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This study was designed to investigate the interaction of staphylococcal enterotoxins and exfoliative toxins with leukocytes and to establish baseline information on the consequences of those interactions. Receptor-ligand interactions were characterized and a new non-MHC class II receptor on the surface of macrophages was discovered. Once these data were at hand, we also prepared an anti-idiotype antibody to class II MHC and have used it to inhibit interaction of toxins with their cognate receptor on macrophage surfaces. These studies have resulted in the publication of 31 manuscripts and 7 published abstracts.

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INTRODUCTION

Staphylococci are probably the most commonly encountered animal pathogen. They are pathogenic to humans as well as virtually all species of animals. Pathogenesis is mediated by the secretion of up to 33 different extracellular enzymes and toxins. This impressive group of invasive factors provides the organism with a mechanism to establish its ubiquitous distribution in nature. However, other than abscess formation, the details of staphylococcal have only slowly surrendered to scientific scrutiny. Over the past 15 years, our group has carried out a comprehensive molecular and immunological investigation of staphylococcal pathogenesis mediated primarily by extracellular virulence factors. For the last 5 years we have established baseline information on the interactions that occur between leukocytes and toxins, we characterized non-class II major histocompatibility complex toxin binding receptors and we have produced novel monoclonal "anti-idiotype" antibodies specific for exfoliative toxin A. These antibodies interfere with T cell activation in a less specific fashion and their mode of action served as part of the focus of this application. These studies set the foundation for investigations on the in vivo pathophysiology mediated by the exfoliative toxins (ETA and ETB) and the structure-function relationship between these proteins and the mammalian host.

Staphylococcal exoproteins are broadly characterized as epidermolytic toxins (ETA and ETB), the enterotoxin family of toxins which includes SEA, SEB, SEC 1, 2 & 3, SED, SEE and SEG as well as streptococcal pyrogenic exotoxin A and toxic shock syndrome toxin and membrane- damaging toxins (α, β, δ, Γ toxins and others). Coagulase and staphylokinase also are important extracellular secretion products of staphylococci (1, 2, 3, 4).

The exfoliative toxins cause Ritter's disease, a bullous exfoliative dermatitis in infants as well as other similar impetigous diseases such as toxic epidermal necrolysis, bullous impetigo and scarlatiniform rash (2). These maladies are commonly referred to as staphylococcal scalded skin syndrome. Exfoliative toxin was first partially purified in 1971, however, the failure to recognize the existence of two toxins led to controversy regarding the physical properties of the two proteins (2). Both ETA and ETB are approximately 27 kDa, have approximately 50% DNA homology between each other, are distinguished by their

genetic locus, (ETA, chromosomal; ETB, plasmid) and their sensitivity to a 30 min. 60°C heat treatment (ETA, stable; ETB, labile) (1). The ability of the exfoliative toxins to cause disease and their powerful effects on immune cells makes the understanding of these molecules extremely important.

Many of the secreted proteins of Staphylococcus aureus were recognized for their "mitogenic" properties in 1975 (5, 6). Morlock et al. subsequently reported on the mitogenic activity of the exfoliative toxins (7). However, our understanding of the consequences of the effects of these molecules has increased enormously in the last 5 years (3, 8, 9). Several groups investigated how these bacterial products affected T cells. Now often called "superantigens" (10), staphylococcal exoproteins stimulate specific +wcell subpopulations based on their interaction with the variable region of the T cell receptor ß chain (10, 11, 12, 13). T cell stimulation by superantigens originally was believed to require antigen presenting cells (14). The presentation of the superantigen was not MHC restricted (15) because the toxin bound to nonpolymorphic regions of class II major histocompatibility (MHCII) molecules (16); perhaps with the Vß portion of the T cell receptor coming into contact with the toxin 13, 17, 18). However, recent studies by our group and others suggest that T cells may function or be activated in the absence of accessory cells or MHCII molecules (19, 20, 21, 22, 23).

Macrophages also are activated by superantigens. Ikejima et al. (24), soon followed by Parsonnet et al. (25, 26) found that human mononuclear phagocytes secreted IL-1 in response to toxic shock syndrome toxin-1 (TSST). Other studies, including our own, demonstrated that TSST, the enterotoxins and the exfoliative toxins activated human, rat, mouse and bovine macrophages (27, 28, 29, 30, Chapes et al. unpublished observations). In addition, macrophages and monocytes can be induced to produce tumor necrosis factor-α (TNF, 28, 29, 31), nitric oxide (29, 30, 32), and interleukin- 6 (29). Furthermore, in the presence of interferon-Γ (INF-Γ) these toxins can induce macrophages to become cytolytic (29, 30).

Several groups demonstrated that MHCII molecules serve as the major toxin receptor (33, 34, 35, 36) on macrophages and additional toxin receptors have been proposed (21, 22, 37).

However, these alternate receptors have been poorly characterized to date. Nevertheless, macrophages contribute to pathogenesis (38); the macrophage cytokine, TNF, appears to be important (38, 39). In vivo studies have demonstrated that T cells are also important for the pathogenic effects of enterotoxins (40, 41). Interestingly, one recent study indicated that MHC binding was not associated with the toxic properties of staphylococcal exotoxins (42). However, Stiles et al. (43) suggested that MHCI and MHCII both contribute to enterotoxin (+LPS) induced lethality. Therefore, the way staphylococcal superantigens stimulate T cells and macrophages, the cell surface receptors these toxins interact with, the cytokines the leukocytes produce (e.g. TNF) and the the biologically active sites of these exoproteins, are of considerable interest and have precipitated many recent investigations (See below).

The results of several investigations, including the recent crystallization of the SEB molecule (44) have contributed to the determination of the biologically important regions of several staphylococcal exoproteins. Among the earliest of these studies, Spero and Morlock, in 1978,(45) used limited tryptic digests to identify a 6.5 kDa fragment from the amino terminus of SEC_1 that had mitogenic activity and a 19 kDa fragment from the COOH-terminal that had the emetic activity. There are two regions of amino acid sequence that are highly conserved among the enterotoxins (AA's 106-119 & 147-163) that were suspected to be responsible for the toxic effects (2) and both are present in that 19 kDa peptide. Indeed, even in SEA, changing the cysteine, at the comparable position 106, to an alanine reduced its toxic effects (38, 42). Other groups have found that the SEA amino terminus is biologically important. Johnson's group used synthetic peptides to determine that the NH_2 terminal region of SEA was important for toxin binding to MHCII molecules (46, 47). Peptides that resembled AA's 39-66 of SEA were the most inhibitory in competitive binding assays, whereas peptides toward the COOH-end, except 121-149, had little inhibitory effect on binding (47).

Kappler et al. mutated the SEB gene and made recombinant proteins with various AA substitutions (41). Substitutions in the SEB amino- terminus resulted in diminished binding to HLA-DR-1. Hedlund, et al. also used recombinant toxins to examine the effects of truncated SEA molecules on MHC binding and T cell activation (48). SEA molecules that had deletions of AA's 180-233 or 125-233 bound poorly to Raji cells. Interestingly, a

recombinant SEA peptide, made up of AA's 107- 233, bound somewhat better to MHCII-expressing cells whereas a molecule consisting of the terminal 126-233 AA's did not. Therefore, Hedlund et al's data suggest that AA's 107-126 may be instrumental in allowing for the COOH-end of SEA to bind to human class II molecules. Mollick et al. (49) using chimeric SEA/SEE molecules concluded that the 70 AA's at the NH_2-terminus were responsible for MHC binding. Although there appears to be some discrepancies among these studies, there is evidence that toxin carboxyl and amino termini are necessary for optimal binding to MHCII molecules. For example, in the study by Griggs et al. (47) an SEA peptide derived from sequence in the COOH- terminus (AA's 121-149) had some apparent binding activity. Furthermore, Hedlund et al.'s recombinant SEA molecule (made up of AA's 107-233) did not bind to Raji cells nearly as well as wild type SEA. In the studies by Mollick et al. there was a 7 AA sequence (200-207) in their construct that was important for MHC binding or stabilization. Therefore, it appears that tertiary structure may be important for maximal binding and that more than one region of the toxin can contribute to toxin binding to human MHCII molecules. The suggestion that two or more sites on SEB have the potential to bind to MHCII (44) also supports the hypothesis that the folding of the molecule is important for receptor binding.

The central portion of staphylococcal exotoxins appears important for mitogenic activity (50, 51, 52). Although Grossman et al. (53) found that reduction and alkylation of the cysteines at AA residues 96 and 106 in SEA destroyed the ability to stimulate T cell proliferation, it did not affect the ability to induce monocyte secretion of TNFα. Curiously, TSST-1 does not have a disulfide loop motif, yet it is still a potent T cell mitogen and macrophage stimulant (24, 25, 29, 30, 54). Therefore, it appears that the portions of enterotoxin molecules that are critical for T cell activation may be distinct from those that are necessary for macrophage activation. Macrophage activation probably is mediated by portions that bind MHCII molecules or alternate toxin-binding receptors. The biologically important regions of ETA and ETB have yet to be studied even though these proteins are just as important.

Before it was learned that staphylococcal exotoxins bound to MHCII molecules, Buxser et al. determined that SEA and SEE probably bound to common sites on murine spleen cells

(55). Furthermore, because of structural similarities between SEA and SEE, SEB was a poorer competitor of SEA binding than SEE. They found that SEA had a K_d of 8 x 10^{-7} M and that there were 3,600 binding sites per cell. Since then it has been suggested that exotoxins of S. aureus have a lower affinity for murine MHCII than human class II molecules (56). The explanation for this is largely teleological, in that, S. aureus is a human pathogen (40, 56), and differences in polymorphism affect the way the toxins bind to MHCII molecules. SEA and SEE bind to human MHCII molecules in an ordered fashion, HLA-DR > HLA-DQ > HLA-DP (56). HLA- DP also had the lowest affinity for TSST when compared to HLA-DR and HLA-DQ (57). Braunstein et al. (58) found that TSST could bind to murine MHCII molecules in a similar, hierarchical fashion. IA^b bound TSST better than IA^d. Both were better receptors than IA^k molecules. This agreed with earlier studies that suggested that SEA was presented by MHCII-positive cells in a genotypic-specific way, $H\text{-}2^S > H2^b > H2^d > H2^k$ or $H2^a$ (59). Vroegop and Buxser also found that anti-IA^d antibodies inhibited mitogenesis induced by SEA better than SEB and that anti-IE^d antibodies inhibited mitogenesis induced by SEB better than SEA. However, that study did not preclude competition by SEA and SEB for similar sites. In fact, Lee and Watts (60) found when IA^d or IE^d was incorporated into planar membranes SEA bound with greater avidity than SEB. The K_d of SEA for IE^d was determined to be approximately 3.5 x 10^{-6} M (60). Our group (61, See Appendix 1) demonstrated that SEA and SEB shared same or closely spaced receptors on murine macrophages that expressed the $H2^b$ haplotype. However, SEA can bind to at least one additional distinct receptor than SEB. We also have found that normal macrophages express more than one type of binding receptor and that a low affinity toxin binding receptor is present on MHCII-deficient macrophages (See Progress Report). In competitive binding experiments, Chintagumpala et al. (62) found that SEA bound to the same epitopes on HLA-DR1 molecules as SEB, however, SEA also bound to receptors that SEB did not. These data are consistent with the earlier observations of Fraser (63) and our own studies with mouse macrophages which found that SEA easily displaced SEB, but SEB did not displace SEA in competitive assays (64).

