
The Medical University of South Carolina
221 Fort Johnson Road, Charleston, S.C. 29412, USA

[1]The Grice Marine Biological Laboratory
The University of Charleston, South Carolina
205 Fort Johnson Road
Charleston, S.C. 29412, USA

ABSTRACT

Numerous studies have suggested that high doses of many environmental metals may suppress vertebrate immune responses while low doses may enhance these responses. Our laboratory is studying signal transduction pathways as targets for these low dose effects in lymphoid cells. Cell signaling pathways have been highly conserved through evolution and evidence from our laboratory suggests that, like their mammalian counterparts, antigen receptors on teleost lymphoid cells utilize the inositol phospholipid pathway. In preliminary studies sub-mitogenic doses of the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) synergized with calcium ionophore A23187 to induce proliferation in *Sciaenops ocellatus* (red drum) leukocytes. In more recent work mercuric chloride ($HgCl_2$) inhibited the proliferation of teleost lymphoid cells in response to mitogenic doses of TPA with an IC_{50} = 3.0 μM. At this toxic dose Hg rapidly (10 min.) induced massive calcium influx. At ten-fold lower doses Hg synergized with sub-mitogenic doses of TPA to induce proliferation. Synergism of TPA and low dose Hg was partially suppressed by Verapamil, suggesting the involvement of voltage-sensitive calcium channels. We hypothesize that, as suggested from mammalian studies, low dose Hg may directly activate PKC by altering the redox state of the cell and/or it may generate a low, sustainable increase in intracellular calcium. Both possibilities are being investigated in our laboratory.

INTRODUCTION

Many environmental contaminants exert a hormetic, or biphasic, effect on biological processes. At high doses these toxicants destroy tissues and suppress or inactivate biological functions. At low doses, these same contaminants may enhance various activities, such as DNA replication and tissue repair (Mehendale, 1994). In mammals, high concentrations of mercury and other thiol-reactive heavy metals elicit immunosuppressive and/or lethal effects while low doses cause

symptoms of autoimmunity or hypersensitivity. The immunotoxicity of mercury has been documented by *in vitro* studies of B cell function (Thaxton and Parkhurst, 1973; Dieter *et al.,* 1983) and *in vivo* studies of thymus dependent antibody responses in chickens, rabbits, and mice (Dieter *et al.,* 1983). In contrast low doses of mercury can induce systemic autoimmunity in susceptible mouse and rat strains. This susceptibility is determined by MHC Class II and requires CD4+ T cells (reviewed by Pelletier *et al.,* 1994). *In vivo* (Roales and Perlmutter, 1977) and *in vitro* (Voccia *et al.,* 1994) studies have documented the immunosuppressive effects of mercury in teleost fish. Literature review also suggests that low doses of heavy metals can enhance immune responses of fish (Zelikoff, 1994).

Various authors have suggested that mercury modulates early events in lymphocyte activation (Daum *et al.,* 1993; Nakashima, 1994), suggesting the involvement of critical signaling pathways. Antigen receptors on both B and T cells are linked to the phosphatidyl inositol (PI) pathway. Activation of the PI pathway stimulates the activity of a critical enzyme, protein kinase C (PKC), and increases intracellular calcium levels. From *in vivo* studies in mice, Saijoh *et al.,* (1993) demonstrated that PKC is inhibited by mercury. However, PKC can also be enhanced by mild oxidation (Kass *et al.,* 1989), a situation which might arise with exposure to low doses of mercurials. In addition, mercuric compounds are known to accumulate in and alter the membrane potential, permeability and thiol redox state of cells in the mammalian central nervous system, kidney, gastrointestinal, and respiratory tract. The possibility that altered thiol redox state and disruption of normal calcium flux could be critical to the immunomodulatory effects of mercury on mammalian lymphocytes is being increasingly recognized (Nicotera *et al.,* 1992; Tan *et al., 1993;* Shenker *et al.,* 1993).

Interest in using lower vertebrates such as teleost fish to study low dose, chronic immunotoxicity of environmental contaminants is increasing (Zelikoff, 1994). Justification and interpretation of data from such animal models would be strengthened by an understanding of the pathways of intracellular activation in teleost lymphocytes. Our laboratory is studying the PI pathway in leukocytes of the teleost fish, *Sciaenops ocellatus*. Known as the red drum or redfish, *S. ocellatus* has been proposed as a candidate sentinel species for monitoring environmental impact in warm water estuaries (Burnett *et al.,* 1994). We are pursuing evidence that the PI pathway is an important target for the immunotoxic effects of mercury on teleost fish, as suggested by studies of mammalian species.

The PI signaling pathway.

When antigen receptors on mammalian T- or B-lymphocytes are engaged by antigen or cross-linked by anti-immunoglobulin cellular activation is initiated within a few minutes. Critical tyrosine residues become phosphorylated on the receptor complex and associated protein tyrosine kinases (PTK). These activated PTK catalyze downstream phosphorylation and activation of phospholipase Cγ2 (PLC). PLC activates the breakdown of membrane phosphatidyl inositides into the second messenger molecules diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG stimulates PKC activity, while IP3 triggers large and immediate increases in free intracellular calcium. These signaling events lead to a series of phosphorylation events on serine/threonine residues and the induction of transcription factors NF-κB and AP-1. This activation cascade ultimately leads to cell type specific manifestations of adhesion, proliferation and differentiation. (Pleiman *et al.,* 1994).

SUMMARY OF RESULTS

Cell signaling in red drum lymphocytes

To investigate the association between antigen receptors and the PI pathway in teleost lymphocytes, we attempted to activate viable red drum B cells by cross-linking surface IgM. The mouse monoclonal antibody, RDG048, generated against affinity-purified red drum immunoglobulin, gave visible evidence of capping B cells, as monitored using a FITC-labeled secondary antibody (MacDougal *et al.*, 1995). van Ginkel *et al.*, (1994) have reported that cross-linking of surface IgM will induce replication in B cells from *Ictalurus punctatus*, the channel catfish. Using similar techniques, we were unable to induce proliferation of red drum B cells. Instead, evidence for the existence of the PI pathway in red drum leukocytes was obtained by demonstrating that low doses of the phorbol ester TPA synergized with calcium ionophore A23187 to induce DNA proliferation (Figure 1). This synergistic activation did not rely on macrophage derived factors (data not shown) and was suppressed by kinase inhibitors at IC50s consistent with mammalian PKCs (Figure 2). One presumptive PKC inhibitor, staurosporine (STAR), failed to inhibit this synergistic activity (Figure 3). Unlike other PKC inhibitors, STAR appears to bind to the ATP binding site in PKC (Huang, 1989). Lack of efficacy in the red drum model suggested that a PKC isoform critical to proliferation in teleost lymphocytes might have a unique ATP-binding region.

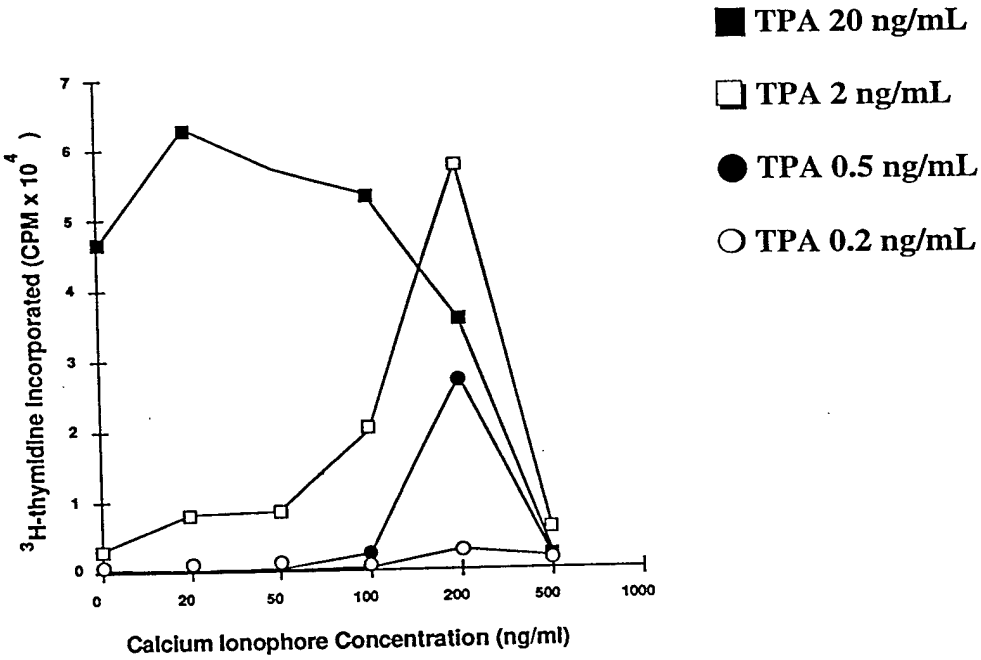


Figure 1. Synergistic action of phorbol ester TPA and calcium ionophore A23187. Freshly isolated red drum PBL were cultured with titrated doses of TPA (0.2 - 20 ng/mL) and calcium ionophore (0 - 500 ng/mL). All mitogens were removed after 18 hrs of culture. After two days in culture, PBL were radiolabeled with 3H-thymidine for 18 hours. Results from a single representative animal are illustrated; each data point represents the average of triplicate values (SE< 0.2) (from Burnett and Schwarz, 1994).

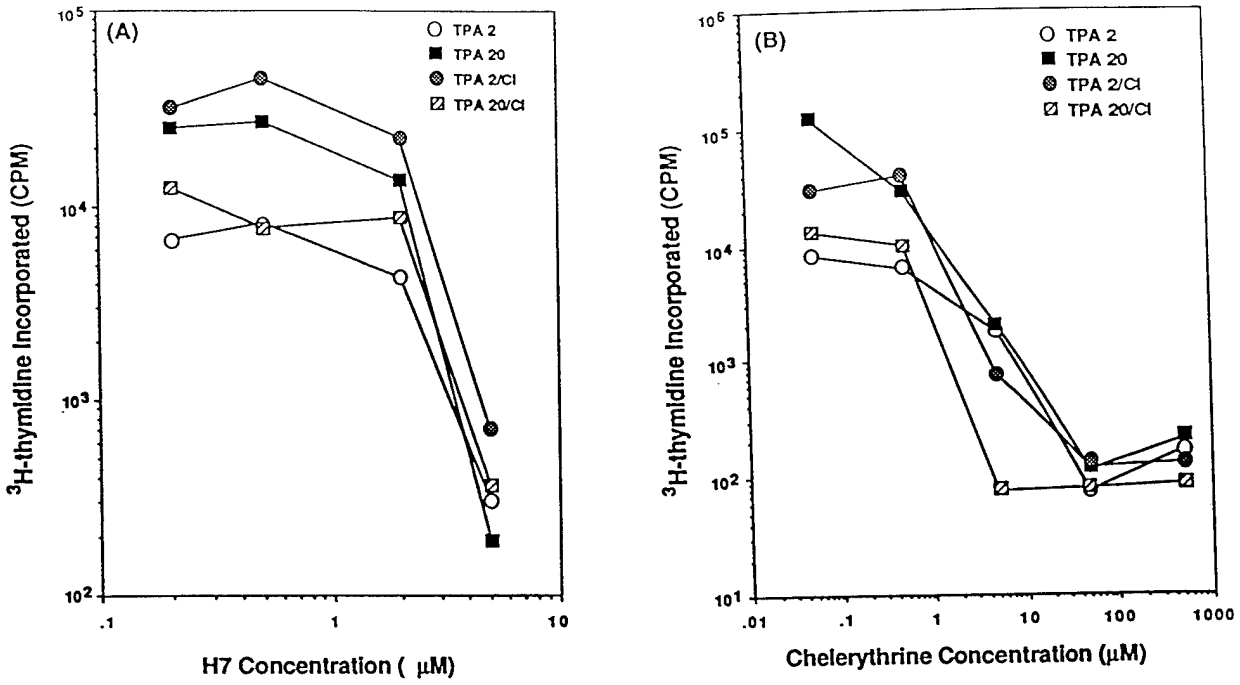


Figure 2. Inhibition profiles for PKC inhibitors H-7 and chelerythrine. Freshly isolated red drum PBL were cultured with TPA or TPA + CI at the indicated concentrations in the presence of titrated doses of kinase inhibitors H-7, chelerythrine and HA-1004. (A) H-7. (B) Chelerythrine. These inhibition profiles are consistent with selective activity against protein kinase C. All data points are the average of triplicate values, SE < 0.2 (from Burnett and Schwarz, 1994).

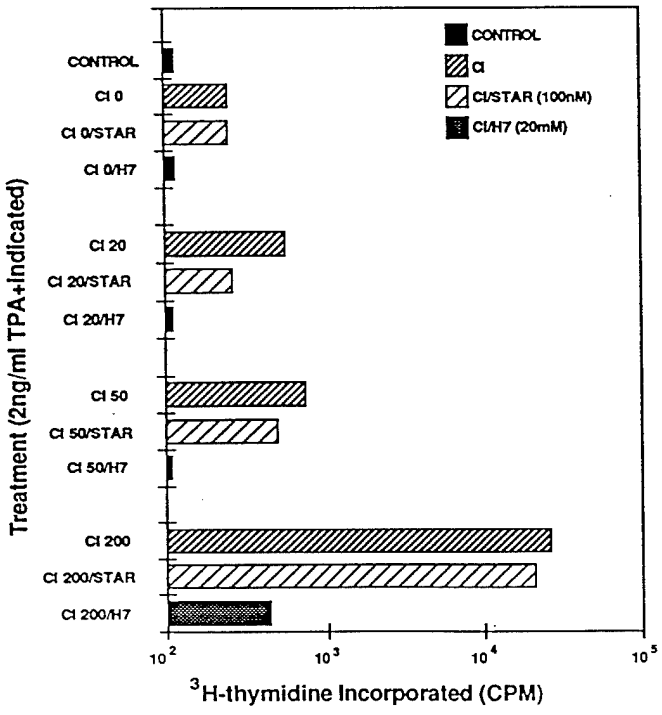


Figure 3. The action of protein kinase inhibitors H-7 and staurosporine (STAR). Freshly isolated red drum PBL were cultured with 2 ng/mL TPA + 200 ng/mL CI in the presence of kinase inhibitors H-7 or STAR. All mitogens and inhibitors were removed after 18 hours of culture. After two days, cultures were labeled with 3H-thymidine and harvested 18 hours later (from Burnett and Schwarz, 1994.)

With this thought in mind, we have begun to describe PKC isoforms and their activities in red drum leukocytes. Conventional isoforms of PKC are located in the cytoplasm and upon activation by DAG or phorbol esters will translocate to the cell membrane. Twelve isoforms of mammalian PKC have been described. These are grouped into four subtypes. Conventional PKCs are activated by both calcium and phospholipid. Novel PKCs are calcium-independent, and activated by phospholipid only. Atypical PKCs are independent of both calcium and phospholipid. Finally, a recently described class of PKCs have hydrophobic sequences suggesting that they are constitutively inserted into the cell membranes (Hug and Sarre, 1993; Johannes *et al.*, 1994). PKCα has been reported in channel catfish non-specific cytotoxic cells by Jaso-Friedman *et al.*, (1995). Using commercially available antibodies to the major isoforms, we have detected conventional PKCs α, β and γ. In addition we have evidence for novel PKCs δ, ε, η and atypical PKC z (Mericko and Burnett, in preparation). Currently, our laboratory is testing the ability of mercury to modulate the translocation and activation of each isoform upon stimulation with phorbol ester or cross-linking of antigen receptors.

Effects of mercuric compounds on calcium flux in teleost PBL

As described above, mammalian toxicology literature has provided a strong basis to suspect that PKC and calcium flux may be important targets for the action of mercury on lymphoid cells. To evaluate whether the PI signal transduction pathway in teleost peripheral blood leukocytes (PBL) is a target for the action of mercury we co-stimulated freshly isolated red drum PBL *in vitro* with 20, 5, 2 or 0 ng/mL TPA and graded doses of $HgCl_2$ (10^{-6} - 10^{-1} M). DNA proliferation was monitored after two days of exposure to mercury. At all three TPA doses the IC_{50} of $HgCl_2$ for red drum PBL was approximately 3 μM (Figure 4). However at the two lowest TPA doses sub-lethal

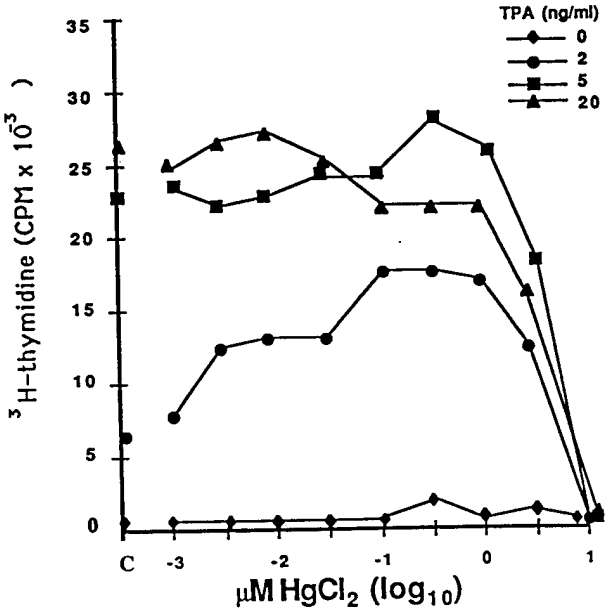


Figure 4. Mercury synergized with sub-mitogenic doses of TPA to induce PBL proliferation. Freshly isolated red drum PBL were treated with the indicated doses of TPA and $HgCl_2$. Proliferative responses were measured as described in Figure 1 (from Burnett *et al.*, manuscript in preparation.)

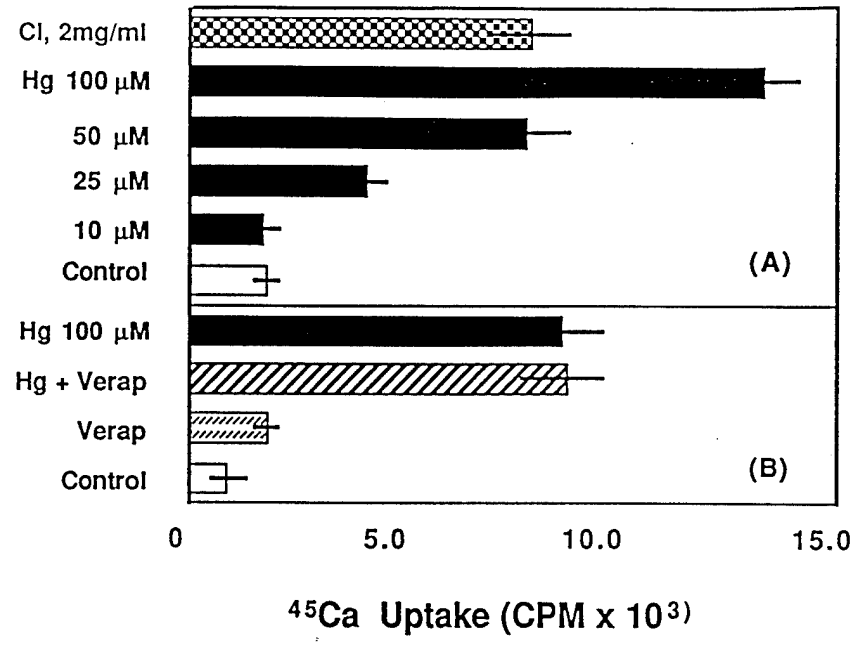


Figure 5. Uptake of 45Ca induced by $HgCl_2$. Freshly isolated red drum PBL were resuspended at 2.5 x 107 cells/ml in the indicated treatment and 15 mCi/ml 45Ca. After 10 min., cells were placed on ice, washed and lysed in 1% SDS + 0.5N NaOH and 45Ca uptake was measured. (A) Dose titration for HgCl2. Treatment with calcium ionophore A23187 (CI) served as a positive control. (B) Effects of calcium channel blocker Verapamil (Verap) on calcium flux. Verapamil (100 mM) was added 10 minutes before addition of 45Ca (from Burnett *et al., manuscript in preparation.)*

levels of $HgCl_2$ synergized with phorbol ester to induce DNA replication, with maximum enhancement at $HgCl_2$ = 1µM (Figure 4). This synergistic response was reminiscent of the previously demonstrated synergy between TPA and CI (Figure 1), and suggested that this *in vitro* system might allow us to investigate the mechanisms for the biphasic activation and suppression of the immune response in teleost fish. Followup studies demonstrated that the toxic action of 10 and 100 µM $HgCl_2$ on red drum PBL could be replicated with a only ten minute exposure to mercury. In contrast, the synergistic action of mercury and TPA required longer exposure times than exposures to $HgCl_2$ alone. This observation was consistent with the possibility that mercury might induce a mildly elevated, but sustained level of intracellular calcium as required to induce cell proliferation. In addition the synergism of 5 ng/mL TPA and 1.0 µM $HgCl_2$ was suppressed by 100 µM Verapamil, an inhibitor of L-type voltage-dependent calcium channels (data not shown, Burnett, MacDougal and Johnson, in preparation).

To directly measure calcium flux in response to mercury, red drum PBL were exposed to 10, 25, 50, or 100 µM $HgCl_2$ for ten minutes in the presence of ^{45}Ca. Immediately following this exposure, cells were cooled on ice, washed and lysed. At doses above 10 µM, $HgCl_2$ induced calcium uptake in excess of sham treated controls in a dose-dependent manner (Figure 5A). No uptake of calcium could be detected at 10 µM $HgCl_2$ for exposure periods up to one hour. The uptake of radiolabelled calcium with higher doses of mercury was not inhibited by Verapamil (Figure 5B). Efforts are now underway to monitor more subtle changes in intracellular calcium levels using the fluorescent calcium indicator dye fura-2.

CONCLUSIONS

Vertebrates from fish to humans share many common elements of immune function. Our studies and the work of others (van Ginkel *et al.*, 1994; Jaso-Friedmann *et al.*, in press) suggest that these commonalities extend both above and below the surface of lymphoid cells. Of themselves, these observations are and will continue to be of phylogenetic interest. Of further importance, these data support the contention that teleosts can serve as useful animal models for immunotoxicology studies and risk assessment for human health.

These data from *in vitro* studies of red drum PBL suggest that synergistic doses of mercury and TPA that induce cell proliferation target an intracellular mechanism distinct from that of toxic doses of mercury. Synergistic effects operate directly or indirectly through an L-type calcium channel and are not accompanied by rapid calcium uptake. Toxic effects are accompanied by massive calcium uptake, and this effect cannot be blocked by treatment with calcium channel inhibitors. However, the primary intracellular targets for the actions of mercuric chloride remain to be clarified.

The studies reviewed here have focused on Hg. Similar mechanisms have been proposed for the biphasic effects of cadmium and other divalent heavy metals on immune function. Cadmium induces calcium flux, enhances turnover rates of membrane phospholipids and induces PKC activity (Smith *et al.*, 1989; Block *et al.*, 1992). Some organic contaminants, such as the dioxins, also may effect early events in the PI pathway, both directly (Kramer, 1987; Luster *et al.*, 1988) and by "cross-talk" with other intracellular signaling pathways (Berghard *et al.*, 1993). Should the intracellular effects of the heavy metals and organic contaminants be elucidated in lower vertebrates, such as fish, it may be possible to formulate predictive models for the effects of mixed contaminants on immune function and to confirm these predictions in inexpensive, large scale immunotoxicology assays with non-mammalian species.

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Chapter 35

Health Status Determination and Monitoring in an Aquatic Model (*Oryzias Latipes*) Used in Immunotoxicological Testing

Lorraine E. Twerdok[1], Joseph R. Beaman, Marianne W. Curry and Judith T. Zelikoff[2]

GEO-CENTERS, INC.
Frederick, MD

Present address:American Petroleum Institute
1220 L. St. NW
Washington, DC 20005

[2]New York University School of Medicine
Nelson Institute of Environmental Medicine
Tuxedo, NY.

INTRODUCTION

There are many reasons for monitoring the health status of animals raised for use in toxicological and immunotoxicity testing. (1.) It is necessary to document that healthy animals are used in toxicological studies. (2.) It is necessary to monitor the health status of animals during chronic toxicity testing, to demonstrate that no confounding health problems arise during the course of the study. This is particularly important when evaluating the immune system as an endpoint for toxicity. (3.) It would be beneficial to anticipate problems before large numbers of animals are committed to studies. (4.) If research facilities supply test animals to other facilities and/or research collaborators, it is necessary to demonstrate and document that the fish are healthy and suitable for study. In many aquatic models, including medaka (*Oryzias latipes*), much of this routine health screening has not been documented. Thus, the purpose of this study was to assess the normal variability in routine health parameters in medaka. The longer term goal of this work is to develop a health screening protocol for small fish models of toxicity, such as the Japanese medaka, which, when performed on a routine basis, is indicative of the health status of the animals.

MATERIALS AND METHODS

Test species

Japanese medaka (*Oryzias latipes*) were reared and maintained in the U.S. Army Biomedical R&D Laboratory (USABRDL, Fort Detrick, MD) in a flow-through aquaria system maintained at 25°C with 16/8 hrs. light/dark cycle, and fed flake food (Tetramin®), brine shrimp and microworms. Animals used for this study were 2-12 months of age.

Weight, length, blood collection

Adult medaka were anesthetized using tricaine methanesulfonate (MS-222, 200 mg/L; Sigma #A-5040) and weights and lengths were measured. Blood was collected from the caudal vein using a microhematocrit tube (20 µL) containing heparin. Blood was spun in a standard microcentifuge for determination of red and white blood cell levels. Plasma was then collected and frozen for future use in media or used immediately for plasma IgM and total protein determinations.

Plasma Ig and total plasma protein

Plasma protein and immunoglobulin was assessed by a modification of the method of Siwicki *et al.*, (1994). Immunoglobulin is separated from plasma by precipitation with polyethylene glycol (PEG). The difference between total plasma protein and plasma protein after removal of Ig-PEG (by centrifugation) is the plasma Ig protein. Briefly, the procedure is as follows. Reserve a small aliquot per sample of whole plasma for protein determinations. Incubate a known volume of plasma with 12% PEG (10,000 kD) for 2 hrs at room temperature, constantly shaking on an orbital shaker to keep the PEG in suspension. Following incubation, centrifuge the plasma/PEG suspension for 10 min. @ 5000 xg; remove supernatant (discard pellet). Protein concentrations were then determined in the supernatant and whole plasma using the Biorad® assay kit (#500-0006). This assay uses linear regression analysis of concurrently assayed bovine serum albumin standards to estimate unknown protein concentrations.

Anterior kidney cell isolation

Anterior kidney cells were isolated by a modification of previously described procedures (Twerdok *et al.*, 1994). Briefly, following anesthesia, fish were decapitated and anterior kidneys removed and pooled in 3 mL fish physiological saline containing 1% glucose (FPS+, pH 7.2). The pooled organs were weighed, and then stored overnight at 4°C. Organs were homogenized using glass/glass homogenizers, and resultant whole cell suspensions filtered through loosely packed glass wool and collected in 15 mL centrifuge tubes. Cell suspensions were then gently centrifuged @350 xg for 5 min, resuspended and counted using a hemacytometer.

In vitro superoxide dismutase-inhibitable extracellular superoxide anion production

In vitro generation of extracellular superoxide anion was assessed by reduction of cytochrome *c* by a modification of previously described procedures (Zelikoff *et al.*, 1991; Twerdok *et al.*, 1994). Briefly, superoxide anion production by anterior kidney cells was measured immediately after

isolation. Cells were plated at 2 x 10^5 cells per well in 96-well microtiter plates, and then stimulated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA, 0.5 µg/mL; Sigma #P-8139). The amount of superoxide dismutase-inhibitable superoxide anion produced was assessed by spectrophotometric measurement in a microtiter plate reader at 550nm; production was measured in stimulated and unstimulated cells for 2 hr.

Bacterial isolation and identification

Internal viscera, excluding intestine, were aseptically removed from fish previously anesthetized with MS-222 (200 mg/L) and streaked onto Trypticase Soy Agar plates (BBL, #11043). Water samples were collected from culture tanks and inlet valves using sterile pipettes. Sample volumes of five and 10 ml were passed through 0.45 µm gridded HA filters (Millipore), each filter was removed, blotted, and placed surface up onto a Trypticase Soy Agar plate. All fish and water sample plates were incubated at 25° C. Bacterial identification was performed by Maryland Medical Laboratories, Frederick, MD.

Histopathology

Medaka were euthanized with an overdose of MS-222 (400 mg/L) and weights and lengths of the fish were determined. A slit was made in the abdominal cavity and whole fish were fixed with Bouins solution (24-48 hr), washed twice in 70% ethanol (24 hr each) and transferred into 10% buffered formalin. Hematoxylin and eosin stained slides were prepared from the fixed fish. Each fish was sampled by cutting five step sections through the whole fish in a longitudinal plane. Histopathological evaluations were conducted by Dr. Marilyn Wolfe of Experimental Pathology Laboratories, Inc., Herndon, VA, and Dr. Tracie Bunton of the Johns Hopkins University, Baltimore, MD.

Statistics

Data were analyzed using SPSS® software using the Student's *t*-test (paired data) or one-way ANOVA and Scheffe Multiple Range Test, when appropriate. Results were considered significantly different with a $p<0.05$.

RESULTS AND DISCUSSION

Characterization of weight and length

These small aquarium fish grow rapidly during the first four months of life, and continue to increase slowly in size through 12 months of age. This slow increase in size most likely continues throughout life, however, in this study we only examined fish up to 12 months of age. Mature medaka typically range from 300 - 450 mg in weight and 25 - 30 mm in length. Females are typically larger than males (data not shown).

Characterization of hematocrit, leukocrit, plasma protein and plasma IgM

Hematocrit, leukocrit, plasma protein and plasma Ig concentrations are presented in Table 1. Data is presented by sampling point, rather than age, since there was no age-related statistical difference in these values. It should be noted that these parameters can only be measured in older/larger fish due to the small volume of blood recoverable from a medaka. Generally, these parameters were measured in animals ≥ 5 months of age. Hematocrit, leukocrit, plasma protein and plasma Ig concentration values were comparable to other teleost species (Blaxhall, 1972; Frange, 1992; Klontz, 1994; Pickering and Pottinger, 1987; Smith, 1968; Summerfelt, 1967; Wedemeyer, 1983). The hematocrit values observed in medaka (≈45-50%) from our facility were similar to those measured by Zelikoff *et al.* (1995). However, these values were at the high end of normal values reported in the literature for teleosts; typical values for other fish species range from 30-40% (Klontz, 1994).

TABLE 1
Characterization of hematologic parameters in the Japanese medaka

	Sampling Timepoint		
	7/94	11/94	1/95
Hematocrit	49.1 ± 6.3[a] (14)[b]	47.9 ± 8.5 (33)	45.5 ± 6.27 (94)
Leukocrit (%)	57 ± 0.59[b] (14)	94 ± 0.56[b] (18)	77 ± 0.47[b] (6)
Total Plasma Protein (mg/mL)	n.d.[c]	18.9 ± 10.7 (18)	16.4 ± 4.3 (6)
Plasma Immunoglobulin (mg/mL)	n.d.[c]	5 ± 6.5 (18)	14.7 ± 3.8 (6)

[a] Values represent Mean ± S.D.; number in parenthesis is number of observations (*n*).
[b] Values significantly different over time (between sampling timepoints) ANOVA at $p<0.05$ and Scheffe Multiple Range Test; F=7.4510.
[c] n.d. = not done

Characterization of anterior kidney weight, cell yields and viability

The anterior kidney is the hematopoietic organ in teleost species, consequently, this organ is harvested as a source of immunological cells used to assess immune status in medaka. Anterior kidney weight, cell yields and viability from animals ≥ 5 months of age are presented in Table 2. There is a trend toward increases of cell number and organ weight with age and size of the animal; the only significant difference noted was in kidney weight between 5-6 and 7-9 month old animals. However, the difference noted may be a result of the small sample sizes assessed. Cell viability was high (≈95%) and did not vary with age.

Table 2.
Anterior kidney health status indices in adult medaka

Fish Age	5-6 mo	7-9 mo	≥10 mo
Cell Yield (10^5 cells/fish)	5.7± 3.2[a] (4)	13 ± 6.5 (5)	17 ± 5.8 (4)
Cell Viability (%)	95 ± 2.6 (4)	95 ± 3.8 (5)	96 ± 1.7 (4)
Ant. Kidney Wet Wt. (mg/fish)	1.92 ± .30[b] (4)	3.32 ± .43b (5)	2.92 ± .48 (4)
Peak Superoxide Production (nmol cyt. *c* reduced/2x10^5cells)	6.7 ± 1.46 (4)	8.3 ± 2.0 (5)	5.7 ± .89 (4)
Time to Peak O_2^- Production (min)	150 ± 52 (4)	60 ± 18 (5)	135 ± 53 (4)

[a] Values represent Mean ± S.D. Number in parenthesis is number of observations (*n*).
[b] Values significantly different between 5-6 and 7-9 mos old animals by ANOVA at $p<0.05$ and Scheffe Multiple Range Test; F=7.4510.

In vitro superoxide dismutase-inhibitable extracellular superoxide anion production

Since we are currently using the medaka in immunotoxicological screening assay development, one functional assay of immune status was selected to incorporate into our health monitoring protocol. We selected to assess *in vitro* generation of superoxide anion production, which is a component of non-specific, bacteriocidal immunity (Zelikoff *et al.*, 1996). We selected this parameter because alterations (i.e. suppression or stimulation) of non-specific immunity in our culture animals could seriously compromise the culture's overall health status and suitability for use in immunotoxicological and general toxicological studies. There were no age-related differences in peak superoxide anion production following stimulation with TPA in animals 5 to 12 months of age; mean production ranged from 5.7 - 8.3 nmol superoxide anion produced per 2x10^5 cells. There was an age-related difference in the time to peak oxidant production, with 7-9 month old animals reaching peak production much faster than younger or older animals. The exact mechanism by which this may be occurring is currently unclear.

Bacterial isolation and identification

Preliminary data indicated that *Aeromonas* and *Pseudomonas* appeared to be common flora in the medaka (data not shown). More work needs to be done before a definitive characterization of the flora in our culture fish will be available.

Histopathology

The most striking histopathological lesions noted to date in our cultured medaka is the increasing incidence of granulomatous lesions in older animals. Preliminary data is presented by sampling timepoint as a convenient method to differentiate between groups of fish, and is presented in Table 3. It is interesting to note that granulomas have been observed in a wide variety of organs, predominantly in male animals. The causative agent for these lesions has yet to be determined, however, acid fast organisms, suggestive of *Mycobacterium*, have been observed in some samples using special staining techniques. The biological significance of these lesions with respect to host immune competence is unknown. However, in a recent study by Abner *et al.* (1994), no correlation was observed in medaka between incidence of granulomas and chemical treatment in long term carcinogenicity studies. The data presented in this paper are derived from initial studies to characterize the normal histopathology of our culture animals, thus it is premature to draw any definitive conclusions concerning the "normal" incidence of granulomatous lesions in medaka. As with our bacteriological studies, more work needs to be done before a definitive characterization of the normal histology and histopathology in medaka are available.

Table 3
Incidence of granuloma by age

Sacrifice Date	Total Incidence (%)	Incidence by age		Incidence by sex (%)
		Age (mos)	Incidence (%)	
4/94	5/34 (15)	11	1/2 (50)	male 5/22 (23)
		10	2/8 (25)	female 0/12 (0)
		9	1/8 (12)	
		8	1/8 (12)	
		5	1/8 (12)	
		3	0/8 (0)	
7/94	2/16 (12)	11	2/2 (100)	male 2/7 (28)[a]
		8	0/4 (0)	female 0/8 (0)
		6	0/2 (0)	
		5	0/4 (0)	
		3	0/4 (0)	
8/94	6/13 (46)	12	6/13 (46)	male 5/9 (56) female 1/4 (25)

[a] One animal in this group could not be sexed histologically, i.e. no gonadal tissue on section examined.

SUMMARY

The purpose of this study was to begin to assemble a database on the normal variability in routine health parameters in the Japanese medaka (*Oryzias latipes*). Hematocrit, leukocrit and plasma IgM levels were within the range for other teleost species. Preliminary results of this study indicate that multiple strains of *Aeromonas* and *Pseudomonas* were the predominant internal flora in medaka. Additionally, a trend toward seasonal variability in bacterial flora was also noted. The most striking histological finding to date was the presence of granulomas, which correlated with age. It should be noted that some of these parameters, particularly bacterial flora, may be facility, water source and diet dependent.

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Chapter 36

Heavy Metal-Induced Changes in Antioxidant Enzymes and Oxyradical Production by Fish Phagocytes: Application as Biomarkers for Predicting the Immunotoxic Effects of Metal-Polluted Aquatic Environments

J.T. Zelikoff*, W. Wang, N. Islam, E. Flescher, and L.E. Twerdok[1]

Institute of Environmental Medicine
New York University School of Medicine
New York, New York 10016 USA

[1]GEO-CENTERS
Fort Detrick
Frederick, MD

[1]present address: American Petroleum Institute
1220 L. Street Northwest
Washington, D.C. 20005 USA

*To whom reprint requests should be addressed

ABSTRACT

Because of increasing social and political pressure to use alternative (non-mammalian) models for predicting human health risks, as well as to develop markers for assessing the biological effects of environmental stress, studies that utilize less traditional animal species are increasing. The study of biochemical responses in aquatic animals comprises a vigorous area of investigation for several reasons, including the need for sensitive biomarkers useful for hazard assessment. Of particular concern to environmental toxicologists in this area of study is the generation of reactive oxygen intermediates (ROI) and subsequent oxidative stress in biological systems. Reactive oxygen intermediates are continuously produced as metabolic by-products by virtually all tissues in both fish and mammalian species, and without adequate protection from these oxygen species, cells/tissues can suffer significant oxidative damage. While superoxide dismutase (SOD), catalase (CAT), and NADPH: quinone reductase (QR) are well-recognized antioxidant/protective enzymes in mammalian cells, little is known concerning these enzymes in cells from aquatic species. In addition, the effects of environmental contaminants on ROI production and on these protective enzymes in fish have not been well-studied. For this investigation, 10 month old medaka (*Oryzias*

latipes) were exposed for 5 days in the water to 6, 60, or 600 ppb cadmium (Cd), a well-studied immunotoxicant and major aquatic pollutant, and the effects on ROI production by kidney phagocytes, and on antioxidant activity by cells recovered from the kidney, spleen, and liver were evaluated. At concentrations ≤ 60 ppb, Cd produced no effects on overall body weight, cell viability, recovered cell number, leukocrit or hematocrit values, total plasma protein levels, or plasma immunoglobulin titers. However, short-term, low-dose exposure to Cd stimulated the production of large quantities of both superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by medaka whole kidney cell homogenates (bone marrow equivalent). Immunotoxic effects of Cd persisted for up to 10 days following placement of fish into clean water. Equimolar concentrations of other inorganic metal cations including zinc, nickel, and mercury also increased O_2^- production, but only exposure to Cd enhanced H_2O_2 production. Waterborne exposure to Cd at a concentration well below the LC_{50} value also altered SOD, CAT, and QR activity in the medaka kidney, spleen, and/or liver. Results of these laboratory studies suggest the applicability of ROI production and antioxidant enzyme activity to serve as biomarkers in field studies to predict the effects of aquatic metal pollution.

INTRODUCTION

Fish are exposed to toxic chemicals mainly from discharges to rivers, lakes, and oceans; marine dumping; and atmospheric fallout. In the North Atlantic ecosystem alone, hundreds of pollutant chemicals have been identified and quantified (Myerson et al. 1981; O'Connor and Huggett, 1987). These chemicals include metals, as well as synthetic and chlorinated organics, and polycyclic aromatic hydrocarbons (PAH) (Meyerson *et al.*, 1981; MacLeod *et al.*, 1981; O'Connor and Huggett, 1987). For example, mean concentrations as high as 400 ppm of lead (Pb), mercury (Hg), and cadmium (Cd) have been measured in Newark Bay sediments (Meyerson *et al.*, 1981). In Puget Sound, arsenic (As), Pb, and Hg concentrations are significantly elevated in the urban embayments of Sinclair Inlet and Commencement and Elliott Bays (compared to nonurban reference areas) (Malins *et al.*, 1984). In other parts of the world, levels of toxic heavy metals over and above those found naturally in the aquatic environment have been measured in the water, sediment, and/or in the tissues of those organisms that reside there. For example, perch (*Perca fluviatilis*) living in the Cd-contaminated Eman river in southeastern Sweden have liver Cd levels 6 - 8 times higher than those measured in reference perch (Sjobeck *et al.*, 1984). Fish taken from the North Sea and distant-water fishing grounds (in the vicinity of England and Wales) have body burdens of Pb 8 - 12 fold above government regulated limits (i.e., 0.5 ppm Pb) (Portmann, 1972). Furthermore, some commercial species of New Zealand sea fish have liver concentrations of Cd as high as 54 ppm Cd (Brook and Rumsey, 1974).

The growing environmental pollution by potentially toxic metals gives rise to particular problems in the aquatic environment. The effects of metal pollution are measurable on both ecological and economic scales. Ecosystem impacts include contamination of sediments and the water column, accumulation of pollutants in biota over a wide area, and apparent increases in pollutant-related anomalies in the species that reside there.

The biological effects of aquatic metal pollutants can be devastating not only for directly-exposed organisms, but also for humans that may consume these affected species. Documented effects of

metallic pollutants on residing aquatic species include alterations in hematological parameters, enzyme functions and/or gene expression, homeostasis, carbohydrate metabolism, and embryonic and/or ova development, as well as an increased incidence of neoplasms (Larsson, 1975; Johannson-Sjobeck and Larsson, 1979; Weis et al. 1981; Sjobeck *et al.,* 1984; Malins *et al.,* 1984). In addition, metallic aquatic pollutants alter host immunocompetence by affecting cell-, humoral-, and/or non-specific macrophage-mediated immunity (Enane *et al.,* 1991; Zelikoff, 1993; Bowser *et al.,* 1994; Zelikoff, 1994; Zelikoff *et al.,* 1995; Zelikoff, 1995). Because of their level of sensitivity to chemical pollutants, fish systems are currently being used for biomonitoring studies and for assessing the toxicological hazards associated with pollutant exposure.

Emphasis has recently been placed on the development of biological markers that can predict exposure to- and effects from- environmental pollutants. As monitors for exposure, biomarkers have the advantage of quantifying only biologically-available pollutants. As measures of effects in the laboratory, biomarkers can integrate the effects of multiple stressors and can assist in elucidating mechanisms of effects.

The immune system is crucial for protection of the host against infectious agents and developing neoplasms. In addition to their importance in host defense, immune responses are exquisitely sensitive for assessing the toxicological hazard of chemicals of environmental concern and any alterations are observed well before the more routine markers of chemical stress and toxicity, i.e., hematological parameters or tissue cellularity. The sophistication, complexity, and other characteristics of the immune system enable it to potentially be the most sensitive and, therefore, most prominent body function to detect the adverse effects from pollutant exposure.

Generation of reactive oxygen intermediates (ROI) by host phagocytes (i.e., macrophages and neutrophils), as well as subsequent oxidative stress in a variety of biological systems, is being investigated in mammalian species as a possible biomarker useful for biomonitoring studies. Superoxide anion (O_2^-), produced both inside and outside mitochondria, is the first radical produced in the reduction of oxygen to water (Drath and Karnovsky, 1975; Bostek, 1989). Mammalian (Johnston, 1978) and fish (DiGuilio *et al.,* 1989; Secombes, 1990; Zelikoff, 1994; Bowser *et al.,* 1994; Zelikoff *et al.,* 1995) phagocytes, when activated by microbes or other stimulants, quickly produce O_2^- and hydrogen ions as part of their anti-microbial defense. These potent cytotoxic free radicals then participate in the ultimate destruction of internalized infectious agents. Chemical-induced alterations in this important biological mediator could, potentially, lead to an increased susceptibility of the host to infectious diseases and/or localized tissue damage. Hydrogen peroxide (H_2O_2) is a powerful oxidant with cytotoxic properties (Bostek, 1989). Like O_2^-, H_2O_2 is produced by activated phagocytes, as well as by mitochondrial NADH and ubiquinone. Probably the most important reaction contributing to the toxic effects of H_2O_2 is its reduction to OH^- radical in the presence of ferrous iron.

The biological potential for oxygen radical production is great. Complex multienzyme systems such as mitochondrial and microsomal electron transport, as well as leukocyte phagocytosis, can generate large quantities of oxygen radicals (Drath and Karnovsky, 1975; DiGuilio, 1989; Bostek, 1989). Certain classes of xenobiotics, such as metals and quinones, represent particularly prolific sources of oxygen radicals because of their abilities to be reduced to their corresponding radicals via NAD(P)H-dependent reductases (NADPH-cytochrome P-450 reductase, xanthine oxidase,

ferredoxin reductase, NADH-ubiquinone oxidoreductase) prior to subsequent redox cycling (DiGuilio, 1989). In addition to the ability of certain xenobiotics to generate ROI based on their chemical reactivites, these toxicants may also affect cellular components (i.e., receptors, protein kinases, and phoshphatases), thereby modulating the response of phagocytes to stimulators of the oxidative burst. The analysis of data is even more complex when fish are exposed *in vivo* and the observed effects on immune cells may be secondary to other xenobiotic-induced alterations such as tissue damage.

An important aspect of free radical-mediated toxicity is that it is modulated in the host by several antioxidant cellular defense mechanisms including enzymatic [i.e., superoxide dismutase (SOD)], and non-enzymatic (vitamin E and C) systems. Antioxidant enzymes such as SOD, catalase (CAT), and quinone reductase (QR) allow the organism to protect itself against xenobiotic oxidants. In addition, these enzymes enable ROI-producing cells and adjacent tissues to withstand the oxidative stress exerted by endogenously-generated active oxygen species. Therefore, efficient elimination of ROI constitutes an essential component of the defense systems used by impacted species exposed to aquatic toxicants. While SOD, CAT, and QR are well-recognized antioxidant/protective enzymes in mammalian cells, little is known concerning these enzymes in cells from aquatic species. In addition, few studies have even examined these same parameters in warm, freshwater fish, or in those species exposed to toxicants under controlled laboratory conditions.

Because of the sensitivity of immune responses to environmental contaminants and their importance in maintaining host resistance against disease, chemical-induced immune dysfunction can be predictive of the toxicological hazards/risks associated with pollutant exposure. Thus, this study was undertaken to develop specific aspects of the fish immune response as a biological system for predicting the health risks associated with polluted aquatic environments. To address the aforementioned goal, this laboratory has been developing and validating *in vitro* bioassays that assess normal immune function of small aquaria fish and that can serve as biomarkers to predict the effects of pollutant exposures in feral fish populations.

MATERIALS AND METHODS

Fish Sacrifice and Cell / Blood Collection

Ten-month old medaka (*Oryzias latipes*), fed twice a day on a diet of flake food (Tetramin) and brine shrimp, were selected randomly from 10-gallon aquaria maintained under static conditionsat a temperature of 25 ± 1°C with a 16/8 hr light/dark cycle. Water quality was closely monitored by measuring ammonia, alkalinity, chlorine, oxygen, hardness, and pH twice daily.

Fish were anaesthetized by placing medaka into 500 mL deionized water containing phosphate-buffered MS-222 (100 mg MS-222/500 mL) for approximately 1 min and then sacrificed by decapitation. Kidney, spleen, and/or liver was then removed and weighed, and the organ either stored overnight at 4°C [in 3-mL 0.5% medaka serum-supplemented fish physiological saline (FPS)] to supply cells used the next day for measuring ROI production, or frozen at -70°C until used to measure antioxidant activity.Blood was collected prior to sacrifice and used to measure hematocrit

and leukocrit values, Total plasma protein and plasma immunoglobulin levels as described by Twerdok, *et al.* (in press).

Like body organs were pooled and ground using glass/glass homogenizers. The resultant homogenates were then passed through a loosely-packed glass wool-syringe column (3-mL syringe barrel) to remove tissue/cellular debris/red blood cells. The recovered single cell suspensions were then centrifuged twice at room temperature for 15 min at 400 x g. After the final wash, the supernatant was removed, the pelleted cells resuspended in 1 mL supplemented FPS (Zelikoff *et al.*, 1996), and the cell number and viability determined by hemocytometer counting and trypan blue exclusion, respectively.