There is a precedent for toxin binding to distinct cell-surface receptors. SEB and TSST bind to distinct sites (62, 65). As already mentioned, staphylococcal exotoxin binding to

MHCII molecules is dependent upon the allotype of the expressed molecule and the specific exotoxin that is used. This fact is also reflected in the relative importance of the alpha and beta chains of the MHCII molecule in toxin binding. For example, Herman et al. (66) found that SEA bound to the beta chain of human DR molecules. They examined DRw53 and DR-1 mutants to find that substitution of a tyrosine for a histidine at residue 81 diminished SEA binding. Johnson's group found that the alpha and beta chains were important for toxin-MHC interactions. They found that synthetic peptides against IA^b-beta chain (AA's 65-85) could inhibit SEA, but not TSST binding to Raji cells (67). Furthermore, they identified peptides encoding for amino acids in an alpha-helical region of the IA^b-alpha chain (AA's 51-80) that were also capable of inhibiting SEA binding (68). Braunstein et al. (58) used exon shuffling to make recombinant MHCII molecules and took advantage of the hierarchy of TSST binding to $H2^b$, $H2^d$ and $H2^k$ to confirm Johnson's data. They found that the alpha helices of MHCII molecule alpha and beta chains were needed to form one conformationally-dependent binding site for TSST. Thus, the staphylococcal exotoxin-MHCII interaction is complex. Generalizations can not be made based on data obtained to date. Furthermore, much of what is known about toxin-receptor interactions has been done with the enterotoxins and with MHCII- expressing cells. Little is known about how ETA and ETB interact with MHC molecules or alternate cellular receptors and which portions of the exfoliative toxins are involved in cellular activation and pathogenesis. Therefore, further studies designed to understand exfoliative toxin- leukocyte interactions and the related pathophysiology appeared to be justified.

BODY OF THE REPORT

This report represents a summary of efforts on USA Contract DAMD17-89-Z-9039. It chronicles data collected over the period from September 15, 1989 through September 14, 1994. In addition to the research conducted, I wish to point out and express my gratitude to the USAMRDC not only for support but also for the vision to allow this vehicle to help train the graduate students and post-doctoral who carried out the work. This work resulted in the training of 7 graduate students and 3 postdoctorals whose research resulted in the publication of 24 papers in refereed Journals, 7 additional manuscripts submitted or in

preparation, 7 published abstracts and 17 papers presented at national and international meetings.

Reference to strains, plasmids, genetic constructions, cloning and sequencing strategies and references are provided in the reprints appended to this report. This work is reported in chronological order of accomplishment.

I. SEA, ETA, ETB and TSST-1 Activate The Macrophage TNF Response Differently than SEB.

Our group has established that mouse macrophages respond to several staphylococcal extracellular proteins including ETA and ETB (29, Appendix 1). There are toxin- and mouse strain-dependent secretion patterns (Table 1 and 4 in Appendix 1). In particular, we found that SEA, ETA, ETB and TSST-1 induced TNF secretion by C3HeB/FeJ macrophages (H-2^k, lps^n) while enterotoxin B (SEB) did not. Interestingly, SEC1, probably because of sequence similarity to SEB, also did not induce TNF secretion whereas in comparison to SEA, SED and SEE induced intermediated responses (Table 2).

Table 1. Summary of exotoxin activation of macrophages

	C3HeB/FeJ-LPS responders				C3H/HeJ-LPS nonresponders			
Exotoxin	Tumoricidal activity	TNF U/ml	IL-6 pg/ml	NO_2	Tumoricidal activity	TNF U/ml	IL-6 pg/ml	NO_2
SEA	+	150	1,318	-	-	0	36	-
SEB	+	12	1,600	-	-	0	19	-
ETA	+	30	1,600	+	-	0	24	-
ETB	+	40	1,600	+	-	0	55	-
TSST-1	+	200	224	-	-	18	28	-

TABLE 2. TNF-alpha secretion by macrophages in response to toxins.

Toxin	TNF (Units/ml)
SEA	35
SEB	<5
SEC1	<5
SED	24
SEE	16
Medium	<5

We used the macrophage cell line B6MP102 (H-2^b) to confirm that the differential TNF response to SEA and SEB was not unique to C3H (lps^n) mouse macrophages. T cells were not required in the SEA-mediated activation process and differential toxin effects occurred at

the transcriptional level. It was not due to secretion anomalies (61, Appendix 1). The toxins both bound to B6MP102 cells and to some extent could compete with one another (Figure 1). Our studies have established that signal transduction occurs in response to SEA, SEB, ETA and ETB because these toxins significantly increased the F-actin concentration in peritoneal macrophages (Figure 2, 61, See Appendix 1) and B6MP102 cells (Figure 3) within 30 seconds of their addition (Figures 2A & B). We also found that toxin-mediated signal transduction and TNF secretion are dependent upon the activation of protein kinase C (69). Inhibitors of PKC activation prevent changes in F actin concentration and increased TNF secretion in response to SEA. Most importantly, only SEA increased TNF mRNA levels (Figure 4, 61, See Appendix 1). However, SEA was a better competitor of SEB than SEB was of SEA as determined by half-maximal inhibitory concentrations and percent maximum inhibition possible with each toxin (Tables 3 and 4, 61, See Appendix 1).

Table 3. Percent maximum inhibition of binding of enterotoxins to macrophage receptors

	Cold Competitor (in µg)	
	SEA	SEB
^{125}I-SEA	66 ± 6[a]	45 ± 7[b]
^{125}I-SEB	56 ± 2	5 3

a)Numbers represent x ± SEM of >4 experiments

b)Statistically different from cold SEA competition of ^{125}I-SEA P<0.05

Table 4. Half-maximal toxin inhibitory concentrations of competitive binding of toxin to macrophage receptors.

	Cold Competitor (in µg)	
Ligand	SEA	SEB
^{125}I-SEA	4.8 ±1.0[a]	12.4 ± 0.6[b]
^{125}I-SEB	6.3 ± 1.4	4.8 ± 1.0

a)Numbers represent x ± SEM of >4 experiments

b)Statistically different from cold SEA competition of ^{125}I-SEA P<0.01

Therefore, it appears that SEA binds to at least one binding site not shared with SEB. Therefore, staphylococcal enterotoxins appear to differentially regulate TNF transcription perhaps because of the engagement of a receptor bound only by SEA that uniquely regulates or activates specific second messengers.

II. Murine Cells Express Toxin Binding Ligands Distinct from Class II MHC Molecules.

Although it is well established that staphylococcal exotoxins bind to class II molecules (33, 34, 35, 36, 63) the existence of alternate toxin-binding receptors has been proposed (9, 21, 22, 37). Our data indicates that functional non MHCII toxin-binding ligands are present on macrophages (70, See Appendix 1). Closer examination of the binding data presented in Figure 1 suggested biphasic binding of SEA and SEB to macrophages. This is indicative of the presence of more than one type of receptor. Furthermore, our group used C2D transgenic mice, which are deficient in the expression of MHCII molecules, to determine if toxins could bind or activate macrophages in the absence of MHCII molecules. Several staphylococcal exotoxins were able to bind to peritoneal macrophages from C2D mice. These include, SEA, SEB, ETA and ETB (70). Figure 5A illustrates that SEB bound to inflammatory, peritoneal macrophages from C2D mice in a concentration dependent manner and was saturable. Binding was inhibited by the addition of increasing concentrations of cold toxin (Figure 5B).

TABLE 5 Comparison of toxin binding receptors on normal and C2D macrophages[a]

	Normal Macrophages				C2D Macrophages[b]	
	MHC Class II Receptor		Alternative Receptor			
Toxin	K_d	Binding Sites	K_d	Binding Sites	K_d	Binding Sites
SEA	$8X10^{-8}M$	$4X10^5$	$8X10^{-5}M$	$1X10^6$	$3X10^{-5}M$	$3X10^6$
SEB	$7X10^{-8}M$	$3X10^5$	$9X10^{-5}M$	$1X10^6$	$5X10^{-5}M$	$4X10^6$

a) Values obtained from Scatchard analysis of at least 3 experiments

b) Values are the average from peritoneal and bone-marrow C2D macrophages

The affinities of SEA and SEB binding to various populations of inflammatory, peritoneal macrophages were calculated. Table 5 illustrates the dissociation constants for the two receptor classes found on normal macrophages and C2D macrophages. Due to the similarity in Kd values reported previously (60), the high affinity receptor is probably the MHCII molecule. Interestingly, the low affinity receptor appears to be quite similar to the toxin binding receptor apparently on C2D macrophages. For example, the low affinity toxin receptor on normal macrophages had an affinity for SEB of 9.0×10^{-5} M compared to an affinity of 5.0×10^{-5} M for the SEB toxin receptor on C2D macrophages. The number of binding sites also was similar ranging from 1.0 to 4×10^{6} receptors per cell. Importantly, C2D macrophages responded to SEA by polymerizing actin (Figure 6) and secreting IL-6 (Figure 7). Thus, toxin binding to C2D macrophages occurred in a concentration-dependent manner, was competitive in nature and could induce a biological response 70, Appendix 1). Therefore, there must be receptors for the staphylococcal exotoxins independent of MHCII. Recent studies by Harris et al. (68) have strongly suggested that toxin binding to MHCII molecules may not be important for toxin-induced pathogenesis. Preliminary studies using iodinated cell surface molecules, chemical cross-linking methodologies and immunoprecipitation have identified several macrophage surface molecules found on C2D macrophages that bind staphylococcal superantigens (Figure 8). Therefore, C2D mice offer us a unique tool to directly investigate the role of MHCII molecules in the pathophysiology of exfoliative toxins. Experiments outlined in this proposal will address this question using model systems developed during the current grant period.

III. Model Systems to Investigate Toxin-Induced T Cell Immune Responses and Pathogenesis.

Superantigens are characterized by their ability to induce T cell subpopulations to proliferate based on the Vß component of the T cell receptor (TCR). During the course of this project we established that ETB behaves similarly to more well established superantigens, SEB and ETA. Exfoliative toxin B induced T cell proliferation similarly to ETA and SEB (23) and it was able to stimulate different populations of T cells. Whereas ETA predominantly activates T cells bearing T cell receptor Vß's 10, 11 and 15 (8), ETB activated T cells

expressing T cell receptor Vß's 7 and 8 (8.1, 8,2 and 8.3 combined). Furthermore, we have established that both ETA and ETB can activate T cells using conventional culture conditions with cells bearing MHCII molecules and can activate T cells and accessory cells from C2D mice that lack MHCII molecules (23). Therefore, ETA and ETB appear similar to other superantigens, like SEB (23).

One of the pathogenic effects of the superantigen, SEB, is induced weight loss by adult mice injected with toxin i.p. Furthermore, T cells were implicated in the pathogenesis (40, 41). Studies by our group have found similar induced weight loss following i.p. injection of 100 µg of either ETA or ETB (Figure 9). Splenic hypertrophy also occurs in response to toxin injection (Table 6), suggesting that a vigorous T cell response occurs in vivo. However, follow up studies, as proposed in this continuation, will be necessary to determine the relative importance of T cells and macrophages to the murine pathophysiological response to exfoliative toxin.

Table 6. Effects of i.p. injection on splenic hypertrophy

Exp.	Treatment	Spleen Wt. [$\bar{x}$ ± SEM (g)]
1.	PBS	0.492 ± 0.042
	ETB	0.583 ± 0.091*
2.	PBS	0.433 ± o.040
	ETB	0.580 ± 0.078*
3.	PBS	0.408 ± 0.039
	ETA	0.511 ± 0.055*

*significantly different from PBS control, $P<0.05$ as determined by a two-tailed student t-test. n=5 mice per treatment. Spleen weights measured 7 days after i.p. injection of 100 µg of toxin.

IV. Toxin Receptor "Anti-idiotypic" Antibodies Recognize ETA and Inhibit T Cell Proliferation.

A project goal was to produce toxin receptor anti-idiotypic antibodies that would bind toxin and interfere with T cell proliferation; indicative of a reagent that binds the paratope/restitope of the TCR. The identification of MHCII molecules as high affinity cellular receptors for staphylococcal exotoxins (superantigens, refs. 33, 34, 35, 36) facilitated our goal because several MHCII-specific monoclonal antibodies already had been produced (American Type Culture Collection, ATCC). C3H.OL and Balb/c mice were immunized with these monoclonal antibodies and were screened to determine if they would make antibodies specific for

staphylococcal exotoxins. B cells from those animals were used to produce hybridomas that secreted monoclonal antibodies specific for toxin. Several clones have been identified using this methodology. Furthermore, One such clone was identified satisfying the criteria outlined in our original proposal. Hybridoma clone 92.13.1.1 was induced after injection of Balb/c mice with monoclonal antibody TIB154 (Obtained from the ATCC, specific for $MHCII^d$). 92.13.1.1 is the product of two subclonings and secretes IgM isotype immunoglobulin specific for ETA (Figure 10). 92.13.1.1 will interfere with the interaction of TIB154 with MHCII ($H\text{-}2^d$)(See Figure 11) and specifically inhibits the proliferation of T cells activated by several T cell mitogens and superantigens (Table 7). The idiotype of 92.13.1.1 must interact with the paratope/restitope of the T cell receptor that interacts with Concanavalin A (Con A), Phytohemagluttinin (PHA) and the superantigens. The mitogens PHA and Con A are thought to bind to Vα and/or Vß domains of the TCR (`4624) whereas superantigens bind to Vß domains (8). Therefore, 92.13.1.1 must recognize portions of the TCR Vα and Vß domains spaced closely due to tertiary and quartenary structure.

Table 7. Specificity of monoclonal antibody 92.13.1.1 for exfoliative toxin A

	Exp. 1		Exp. 2		
Antigen[a]	92.13.1.1	HB423	92.13.1.1	αETB[b]	αSEA[b]
ETA	0.143[c]	0.100	0.553	0.096	0.935
ETB	0.116	0.129	0.128	0.902	0.507
SEA	0.061	0.005	0.109	0.123	1.397
SEB	0.061	0.005	0.102	0.080	0.456
BSA	NT	NT	0.086	0.092	0.062

a) Toxins at 3 µg/ well

b) Rabbit anti-ETB or SEA

c) Values represent Absorbance at 450 nm.

The precise sequence recognized by 92.13.1.1 has yet to be determined and will require additional study. However, possession of an antibody capable of abrogating the T cell response to superantigens may offer a novel reagent capable of reversing the pathophysiological effects of staphylococcal superantigens.

V. Summary of Accomplishment of Technical Objectives.

The experiments described in sections CI and CIII were directed toward the completion of the first technical objective of our project; to characterize the receptor-ligand interactions between enterotoxins and exfoliative toxins with leukocytes and to establish baseline information on the consequences of those interactions. The experiments described in section CII were directed toward the completion of the second and third technical objectives of our project; to characterize staphylococcal exotoxin receptors on immune cells and to carry out in vitro assays of binding activity. The experiments described in section CIV were directed toward the completion of the fourth technical objective; to prepare anti-idiotype antibody to the receptor protein.

VI. Regulation of the expression of macrophage stimulating toxins.

We have continued experiments to characterize the global exoprotein gene regulator, *xpr*, that we have discovered. Over the past year, we have localized the Tn*551* insertion site to a region distinct from *agr* and from the lipase structural gene, *geh*. The location has been mapped and designated Ω1058. In addition, the structural genes for the Agr region and for lipase were shown to be intact in the Xpr- strain KSI9051. A phenotypic comparison between Xpr- and Agr- strains and their respective parent strains showed that the mutant strains produced greatly reduced amounts of lipase, α-toxin, ∂-toxin, protease, and nuclease and where appropriate enterotoxin B. As expected, coagulase was increased in the mutant strains. Using a mouse lethality model, we also demonstrated that both mutant strains were far less virulent than their respective parents. Northern blot analysis of transcription confirmed the phenotypic analysis and showed concomitant decreases in message for the reduced exoproteins as well as higher amounts for increased exoproteins. In addition and as expected, the transcript for protein A was elevated and that for Sea was unchanged. Northern analysis of total cellular RNA hybridized with RNAII and RNAIII specific pres showed that both transcripts were reduced 16 to 32 fold. These data confirm that 2 regulatory loci, *xpr* and *agr* are interactive at the genotypic level to control the expression of exoproteins that play an important role in staphylococcal pathogenesis.

The chromosome of *Staphylococcus aureus* strain S6C was shown to contain a prophage

inserted within the ß-toxin structural gene (*hlb*). The phage *att* site was identical to that reported for the ß-toxin-converting phages ϕ13 and ϕ42. The prophage carried the genes encoding staphylokinase (*sak*) and enterotoxin A (*sea*), which suggests that it is similar to ϕ42. However, it was not induced in the presence of mitomycin C and appears to be defective. Chromosomal mapping studies revealed that the genomes of the ß-toxin-converting phages present in strains S6C and PS42D (a ϕ42 lysogen) encode at least one *Sma*I restriction site. Although the ß-toxin-converting phage present in strain S6C could not be induced, a phage was induced from strain S6C using mitomycin C. Southern blots suggest that it is similar to ϕ11; however, the restriction patterns of DNA from the induced phage and ϕ11 were clearly distinct. We have designated the inducible phage present in strain S6C, as ϕ15, to denote that distinction. Relatively weak hybridization signals were also observed when ϕ15 DNA was used to probe chromosomal DNA from *S. aureus* strains lysogenized with the ß-toxin-converting phages, ϕ13, 42 and 42E. Taken together, our results demonstrate that all of the *Sma*I-defined restriction fragment-length polymorphisms observed among the genomes of the strains examined can be accounted for by the presence of prophage DNA within the staphylococcal chromosome.

In conclusion, we feel we have made significant progress toward the completion of the technical objectives as exemplified by our manuscripts supported in part by DAMD-17-89-Z-9039.

FIGURE LEGENDS:

1. Competitive inhibition of ^{125}I-SEA (A and B) and ^{125}I-SEB (C and D) binding to B6MP102 cells by cold SEA (A and D) or cold SEB (C and D). Scatchard plots (insets) of these data were used to determine apparent K_d values and valence (receptors/cell). These graphs are representative of 4 to 6 experiments.
2. (A) Polymerized actin in peritoneal macrophages was detected using NBD-phallacidin and flow cytometry. Macrophages were incubated in PBS (panel labelled FITC) or in

PBS containing f-Met-Leu-Phe, SEA, SEB, ETA, or ETB (panels labeled as such). Cells were stimulated for 30s. (B) Same experiment but cells were treated with H7 (a protein kinase C inhibitor) to show involvement of phosphorylation.