EXPERIMENTAL DESIGN

Fish were exposed for 5 days, using a static-renewal exposure system, to either clean water or to water containing chlorides of either cadmium (Cd), nickel (Ni), inorganic mercury (Hg), or zinc (Zn). Fish were exposed to waterborne Cd at 6, 60, or 600 ppb, or to the other three metals at a concentration equal to the lowest Cd dose. In some Cd experiments, medaka were removed at the end of 5 days and placed in "clean water" for either 3 or 10 days to determine the persistence of Cd-induced effects on ROI production. Following metal/clean water exposures, kidney cells were recovered from medaka as described above and extracellular and intracellular superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) production were evaluated. To determine the effects of Cd on antioxidant activity, additional groups of fish exposed to Cd at 60 ppb for 5 days were sacrificed and the effects on superoxide dismutase (SOD), catalase (CAT), and quinone reductase (QR) activity in the kidney, spleen, and liver were determined. Metal body burdens and water concentrations were determined as described previously by Zelikoff *et al.* (1995), using atomic absorption spectrophotometry (AAS).

Superoxide (O_2^-) Production

Extracellular

Kidneys were removed from medaka and placed in a 35-mm Petri dish containing 3 mL cold medaka serum-supplemented FPS. Following tissue disruption and passage of the cells through a loosely-packed glass wool column, single cell suspensions were adjusted to a cell concentration of 4×10^6 kidney cells/mL.

The reaction mixtures used to measure extracellular O_2^- production were prepared by adding 10^6 kidney cells (in a total volume of 250 µL) to each of 4 sterile polypropylene tubes containing 500 µL ferricytochrome C (final concentration = 2 mg/mL). The second and fourth tubes received 125 µL of SOD (final concentration = 37.5 µg/mL) and the third and fourth tubes received 50 µL of phorbol myristate acetate (PMA) at a final concentration of 0.5 µg/mL to measure stimulated production. Fish physiological saline was then added to each tube to bring the final volume up to 1 mL. An additional tube that contained all of the reagents, but without cells, served as the reaction blank. After vortexing, 2×10^5 cells were then placed into the individual wells of a 96-well microtiter plate and the absorbance measured at 550 nm for up to 2 hr. Change in absorbance was

calculated by subtracting the mean of the "blank" wells and the wells containing SOD from the absorbance measured in the non-SOD-containing wells. By multiplying the change in absorbance by 15.87 (Pick and Mizel, 1981), the nmol concentration of SOD-inhibitable O_2^- was computed. Data was expressed as nmol O_2^-/2 x 10^5 cells/60 min (Zelikoff *et al.*, 1996).

Intracellular

Kidney cells were counted using a hemocytometer and the cell concentration adjusted to 6 x 10^6 cells/mL in 0.5% medaka plasma-supplemented L-15 medium. One-hundred µl of the cell suspension was then added to each of 12 wells of a microtiter dish and the plate incubated (at 30°C) in a humidified environment for 90 min to allow for cell attachment. During incubation, reaction solutions were prepared in each of 4-sterile polystyrene tubes. Tubes 1 and 2 were used to measure unstimulated (basal/background) intracellular O_2^- production, while tubes 3 and 4 contained PMA and were used to measure stimulated production. Nitroblue tetrazolium (NBT) at a final concentration of 0.8 mg/mL was added to all 4 tubes. Two hundred µL of SOD was then added to tubes No. 2 and 4; tubes 3 and 4 received 100 µL of PMA. Following cell incubation, the supernatant was removed and the cells washed once with warm (30°C) Hank's balanced salt solution (HBSS). The covering medium (CM) and HBSS wash was saved to determine the actual numbers of remaining attached cells. Reaction mixtures (100 µL) were then added to quadruplicate wells. The cells were incubated at 30°C for 60 min and then washed three times with 70% methanol and air-dried at room temperature. Aliquots of 2M KOH (120 µL) and DMSO (140 µL) were then added to each well and mixed thoroughly to dissolve the dried formazan; KOH and DMSO were also added to wells without cells that served as the reaction blanks. The absorbance in each well was measured at 630 nm in a microtiter platereader and the OD values measured in both the "blank" wells and those wells containing SOD were subtracted from those values measured in the wells with cells, but without SOD. The nmole concentration of intracellular O_2^- was calculated by multiplying the final OD values by 15.87 (Pick and Mizel, 1981) and adjusting for any cell detachments (Zelikoff *et al.*, 1996).

Hydrogen Peroxide (H_2O_2) Production

Medaka kidney cells were adjusted to 6 x 10^6 cells/mL in medaka plasma-supplemented L-15 medium. Cell suspension (100 µL) was then added to each of 8 wells of a microtiter dish and the plates incubated for 90 min at 30°C to allow for cell attachment. Following incubation, the CM was removed and the cells rinsed one time with warm HBSS. Both the CM and rinsing medium were saved for determining the total numbers of detached cells. One hundred µL of a previously prepared phenol red (PR), horseradish peroxidase (HRPO) solution (PR/HRPO = 2.4 mL; HBSS = 0.6 mL) was added into each of 3 wells containing the attached medaka kidney cells. The remaining 4 wells were used to measure stimulated production of H_2O_2 and received 100 µL of the same solution supplemented with PMA (PR/HRPO = 2.4 mL; 0.3 mL PMA; HBSS = 0.3 mL). Three wells without cells also received 100 µL of the reaction mixture (with and without PMA) and served as the reaction blanks. Immediately following addition of the reaction mixture to the individual wells, the plates were incubated for 60 min. Following incubation, 10 µL of 1 N NaOH was added to every well and the plates reincubated at 30°C for an additional 3 min. Absorbance in each well was determined in a microtiter platereader using a 620 nm filter. The final nmole concentration of H_2O_2 was

determined against a freshly-prepared standard curve using commercial H_2O_2 and the results expressed as nmole $H_2O_2/10^6$ cells after adjustment for attachment (Zelikoff *et al.*, 1996).

Antioxidant Enzyme Activity

Catalase (CAT)

To measure CAT, an antioxidant enzyme localized in the cytoplasmic peroxisomes that directly catalyzes the decomposition of H_2O_2, 5 X 10^6 cells from medaka liver, kidney, or spleen were suspended in 0.5 mL 50 mM PBS and sonicated for 20 sec (Zelikoff *et al.*, 1996). The sonicate was centrifuged at 20,000 x g for 10 minutes (at 4°C) and 50 µL of the resulting supernatant was then added to a solution of 10 mM H_2O_2 in PBS. The decrease in absorption (due to H_2O_2 decomposition by CAT) was then measured at 240 nm over a 3 min period. It is not possible to define CAT units according to international conventions due to aberrant kinetics. However, enzymatic activity can be expressed as the rate constant of a first-order reaction (k). A1 and A2 refer to the absorbance before and after a given time interval of measurement, respectively. Equation 1 is an example for a 15 sec time interval:

$$(1)\ k = (2.3/15)(\log A_1/A_2) = 0.153(\log A_1/A_2)(sec^{-1})$$

Superoxide Dismutase (SOD)

Superoxide dismutase, found in mitochondria where it protects cells from oxidation damage by catalyzing the dismutation of O_2^- to H_2O_2, is measured using the assay method described by Zelikoff et al. (1996) for medaka and based upon the inhibition by SOD of O_2^--dependent auto-oxidation of pyrogallol (Marklund and Marklund, 1974). Briefly, kidney cells were suspended in 0.1 mL lysing solution and incubated for 20 min at 30°C. The cell lysate was then centrifuged at 250 x g for 10 min (at 4°C), the supernatant collected, and 0.1 mL plasma-supplemented FPS added. Enzymatic activity in the supernatant was measured by adding to solution A (9.8 mL; 50 mM Tris acetate buffer with 1 mM EDTA) and to 0.1 mL of solution B (0.2 mM pyrogallol in 50 mM Tris acetate buffer) either: (a) 0.1 mL water to determine the maximal rate of auto-oxidation of pyrogallol, (b) 0.1 mL of a known SOD concentration to generate the standard curve, or (c) 0.1 mL of an unknown sample. Samples from these mixtures were then transferred to a cuvette and their absorbance measured for 3 min at 240 nm in a spectrophotometer. This particular time range was selected because of the linear correlation between decreased absorbance and time. A standard curve was generated from a set of known SOD concentrations and was used to derive the concentration of SOD in the test sample. Superoxide dismutase used for generating the standard curve was prepared by diluting a 1 mg/mL stock solution in sterile, double-distilled water to yield a final working concentration between 2.5 - 15 µg/mL.

NAD(P)H: Quinone Reductase (QR)

NAD(P)H:QR, a phase II enzyme that catalyzes the reduction of quinones and quinoneimines in mammalian systems (Prochaska and Santamaria, 1988), is measured by plating 2.5 x 10^6 medaka kidney cells/0.2 mL/well into a 96-well microtiter plate. The cells were then lysed with 0.08% digitonin in 2 mM EDTA (pH 7.8) for 10 min and the plate centrifuged at 250 x g and the supernatants

collected and added to wells of a second microtiter dish. In a final volume of 230 µL, each well in the second plate received 40 µL cell lysate supernatant, 40 µL 0.3 mM dicoumarol [NAD(P)H:QR inhibitor] or its vehicle (0.5% DMSO/5 mM potassium phosphate, pH 7.4), and 150 µl of the assay solution. The assay solution consisted of: 0.025 M Tris-HCl, pH 7.4; 0.67 mg/mL BSA; 50 mM menadione in acetonitrile; 0.01% Tween-20; 5 mM FAD; 1 mM glucose-6-phosphate; 0.12 mM NADP; 2 U/mL crystalline yeast glucose-6-phosphate dehydrogenase and 0.3 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After a 1 hr incubation at 37°C, the absorbance in each well was measured at 610 nm in a microtiter platereader. Specific activity was expressed as nmol MTT reduced (calculated on the basis of extinction coefficient = 11,300 M^{-1} cm^{-1}) per min per mg of protein. Non-specific quinone reduction, in the presence of dicoumarol, was subtracted from the enzymatic measurements (Zelikoff *et al.*, 1996).

Protein Determination

The activities of all three antioxidant enzymes were determined on a per mg cellular protein basis to allow distinctions to be made between effects of Cd on enzymatic activities and general effects on cell protein levels. Protein concentration was measured in the cytosolic preparation in which the corresponding enzymatic activity was also measured. The same assay was also used to measure plasma immunoglobulin levels and total plasma protein (data not shown). The Bio-Rad assay used to measure protein is based on a modification of the Lowry reaction (Lowry *et al.*, 1951). In this assay, color development takes only 15 min and the product is stable for 1-2 hours (only 5-10% change). The final absorbance was measured on a spectrophotometer at 750 nm. A standard curve was prepared with BSA and readings from each sample were inserted into the curve's formula to quantitate sample protein concentrations.

Statistics

Differences in tissue Cd burdens, phagocyte-mediated ROI production, and enzyme activities between cells from control and metal-exposed fish were assessed using two-way (treatment, dose) analysis of variance (ANOVA), followed when appropriate, by the Dunnett test. Statistical significance was accepted at $p \leq 0.05$.

RESULTS AND DISCUSSION

Effects of waterborne Cd exposure for 5 days on medaka survival are shown in Figure 1. Survival decreased with increasing Cd concentration and, although not precisely defined, the LC_{50} value was approximated at 620 ppb. Although Cd exposure had no effect on overall fish body weight (~500 mg), kidney, gill, and spleen weights increased approximately 30, 22, and 21%, respectively, at all exposure concentrations. Body burdens of Cd also increased with increasing metal concentration in these same organ systems (Figure 2). At the two lowest Cd concentrations (6 and 60 ppb), the net gain of metal was greatest in the gills. Interestingly, spleens from unexposed fish contained relatively high levels of Cd (compared to that measured in the gills and kidneys from unexposed fish). Trace levels of Cd in the shrimp diet may have been responsible for this accumulation. Furthermore, while the kidney appears to be a Cd "sink" in mammalian species (Squibb *et al.*, 1977), levels of Cd in the medaka kidney were low compared to that measured in the gills or liver. Since

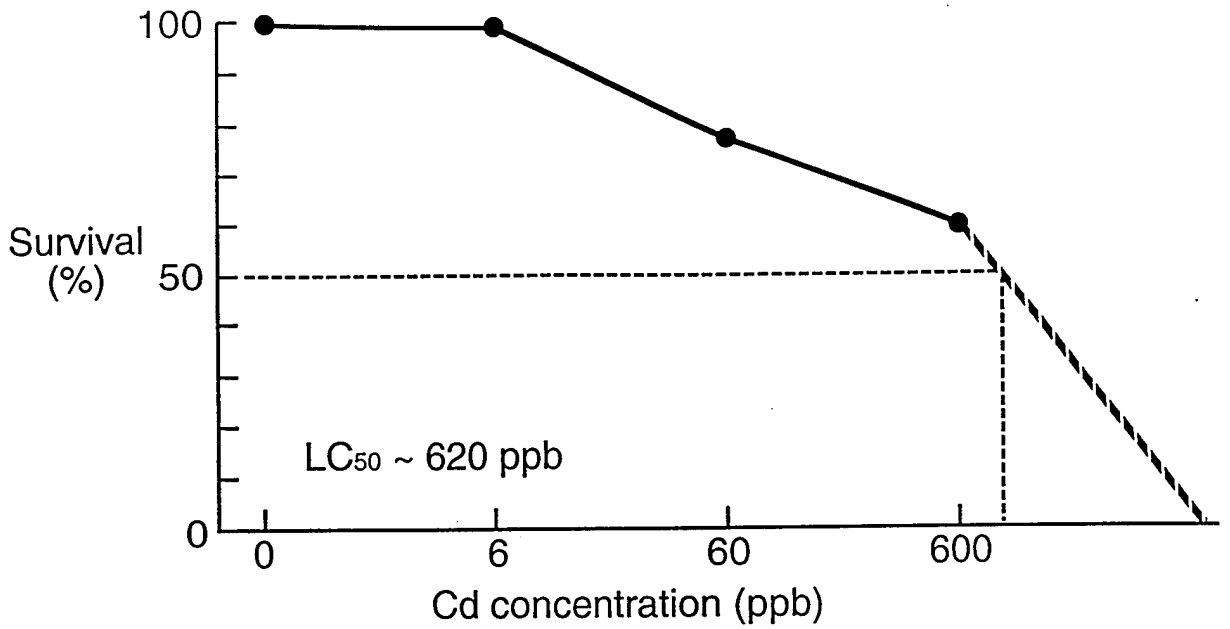


Figure 1. Effects of waterborne cadmium (Cd) exposure at 6, 60, and 600 ppb for 5 days on medaka survival. Survival decreased with increasing Cd concentrations. Although not exactly defined, the estimated LC_{50} value is 620 ppb. Values represent the mean ± SEM of 4 separate experiments.

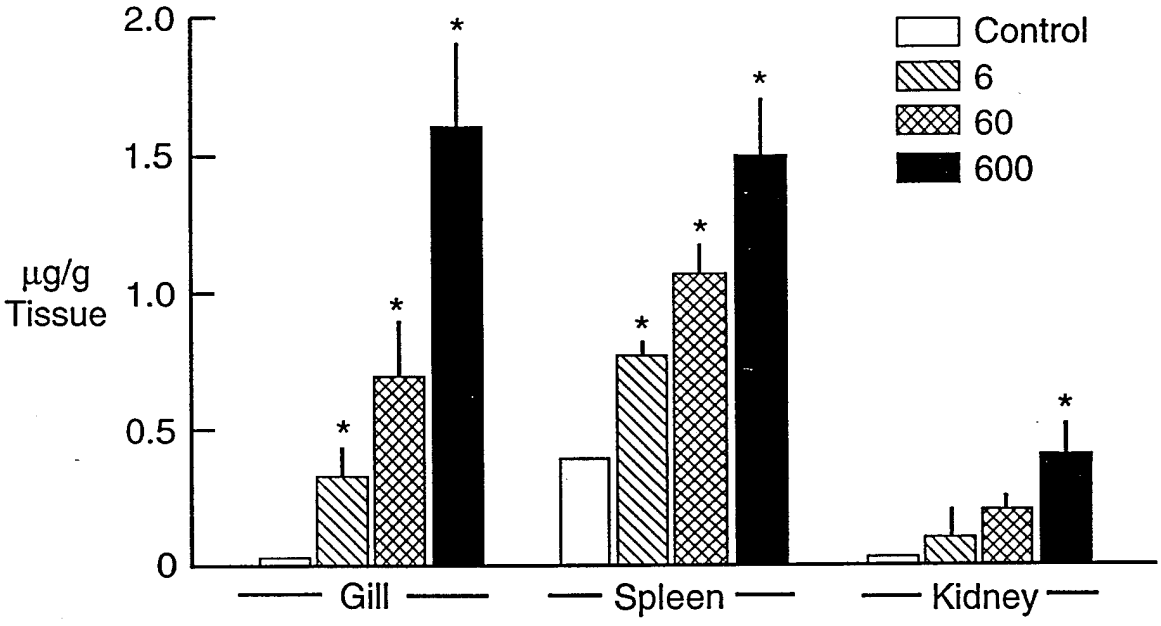


Figure 2. Body burdens of cadmium (Cd) in medaka after 5 days exposure to 6, 60, and 600 ppb Cd. As measured by atomic absorption spectrophotometry (AAS), Cd burdens increased in the gills, spleen, and kidney with increasing metal concentration. Exposure to all three Cd concentrations significantly (*$p < 0.05$) increased (compared to clean water control values) metal burdens in the gills and spleen; Cd levels in the kidney reached significance (*$p \leq 0.05$) only following exposure to the highest metal concentration. Values represent the mean ± SEM of 3 separate experiments.

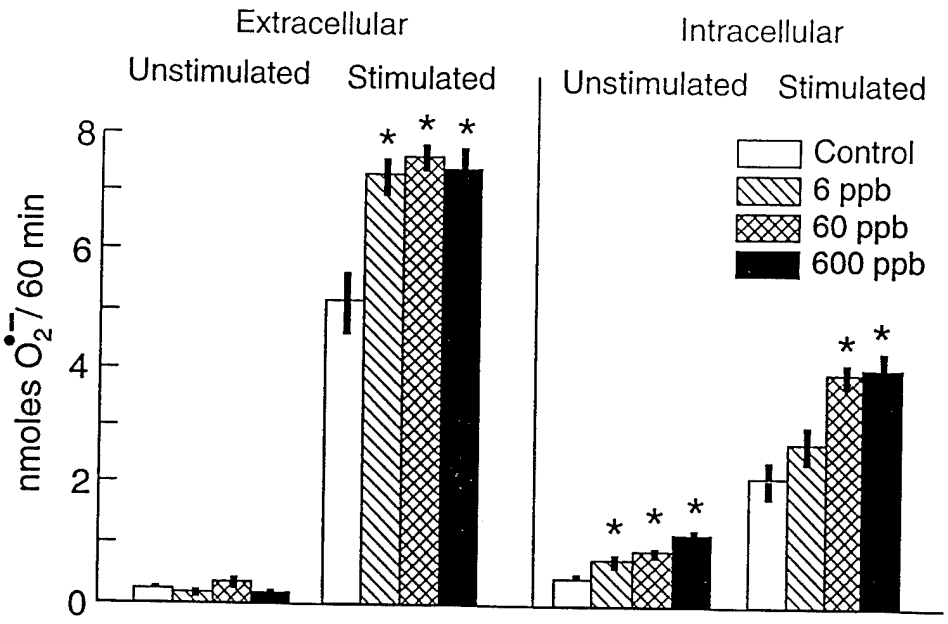


Figure 3. Effects of cadmium (Cd) exposure for 5 days on superoxide (O_2^-) production by medaka whole kidney cell homogenates. Waterborne exposure to Cd signficantly (*$p<0.05$) enhanced extracellular and intracellular production of O_2^- (compared to control levels) by kidney phagocytes. While extracellular basal levels were unaffected by Cd exposure, phorbol myristate acetate (PMA)-stimulated production of extracellular O_2^- was significantly (*$p< 0.05$) increased above control values; changes in production were independent of metal concentration. Phorbol myristate acetate-stimulated intracellular production of O_2^- was increased by Cd concentrations of 60 ppb and above, while basal levels were elevated by Cd concentrations as low as 6 ppb. Unlike changes in extracellular O_2^- production which were independent of Cd concentration, intracellular production increased with increasing metal concentration. Values represent the mean ± SEM of 4 separate experiments.

the pharmacokinetics of Cd in medaka are not yet known, it is possible that Cd has either not yet reached the kidney or has already started to be cleared.

Exposure to Cd at the near LC_{50} value of 600 ppb reduced kidney cell numbers, cell viability, and hematocrit levels approximately 30, 15, and 16%, respectively. Plasma immunoglobulin titers and plasma protein levels, a general marker of cell damage, was unchanged by short-term Cd exposure. These results demonstrated that, although body burdens of Cd increased, exposure of medaka (data not shown) for 5 days at concentrations of Cd as high as 60 ppb produced no overt toxic effects.

In the absence of any overt toxicological effects, exposure to Cd at near environmentally-relevant levels (6 ppb) and above (60 ppb), altered ROI production by kidney phagocytes (Figure 3). Exposure to Cd significantly increased extracellular and intracellular PMA-stimulated production of O_2^- compared to clean-water control values; extracellular production was increased (by ~31%) independent of Cd concentration. Intracellular O_2^- production was also increased by Cd exposure. Waterborne exposure to the two highest Cd concentrations increased intracellular O_2^- production

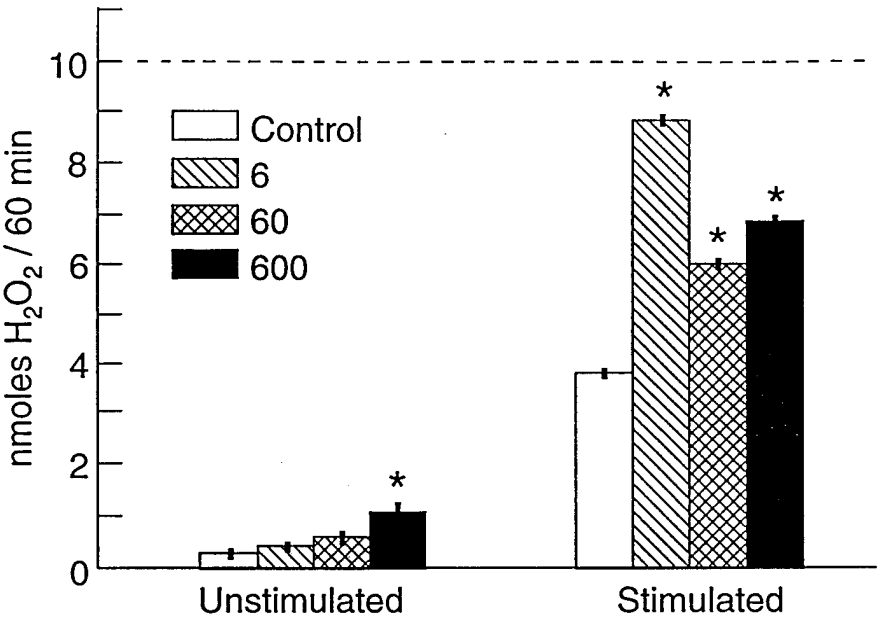


Figure 4. Effects of cadmium (Cd) exposure for 5 days on hydrogen peroxide (H_2O_2) production by medaka kidney cells. Waterborne exposure to Cd significantly (*$P \leq 0.05$) enhanced H_2O_2 production (compared to the clean water control value) by kidney phagocytes. While basal levels of H_2O_2 production were elevated only following exposure to Cd at 600 ppb, phorbol myristate acetate (PMA)-stimulated production was enhanced by all waterborne exposure concentrations; the greatest effect on PMA-stimulated H_2O_2 production was produced by the lowest Cd concentration (6 ppb). Values represent the mean ± SEM of 4 separate experiments.

by PMA-stimulated kidney phagocytes by ~10%; a trend toward increasing basal levels with increasing Cd concentration was also observed.

Hydrogen peroxide production was also enhanced by waterborne exposure to Cd; production by PMA-stimulated kidney cells was greatest following exposure to the lowest Cd concentration (Figure 4). Given that mammalian studies have demonstrated that exposure to metals at low, environmentally-relevant concentrations augment immune responses (Zelikoff and Cohen, 1995), and our own studies with rainbow trout that demonstrated augmented macrophage movement and H_2O_2 production following *in vitro* exposure to Ni (Bowser *et al.*, 1994) and Cd (Enane *et al.*, 1991), as well as enhanced macrophage phagocytic activity following waterborne Cd exposure (Zelikoff *et al.*, 1994), this finding is not surprising.

Optimally biomarkers should be persistent in nature, thus the duration of the alterations in ROI production were examined by placing fish exposed to Cd at 60 ppb in clean water for 3 or 10 days (Figure 5). Results demonstrated that while intracellular O_2^- production declined to control values after 3 days in clean water, extracellular O_2^- and H_2O_2 production remained significantly elevated (compared to unexposed control values). After 10 days, production of H_2O_2 and intracellular and extracellular O_2^- was depressed by approximately 30, 40, and 20%, respectively compared to PMA-stimulated production by cells from unexposed control fish. Even though the effects on ROI production observed 10 days following Cd exposure were opposite to those observed immediately

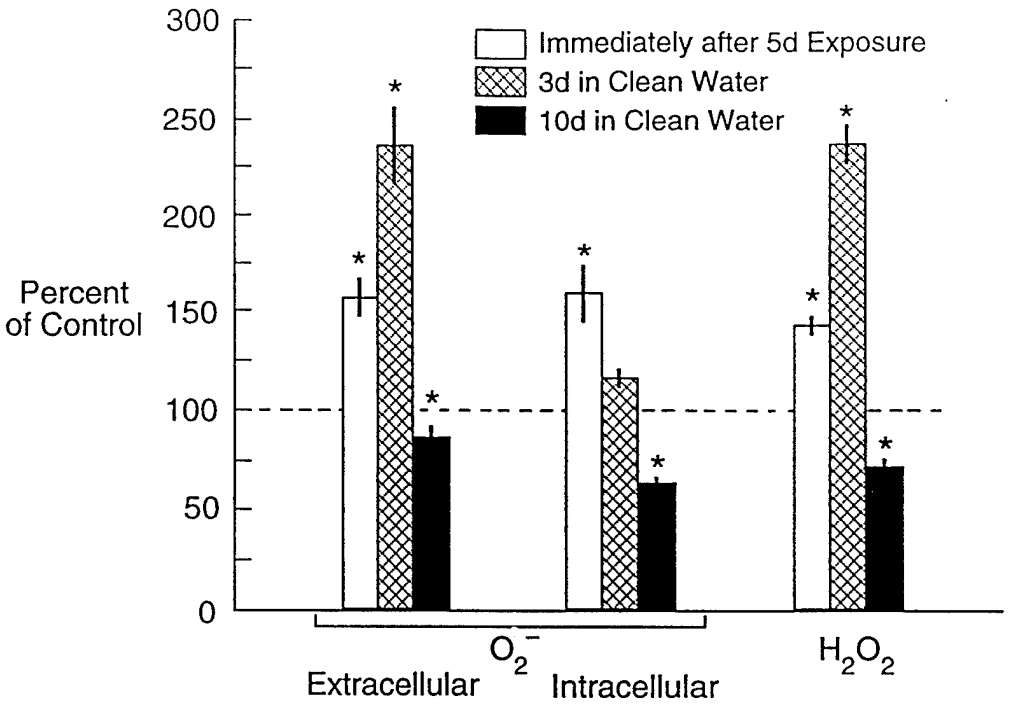


Figure 5. Persistence of cadmium (Cd)-induced effects on phorbol myristate acetate (PMA)-stimulated oxyradical production. Medaka exposed to Cd at 60 ppb for 5 days were placed in clean water for 3 or 10 days and the effects on O_2^- and H_2O_2 production evaluated. Production of H_2O_2 and extracellular O_2^- remained significantly (*$p \leq 0.05$) elevated (compared to PMA-stimulated control values) after 3 days in clean water. After 10 days, oxyradical production declined significantly (*$p \leq 0.05$) below unexposed PMA-stimulated control values. Values represent the mean ± SEM of 4 separate experiments.

and 3 days following metal exposure, ROI production remained modified at this later time point which demonstrates persistence of the altered phenotype. The persistent Cd-induced alterations observed after placement in clean water correlate with changes in macrophage activation (measured by 5'-nucleotidase activity) also observed in this study (data not shown).

To determine the specificity of Cd to bring about the aforementioned changes in ROI production, medaka were exposed under the same exposure conditions (i.e., 6 ppb Cd for 5 days) to related divalent cations, including inorganic chloride salts of Hg, Ni, and Zn, and effects on ROI production evaluated (Figure 6 and 7). Exposure for 5 days to an equimolar concentration of Hg, Ni, and Zn increased PMA-stimulated extracellular O_2^- production compared to control, similar to that observed for Cd. The greatest increase in respiratory burst (above control) was produced by Cd (32%) followed by Hg and Ni (22%) and then Zn (16%). With the exception of Zn, intracellular O_2^- production was also significantly increased by exposure to the other cations. Interestingly, in contrast to the non-specific metal-induced stimulatory effects observed on O_2^- production, H_2O_2 production was increased (by ~67%) only in response to short-term, low-dose Cd exposure. This finding suggests that H_2O_2 may not be generated by SOD activity following exposure to metals other than Cd and that elevated H_2O_2 production by medaka kidney phagocytes may be specific for Cd. This finding may also be related to the observed decrease in kidney CAT activity following Cd exposure (Figure 8).

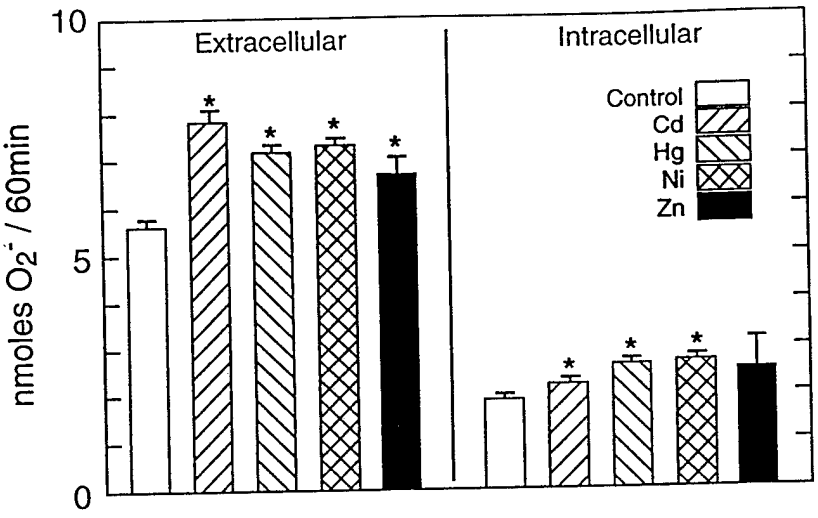


Figure 6. Effects of waterborne exposure to cadmium (Cd), inorganic mercury (Hg), nickel (Ni), and zinc (Zn) at 6 ppb for 5 days on phorbol myristate acetate (PMA)-stimulated O_2^- production by medaka kidney phagocytes. With the exception of Zn, exposure to equimolar concentrations of all of the selected metals significantly (*$p \leq 0.05$) enhanced (compared to clean water control values) both extracellular and intracellular O_2^- production. Exposure to Cd produced the greatest effect on extracellular production followed by Hg and Ni and then Zn; Hg and Ni proved to be the most effective stimulators of intracellular O_2^- production. Values represent the mean ± SEM of 4 separate experiments.

While it is difficult to determine the implications of these alterations in regards to overall fish health, because of the crucial nature of the immune system for protection of the host against infectious agents and developing neoplasms, chemical pollutant-induced changes of any essential immune function could offset the balance necessary for immunoregulation in the host and, thus, lead to a cascade of detrimental secondary events, including hypersensitivity reactions, autoimmune responses, or localized tissue damage. In addition, the lack of effect of Cd at concentrations ≤ 60 ppb on the more general markers of toxicity (i.e., body weight, cell viability, hematocrit levels, data not shown) demonstrates the sensitivity of the selected immune endpoints for predicting the adverse effects of pollutant exposure at environmentally-relevant concentrations.

Recently, antioxidant enzymes in fish, specifically SOD and glutathione transferases, have been proposed as bioindicators for assessing the environmental impact of marine aquatic pollutants that generate oxidative stress (Rodriguez-Ariza *et al.*, 1991; Gabryelak and Tawfek, 1991; Pedrajas *et al.*, 1993; Hasspieler and DiGiulio, 1994). Thus, using medaka previously exposed to 60 ppb Cd, effects on SOD, CAT, and QR activity in the kidney, liver, and spleen were determined (Figure 8). Exposure to a concentration of Cd well below the LC_{50} value significantly altered antioxidant enzyme activity; Cd depressed CAT activity 50 - 80%, depending upon the organ system studied. The Cd-induced suppression in CAT activity may have occurred by any number of different

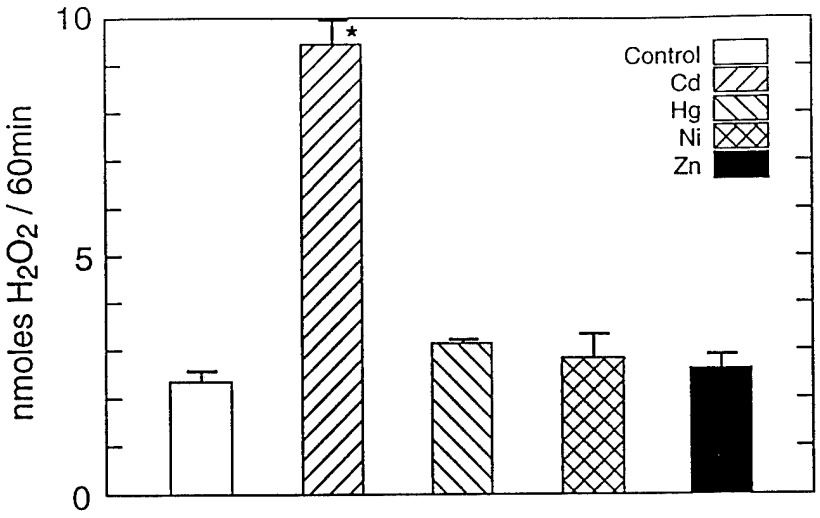


Figure 7. Effects of cadmium (Cd), inorganic mercury (Hg), nickel (Ni), and zinc (Zn) at 6 ppb for 5 days on phorbol myristateacetate (PMA)-stimulated H_2O_2 production by medaka kidney phagocytes. Waterborne exposure of medaka to Hg, Ni, or Zn, at or near-environmental levels, had no effect on H_2O_2 production (compared to the clean water control value). In contrast, exposure to an equimolar concentration of Cd (6 ppb) increased PMA-stimulated production 4-fold. Values represent the mean ± SEM of 4 separate experiments.

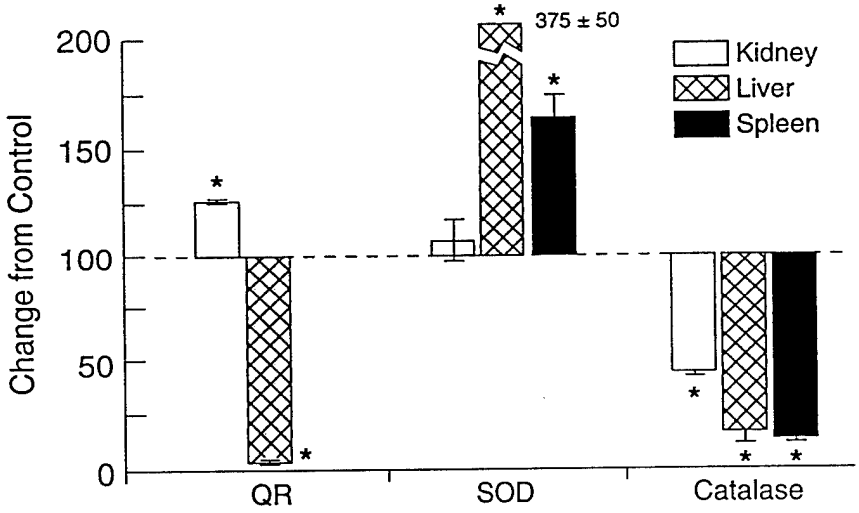


Figure 8. Activity of antioxidant/protective enzymes in medaka tissues and/or immune cells following waterborne exposure to cadmium (Cd) at 60 ppb for 5 days. Exposure to Cd significantly (*$p < 0.05$) depressed catalase (CAT) activity in the kidney, liver, and spleen; superoxide dismutase (SOD) activity was enhanced 3.7-fold and 1.7 fold in the liver and spleen, respectively. Effects of Cd exposure on quinone-reductase (QR) activity were dependent upon the target organ examined; activity was increased by 25% in the kidney and depressed by 95% in the liver. Values represent the mean ± SEM of 3 separate experiments.

mechanisms including protein denaturation, reduced enzyme production, or inactivation of the functional protein. Nonetheless, given the importance of this antioxidant enzyme for reducing H_2O_2-mediated stress within the host, this suppression could, potentially, lead to localized pathogenesis, and/or oxyradical-mediated tissue damage. Furthermore, given the association between H_2O_2 and tumor promotion, decreased CAT activity and, thus, increased H_2O_2 levels, could, potentially, influence tumor incidence. On the other hand, SOD activity in the liver and spleen was increased significantly by Cd exposure; liver SOD activity increased ~37-fold and that in the spleen was increased 50% above control values. Effects of waterborne exposure to Cd on QR activity was dependent upon the site of production; QR activity in the kidney was significantly enhanced by Cd exposure, while that in the liver was reduced by 95%. These results, demonstrate the presence of protective/antioxidant enzymes in medaka tissues and immune cells, and suggest the applicability of these endpoints as biomarkers of aquatic pollution.

In summary, in the absence of overt toxicological alterations, exposure of fish to a low (near-environmentally relevant) concentration of Cd and above increased ROI production by fish kidney cells. Alterations in ROI production were persistent and appear to be a useful marker for biomonitoring studies and (possibly) as a means for detecting human health impacts arising from metal-contaminated aquatic environments. Furthermore, antioxidant enzymes present in the medaka kidney, liver, and spleen were altered by exposure to Cd at a level well below the LC_{50}, value which suggests that some Cd-induced toxicity in fish may be mediated by oxidant stress.

Although additional laboratory and field studies need to be carried out to support these findings, the results of this study provide evidence that alterations in non-specific immune functions (i.e., oxyradical production by cells equivalent to those found in the mammalian bone marrow) are sensitive indicators of the immunotoxic effects of waterborne Cd exposure, and that these endpoints could, potentially, serve as biomarkers to predict the effects and biological hazards associated with metal pollution. In addition, while to date the aforementioned assays have only been shown to be sensitive to the immunotoxic effects of metals, studies performed by our laboratory have demonstrated the applicability of these same endpoints to predict the effects of organic pollutants (e.g. PCBs) on feral fish populations.

Acknowledgment

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Chapter 37

Serum Factors as Indicators of Environmental Stress: Optimization of Methodologies for Striped Bass Serum

V.S. Blazer, D.L. Higginbotham[1] and J.W. Fournie[2]

National Biological Service
National Fish Health Research Laboratory
1700 Leetown Rd
Kearneysville, WV 25430

[1]School of Forest Resources
University of Georgia
Athens, GA 30602

[2]U.S. Environmental Protection Agency
National Health and Environmental Effects Research Laboratory
Gulf Ecology Division
Gulf Breeze, FL 32561

ABSTRACT

With the increasing interest in "ecosystem health", many federal and state agencies are routinely monitoring biological responses of various fish species. Numerous physiological, histopathological and biochemical changes have been investigated as biomarkers of contaminant exposure. The immune system of animals, including fish, is very sensitive to some contaminant exposures, hence there is interest in developing markers of immune function/disease resistance. Our laboratories have been involved in developing soluble and cellular assays to measure the effects of contaminant exposures in the laboratory and attempting to field validate these assays. Striped bass were chosen because of their commercial value and previous indications that environmental contaminants are affecting wild populations. Serum lysozyme, spontaneous hemolytic activity and cortisol were identified as potential indicators. Methods were standardized for these assays and the effects of clotting time, use of plasma versus serum and freezing at either -20 or -70°C were evaluated.

INTRODUCTION

Many federal and state agencies are involved in monitoring programs which include some type of fish health assessment. These assessments range from simply noting the proportion of fish with external abnormalities as part of the index of biotic integrity (Leonard and Orth, 1986) to the use of histopathology and various biomarkers (Huggett *et al.*, 1992). Physiological, histological and biochemical measurements attempt to indicate early effects on fish health and to determine some degree of risk and hazard assessment (Peakall, 1992), prior to population level effects. The immune response of animals is known to be extremely sensitive to a variety of environmental contaminants. For this reason there is interest in developing measures of immunity and disease resistance for fish, which can be used in field monitoring programs (Weeks *et al.*, 1992; Wester *et al.*, 1994). Many of the assays, particularly cellular assays, used in laboratory studies are not amenable to field monitoring programs because of specialized equipment needs, requirement for live and optimally functioning cells, sterile technique, time required for the completion of the tests and lack of knowledge concerning previous antigenic exposure. Soluble factors have potential as field indicators since blood can easily be drawn in the field, kept cold while clotting and serum collected with minimum equipment need. Serum can then be frozen (in liquid nitrogen, if necessary) and transported to a laboratory for analysis. However, in order to validate these soluble factors in both laboratory and field studies, optimum assay conditions, effects of freezing (temperature and storage time), effects of clotting time and effects of using serum versus plasma must first be determined.

The factors we chose are lysozyme, spontaneous hemolytic activity as a measure of the alternative complement pathway and cortisol, a stress hormone. Lysozyme is an enzyme with antibacterial and antiviral activity (Jollès and Jollès, 1984). It acts on peptidoglycan in bacterial cell walls, causing lysis of the cells (Chipman and Sharon, 1969). Gram positive bacteria are most susceptible to its action. However, rainbow trout lysozyme has been shown to be capable of lysing a number of gram-negative bacteria (Grinde, 1989). It is believed that lysozyme of mammals as well as fish, works in conjunction with complement and other proteolytic enzymes to lyse gram negative organisms (Neeman *et al.*, 1974; Hjelmeland *et al.*, 1983).

Numerous fish species are known to have complement systems: both the classical or immunoglobulin-dependent and the alternative or immunoglobulin-independent (reviewed by Sakai, 1992). The natural hemolytic activity to heterologous red blood cells is suggested to utilize the alternate pathway for complement activation and to be an important defense mechanism (Ingram, 1987; Sakai, 1981; Sakai, 1992). Inactiviation of certain viruses (Sakai, 1992), detoxification of lethal exotoxins of *Aeromonas salmonicida* (Sakai, 1984), and bactericidal activity against *Escherichia coli* and *Pseudomonas fluorescens* (Jenkins *et al.*, 1991) have been reported. Indeed, this nonspecific hemolytic activity has been suggested as a promising parameter for the assessment of fish health (Sakai, 1983).

Cortisol is the major hormone secreted by the adrenocortical tissue of fish. Exposure to numerous waterborne contaminants causes elevation of plasma cortisol (reviewed by Donaldson *et al.*, 1984; Brown, 1993). In some cases this is a transitory increase followed by acclimation with a decline of circulating cortisol, in other cases there is a more chronic elevation. The fact that cortisol is rapidly elevated as a result of capture and handling complicates the interpretation of cortisol results from

field studies. Recently it has been suggested that fish collected at polluted sites have lower cortisol responses to capture due to an exhaustion of the adrenocortical response by chronic exposure to chemical pollutants (Hontela *et al.*, 1992). In addition, there are a number of studies which suggest that cortisol affects the spontaneous hemolytic activity and lysozyme (Carlson *et al.*, 1993; Fevolden *et al.*, 1994).

Striped bass (*Morone saxitilis*) is a commercially important sport and food fish. The original range of the striped bass was in nearshore waters, bays and coastal rivers from the St. Lawrence River, Canada to northern Florida, and along the Gulf coast from western Florida to Louisiana. However, they have been transplanted to the west coast of the United States and into many freshwater impoundments (Harrell *et al.* 1990). A decline in the abundance of this species has been observed and contaminant stress is suggested as a contributing factor (Goodyear 1985; Hall *et al.* 1989). Despite the importance of striped bass, very little research has been directed toward disease resistance factors or laboratory-controlled contaminant exposures. The purpose of this research was 1) to optimize these assays for striped bass; 2) to determine if acute changes (due to sampling stress) in serum cortisol concentrations influence lysozyme or spontaneous hemolytic activity; and 3) to use these assays in laboratory exposed fish to determine their sensitivity to contaminants. The results of objectives one and two are included in this report.

METHODS

Fish and Experimental Conditions

Serum was obtained from striped bass (*Morone saxitilis*), mean weight of 275-290 g. These fish had been maintained in a 90% reuse system for approximately two years. During that time there had been no disease outbreaks, no exposure to chemicals (other than NaCl), all fish appeared healthy and ate well. Fish were anesthetized with MS222 (Finquel, Argent Chemical Co., WA), bled (after being fasted for at least 24 hr), blood was allowed to clot at 4°C, and serum collected by centrifugation. Generally, eight replicates, each composed of pooled serum from four fish, were used for each experiment.

Serum Factors

We evaluated the effects of pH, ionic strength, clotting time, addition of an anticoagulant and freezing at either -20 or -70°C for 1, 2, 3 and 6 weeks on striped bass serum lysozyme. Since many investigators use a standard curve produced with hen egg white lysozyme (HEWL), we first investigated the effects of pH and ionic strength on this activity. Sorensen's phosphate buffers (Luna, 1992) of 0.05, 0.067 and 0.10M with 0.1% NaCl, at pHs of 5.5, 6.5 and 7.5 were used to evaluate effects of ionic strength and pH on HEWL (Sigma Chemical Co., St. Louis, MO) activity. The method described by Tahir *et al.* (1993) with some modifications was used for all experiments. A 0.075% suspension of *Micrococcus lysodeikticus* (Sigma Chem. Co., St. Louis, MO) was prepared in the appropriate buffer. A 175 µL aliquot of this suspension was added to wells of a 96-well, untreated, flat-bottomed microtiter plate. Serum samples or standard concentrations (25 µL) were added, the plate immediately shaken and read every 15 sec for 5 min. on a Molecular Devices V_{max}

kinetic microplate reader at 450 nm. Values were calculated using Softmax software. Triplicate wells were used for each measurement.

The effects of pH on striped bass serum were evaluated using 0.067 M phosphate buffer with 0.1% NaCl at pH's of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. A standard curve was produced using HEWL with the 0.067M pH 6.5 buffer. We also evaluated a variety of ionic strengths (0.01 to 0.1M) of the phosphate buffer with and without the 0.1% NaCl. A 0.02M acetate buffer has been used by some investigators (Siwicki *et al.* 1990; Studnicka *et al.* 1986) and this was also tested.

Serum for measurement of spontaneous hemolytic activity was collected as described above and the protocol described by Carlson *et al.* (1993) with minor modifications was followed. Each well of a 96-well round bottom microtiter plate (Dynatech Laboratories, Inc., Chantilly, VA) received 100 µL of the appropriate buffer. An equal volume of serum was added to the first row, serially diluted and 10 µL of a 3% (v/v) solution of washed, unsensitized sheep red blood cells (SRBC) or rabbit red blood cells (RRBC) added to each well. Hemolysis titers were determined as the highest dilution showing 100% lysis of red cells after a two hour incubation at 20°C. SRBC (40% in Alsever's solution) were obtained from BioWhittaker, Walkersville, MD and stored at 4°C. They were used within three weeks of the bleed date and washed three times in cold PBS, pH 7.2 immediately prior to use. RRBC were obtained from laboratory-held rabbits, blood was added to Alsever's solution and stored at 4°C for not more than three weeks. We compared a variety of buffer solutions including: 0.1M Sorensen's phosphate buffer with 0.1% NaCl (PBS) at pH 7.2 and 7.4 (Luna, 1992) or a gelatin-veronal buffer (gelatin 1 g; NaCl 8.5 g; 5,5-diethylbarituric acid 0.757 g; Na-5,5-diethylbarbiturate 0.375 g; $CaCl_2.2H_2O$ 0.022 g), at pH's 6.5, 7.0, 7.5 and 8.0, with varying concentrations of magnesium, $MgCl_2.6H_2O$ (Sakai, 1981).

The effect of clotting time was evaluated by splitting blood samples immediately after withdrawal from the fish. Matched samples were allowed to clot for 2 or 24 hrs at 4°C. The effect of freezing was tested by analyzing samples within two hours of collection and then freezing subsamples at either -20 or -70°C. Subsamples were removed and analyzed at 1, 2, 3 and 6 weeks. For the comparison of plasma and serum, sixteen individual fish were bled. Two to three mL of blood was placed in a centrifuge tube and an equal amount from the same fish in a heparinized Vacutainer.