3. Polymerized actin in B6MP102 cells detected with NBD-phallacidin and flow cytometry. B6MP102 cells were incubated in PBS (panel labeled plus FITC) or in PBS containing f-Met-Leu-Phe, SEA, SEB, ETA or ETB (panels labeled as such). Cells were stimulated for 30s.
4. Northern blot of B6MP102 RNA. Probes were specific for murine TNFα and the ribosomal protein gene S14. B6MP102 cells were incubated for 3h in medium or medium containing 10μg/ml SEA or SEB ± 10 U/ml recombinant murine IFN–γ or authentic IFN–γ. The amount of TNF secreted by these cells was as follows: medium <5 U/ml, IFN–γ < 5 U/ml, SEB < 5 U/ml, SEA 17 U/ml, SEA + IFN–γ 18 U/ml.
5. Dose response curves demonstrating specific and saturable binding of ^{125}I-SEA, ^{125}I-SEB and ^{125}I-ETA to 10^6 C2D, C1D or C57BL/6 peritoneal macrophages. Specific binding is the difference between the signal obtained with labeled toxin alone and the signal obtained in the presence of a 100-fold excess of unlabeled toxin. These graphs represent results of at least three experiments.
6. Competitive inhibition of ^{125}I-SEA (Left Panel) and ^{125}I-SEB (Right Panel) binding to C2D macrophages by cold SEA or SEB. Scatchard plots (insets) of these data were used to determine apparent K_ds and valences (receptors/cell). These graphs are representative of the results of at least three experiments.
7. Polymerized actin in C2D peritoneal macrophages was detected by using NBD-phallacidin and flow cytometry. Macrophages were incubated in PBS (A) in PBS with f-Met-Leu-Phe (10^{-6} M) or in 10 μg of SEA, SEB, ETA or ETB per ml (panels B, C, D, E and F). Macrophages were stimulated for 30s and fixed.
8. Secretion of IL-6 by C2D and C57BL/6 bone marrow-derived macrophages in response to staphylococcal exotoxins (10μg/ml) or LPS (12.5 μg/ml). Cells were stimulated for 18h. IL-6 concentration was determined by linear regression of recombinant IL-6 standards with unknown samples. Columns with different superscripts differ (P<0.05).

9. Cell surface ^{125}I-labeled B6MP102 cells and C2D macrophages alone (lanes 1 and 6) or incubated with unlabeled SEA (lanes 3 and 4) were immunoprecipitated with rabbit anti-SEA serum. Additionally, labeled cells were incubated with SEA and immunoprecipitated with Pansorbin (lanes 2 and 5). The arrow points to the 28 Kda band.
10. Effects of ETA and ETB injection on mouse weight loss. (Left) Controls and ETA. (Right) Controls and ETB.
11. Spot blot and ELISA detection of exotoxins by antibody 92.13.1.1.
12. Competition of TIB154 and 92.13.1.1 with unlabeled TIB154. (A) TIB154 against itself and (B) against 92.13.1.1.

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2. Fleming, S.D., L.S. Edelman and S.K. Chapes. 1991. Effects of corticosterone and microgravity on inflammatory cell production of superoxide. J. Leukoc. Biol. 50:69-76.
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26. J. W. Armstrong, R. A. Gerren and S. K. Chapes. 1994. The effect of space and parabolic flight on macrophage hematopoiesis and function. Ms. in preparation (submitted).
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28. M. E. Hart and J. J. Iandolo. 1994. Analysis of exoprotein gene expression in an *xpr* mutant strain of *Staphylococcus aureus* when RNAIII is provided in *trans*. Ms. in preparation. (submitted).

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1. Fleming, S.D., J.J. Iandolo and S.K. Chapes. 1991. Macrophage activation by staphylococcal exotoxins. FASEB J. 5:A1345
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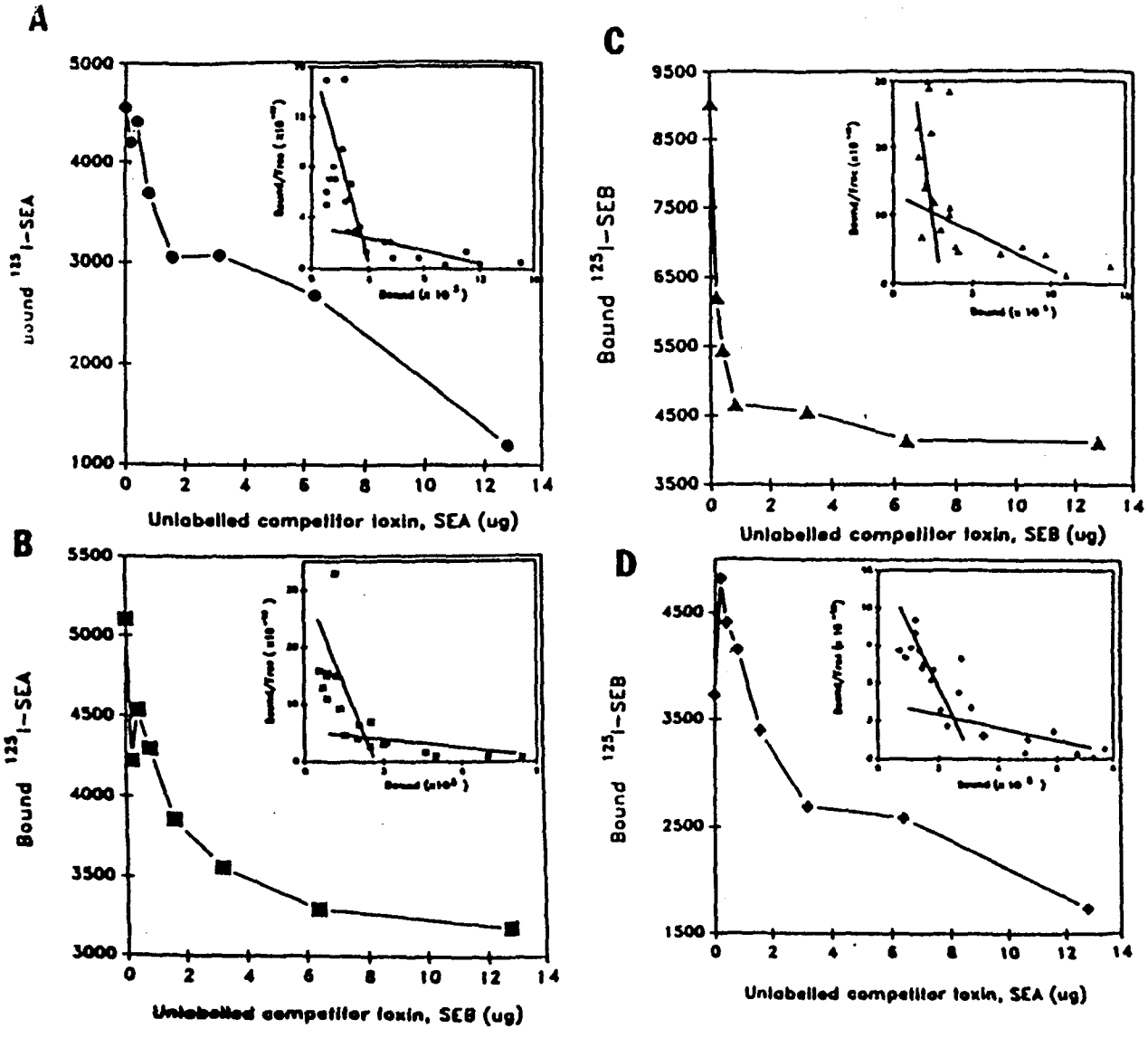


Fig. 1. Competitive inhibition of ^{125}I-SEA (A and B) and ^{125}I-SEB (C and D) binding to B6MP102 cells by cold SEA (A and D) or cold SEB (B and C). Scatchard plots (inserts) of these data were used to determine apparent K_d values and valence (receptors/cell). These graphs are representative of four to six experiments.

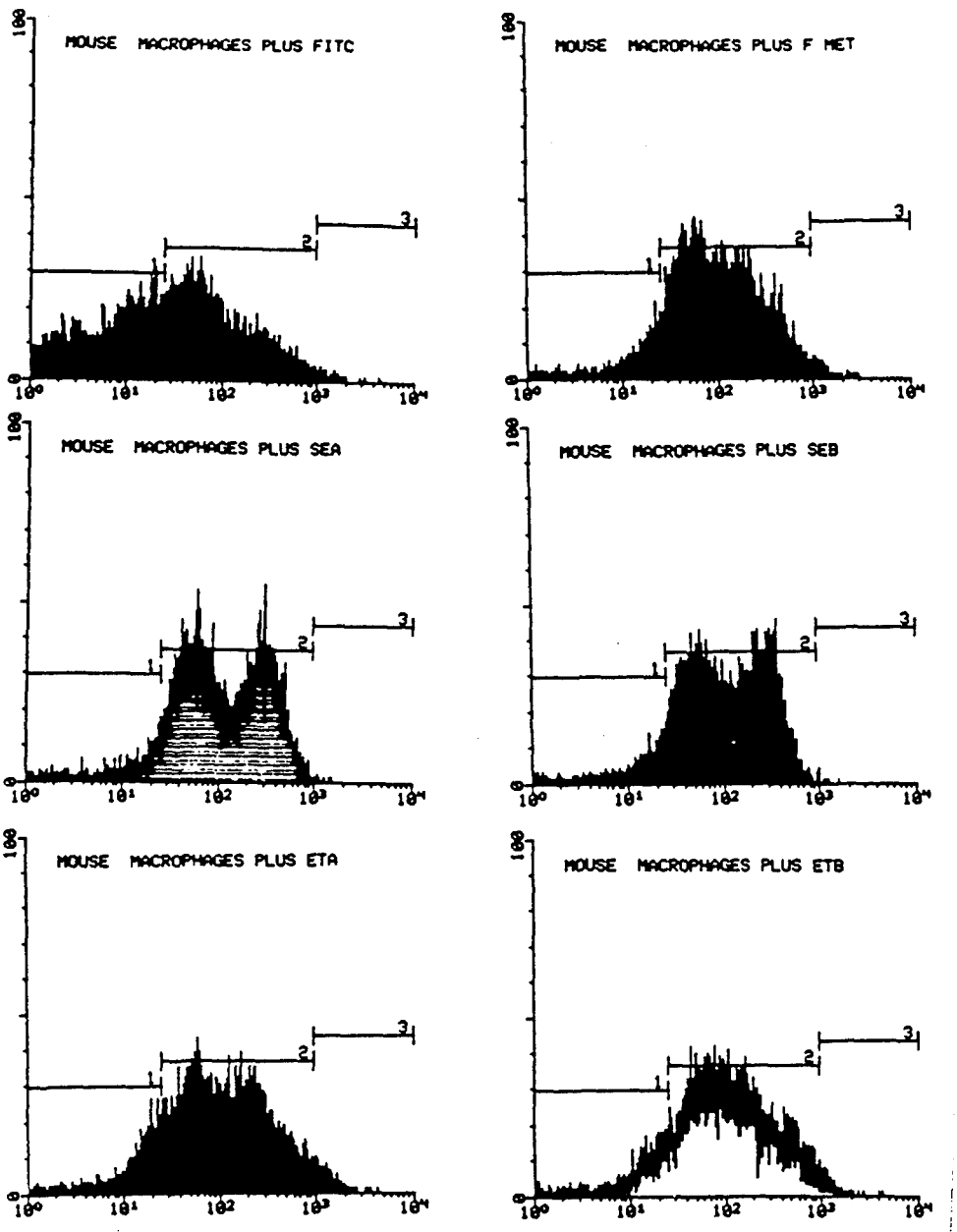


Fig.2A Polymerized actin in peritoneal macrophages was detected using NBD-phallacidin and flow cytometry. Macrophages were incubated in PBS (panel labeled plus FITC) or in PBS containing f-Met-Leu-Phe, SEA, SEB, ETA, or ETB (panels labeled as such). Cells were stimulated for 30 s.