We used a modification of the ELISA technique described by Barry *et al.* (1993) for cortisol measurements. The antibody was raised against cortisol-3-carboxymethyloxime (CMO)/bovine serum albumin (BSA) and used at a titer of 1:8500 (50 µL/well). The enzyme conjugate was horseradish peroxidase coupled to cortisol through a CMO bridge and was used at 1:30,000. Both the antibody and conjugate were supplied by Ms. Coralie Munro, University of California, Davis, CA. The substrate was the ABTS substrate system (Kirkegaard & Perry Laboratories, Inc.). This system consists of 2,2'-azino-di[3ethyl-benzthiazoline sulfonate (6)] and H_2O_2. To validate the method we used standard curves produced by diluting a known amount of hydrocortisone (Sigma Chem. Co., St. Louis, MO) dissolved in either ethanol or charcoal-stripped striped bass serum.

The possible effect of short term (capture) stress on lysozyme and spontaneous hemolytic activity was evaluated by using 60 serum samples which had been collected from bass captured 4 or 6 at a time, rapidly anesthetized and bled.

Statistical Analyses

Statistical comparisons were made using SigmaStat software (Jandel Scientific, San Rafael, CA). One way analysis of variance (ANOVA) and the Student Newman Keuls procedure for pairwise multiple comparisons was used. For comparison of spontaneous hemolytic activity after various treatments both the t-test and the Mann-Whitney rank sum test were used. Values were considered statistically different at $p \leq 0.05$. Pearson product moment correlation and Spearman rank order correlation tests were used to test for relationships between cortisol and lysozyme or spontaneous hemolytic activity.

RESULTS

Lysozyme

Standard solutions (3.906 to 250 µg/mL) of hen egg white lysozyme (EC 3.2.1.17 from Sigma Chemical Co., St. Louis, MO) were produced in buffers of pH 5.5, 6.5 or 7.5 and molarities of 0.05, 0.067 or 0.100. A significant effect of pH was noted, with maximum activity at pH 6.5 (Figure 1a). Molarity had a slight effect on activity. No significant differences were observed, particularly at the lower concentrations of lysozyme, however 0.067M generally resulted in the highest activity (Figure 1b).

Fish serum was subsequently evaluated using a standard curve of hen egg white lysozyme prepared in a 0.067M buffer at pH 6.5. Allowing the blood to clot overnight at 4°C versus removing serum after 2 hr at 4°C and immediately analyzing for lysozyme generally resulted in a slightly decreased activity however, the means (± S.D.) for 16 fish, 56.7 ± 14.1 at 2 hr and 53.3 ± 13.5 at 24 hr, were not statistically different. Nor was there a difference between plasma (62.3 ± 21.6) and serum (60.5 ± 18.5).

The effect of pH on striped bass serum was marked (Figure 2a). The optimum pH for striped bass lysozyme activity is between 5.5 and 6.0. Ionic strength also significantly affected striped bass lysozyme activity with higher molarities resulting in a lower lysozyme activity, at each of the pH levels used (Figure 2b). Comparing buffers of molarities 0.01, 0.02 and 0.05 with and without the 0.1% NaCl again indicated that the lower the ionic strength the higher the lysozyme activity (Table 1). The optimum buffer for striped bass serum lysozyme was a 0.01M phosphate buffer without additional salt, at pH 5.5.

Freezing at -70°C did not have a significant effect on striped bass lysozyme activity for a six week storage time. However, storage at -20°C did significantly affect this activity (Table 2). The first eight samples analyzed showed significantly reduced activities after one and two weeks storage, however, at three weeks the activity appeared to increase and was not significantly different than the initial sample. At six weeks the activity was greatly reduced. A second set of samples showed the same trend.

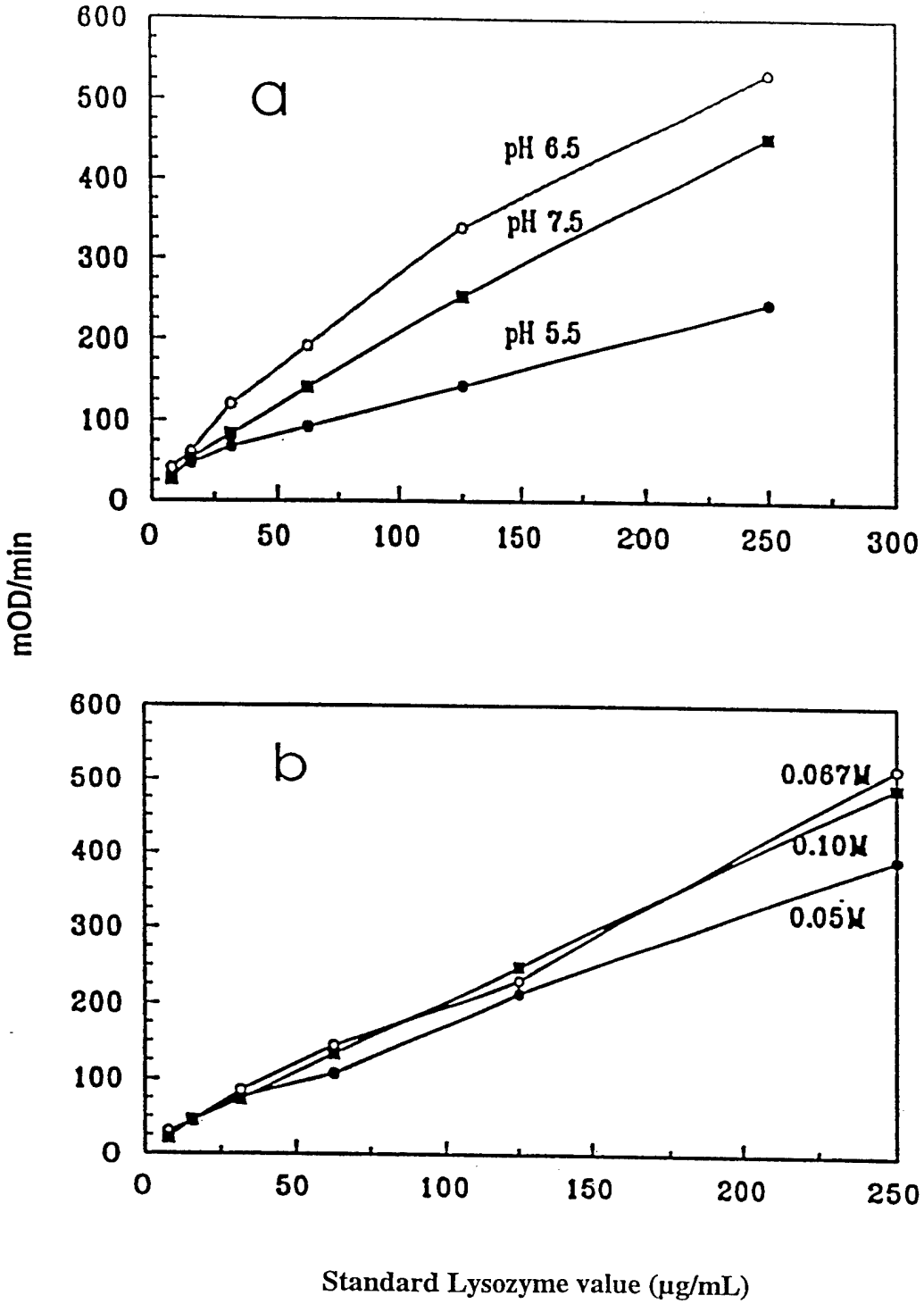


Figure 1. (a). Hen egg white lysozyme standard curves produced with different pH buffers (0.067M). (b). Hen egg white lysozyme standard curves produced with different molarities at pH 6.5.

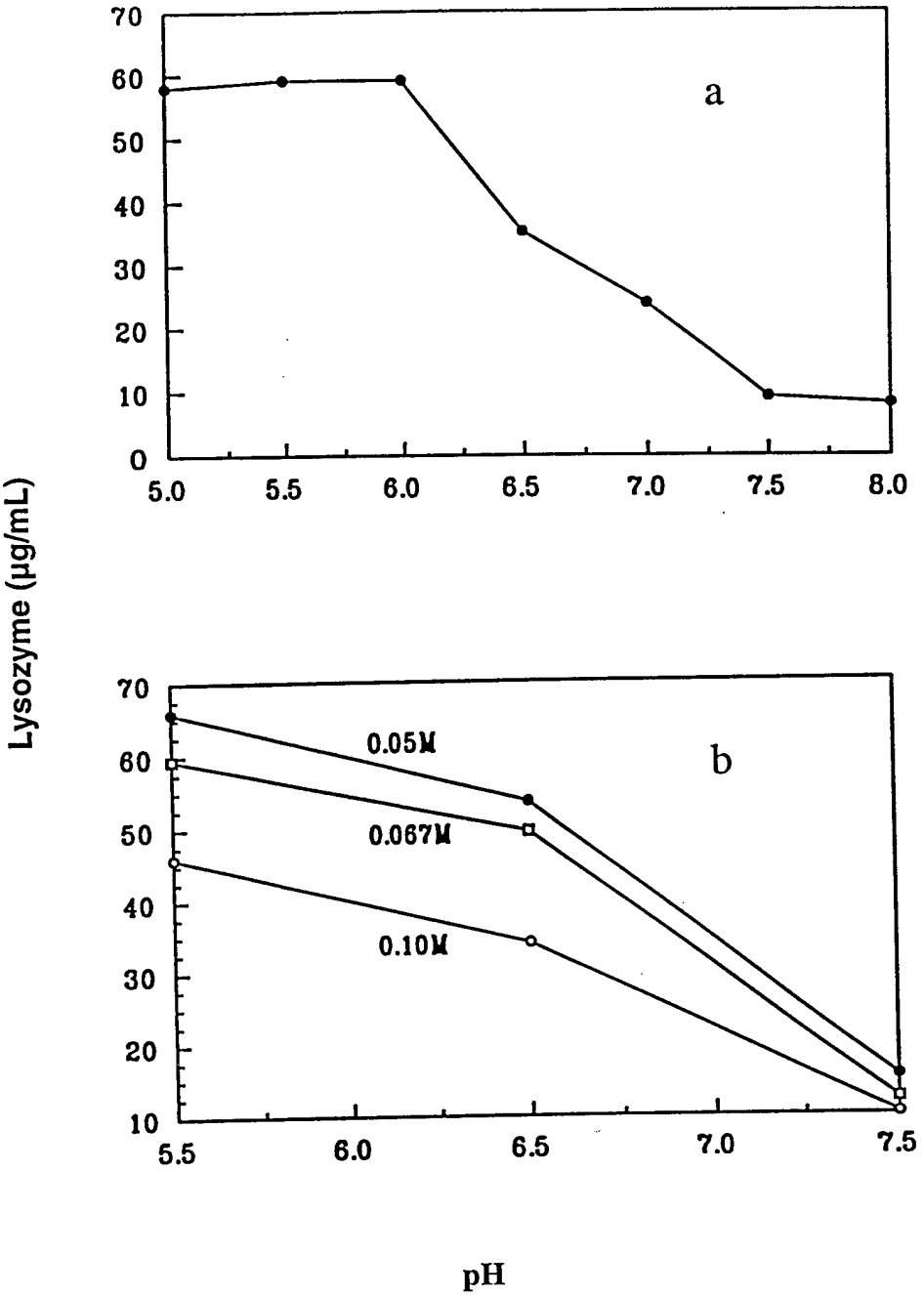


Figure 2. **a.** pH effects on striped bass serum lysozyme activity. **b.** Effects of ionic strength and pH on striped bass serum..

Table 1
Buffer effects on striped bass serum lysozyme evaluated at pH 5.5

Buffer[1]	Lysozyme (mOD/min)[2]
Phosphate	
0.01M	92.8 ± 10.9 a
0.01M +	78.3 ± 13.5 b
0.02M +	71.8 ± 10.4 b,d
0.05M +	55.8 ± 11.1 c
Acetate	
0.02M	61.0 ± 5.1 c,d
0.02M +	62.4 ± 7.7 c,d

[1] + indicates the buffer contained 0.1% NaCl.

[2] Each value represents the mean of eight samples, each composed of pooled serum from two fish, run in triplicate. Data is presented as means ± S.D. and values followed by the same letter are not significantly different at $p \leq 0.05$.

Table 2
Effects of storage temperature and time on lysozyme activity of striped bass serum

Lysozyme Activity (µg/mL)[1]				
Initial	1 week	2 week	3 week	6 week
65.7 ± 18.2 a	46.5 ± 16.0 b	47.0 ± 9.3 b	52.5 ± 14.4 ab	35.8 ± 7.3 b
62.8 ± 9.8 a	47.3 ± 12.8 b	45.5 ± 8.6 b	52.8 ± 15.0 ab	N.D.
Samples stored at -70 ° C				
66.2 ± 17.6	64.1 ± 18.3	72.8 ± 31.8	N.D.	68.3 ± 15.5

[1] Each value represents the mean of eight samples, composed of pooled serum from four fish, each sample run in triplicate. Data is presented as means ± S.D. and values followed by the same letter are not significantly different.

Spontaneous Hemolytic Activity

Preliminary results did not show significant effects of buffer when using SRBC as the target cell. However, the mean titer was highest using gelatin-veronal buffer with magnesium and calcium at pH 7.2. Striped bass serum showed a significantly greater lytic activity to RRBC than to SRBC (Table 3). We further characterized the optimal assay conditions for the activity against RRBC. No significant effects of buffer pH, between 6.5 and 8.0, or buffer magnesium concentration, between 0.5 and 10 mM, were observed (Table 4).

Table 3
Effects of buffer on spontaneous hemolytic activity of striped bass serum

Buffer[1]	Hemolytic Titer (log_2) Means ± S.D.[2]
PBS pH 7.2	3.0 ± 0.5
PBS pH 7.4	2.8 ± 0.5
GVB pH 7.2	3.1 ± 0.6
GVB pH 7.4	3.4 ± 0.5
GVB+ pH 7.2	3.6 ± 0.5
GVB+ pH 7.4	3.5 ± 0.5
SRBC versus RRBC (GVB+ pH 7.2 buffer)	
SRBC	3.6 ± 0.5 a
RRBC	8.1 ± 0.8 b

[1] Buffers were PBS - Sorensen's phosphate buffered saline; GVB - gelatin-veronal buffer; GVB+ - gelatin-veronal buffer with magnesium.
[2] Each value represents the mean of eight individual fish.
[a]Values followed by a different letter are significantly different at $p< 0.05$.

Table 4
Buffer effects on spontaneous hemolytic activity against rabbit red blood cells

Buffer[1]	Titer (log_2) Mean ± S.D.
pH 6.5	8.4 ± 0.9
pH 7.0	8.1 ± 0.8
pH 7.5	7.6 ± 0.7
pH 8.0	7.5 ± 0.8
0.5 mM magnesium	8.1 ± 0.8
5 mM magnesium	7.9 ± 0.8
10 mM magnesium	8.3 ± 0.5

[1] Buffer to test pH effects was gelatin veronal buffer with 0.5 mM magnesium and calcium. Buffer to test effect of magnesium concentration was pH 7.0.

The spontaneous hemolytic activity against both SRBC and RRBC was totally destroyed by heating the serum at 44°C for 20 min. There was no significant loss of activity against RRBC after storage at -70°C for 3 weeks. There was a significant difference in the mean titer (log_2) when comparing serum and plasma from the same fish (n=16): plasma (6.9 ± 1.3) was significantly lower than serum (8.3 ± 0.6). However, there was no difference when blood was allowed to clot for 2 hr at 4°C (5.9 ± 1.3) versus clotting for 24 hr (5.8 ± 1.1).

Cortisol

We were able to validate the ELISA method and obtain reproducible results with a sensitivity as low as 5 ng/mL (Figure 3). Data from 60 control fish which had been collected to assess effects of capture and MS222 on circulating cortisol concentrations were used to determine if cortisol levels correlated with lysozyme or spontaneous hemolytic activity. Values for serum cortisol ranged from 35.6 to 802.8 ng/mL. Pearson product moment correlation or Spearman rank order correlation tests were used and no significant correlation was found with cortisol concentrations and lysozyme or spontaneous hemolytic activity. There was an increase in circulating cortisol over the few minutes (≤ 10 min) it took to bleed the four fish (Table 5). This pattern was observed in about 90% of the samples. Table 5 illustrates three sampling periods and the lack of correlation between cortisol and other serum factors from each fish.

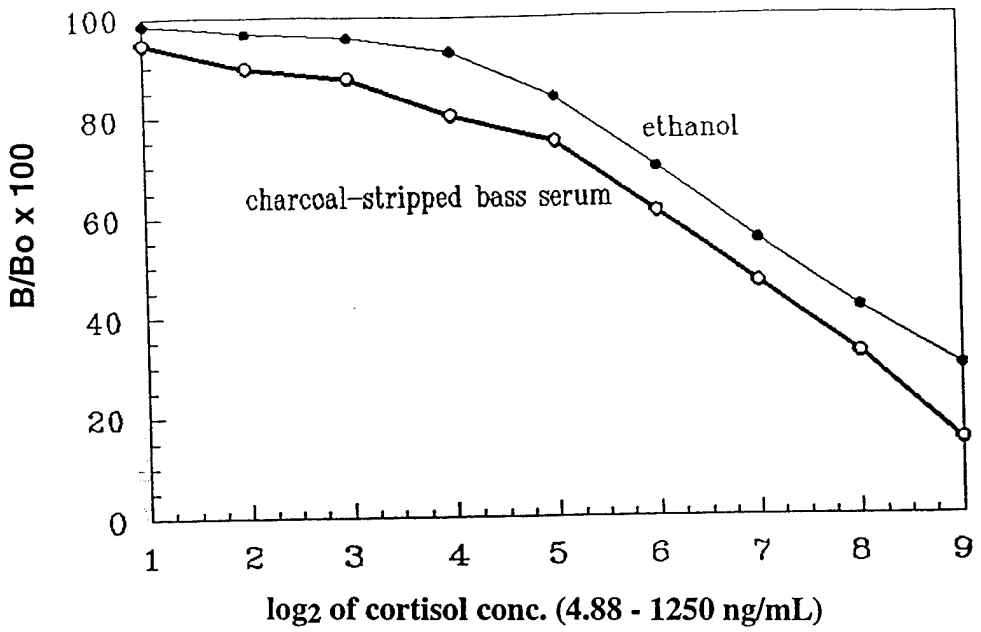


Figure 3. Standard curve ètisol dissolved in ethanol versus charcoal stripped bass serum. Values were obtained using a four parameter (log logit) curve fitting equation (Softmax software, Molecular Devices Corp., Menlo Park, CA). B = O.D. of triplicate wells at each concentration, B0 = O.D. of the maximum-binding wells. Line with solid circles is standards dissolved in ethanol; line with open circles is standards dissolved in charcoal-stripped serum.

Table 5.
Effect of sampling stress on serum factors

Fish[1]	Cortisol (ng/mL)	Lysozyme (µg/mL)	Hemolytic titer[2] log_2
1	54.2	38.6	6
2	115.0	51.2	7
3	141.7	40.2	6
4	318.6	34.8	6
5	112.7	29.2	6
6	194.8	28.3	5
7	303.4	18.5	6
8	577.6	23.3	6
9	259.2	29.3	6
10	222.3	31.3	6
11	347.1	19.9	5
12	353.8	35.4	5

[1] Fish were sampled during three periods, four at a time, anesthetized and bled as rapidly as possible.
[2] Hemolytic titer against rabbit red blood cells.

DISCUSSION

Two methodologies are commonly used to assess lysozyme activity in fish tissues. Variations of a photometric determination based on the method of Litwack (1955) and of a lysoplate assay described by Osserman and Lawlor (1966) have both been used with fish serum. Hen egg white lysozyme is generally used to produce a standard curve. The pH optimum of HEWL is reported to be 7.5 (Möck and Peters, 1990). Most fish species that have been investigated to date have shown a pH optimum between 5.0 and 6.0 (Fletcher and White, 1973; Lundblad *et al.*, 1979; Möck and Peters, 1990). Investigators have recognized this difference in pH optimum between the standard and fish samples, however, there is no consistency in approaches to deal with the difference. Many investigators use the same buffer system for both standard and unknowns (Ellis, 1990; Tahir *et al.*, 1993), while others have suggested the use of an internal standard (Möck and Peters, 1990; Røed *et al.*, 1993). In addition, there has been little comparison or standardization of methods in terms of buffer ionic strength. Reported buffer molarities range from 0.01 (Takahashi *et al.*, 1986) to 0.10M (Tahir *et al.*, 1993) and both phosphate and acetate buffers have been used.

A unit of the HEWL used in this study was defined by the manufacturer as that which will produce a 0.001 change in absorbance (at 450 nm) per minute at pH 6.24 at 25°C. The optimum activity, with the buffers used in this study, was observed at pH 6.5. This differs from a previous report in which 7.5 was found to be optimum (Möck and Peters, 1990). Since different sources of HEWL were used in the two studies it is possible different preparations may have different pH optimas. If HEWL is to be used as a standard, the optimum conditions should be determined and then used to produce the standard curve. Conversely, with the kinetic method, groups of fish could be compared using the mOD/min or the kinetics of the reaction rather than an absolute concentration. The optimum buffer for striped bass serum lysozyme is a 0.01 M, pH 5.5 phosphate buffer. If serum must be frozen at -20°C prior to measuring lysozyme it should be analyzed at approximately 3 weeks. It is best if serum samples can be stored at -70 to -80°C.

Many investigators have used SRBC as the target cell for measurement of both the classical and alternative complement activity (Carlson *et al.* 1993; Fevolden *et al.*, 1994; Ingram, 1987; Røed *et al.*, 1993). Hence, in our initial experiments to determine optimum assay conditions SRBC were used. Preliminary results indicate a gelatin-veronal buffer with magnesium at pH 7.2 is optimum for analysis of striped bass spontaneous hemolytic activity against SRBC. However, in mammalian systems RRBC have historically been used to activate the alternative complement pathway and so can be used as both an activator and a target cell (Fearon and Austen, 1977). This is apparently due to the lower concentrations of sialic acid, an alternative complement regulator, in RRBC membranes when compared to sheep and other mammalian erythrocytes (Fearon 1982). Collazos *et al.* (1994) and Matsuyama *et al.* (1988) found significantly higher alternative complement activity in cyprinid fish when using RRBC versus SRBC as the target cell. RRBC also appear to be the preferred target for measurement of striped bass alternative complement. Buffer pH and magnesium content did not have significant effects on the hemolytic activity as measured by the highest titer showing complete lysis. However, a buffer of pH between 6.5 and 7.0 with 10 mM magnesium appears optimum. As with lysozyme, it is preferable to store samples at -70 to -80°C. Sakai (1981) reported no significant loss of hemolytic activity after storage at -80°C for 2 months. Niether factor was significantly

affected by allowing blood to clot overnight versus 2 hr at 4°C, however, there were differences observed between plasma and serum.

Cortisol has been routinely measured in fish serum by radioimmunoassay (Carlson *et al.*, 1993; White and Fletcher, 1984). ELISA methods have the advantage of being faster, less expensive and do not require the use of radioisotopes. Previously, a solid-phase enzyme immunoassay technique was used to measure plasma cortisol in rainbow trout and found to have good correlation (r = 0.98) with the radioimmunoassay technique (Caldwell *et al.* 1990). The method we used was developed for mammalian species and also was shown to compare favorably (r = 0.998) with radioimmunoassy (Munro and Stabenfeldt 1985). It has since been validated for rainbow trout, *Oncorhynchus mykiss*, and lake trout, *Salvelinus namaycush* (Barry *et al.* 1993). The use of charcoal-stripped serum for production of a standard curve, gave slightly higher cortisol readings than did the use of ethanol. However, the curves themselves were not significantly different. Further work is in progress to determine the reason for this finding.

Stressors of various types have been reported to influence serum lysozyme and hemolytic activity. However, most of these studies have looked at relatively long-term stress. Carlson *et al.* (1993) reported a significant decrease in serum hemolytic activity in salmon implanted with cortisol and a correlation between serum cortisol levels and hemolytic activity. Möck and Peters (1990) found the effects of a short-term (30 min) handling stress on rainbow trout lysozyme to be mixed. In two experiments there was no significant difference between control and stressed fish while in two other experiments, serum lysozyme was actually significantly higher in stressed fish when compared to controls. Conversely, a 2 hr confinement and transport stress resulted in significantly decreased lysozyme activities (Möck and Peters, 1990). The stress involved in the present study was very short term. It involved netting and anesthetizing four to six fish at a time with buffered MS222 and bleeding within 10 minutes of initial capture. Despite this short time, fish did have significantly elevated circulating cortisol levels. However, we found no correlation between cortisol and lysozyme or hemolytic activity.

In conclusion, methods have been standardized for serum lysozyme, alternative complement activation and cortisol, in striped bass. We feel these factors are potential indicators of environmental stress and are using them as part of a battery of assays for evaluating the effects of selected contaminants in laboratory and field studies.

Acknowledgements

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Chapter 38

Avian Immunotoxicology

Rodney R. Dietert, Karen A. Golemboski and Thomas E. Miller

Institute for Comparative and Environmental Toxicology and Unit of Avian Medicine
Department of Microbiology and Immunology
Cornell University
Ithaca, NY 14853

ABSTRACT

Immunotoxicology research and assessment using avian species have the opportunity to address three significant societal needs. Assessment using animal models where results are directed toward human health protection is most effective when multi-species validation can be performed. In this regard, the chicken represents a potential second species (non-mammalian representative) model for extrapolation to humans. A second direct use of avian immunotoxicological information pertains to the importance of several species (e.g., chicken, turkey, duck, goose, ostrich) in the agricultural setting. Finally, immunotoxicology efforts on avian species can provide an important means of protecting avian wildlife from environmental hazard, thereby enhancing the preservation of our natural resources. The development of avian reagents and probes, the existence of specialized genetic strains, and the current knowledge of the avian immune system offer a timely opportunity for cost-effective immunotoxicological assessment. This review will consider the specialized features of the avian immune system, the range of environmental factors known to modulate avian immunity, the status of avian immunotoxicology, and the potential benefits of comprehensive immunotoxicological assessment.

INTRODUCTION: THREE ARENAS FOR AVIAN IMMUNOTOXICOLOGY

Avian immunotoxicology is positioned such that it can potentially contribute in three very diverse societal arenas. First, there is an opportunity to contribute to enhanced human preventative medicine via the identification and examination of immunotoxins. There currently exists a need to validate environmentally-induced immune alterations in second (non-rodent and/or non-mammalian) species. In the absence of direct human immunotoxicological data, such two-species validation information among phylogenetically-distinct animals can provide additional evidence for the likely extrapolability of results to humans. Two-species data can be important in confirming the universality of chemical-immune interactions and can be designed to take advantage of certain specific characteristics.

When the non-mammalian option is sought in such validations, the chicken represents a species with appropriate inbred lines (Abplanalp, 1986), crosses (Martin *et al.*, 1990; Pinard *et al.*, 1992), congenic lines (Bacon *et al.*, 1986), and recombinant strains (Miller *et al.*, 1988) to provide the

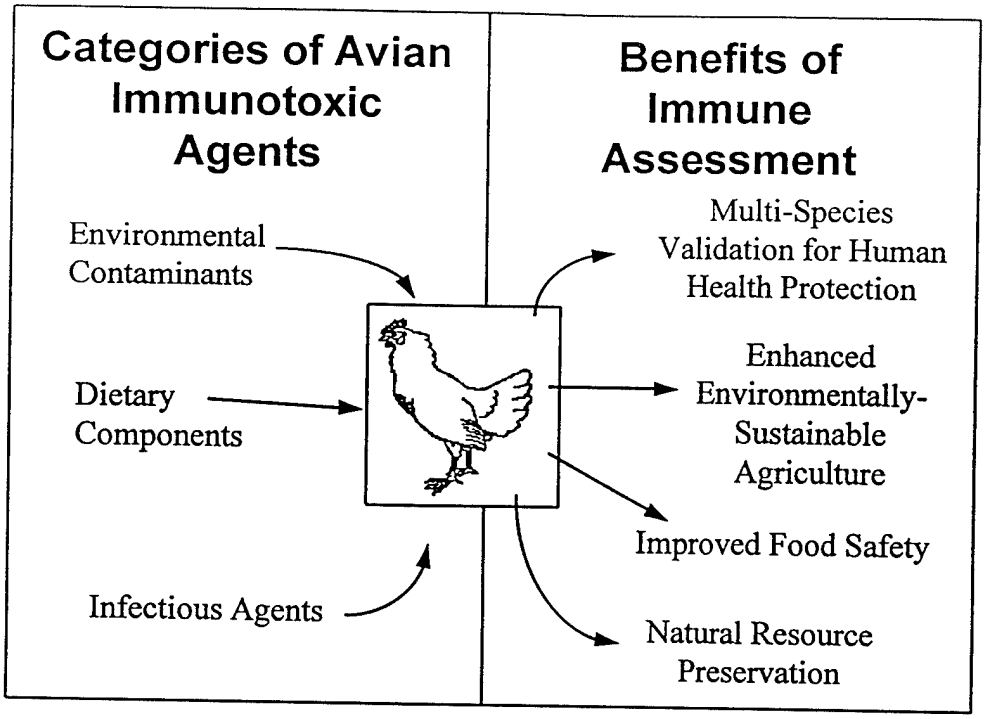


Figure 1. Sample categories of environmental factors known to influence avian immune status and the potential benefits of avian immune assessment are illustrated.

When the non-mammalian option is sought in such validations, the chicken represents a species with appropriate inbred lines (Abplanalp, 1986), crosses (Martin *et al.*, 1990; Pinard *et al.*, 1992), congenic lines (Bacon *et al.*, 1986), and recombinant strains (Miller *et al.*, 1988) to provide the genetic base necessary for effective immune evaluation. Additionally, the fundamental knowledge of the avian immune system with excellent avian disease models and increasing availability of reagents (Chan *et al.*, 1988; Chen *et al.*, 1986; Ratcliffe *et al.*, 1993; Trembicki *et al.*, 1986; Lillehoj *et al.*, 1993) and molecular probes (Miller and Goto, 1993; Lahti *et al.*, 1991; Leutz *et al.*, 1989; Reynaud *et al.*, 1985; Zoorob *et al.*, 1993; Pharr *et al.*, 1993; Emara *et al.*, 1992) makes comprehensive avian immune assessment a reality.

A second and paramount importance of avian immunotoxicology concerns the challenge of providing an environmentally-sustainable nutrient source for an ever increasing human population. Because the chicken and certain other avian species (e.g., turkey, duck, ostrich) constitute a group of economically-significant animal species, avian immunotoxicology expands beyond a preventative health mode to include pro-active economic considerations. Poultry consumption and production have increased dramatically during the last decade in the US (USDA, 1993), and similar increases are occurring on a world-wide basis (FAO, 1992). As a result, environmentally-induced influences on the chicken immune system translated across billions of chickens per year can have a profound effect on agricultural productivity and efficiency. Additionally, as poultry management designs incorporate more information from avian immunotoxicology to achieve immune optimization, there is an opportunity to shift the paradigm of agricultural practices from a historic driving force of sheer growth to a new balance of growth and health. This paradigm shift is central among efforts to achieve environmentally-sustainable food production (Dietert *et al.*, 1995).

immunotoxicology in a potentially pivotal environmental position. The impact of environmental contaminants on wildlife species has gained increasing public attention resulting in increased scientific efforts in ecotoxicology. The potential for differential species susceptibilities to environmental contaminants and for reduced biodiversity through ecotoxicological damage has stimulated additional immunotoxicological assessment in animals that can predict the impact of environmental factors on wildlife. Within avian immunotoxicology this has resulted in two tracks of investigation. First, recent efforts have been directed toward the definition of avian sentry species for immunotoxicological assessment. In general, these species would be among those experiencing front-line exposure to environmental contaminants (e.g., herring gull) (Braune *et al.*, 1993). In the absence of extensive sentry animal information, immunotoxicity models using domesticated avian species have been developed to extrapolate effects to indigenous birds (Baecher-Steppan *et al.*, 1989).

Therefore, avian immunotoxicology is supported in societal infrastructure by concerns over human health, effective food production, and the preservation of natural resources. The potential sources of immunotoxicants and the value of avian immunotoxicological assessment are illustrated in Figure 1. The following sections of this review provide an overview of the state of avian immunotoxicology and the opportunities for future development.

Comparative Avian Immunology

While the avian immune system has basic similarities with that of mammalian species, it also possesses certain novel features that can be important in approaching immune assessment. Such opportunity for comparative immunological analysis of the avian immune system has been important in approaching issues of environmental immunomodulation. Clearly, the progressive accumulation of information within fundamental avian immunology is vital not only for the utility of the various species as animal models, but also for the enhanced management of economically significant species (Dietert and Lamont, 1994).

Among these features are the existence of discrete organs, the bursa of Fabricius and thymus, for the differentiation of B and T lymphocytes, respectively. The location of the bursa near the cloacal vent means that the differentiating bursal lymphocytes are exposed to environmental antigens and chemicals (Bloom *et al.*, 1987; Sovari and Sovari, 1977). In this regard, the environmental exposure of the bursa is more similar to that of the lung than to the relatively protected areas of mammalian B lymphocyte differentiation.

The generation of immunoglobulin diversity in avian species is distinct from that of rodents and humans. While opportunities for gene conversion exist within some mammalian species such as the rabbit, the chicken takes this vehicle of generation of antibody diversity to an extreme. Somatic recombination can utilize only single V_H and V_L genes and produce, even with imprecise joining, only a handful of distinct antibodies (Reynaud *et al.*, 1985; 1989; Thompson and Neiman, 1987). However, the process of gene conversion in the bursa of Fabricius enables the chicken to develop a repertoire of antibodies that is comparable in diversity to that of mammalian species (Masteller and Thompson, 1994).

The avian T lymphocyte is also distinct from that of mammals in that two different $\alpha\beta$ T cell receptors occurring on distinct subpopulations of T lymphocytes have been reported for the chicken

(Chen *et al.*, 1989). These receptors are designated as TCR2 and TCR3 with gamma-delta chain positive T cells designated as TCR1 (Bucy *et al.*, 1990). Studies using monoclonal antibody depletion of T lymphocyte subpopulations in conjunction with thymectomy demonstrated that TCR2-positive T lymphocytes are important for the production of IgA (Cihak *et al.*, 1991). The topic of T cell development in the chicken was recently reviewed by Chen *et al.* (1994).

Even among non-lymphoid leukocytes, some distinctions between the mouse and chicken appear to exist. For example, the chicken has few harvestable resident peritoneal macrophages in contrast with the mouse (Rose and Hesketh, 1974). This means that macrophages harvested after introduction of a stimulus reflect a relatively purified population of cells which have responded to the stimulus and/or secondary biochemical signals *in vivo*. As a result, it is possible to perform *in situ* macrophage recruitment and activation analysis that is more problematic in species with a significant contaminating resident macrophage population (Chu and Dietert, 1988; Golemboski *et al.*, 1990a,b). Based on evidence to date, it also appears likely that the specific acquisition and loss of functional capacities during chicken macrophage activation is not identical to that observed for mouse macrophages (Golemboski *et al.*, 1990b).

The chicken major histocompatibility complex (MHC), also known as the B complex, presents an interesting contrast from the mouse H-2 or human HLA complexes. The B complex is located on a microchromosome (#16) adjacent to the nucleolar organizer region (NOR) (Bloom and Bacon, 1985). While the B complex contains the prerequisite composition of Class I and Class II genes, it also has some novel multigene families such as the so-called Class IV or B-G genes. One ramification of the different avian vs. mammalian MHC composition is that the chicken MHC appear to exert more influence over innate immune function and disease resistance mediated by innate responses than do the mouse H-2 or human HLA complexes (Puzzi *et al.*, 1990; Lamont *et al.*, 1987; Lin *et al.*, 1992). This can have important ramifications as immune assessment strategies are considered.

The avian respiratory system (e.g., the air sac) is also quite distinct in structure from the mammalian lung (Fangerland and Arp, 1993). As a result, methodologies for assessing respiratory-induced immunotoxicity are likely to require species-tailored evaluations of environmentally-induced immunomodulation. For example, the avian air sac lacks a readily harvestable resident population of macrophages, in contrast to mammalian alveoli (Toth and Siegel, 1986).

A final difference reported for the chicken vs. mouse immune systems concerns the capacity for specific enzymatic metabolism of arachidonic acid. Elicited chicken macrophages appear to lack the capacity to produce certain key leukotrienes (e.g., LTC_4, LTD_4, LTE_4) when compared with mouse macrophages elicited under the same protocols (Whelan *et al.*, 1994). This raises intriguing questions about potential avian alternative mediators of biological responses attributed from mammalian data to certain leukotrienes.

GOALS AND DIRECTION OF AVIAN IMMUNOTOXICOLOGICAL ASSESSMENT

Avian immune assessment historically has been hallmarked by the application of a wide array of assays with few standard comparisons available in the literature. The assays utilized in individual studies tended to be those available to individual laboratories rather than a package of assays that, when used in combination, conveyed an effective picture of avian immune status. For many situations, single disease challenges were used to evaluate environmental parameters including the effectiveness of vaccines. Just as with the NTP approach to immunotoxicological assessment (Luster *et al.*, 1988), a standard package of assays used to screen numerous environmental factors offers the opportunity for relative comparisons of immunotoxic potential. It can also be applied to determine the environmental conditions required to optimize immune status. For this reason, the development of a standardized package of avian immune assays is necessary for comprehensive immunotoxicological assessment.

Concepts of comprehensive avian immune assessment emerged during the 1980s. Early proposals tended to provide only general guidelines for examination and were oriented solely toward the detection of immunosuppression (Dohms and Saif, 1984). Among the first detailed proposal for chicken immune assessment was the validation study of Kerkvliet and colleagues (Baecher-Steppan *et al.*, 1989) for application in broiler chickens. The proposed combination of assays offered effective measures of both acquired and innate immune parameters. The topic of immune competence was recently reviewed by Bacon (1992) and applications for the commercial setting were discussed by Roth (1992).

Recently, additional immune assessment packages have been proposed both for research birds (Dietert *et al.*, 1993, 1994) and for field trials (Dietert *et al.*, 1994). These proposed assessment packages are similar to those of Baecher-Steppan *et al.* (1989) in that plaque-forming cell analysis, delayed-type hypersensitivity, and natural killer cell activity are given a high priority. However, the new assessment proposals draw more heavily on nitric oxide as a measure of macrophage activation and on T lymphocyte subpopulation analysis. The latter parameter has proven to be an effective immunotoxicological predictor in murine studies (Luster *et al.*, 1992). Additionally, the production of nitric oxide by macrophages, particularly in a uricotelic animal such as the chicken, has emerged as a sensitive parameter that is directly related to innate immune capabilities (Sung *et al.*, 1991, 1992, 1994; Sung and Dietert, 1994).

A final issue concerns genetics and immunotoxicological assessment and pertains both to avian and other species. An ongoing problem is that immunotoxicology testing must ultimately be performed on a subset of genotypes within the species. However, the results are usually extrapolated to the entire species or beyond to other species for xenogeneic applications. Prime examples exist where genotypically-determined problems arise in such extrapolations. For example, ultraviolet B radiation is known to be immunosuppressive in most but not all genotypes of mouse (Yoshikawa and Streilein, 1990). Likewise, the capacity of nickel to induce contact dermatitis in humans is dependent upon HLA genotype (Olerupa and Emtestam, 1988). In the avian arena, examples of both chickens (Manning *et al.*, 1990) and Japanese quail (Marks and Wyatt, 1979) which are highly resistant to aflatoxin-induced toxicity are well known. This suggests that the utility of avian

immunotoxicological data will face the same sobering genetic restrictions as already exists for mammalian data.

CATEGORIES OF AVIAN ENVIRONMENTAL IMMUNOMODULATORS

The avian immune system is subject to significant immunomodulation from several distinct categories of environmental factors, three of which are considered in this review. Environmental contaminants and model toxicants represent one group of immunotoxic agents that have received considerable research attention (Table 1). These include immunotoxic agents of prime mammalian concern (e.g., TCDD, PCBs, lead), other naturally-occurring toxicants that are particularly problematic in the agricultural setting (e.g., T-2 toxin, fusarium-B1), and model toxic chemicals (cyclophosphamide, methane methylsulfonate). One contrast with similar mammalian studies is that a significant number of avian investigations have utilized *in ovo* exposure to examine developmental immunotoxicity or have emphasized the exposure of very young animals. This is in contrast with the majority of mammalian immunotoxicity studies which have been performed in juvenile or adult animals.

Table 1
Examples of Avian Immunotoxicants

Chemical	Species	Effects	Reference
TCDD[1]	Chicken Turkey Duck	Inhibition of thymic lymphoid development	Nikolaidis *et al.*, 1988
TCDD[1]	Chicken	Inhibition of bursal lymphoid development	Nikolaidis *et al.*, 1990
PCBs[2]	Chicken	Thymic and bursal impairment	Andersson *et al.*, 1991
Lead acetate	Chicken	Macrophage heat shock protein expression	Miller and Qureshi, 1992
Silica	Chicken	Macrophage depletion tumor growth	Hala and Schnegg, 1993
Cyclophosphamide	Chicken	Bursa lymphoid depletion	Misra and Bloom, 1991
Methyl Methanesulfonate	Chicken	Reduced macrophage function	Qureshi *et al.*, 1989
Aflatoxin-B1	Chicken	Reduced cell mediated immunity	Dietert *et al.*, 1985
Aflatoxin-B1 metabolites	Turkey	Reduced macrophage activity	Neldon-Ortiz and Qureshi,1991
Fumonison-B1	Chicken	Reduced macrophage function	Qureshi and Hagler, 1992
Ochratoxin A	Chicken	Thymus and bursa lymphoid development	Dwivedi and Burns, 1984
T-2 toxin	Mallard Duck	Decreased thymic cortical lymphocyte numbers	Neiger *et al.*, 1994
Trichothecene mycotoxins	Turkey	Altered macrophage function	Kidd *et al.*, 1995

[1] TCDD = 2,3,7,8-Tetrachlorodibenzo-p-dioxin
[2] PCBs = polychlorobiphenyls

Table 2
Examples of Avian Dietary Immunomodulators

Chemical	Species	Effect	Reference
Arginine	Chicken	Increased T-cell-dependent responses Increased interleukin-2 production Increased nitric oxide production Reduced tumor growth	Kwak *et al.*, 1996 Taylor *et al.*, 1991
Cysteine	Chicken	Altered antibody production and T-dependent responses	Tsiagbe *et al.*, 1987
Leucine	Chicken	Altered antibody production	Tinker and Gous, 1986
Methionine	Chicken	Altered antibody production and T-dependent responses	Tsiagbe *et al.*, 1987
Zinc and Methionine	Turkey	Enhanced phagocytic cell function	Kidd *et al.*, 1994
Threonine	Chicken	Altered antibody production	Bhargava *et al.*, 1971
Valine	Chicken	Altered antibody production	Bhargava *et al.*, 1970
Chloride	Chicken	Altered antibody production	Pimentel and Cook, 1987
Copper	Chicken	Altered antibody production	Rangaachar and Hedge, 1974
Selenium	Chicken	Bursal organ status	Marsh *et al.*, 1986
Zinc	Chicken	Enhanced antibody production	Stahl *et al.*, 1989
Vitamin A	Chicken	Optimized antibody responses Optimized T cell mitogenesis	Sklan *et al.*, 1993
Vitamin B-6	Chicken	Altered humoral responses	Blalock *et al.* 1994
Vitamin C	Chicken	Heterophil : Lymphocyte ratio Disease resistance	Gross, 1992
Vitamin E	Chicken	Increased antibody production Increased T cell proliferation Enhanced macrophage function	Dietert *et al.*, 1990
Fatty Acid Balance	Chicken	Macrophage production of PGE2 Antibody production	Fritsche *et al.*, 1991

A second significant category of environmental factors controlling avian immune status includes dietary chemicals (Table 2). This area was the subject of several recent reviews (Austic *et al.*, 1991; Cook, 1991; Klasing, 1991). Dietary immunomodulators can be grouped into three principal dietary components: the vitamins and minerals (e.g., selenium, vitamin C and vitamin E), the amino acids (e.g., arginine, leucine, lysine, methionine), and the fatty acids (e.g., n-3 vs. n-6 series). Some of these dietary factors have potential abilities to exert a greater influence over the avian immune system than might occur in mammals. For example, the chicken is uricotelic and unable to synthesize L-arginine (Tamir and Ratner, 1963). As a result, arginine-dependent immunological activity (e.g., nitric oxide production) might be expected to be more sensitive to dietary alterations in arginine concentration than would occur in mammals. A recent study suggests that levels of

Table 3
Examples of Immunomodulatory Infectious Agents and Vaccines

Infectious Agent	Species	Effects	Reference
Adenovirus	pheasant	Reduced antibody response Reduced T cell response	Fitzgerald *et al.*, 1992
Chicken anemia virus	Chicken	Reduced viral vaccine response Altered macrophage function	Okai *et al.*, 1992 McConnell *et al.*, 1993
Marek's disease virus	Chicken	Increased lymphoid leukosis	Fadley and Witter, 1993
Marek's disease virus vaccine	Chicken	Reduced B lymphocyte function Increased bacterial susceptibility	Friedman *et al.*, 1992
Infectious bronchitis vaccine	Chicken	Reduced antibody titer to bacteria	Montgomery *et al.*, 1991
Infectious bursal disease virus	Chicken	B cells depletion T cell suppression	Saif, 1991
Reovirus	Chicken	Reduced T cell mitogenesis Altered macrophage function	Sharma *et al.*, 1994
Retrovirus	Chicken	Increased Listeria susceptibility	Cummins *et al.*, 1988
Newcastle disease virus	Turkey	Reduced bacterial clearance	Ficken *et al.*, 1987
Hemorrhagic enteritis virus	Turkey	Reduced antibody production	Nagaraja *et al.*, 1985
Eimeria tenella Eimeria adenoeides	Chicken	Reduced Salmonella infection	Tellez *et al.*, 1994
Influenza	Chicken	Reduced nitric oxide production	Lyon and Hinshaw, 1993
Influenza	Turkey	Reduced macrophage function	Kodihalli *et al.*, 1994
Rhinotrachealis virus	Turkey	Increased Pasteurella infection	Cook *et al.*, *1991*

dietary arginine thought to be adequate for growth of young chicks are not necessarily appropriate for optimum immune function (Kwak *et al.*, 1996).

Within a final category of immunomodulatory factors are the avian infectious agents and vaccines (Table 3). The list includes infectious viruses, viral vaccines and parasites. Some of the infectious agents such as infectious bursal disease virus and reoviruses actually target lymphoid organs and/or immune cells and produce severe immunodeficiencies (Smyth and McNulty, 1994; Bülow and Klasen, 1983; Okoye and Uzoukwu, 1990).

In contrast, other viruses produce significant immune alterations during the course of the immune response to the infection. A common thread among the examples of virally-induced immune changes is the reduction in innate immune capabilities. This contributes to a reduced resistance to bacterial challenge. One vehicle for this change may be an alteration in macrophage function and related changes in both bacterial phagocytosis and the production of bactericidal metabolites, such as nitric oxide. Such alterations have been observed with influenza virus infection in both chickens

(Lyon and Hinshaw, 1993) and turkeys (Kodihalli *et al.*, 1994). A particularly interesting finding is the capacity of universally-employed Marek's disease vaccines to increase susceptibility to bacterial challenge. This suggests that the selection of effective field vaccines requires the use of comprehensive immune assessment rather than the historic approach of single disease evaluations. Additionally, the increased use of genetically-determined natural immunity against certain diseases is essential for agricultural practices to achieve environmental sustainability (Dietert *et al.*, 1995).

CONCLUSIONS

Knowledge of fundamental avian immunology, availability of reagents and probes, and development of specialized genetic research strains have progressed to the extent that avian immune assessment can be conducted in a cost effective manner. Additionally, recent examination of available methodologies suggests that a standardized and comprehensive avian immune assessment is readily attainable. Such immune assessment has the opportunity to address three societal problems: the protection of human populations from environmental insult, the production of vital human food resources in an environmentally-sustainable manner, and the preservation of avian natural resources.

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Chapter 39

Immune Cell Phenotype Analysis by Flow Cytometry For Identifying Biomarkers of Chemical Immunotoxicity

N. I. Kerkvliet and J. A. Oughton

Department of Agricultural Chemistry
Environmental Health Sciences Center
Oregon State University
Corvallis, OR 97331

ABSTRACT

Multiparameter flow cytometric analysis of cell surface proteins ("markers") on immune cells offers a promising approach to the field of immunotoxicology for identifying immunotoxic chemicals as well as elucidating their mechanisms of action. However, in order to apply data from laboratory animal studies to the assessment of human health risk from immunotoxic chemicals, research needs to focus on the identification of markers in animal models that are sensitive to environmental chemical exposure and measurable in humans. In addition, since rodent studies routinely use cells isolated from lymphoid organs (e.g., spleen, lymph nodes, thymus) for immunophenotyping while human studies utilize peripheral blood, research is needed to determine if phenotypic marker changes identified in lymphoid organs correlate with similar changes in the peripheral blood. In this report, we summarize the results of several studies using 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in a mouse model that begin to address these issues. First, we have shown that acute exposure to a known immunotoxic dose of TCDD does not alter the phenotypic profile of spleen cells based on analysis of B cells and T cell subsets defined by CD4 and CD8. Similarly, these subsets were not altered following challenge of the mice with SRBC even though the antibody response was profoundly suppressed by the same exposure. Thus, analysis of these major subsets was not sensitive to detect the immunotoxic effects of acute exposure to TCDD. In contrast, using an allogeneic tumor model where the responding population of cells is much larger than the response to SRBC, $CD8^+$ T cells and $B220^+$ B cells were significantly decreased following acute exposure to TCDD, and this decrease correlated with the suppression of CTL activity and alloantibody titers in TCDD-treated mice. In other studies, we have shown that long-term (15 month) exposure of naive but non-barrier-reared C57Bl/6 mice to a low weekly dose of TCDD did not alter the frequency of $CD4^+$ or $CD8^+$ T cells, B cells, or macrophages. However, TCDD did affect functionally discrete subpopulations within the $CD4^+$ T cell compartment defined as naive or memory cells by the correlated expression of CD44 and CD45RB. Specifically, TCDD exposure resulted in a significant decrease in the frequency of memory T helper cells, with a concomitant increase in the proportion of naive T helper cells. This change is consistent with an immunosuppressive effect of TCDD and may reflect a decreased lifelong responsiveness of the mice to

environmental antigens. In addition to TCDD-induced changes, several age-dependent changes in immune phenotypes were identified in this study. These changes were highly correlated in spleen and blood, providing preliminary evidence in support of the use of spleen cells as surrogates for blood cells in the development of phenotypic biomarkers of immunotoxicity in the mouse.

INTRODUCTION

Humans are often and unavoidably exposed to numerous xenobiotics in their environment that may impact their state of health. Several of these chemicals have been shown to influence the immune system of laboratory animals and to render such animals more susceptible to infectious and neoplastic diseases. While these effects in laboratory animal studies have been described following exposure to relatively high doses of such chemicals, there is a growing concern over the possible long-term human health effects that might result from low level, chronic exposure. This concern has led to the realization among the scientific and regulatory communities of a need to develop biomarkers that can be used to identify human exposure to chemicals and/or predict the effect of such chemicals prior to overt toxicity or disease development.

For chemicals that affect the immune system, flow cytometric analysis of immune cell phenotypes in the blood offers a promising approach to biomarker development. The accessibility of blood as a human tissue sample, the relative simplicity of modern-day flow cytometry, and the ever-expanding array of monoclonal antibodies available to phenotype cells combine to justify considerable effort on this front. However, before the full potential of lymphocyte phenotyping can be realized, broad-based studies are needed to establish the sensitivity of various phenotypes to chemical-induced changes and to understand the relationships between changes in surface antigen expression and functional immunity as related to immunotoxicant exposure. This overview will summarize the current status of the field.

IMMUNOPHENOTYPES AND CD ANTIGENS

Research in immunology was revolutionized in the late 1970s by the discovery that functionally distinct lymphocyte subpopulations could be identified on the basis of their expression of different cell membrane proteins (Reinherz and Schlossman, 1982). These molecules function as cell surface receptors for essential growth factors (e.g., cytokines), as membrane enzymes, or as cell adhesion proteins which facilitate binding to other cells or to the extracellular matrix. Expression of many of these molecules is induced or modulated by antigenic activation of the cell. Thus, their level of expression may serve as surrogate markers of the functional status of the immune system.

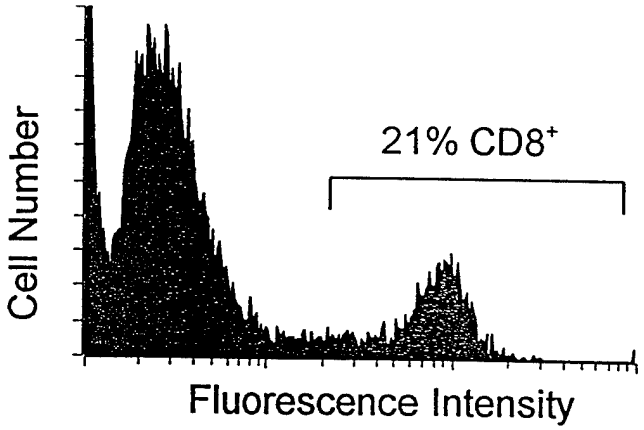
Many of the cell-surface proteins associated with hematopoietic cells have been identified by different monoclonal antibodies in different species. The cell surface molecule that is recognized by a group of monoclonal antibodies is designated CD (cluster of differentiation) followed by a number (e.g., CD1, CD2, CD3, CD4, etc.). Currently, 130 different CDs have been defined. Some of the more widely recognized CD antigens are listed in Table 1 along with functions that have been associated with these different proteins. Although not assigned a CD designation, surface immunoglobulin molecules expressed on B lymphocytes can also be recognized by antibodies directed against their constant regions and are often used to immunophenotype B cells.

FLOW CYTOMETRY AS A TECHNIQUE FOR IMMUNOPHENOTYPING

The differential expression of CD antigens on the surface of immune cells can be used to identify, quantify and, if desired, separate distinct subpopulations using the powerful techniques of flow cytometry and fluorescence-activated cell sorting (FACS). Monoclonal antibodies to CD antigens or other cell-surface molecules are conjugated with fluorochromes and used to specifically "stain" individual cells within a mixed population of cells. The suspension of labeled cells is then analyzed by laser-activated flow cytometry. Each time that a cell labeled with one or more fluorochromes passes by the laser, the fluorescent emission generated from the cell is differentially filtered, collected by photomultiplier tubes, amplified, and sent to a computer for analysis. Since flow cytometers can analyze thousands of cells per second, data can be quickly collected on large numbers of cells. Cells can be identified as either positive or negative for expression of a particular protein. In addition, the intensity of fluorescence associated with a specific dye provides data on the relative amount of a specific protein present on the positive cells. Recent advances in fluorescent dye development have made three-color analysis a relatively routine procedure using a flow cytometer that is minimally equipped with a single argon ion laser. Three-color combinations usually involve fluorescein isothiocyanate (FITC), phycoerythrin (PE), and a tandem complex of PE with Cy-5 (PE-Cy5) which are all excitable by the laser tuned to a wavelength of 488 nm. In addition to fluorescence from fluorochromes, laser light that is scattered by a cell in a forward and side angle direction is also collected and analyzed. These light scatter measurements are proportional to cell size and granularity and can be used to differentiate lymphocytes, monocytes, and granulocytes in peripheral blood as well as to electronically gate out erythrocytes, dead cells and debris. This ability to electronically gate on the population(s) of interest is one of the major advantages of flow cytometry.

Figure 1 illustrates data obtained from mouse spleen cells stained with monoclonal antibodies to CD8 and CD28. In Figure 1A, the data (gated to exclude erythrocytes and debris) are displayed as a single-parameter histogram with the intensity of fluorescence associated with CD8 staining increasing along the X axis, and the relative number of cells increasing on the Y axis. The $CD8^+$ cell population is delineated in brackets and represents 21% of the mouse splenic lymphocyte population. In Figure 1B, a two-parameter histogram illustrates the correlated expression of CD8 and CD28 on mouse spleen cells. Only 6.2% of the mouse spleen cells stained positive for both CD8 and CD28 while 15.0% stained positive for CD8 but negative for CD28.

A. Single-parameter histogram



B. Two-parameter histogram

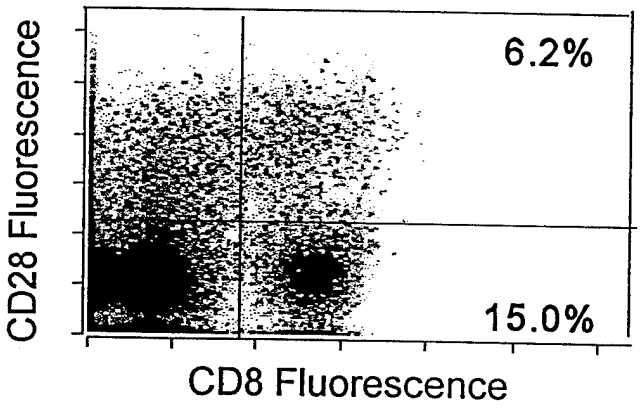


Figure 1. Representative data illustrating (A) single-parameter and (B) two-parameter histograms. Prior to flow cytometric analysis, mouse spleen cells were stained with (A) PE-labeled anti-CD8 or (B) FITC-labeled anti-CD8 and PE-labeled anti-CD28. Histograms represent fluorescence gated on forward angle light scatter to exclude debris, erythrocytes, and cell clumps. The region defined in the single-parameter histogram (A) shows that 21% of the mouse spleen cells stained positive for CD8. The regions defined in the two-parameter histogram (B) show that 6.2% of the $CD8^+$ cells stained positive for CD28 while 15.0% stained negative for CD28.

CLINICAL APPLICATIONS OF LYMPHOCYTE PHENOTYPING

There are three major clinical applications for the analysis of cell-surface markers on cells of the immune system (Check and Maxwell, 1991):

- to classify leukemias and lymphomas or monitor their recurrence or response to therapy,
- to diagnose or follow patients with immuodeficiency diseases, and
- to monitor organ transplant recipients who are treated with immunosuppressive drugs to prevent organ rejection.

By far, the most successful application of lymphocyte phenotyping has been in the diagnosis of Acquired Immune Deficiency Syndrome (AIDS) and the correlation of $CD4^+$ cell count in the blood with disease progression (Giorgi *et al.*, 1992). Immunophenotyping has also been fairly successful in the diagnosis of congenital immunodeficiency diseases (Giorgi *et al.*, 1992). However, other clinical applications of immunophenotyping such as the diagnosis of autoimmune disease or monitoring of transplant patients treated with immunosuppressive drugs have met with less success (Zola, 1992). This is most likely due to our lack of full understanding of the mechanisms of the biological processes that signal early transplant rejection or autoimmune disease, which is necessary for selecting the appropriate cells and markers to monitor. In addition, the heterogeneity of disease processes in a heterogeneous human population makes it difficult to identify common and consistent changes that may serve as diagnostically useful tools. However, with continued advances being made in quality control and methods standardization, and a candid recognition of the many potential confounding variables that must be considered when interpreting the data, immune cell phenotyping still holds great promise as an important diagnostic and prognostic indicator of disease status.

IMMUNE PHENOTYPING IN IMMUNOTOXICOLOGY RESEARCH

Immune cell phenotyping was recognized several years ago as a potential approach to identify and study the mechanisms of immunotoxicity of xenobiotics in laboratory rodents (Burchiel *et al.*, 1987; 1988). As a general screening technique for detecting immunotoxicants, this approach was further strengthened following reports by Luster *et al.* (1992; 1993) that correlated changes in spleen cell surface markers induced by chemicals with functional immunotoxicity and altered host resistance to disease challenge. Specifically, many chemicals that altered at least one of several immune function assays also altered B cells or T cell subsets (CD4/CD8) in the spleen of mice. In many cases, cell markers were altered without a parallel effect on peripheral blood counts or spleen cellularity, suggesting that immunophenotyping detected changes induced by chemicals that were more subtle than overt cytotoxicity. However, most of these effects were observed following exposure to relatively high levels of the chemicals. These studies did not address the sensitivity of the technique for detecting immunotoxicity in relation to other immunoassays or in relation to toxicity in other target organs. Likewise, the fact that some chemicals produced immune suppression in the absence of changes in B and T cell subsets indicated that measuring only these major subsets was not sufficient to identify all immunotoxicants. Thus, more recent work in many laboratories has focused on utilizing cell surface markers that are induced upon antigenic activation

Table 1
Examples of CD antigens, their expression on different cells, and their association with cellular functions

CD ANTIGEN	CELLULAR EXPRESSION	FUNCTIONS
CD3	T cells, thymocytes	part of the T cell antigen receptor
CD4	T cell and thymocyte subsets monocytes, macrophages in rats and humans	co-receptor for MHC class II molecules associated with helper T cell subset
CD8	T cell and thymocyte subsets	co-receptor for MHC class I molecules as sociated with cytotoxic and suppressor T cell subsets
CD25	activated T cells, B cells, monocytes	IL-2 receptor α chain
CD28	T cell subsets, activated B cells	activation of naive T cells, receptor for CD80 (B7-1) and CD86 (B7-2)
CD44	leukocytes	binds hyaluronic acid, mediates adhesion
CD45	leukocytes	tyrosine phosphatase, distinct isoforms associated with naive, activated and memory subpopulations of Tcells
CD56	natural killer cells	adhesion molecule

of the cells as more sensitive and functionally relevant endpoints than markers that delineate lineage-specific immunocyte subpopulations.

There have also been attempts to use immune phenotyping to "diagnose" environmental contaminant-induced immunotoxicity in humans (Hoffman *et al.*, 1986; Fiore *et al.*, 1986; Knutson *et al.*, 1987; Webb *et al.*, 1989; McConnachie and Zahalsky, 1991; 1992). In most cases, the results of these studies have shown either no significant alterations in the markers analyzed or small changes that have questionable biological significance. Unfortunately, in most of these studies, the actual exposure status of the exposed cohort was poorly defined. Thus, lack of effects in a truly exposed population could result from the inclusion of subjects that were not really exposed. On the other hand, some studies have reported significant phenotypic alterations in people that were presumptively exposed to a suspected immunotoxic chemical (e.g., pentachlorophenol, chlordane, dioxin). However, these subjects were "phenotyped" for the purpose of litigation over perceived health risks of exposure to an industrial contaminant. The conclusions drawn from these data were derived from statistical comparisons between the litigants and a poorly matched, poorly defined "laboratory control" population. Thus, many scientists reject any conclusions from these studies as invalid.

In order for immune phenotyping to evolve as an useful biomarker for determining public health risks from immunotoxic chemicals, the approach must be validated for specificity and sensitivity, as discussed by the National Research Council's Committee on Biologic Markers, Subcommittee on Immunotoxicology (1992). Foremost, controlled animal studies are needed to identify what phenotypes are altered by specific chemicals and the degree to which the altered phenotype correlates with altered immune function. Secondly, the phenotypes need to be shown to be affected

by the chemical in a dose-responsive fashion and to occur at lower doses than other toxicity symptoms or overt clinical disease Thirdly, animal studies need to address if the same phenotypic changes occur after acute or chronic exposure, and the relationship of the changes to antigenic challenge. It is not unreasonable to assume that immunotoxic chemicals that are not lymphotoxic (i.e., cytoreductive) may influence the induction of markers during an ongoing immune response without altering preexisting marker expression on resting cell subsets. Thus, exposure of laboratory rodents reared under barrier conditions to an immunotoxic chemical may not affect the immune system unless antigenic stimuli are intentionally introduced. In contrast, humans exist in an environment replete with antigenic challenges and thus their immune system may be more vulnerable to exposure to immunotoxic chemicals. Finally, it is also important for laboratory animal studies to establish that any immunophenotypic changes that occur in lymphoid tissues due to immunotoxicant exposure are reflected by similar changes in the blood. While most immunotoxicity studies in laboratory rodents utilize cells from the spleen and lymph nodes, human studies rely almost entirely on sampling peripheral blood. As discussed by Westermann and Pabst (1990), since the blood represents only about 2% of the total lymphocyte pool in the normal adult human body, it is unlikely that the cells are representative of the other 98%. On the other hand, because roughly 500 x 10^9 lymphocytes migrate in and out of blood each day with a mean transit time of approximately 30 minutes and migrate through most peripheral tissues, the blood may indeed reflect alterations in lymphocyte composition within the organs that are traversed (Westermann and Pabst, 1990).

EFFECTS OF TCDD ON LYMPHOCYTE PHENOTYPES

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a ubiquitous and persistent environmental contaminant that produces a broad spectrum of toxic effects at very low levels of exposure in many animal species. A number of animal studies conducted over the past 25 years have identified the immune system as one of the primary target organs of TCDD. For example, in C57Bl/6 mice, TCDD produces a dose-dependent suppression of the antibody response to sheep erythrocytes (SRBC), with an ED_{50} of 0.7 μg/kg (Figure 2A). Likewise, cell-mediated immunity as measured by the cytotoxic T lymphocyte (CTL) response to allogeneic P815 mastocytoma cells is dose-dependently suppressed by TCDD with an ED_{50} of approximately 7 μg/kg (Figure 2B). TCDD is also one of the first environmental chemicals to be studied for its possible immunotoxic effects in humans, namely the Vietnam veterans exposed to Agent Orange. However, the results of extensive studies failed to identify any consistently significant change in lymphocyte subsets, leading some scientists to conclude that the human immune system is resistant to the toxicity of TCDD. This conclusion, however, was premature given that controlled animal studies had not yet established the sensitivity of the markers used in these investigations to alterations by TCDD.

In order to address this data gap, our laboratory has conducted numerous studies in C57Bl/6 mice in an attempt to identify changes in immunophenotypes that correlate with and provide mechanistic insight into the functional abnormalities that underlie TCDD immunotoxicity. The results of these studies provide an interesting perspective on the potential utility of using immunophenotyping as biomarkers of immunotoxicity. The results of these studies will be briefly reviewed in the remainder of this paper.

Acute exposure of naive mice

Our first study focused on the changes in immune phenotypes following an acute exposure to a single dose of TCDD that was known to significantly suppress the antibody response to SRBC (Kerkvliet and Brauner, 1990). Mice were treated with 0 or 2 μg/kg TCDD and killed two days later for analysis of B and T cells in the spleen (identified by surface Ig and Thy 1.2 markers, respectively) as well as T cell subsets defined by CD4 and CD8 expression. Results of these studies indicated that an acute immunotoxic dose of TCDD did not alter the relative frequency of these major lymphocyte subsets in the spleen of mice.

Acute exposure of SRBC-challenged mice

We next asked whether or not phenotypic changes could be induced if the mice were challenged with antigen. Because the primary antibody response to SRBC was so sensitive to suppression by TCDD, we used this model to evaluate phenotypic alterations. Mice were treated with 0, 2 or 5 μg/kg TCDD, injected 2 days later with SRBC, and then killed on the following 4 days for subset analysis. However, as with unchallenged mice, TCDD exposure failed to induce any significant alterations in splenic lymphocyte subsets except for a small decrease in the percentage of $CD8^+$ cells on day 3 in the TCDD-treated groups (Kerkvliet and Brauner, 1990). However, this effect appeared to result from an elevated percentage of $CD8^+$ cells in the controls rather than a reduction in TCDD-treated mice. In retrospect, the SRBC model was not the best choice for testing the role of antigen stimulation in immunophenotyping. If one considers that a fully developed primary antibody response to SRBC in C57Bl/6 mice constitutes approximately 2500 antibody-producing B cells/10^6 spleen cells on day 5 of the response, this represents only 0.25% of the total spleen cell population. If one further assumes that the T cell response to SRBC would be even smaller than

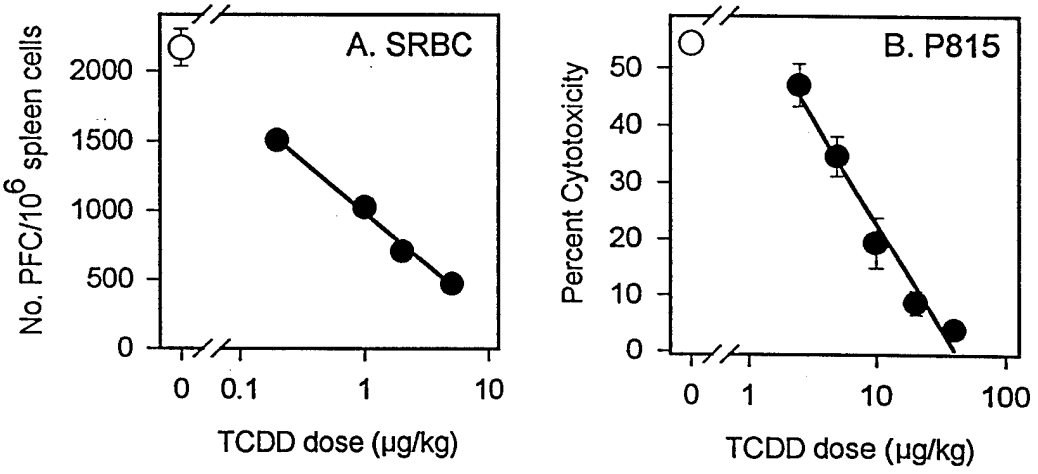


Figure 2. Effect of acute TCDD exposure on the (A) splenic plaque-forming cell (PFC) response to SRBC and (B) splenic cytotoxic T lymphocyte (CTL) response to P815 allogeneic tumor cells. C57Bl/6 mice were given a single oral dose of vehicle (○) or TCDD (●) prior to the injection of SRBC or P815 tumor cells. The PFC response was measured 5 days after injection of SRBC in a modified Cunningham assay as described by Kerkvliet and Brauner (1990). CTL response was measured 10 days after injection of P815 tumor cells in a standard 4-hr ^{51}Cr release assay as described by DeKrey and Kerkvliet (1995).

the clonally expanded B cell population, then it is obvious that even flow cytometry would not be sensitive enough to detect changes in the frequencies of the responding cells.

Acute exposure of mice injected with allogeneic tumor cells

In order to address the problem of detection sensitivity, we turned our focus to a tumor allograft model. This model provided the important advantage of having a relatively large number of alloantigen-reactive precursor T cells present in the peripheral lymphoid tissue of naive mice, unlike conventional antigens like SRBC (Orosz *et al.*, 1986). As shown in Figure 3, the number of splenic $CD8^+$ T cells and $B220^+$ B cells, but not $CD4^+$ T cells, increased in vehicle-treated C57Bl/6 mice following the injection of allogeneic P815 mastocytoma cells. These phenotypic changes correlated with the development of CTL activity and alloantibody production in response to P815 tumor challenge (data not shown). In contrast, in TCDD-treated mice, the increases in $CD8^+$ and $B220^+$ cells failed to occur, correlating with the immunosuppressive effect of TCDD on the CTL and alloantibody responses.

Allospecific CTL activity has been shown to be associated with a specific subset of $CD8^+$ T cells that express high levels of CD44 and low levels of CD45RB, a so-called "CTL effector" (CTL_E) phenotype (Mobley and Dailey, 1992). We used these markers to further analyze the effects of TCDD on CTL activity. As shown in Figure 4, spleen cells expressing the CTL_E phenotype represented a minor population in naive mice while the majority of the $CD8^+$ cells were CTL_E in mice on day 10 after P815 challenge. TCDD treatment of mice resulted in a significant reduction in the proportion of cells expressing the effector phenotype. Furthermore, as shown in Figure 5, the percentage of $CD8^+$ cells that expressed the CTL_E phenotype in individual mice treated with different doses of TCDD was highly correlated with the amount of CTL activity as measured in a

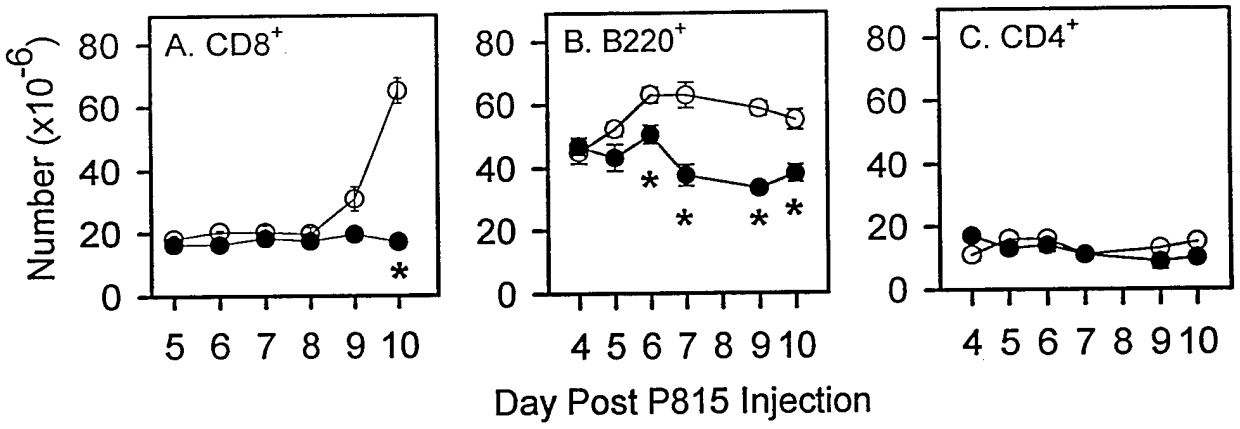


Figure 3. Effect of acute TCDD exposure on the major lymphocyte subpopulations in the spleen of mice injected with P815 tumor cells. C57Bl/6 mice were given a single oral dose of vehicle (○) or 15 µg TCDD/kg body weight (●) one day prior to the injection of P815 tumor cells. Four to 10 days later, spleen cells were stained with monoclonal antibodies to (A) CD8, (B) B220, or (C) CD4 and analyzed by flow cytometry. * notes a statistically significant difference at $p<0.05$. Data represent the mean ± SEM of 6 mice per treatment per day. In some cases, error bars are smaller than the symbols.

^{51}Cr release assay. Thus, one can use phenotypic analysis of CTL_E as a marker for functional CTL activity.

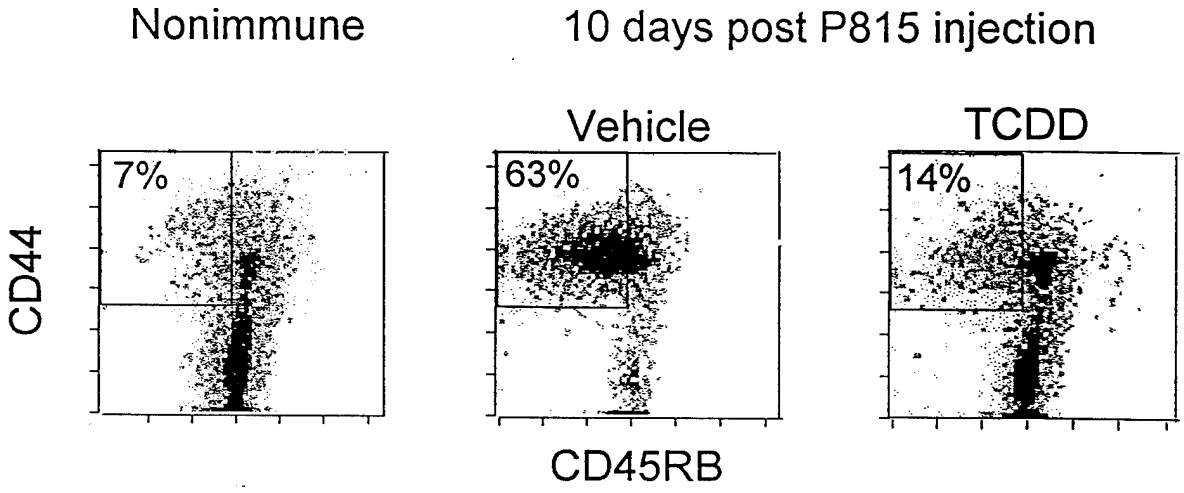


Figure 4. Representative histograms illustrating the proportion of $CD8^+$ spleen cells that express the CTL effector (CTL_E) phenotype in nonimmune and P815-injected mice. C57Bl/6 mice were given a single oral dose of vehicle or 15 µg TCDD/kg body weight one day prior to the injection of P815 tumor cells. Ten days later, spleen cells were stained with monoclonal antibodies to CD8, CD44, and CD45RB and analyzed by three-color flow cytometry. Two parameter histograms representing CD44 versus CD45RB immunofluorescence were derived by gating on $CD8^+$ spleen cells. The region defined in the histograms identifies the proportion of $CD8^+$ cells that express the CTL_E phenotype ($CD44^{hi}CD45RB^{lo}$).

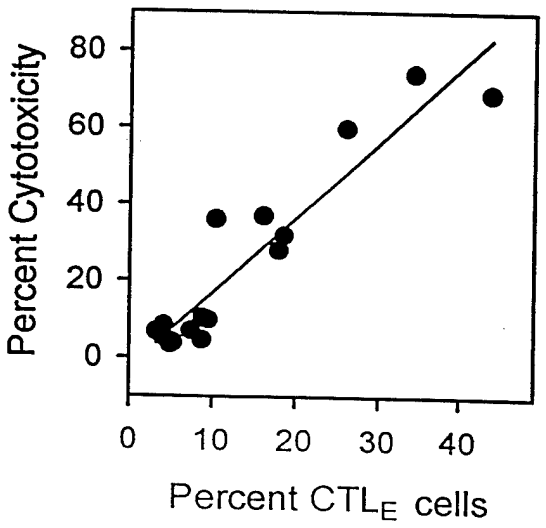


Figure 5. Correlation between CTL activity and the percentage of $CD8^+$ spleen cells that express the CTL_E phenotype. C57Bl/6 mice were given a single dose of 0, 3.75, 7.5, or 15 µg TCDD/kg body weight one day prior to the injection of P815 tumor cells. Nine days later, spleen cells were stained with monoclonal antibodies to CD8, CD44, and CD45RB and analyzed by flow cytometry for the proportion of $CD8^+$ that expressed the CTL_E phenotype (as described in Figure 4). CTL activity was also measured in a standard 4-hr ^{51}Cr release assay as described by DeKrey and Kerkvliet (1995). A highly significant correlation between CTL activity and the proportion of $CD8^+$ cells expressing the CTL_E phenotype was observed (R=0.95, $p<0.0001$).

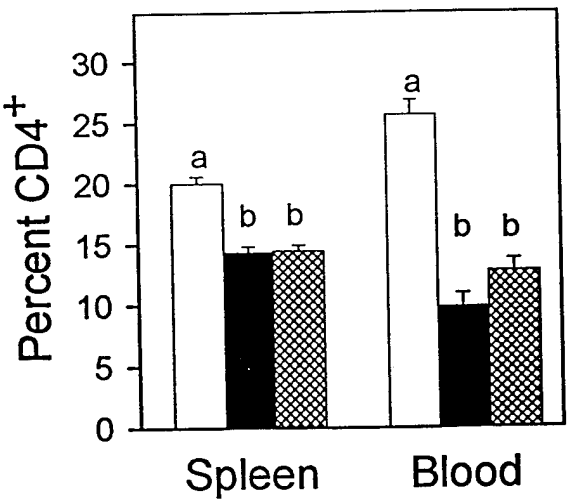


Figure 6. Effect of aging and long-term exposure to TCDD on the proportion of $CD4^+$ cells in the spleen and peripheral blood. Mice were treated with vehicle (solid bars) or 0.2 µg TCDD/kg body weight (cross-hatched bars) by gavage once a week for 14-15 months. A group of 4-month old mice (open bars) was also evaluated to control for the effect of aging. Spleen cells and peripheral blood lymphocytes were stained with monoclonal antibody to CD4 and analyzed by flow cytometry as described by Oughton *et al.* (1995). Each bar represents the least squares mean ± SEM. Bars with different letters represent statistically significant differences at $p<0.0001$.

chronic exposure of naive mice

While the preceding type of data was important for validating phenotypic markers in terms of functional correlates and sensitivity to TCDD, the data were difficult to relate to the use of phenotypic biomarkers for human epidemiology studies. Therefore, a long-term study was conducted in which C57Bl/6 mice were treated weekly with 0 or 0.2 µg TCDD/kg body weight for 15-16 months prior to immune phenotyping (Oughton *et al.*, 1995). (This dose of TCDD was selected on the basis of representing the lowest single dose that significantly suppresses the antibody response to SRBC.) Since the mice were 16-17 months old at the time of sacrifice, a group of 4-month-old mice was analyzed concurrently in order to document immunophenotypic alterations that occur with aging. Major spleen cell phenotypes were defined by the expression of CD4 or CD8 on T cells, B220 on B cells and Mac-1 on macrophages and granulocytes. Mac-1^+ and $B220^+$ cells were also evaluated for their expression of the activation antigen I-A, while T cell subsets were

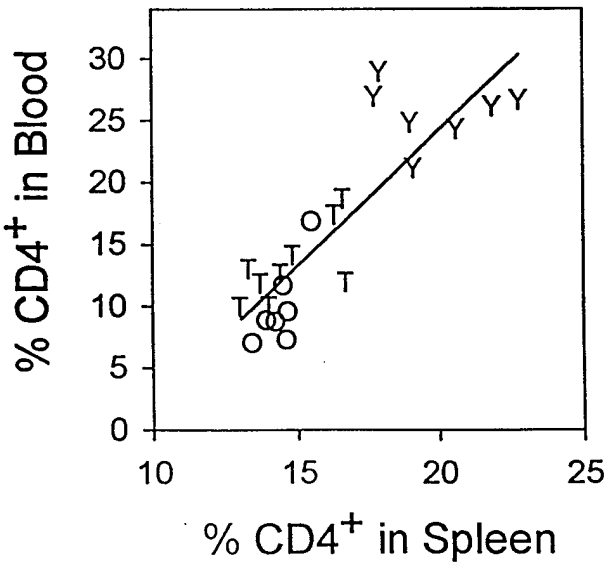


Figure 7. Correlation between the proportion of $CD4^+$ cells in the spleen and peripheral blood of mice. Mice were treated as described in Figure 6. Each symbol represents an individual young (Y), aged vehicle-treated (O), or aged TCDD-treated (T) mouse. A highly significant correlation ($R=0.86$; $p<0.0001$) was observed when all mice were treated as one homogeneous group.

characterized as naive, effector, or memory subpopulations based on their level of expression of CD44 and CD45RB. We also attempted to address the feasibility of using mouse blood for phenotypic analysis and to correlate any changes in phenotypes in the spleen with similar changes in the blood.

The results of this study revealed several changes in phenotypes associated with aging but few that could be associated with TCDD exposure (see Oughton *et al.*, 1995 for full discussion of the results). As shown in Figure 6, aging was associated with a significant decrease in the proportion of $CD4^+$ cells in both the spleen and the blood. On an individual animal basis, there was a good correlation ($r = 0.86$) between the frequency of $CD4^+$ cells in the spleen and their frequency in the blood (Figure 7). The age-dependent changes in $CD4^+$ cells also included a significant shift in the frequency distribution from mostly naive cells in young mice to mostly memory cells in old mice (Figure 8), as has been previously reported (Ernst *et al.*, 1990). Although TCDD exposure did not alter the frequency of $CD4^+$ cells (Figure 6), there was a significant reduction in the percentage of memory $CD4^+$ cells and a concomitant increase in the percentage of naive cells (Table 2). This change is consistent with an immunosuppressive effect of TCDD and implies an overall reduction in responsiveness of the mice to environmentally encountered antigens. (It should be noted that these mice were housed under conventional conditions and were presumably exposed to more environmental antigens than if reared under barrier conditions.) Long-term TCDD exposure did not alter the frequency of $CD8^+$, $B220^+$ or Mac-1^+ cells (data not shown).

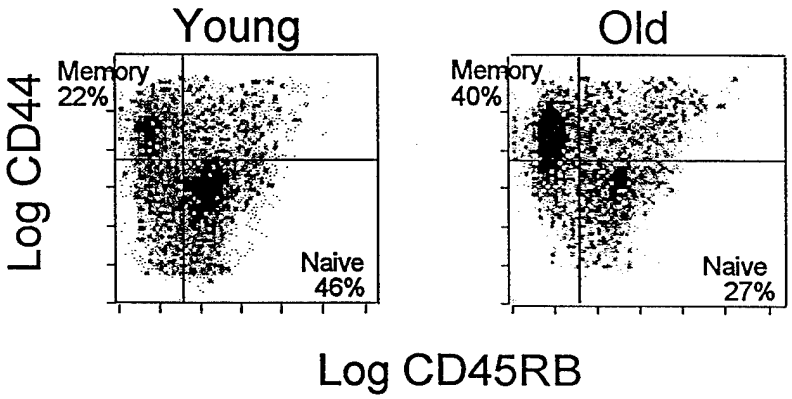


Figure 8. Representative histograms illustrating the effect of aging on $CD4^+$ subsets in the spleen of mice. Spleen cells from young adult (4 months old) and old mice (15-16 months old) were stained with monoclonal antibodies to CD4, CD44, and CD45RB and analyzed by three-color flow cytometry as described by Oughton *et al.* (1995). Two parameter histograms showing the staining patterns of CD44 versus CD45RB immunofluorescence were derived by gating on $CD4^+$ cells. On the basis of the correlated expression of CD44 and CD45RB, $CD4^+$ can be divided into naive ($CD44^{lo}CD45RB^{hi}$) and memory ($CD44^{hi}CD45RB^{lo}$) subsets, as shown.

Table 2
Effect of long-term exposure to TCDD on $CD4^+$ subsets in the spleen of mice[1]

Subset	Percent Positive	
	Vehicle	TCDD
Naive	18±2	24±1*
Memory	42±1	36±1*

[1]C57BV6 mice were administered vehicle or 0.2 µg TCDD/kg body weight by gavage once a week for 14-15 months. Spleen cells were stained with monoclonal antibodies to CD4, CD44, and CD45RB and analyzed by flow cytometry as described in Figure 8.
* Values are statistically significant at $p<0.01$.

CONCLUSIONS

From these results, we conclude that immunophenotyping of major T, B and macrophage subpopulations is not sensitive to detect the immunotoxicity of TCDD in mice and are, thus, not good candidates for biomarker development in humans. On the other hand, the "memory markers" on $CD4^+$ cells appeared to be quite sensitive to TCDD in the mouse. Furthermore, Neubert *et al.* (1990) also identified the memory T helper cell subset as sensitive to low-dose TCDD effects in the marmoset. Thus, CD44 and CD45RB appear to represent potential biomarkers of TCDD immunotoxicity.

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Chapter 40

Resistance to *Trichinella spiralis* in Mice and Rats Exposed to 2,3,7,8-Tetrachlorodibenzo- p-Dioxin (TCDD):Species Dependent Differences.

Robert W. Luebke

Immunotoxicology Branch (MD-92)
Environmental Toxicology Division
National Health and Environmental Effects Research Laboratory
US EPA
Research Triangle Park, NC 27711

ABSTRACT

Exposure to TCDD has been reported to decrease resistance to infection with certain organisms. However, strain differences in sensitivity to TCDD-induced immunotoxicity have been reported. These experiments were conducted to evaluate resistance to *Trichinella spiralis* (Ts) infection in mice (B6C3F1) and rats (F344) of both sexes, following TCDD exposure. Animals were administered 0 (corn oil), 1, 10 or 30 µg TCDD/kg intraperitoneally and infected 7 d later with 200 (mice) or 1000 (rats) Ts larvae. Animals were killed at various times after infection for parasite counts or to assess parasite fecundity. Lymphoproliferative (LP) responses to parasite antigen (TsE) were evaluated 7, 14 and 28 d after infection. In female rats, TCDD exposure slowed adult parasite elimination from the small intestine and did not affect the number of encysted larvae in the muscle, although production of newborn larvae was elevated in female parasites isolated from the highest dose group. Parasite expulsion was suppressed in male rats and numbers of encysted larvae were increased by TCDD exposure. Male rats have been shown to be less resistant to Ts infection than females; however, in a single experiment, we found no differences in resistance to infection between castrated and sham operated male rats exposed to dioxin. Thus, gender-related differences in resistance to Ts infection may be associated with decreased resistance following TCDD exposure, but castration alone does not appear to be protective. In both male and female rats, proliferative responses of lymphocytes cultured with parasite antigen were enhanced in groups of rats exposed to 30 µg TCDD/kg. In female mice, TCDD dosing delayed parasite expulsion, increased parasite fecundity and increased body burdens of encysted larvae. Expulsion of parasites was delayed, but parasite fecundity and burdens of encysted muscle larvae were not affected by exposure of male mice to TCDD. In both male and female mice dosed with TCDD, the lymphoproliferative response to parasite antigen was suppressed for the first 2 weeks of infection. These studies provide an example of the possible differences in experimental outcomes that can be observed between species and highlight the fact that males and females of the same species may or may not be equally affected by exposure to a given chemical.

INTRODUCTION

Twenty five years ago, Friend and Trainer (1970) published their work describing decreased resistance to infection following exposure to polychlorinated biphenyls. Since that time, a great deal of effort has been put forth to identify changes in the immune system brought about by exposure to xenobiotics. As the field of immunotoxicology has matured, and as the focus of the discipline has moved from descriptive to mechanistic studies, increasingly sophisticated immunological techniques are being employed which are capable of demonstrating chemically-induced changes at the molecular level. However, the majority of immunotoxicologists agree that the ability of the host to resist infection or to destroy injected tumor cells remains the best indicator of normal immune system function.

Resistance to infection may be the result of T lymphocyte-mediated destruction of the infectious agent and infected cells, or antibody coating of the agent, leading to lysis, agglutination, neutralization or opsonization. Acting in concert with these responses, the non-antigen-specific activity of natural killer cells, granulocytes and macrophages may make significant contributions to recovery from infection. The nature of the protective response made by the host usually depends on the characteristics of the infectious agent. In general, resistance to organisms that reproduce within host cells (e.g., *Listeria*, *Mycobacterium*) is mediated by T cells, and resistance to extracellular organisms (e.g., *Staphylococcus* and *Streptococcus*) is mediated by antibody. Destruction and clearance of parasitic protozoa and helminths may depend on both cell mediated and humoral immunity, as well as the activity of granulocytes and macrophages, depending on the parasite and the complexity of the parasite life cycle.

The genotype, and to an extent, the phenotype of the host influences the ability to mount an effective defense against infection. This has been shown repeatedly in rodent models infected with certain bacteria and protozoan or metazoan parasites. Host gender may also influence the outcome of infections, with females generally more resistant to infection than males (Reddington *et al.,* 1981; Alexander and Stimson, 1988). The gender of the host is also known to influence resistance to the parasitic nematode *Trichinella spiralis*, with females generally reported to be more resistant to infection. For example, gender-specific differences in resistance to infection have been linked to levels of sex hormones; castration and/or administration of the opposite gender hormones can switch the gender-related resistance profile (Mankau and Hamilton, 1972).

In my laboratory, we have been evaluating resistance to infection with Ts in both mice and rats exposed to various xenobiotics. As a host resistance model, Ts infection has a number of advantages: the immune response to infection is well characterized (Wakelin, 1993); resistance to infection is susceptible to alteration by a variety of environmental chemicals, including benzo-*a*-pyrene (Dean *et al.,* 1982), diethylstilbestrol (Luebke *et al.,* 1984) and tributyltin oxide (Vos *et al.,* 1984); the infection is not lethal; and, there is no transmission between laboratory animals under normal husbandry conditions. Natural infections are initiated by consuming meat containing viable encysted *T. spiralis* larvae. The larvae hatch in the acid/pepsin environment of the stomach, migrate to the small intestine, and reach sexual maturity within 72 - 96 h of infection. After mating, female parasites give birth to living larvae that migrate to and encyst in striated muscle (Brown, 1965). The protective immune response to the parasite is life cycle stage-specific; elimination of adult worms

during a primary infection is mediated by T cells (Ruitenberg *et al.*, 1977a,b), without any apparent contribution made by antibodies (Almond and Parkhouse, 1987); the number of live larvae produced by female parasites is influenced by a T cell-dependent antibody response (Love *et al.*, 1976); and finally, destruction of newborn larvae migrating to host tissues is believed to be mediated by antibodies, macrophages and/or monocytes plus granulocytes (Wakelin, 1993). With few exceptions, delayed expulsion of adult parasites from the intestine increases the total number of migrating larvae; decreased host control of parasite fecundity likewise increases the number of migrating larvae. Neither condition is favorable for the host since the migrating larvae are the most deleterious phase of the life cycle. Although larvae only survive in striated muscle, some enter the brain and heart. The ensuing inflammatory response in these critical tissues can, in heavy infections, cause the death of the host (Brown, 1965).

These studies were undertaken to determine the effects of a single dose of TCDD on host resistance to infection with *T. spiralis* in adult animals. The experiments were conducted in both male and female mice and rats, to determine whether the higher constitutive resistance of females protected them from the immunosuppressive effects of TCDD and whether species differences existed in the response to infection following TCDD exposure. The same TCDD exposure/infection paradigm which was reported by House et al. (1990) to suppress host resistance to influenza virus challenge (a single intraperitoneal injection 7 days before infection) was used in the present set of experiments.

METHODS

Animals

B6C3F1 mice (C57BL/6 X C3H F1, Charles River, Portage, MI), F344 rats (Charles River, Raleigh, NC) and male F344 rats castrated at 5 weeks of age (Charles River) were housed under standard conditions in an AALAS-approved animal facility. Quarterly monitoring of sentinel animals kept in the same room for antibodies to selected viruses, plus internal and external parasites, were negative. Animals had free access to food and water, unless noted otherwise and were 8 - 10 weeks old at the time of dosing. All treatments and manipulations of animals were approved by the Institutional Animal Care and Use Committee before experiments were started.

Chemical Exposure

TCDD (purity 98% by gas chromatograph-mass spectroscopy analysis, Radian Corp., Austin, TX) was dissolved in acetone and added to corn oil (Sigma Chemical Co., St. Louis, MO). The acetone was removed under vacuum and stock solutions of TCDD were diluted with corn oil to prepare dosing solutions. Animals were dosed by intraperitoneal injection; mice received 100 µL of dosing solution/10 g of body weight and rats were given 5 µL of dosing solution/g of body weight. The dose levels for both mice and rats were 0 (corn oil) 1, 10 or 30 µg TCDD/kg of body weight.

Infection with *T. spiralis*

One week after TCDD exposure, animals were infected with 200 (mice) or 1000 (rats) L_1 larvae obtained by 1% pepsin/1% HCl digestion of muscle from skinned, eviscerated donors of the same

species, infected for at least 6 weeks. Larvae were suspended in 2% gelatin in nutrient broth (Difco, Detroit, MI); animals were infected by oral gavage with 200 µL (mice) or 500 µL (rats) of suspended larvae as previously described (Luebke *et al.*, 1992).

Parasite Endpoints

Adult parasites were isolated and counted 7, 9, 11 and 14 days after infection; larvae counts were done on d 28 of infection. Adult parasite fecundity was evaluated on days 5 and 8 in rats and 6 and 9 in mice. All procedures were performed as previously described (Luebke *et al.*, 1992). Results were expressed as the number of adult parasites recovered from the intestine, as larvae per gram of tongue muscle, or larvae released per female parasite/18 hours, respectively.

Lymphocyte Proliferation Assays

Spleens and mesenteric lymph nodes were removed aseptically from animals killed 7, 14 or 28 days post-infection for parasite endpoints. Proliferative responses of 2 X 10^5 viable splenocytes or mesenteric lymph node cells (MLNC) were induced in microcultures with a crude extract of Ts muscle larvae (TsE) as previously described (Luebke *et al.*, 1992). Additional cultures were stimulated with Con A (Sigma Chemical Co., St. Louis, MO.) or lipopolysaccharide (LPS, Difco, Detroit, MI) for mouse cells and *S. thyphimurium* mitogen (STM, Ribi ImmunoChem Research, Hamilton, MT) for rat cells as positive proliferation controls (Luebke *et al.*, 1992). Results for spleen and MLNC were standardized to percent of control to facilitate the visual comparison of data.