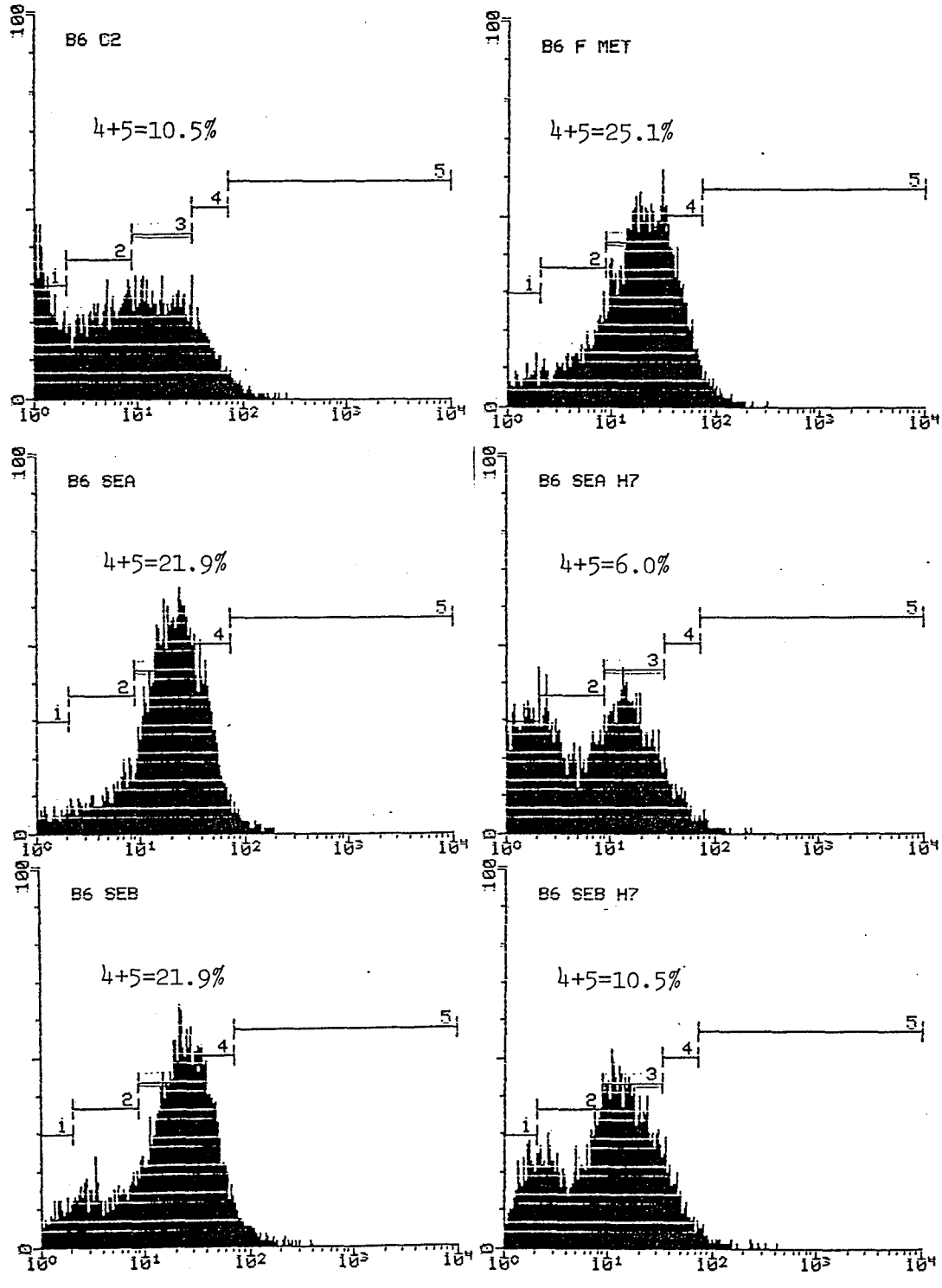

B6 C2
4+5=10.5%
B6 F MET
4+5=25.1%
B6 SEA
4+5=21.9%
B6 SEA H7
4+5=6.0%
B6 SEB
4+5=21.9%
B6 SEB H7
4+5=10.5%

FIG. 2B

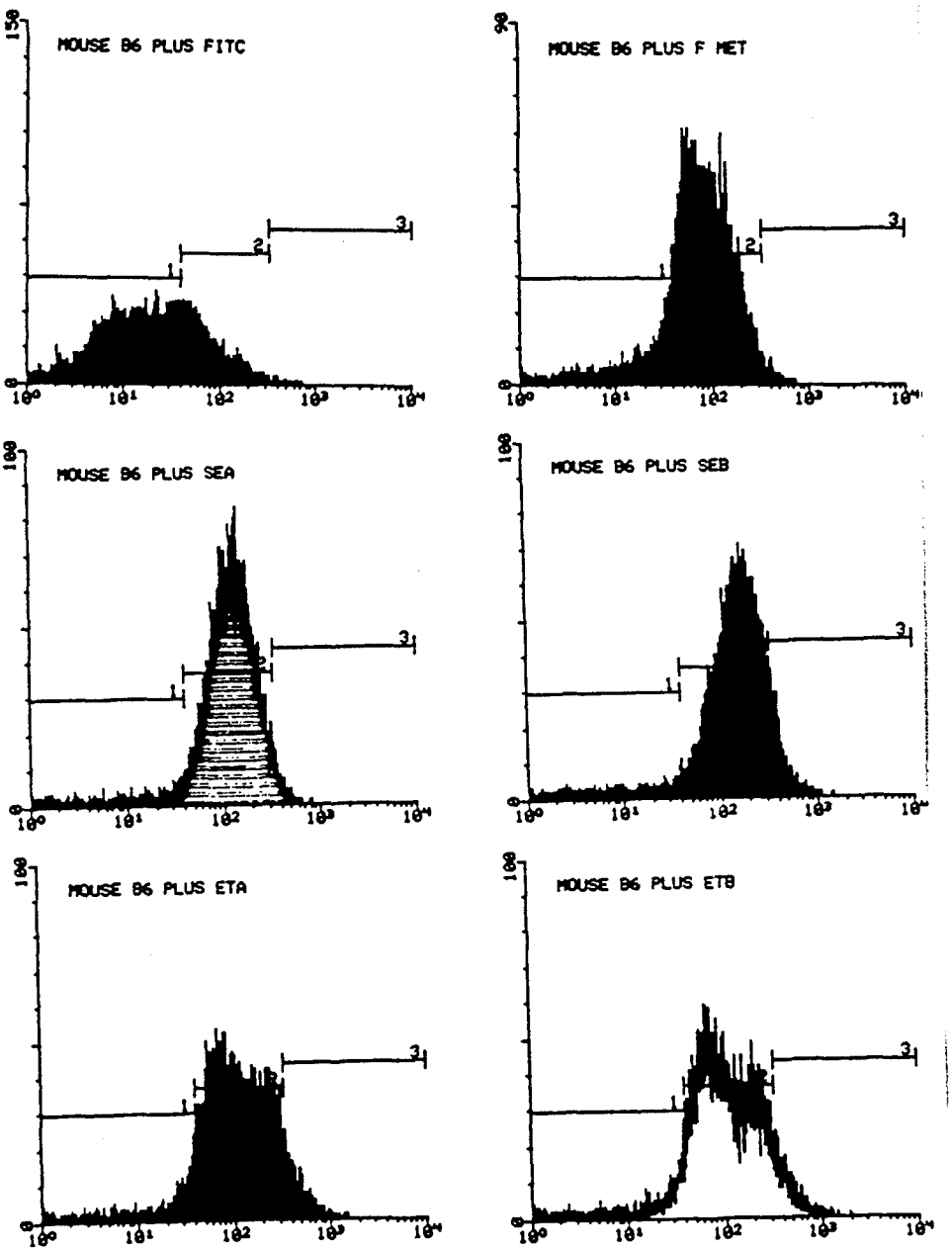


FIG. 3 Polymerized actin in B6MP102 cells detected with NBD-phallacidin and flow cytometry. B6MP102 cells were incubated in PBS (panel labeled plus FITC) or in PBS containing f-Met-Leu-Phe, SEA, SEB, ETA, or ETB (panels labeled as such). Cells were stimulated for 30 s.