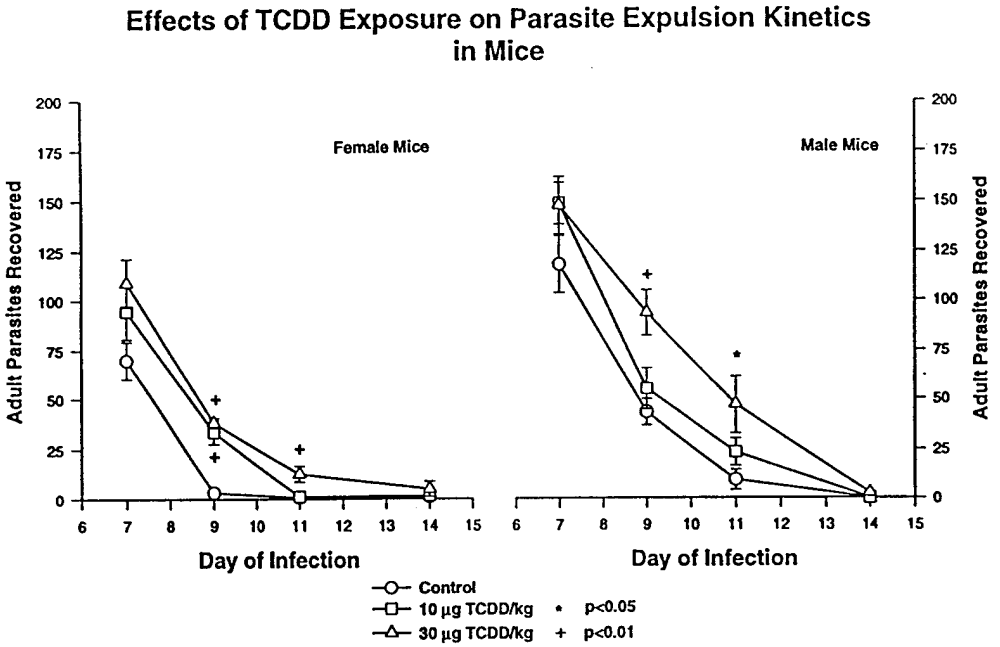
Statistical Analysis

Mean values for parasite endpoints and proliferative responses in intact animals were analyzed for significant differences between control and treated groups by Dunnett's *t* test. The effect of castration on resistance to Ts in male rats was analyzed by a two-way analysis of variance with post hoc analysis by bootstrap resampling.

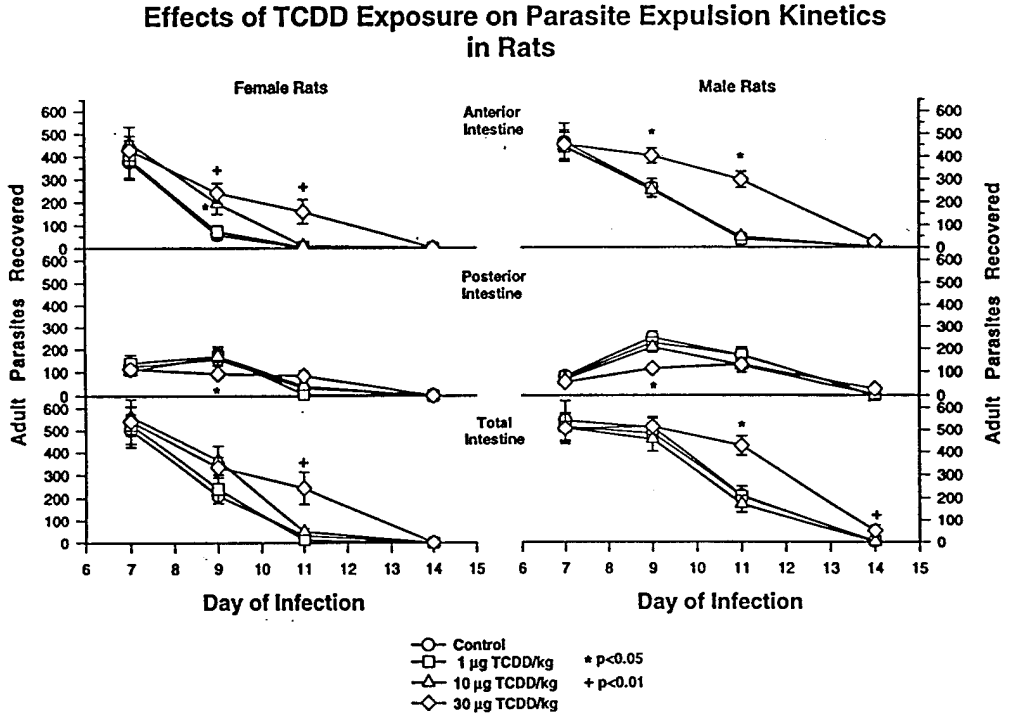
RESULTS

A single injection of 10 or 30 µg TCDD/kg one week before infection slowed the process of adult parasite elimination in mice (Figure 1) and rats (Figure 2). Female control mice and rats expelled a greater proportion of the adult parasite load between days 7 and 9 of infection than did male animals. Both sham operated and castrated male rats that were dosed with TCDD had significantly higher intestinal worm counts 11 days after infection than did sham and castrated corn oil-exposed males (Figure 3). At the 30 µg TCDD/kg dose level, body burdens of encysted larvae were greater in female mice (Figure 4), showed a trend toward an increase in male mice (Figure 4), were not affected in female rats (Figure 5) and were increased in male rats (Figure 5). Exposure of sham operated and castrated male rats to TCDD produced similar burdens of encysted larvae, which were significantly greater than burdens of vehicle-exposed animals (Figure 6). Results of parasite fecundity assays are shown for mice and rats in Figure 7 and 8, respectively. Production of newborn larvae was increased 6 days after infection in female mice exposed to 30 µg TCDD/kg and in female

1



2



Figures 1-2. Effects of TCDD exposure on parasite expulsion kinetics. Mice (Figure 1) were infected with 200, and rats (Figure 2) were infected with 1000 *Trichinella spiralis* (Ts) larvae 7 days after a single i.p. injection of TCDD. Animals were killed at the indicated days post-infection and adult parasites remaining in the small intestine were counted.

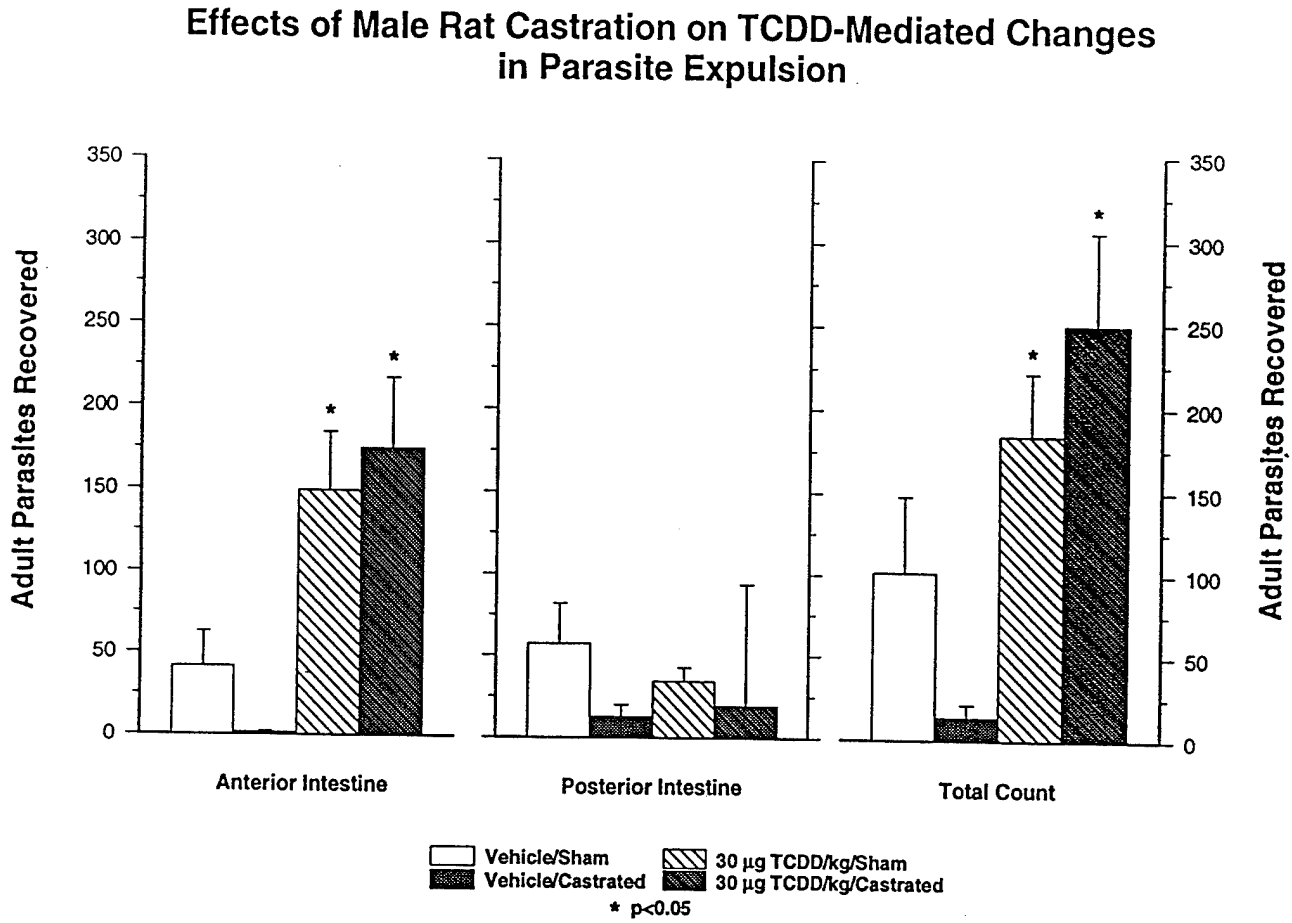
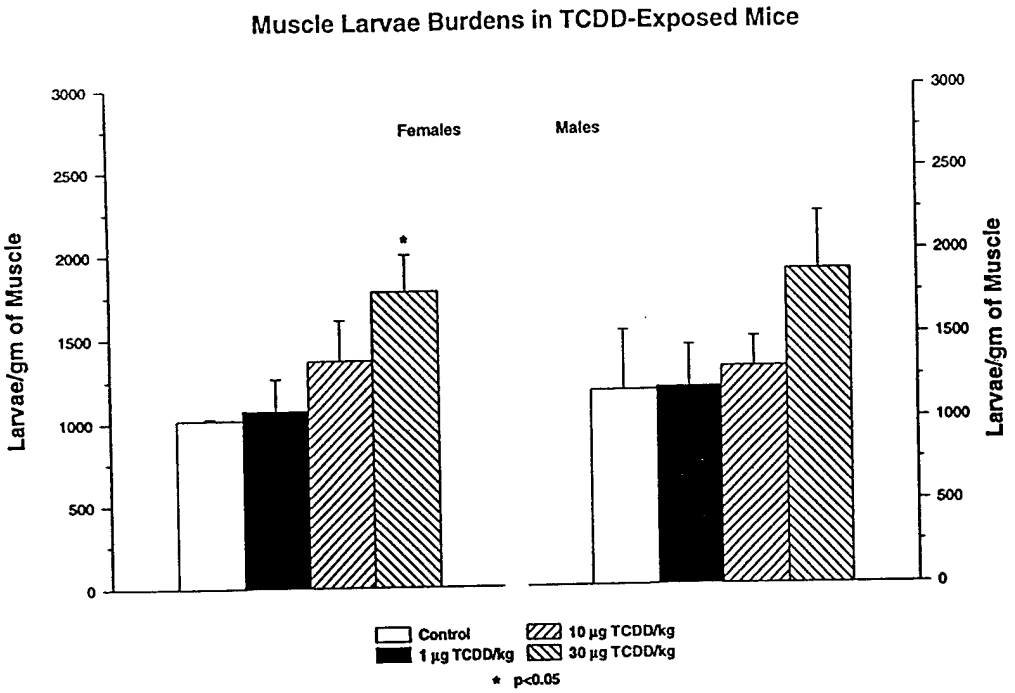


Figure 3. Effects of castration on TCDD-mediated changes in parasite expulsion. Male rats were castrated at 5 weeks of age, 5 weeks prior to a single i.p. injection of TCDD. Seven days after exposure, rats were infected with 1000 Ts larvae. Castrated and sham operated animals were killed on d 11 of infection for adult parasite counts.

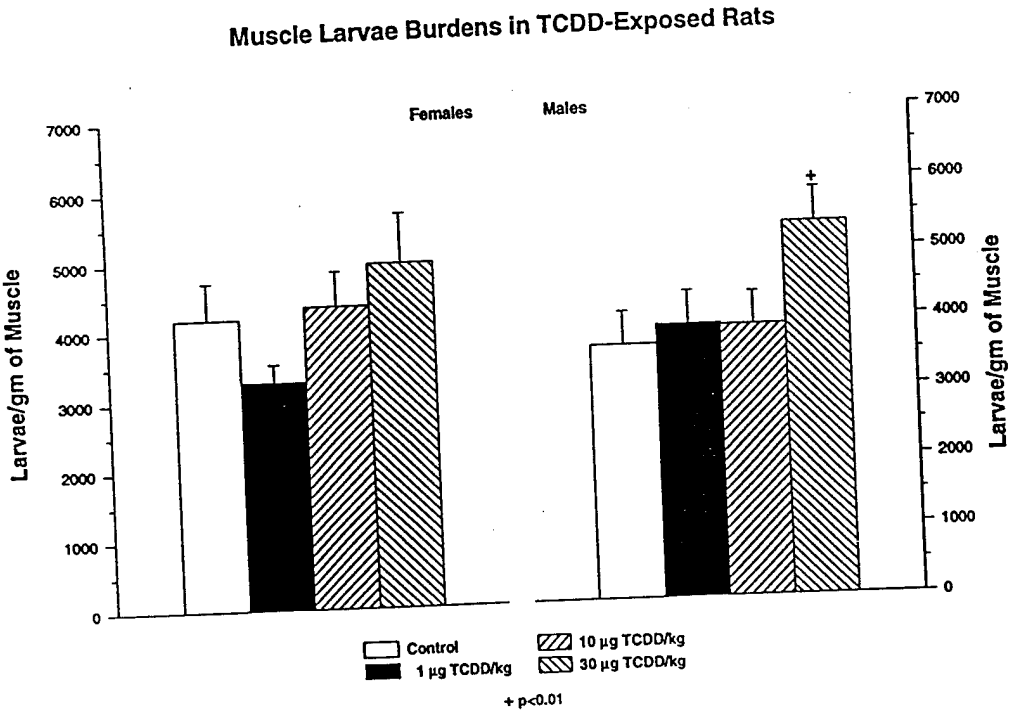
rats 9 days after infection which had been given 10 or 30 µg TCDD/kg; fecundity was not affected in other groups of animals, regardless of gender or exposure level.

Proliferative responses of spleen cells and MLNC are shown in Figure 9 - 12 for female and male mice and for female and male rats, respectively. In mice, the LP response to parasite antigen was suppressed in both the spleen and MLNC 7 and 14 d after infection whereas the nonspecific response to mitogens was generally unaffected. However, in rats, proliferative responses to TsE and mitogens were enhanced in the spleens of animals exposed to 30 µg TCDD/kg. Slight but significant enhancement of Con A stimulated responses were also observed in the MLN of female rats.

4



5



Figures 4-5. Muscle larvae burdens in TCDD-exposed animals. Mice (Figure 4) were infected with 200, and rats (Figure 5) were infected with 1000 *Trichinella spiralis* (Ts) larvae 7 days after a single i.p. injection of TCDD. Animals were killed 28 days post-infection and the numbers of larvae encysted/g of tongue muscle were determined.

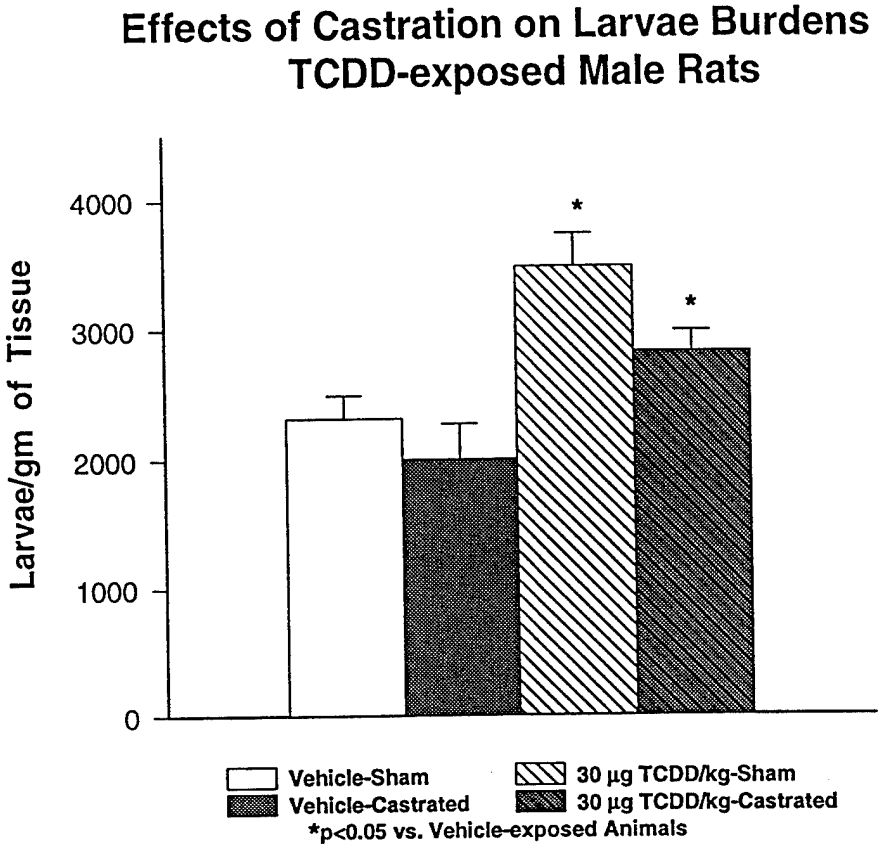
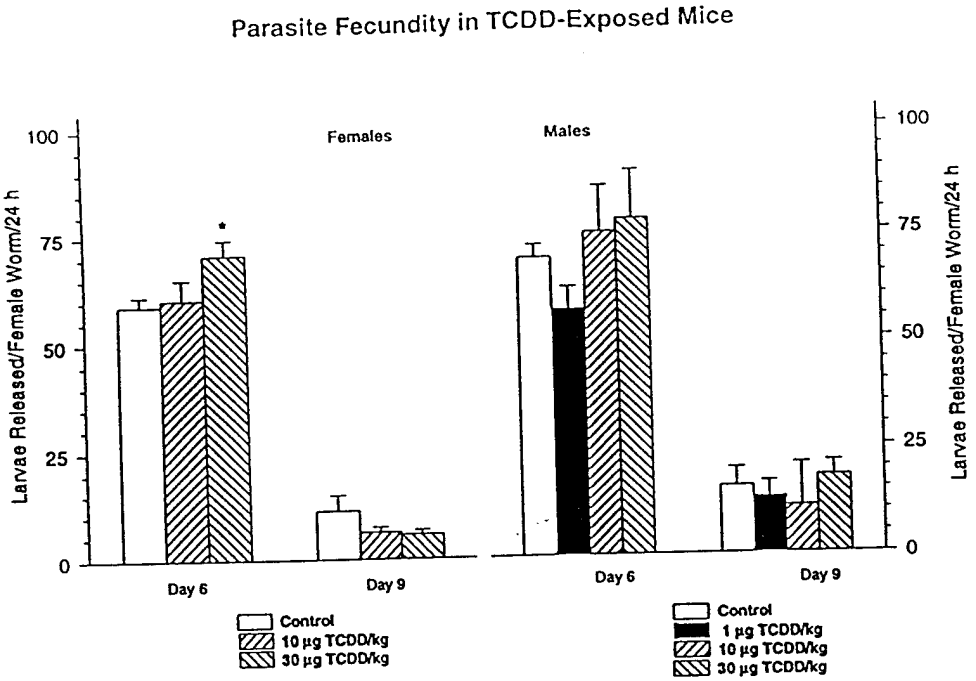
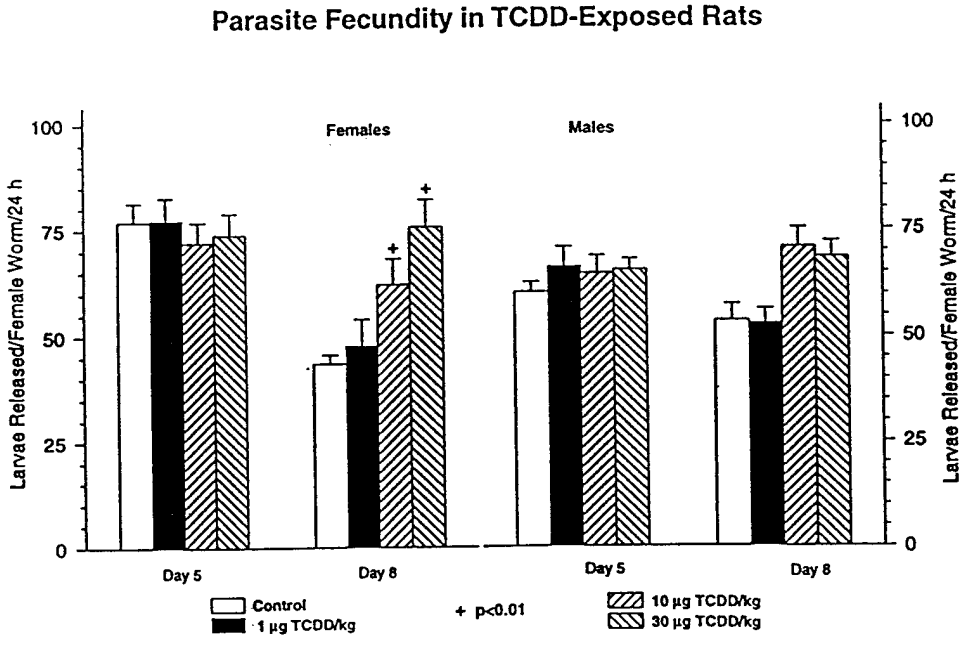


Figure 6. Effects of castration on larvae burdens in TCDD-exposed male rats. Male rats were castrated at 5 weeks of age, 5 weeks prior to a single i.p. injection of TCDD. Castrated and sham operated animals were killed 28 days post-infection and the numbers of larvae encysted/g of tongue muscle were determined.

7

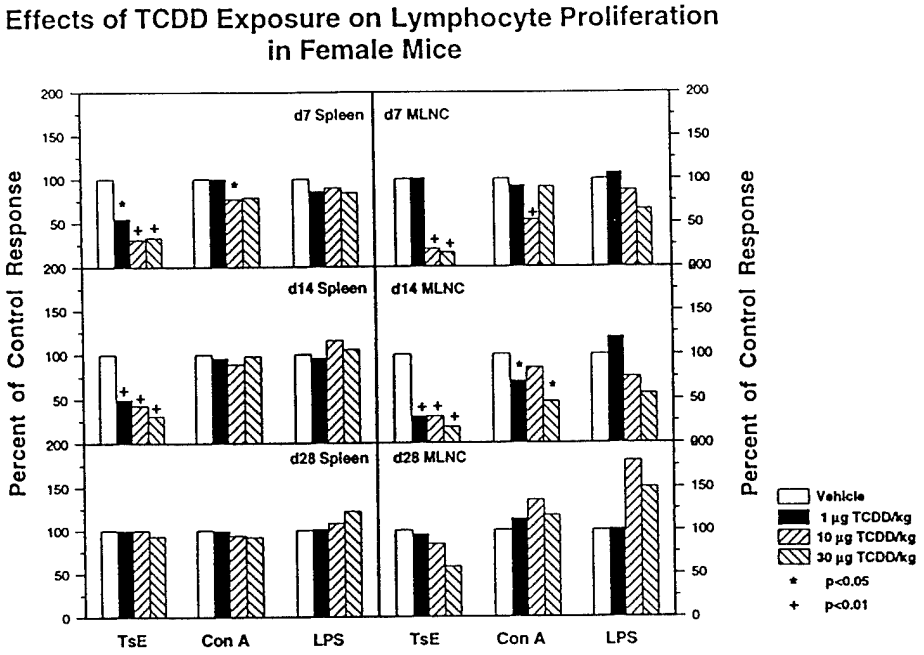


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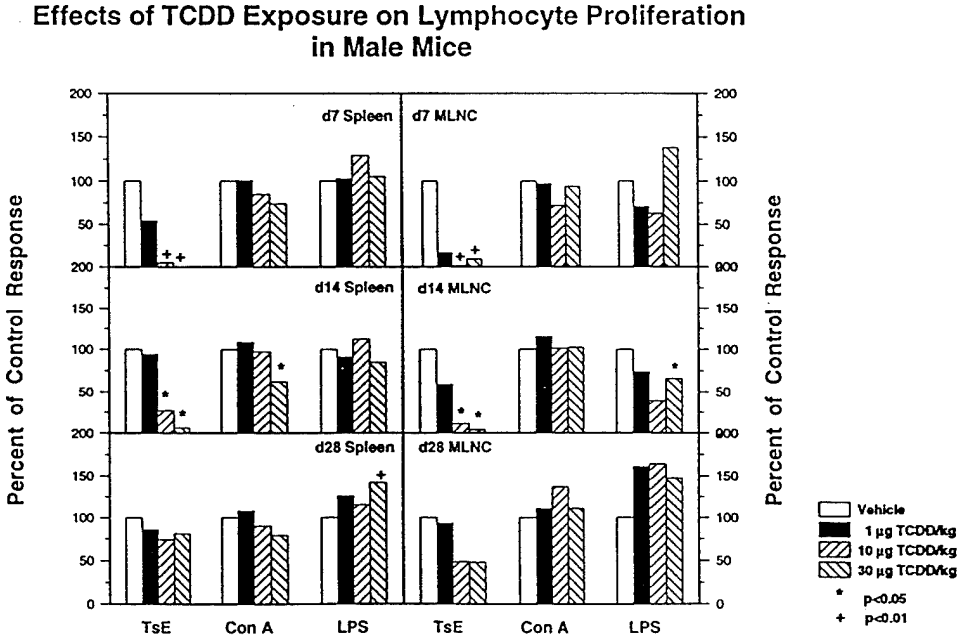


Figures 7-8. Mice and rats were exposed to TCDD and infected as described in the legend for Figure 1-2. At the indicated times after infection, female parasites were isolated from the intestine and the number live larvae released/female was determined.

9

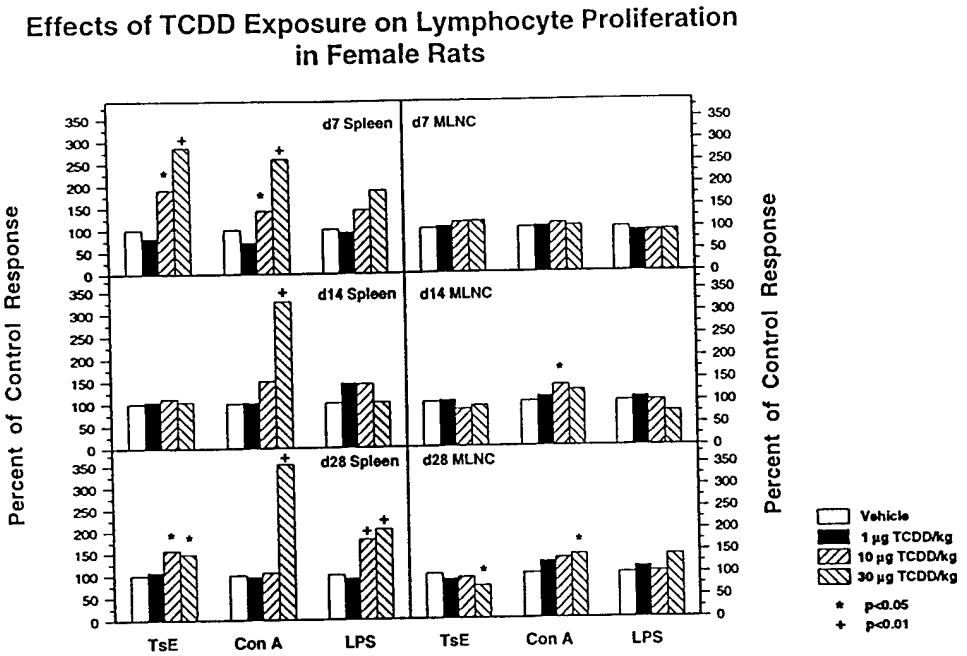


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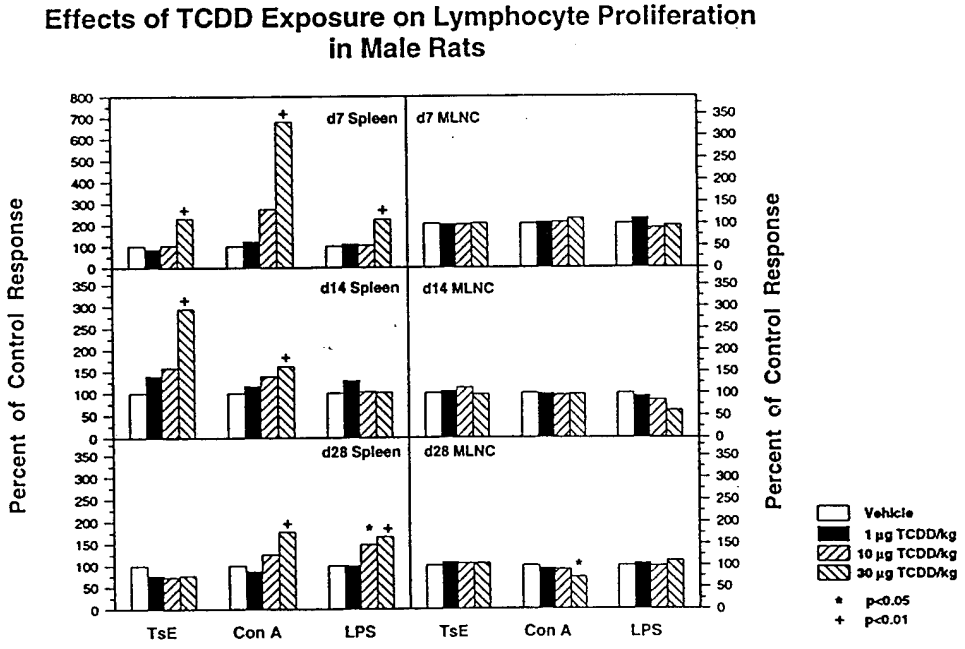


Figures 9-10. Splenocytes and mesenteric lymph node cells were isolated from the animals referred to in Figures 1-2 (day 7 and 14) and Figures 4-5 (day 28) and placed in culture for 72 hr with parasite antigen (TsE) and the indicated nonspecific mitogens.

11



12



Figures 11-12. Splenocytes and mesenteric lymph node cells were isolated from the animals referred to in Figures 1-2 (day 7 and 14) and Figures 4-5 (day 28) and placed in culture for 72 hr with parasite antigen (TsE) and the indicated nonspecific mitogens.

DISCUSSION

Our goal in these studies was to determine the extent to which the gender and species of the host influences the outcome of a host resistance assay in animals exposed to a known immunosuppressive xenobiotic, TCDD. To date, most host resistance experiments toxicologists have been conducted in a single gender of a single species, with little or no consideration given to constitutive levels of resistance to infection when interpreting results. To determine just how much variability one can attribute to the strain and gender of host, we compared the responses of male and female F344 rats and B6C3F1 mice, the strains used by the National Toxicology Program in the United States.

Exposure to TCDD slowed the expulsion of adult parasites from the small intestine of rats and mice of both genders, but did not have a marked effect on when the remaining adult worms were eliminated from the intestine. Nevertheless, in all but the female rats, elevated burdens of muscle larvae were found in animals exposed to 30 μg TCDD/kg, indicating that the cumulative effects of TCDD exposure on resistance to infection lead to a greater number of migrating larvae in the exposed animals. This was most likely the result of longer residence time of adult parasites in the intestine of exposed animals, and is clearly an adverse outcome, as migrating larvae cause the most damage to the host (Brown, 1965). These results also strongly suggest a defect in T cell mediated responses, since in both rats (Vos *et al.,* 1983) and mice (Ruitenberg *et al.,* 1977a,b), expelling the adult parasites of a primary Ts infection is strictly dependent on T cell function. While rats had enhanced splenic proliferative responses to both parasite antigen and nonspecific T and B cell mitogens, proliferative responses of MLNC were essentially unaffected, suggesting that the apparent enhancement was not a direct effect of TCDD exposure on lymphocytes. The splenic cellular changes which led to enhanced responses remains to be determined.

Gender influences the pattern of resistance to Ts infection in both rats (Mankau and Hamilton, 1972) and mice (Reddington *et al.,* 1981), due at least in part to the T cell-dependent nature of *T. spiralis* expulsion from the intestine, and the sensitivity of T cell function to male hormones (Ansar Ahmed *et al.,* 1985). Castration of male rats prior to TCDD exposure and infection with Ts did not mitigate the effect of TCDD on resistance to infection, although castrated males were more resistant to infection than sham operated males. There was no significant interaction between castration and exposure to TCDD. This suggests that the influence of androgens on resistance to Ts infection is not coupled to the resistance mechanisms that are down-regulated following TCDD exposure. Alternatively, the antiandrogenic effects of TCDD, caused by inhibition of free cholesterol mobilization (Moore *et al.,* 1991), which could potentially increase resistance to Ts infection, may have been overpowered by the immunosuppressive effects of TCDD exposure.

TCDD is reported to have antiestrogenic activity via antagonism of estrogen receptor expression (see DeVito *et al.,* 1992); thus, females exposed to TCDD could have a more male-like pattern of decreased resistance to Ts infection. If the rate of parasite expulsion in TCDD-exposed females is compared with that of control males, one sees similar kinetics of expulsion, suggesting that chemical exposure did indeed produce a male-like pattern of resistance. Interestingly, 30 μg TCDD/kg exposure of female mice and rats, but not male mice and rats, increased female parasite fecundity. At this point, it is not clear why this occurred, although females make a more robust antibody

response than do males (Ansar Ahmed *et al.*; 1985) and host control of parasite fecundity is believed to be mediated by antibodies (Love *et al.*, 1976). Studies are in progress to determine the relationship between TCDD exposure, antiestrogenic activity, and increased parasite fecundity.

Measuring parasite endpoints in the Ts model provides specific information on the overall status of the host. However, like many resistance assays, the Ts model is labor intensive, and, as a result, expensive. The parasite antigen-specific proliferative response of spleen and mesenteric lymph node cells was evaluated as a substitute for counting parasites. The lymphocytes isolated from Ts-infected mice which undergo blastogenesis when cultured with Ts antigen are T cells, specifically those of the $L3T4^+$, $Lyt2^-$ phenotype (i.e., $CD4^+$, $CD8^-$) (Grencis *et al.*, 1985). These cells, when injected into a naive recipient, will confer immunity to reinfection (Wakelin *et al.*, 1982; Grencis *et al.*, 1985). In this study, TsE-driven blastogenesis was suppressed at both 7 and 14 d post-infection in male and female mice, reflecting reduced resistance to infection. In fact, the proliferative response was even more sensitive to TCDD-mediated suppression, with suppression observed at 1 μg TCDD/kg, putting this response in the sensitivity range of the antibody response to sheep erythrocytes. In marked contrast, the responses of spleen cells, but not MLNCs, to Ts antigen were elevated in male and female rats, as were the responses to T and B cell mitogens. These data suggest the elimination of a suppressor cell population in the spleen that is not present in the MLN. In support of this possibility, Smialowicz *et al.* (1994) reported an increase in the number of antibody producing cells in the spleens of female F344 rats given a single injection of TCDD 7 days before immunization with sheep erythrocytes, as well as a decrease in the proportion of splenic $CD8^+$ cells. The differences observed in TCDD effects on antigen driven responses between mice and rats highlight the need to measure actual resistance endpoints, at least until a large enough multi-species data base exits to allow more certainty in the extrapolation of immune function endpoints as predictor of significant changes in resistance to infection.

In these studies, we found that there were differences in the host response to infection that could be attributed to the gender and the species of the host. Others have shown that host genotype may have a marked effect on the response to Ts infection. Particularly in mice, the rate of parasite expulsion is under the control of genes both within and outside the major histocompatibility locus (Wakelin, 1992). Additionally, the effects of sex steroids on immune function are also under the influence of genes within the major histocompatibility complex (Ansar Ahmed *et al.*, 1987). Taken together, these results suggest that it may be inappropriate to draw conclusions about the effect of environmental chemicals on resistance to infection based on the results obtained in a single species/gender model.

Disclaimer

This report has been reviewed by the Environmental Protection Agency's Office of Research and Development, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Chapter 41

Laboratory Rodents as Models For Human Immune Function in Immunotoxicity Testing

Robert V. House

Life Sciences Department, IIT Research Institute
10 West 35th Street
Chicago, IL 60616

INTRODUCTION: RODENTS AS A MODEL SPECIES

From its inception, the practice of immunotoxicology has utilized rodents -primarily mice - as a model species. Rodents are a logical choice as laboratory animals for a number of important reasons. First and most importantly is the high degree of conservation in the vertebrate immune system. This results in a close similarity between rodent and human immune system structure and function, allowing for reasonable extrapolations of experimental results to the human condition. In general, primary differences between rodents and humans occurs in cytokine production profiles, immunoglobulin switching and subclasses, and mononuclear cell surface markers. A particularly useful development in rodent immunology has been the description of immunological mutants (Table 1) and models for autoimmunity using mice and rats. In some cases the use of these variant models may allow a closer approximation to human disease than would be possible using "healthy" animals.

Table 1
Immunological mutations in rodents (adapted from Schultz *et al.*, 1991)

Designation	Nature of Defect	Phenotype
Nude (nu) (rats and mice)	Thymic stroma lack class II MHC antigens	Athymic; lack functional T-cells; enhanced NK function
Beige (bg)	Altered lysosomal biogenesis	Decreased NK/CTL function
X-linked immunodeficiency (xid)	Lack subset of functional B-cells	Abnormal B-cell responses
Severe combined immunodeficient (scid)	Defective recombinase for heavy chain genes	Severe immune defect; lack functional T- and B-cells

A second advantage of rodent models involves ethical considerations regarding animal experimentation. Studies utilizing rodents are, at present, more socially acceptable than similar studies using nonhuman primates or companion animals. Another important consideration is the relatively short life span of rodents. This short life span provides great flexibility in designing long-term studies including prenatal exposure, recovery from treatment effects, etc. Finally, rodents are inexpensive to obtain and maintain, effectively increasing the number of studies which may be performed.

Given the advantages listed above, there are several other important considerations to be made when selecting rodents as a model species for immunotoxicology studies. For example, relative differences in pharmacokinetics/pharmacodynamics must be considered, as well as the equivalence of dose and route of administration. In addition, the selection of immune function assays must be carefully considered. Our laboratory and others spent a considerable amount of time and effort developing a panel of immune function and host resistance assays for rodent immune system assessment. This work has been recently summarized (Luster *et al.*, 1994). Table 2 lists the assays included in the most recent version of this immune testing approach. In general, animals are exposed to test materials; the exact protocol, including route of administration, duration of exposure and doses examined, will vary depending on the test material. Following exposure, animals are sacrificed and immune function is assessed using *in vitro* assays. Alternatively, control or test material exposed animals will be exposed to microorganisms or transplantable tumor cells to evaluate potential changes in host resistance. To date, a number of compounds have been examined in this system. Results obtained in these assays indicate that alterations in rodent immune function correlate well with similar observations in humans (Table 3).

Table 2
Assays for measuring immune function (adapted from Luster *et al.*, 1994)

Basic/Screen/Tier I	Advanced/Comprehensive/Tier II
Hematology (CBC and differential)	Natural killer (NK) cell assay
Body and lymphoid organ weights	IgG AFC
Spleen and bone marrow cellularity	Cytotoxic T-lymphocyte (CTL) response or Delayed-type hypersensitivity (DTH)
Lymphoid organ histology	Mixed leukocyte response (MRL)
IgM antibody-forming cell (AFC) assay	Host resistance assays
Surface marker analysis	RES clearance

Table 3
Immune abnormalities caused by various agents tested in animals and humans (adapted from Luster *et al.*, 1989)

Compound tested	Animals	Humans
TCDD	+	±
PCB	+	+
PBB	+	+
LEAD	+	-
CADMIUM	+	-
BENZENE	+	+
TOLUENE	+	+
OZONE	+	+
ASBESTOS	+	+

ASSAYS FOR MEASURING HUMAN IMMUNE FUNCTION

In vitro assays for measuring human immune function are similar to those employed in rodent models, with several important exceptions. First, the range of sources of immune system cells are more restricted in humans than in rodents. For example, any and all rodent tissues are made available at sacrifice; human tissue, on the other hand, is normally limited to peripheral blood mononuclear cells (PBMC), although thymus, bone marrow or lymph node tissue may occasionally be available. Likewise, the amount of available tissue is limited in humans. Another important consideration is the degree of variability associated with human immune responses. Inbred rodents are routinely employed in immunotoxicology studies, minimizing any genetically related variations in immune responsiveness. In addition, laboratory rodents are pathogen-free and are maintained under rigidly defined environmental conditions, thus holding interanimal variability relatively constant. This situation is not true for humans, whose individual backgrounds and lifestyles may introduce pronounced differences in immune function. As more investigators publish "standard" reference ranges for various human immune parameters, this uncertainty should diminish. The following paragraphs described some commonly used human immune function assays.

Immunopathology

Whereas many of the measures of immune system pathology routinely examined in rodents are not generally practical in human studies, a wealth of valuable information may be gained from routine hematology (e.g., CBC, differentials) as well as from cell surface marker analysis by flow cytometry. Although cell surface marker analysis has facilitated enormous advances in immunology, there are a number of potential problems in interpreting surface marker analysis (Westermann and Pabst, 1990). Some of these potential problems include:

- alterations in blood lymphocyte profiles may not reflect organ pathology
- only a small number of total body lymphocytes are found in blood at any given time
- functional alterations may be difficult to infer from available data

B-lymphocyte function

There are at least two main approaches for assessing B-lymphocyte function. The first approach is to measure the proliferative capacity of the cells following activation (i.e., the mitogenic response). PBMC are exposed *in vitro* to either mitogens such as bacterial lipopolysaccharide (a nonspecific activator) or to a specific recall antigen. The degree of proliferative response is subsequently measured. A more advanced approach may be to measure B-cell size by flow cytometry. Although these methods may provide an indication of B-lymphocyte responsiveness, they do not necessarily measure immune function. A more specific approach is to assess antibody production, which is the primary function of B-cells. Perhaps one of the best indicators is the production of specific antibody following challenge with antigen. Other options include the quantitation of IgG subclasses, as well as polyclonal antibody production following stimulation with mitogens or other cellular activators.

T-lymphocyte function

In vitro assays are available to determine both effector and helper functions served by T-cells, as well as to determine the overall functional competence of these cells. Functional competence is generally measured by T-cell proliferative response to either recall antigens or nonspecific activators such as mitogens or anti-CD3 monoclonal antibody. Helper cell function may be assessed by measuring cytokine production. A reasonable choice for measurement is interleukin-2 (IL-2), a cytokine involved in many immune states. Cytokine production may be measured in cultures of isolated cells (either resting or following activation), or in body fluids such as serum. Effector T-cell function may be assessed by examining the action of either cytolytic cells (CTL), or by measuring delayed-type hypersensitivity (DTH). CTL function may be either antigen-specific (relatively difficult in humans), or by "redirected" cytolysis. Finally, DTH may be measured by response to exposure to common antigens including *Candida albicans*, tetanus toxoid, or tuberculin PPD.

Natural immunity

As with rodents, natural immunity in humans is routinely evaluated by measuring NK cell function. Human NK cell activity is measured by a standard 4-hr chromium release assay, with the exception that the human K562 tumor cell line is used as a target. K562 cells are sensitive to lysis by NK cells; however, if a negative control is desired, the investigator may also evaluate lysis of DAUDI cells, an NK-insensitive tumor cell line. A related function is known as lymphokine-activated killer (LAK) cell activity. LAK cells appear to be related (if not identical) to NK cells; however, LAK activity is induced by a more prolonged stimulation by cytokines such as IL-2. Although the role LAK cells play *in vivo* has not yet been completely defined, they do exhibit a pronounced cytolytic effect on tumor cells *in vitro*.

Macrophage Function

Due to the diversity of functions ascribed to the macrophage (covering both acquired immunity as well as nonspecific host resistance), a wide variety of *in vitro* assays are available for assessing the functional status of this cell. These include cytokine production, phagocytic function, chemotaxis, and biochemical reactions, among others.

FUTURE DIRECTIONS

As with all scientific disciplines, the growth of immunotoxicology depends on the continued development, validation and implementation of new experimental techniques. Following are examples of experimental or nonvalidated techniques for immune function assessment. Some of these techniques have already been utilized for immunotoxicity testing, whereas others are still developmental.

Immunopathology

As previously described, surface marker analysis will be an increasingly vital tool for immune function assessment in the future. Other important tools will include evaluation of genetic regulation and dysregulation of immune activation and function, as well as the role of apoptosis in immune function.

Humoral immunity

The anti-SRBC AFC assay is a well validated, predictive model for measuring immune function. However, the assay is highly labor-intensive, requires skilled technical assistance, and does not allow for long-term functional assessment since it is a terminal assay. An alternative is the anti-SRBC ELISA described by Temple *et al.* (1993). The microplate ELISA format of this assay simplifies the assay, and the use of serum (rather than splenocytes) allows for options such as recovery studies. The assay has not yet been completely validated, and a widespread adoption of

this technique will require the development of standardized reagents, particularly anti-SRBC antibodies.

Cell-mediated immunity

The methodologies previously described for assessing cell-mediated immune function (e.g., CTL) have been validated for effector function. However, the regulatory functions served by CD4+ T-cells are crucial for normal immune function. One approach for assessing T-cell regulatory function is the quantitation of cytokine production following cellular activation. Although approaches for including cytokine measurement in immunotoxicology studies have been described (House, 1995) the full potential of this technology has not yet been realized. Another technique for measuring cell-mediated immunity is the murine local lymph node assay (MLLNA) originally described by Kimber and Weisenberger (1989). This assay may be employed in at least two ways. The first is its intended use, which is as an alternative model for determining the contact sensitizing potential of test materials. Another use is as an alternative to the delayed-type hypersensitivity model originally included in tier-type testing. Both applications should receive greater attention in the coming years.

Host resistance assays

A number of well described bacterial (e.g., *Listeria monocytogenes*, *Streptococcus sp.*) and viral (e.g., Influenza) host resistance models have been employed in immunotoxicology studies with good results. Increasingly, however, it is recognized that infections with protozoan (e.g., *Toxoplasma gondii*) and metazoan (e.g. nematode) parasites are important pathogens as well. Unfortunately, to date most host resistance models utilizing parasites have been notorious for their labor-intensive nature; this often precludes their use in routine screening-type studies. Another host resistance model which may be applicable to immunotoxicology studies is the retroviral infection commonly referred to as murine AIDS (MAIDS). This condition follows infection with the LP-BM5 mixture of retroviruses, and is characterized by splenic hypertrophy and immunosuppression. MAIDS is not completely analogous to human AIDS (Cunningham *et al.*, 1994), but it may represent a useful model nonetheless. More study is required before the true utility of this model is known.

SUMMARY

Rodents provide a practical model for assessment of human immune function in the context of immunotoxicology assessment. The immune system of rodents is analogous to that of humans in most respects; in addition, immunological variants (including immunodeficient and autoimmune) are available to more closely approximate human immune function. Similar *in vitro*/ex *vivo* assays are available for both rodents and humans, allowing confirmation or follow-up studies to be performed. Finally, new technologies under development are expected to further enhance our understanding of both normal and dysfunctional immune system status.

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Chapter 42

Differential Modulation of Earthworm Cellular and Humoral Immune-Related Functions by Polychlorinated Biphenyls (PCB)

Philippe Roch[1], Patrice Ville[2], Edwin L. Cooper[3] and Jean-François Narbonne[1]

[1] Défense et Résistance chez les Invertébrés Marins (DRIM)
Université de Montpellier 2, cc 80,
34095 Montpellier Cédex 05, France.

[2] Laboratoire de Toxicologie Alimentaire
Université de Bordeaux 1, Avenue des Facultés
33405 Talence Cédex, France.

[3] Laboratory of Comparative Immunology
Department of Neurobiology
University of California
10833 Le Conte Avenue
Los Angeles, Ca 90024-1763, USA.

ABSTRACT

The possible site of action of PCB in three invertebrate lumbricid species, *Eisenia fetida andrei, Eisenia hortensis* and *Lumbricus terrestris* were studied to better understand the relationship between this compound and molecular and cellular immune responses. PCB did not decrease the *in vivo* sensitivity to pathogenic bacteria nor the humoral antibacterial growth capacity as measured *in vitro*. As a consequence, this chemical pollutant did not exert any negative effects on antibacterial coelomic fluid proteins nor on the chloragogue cells involved in their synthesis. Cell-free related activities, such as lysozyme and protease, were also not inhibited by PCB exposure of the earthworms. In contrast, phagocytosis and wound healing were dramatically inhibited. This is probably the consequence of cell surface modifications and alterations in cell physiological capacities. We postulated that PCB inhibits those macrophage functions which interact with other cells leading to the suppression of cell-to-cell co-operation.

INTRODUCTION

The immune defense system of earthworms consists of two major components, humoral and cellular (Cooper 1974, Vetvicka *et al.* 1994). Lysozyme and multifunctional proteins released by chloragocytes and leukocytes are also part of the humoral system (Roch 1979, Kauchke and Mohrig 1987, Lassalle *et al.* 1988). Cellular defense mainly involves the activity of free coelomic cells, i.e., macrophages, leukocytes and chloragocytes, according to the terminology of Cooper *et al.* (1982).

The present study was undertaken to:

- Investigate the effects of PCB on molecular activity and/or cellular functions of earthworm immune defense
- To detect interrelationships between humoral and cellular immune compartments in an invertebrate
- To collect information on the toxicity of micro-pollutants that can act directly on animals that live in the soil.

It constitutes a synopsis of some of ours previously published results: Roch and Cooper, (1991) Ecotoxicol. Environ. Safety 3:283-290; Cooper and Roch, (1992) J. Invertebr. Pathol. 60:59-63; Ville et al. (1995) J. Invertebr. Pathol. 65:217-224.

EARTHWORMS AND PCB EXPOSURE

Eisenia fetida andrei, *Eisenia hortensis* and *Lumbricus terrestris*, belong to the Lumbricidae family, Annelida, Oligochaeta. They were maintained at 22°C (both *Eisenia*) or 15°C (*L. terrestris*) in the lab and regularly fed prior to experiment. Coelomic fluids were collected by puncturing individual *L. terrestris* with sterile Pasteur pipettes. Both *Eisenia* were excited with a 5 volts stimulation (Roch 1979). Coelomic cell sub-populations were isolated by differential centrifugation (Roch 1977). Cell lysates refer to supernatants of pure cell preparations previously sonicated at 4°C.

Polychlorinated biphenyl Aroclor 1254 (PCB) was used as xenobiotic in a contact assay. Earthworms were exposed individually for 5 days in Petri dishes containing moistened filter paper that received a defined concentration of PCB according to the protocol of Roberts and Dorough (1984). Controls consisted of unexposed worms starved for 5 days on filter paper. PCB doses are expresses as µg PCB/cm^2 of filter paper.

Antibacterial activity

Aeromonas hydrophila at 2x10^2 in 100 µL liquid nutrient broth (LNB) were incubated with 100 µL of sterile earthworm coelomic fluid for 30 min at 20°C. Serial dilutions were plated between two layers of nutritive agar and emerging colonies counted after 24 hr at 30°C. The percentage of inhibition of bacterial growth was calculated as differences between numbers of colonies in controls

(bacteria grown in LNB) and in experimentals (bacteria grown in LNB supplemented with coelomic fluid).

In *E.f. andrei,* the presence of 1 μg/cm^2 of PCB did not significantly change the anti-*A. hydrophila* activity. In contrast, in *L. terrestris*, 10 μg/cm^2 of PCB almost completely suppressed the activity. Immunization of both *E.f. andrei* and *L. terrestris* resulted in increased LD_{50} values, especially in *E.f. andrei* (more than 3.5 times). Immunization of the PCB-exposed worms also resulted in increased LD_{50}. Meanwhile, these values were lower than those obtained after immunizing unexposed earthworms.

Healing defects

Wound healing assays were performed by cutting longitudinally 3 mm in the dorsal body wall of *E.f. andrei* and looking for the presence of healing at 24 hr post operation (Cooper and Roch, 1984). All of the naïve *E.f. andrei* were able to heal wounds in less than a day. Unexposed *E.f. andrei* had healing defects calculated to be 7%, probably due to starvation. However, 1 μg/cm^2 of PCB increased the defect level to 17 % and to 36 % with 20 μg/cm^2.

Allograft rejections

Allografts were performed on anesthesized *L. terrestris* by transplanting 4 mm² of body-wall 10 segments posterior to the clitellum and allowed to heal without suturing (Cooper and Roch, 1984). Assessment of graft rejection was done by scoring the worms daily for 25 days post-operation using a previously defined, gradual scale (Cooper and Roch, 1986). The percentage of *L. terrestris* exhibiting gross features of allograft rejection increased more rapidly in PCB exposed worms than in unexposed ones. Maximum percentage appeared at 8 and 16 days post-grafting respectively, but the percentages were not significantly different.

Phagocytosis

Coelomocytes of individual earthworms were collected in the presence of 5 mM EDTA and chloragocytes were eliminated by centrifugation. Leukocytes and macrophages were supplemented with $CaCl_2$ and incubated for 30 min at 20°C on glass slides. Leukocytes were eliminated by gentle washing as non-adherent cells. Phagocytosis of 5x10^6 heat-killed yeasts (at least two yeasts per cell)

TABLE 1
Percentages of macrophages containing two or more yeasts per cell. Star indicates percentages statistically different from unexposed ones ($p < 0.0001$).

	E.f. andrei	*E. hortensis*	*L. terrestris*
Unexposed	55.2 ± 2.3	63.8 ± 0.9	79.5 ± 1.4
PCB exposed	14.8 0± 1.1*	11.6 ± 1.6*	28.8 ± 1.8*

was carried out using monolayers of 10^5 adherent macrophages during 45 min at 20°C (Toupin *et al.* 1977).

In unexposed earthworms, the percentages of macrophages able to phagocytose yeasts ranged from 55 % for *E.f. andrei* to 63 % for *E. hortensis* and 79 % for *L. terrestris* (Table 1). Collected from PCBs-exposed worms, the percentages of phagocytes were as low as 11% (*E. hortensis*), 14 % (*E.f. andrei*) and 28 % (*L. terrestris*). Moreover, macrophages did not spread onto glass as avidly as those derived from naïve or unexposed worms. Instead, they appeared more spherical with less pseudopodia which were short, blunt and more like filopodia.

Lysozyme

Lysozyme activity was measured in agarose containing 5 mg (for 100 mL) of *Micrococcus luteus (M. luteus)* as diameter of cell wall lysis around wells filled with 50 µL of leukocyte lysate after 4 hr incubation at 37°C. All three earthworm species possessed natural lysozyme activity, with the highest recorded in *E. hortensis*. In both *Eisenia,* 1 µg/cm^2 of PCBs was sufficient to produce significantly higher activities. Increasing the concentrations of PCBs to 5 and 10 µg/cm^2 regularly caused increased lysozyme activity. In *L. terrestris,* 10 µg/cm^2 of PCBs resulted in increased lysozyme activity. After being injected with *A. hydrophila, E.f. andrei,* previously exposed to a concentration of 1 µg/cm^2, showed significantly higher lysozyme activity than in non-injected worms. Maximum activity was obtained 4 hr after injection then decreased rapidly to reach the background level after 5 to 6 hr, a kinetics that resembled those of unexposed earthworms.

Cytolytic activity

Hemolysis was evaluated by mixing 100 µL of different dilutions of *E.f. andrei* and *E. hortensis* chloragocyte lysates with 100 µL of a 2 % suspension of sheep red blood cells (SRBC). The reaction mixture was incubated for 60 min at 37°C and the hemoglobin content of the supernatants evaluated spectrophotometrically at 541 nm (Roch *et al.*, 1989). Exposure to 5 µg/cm^2 of PCBs, significantly increased the hemolytic activities of both *Eisenia*, reaching 43 % for *E.f. andrei* and 85 % for *E. hortensis*.

Protease activity

Solutions of 1% gelatin in 1.5 % agarose were prepared in 0.2 M TRIS pH 8 (Maskel and Di Capua's, 1988). Ten mL were poured into a 9 cm diameter Petri dish. Wells of 6 mm were cut in the solid agarose and filled with 30 µL of chloragocyte lysate. After incubation for 4 hr at 37°C, undigested gelatin was precipitated with acidified $HgCl_2$, and diameters of clear circles around all wells were measured. Protease activity, probably of serine-protease nature, was detected in leukocyte lysates of *E. hortensis*. Exposure to 1 µg/cm^2 did not significantly modify this natural activity. In contrast, 5 µg/cm^2 resulted in stimulated activity that increased gradually, at least until 30 µg/cm^2

DISCUSSION

With respect to the cellular system, earthworms that have been exposed to PCB are less reactive than non-exposed earthworms (Goven *et al.* 1988, Rodriguez-Grau *et al.* 1989, Fitzpatrick, *et al.* 1992). For example, PCB reduced the ability to form secretory rosettes in *L. terrestris*, a response that overlaps both the humoral and cellular systems. To better understand the relationship between molecular and cellular immune responses and to determine the possible site of action of PCB, we have tested several immune-related functional assays and compared the responses in three earthworm species: *E.f. andrei, E. hortensis* and *L. terrestris*.

A. hydrophila is pathogenic for *E.f. andrei* and stimulates antibacterial activity of the coelomic fluid (Lassègues *et al.* 1981; 1989). PCB did not modify the immunization capability. It is also possible to immunize *L. terrestris,* but PCB dramatically decreased the antibacterial activity in both naïve and immunized worms. We interpret such difference as the consequence of different elimination mechanisms of bacteria according to their pathogenicity: pathogenic bacteria are first attacked by humoral components (Valembois *et al.* 1985), but non-pathogenic species are slowly aggregated (Stein *et al.* 1986) then eliminated, mainly by phagocytosis.

TABLE 2
Interpretative effects of PCB on earthworm immuno defense according to several functional tests (modified from Ville *et al.* 1995).

Cellular Immunity	PCB effect	Active Cells
Wound healing	Inhibition	Macrophages
Phagocytosis	Inhibition	Macrophages
Graft rejection	Stimulation	Lymphocytes
Humoral immunity	**PCB effect**	**Origin of activity**
Pathogenic bacteria	Stimulation	Mainly chloragocytes
Cytolysis	Stimulation	Chloragocytes
Lysozyme	Stimulation	Leukocytes
Proteolysis	Stimulation	Chloragocytes

PCB dramatically decreased the percentage of macrophages capable of phagocytosis and affected cell membranes, since the shapes of macrophages and leukocytes were altered considerably (Table 2). This is probably the consequence of cell surface modifications and alterations in physiological capacity. Wound healing was also reported as inhibited by PCB in *E.f. andrei* as it was in *L. terrestris* (Cooper and Roch 1992). In fact, the higher the concentrations of PCB, the more serious were the healing defects. We postulated that PCB inhibits those macrophage functions which interact with other cells to affect wound-healing (Cooper and Roch 1984) and phagocytosis (Goven *et al.* 1988).

In addition, allografts performed on PCB exposed *L. terrestris* confirmed our previous observations obtained by simultaneously exposing earthworms to several antigens (Cooper and Roch 1984). At that time, it was hypothesized that at least two subpopulations of leukocytes are engaged in recognition processes, whereas macrophages are acting in early wound healing and, at later stages, in tissue damage cleaning. Increased healing defects oposed to accelerated allograft rejection are consistent with negative effect of PCB only on macrophages.

Interestingly, in all three earthworm species, exposure to PCB resulted in higher levels of lysozyme activity. Since lysozyme is involved in innate immuno defense responses and PCB alters certain basic immunological functions, we suggest that elevated lysozyme could represent the host's attempt to compensate for a decrease in other defense mechanisms.

PCB did not suppress hemolytic activity, a function mediated by chloragocytes, but stimulated it in both *Eisenia.* In connection with hemolytic activity, PCB also stimulated protease activity of chloragogue cells from *E. hortensis*. We presumed that protease activity is a component of the same system that regulates the cytolysins of *E.f. andrei* (Roch *et al.* 1991). The increased protease activity induced by PCB could be, at least in part, responsible for the increased hemolytic activity.

In conclusion, modulations of cellular and molecular-related immuno defense activities in earthworms by PCB include: 1) a strong inhibitory effect on macrophage-mediated functions as reflected by wound healing and phagocytosis, 2) no negative effect on antibacterial coelomic fluid proteins nor on the chloragogue cells involved in their synthesis and, 3) stimulation of cell-free-related activities, such as lysozyme and protease.

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Chapter 43

Effects of Methyl Parathion on Cellular Immune Responses of Sea Bass (*Lates calcarifer*)

Nantarika Chansue and Aranya Ponpornpisit

Veterinary Medical Aquatic Animal Research Center
Faculty of Veterinary Science
Chulalongkorn University
Henri Dunant Rd.
Pratumwan, Bangkok 10330, Thailand

ABSTRACT

There has been a great increase in sea bass (*Lates calcarifer*) culture in Southeast Asia for commercial purposes. During the growout period, there has been serious damages due to diseases caused by many kinds of pathogens. From regular surveys of water and sediment qualities, we discovered contamination of pesticides in many areas of Thailand, especially, methyl parathion. Therefore, the study of effects of methyl parathion on sea bass cellular immune responses was carried out in 150 gram fish. Lethal concentration of 50% at 96 hours was found to be 0.85 mg/L. Chemotaxis and phagocytic activities were measured by using phagocytes isolated from anterior kidney. Macrophage of sea bass was found between 1.050-1.060 g/dL band by discontinuous density gradient centrifugation. Fish were exposed to 0.6, 1.2, 1.8, and 2.4 mg/L methyl parathion in static renewal system. The results showed significant reduction of chemotaxis of all experimental groups, while phagocytic activity reduced significantly at the concentration above 0.6 mg/L when compared to the control groups. Clinical signs of methyl parathion exposed fish showed dramatic reduction of motion and response to external stimulation. Fish were weak, lay laterally on the bottom with spastic movement and widely open operculum prior to death. Histopathological study of gill showed fused secondary lamellae, epithelial cell hyperplasia, mucous cell hypertrophy and generalized inflammation of gill filament. Liver cells were necrotic and numerous vacuoles were noted in the cytoplasm of hepatocyte.

INTRODUCTION

Methyl parathion has been one of the most widely used organophosphate pesticide in the world. The contamination of this substance in water was detected in many parts of Thailand's aquatic system (Tangtrongpiros *et al.*, 1995). Sea Bass (*Lates calcarifer*) culture, among other aquaculture species, also was affected by the contamination. In heavily methyl parathion contaminated area, fish died with obvious clinical signs of pesticide intoxication. But in lightly contaminated areas,

most mortality was caused by several pathogens. The objective of this study is to evaluate the effects of methyl parathion on Sea Bass immune responses which may be one of the main factors that increase the susceptibility of contaminated fish to infectious diseases. The results of this experiment can also be used in biomarker studies in the future.

MATERIALS AND METHODS

One hundred and fifty Sea bass (*Lates calcarifer*) with an average weight of 150 grams and an average length of 10 cm. was divided into 15 fifty liter tanks. Water in the tanks measured 5 ppt. salinity, pH 7.8, and a temperature of 28-30^{o}C.

Methyl parathion (O, O-Dimethyl, O-P-Nitrophenyl phosphorothiate) technical grade (92.4%) (Bayer, Co., Ltd.) was used to expose the fish in the laboratory in a static renewal system. The exposure concentrations were 0, 0.6, 1.2, 1.8 and 2.4 mg/L. After 96 hours, fish were sacrificed and the macrophages isolated from the kidney; while histopathological studies were performed on the gill and liver. All experiments were done in triplicate and the LC_{50} was evaluated in a separate experimental system.

Isolation of macrophages from the anterior kidney was performed by teasing the tissue with a glass rod over a stainless steel mesh in a petridish. After washing the cells 3 times with PBS, the cells are separated on a density gradient (Percoll, Pharmacia) and then centrifuged at 3000 rpm for 20 min.. Macrophages were recovered between 1.050-1.060 g/dL band. The cells are recovered using a pasture pipette, washed twice and the concentration of cells adjusted to 10^7 cells per mL to be used in the chemotaxis and phagocytosis assays. Cell viability was over 90% as measured by trypan blue exclusion method (Hudson and Hay, 1989). All experiments was performed on ice.

The chemotaxis assay was performed according to Boyden's double chamber method (Weeks *et al.*, 1986). *Escherichia coli* opsonized with sea bass serum was used as the antigen in the lower chamber. Sea bass macrophages were added into the upper chamber after placing the polycarbonated membrane filter (0.5 µm pore sized; Nucleopore corp.) on top of the lower chamber and then incubated in a humidified chamber for 20 min. Following incubation, the membrane was air-dried, fixed with methanol, and stained with eosin-methylene blue (Arnaparn Co, Ltd.). The filters were mounted on microscope slides and examined using light microscopy in order to count the number of cells on the upper and lower surfaces of the filter. Percent chemotaxis was derived from the number of migrated cells divided by the total number of cells times 100.

The phagocytosis assay was performed according to the slide method of Bodhipaksha (1993). Opsonized *E. coli* was used as the antigen. After incubation of the antigen and the macrophage for 20 min. in a humidified chamber, the slides were stained with eosin-methylene blue and examined under light microscope. The percent phagocytosis was calculated from the number of phagocytic macrophage divided by the total number of cells in the field times 100.

Statistical analysis was performed using one way analysis of variance and Duncan's new multiple range test in both chemotaxis and phagocytosis assays.

RESULTS

From the study of lethal concentration 50, the concentration of methyl parathion used to kill 50% of the fish was 0.85 mg/L after 96 hours. Exposed fish showed signs of distress and change in behavior. They were lethargic and over-sensitive to external stimulation. At higher concentrations, dead fish showed extension of fins and a widely open operculum.

Chemotaxis assay

Percent chemotaxis of the control group was 32.65% which was significantly higher than the treatment groups ($P<0.0001$). However, average percent chemotaxis of methyl parathion exposed groups decreased with the increase of methyl parathion concentration (Figure 1). There was no significantly difference in average percent chemotaxis among the treatment groups ($P<0.01$) as demonstrated in Table 1.

Table 1
Average percent chemotaxis of macrophage of fish exposed to methyl parathion

Methyl parathion Concentration (mg/L.)	Average Percent Chemotaxis (%) ± SD
0	32.65^{a} ± 35.71
0.6	9.14^{b} ±5.71
1.2	5.07^{b} ±5.79
1.8	5.46^{b} ± 3.76
2.4	5.21^{b} ± 4.12

a and b indicate the stastistically significant difference of each value ($p<0.01$).

Phagocytosis assay

Fish exposed to methyl parathion at and above 1.2 mg/L showed significantly lower phagocytic activity than the control groups (Figure 1). But there was no significant difference between the control group and shrimp exposed to 0.6 mg/L of methyl parathion. The decrease in average percent phagocytosis was significantly different between the exposed concentration of 0.6, 1.2, and 1.8 mg/L ($P<.01$). At the concentration of 2.4 mg/L, the average percent phagocytosis was not significantly different than the 1.8 mg/L methyl parathion exposed group. Average percent phagocytosis at 0, 0.6, 1.2, 1.8 and 2.4 mg/L was 52.25, 46.12, 33.35, 24.97 and 20.12 percent respectively (Table 2).

Table 2.
Average percent phagocytosis of macrophage of fish exposed to methyl parathion

Methyl parathion concentration (mg/L)	Average phagocytosis (%) ± SD
0	$52.25^{a} \pm 11.14$
0.6	$46.12^{a} \pm 13.65$
1.2	$33.35^{b} \pm 9.80$
1.8	$24.97^{c} \pm 10.25$
2.4	$20.12^{c} \pm 11.37$

a, b, and c are statistically significant differences among groups.

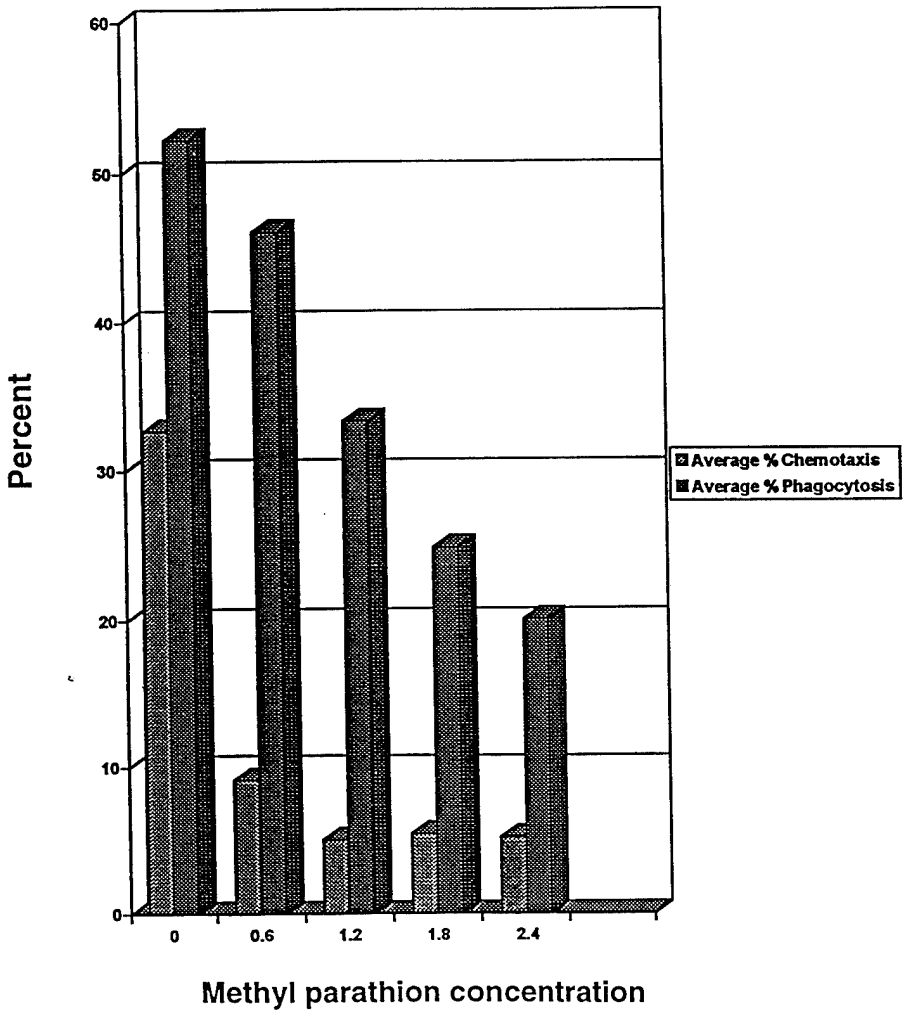


Figure 1. Average percent chemotaxis and phagocytosis of sea bass macrophage exposed to methyl parathion

Histopathological studies

Liver

- No pathological lesion was observable in the liver of fish exposed to 0, 0.6 and 1.2 mg/L of methyl parathion.
- At concentrations of 1.8 and 2.4 mg/L, vacuolation in hepatic cells and pyknotic nuclei were evidenced. Degree of damage increased with increasing methyl parathion concentrations.

Gill

- All exposed fish showed damaged gill lamellae, fusion of secondary lamellae, and swelling and inflammation of gill epithelial cells. Some areas were sloughed off. Hyperplasia and enlarged mucous cells were also noted.
- The degree of damage increased with increasing methyl parathion concentrations.

DISCUSSION

The results of this study indicated that fish exposed *in vivo* to methyl parathion in water had reduced macrophage mediated immune responses when measured by chemotactic and phagocytic activities. The LC_{50} of 0.85 mg/L was correlated with Harnjariyakul (1993). Other reports showed that the sensitivity of fish to methyl parathion varied with the individual species (Srivastava, 1987, Pigmental, 1971). Many factors involved in the severity of the effects include other environmental stresses, fish health and the nutritional state of the fish (Bryant, 1992).

Direct effects of methyl parathion produce over-stimulation of the nervous system by inhibiting the acetylcholinesterase activity (Hassall, 1990). Fish showed signs of stress and alteration of behavior. Spastic movement and over-sensitivity to external stimulation was seen, especially, at the higher methyl parathion concentrations. Since methyl parathion exposed fish showed signs of stress, blood glucocorticoid level was increased. From the increase of glucocorticoid, previous studies showed evidence of monocytopenia, and reduction of elastase, collagenase, non-specific neutral protease, and endogenous pyrogen and prostaglandins of macrophages (Ellis, 1981). The reduction of these enzymes and protein caused by stress may also be a factor of decreased macrophage functions.

Since methyl parathion and its metabolites can alter the factors generated in serum and the composition of enzymes which effect mitochondria and other organelles, cellular mechanisms such as surface antigen recognition would also change, thus, cells may be unavailable for subsequent recognition and, hence, these cells demonstrated reduced chemotactic and phagocytic activities (Chung and Secombes, 1987).

In general, immunological effects of methyl parathion have been studied mainly in higher vertebrates (Descotes, 1988). For example, mice under dietary treatment with this compound had enhanced immune responses to bacterial infections. Bacterial clearance rates were decreased and phagocytic as well as bactericidal activities were reduced. Reduced opsonin levels and a concomitant decrease in IgG_2, which are likely to be involved in decreased opsonization and phagocytic processes have been noted (Street, 1981). Some organophosphorus compounds such as malathion have been shown to suppress the humoral immune response in channel catfish following chronic exposure (Plumb and Areechon, 1990). In carp, *Cyprinus carpio*, leukopenia as well as decreases in phagocytic ability and phagocytic index of neutrophils were observed following intoxication with trichlorfos. (Siwicki *et al.*, 1990)

From histopathological studies, the effects on liver and gill were observed to be a function of the concentration of methyl parathion exposure. Since this experiment was done in only 96 hours, the lesion at lower concentration was not obvious. Harnjariyakul (1993) found that sea bass exposed to 0.5-2.5 mg/L. of methyl parathion showed necrosis of liver and muscle tissue. Gill damage also increased with increasing pesticide concentration. Fusion and erosion of gill lamellae with hypertrophic mucous cells were seen. Similar results were also observed in carp, *Cyprinus carpio*, exposed to lethal and sublethal concentrations of methyl parathion (Ramamurthy, 1987). This results in respiratory distress which is also seen in other fish (Areechon and Plumb, 1990; Walsh and Ribelin, 1975). The activation of mucous cells may be one of the protective mechanism to reduce the absorption of pesticide into the body.

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Chapter 44

Response of Fish Immune Cells to *in vitro* Organotin Exposures

K. O'Halloran, J.T. Ahokas and P.F.A. Wright.

Key Centre for Applied and Nutritional Toxicology
RMIT University
Melbourne, Victoria. 3000 Australia

Organotins are examples of widespread industrial and agricultural compounds which have the potential to persist in the environment. A common source of organotin in the aquatic environment is the leachate from antifouling paints applied to the hulls of boats. Tributyltin (TBT), the common organotin component of antifouling paints, was shown to be highly toxic to sensitive non target species (Thompson *et al.*, 1985), causing adverse effects at part per billion (ppb) levels. Legislation was introduced in the 1980's banning the use of organotin antifouling paint in many countries, however concern over the toxicity of TBT still remains due to its persistent nature and potential for bioaccumulation in aquatic species.

The health and survival of fish in the presence of infectious diseases and tumours is fundamentally dependent on their immune function. The presence of contaminants in aquatic environments has been correlated with increases in the incidence of infectious disease outbreaks and tumours in fish populations (Snieszko, 1974; Malins *et al.*, 1988). Since organotins are known immunosuppresant agents in mammalian species (Snoeij *et al.*, 1987), it is feasible that the presence of these compounds in the aquatic environment may compromise the immune systems of fish and thus enhance their susceptibilty to disease.

This study investigated the *in vitro* effects of TBT and its dealkylated metabolite dibutyltin (DBT) on fish immune function at 2.5, 50 and 500 ppb. Immune cells were isolated from the spleen and head kidney of juvenile rainbow trout (*Oncorhynchus mykiss*). Cells were resuspended in tissue culture media (RPMI plus 10% fetal calf serum) and their capacity to proliferate in the presence of mitogens and express natural killer activity were assessed during exposure to organotins. Differences between dose groups were assessed by analysis of variance at a 5% significance level. A post-hoc Turkey's compromise test was utilized to identify those groups which were significantly different.

T- and B-lymphocyte mitogenesis were quantified by tritiated thymidine incorporation into cells cultured (for 7 days at 15°C) with the mitogens concanavalin A or lipopolysaccharide, respectively. Exposure to the lowest doses of TBT and DBT did not alter lymphoproliferation in spleen and head kidney lymphocytes (Figures 1 and 2). T-lymphocyte mitogenesis (Figure 1) was significantly suppressed by 85% in spleen cells with exposure to 50 ppb DBT. B-lymphocyte mitogenesis (Figure 2) was significantly suppressed by 96% in spleen cells and by 58% in head kidney cells with 50

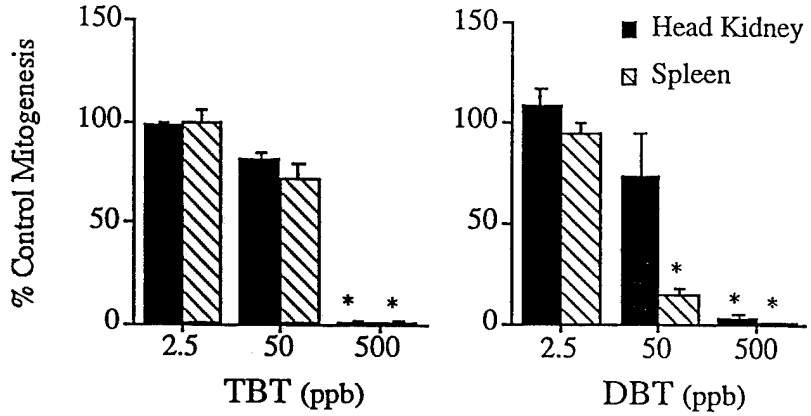


Figure 1. T-cell mitogenesis in head kidney and spleen immune cells exposed to TBT and DBT. Control peak mitogenesis was 9800 ± 2600 cpm for spleen and 8450 ± 1600 cpm for head kidney cells. Basal mitogenesis was 650 ± 100 cpm for spleen and 1200 ± 200 cpm for head kidney cells. Values are mean ± standard error. *Values are significantly different to controls, (p 0.05) for TBT (n = 8 fish) and DBT (n = 4 fish).

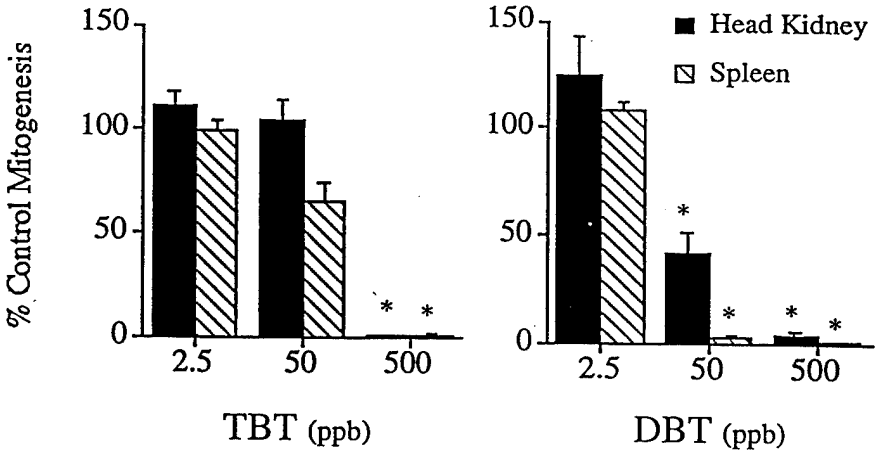


Figure 2. B-cell mitogenesis in head kidney and spleen immune cells exposed to TBT and DBT. Control peak mitogenesis was 2700 ± 550 cpm for spleen and 3350 ± 850 cpm for head kidney cells. Basal mitogenesis was 650 ± 100 cpm for spleen and 1200 ± 200 cpm for head kidney cells. Values are mean ± standard error. *Values are significantly different to controls, (p <0.05) for TBT (n = 8 fish) and DBT (n = 4 fish).

ppb DBT. Exposure to 50 ppb TBT did not cause significant suppression of either T- or B-lymphocyte mitogenesis (Figures 1 and 2). Both TBT and DBT abolished *in vitro* lymphocyte proliferation at 500 ppb (Figures 1 and 2).

Neither TBT or DBT were significantly cytotoxic to the immune cells, as determined by trypan blue exclusion at the conclusion of the functional assay. Natural killer activity was not inhibited by *in vitro* exposure to either compound, as determined by the lysis of chromium-51 labelled K562 human erythroleukemia cells and P815 mouse mastocytoma cells (during an 18 hour incubation at 15°C).

Since TBT levels of up to 1-3 ppb are measured in polluted water bodies and fish have been shown to bioaccumulate TBT by 2-3 orders of magnitude, we believe that the concentrations of organotins used for this *in vitro* experiment can be related to real environmental exposures. In addition, these concentrations compare well with those used to demonstrate TBT-induced effects in fish macrophages (Wishkovsky *et al.,* 1989; Rice and Weeks, 1989).

These results show that organotins have both functional- and tissue-specific effects on the fish immune system, i.e. lymphocytes isolated from splenic tissue appear more sensitive than those isolated from the head kidney, and mitogenesis in B-lymphocytes was suppressed to a larger extent than in T-lymphocytes. In mammals, organotins appear to have a selective action on the thymus, affecting T-cell-mediated immune functions (Snoeij *et al.,* 1987). Conflicting results obtained from *in vivo* organotin exposure studies in two fish species (i.e. guppy and medaka) suggest that the thymotoxicity of butyltins is a species-specific phenomenon (Wester *et al.,* 1990). In our study, B-lymphocyte mitogenesis was more sensitive to the organotins indicating that the selecitve immunotoxicity to T-cell functions demonstrated in mammals may not be the case for aquatic organisms. However, due to the dependent nature of B-lymphocytes on T-helper cells it is difficult to exclude the possibility of a selective action of organotins on a T-cell sub-population.

In mammals, the toxic potential generally decreases with progressive dealkylation from tri- to mono-alkyl substituted organotins. However, dialkyltin compounds appear to be selectively immunotoxic (Snoeij *et al.,*1987) and are thought to be responsible for the immunotoxicity observed in mammals exposed to tri-alkyltins. Previous aquatic studies examining the effects of TBT and DBT did not find DBT to be selectively immunotoxic, as measured by thymus atrophy in guppies and medaka (Wester *et al.,* 1990) and rainbow yolk sac fry (de Vries *et al.,* 1991). Contrary to these findings, our *in vitro* results indicate that DBT is indeed the more potent immunotoxin and suggests a need for reassessing DBT's potential toxicity to aquatic organisms.

Acknowledgments

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Chapter 45

In Vitro Responses of Fish Immune Cells to Three Classes of Pesticides

K. O'Halloran, J.T. Ahokas and P.F.A. Wright

Key Centre for Applied and Nutritional Toxicology
RMIT University
Melbourne, Victoria. 3000, Australia

Heavy rains and flooding after the application of pesticides can result in the contamination of aquatic environments. The presence of contaminants in aquatic environments has been correlated with increases in the incidence of infectious disease outbreaks and tumours in fish populations (Snieszko, 1974; Malins *et al.*, 1988). Therefore the potential for pesticides to adversely affect fish immune systems needs to be determined.

Organochlorine (OC) and organophosphate (OP) pesticides are known mammalian immunotoxins (Dean *et al.*, 1989). Recent studies indicate that these compounds also modulate the immune system of fish. Lindane, an OC pesticide, has been shown to cause a suppression of both humoral and cell mediated responses in rainbow trout (Dunier and Siwiki, 1994; Dunier *et al.*, 1994) whereas carp are relatively insensitive (Cossarini-Dunier *et al.*, 1987; Cossarini-Dunier, 1987). Immunotoxic effects of various OP pesticides have also been demonstrated in fish. Malathion inhibited the humoral responses in channel catfish (Plumb and Areechon, 1990). However another OP, trichlorophon suppressed both non-specific call activities and T-lymphocyte proliferation in carp, but had no effect on humoral responses (Siwicki *et al.*, 1990; Dunier and Siwicki, 1993).

In this study we investigated the *in vitro* effects of three different classes of pesticides on rainbow trout immune function. The OC pesticide endosulfan is widely used in the cotton growing areas of Australia, and although its persistence is low relative to other OC compounds, it can accumulate in the tissue of fish living in enclosed water bodies to part per billion (ppb) levels (Nowak and Julli, 1991). Malathion is one of the most widely used pesticides in North America (Plumb and Areechon, 1990). Esfenvalerate, a synthetic pyrethroid, was also investigated as a representative of a pesticide class which is considered to be relatively low in toxicity and persistence compared to OC and OP pesticides, but which also appears to be highly toxic to several non-target species (Anderson, 1989).

Head kidney and splenic tissue were removed from juvenile rainbow trout. Immune tissues were pressed through nylon mesh and washed with RPMI-1640 media. Head kidney and splenic leukocyte suspensions were obtained by density gradient centrifugation and hypotonic lysis, respectively. The leukocytes were resuspended in tissue culture media (RPMI plus 10% fetal calf serum) and were examined for mitogen-stimulated lymphoproliferation and natural killer cell activity during in vitro exposure to 0, 0.1, 1.0 and 10 ppm of each pesticide. Differences between

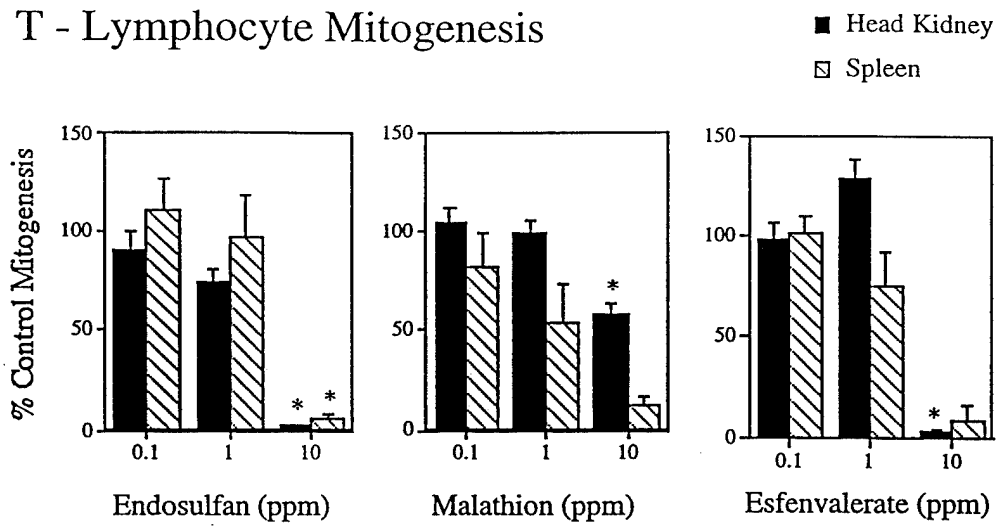


Figure 1. T-lymphocyte mitogenesis in head kidney and spleen immune cells. Control peak mitogenesis was 1600 ± 450 cpm for spleen and 5750 ± 650 cpm for head kidney cells. Basal mitogenesis was 200 ± 50 cpm for spleen and 700 ± 200 cpm for head kidney cells. Values are mean ± standard error. * Values are significantly different from controls ($p < 0.05$, n = 3 - 6 fish).

dose groups were assessed by analysis of variance at a 5% significance level. A post-hoc Turkey's compromise test was utilized to identify those groups which were signifcantly different.

T- and B-lymphocyte mitogenesis was quantified by tritiated thymidine incorporation into cells cultured (for 7 days at 15°C) with the mitogens concanavalin A or lipopolysaccharide, respectively.

Immunotoxic potencies at the highest concentration (10 ppm) were: endosulfan = esfenvalerate> > malathion. At this pesticide concentration, endosulfan and esfenvalerate reduced T-lymphocyte mitogenesis (Figure 1) and B-lymphocyte mitogenesis (Figure 2) to less than 5% of control values. Malathion reduced T-lymphocyte mitogenesis by 42% in head kidney cells (Figure 1) and B-lymphocyte mitogenesis was reduced by 57% and 75% in head kidney and spleen cells, respectively.

At the medium concentration (1 ppm) immunotoxic potencies were: endosulfan ≥ malathion> esfenvalerate. T-lymphocyte mitogenesis was not significantly affected by any of the pesticides at this concentration (Figure 1), however B-lymphocyte mitogenesis (Figure 2) was suppressed by 44% in head kidney cells with endosulfan exposure and by 14% in spleen cells exposed to malathion. Esfenvalerate did not produce any significant effects at this dose. There were no immunotoxic effects resulting from exposure to the lowest pesticide concentration (0.1 ppm).

None of the pesticide exposures were significantly cytotoxic to the immune cells, as determined by trypan blue exclusion at the conclusion of the mitogenesis assay. Natural killer activity was not inhibited by *in vitro* exposure to any of the pesticide compounds, as determined by the lysis of chromium-51 labelled P815 mouse mastocytoma cells. Endosulfan at 10 ppm was directly toxic to the P815 tumor target cells, preventing the accurate determination of natural killer cell activity at this concentration.

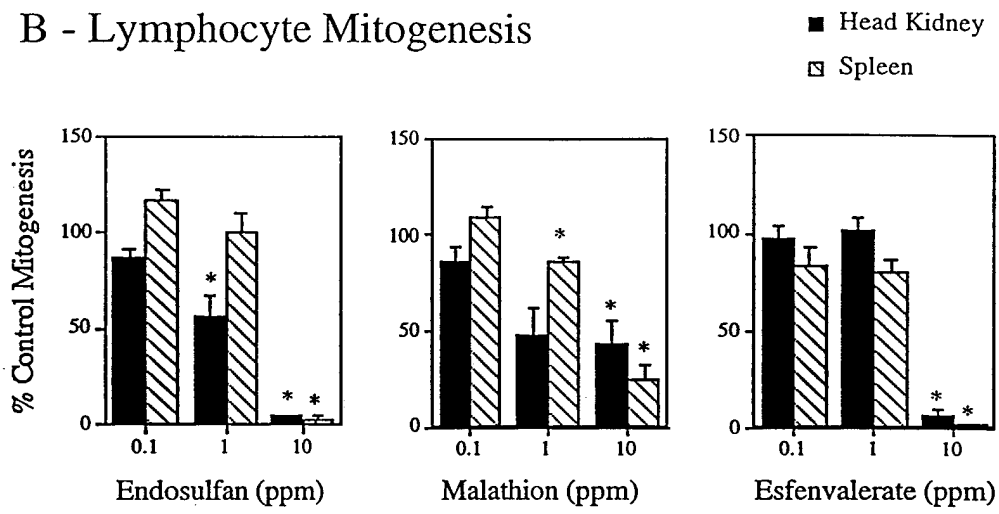


Figure 2. B-lymphocyte mitogenesis in head kidney and spleen immune cells. Control peak mitogenesis was 2200 ± 300 cpm for spleen and 2650 ± 300 cpm for head kidney cells. Basal mitogenesis was 200 ± 50 cpm for spleen and 700 ± 200 cpm for head kidney cells. Values are mean ± standard error. * Values are significantly different from controls ($p < 0.05$, $n = 3 - 6$ fish).

Overall, B-lymphocyte mitogenesis appeared to be more sensitive to pesticide exposure than T-lymphocyte mitogenesis. Neither malathion or esfenvalerate are particularly persistent contaminants in aquatic environments, and are therefore unlikely to be found in fish at concentrations which were shown in this study to significantly suppress lymphocyte mitogenesis *in vitro*. However, it is possible that some aspects of fish immunity may be adversely affected in OC-pesticide contaminated environments, since tissue levels of endosulfan have been reported in Australian freshwater fish (Nowak and Julli, 1991) at levels which suppress lymphoproliferation *in vitro*.

The results of this study show that these three representative pesticides are capable of modulating fish immune responses at relatively high concentrations *in vitro*. However, only endosulfan is likely to be present at immunotoxic levels in pesticide-contaminated environments.

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Chapter 46

Characterization of Macrophages Isolated from Fish Kidney

Suzanne V. Jacobson and Renate Reimschuessel

University of Maryland School of Medicine Department of Pathology
Aquatic Pathobiology Center
Program in Toxicology
10 S. Pine Street Baltimore, MD 21201

ABSTRACT

Fish are becoming important models for higher vertebrates and biomarkers for studying the effects of environmental toxicants on the immune system. Based upon the methods described by other investigators, we compared several methods of macrophage isolation using goldfish (*Carassius auratus*) anterior kidney in order to identify those methods which yield the greatest number of viable macrophages. In addition, we have developed a method which optimizes certain parameters of Hemacolor staining and alpha-naphthyl acetate esterase activity that preserves cell morphology and facilitates the characterization of macrophages.

INTRODUCTION

Teleosts are highly developed aquatic vertebrates, whose immune systems share similar structural and functional characteristics with those of mammals. Due to these similarities and the immunotoxic potential of many waterborne pollutants, fish are used as models for higher vertebrates in immunotoxicological studies (Zelikoff *et al*, 1991) and as biomarkers for immunotoxicology (Wester *et al*, 1994). The immune system of fish, like that of mammals and birds, is comprised of two separate but interacting branches of immunity, specific and non-specific. Specific mechanisms include humoral immunity, cell-mediated immunity (CMI), and memory. Non-specific defense mechanisms are constitutively present and in fish are the more important branch of immunity since they have fewer complex specific immune capabilities compared to higher vertebrates (Anderson and Zeeman, 1995). Non-specific defense mechanisms include physicochemical barriers; soluble messengers (Grondel *et al,* 1985; De Sena and Rio, 1975; De Kinkelin *et al*, 1982); and phagocytic cells such as granulocytes and macrophages. Phagocytes function to recognize, bind, engulf, and destroy potentially harmful particles (Kennedy-Stoskopf, 1993). In addition, phagocytes present ingested material to lymphocytes signaling the specific immune system to participate in the host's defense (Anderson and Zeeman, 1995).

There are, however, some major differences between mammalian and fish lymphoid tissues. In mammals, the primary hematopoeitic tissue includes the bone marrow, spleen, thymus, and lymph

nodes. In contrast, hematopoeitic tissue of teleosts is primarily located in the interstitum of the kidney and stroma of the spleen. To a lesser degree, hematopoeitic tissue is also located in the thymus, intestinal submucosa, and periportal areas of the liver (Ellis *et al.*, 1989). Teleost kidney is morphologically divided into a posterior part (trunk kidney) and an anterior part (head kidney). The posterior kidney is predominantly composed of nephrons. The anterior kidney contains hematopoeitic cells and lymphoid cells in different stages of maturation including undifferentiated stem cells, blast cells, erythrocytes and leukocytes (Amin *et al*, 1992). In addition, the anterior kidney contains cells with endocrine function including interrenal cells and chromaffin cells. At the junction of the anterior and posterior portions of the kidney are Corpuscles of Stannius. These cells are unique to fish and are thought to be involved in the regulation of calcium (Ferguson, 1989). Due to the numerous types of cells in different stages of development that are present in the fish head kidney, it is necessary to optimize methods of macrophage isolation which preserves cell morphology for characterization. We have evaluated different methods of macrophage isolation and techniques for identification including buffer composition, cell substrate, time and temperature for adherence, and conditions for testing alpha-naphthyl acetate esterase activity.

MATERIALS AND METHODS

Experimental animals

Goldfish (*Carassius auratus)* were acquired from commercial sources (Hunting Creek Fisheries, Thurmont MD). Fish were acclimated for a minimum of 4 weeks and maintained at water temperatures 18-20° C (± 2°) with a 16 hr: 8 hr dark photoperiod. Fish were held in 50 gallon glass aquaria. Water quality was monitored on a routine basis for temperature [18-20° C (± 2°)], pH [7.4 (± 0.2)], salinity [<1 g/L], nitrites [0-20 µg/L], and hardness [80-85 mg/L as $CaCO_3$]. Water changes were effected by a flow through system. The goldfish received trout grower diet (5/32" pellets, 38% protein, 8% fat) (Zeigler Bros., Inc., Gardners, PA). Fish were overdosed with MS222 (tricaine methanosulfonate) and sacrificed by severing the spine. The tissues were removed and processed. Any animal which appeared to be moribund during the course of the study was sacrificed as described above.

Slides

Slides for light microscopic evaluation of cell morphology and esterase activity were either (1) uncoated; (2) coated with (a) undiluted fetal bovine serum (FBS) (Sigma), (b) 0.1 mg/mL poly L-lysine (Sigma), or (c) 2.5% silane (Sigma); or (3) positively charged (Erie Scientific, Portsmouth, NH). Adequate volumes of FBS, poly L-lysine, and silane were used to completely cover the slides. Coated slides were kept at 4°C and used within 48 hr of coating. All slides were at room temperature when used.