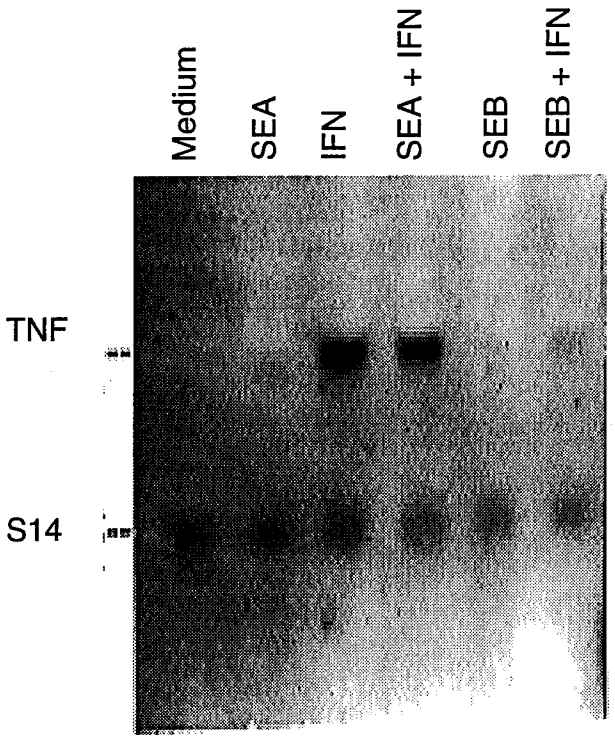
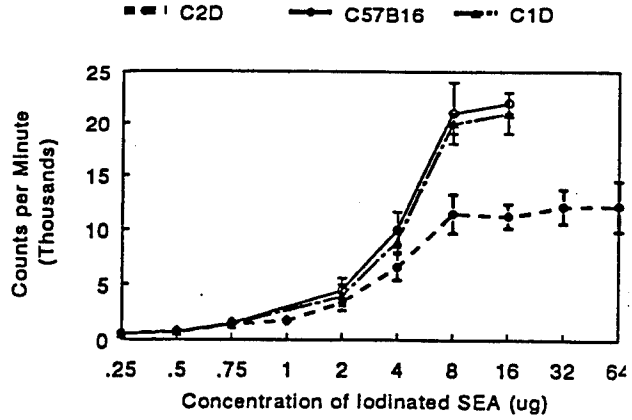
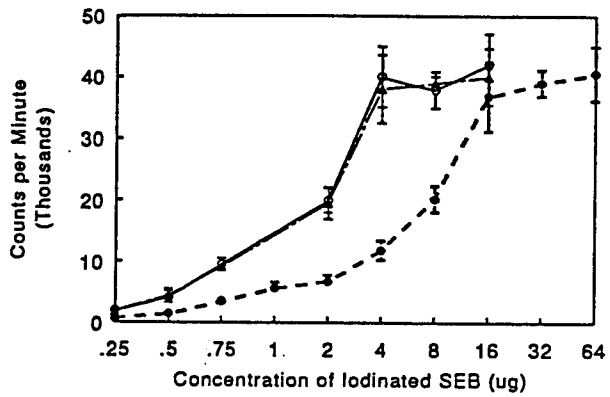


FIG. 4

FIG. 5


C2D
C57B16
C1D
Counts per Minute (Thousands)
25
20
15
10
5
0
.25 .5 .75 1 2 4 8 16 32 64
Concentration of Iodinated SEA (ug)


Counts per Minute (Thousands)
50
40
30
20
10
0
.25 .5 .75 1 2 4 8 16 32 64
Concentration of Iodinated SEB (ug)

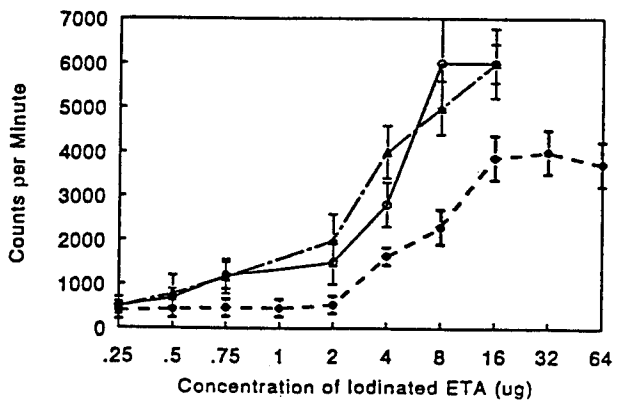
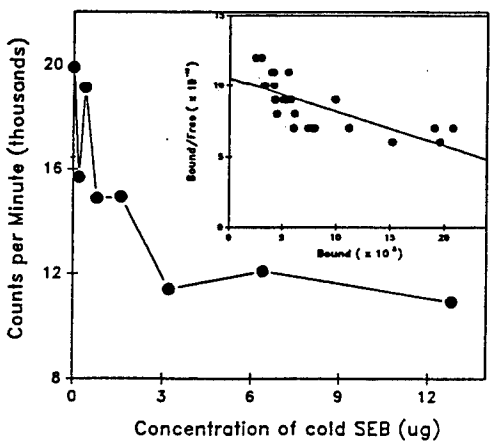
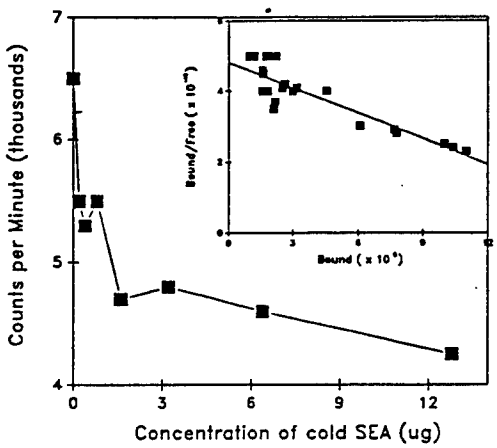

Counts per Minute
7000
6000
5000
4000
3000
2000
1000
0
.25 .5 .75 1 2 4 8 16 32 64
Concentration of Iodinated ETA (ug)

FIG. 6


Counts per Minute (thousands)
20
16
12
8
0 3 6 9 12
Concentration of cold SEB (ug)


Counts per Minute (thousands)
7
6
5
4
0 3 6 9 12
Concentration of cold SEA (ug)

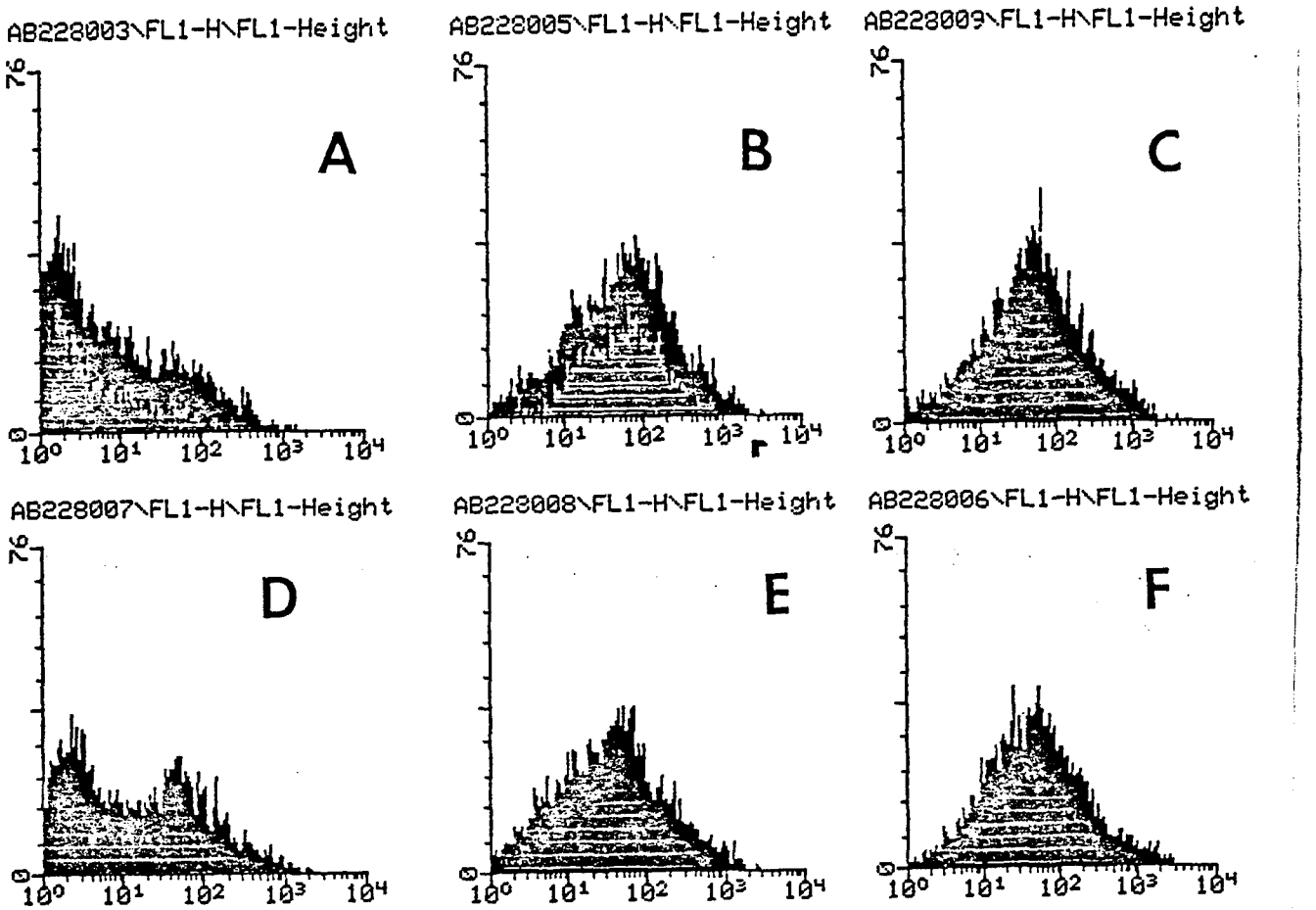

AB228003\FL1-H\FL1-Height
A
AB228005\FL1-H\FL1-Height
B
AB228009\FL1-H\FL1-Height
C
AB228007\FL1-H\FL1-Height
D
AB228008\FL1-H\FL1-Height
E
AB228006\FL1-H\FL1-Height
F
76
0
10⁰ 10¹ 10² 10³ 10⁴