Cell isolation

The head kidney was removed using sterile forceps and lightly pressed onto slides for tissue impressions. To determine if macrophage yield varied by method of isolation, each head kidney from several fish was divided into three sections and evenly distributed by weight into three different groups. The pooled tissue in each group was then isolated by one of the three following methods: (1) the tissue was homogenized in approximately 3 mL of Hanks Balanced Salt Solution (HBSS) containing 1% penicillin (10,000 U)/streptomycin (10 mg/mL); 5% FBS; and ± 5 mM glucose using 10-15 small clearance strokes in a Dounce homogenizer then layered onto 3 mL Histopaque (d=1.077 g/cm^3) (Sigma) and centrifuged at 400 xg for 20 min at 10-15°C. The layer above the buffy coat was removed and washed with 5 mL HBSS at 200 xg for 10 min at 10-15°C (Dounce homogenization/Histopaque method); (2) the tissue was homogenized as described above then filtered through sterile glass wool in a sterile 5 mL syringe to remove the erythrocytes and centrifuged for 5 min at 200 xg (Dounce homogenization/Glass wool method) (Twerdok, personal communication); or (3) the whole kidney was pushed through a sieve (mesh 784 x 1344 micron) (Rowley, 1990) into a petri dish containing HBSS and separated on Histopaque as described above (Sieve/Histopaque method).

Cell Identification

Cell adherence

Several variables were tested in the adherence process including (1) length of time from cell resuspension to adherence, (2) temperature and length of time of adherence, and (3) length of time from cell resuspension to slide processing for cell identification. A drop of the isolated cell solution was placed onto slides either immediately following resuspension of the pellet (one-step) or after 2 hr during which time the cells were kept at ambient temperature or 4°C (two-step). Cells adhered onto slides in a humidified chamber at either ambient temperature or 37°C. The length of time from initially placing the cell isolate onto slides to processing the adhered cells was also evaluated. For the one-step method cells either (1) adhered 1 hr then were processed immediately; (2) adhered 3 hr then were processed; or for the two-step method cells (3) adhered 1 hr after the 2hr delay and then were processed. The first method in which the slides with adhered cells were processed 1 hr following resuspension is referred to as the "immediate method". The second and third methods in which the slides with adhered cells were processed 3 hr following resuspension is referred to as the "delayed method".

Processing the slides

Adhered cells were either stained with Hemacolor (EM Diagnostics Systems, Gibbstown, NJ) or tested for alpha-naphthyl acetate esterase activity (Sigma). Modifications to the manufacturer's protocols for esterase testing include (1) incubating the slides at ambient temperature (2) horizontally in 5 mL of esterase solution and (3) counterstaining 2 to 4 s in phosphate buffered thiazine (Hemacolor #3). Tissue impressions incubated in esterase solution were used as the positive control, impressions incubated in esterase solution lacking substrate (Fast Blue) were used as the negative

control. Macrophages were identified on the basis of cell morphology and alpha-naphthyl acetate esterase activity. Cells stained with Hemacolor were identified as macrophages if they were large with an irregular, horse-shoe shape nucleus and pale blue cytoplasm. Cells incubated in esterase solution were considered macrophages if the cytoplasm contained black granulation indicating sites of enzyme activity.

Cell counts

Cells were counted in a hemocytometer by trypan blue exclusion (100 µL cells:500 µL 50% trypan blue in HBSS) for viability. The percentage of macrophages was determined by counting the number of cells that were esterase-positive per 100 cells per 100x field.

Cell morphology

Fixed cells stained with Hemacolor were evaluated by two separate investigators and rated on a scale of 1-4 according to the guidelines below:

Grade	Description
1	Cell borders: well defined, Shape: flattened circular cells with basophilic cytoplasm, excentric nuclei.
2	Cell borders: less well-defined, Shape: less flattened, with a more centrally located nuclei.
3	Cell borders: intact but poorly defined, Shape: rounded, with a condensed darker basophilic cytoplasm, central nuclei.
4	Cell borders: deteriorated, cells lysed.

It should be noted that the degree of flattening and rounding may be proportional to cell attachment to the substrate. Hence, cells that are rounded may appear as lymphocytes in cases of poor attachment.

RESULTS AND DISCUSSION

Cell isolation

There was no significant difference in the morphology of the cells isolated by the following three methods: (1) Dounce homogenization/Histopaque, (2) Dounce homogenization/Glass wool, and (3) Sieve/Histopaque. Initially, the Dounce homogenate cell morphology appeared better than the sieve filtrate, however, after centrifugation there was no difference. We have also isolated cells on Percoll (d=1.05, 1.06, 1.07, 1.08 g/cm^3) but found the method to be more time consuming without any appreciable difference in results compared to Histopaque. On average, macrophages represented 30.5%, 14.5%, and 19.0% of the cells isolated by the Dounce homogenization/ Histopaque, Dounce homogenization/Glass wool, and Sieve/Histopaque methods, respectively (Table 1). Viability was between 90-95% in all methods of isolation.

Table 1.
Percentage of esterase positive cells per 100 cells counted on two different 100x fields.

Method of Macrophage Isolation	Esterase Positive cells /100 cells (1st field)	Esterase Positive cells /100 cells (2nd field)	Percentage of Esterase Positive cells (Average)
Dounce Homogenizer/Histopaque	26	35	30.5%
Dounce Homogenizer/Glasswool filter	12	17	14.5%
Sieve/Histopaque	15	23	19.0%

Cell Morphology

When comparing the cell morphology of the tissue impressions to that of the isolated cells, we found the morphology of the isolated cells to be deteriorated in that the cells appeared shrunken with indistinct borders and increased amounts of cell debris was present. These differences were, however, resolved when all procedures after centrifugation were carried out at ambient temperature (versus 4°C or 37°C) and glucose (5 mM) was added to the buffer (Table 2). The addition of glucose to the buffer also improved adherence and cell spreading and enhanced the esterase activity. Cell morphology further improved when the resuspended cells were kept at room temperature 3 hr ("delayed") instead of 1 hr ("immediate") before Hemacolor staining (Table 2) or testing for esterase activity (Figure 1). There was no significant difference in the cell morphology if the resuspended cells were kept in a polypropylene tube for 2 hr and then adhered onto slides for 1 hr (two-step method) versus adhering on slides in one step for 3 hr (one-step method) (Table 2).



Figure 1. The effect of delaying test for esterase activity on goldfish (*C. auratus*) macrophages. (A) **Immediate:** Resuspended cells kept at room temperature 1 hr (1 hr adherence on slide) then tested for esterase activity. (B) **Delayed:** Resuspended cells kept at room temperature 3 hr (2 hr in polypropylene tube, 1 hr adherence on slide) before testing for esterase activity. All cells resuspended in HBSS containing 5 mM glucose and adhered on FBS-coated slides. Bar=20 microns.

Table 2
Macrophage morphology. Scores represent the average morphology grade based on the evaluation of Hemacolor-stained macrophages by two separate investigators. See Methods section for guideline to scale. All cells isolated by Dounce homogenizer/Histopaque method and adhered onto FBS-coated slides

Variations in Methods	Macrophage Morphology Grade (Scale 1-4)
Ø Glucose	2.50
+ Glucose (5mM)	1.25
Adherence at 37°C	4.00
Adherence at T_A	2.28
"Immediate": Cells placed on slides immediately after resuspension, adhere 1hr, stain	2.75
"Delayed": Resuspended cells kept at room temperature 3 hr before staining	1.25
"One-step": Resuspended cells adhere on slides in one step for 3 hr	1.33
"Two-step": Resuspended cells kept in a polypropylene tube for 2 hr and then adhered on slide for 1 hr	1.43

Adherence and Substrate

Resuspending the pellet in very small quantities of buffer, ≤ 500 µL, improved the number of cells that adhered. However, if cells are isolated by the glass wool method, it may be necessary to dilute the cell suspension with 3 mL HBSS as the isolates appeared very dense on the slides and were difficult to interpret. The substrate the cells were placed onto affected their adherence. Plain non-coated slides resulted in the greatest loss of cells. The poly L-lysine coated slides were useful in improving cell adherence but tended to have greater amounts of debris present. Silane-treated slides and FBS-coated slides provided adequate cell adherence. The positively charged slides, however, yielded the greatest amount of adhered cells. Adherence was better in the one-step procedure in which the cells adhered for 3 hr versus the two-step procedure in which cells adhered for only 1hr. In terms of morphology, the slides coated in FBS or the positively charged slides provided the best Hemacolor staining results. Esterase activity was more easily detected in cells adhered to either FBS-coated slides or positively charges slides.

Alpha-Naphthyl Esterase Activity

The esterase solution must be used within 60 min or reactions become negative or weak. The best results were obtained when the esterase solution was made up after the slides were fixed and the solution was kept at 37°C protected from light until use. The manufacturer's protocol requires the slides to be incubated in esterase solution for 30 min at 37°C. We have altered that protocol for

goldfish macrophages which are normally at temperatures between 18-22°C *in vivo*. The fixed cells were incubated at ambient temperature for 45 min on a moist paper towel in a humidified chamber protected from light. The longer amount of time allowed for slower reaction rates at lower temperatures. The esterase activity is not significantly affected under these conditions, however, the most intense reaction was obtained from cells adhered onto FBS-coated slides by the one-step method and incubated in esterase solution at 37°C for 30 min. Incubation temperature had no effect on intensity of esterase activity in cells adhered to positively charged slides. Morphology was slightly better in cells incubated at ambient temperature. It may be possible to decrease morphology by reducing the incubation time to 30 min as 45 min at ambient temperature to achieve sufficient results. To conserve reagents, the slides may be incubated horizontally in trays covered with 5 mL of esterase solution rather than vertically in coplin jars in 50 mL of esterase solution. The recommended counterstain, 2 min Gills hematoxylin, completely obliterated the black granulation in esterase-positive cells. Decreasing the amount of time to 15 s still masks the black granulation. We found that 2-4 s in phosphate buffered thiazine (Hemacolor #3) gave the best definition of cell morphology without masking the black granulation.

CONCLUSIONS

Based on the findings from this research, we have adopted the following method of cell isolation and identification based on esterase activity. The anterior kidney is disrupted in HBSS buffer supplemented with glucose (5 mM) at 4°C using a glass Dounce homogenizer. The homogenate is layered over a Histopaque density gradient and centrifuged at 400 xg 10-15°C for 20 min, buffy coat is removed and centrifuged at 200 xg 10-15°C for 10 min. The pellet is resuspended in ≤ 500 µL HBSS. A drop of the resuspended cells is placed onto the positively charged slides to adhere in one-step for up to 3 hr at ambient temperature. To test for esterase activity, adhered cells are incubated horizontally at ambient temperature for ≤45 min in 5 mL esterase solution that is prepared after the cells are fixed and protected from light at 37°C. Cells are counterstained for 2-4 s in phosphate buffered thiazine. These methods were developed for use in optimizing the isolation and identification of macrophages from goldfish anterior kidney. They may also prove useful in isolating cells for functional assays, however, the time and temperature of delay may need to be modified to preserve enzyme activity.

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Chapter 47

In Vivo Effects of Cadmium Chloride on the Immune Response and Plasma Cortisol of Rainbow Trout (*Oncorhynchus mykiss*)

Isabelle Voccia[1], Jaime Sanchez-Dardon[1], Muriel Dunier[2], Perry Anderson[3], Michel Fournier[1] and Alice Hontela[1].

[1]TOXEN
Dept. des Sciences Biologiques
Université du Québec à Montréal
C.P.8888, Suc.Centre-Ville
Montréal, Québec, H3C 3P8, Canada.

[2]Lab. de Toxicologie Métabolique et d'Ecotoxicologie
I.N.R.A.-Ecole Nationale Vétérinaire de Lyon B.P.83
69280 Marcy l'Etoile, France

[3]Department of Biology
Concordia University
1455 DeMaisonneuve West
Montréal, Québec Canada

ABSTRACT

Rainbow trout were exposed to 1 and 5 µg/L of cadmium chloride ($CdCl_2$) through water for 30 days. Phagocytosis and the resporatory burst of head kidney macrophages (non-specific immune parameters) and the lymphoblastic proliferation of head kidney and thymus leukocytes (specific immune parameters) and plasma cortisol levels were measured.

Phagocytosis was significantly inhibited at both concentrations tested in a dose related fashion while the respiratory burst was inhibited at the higher concentration only. The lymphoblastic proliferation of head kidney leukocytes was inhibited at 5 µg/L while both concentrations produced an inhibition in the thymus. These results suggest that phagocytosis is the most sensitive parameter tested and that the thymus, which is directly in contact with the environment, is more sensitive to cadmium than the head kidney. Plasma cortisol levels were higher than the controls at 1µg/L indicating a stress response to cadmium while at 5 µg/L the cortisol levels were significantly reduced.

INTRODUCTION

Industrial and agricultural discharges are considered the primary source of metal poisoning of fish in many countries including Canada. Cadmium (Cd), a non degradable cumulative heavy metal, present in many contaminated aquatic systems is considered extremely toxic to fish (Sorensen, 1991).

Its effects on the immune system have been studied mainly in mammals and conflicting findings have been obtained in different laboratories. For instance, the effect on macrophage function varies from no effect, to suppression or stimulation (Exon, 1984). Evidence for inhibited or stimulated resistance was also reported (Nelson *et al.*, 1982, Gray *et al.*, 1982).

Contradictory results were also observed for antibody production; fish such as brown trout (*Salmo trutta*) (O'Neil, 1981) and cunner (*Tautogolabrus adspersus*) (Robhom,1986) showing a suppression while in rainbow trout (Thuvander, 1987) and striped bass (*Morone saxatilis*) (Robhom,1986) a stimulation was detected. Thuvander (1987) showed a decrease in the proliferative response of splenocytes from rainbow trout exposed 9 weeks to 3.6 µg/L of Cd. However, Elsasser *et al.* (1986) showed a stimulation *in vitro* of the respiratory burst by head kidney macrophages after one hour.

Cadmium is also known to induce an elevation of plasma cortisol in rainbow trout (James and Wigham, 1986). The immunosuppressive effect of cortisol in fish is well documented and may accentuate the effect of Cd (Bennett and Wolke, 1987, Carlson *et al.*, 1993).

The purpose of this study was to examine the effect of 1 and 5 µg/L of Cd on the non specific and specific immune response and plasma cortisol levels in rainbow trout and to identify the most sensitive immunological parameter for determining the toxicity of Cd.

MATERIAL AND METHODS

Experimental Animals

Juvenile rainbow trout were acclimated two weeks in chlorine free tap water at a temperature of 15 °C. They were fed daily at 1 % body weight and were kept on a photoperiod of 12 hr day/12 hr night. The fish were exposed for 30 days to 1 or 5 µg/L of cadmium ($CdCl_2$). Cadmium was delivered to the tanks at a constant flow using Mariot bottles (Leduc, 1966).

Collection of Samples

After 30 days, the fish were sacrificed with MS-222 (100 µg/L) and the blood was collected with an heparinized syringe from the caudal peduncle. The thymus and the head kidney were removed and a single cell suspension was prepared in sterile condition by grinding the tissues on a metal mesh with cold HBSS medium (Gibco BRL) supplemented with 10 units/mL of heparin and 1 % of a solution of penicillin and streptomycin (Gibco, BRL). The suspensions were then placed over a Ficoll gradient (d=1.077g/mL, Pharmacia) and spun 20 min at 1000 xg to remove erythrocytes and debris. The cells located at the interphase of the medium and the Ficoll were then collected and

washed once in cold HBSS. The pellet was resuspended in RPMI-1640 (Gibco, BRL) medium and a cell and viability counts were performed with the exclusion dye Trypan blue.

The plasma was obtained by centrifugating the blood at 400 xg for 20 min and kept at -20°C until analysed.

Phagocytosis

The phagocytic activity of head kidney macrophages was determined by flow cytometric determination of the cells engulfing fluorescent latex microspheres (1.52 µm diameter, Polysciences) as described by Voccia *et al.* (1994). Cell suspension of $1x10^6$ cells/mL were made. Negative controls were pretreated 30 min with 0.2% sodium azide and all samples were incubated with fluorescent beads in a proportion of 1 cell:100 beads for 18 hrs at 22 °C. Next, the cells were centrifuged for 5 min at 100 xg through a gradient mixture of 3% bovine serum albumin (BSA, Sigma Chemical Co) and RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco) to remove unphagocytosed beads. The samples were then analysed with a FACscan (Becton Dickinson) and the results were expressed as the percentage of cells which phagocytosed 3 beads or more.

Respiratory Burst

The production of H_2O_2 was measured as described (Voccia *et al.*, 1994). Head kidney macrophages were resuspended in RPMI-1640 at a concentration of $2x10^6$ cells/mL and incubated 15 min with 5µM of the fluorescent probe 2',7'-dichloro-fluorescein diacetate (DCFDA) (Molecular Probes Inc). They were then stimulated with 3 µg/mL of phorbol myristate acetate (PMA, Molecular Probes). The production of hydrogen peroxide by activated macrophages hydrolyses the probe which becomes fluorescent. The fluorescence was determined by flow cytometry on 1×10^4 cells and the results are expressed as the difference between the mean fluorescence produced by stimulated cells (PMA) and unstimulated cells.

Lymphoblastic Proliferation

Head kidney and thymic leukocytes were resuspended in RPMI-1640 medium supplemented with 4 % fetal calf serum and 50 mg/mL streptomycin and 50 U/mL penicillin to a concentration of $5x10^6$ cells/ml. In 96 well microplates 100 µL of head kidney cells were incubated with 20 µg/mL of phytohaemagglutinin (PHA-P; Sigma) and thymic cells were incubated with 10 µg/mL of Concanavalin A (Con A; Sigma) or RPMI-1640 for the controls. All samples were tested in triplicates and were incubated 4 days. At 18 h prior to culture termination, 0.5 Ci/well (^{3}H)thymidine (ICN Biomedicals, specific activity 6.7 Ci/mmol) was added. The radioactivity of cells was determined by liquid scintillation and the data expressed as stimulation indices (SI).

SI=DPM of stimulated cultures/DPM of controls.

Cortisol

Cortisol level in the plasma was measured by the radioimmunoassay (RIA) method with the Cortisol kit Immunocorp # 07-221102 (ICN, Biomedical)

Statistics

The data were first tested for normal distribution and for homoscedasticity with the Bartlett's test. Then with an analysis of variance, we determined if there was a difference between groups, if so the Tukey test was performed. However, when the data set had unequal replicates, the Bonferonni t-test was used. (ZAR, 1984).

RESULTS

Cadmium chloride affects the phagocytic capacity of head kidney macrophages in a dose response manner. Both concentrations tested inhibited this parameter, as is shown in Figure 1. The respiratory burst (Figure 2), measured by the production of hydrogen peroxide, was also affected in a dose response fashion but only the highest concentration of cadmium affected this parameter significantly. Hence, phagocytosis seems more susceptible to cadmium than the respiratory burst.

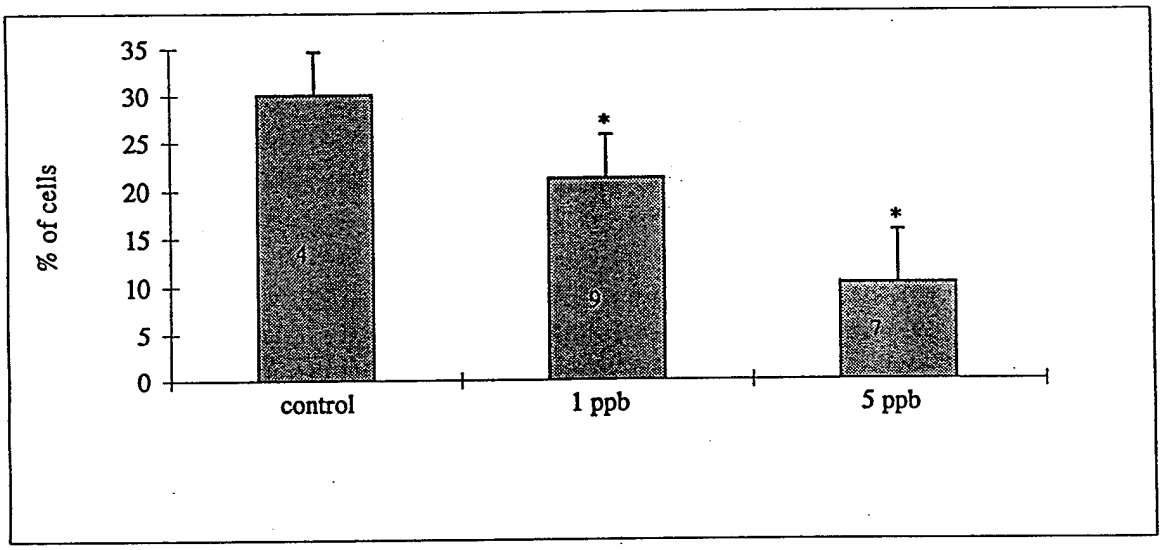


Figure 1. Effects of cadmium chloride ($CdCl_2$) on the phagocytosis of latex beads by head kidney leukocytes of rainbow trout exposed 30 days to 1 and 5 µg/L. N=number of fish tested. Results are expressed as percentage (mean ± SE) of cells that phagocytosed 3 beads or more. *P ≤ 0.05 (significantly different from controls).

For the specific immune response, we tested the lymphoproliferation of head kidney (Figure 3a) and thymic (Figure 3b) leukocytes stimulated with the mitogens, PHA and Con A, respectively. Our results show that at the highest concentration, both are significantly inhibited. However at 1 µg/L, even if we observed an inhibition for both organs, only the thymus was significantly different from the control.

Finally as shown in Figure 4, the cortisol level in the plasma was significantly higher at 1 µg/L but significantly lower at 5 µg/L.

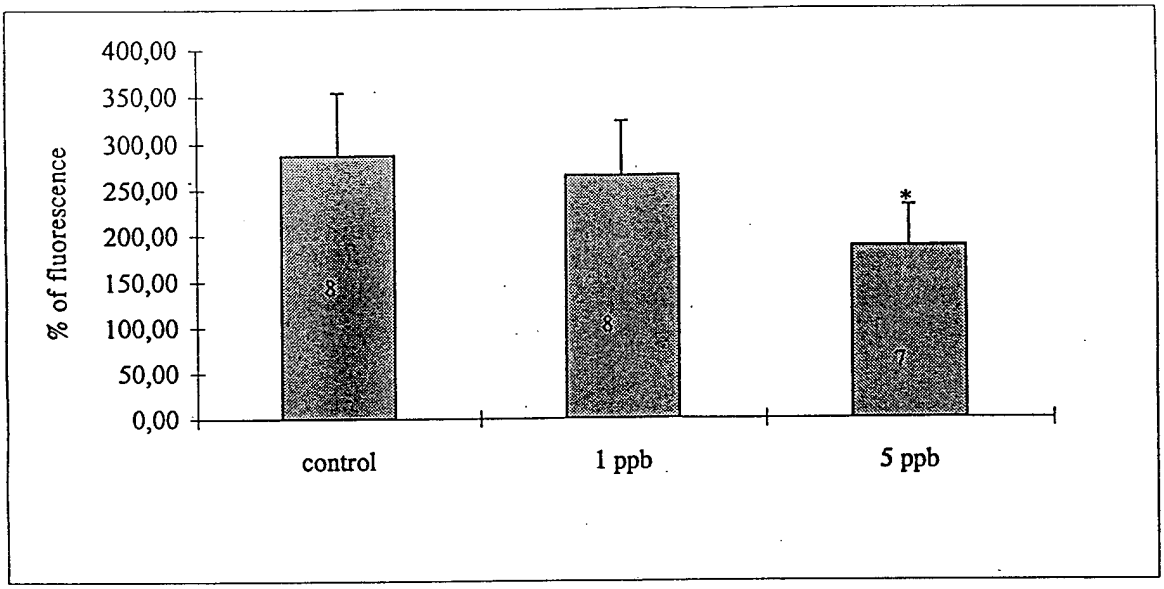


Figure 2. Effects of cadmium chloride ($CdCl_2$) on the respiratory burst by head kidney leukocytes of rainbow trout exposed 30 days to 1 and 5 µg/L. N=number of fish tested. Results are expressed as mean fluorescence (mean ± SE) (FL1). *P ≤ 0.05 (significantly different from controls).

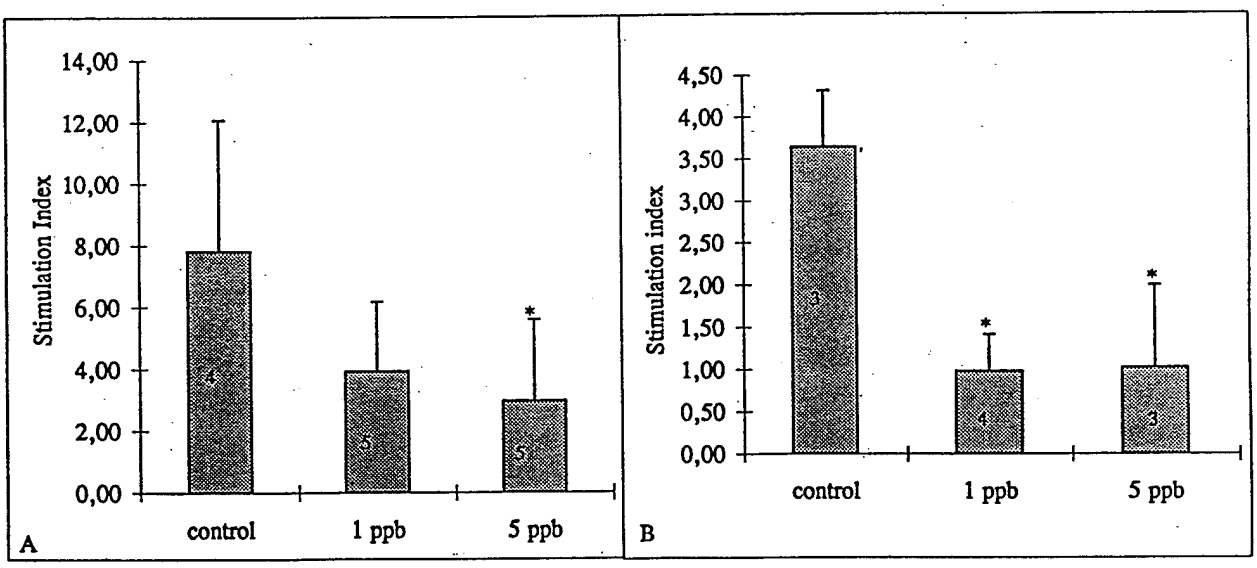


Figure 3. Effects of cadmium chloride ($CdCl_2$) on the proliferation of head kidney leukocytes of rainbow trout (**A**) stimulated with PHA (20 µg/mL) and thymus leukocytes (**B**) stimulated with Con A (10 µg/mL). Fish were exposed 30 days to 1 and 5 µg/L. N=number of fish tested. Results are expressed as stimulation index (mean ± SE). *P ≤ 0.05 (significantly different from controls).

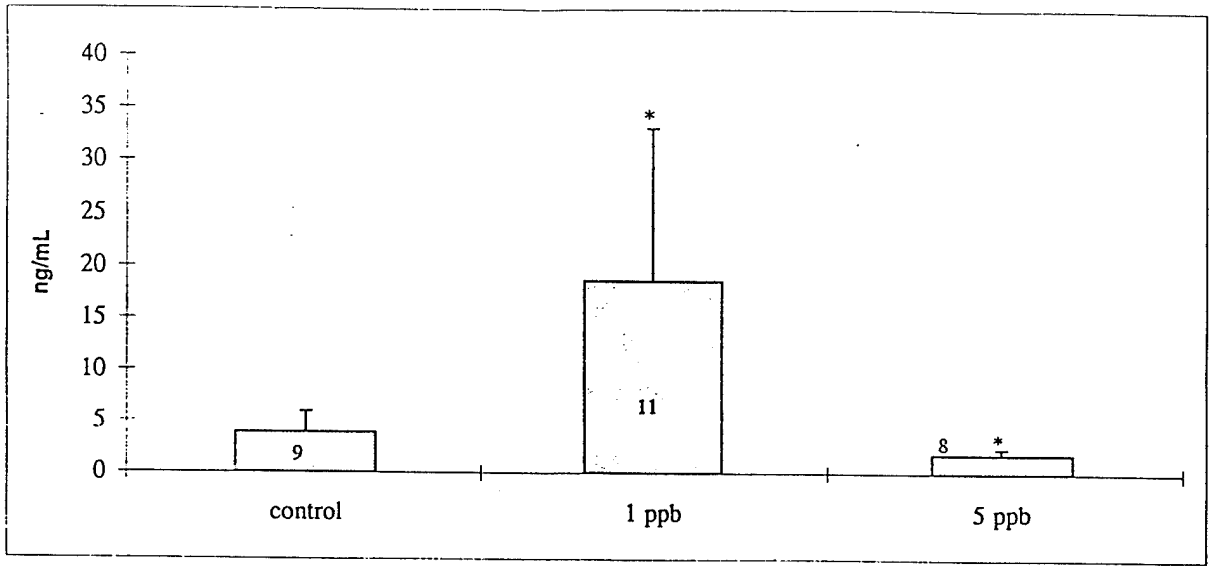


Figure 4. Effects of cadmium chloride ($CdCl_2$) on the cortisol levels (mean ± SE) in the plasma of rainbow trout exposed 30 days to 1 and 5 ppb. Number of fish tested is written in the bars. REsults are expressed as ng/mL. *P ≤ 0.05 (significantly different from controls).

DISCUSSION

Phagocytosis is the first line of cellular defense mechanism in fish and is crucial for establishing and initiating the specific immune response. Foreign matter is engulfed and subsequently degraded in a process called the respiratory burst which involves among other processes the production of hydrogen peroxide. An *in vitro* study done by Elsasser *et al.* (1986) showed in rainbow trout that a short exposure (1 hour) to cadmium chloride stimulated the respiratory burst while a longer exposure (24 hour) inhibited it. However, in another study done with medaka (*Oryzias latipes*) exposed 5 days to 6 ppb of cadmium chloride, Zelikoff *et al.* (1995) it was found that head kidney leukocytes homogenate had an increased production of hydrogen peroxide. Our results showed that in rainbow trout exposed 30 days to 1 and 5 µg/L of cadmium chloride, phagocytosis by head kidney macrophages was significantly inhibited by cadmium at both concentrations in a dose response manner. The respiratory burst by the same cells was significantly inhibited by 5 µg/L but not 1 µg/L, indicating that at this concentration both the engulfing and the degradation of foreign matter was affected. Therefore, the non-specific immune response is significantly inhibited by cadmium chloride at concentrations sublethal to rainbow trout, with phagocytosis being more sensitive to exposure to cadmium than the respiratory burst. The comparaison between studies is difficult because of the heterogeneity in exposure (time and concentration) and the different fish species used. Even if they all demonstrated that cadmium is immunomodulatory there is a need for standardizing the tests in fish immunotoxicity. The heterogeneity of the results emphasize the complexity of cadmium toxicity and the need for further studies to fully understand the mechanism of action of cadmium in aquatic fauna.

The second part of this study tested the effect of an *in vivo* exposure to cadmium on the specific immune response, and more specifically the cell-mediated immunity. Immunocompetent cells require continued proliferation and differentiation for self-renewal and for adequate host defense against infectious agents (Dean *et al.*, 1986).

In mammals, Gaworsky and Sharma (1978) observed depressed lymphoproliferative responses to the mitogen PHA. Our results confirm what was found in mammals with a tendency toward inhibition for head kidney leukocytes at 1 µg/L and a very significant inhibition at 5 µg/L. For the thymus, both concentrations inhibited proliferation. In fish, the thymus is directly exposed to the external environment and it is directly accessible to xenobiotics. It can be expected that this organ be more sensitive than the head kidney to xenobiotics and our results support this hypothesis. Thus, for *in vivo* studies, the thymus appears to be more sensitive than the head kidney for the evaluation of cell-mediated immunity.

In salmonids, cortisol is secreted into the blood stream following a stressful situation via the HPI (hypothalamo-pituitary-interrenal) axis from the interrenal tissue located in the head kidney. Cortisol has been shown to increase the susceptibility of rainbow trout to the hemaflagellate *Cryptobia salmositica* (Woo *et al.*, 1987) and to PKD (proliferative kidney disease) (Kent and Hedrick, 1987). As for cadmium, a study conducted by Pratap and Bonga (1990) on tilapia exposed 35 days to 10 µg/L of cadmium reported that after 14 days the cortisol level increased but after 35 days returned to control levels. These results were confirmed by another study where a chronic elevation of plasma cortisol was observed in the american eel (Gill *et al.*, 1993) exposed 16 weeks to 150 µg/L of Cd. We have also observed an elevation of cortisol in the plasma at 1 µg/L however at 5 µg/L the plasma cortisol levels fell below those of controls. A reduced capacity to elevate plasma cortisol in response to an acute stress of capture has been reported by Hontela *et al.* (1995) in feral fish from polluted waters containing cadmium and organic pollutants.

At 1 µg/L, cortisol levels were elevated but only phagocytosis and thymic proliferation were significantly inhibited. All immune parameters tested were significantly altered at 5 µg/L but the cortisol levels were lower than the controls. The mechanism by which cadmium can reduce blood levels of cortisol is not known. One testable hypothesis is that cadmium exerts its effects on the HPI axis by inhibiting the production of cortisol, as was shown in one teleost species injected with o,p'-DDD (Ilan and Yaron, 1983). Nonetheless, since the immunosuppression observed was stronger at a concentration when cortisol was at its lowest, it seems that in this case, cortisol had no direct effect on the immune response of the rainbow trout. Future studies should further characterize the interaction between cortisol and the immune response in fish exposed to xenobiotics and elucidate the mechanisms of action of cadmium.

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Chapter 48

Phylogenetic Conservation of the Immune Response

E.M. Boncavage-Hennessey[1], M.W. Curry[1], H.S. Gardner[2], R.A. Finch[2] and L.E. Twerdok[1,3]

[1]GEO-CENTERS, INC
Frederick, MD USA

[2] U.S. Army Biomedical Research and Development Laboratory
Fort Detrick, MD USA

[3]currently at: American Petroleum Institute
Washington DC USA

ABSTRACT

The use of fish as a model to predict immune dysfunction in response to environmental contaminants is a relatively new concept when compared to the use of rodent models. The phylogenetic conservation of many immune functions has prompted the development of a fish model for immunotoxicity. We have compared mononuclear cell reactivity following *in vitro* stimulation between 5 species; Japanese medaka (*Oryzias latipes*), rainbow trout (*Oncorhynchus mykiss*), DBA/2 mice, Sprague-Dawley rats and a human mononuclear cell line (ML-1). Mononuclear cells were isolated from the teleost head kidney (an organ that closely resemble mammalian hematopoietic tissue) and from the rodent bone marrow. Populations from both species were allowed to attach and differentiate for a 24 hour period. ML-1 cells are a myeloblastic cell line differentiated *in vitro* over 6 days to monocytes/macrophages. Cell populations were then characterized using myeloperoxidase and non-specific esterase special stains. Reactive oxidant production was measured following *in vitro* stimulation with a phorbol ester using the following assays: the reduction of cytochrome c (detection of extracellular superoxide), the reduction of nitroblue tetrazolium (detection of intracellular superoxide), and the oxidation of phenol red in the presence of horseradish peroxidase (hydrogen peroxide production). In general, for the superoxide anion assays, the teleost species elicited the greatest response; rodent cells demonstrated the greatest response for hydrogen peroxide.

INTRODUCTION

A result of the increasing levels of urbanization, industrialization, and technology in the world today is increasing pollution of the air, soil and water. While the use of mammalian test models for evaluating the potential human health risks associated with environmental contaminants has its advantages in so much as the test animals are phylogenetically close to humans; they also have several drawbacks. Ever increasing regulatory requirements, increasing costs of animal care and

husbandry, the relatively large investment of time required for testing, and a growing social concern over the use of mammals for research are all factors which have resulted in a growing effort to develop alternative test models (Cairns and van der Schalie, 1982).

The hypothesis under investigation in our laboratory is that some teleost models can respond in a similar manner to rodent models and be used successfully for predicting the response of human inflammatory cells. These studies compare the results of inflammatory cell function of two rodent models, DBA mice and Sprague-Dawley rats to those of rainbow trout and medaka. The results from both the teleosts and rodents were compared to results obtained using an existing mononuclear cell line of human origin (ML-1 cells).

MATERIALS AND METHODS

Test Species

Japanese medaka (*Oryzias latipes*) were reared in the laboratory in a flow-through well-water system maintained at 25±2°C with 16/8 hr light/dark cycle, and fed flake food (Tetramin®), brine shrimp and microworms. Water quality parameters were measured on a weekly basis. Six month old medaka (500-600 mg) which had received no chemical treatment were used in all studies unless otherwise noted. Only fish which appeared healthy and active were used in these experiments and groups were selected by age, without regard to sex.

Rainbow trout (*Oncorhynchus mykiss*), approximately 6-8 inches in length were transported from the National Fish Health Research Laboratory, Leetown, Kearneysville, WV to Ft. Detrick, Frederick, MD. The fish were then transferred to a 340 L fiberglass tank equipped with a flow-through well-water system maintained at 14±3°C with a 16/8 hr light/dark cycle, and were fed commercial trout chow. Water quality parameters were measured on a weekly basis. The fish were allowed to acclimate for 2 weeks prior to testing.

Inbred, male DBA/2 mice (25-30 g) were obtained from Jackson Laboratories, Bar Harbor, ME, and housed in a temperature-controlled room with a 12 hr light/dark cycle. Adult, inbred, male Sprague-Dawley rats (220-250 g) obtained from Harlan Industries, Indianapolis, IN, and housed in a temperature-controlled room with a 14/10 hr light/dark cycle. All animals were fed Purina Rodent Chow® and water *ad libitum*. Only healthy, robust animals were selected for experimental purposes.

Cell Isolation

Aquatic species

The procedure used to collect anterior kidney from medaka follows the protocol described in Twerdok *et al* (1994) with only minor changes. Briefly, adult medaka were anesthetized using tricaine methanesulfonate (MS-222, 200 mg/L)(Sigma Chemical Co, St. Louis, MO). Fish were then decapitated and anterior kidneys removed and pooled in Hank's Balanced Salt Solution containing 1% glucose (HBSS+, pH 7.2). The pooled organs were homogenized and resultant whole cell suspensions were filtered through glass wool and collected in 15 mL polypropylene centrifuge

tubes. Cell suspensions were then gently centrifuged at 350 xg for 10 min, resuspended and counted using a hemacytometer. Cells were then plated into 96-well microtiter plates at 1 x 10^5 cells per well, and allowed to attach in L-15 media (Sigma Chemical Co, St. Louis, MO) containing 0.5% medaka sera (Twerdok *et al.*, 1994). After 90 min attachment, the minimal media was replaced with a fully supplemented L-15 media (containing 5% fetal. bovine serum, 1% pen/strep and 1% L-glutamine). Cells were cultured for 24 hrs and then washed immediately prior to assays to remove unattached cells and debris.

Trout cells were prepared as described by Secombes (1988), with only minor changes. Briefly, trout were anesthetized using tricaine methanesulfonate (MS-222, 200 mg/l)(Sigma Chemical Co, St. Louis, MO). The anterior kidneys were removed and pooled in Hank's Balanced Salt Solution containing 1% glucose (HBSS+, pH 7.2). Due to the large amount of blood contained within the trout head kidney, the pooled organs were homogenized and resultant whole cell suspensions were enriched for monocytes by layering the cell suspension atop a 54% Percoll gradient and centrifuging at 400 xg for 20 min. The enriched cell population was washed twice in HBSS+ and after the second wash the cells were resuspended in L-15 + 0.1% FBS and counted using a hemacytometer. Cells were then plated into 96-well microtiter plates at 1 x 10^5 cells per well, and allowed to attach. After 90 min, the minimal media was replaced with a fully supplemented L-15 media (containing 5% fetal. bovine serum, 1% pen/strep and 1% L-glutamine). Cells were cultured for 24 hrs, then washed immediately prior to assays to remove unattached cells and debris.

Rodent Species

The following procedure for sacrificing and collecting cells, was adapted from Trush *et al* (1990). Animals were first anesthetized and then sacrificed via cervical dislocation; femurs were removed and bone marrow cells flushed out with RPMI media (Sigma Chemical Co, St. Louis, MO); pooled cells were counted by hemacytometer. Cells were then plated into 96-well microtiter plates at 1 x 10^5 cells per well and allowed to attach for 24 hrs at 37°C. Cultured cells were washed immediately prior to assays to remove unattached cells and debris.

Human ML-1 cells

Cells were differentiated *in vitro* to monocytes/macrophages by incubating the cells in RPMI media containing 30 ng/mL 12-O-tetradecanoyl-phorbol-13-acetate (TPA) for 3 days at 37°C. After 72 hrs, cells were counted and plated at 1 x 10^5 into 96-well microtiter plates in TPA-free media. Cells were then allowed to differentiate an additional 72 hrs at 37°C. Cultured cells were washed immediately prior to assays to remove unattached cells and debris.

Enzyme Activity

Cell preparations were characterized with respect to enzymatic activities associated with inflammatory cells. Cells ($1x10^6$) were cultured (in duplicate) in chamber slides for 24 hrs, washed, fixed and stained for esterase and myeloperoxidase activity using Sigma Procedure No. 91 and Sigma Procedure 390, respectively.

In Vitro Reactive Oxidant Generation

For each test an n≥3 was performed for all assays; each n was performed in triplicate. A single n for each species consisted of cells from: 15 medaka; 2 trout; 2 DBA mice, 1 Sprague-Dawley rat, or a single culture of human ML-1 cells. Although trout are outbred, it was necessary to pool cells from 2 trout to obtain sufficient numbers of cells for the assays.

Extracellular superoxide anion production

Following a procedure similar to that described by Secombes (1988), cells were stimulated with TPA (0.5 µg/mL for teleosts and rodents and 0.1 µg/mL for ML-1 cells). It should be noted that all assays were initiated by the addition of TPA. The TPA remained in the reaction mixture throughout the entire incubation period. The amount of superoxide anion produced in stimulated and unstimulated cells was quantified for a total of 2 hrs (15 min intervals) using a microtiter plate reader set to read at 550 nm. Superoxide dismutase-inhibitable extracellular superoxide anion production was detected by assessing reduction of cytochrome *c*. All of the results have been corrected to protein content.

Intracellular superoxide anion production

The procedure for measuring intracellular superoxide anion production was adapted from Rook *et al* (1985). Cells were stimulated with TPA using the same concentration as that used to stimulate extracellular superoxide anion production. The amount of nitroblue tetrazolium reduced was measured 30, 60 and 90 min after stimulation using a microtiter plate reader set to read at 620 nm. Superoxide dismutase was added to all samples to control for extracellular superoxide anion production.

Hydrogen Peroxide Production

H_2O_2 production following TPA-stimulation is assessed by measuring peroxidase-dependent oxidation of phenol red as originally described by Pick and Kesari (1980). Assay conditions were the same as those described previously for measuring intracellular superoxide anion production by NBT reduction.

RESULTS AND DISCUSSION

Staining

Staining

Microscopic analysis of cells stained for the presence of alpha-napthyl esterase demonstrated that for each species, approximately 75% of the cells stained positive. Myeloperoxidase-postive cells comprised 10% or less of the cellular population for each species (Table 1). These preliminary results indicate that rodent and teleost cell populations derived from hematopoietic origin and enriched for monocytes/macrophages were high in esterase activity, suggesting that the population

Table 1
Staining characteristics of cells from hematopoietic origin expressed as Mean ± S.E (n=3)

Species	Esterase % Positive	MPO % Positive
Medaka	91.6 ± 0.70	9.8 ± 2.30[a]
Trout	99.0 ± 0.07	10.3 ± 4.52
Mouse	72.6 ± 12.25	5.3 ± 1.15
Rat	86.4 ± 7.85	8.9 ± 0.85
Human (ML-1)	99.0 ± 0.15	0.4 ± 0.23

[a]values represent mean ± S.E.

being assayed consists primarily of monocytic cells. The low percentage of myeloperoxidase positive cells suggests neutrophil contamination in the cell preparations was minimal.

In Vitro Reactive Oxidant Production

Extracellular superoxide anion production

The extracellular superoxide anion assay produced very reproducible results. Table 2 illustrates the greatest amount of superoxide produced for each species. The results reported here may appear low in comparison to previously published literature where results are corrected to the number of cells assayed (Zelikoff *et al.*, 1991b). Values reported in this paper are corrected to µg protein. These values for medaka are very similar to those previously reported by our lab when presented in this manner (Twerdok, 1994). There was no significant difference in extracellular superoxide anion

Table 2
Peak values and time points for extracellular superoxide anion production in TPA stimulated cells (n=3)

Species	Peak Value ± S.E.
Medaka	0.52 ± 0.054[a,b]
Trout	0.84 ± 0.012[c]
Mouse	0.16 ± 0.049[d]
Rat	0.25 ± 0.023[d]
Human	0.24 ± 0.085[d]

[a] Values are expressed as nmol cytochrome c reduced per µg protein
[b] Statistically significantly different from trout and mammal values ($p<.05$)
[c] Statistically significantly different from medaka and mammal values ($p<.05$)
[d] Statistically significantly different from trout and medaka values ($p<.05$)

production between mouse, rat and human cells of hematopoietic origin. The trout and medaka results were statistically different from each other and from the mammalian species as proven by the Student-Neuman-Keuls comparison ($p<0.05$). In comparing means, the order of most reactive to least reactive is as follows: Trout> Medaka >Rat>Human>Mouse. It should be noted, however, that protein content did not correspond directly with cell size and, therefore, may inflate the values of superoxide anion produced.

Intracellular superoxide anion and hydrogen peroxide production

The nitroblue tetrazolium assay to detect intracellular production of superoxide anion demonstrated that the teleost species have the greatest overall activity; rodents displayed the greatest values for hydrogen peroxide production (data not shown).

CONCLUSIONS

Overall, the results from these studies appear to validate what other investigators have found with respect to the use of the teleost as an alternative animal model for immunological and immunotoxicological studies (Zelikoff, *et al.* 1991a,b). We have demonstrated that teleost and mammalian hematopoietic cell populations cultured as described here, have similar cell staining characteristics and display similar *in vitro* generation of reactive oxidants following TPA stimulation.

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Chapter 49

Measurements of Several Aspects of Immune Function in Toads (*Bufo marinus*) after Exposure to Low pH

Cynthia Carey, Gregory D. Maniero, Craig W. Harper, and Gregory K. Snyder

Dept. of EPO Biology
University of Colorado
Boulder, Colorado

Address all correspondence to: Dr. Cynthia Carey
Dept. of EPO Biology
University of Colorado
Boulder, CO 80309

ABSTRACT

The effect of exposure to low pH on lymphocyte proliferation, complement levels, and white blood cell counts was examined in the toad, *Bufo marinus*. Compared to values of control animals maintained at pH 7.0, only lymphocyte proliferation stimulated by phytohemagglutinin (PHA) changed significantly following 14 days of exposure of the toads to pH 3.8 water. Because blood pH was not significantly altered by exposure of the toads to low pH water, the change in lymphocyte proliferation may be a secondary effect of a physiological response to low pH water.

INTRODUCTION

Over the last 20 years, amphibian populations have been declining and even becoming extinct in some locations (Wake, 1991). In most instances, the cause or causes are unknown but are assumed to be man-made because of the rapidity with which the declines have occurred and because of the world-wide extent of these declines. Acid precipitation was one of the first potential causative agents to be evaluated, because it occurs on many continents and because the time-course of the increase in severity of acid precipitation has paralleled the number of amphibian declines (Pierce, 1987). Because the acidity of precipitation has not reached lethal levels of amphibians in many areas in which declines and extinctions have occurred, some workers have dismissed acid precipitation as a direct causal agent in amphibian declines (Corn *et al.*, 1989; Corn and Vertucci, 1992; Bradford *et al.*, 1994). However, these studies have failed to consider several important observations: 1) a variety of sublethal stresses can cause immunosuppression in vertebrates (Moynihan *et al.*, 1994) and 2) the proximate cause of death in many amphibian populations appears to be infections by opportunistic fungi and bacteria that are normally held in check by healthy immune systems

(reviewed in Carey, 1993). These observations suggest that environmental changes severe enough to be directly lethal are not necessary to cause the demise of amphibian populations: sublethal environmental changes could be sufficient to cause immunosuppression and increased vulnerability to infection (Carey, 1993). Therefore, the question addressed by this study was: how does sublethal exposure to low pH affect immune characteristics in amphibians?

To our knowledge, no existing studies address how exposure to low pH affects immune characteristics in amphibians. We chose to test aspects of the peripheral immune system: complement levels (CH_{50}), proliferative responses of lymphocytes to a mitogenic chemical, and differential white blood cell counts. These characteristics reflect both humoral and cellular aspects of immune function and they do not require the sacrifice of the animals. The latter two tests were selected because they are aspects of the immune system which are significantly altered by various types of stresses, such as handling, in fish (Ellsaesser and Clem, 1986).