FIG. 7

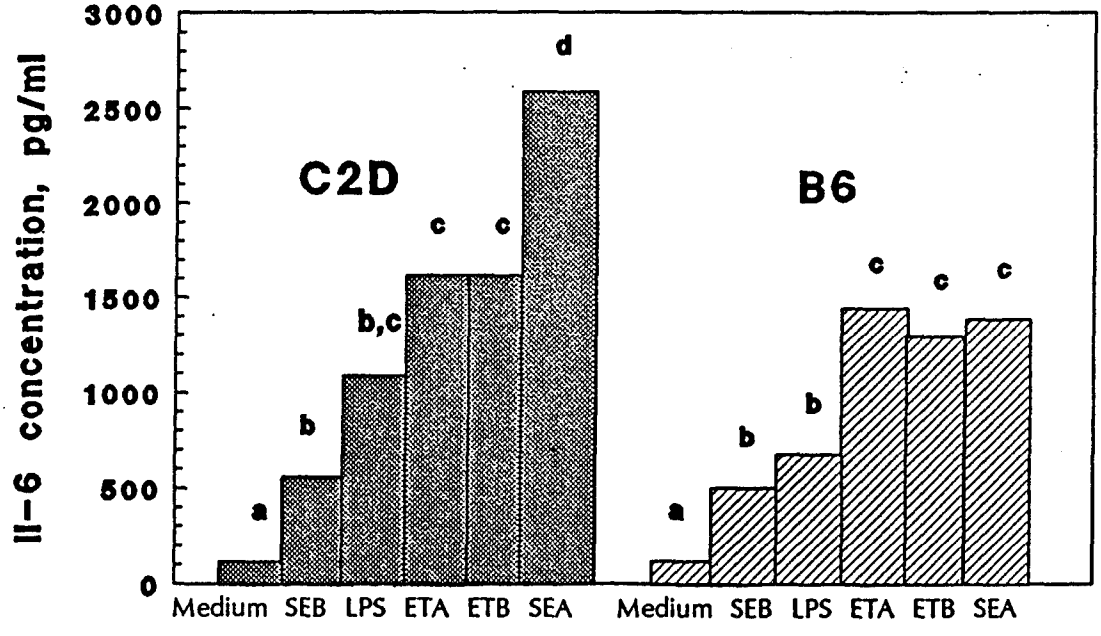

Il-6 concentration, pg/ml
3000
2500
2000
1500
1000
500
0
C2D
B6
a
b
b,c
c
c
d
a
b
b
c
c
c
Medium
SEB
LPS
ETA
ETB
SEA
Medium
SEB
LPS
ETA
ETB
SEA

FIG. 8

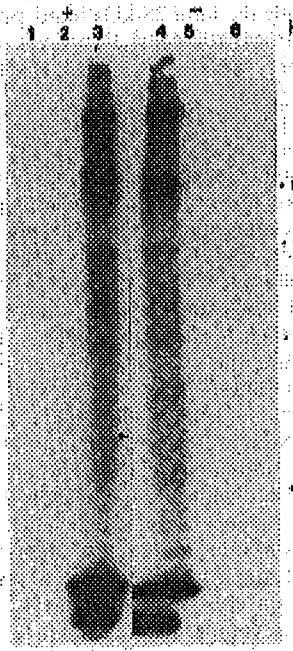
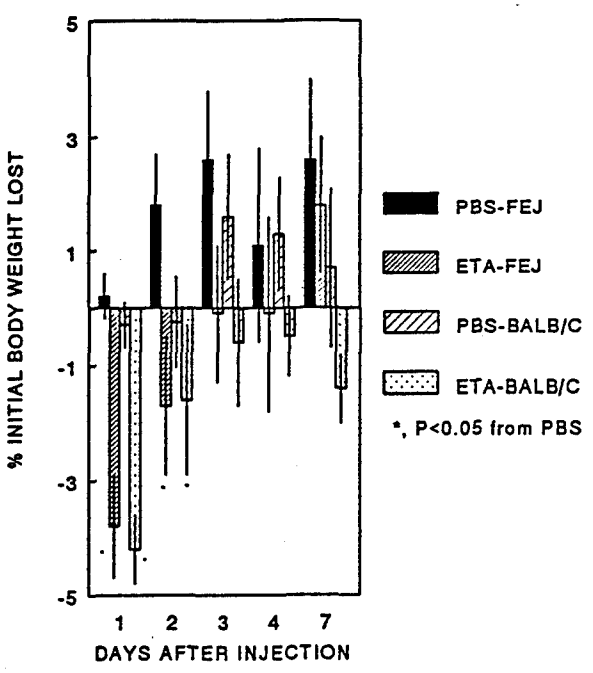


FIG. 9 Cell surface ^{125}I-labeled B6MP102 and C2D macrophages alone (lanes 1 and 6, respectively) or incubated with unlabeled SEA (lanes 3 and 4, respectively) were immunoprecipitated with rabbit anti-SEA-serum. Additionally, labeled cells were incubated with SEA and immunoprecipitated with Pansorbin (lanes 2 and 5, respectively). The arrow points to the 28-kDa band.

FIG. 10

EFFECTS OF ETA AND ETB INJECTION ON MOUSE WEIGHT LOSS



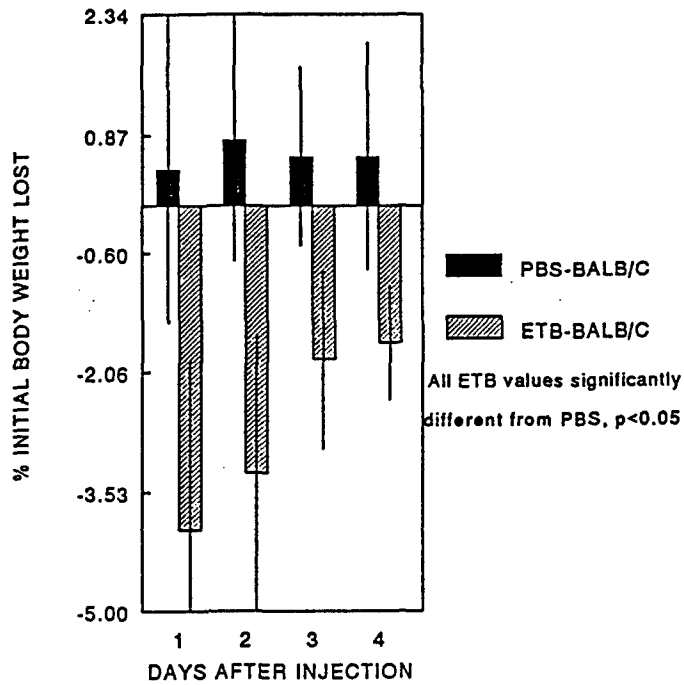


FIG. 11 92.13.1.1 DETECTION OF EXOTOXINS

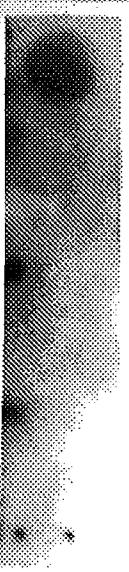
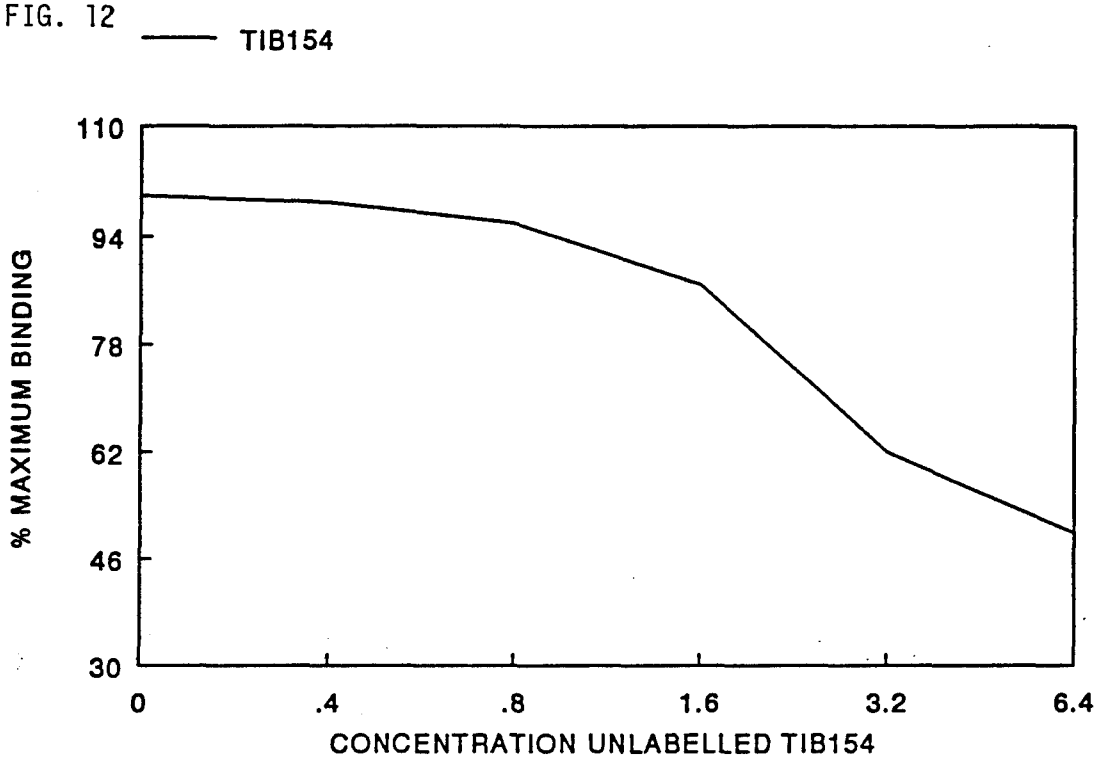
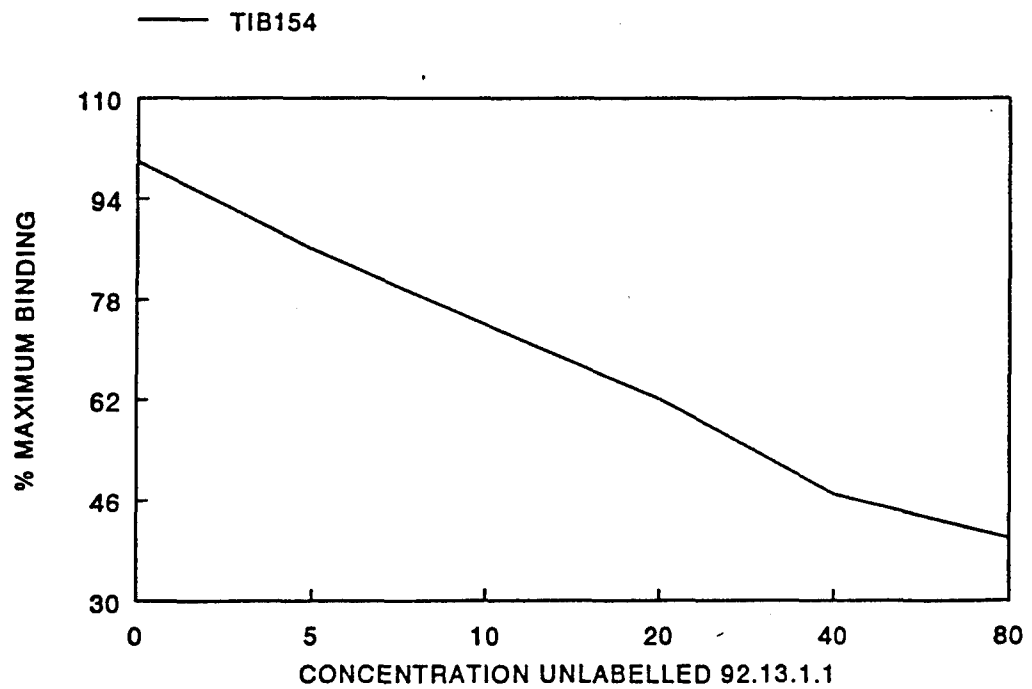
PLATE ELIZA ASSAY (A450)		SPOT BLOT
.553	ETA	
.128	ETB	
.109	SEA	
.102	SEB	
.086	BSA	