One of the most perplexing aspects of the decline in amphibian populations is that only some species, but not all, have declined in many geographical areas. For instance, boreal toads *(Bufo boreas boreas)* and leopard frogs *(Rana pipiens)* became extinct throughout much of the Colorado Rockies in the late 1970's but chorus frogs *(Pseudacris triseriata)* and tiger salamander *(Ambystoma tigrinum)* populations in the same habitats appeared unaffected (Corn *et al.,* 1989; Carey, 1993). It is unknown why some species may be more vulnerable to environmental change than others. *Bufo marinus,* known as the cane toad in Australia or the marine toad elsewhere, was selected for this study because this species is not known to be declining anywhere in its distribution. In fact, this toad is expanding its range in Australia (Covacevich and Archer, 1970). Since it is classified as a pest species in Australia, considerable interest exists concerning how pathogens or man-made environmental change might cause its demise in that country. A few studies have investigated aspects of the immune system of these toads (Evans *et al.,* 1966; Diener and Marchalonis, 1970; Weinheimer *et al,* 1971) and some information is available about its capacity for acid-base regulation (Boutilier *et al.,* 1979; Toews and Heisler, 1982, Boutilier and Heisler, 1988; Snyder and Nestler, 1991). The results from this species will be compared with those gathered in a similar study on the leopard frog *(Rana pipiens)* , a species that has declined sharply over much of its range (*Carey et al.,* 1996).

METHODS

Animal Care

Bufo marinus used in preliminary studies were obtained from suppliers in the United States. Those used in the pH 7.0 and 3.8 experiment were collected near Brisbane, Australia in December, 1994 and shipped by air to the University of Colorado.The toads were held individually in 17 x 34 x 12 cm plastic cages at 20° C in a walk-in environmental chamber . Except during experiments, cages were tilted at a slant so that toads could sit either in tap water at one end or on the dry cage floor at the other end. Photoperiod was 12L: 12D. Toads were fed crickets (dusted with vitamins once per week) two times per week and meal worms *(Tenebrio* larvae) once per week.

In preliminary experiments in which toads were exposed to either low pH or pH 7.0 water, it became apparent that the toads would avoid the low pH water and simply sit at the dry end of the cage. They became considerably dehydrated before they finally contacted the low pH water. In order to avoid additional variables (i.e. the physiological changes associated with dehydration), the cages of both control (pH 7.0) and experimental (various low pH's) animals were laid flat during experiments so that the toads could not avoid sitting in the water covering the entire floor of the cage. It also was apparent in preliminary experiments that the low pH water in the cages did not remain at the initial pH: over a period of hours, the toads changed the water pH toward less acidic values. Therefore, water in each cage (1250 mL of tap water, adjusted to pH 7.0 or low pH with concentrated sulfuric acid [H_2SO_4]), was changed every 12 hr for the duration of an experiment. Since toads could not eat during experiments because live food rapidly drowned in cages in which no dry floor surface existed, toads were not fed during experiments. Both lack of food and continual contact with water are situations which the toads would undoubtedly not encounter in the wild, but experimental conditions for both control and experimental (low pH) animals were identical.

Analyses of Immune Function

Blood Sampling

Blood was collected by heart puncture with a 1 mL syringe using a 20 gauge needle. For differential blood count and mitogen studies, the syringes were heparinized with sterile heparin. Syringes were not heparinized for blood collection or for complement analysis. Due to the fact that sampling with two different syringes was needed from the same animal, each animal experienced two heart punctures on the day it was sampled. No toads died in the two weeks following cardiac punctures.

Responsiveness of lymphocytes to a mitogen

About 0.5 to 0.8 mL blood was placed in sterile nutrient broth (RPMI [Sigma] containing 5 % fetal calf serum). Lymphocytes were separated by centrifugation for 30 min at 1200 rpm on a Percoll density gradient. After three washes in sterile amphibian phosphate buffered saline, cells were counted and the viability of the cells was checked by trypan-blue exclusion. The cells were then reconstituted in a volume of RPMI to reach a final concentration of 5×10^4 cells/ 100 µL. Cells were pipetted into 96-well flat-bottomed plates and were incubated in the presence or absence of 1.25 µg/mL PHA (phytohemagglutinin, a T-cell mitogen) added at the initiation of the culture in 100 µL of complete RPMI. This concentration of PHA was found in preliminary tests to cause the greatest amount of stimulation of toad lymphocytes. Six control and six mitogen wells are run per animal. After incubation for 72 hr at 29°C in a 5% CO_2/95% air environment, the cells were pulsed with ^{3}H- thymidine (0.5 µci/well) for 18 hr and then harvested onto a glass fiber filter using a Harvard cell harvester. ^{3}H-thymidine uptake by the cells was quantified by liquid-scintillation spectrometry. The average counts per minute of cells stimulated by PHA were averaged for each animal and then divided by the average value of unstimulated control cells for that animal. These averages, comprising the "stimulation index" for each animal, were then averaged to arrive at the mean for each test group.

Complement (CH_{50}) levels

Approximately 0.2-0.4 mL of blood per animal were placed in 1.7 mL Eppendorf centrifuge tubes and allowed to clot for at least 1 hr on ice. Plasma was removed by centrifugation and stored at -70°C. Sheep erythrocytes were standardized to a standard optical density and then sensitized by coating with rabbit anti-sheep erythrocyte antibody (Sigma) at 18°C (Hudson and Hay 1980). Varying dilutions of toad plasma were added to the erythrocytes and the mixture was incubated at 29°C for 1 hr. Tubes were then centrifuged and the OD of the supernatant determined at 541 nm. The CH_{50} (amount of complement necessary to lyse 50% of the erythrocytes) titer was calculated as specified by Green and Cohen (1977).

Differential white blood cell count

Blood smears were allowed to dry and then stained with Leishman-Wright's stain. Differential cell counts were made on 100 leukocytes per slide. Identification of various types of white blood cells was made according to descriptions given in Hillman (1968) and Hadji-Azimi (1987).

Exposure to pH 7.0 and 3.8.

After the toads had been in captivity for about 3 months, 30 toads were divided randomly into 5 groups of 6 toads each. The average body mass of the toads was approximately 200 g and did not differ significantly among groups.

Blood samples were taken from each group only once. One control group was sampled on day 0 to obtain an estimate of the typical values of all the toads at the beginning of the experiment. Another control group was sampled after 14 days at pH 7.0; this group was held under identical conditions to the experimental groups (no food, continual exposure to water), except for the pH of the water. Three experimental groups were exposed to pH 3.8 water on day 0. Blood samples were obtained from one experimental group on day 7, and other samples from another experimental group on day 14. On day 14, the last experimental group was transferred to water at pH 7.0. Blood samples were obtained from this last group after 7 days at pH 7.0 to test the effects, if any, of recovery from effects of 14 days exposure to low pH.

Blood and urine pH.

Because it was important to know whether blood pH of the toads exposed to pH 3.8 water varied significantly from that of toads held at pH 7.0, two groups of 5 toads that had not been sampled for other assays were maintained under the same conditions as the previous experiment at pH 7.0 or 3.8 for 14 days. Blood sampling was done on day 14 by heart puncture with heparinized syringes; sampling required about 30 sec to 2 min per animal. Blood pH and PCO_2 was measured in a Radiometer BMS3 Mk2 Blood Micro System at 25°C (Snyder and Nestler, 1991). Samples from each animal were run within 5 min after sampling. Samples of blood were run in duplicate for each animal. Since toads urinate during handling, a urine sample was obtained by collecting the urine in a beaker as it was voided. Urine pH was measured with pH test strips accurate to 0.2.

Modification of Ambient pH

Because preliminary experiments determined that toads could modify the pH of the water in which they were sitting, a test was run to characterize the extent of pH modification during a 12-hr period (the time period between water changes) and to determine whether the extent of pH modification was the same on day 0 as at the end of the exposure period. Two groups of 5 toads were exposed to pH 7.0 or 3.8, respectively, for 15 days. The pH of the water in each cage was measured hourly for 12 hrs on day 0, the first day on which the toads were exposed to their respective pH's, and again on day 15.

Statistics

Averages are given ± SE unless otherwise noted. One-way analysis of variance was used for comparisons among average values of various control and experimental groups. If an ANOVA was significant, Fischer's PLSD test was used *a posteriori* to determine which particular means differed significantly from others.

RESULTS

Preliminary Experiments

Although considerable information exists concerning lethal levels at low and high pH of larval amphibians, to our knowledge no studies exist on adult amphibians. We did not know what the lethal, or therefore sublethal, levels of pH were for *Bufo marinus*. Because sacrificing a number of animals to determine lethal levels was not desirable, we conducted several preliminary experiments to determine if exposure of *Bufo marinus* to either pH 7.0, 5.5, or 4.2 for 5 days would result in a mitogenic response of lymphocytes. Lymphoroliferative responsesto the mitogen PHA were measured and no significant effects of pH exposure were found (data not shown).

Exposure to pH 7.0 and 3.8.

Responsiveness of lymphocytes to mitogens

The lymphocyte stimulation index of the control group of toads on day 0 of the test averaged 8.30 ± 1.09 (Figure 1). Values for lymphocytes of toads exposed to pH 3.8 for 7 days did not differ significantly from this value. However, the stimulation index (14.00 ± 1.38) of lymphocytes of toads exposed to pH 3.8 for 14 days was significantly ($P < 0.0001$) higher than means for both the day 0 pH 7.0 and day 14 pH 7.0 controls. One week after a group of toads exposed for 14 days to 3.8 were returned to pH 7.0 water, the mean lymphocyte stimulation index (8.89 ± 1.00) did not differ significantly from day 0 or day 14 controls.

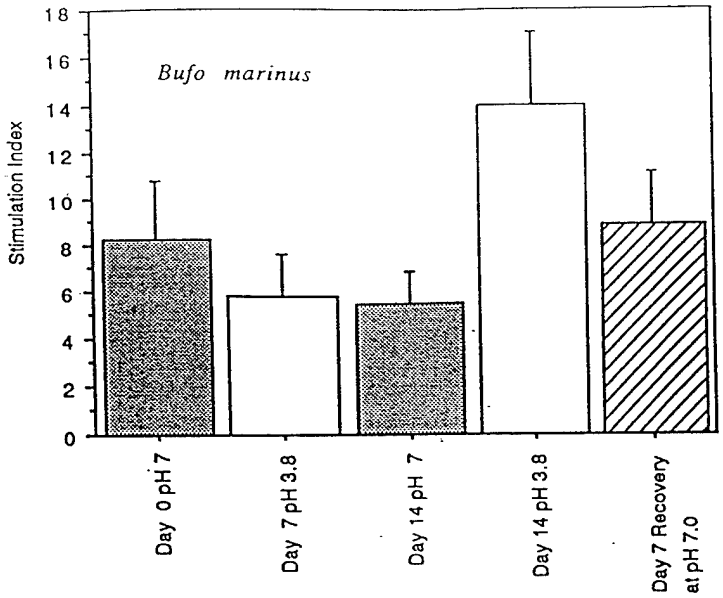


Figure 1. Mean and 95% confidence intervals for stimulation index (mean counts per minute of cells exposed to PHA divided by mean counts of non-stimulated control cells) of lymphocytes of *Bufo marinus* exposed to either pH 7 or 3.8 for varying amounts of time. N = 6 for each mean. $p<0.0001$

Complement levels

Values for the control group held at pH 7.0 for 14 days averaged 63.78 ± 4.81 CH_{50} units/mL (Figure 2). Exposure to pH 3.8 for 7 or 14 days or recovery from exposure to low pH for 7 days did not result in significant (P = 0.87) changes in complement levels.

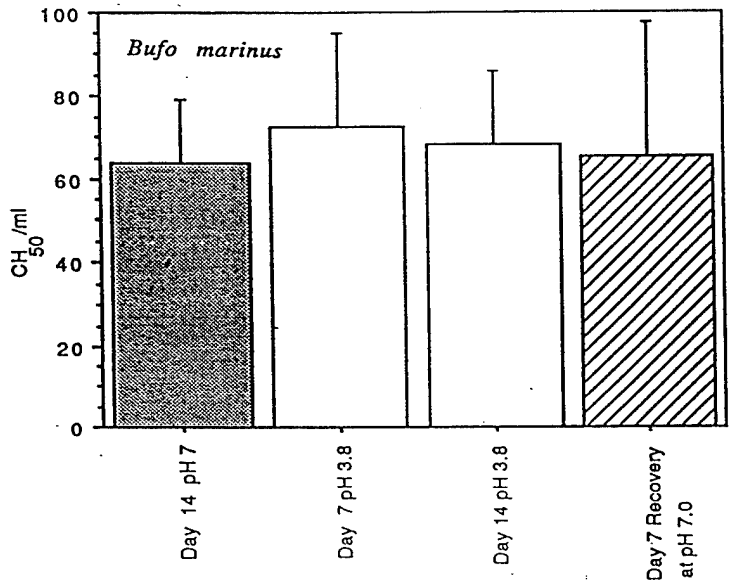


Figure 2. Mean and 95% confidence intervals for complement (CH_{50} units/mL) for *Bufo marinus* exposed to pH 7 or 3.8 for varying amounts of time. N = 6 for each mean, P = 0.87.

Differential cell counts

Exposure of toads to pH 3.8 had no significant effect on differential cell counts. On day 0, lymphocytes of toads comprised 25.5 ± 6.04 percent of total white blood cells, neutrophils 23.67 ± 4.33 percent, eosinophils 12.50 ± 2.14 percent, monocytes 17.33 ± 3.60 percent, and basophils 20.50 ± 4.12 percent. The average values for each type of cell after exposure to pH 3.8 after 7, 14 days or during recovery at pH 7.0 did not vary significantly from control values for each cell type on day 0 or day 14 (Figure 3).

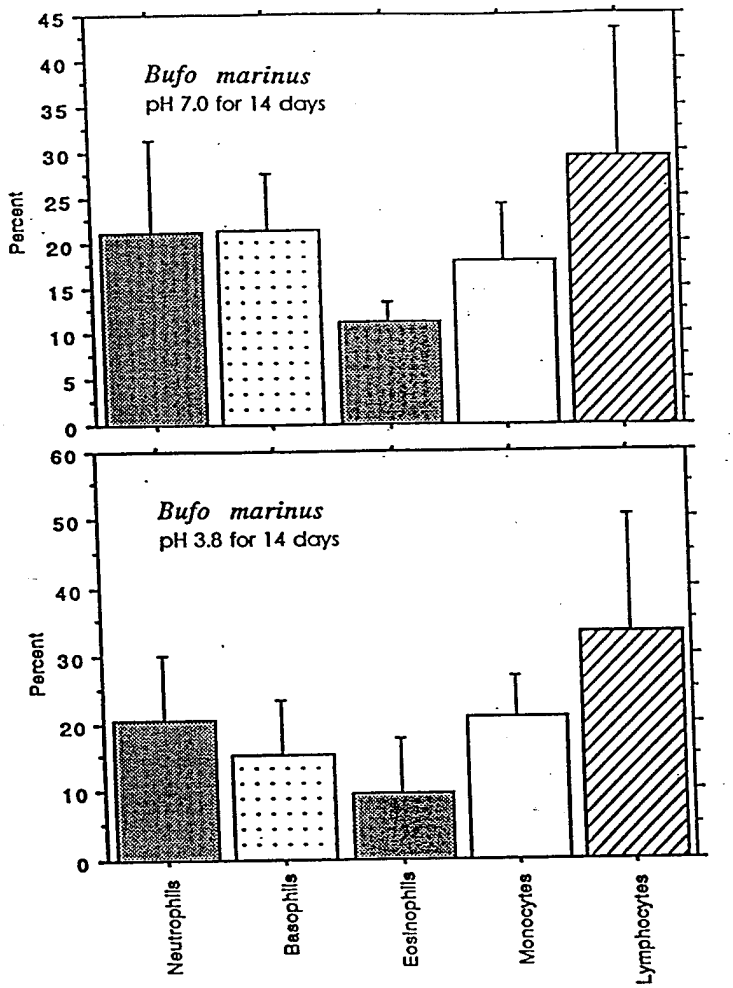


Figure 3. Mean and 95% confidence intervals for counts of different types of cells, plotted as a proportion of total leukocytes of *Bufo marinus* exposed to pH 7.0 for 14 days (top) and pH 3.8 for 14 days (bottom). N = 6 for each mean.

Blood pH

Blood pH of toads maintained at pH 7.0 or 3.8 for 14 days averaged 7.64 ± 0.066 and 7.54 ± 0.059, respectively (n = 5 in each group). These values did not vary significantly (P = 0.31). Similarly, blood PCO_2 of the toads at pH 7 (13.34 ± 1.56 torr) did not vary significantly (P = 0.34) from the mean value of the toads at pH 3.8 (16.44 ± 2.65 torr). However, the mean urine pH of the toads at pH 3.8 (4.46 ± 0.163) was significantly lower (P = 0.0001) than the average value for urine of toads at pH 7.0 (6.44 ± 0.117).

Modification of Ambient pH

The ability of toads to modify the pH of water in which they were sitting varied with the duration of time they were exposed to low pH. Toads that were exposed to pH 3.8 water for 12 hr on day 0 modified the pH of the water to an average pH of 5.2 by the end of the 12-hr period (Figure 4). In contrast, the pH of the water of toads exposed to pH 3.8 for 15 d varied relatively little over a 12-hr interval at the end of the 15-day period (Figure 4). The toads exposed to pH 7.0 on day 0 or for 15 days varied the pH of their water only slightly during the 12-hr period in which pH was measured (Figure 4).

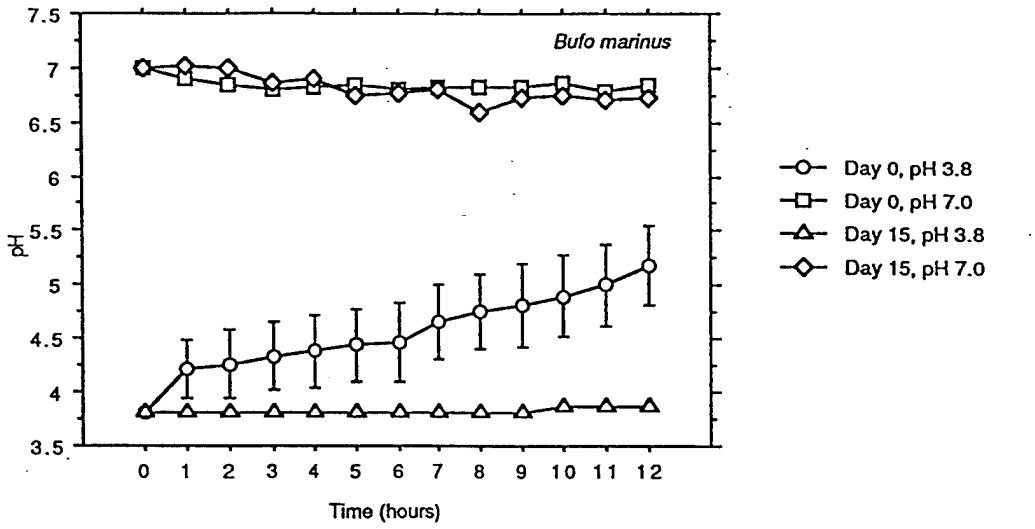


Figure 4. Mean ± SE water pH in cages of *Bufo marinus* during 12 hr period. Day 0 values were obtained the first 12 hrs that toads were exposed to each pH and the Day 15 values were measured immediately after a water change on the first 12 hr of day 15. N = 5 for each mean.

Mortality and Morbidity

No toads became sick or died during or after the 14-day exposure or during the recovery period. Within the subsequent 2 months following the experiment, mortality was less than 5% and did not differ between animals that had been used for controls or experimental animals.

DISCUSSION

Our findings indicate that a 2-wk exposure to pH 3.8 had a significant effect on peripheral blood lymphocyte proliferation of these toads, but it had no effect on blood pH, complement levels, or differential white blood cell counts. The finding that proliferative responses of lymphocytes to PHA increased significantly by day 14 at pH 3.8 has been duplicated in another amphibian, the leopard frog *(Rana pipiens)* in one of two tests using similar protocols to that used in this study, except that the volume of water in the cages differed between tests (Carey *et al.*, 1996). Since the response of these two species, one declining throughout its geographical distribution (frogs) and one stable or increasing (toads), to low pH are somewhat similar, the mechanisms resulting in their differential survival are not resolved by these experiments.

When an animal is exposed to a change in the pH of the external medium, it is important to learn: 1) whether the environmental pH change resulted in significant variation in the pH of blood and extracellular fluid, the milieu in which the peripheral immune system functions, 2) how *in vivo* pH variation affects the performance of various aspects of the immune system, and 3) whether any observed effects are caused by direct action of H^+ on cellular and humoral components of the immune system or indirectly via neuroendocrine mediation. We conclude that the blood pH of toads was minimally affected by exposure to water at pH 3.8. While average blood pH of control toads and those exposed to pH 3.8 for 14 days did not differ significantly in this study, both means for blood pH are more acidic than that found by Snyder and Nestler (1991). Snyder and Nestler (1991) sampled blood via an indwelling cannula. Our method of blood sampling by heart puncture may have caused a build-up of blood lactic acid or contamination with air which might have hidden any small differences in blood pH existing between control and low pH groups.

The few data available lead us to speculate that the exposure of toads to low pH probably had minimal effect on immune function in these toads because blood pH, even if affected by lactic acid accumulation during sampling, did not vary sufficiently to affect immune function. Several lines of evidence bear on this issue. First, the pH of vertebrate blood normally varies between 8.2 at 0° C and 7.5 at 35°C because temperature causes a change in the pH of normality of water over that range of temperature and vertebrates maintain pH in a state of relative alkalinity to the normality of water as body temperature changes (Rahn and Baumgardner, 1972). Therefore, immune systems of ectothermic animals should be able to function effectively over the range of normal blood pH they naturally experience during daily and seasonal fluctuations in body temperatures. Furthermore, since an acidic extracellular medium frequently accompanies inflammatory responses (Geffner *et al.*, 1993), it may not be surprising that various aspects of immune function can operate effectively over a broad range of pH's. Data on human immune systems indicate that certain immune functions appear to be unaffected by pH change over a broader range of blood pH's than observed in controls and experimental toads in this study and also that certain immune functions can even be stimulated

by lower blood pH's than measured in the toads at pH 3.8. Mobility of human neutrophils does not differ significantly between pH 6.5 and 7.4 (Nahas *et al.,* 1971). Oxygen-dependent cytotoxic responses of human neutrophils and monocytes to ConA, immune complexes, zymosan, and N-formyl-methionyl-leucyl-phenylalanine (FMLP) are markedly increased at pH 6.2 compared to those at 7.4 (Geffner, 1993). Activation of neutrophil NADPH oxidase, an enzyme involved in respiratory bursts, by arachidonic acid occurs optimally between pH 7.0 and 7.5 (McPhail *et al.,,* 1985). Direct exposure of circulating immune complexes derived from humans suffering from pulmonary tuberculosis (*Mycobacterium tuberculosis)* to pH 2.8 results in significant enhancement of antibody titers to this bacteria (Udaykumar and Saxena, 1992). However, low pH does have a detrimental effect on other aspects of immune function. The cytotoxic responses of human neutrophils to phorbol myristate acetate was significantly decreased at pH 6.2 compared to 7.4 (Geffner, 1993). By creating a microenvironment in which pH's range between 6.6 and 7.2, some solid tumors exploit the fact that the effectiveness of some natural immune responses, such as lymphokine-activated killer cells, are markedly reduced at pH's below 7.0 (Severin *et al.,* 1994). Thus studies on the effects of variation in blood and extracellular pH on components of the amphibian immune system are badly needed.

Why were lymphocytic proliferative responses significantly higher on day 14 than on day 7 in both toads and frogs? One simple explanation might be that up to two weeks may be required for amphibians at 20°C to manifest a lymphocytic response to any environmental perturbation. Another possibility may somehow be related to the change in ability of these animals to modify the pH of the ambient water. In our tests, both toads and frogs modified the pH of the water in which they were sitting on day 0, and in both cases, the capacity to modify ambient pH was decreased markedly on day 14 compared to day 0. The mechanism used to modify ambient pH is unknown, but a similar capacity has also been observed in a crustacean, *Cyzicus hierosolymitanus* (Ar, 1970). This ability may prove to be widespread in amphibians, because almost all amphibians compensate for high rates of evaporation of water by rehydrating frequently in standing water. The ability to manipulate the pH of the water in which they are sitting may foster more rapid water uptake or may serve to protect the ventral skin from damage from H^+ ions. Since modification of ambient pH undoubtedly requires expenditure of energy for active transport of ions, the loss of the capacity to alter ambient pH by day 14 may reflect an energetic exhaustion of the mechanism (the toads were unfed for 14 days), a progressive inactivation of proteins involved in the process by low pH, or other possible causes. Therefore, the increase in lymphocytic proliferative response by day 14 may occur in response to any or all of the physiological events associated with the energetic expenditure necessary to manipulate ambient pH, or to hormones involved either in generating the ambient pH manipulation or in responding to the consequences of the reduction in the manipulative capacity near day 14. Adrenocortical hormones, such as cortisol, are probably secreted during exposure of amphibians to low pH. Cortisol levels rise rapidly after exposure of fish *(Clarias lazera)* to pH 5.4 and 4.7 and remain elevated for up to 12 weeks (Sabry *et al., 1994).*

The fact that significant effects of low pH occurred on lymphocyte proliferation but not on complement levels or differential cell count of these toads is not unanticipated. A number of studies indicate that different kinds and intensities of stresses can affect various components of the immune system to varying degrees or even can be stimulatory to certain immune characteristics and inhibitory to others (Moynihan *et al.,* 1994). It is also not unusual for a stress to cause stimulation of one or more aspects of immune function (Moynihan *et al.,* 1994). However, heightened levels

of lymphocyte proliferation *in vitro* do not necessarily mean enhanced immunocompetence and resistance to disease *in vivo* (Moynihan *et al.*, 1994). While it is possible that exposure to low pH may have deleteriously affected components of the immune system that we have not tested, our data suggest that it is unlikely that a 14-day exposure to pH 3.8 would increase susceptibility of these toads to disease. However, we cannot rule out that long term exposure to acidic conditions or to synergistic effects of low pH and other environmental factors such as cold, toxicants, heavy metals, etc. have contributed to amphibian declines. Nor can we rule out that a significant increase in immune characteristics, such as shown here and in our companion study, can ultimately prove detrimental for disease resistance in amphibians during prolonged exposure to low pH.

Acknowledgements

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Chapter 50

Immunotoxicological Studies in the Harbour Seal (*Phoca vitulina*)

Peter S. Ross[1,2], Rik L. de Swart[1,3], Helga H. Timmerman[2], Henk van Loveren[2], Joseph G. Vos[2] and Albert D.M.E. Osterhaus[1,3]

[1] Seal Rehabilitation and Research Centre
Hoofdstraat 94a
9968 AG Pieterburen
The Netherlands

[2] National Institute of Public Health and Environmental Protection
P.O. Box 1
3720 BA Bilthoven
The Netherlands

[3]Institute of Virology
Erasmus University
P.O. Box 1738
3000 DR Rotterdam
The Netherlands

INTRODUCTION

Piscivorous marine mammals occupy a high trophic level in the marine environment, and as such, readily accumulate high levels of environmental contaminants. In certain areas, such as the highly contaminated Baltic Sea, several contaminant-related abnormalities have been observed in seals, including skeletal deformations, reproductive toxicity and hormonal alterations. Recent morbillivirus-induced mass mortalities among seals and dolphins (Visser *et al.*, 1993) have led to speculation about a possible increased susceptibility to virus infections as a result of contaminant-induced immunosuppression. While many lipophilic environmental contaminants have been shown to be immunotoxic in laboratory animals, immunotoxicity as a consequence of exposure to environmental contaminants has not previously been demonstrated in wildlife. Following the 1988 phocine distemper virus-related mass mortality of 20,000 harbour (*Phoca vitulina*) and grey (*Halichoerus grypus*) seals in Europe, we assessed the effects that contaminants may have on immune function in harbour seals. During a 2½-year immunotoxicological experiment, we fed herring from the heavily polluted Baltic Sea or from the relatively uncontaminated Atlantic Ocean to two groups of 11 captive seals each. Blood samples were taken every six to nine weeks for measurement of immunological parameters.

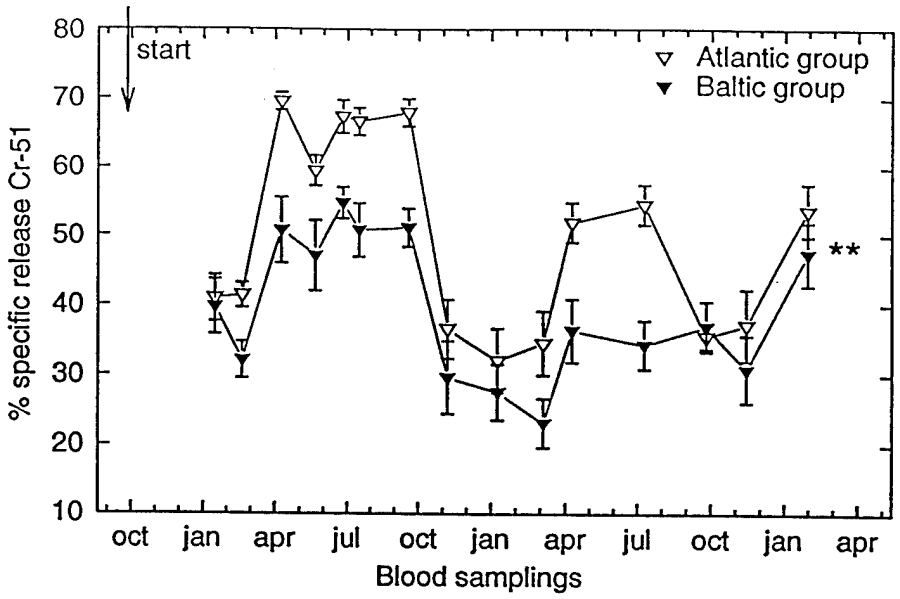


Figure 1. Natural killer cell activity is impaired in harbour seals fed herring from the contaminated Baltic Sea (split plot ANOVA $p<0.01$; n=11 seals per group; mean ± SE; adapted from Ross *et al.* 1996).

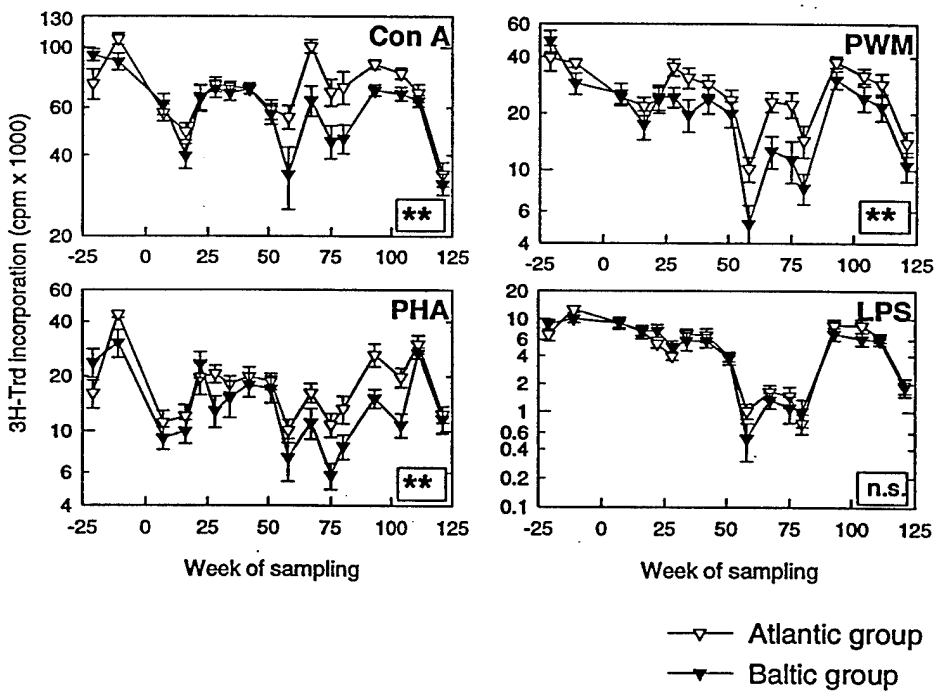


Figure 2. *In vitro* T-cell mitogen-induced lymphocyte proliferation is significantly lower in harbour seals fed herring from the contaiminated Baltic Sea (split plot ANOVA; n=11 seals per group; mean ± SE; adapted from de Swart *et al.*, 1995)

RESULTS AND DISCUSSION

Chemical residue analyses of the two herring batches indicated that estimated daily intakes of potential immunotoxic chemicals, including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) were three to ten times higher in seals fed on Baltic herring. While the Baltic Sea herring contained a complex mixture of contaminants, the lipophilic compounds were considered to pose a greater potential threat owing to their biomagnification in the aquatic food chain. In addition, environmental contaminant-induced immunotoxicity can be expected to be largely mediated via the *Ah*-receptor in free-ranging mammals, enabling a rough simplification of the cumulative toxicity of the complex mixture of the contaminants in the Baltic Sea herring. Daily intakes of 2,3,7,8-TCDD toxic equivalents (TEQ) of seals in this group were estimated to be 288 ng TEQ/day, compared to 29 ng TEQ/day for seals fed on Atlantic herring (De Swart *et al.*, 1994). Following two years on the respective diets, TEQ concentrations in the blubber of study seals were 209 ± 12 and 62 ± 4 ng TEQ / kg lipid in the Baltic and Atlantic seals respectively (Ross *et al.*, 1995).

While there were no significant differences in the results of immune function tests carried out before the start of the feeding experiment, an impairment in several parameters became evident during the course of the feeding experiment. Natural killer (NK) cell activity was consistently lower in seals fed Baltic herring, as assessed by a chromium release assay using the YAC-1 tumour cell line as a target (Ross *et al.*, in press; see Figure 1). A distinct seasonal pattern was evident in the NK cell activity of both groups, perhaps reflecting hormonal cycles. Since natural killer cell activity had not been previously reported for a marine mammal species, we characterized the functional characteristics of these cells in the harbour seal. We found the cytotoxic activity of effector leukocytes in harbour seal blood to be heightened by interleukin-2 treatment, inhibited by anti-asialo treatment, and higher against a virus-infected target cell as compared to a non-infected cell, like natural killer cells described for other species (Ross *et al.*, 1996).

T cell mitogen- (see Figure 2) and antigen- (see Figure 3) induced lymphoproliferative responses were also impaired in the Baltic group of seals (De Swart *et al.*, 1994; De Swart *et al.*, 1995). These *in vitro* results were confirmed when seals of the Baltic group were less able to mount a specific immunological response to ovalbumin, as indicated by an *in vivo* delayed-type hypersensitivity (DTH) test following immunization with this antigen (Ross *et al.*, 1995; see Figure 4). Histological sections of DTH swelling biopsies supported a mediating role for T-lymphocytes, as the cellular inflitrate was characterized by mononuclear cells. A significant, positive correlation between the DTH response (skin thickness) and mean *in vitro in vitro* lymphocyte stimulation (mitogen-induced lymphocyte proliferation) was found for the T-dependent mitogens concanavalin A and phytohaemagglutinin, but not for pokeweed mitogen or lipopolysacchharide, also supporting the notion of a role for T-lymphocytes in the DTH responses. These results suggest a clear effect of contaminants on T-lymphocytes in the harbour seals fed the Baltic Sea herring, consistent with observations in immunotoxicological studies in laboratory animals. However, it is not possible to ascribe these observations to a a particular mechanism of action. While the thymus is a sensitive organ for the action of 2,3,7,8-TCDD, effects at the level of the bone marrow or on the mature T cell cannot be ruled out.

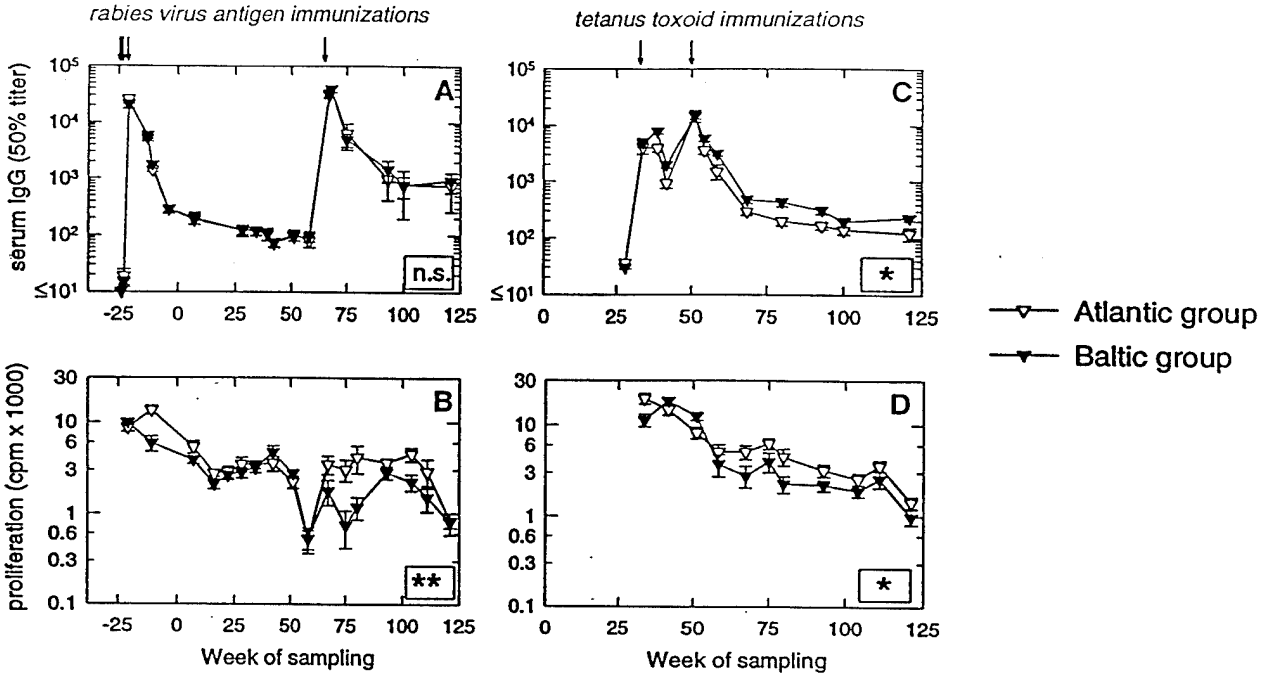


Figure 3. Antigen-specific proliferation responses of lymphocytes *in vitro* are significantly lower in the Baltic Sea group (n=11 per group; split plat ANOVA; adapted from de Swart *et al.*, 1995).

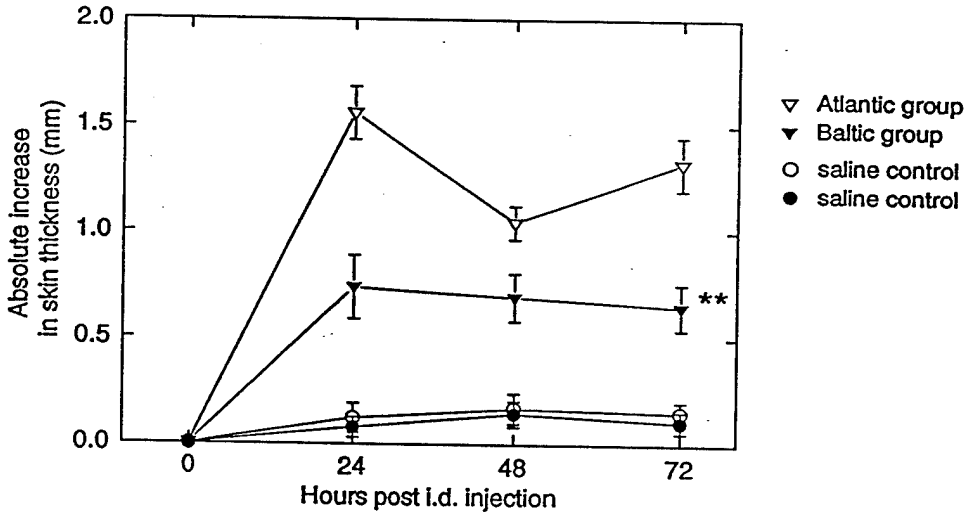


Figure 4. Delayed-type hypersensitivity responses are impaired in harbour seals fed herring from the Baltic Sea (ovalbumin as antigen; repeated measures ANOVA; n=11 for each group; adapted from Ross *et al.*, 1995).

There were few differences in gross haematological parameters measured in the two study groups. However, an increase in the number of circulating neutrophils (De Swart *et al.*,1995; see Figure 5) may have been related to a possible increased incidence of bacterial infections in the Baltic group, or to a disturbance in leukocyte differentiation in the bone marrow.

Our results indicate that environmental contaminants are immunotoxic to harbour seals at levels currently found in the aquatic food chain. The observed immunosuppression may have implications

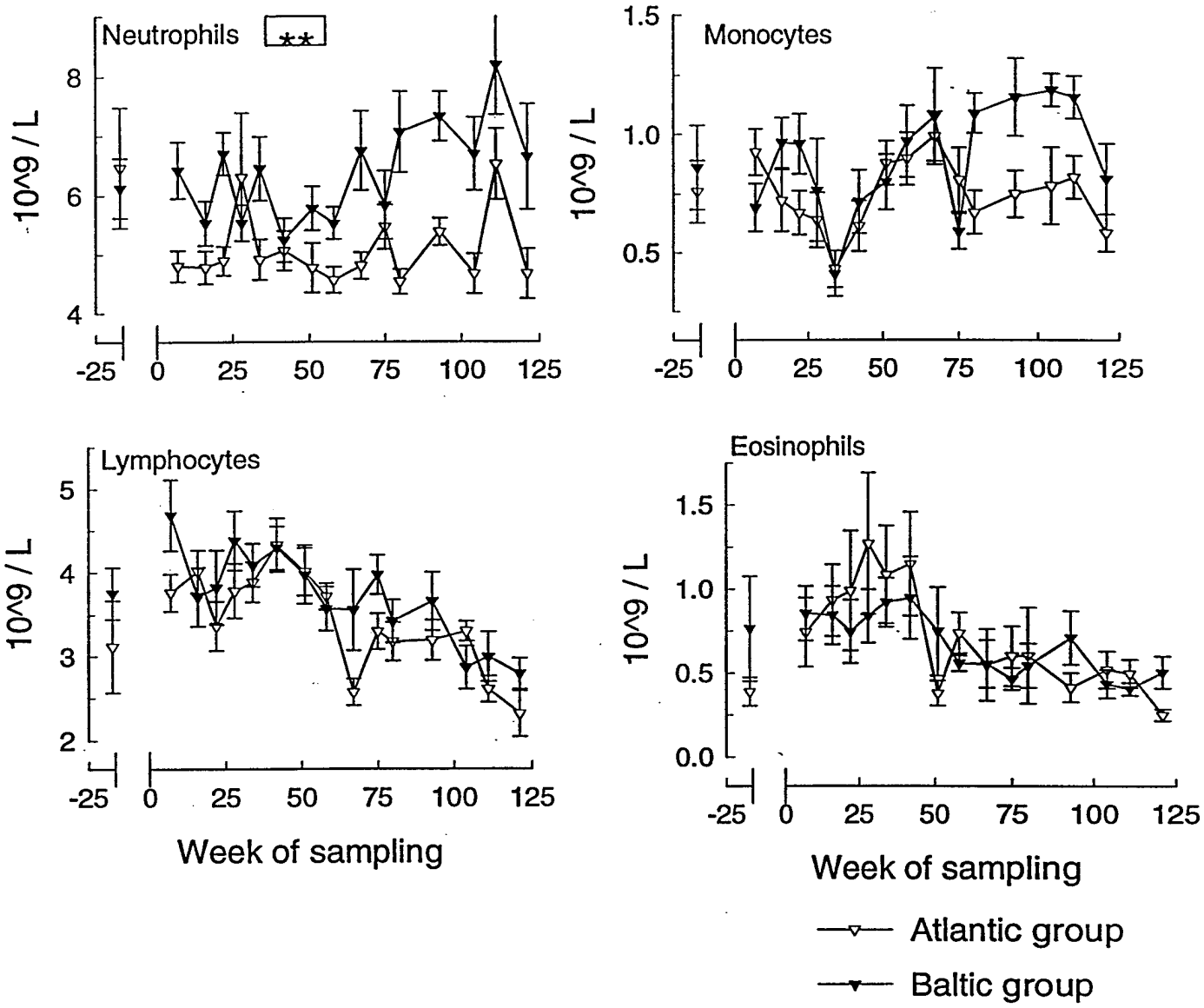


Figure 5. Neutrophils are significantly higher in harbour seals fed herring from the Baltic Sea (split plot ANOVA; n=11 seals per group; mean ± SE; adapted from de Swart *et al.*, 1995).

for the resistance of these animals to infectious disease. NK cells represent an important first line of defence against virus infections and tumours, while T lymphocytes are important in the specific clearance of virus infections. Our results suggest that environmental contaminants may have contributed to the severity and extent of recent virus-related mass mortalities among marine mammals, including the 1988 epizootic among harbour and grey seals in Europe. Since PCBs contributed the majority of the toxic equivalents to the total TEQ in the herring, this class of chemical may be considered as the most problemmatic in the aquatic food chain. The chemical stability of PCBs, coupled with the long lifespan of seals (30-40 years) and the transfer of considerable quantities from mother to pup suggests that environmental contaminant-induced immunotoxicity may remain a threat to the aquatic ecosystem for several decades yet.

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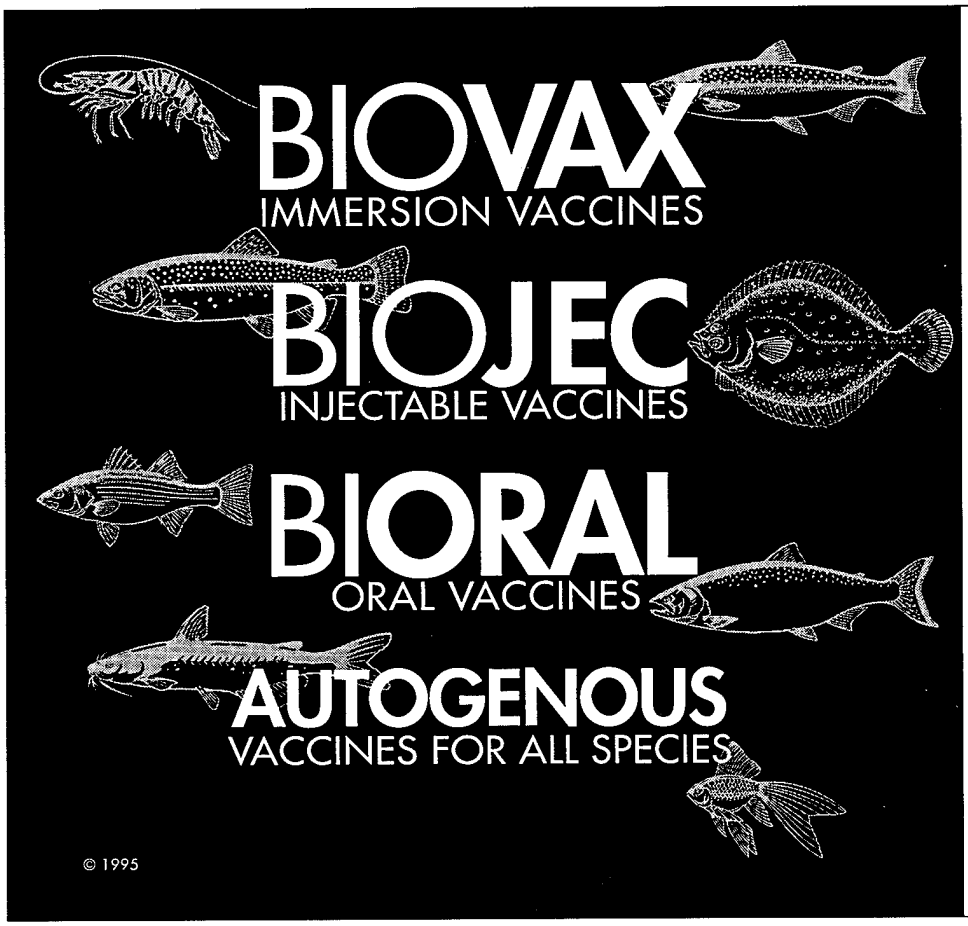
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BIOMED INC.
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U.S.A.:
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Unit 15, Sandleheath Industrial Estate
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Fax: 44 0425 655309

Norway Agent:
Apothekernes Laboratorium A.S
Harbitzalléen 3
0212 Oslo-Norway
Tel: 47 2252 9000
Fax: 47 2252 9080