FIG. 12


TIB154
110
94
78
62
46
30
% MAXIMUM BINDING
0
.4
.8
1.6
3.2
6.4
CONCENTRATION UNLABELLED TIB154


TIB154
110
94
78
62
46
30
% MAXIMUM BINDING
0
5
10
20
40
80
CONCENTRATION UNLABELLED 92.13.1.1

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Apendices

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Glucocorticoid Effects on Immune Cell Activation by Staphylococcal Exotoxins and Lipopolysaccharide

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ABSTRACT

Experiments were conducted to determine the effects of physiologically elevated corticosterone on the activation of macrophages and T cells. These studies find that the elevation of corticosterone does not affect the expression of membrane receptors on macrophages and does not affect the activation of macrophages to produce cytokines. In contrast, elevated corticosterone levels correlate with enhanced T cell proliferation to both mitogens and superantigens.

INTRODUCTION

The activation of macrophages (Møs) and T cells is mediated by receptor ligand interactions subsequently followed by signal transduction processes (Adams et al., 1990; Hesketh et al., 1990). These processes can be altered in vivo by stress hormones such as corticosterone (CS) (Claman, 1975). Space travel is associated with a number of physiological changes in animals and humans that can affect health. One of these changes is the elevation of glucocorticoids (Grigoriev et al., 1987). Antiorthostatic suspension (AOS) is being used to stress mice and to elevate CS to determine the effects on T cells and Møs. This paper briefly reviews some findings to date.

RESULTS AND DISCUSSION

We used the AOS technique previously described by Fleming et al. (1990). Each suspension experiment involved three suspension categories: AOS (experimental), orthostatic suspension (control), and non-suspended (normal control). C3HeB/FeJ Mice were age, weight and gender matched among these three categories. Mice were suspended for 11 days for the experiments described here.

The amount of stress incurred from AOS was determined by measuring body weights, spleen weights, total spleen cell numbers and plasma CA

Table 1. Effect of antiorthostatic suspension on body weight, spleen weight, spleen cell numbers and plasma corticosterone levels.

Suspension[a]	Body weight as % initial body weight[b]	Spleen weight as % final body weight[c]	Total spleen cell count (10^3)	Corticosterone[d] ng/ml
Antiorthostatic	89 ± 0.6[e,f]	0.67 ± 0.02	1.6 ± 0.1	273 ± 26
Orthostatic	93 ± 0.8	0.85 ± 0.02	1.8 ± 0.1	135 ± 16
None	99 ± 0.6	0.91 ± 0.03	2.0 ± 0.1	94 ± 18

[a] Mice were suspended or caged normally for 11 days prior to sacrifice.

[b] Percentage is calculated by: $\frac{\text{wt. mouse post-suspension}}{\text{wt. mouse pre-suspension}} \times 100.$

[c] Percentage is calculated by: $\frac{\text{wt. spleen post-suspension}}{\text{wt. mouse post-suspension}} \times 100.$

[d] Corticosterone levels in plasma were determined by RIA.

[e] Numbers represent mean ± S.E.M. N = 43–51 mice per treatment group.

[f] Significant differences between antiorthostatic vs. orthostatic vs. none (normal control) as determined by two-tailed matched T-test analysis. $P < 0.01$ for Body Weight, Spleen Weight and Corticosterone. $P < 0.05$ for spleen cell count.

concentrations. The data in Table 1 show that antiorthostatically suspended mice are stressed significantly more than orthostatically suspended mice as determined by CS concentrations, spleen weights and weight loss. Total spleen cell numbers were also significantly lower in antiorthostatically suspended mice. CS concentrations were elevated in antiorthostatically suspended animals to an average of 3 times the concentration found in normal mice.

Because cell surface molecules are essential to Mø function the effects of AOS and elevated CS levels were determined on the expression of various Mø membrane receptors. Major histocompatibility class II molecules were examined because of their importance in antigen presentation to T cells. Also examined were the expression of two lectin receptors because of the importance of lectin receptors in phagocytosis of microorganisms. Table 2

Table 2. Expression of Class II molecules and lectin receptors on peritoneal exudate cells of antiorthostatically suspended mice.

Suspension[a,c]	Mean % positive cells[b]		
	anti-I-Ak	ConA	BSI-B4
Antiorthostatic	52 ± 3[d]	96 ± 1	62 ± 3
Orthostatic	49 ± 3	97 ± 1	63 ± 4
None	53 ± 4	97 ± 1	53 ± 4

[a] Peritoneal exudate cells were elicited by i.p. injection of C3H3B/FeJ with 700 g *P. acnes* 4 days prior to sacrifice.

[b] Percent fluorescence is expressed as the mean ± S.E.M. N = 9 mice per treatment group.

[c] Mice were suspended or caged normally for 11 days prior to sacrifice.

[d] No significant difference between any treatment as determined by two-tailed matched T-test analysis.

Table 3. Role of protein kinase C in the secretion of tumor necrosis factor.

Activator[a]	Treatment	% lysis of LM929 cells[b]	% inhibition
Eta	None	63 ± 2	
Eta	H-7[c]	0 ± 2	100
Etb	None	75 ± 2	
Etb	H-7	5 ± 4	93
Sea	None	74 ± 5	
Sea	H-7	0 ± 2	100
LPS	None	75 ± 4	
LPS	H-7	15 ± 14	80
Medium	None	0 ± 6	
Eta	None	71 ± 0	
Eta	PMA[d]	45 ± 1	37
Etb	None	40 ± 1	
Etb	PMA	10 ± 2	75

[a] Peritoneal macrophages from C3HeB/FeJ mice stimulated with 1 μg/ml Eta, Etb or Sea and 12 μg/ml LPs for 20 hours.

[b] Lysis of LM929 cells was measured by colorimetric analysis at 570 nM of formazin crystal formation after cell incubation for 3 hours with MTT.

[c] Cells were incubated in the presence of 20 μM H-7.

[d] Cells were preincubated with 10 μg/ml of PMA for 5 minutes before the addition of the activators.

shows that elevated CS concentrations did not affect the expression of class II molecules or the concanavalin A (Con A) or *Bandeiraea simplicifolia* (BSI-B_4) receptors.

The activation of Møs by staphylococcal exotoxins (Sea, Eta or Etb) or by lipopolysaccharide (LPS) to produce cytokines like TNF requires second messengers. One of the important second messenger systems important in the activation of Møs to produce TNF is the activation of protein kinase C (PKC). The treatment of Møs with the PKC inhibitor H-7 prevents the secretion of TNF by Møs activated with Eta, Etb, Sea or LPS. Pretreatment of Møs with the PKC activator, phorbol-myristate-13-acetate, also results in suppressed TNF secretion. We have investigated whether elevated CS concentrations or AOS would affect processes dependent on PKC; such as the secretion of TNF. The data in Table 4 show that AOS and the accom-

Table 4. Tumor necrosis factor-alpha production by peritoneal macrophages of antiorthostatically suspended mice.

Suspension[a]	TNF (total units made)		
	Antiorthostatic	Orthostatic	None
	104 ± 7[b]	106 ± 6	99 ± 8

[a] Peritoneal macrophages (LPS-activated, 12.5 μg/ml) and suspension as in Table 2.

[b] Numbers represent $\bar{x}$ ± SEM, N = 16, no significant differences as determined by two-tailed matched T-test analysis.

Table 5. Summary of macrophage activation.

Activity[a]	LPS	Sea	Seb	Eta	Etb	TSST-1
Tumoricidal	+	+	+	+	+	+
TNF	+	+	−	+	+	+
IL-6	+	+	+	+	+	+
NO_2^-	+	−	−	+	+	−

[a] Adapted from Fleming et al. 1991.

panying elevated CS concentrations did not inhibit TNF secretion by LPS-activated Møs. Despite numerous studies demonstrating the suppressive effects of glucocorticoids on Møs, our results did not show any correlation between CS concentration, TNF secretion or membrane molecule expression. The CS concentrations present in our animals have been shown to be inhibitory concentrations in other experiments (Norman et al., 1988). Theoretically we have achieved suppressive concentrations. It is possible that the conditions under which Møs are activated or exposed to corticosteroids can influence whether glucocorticoids are inhibitory, stimulatory or ineffective. It is also possible that the Møs became desensitized or habituated during the suspension process (Lysle et al., 1987). Studies on LPS and toxin activation of Møs may help to resolve this issue. For example, we have found that Møs respond differently to various staphylococcal exotoxins (summarized in Table 5). All of the toxins used stimulate the secretion of interleukin-6 (IL-6). In contrast only Eta and Etb stimulate nitric oxide metabolism. Seb is not able to activate Møs to secrete TNF. One explanation for this is that different second messenger systems are activated by these toxins. Future studies will investigate this hypothesis and the effects of AOS and CS on these processes.

Space flight has a profound depression on T cell responsiveness to mi-

Table 6. Suspension mouse spleen cell proliferation in response to mitogens and staphylococcal exotoxins.

	Stimulation index[a]			
Suspension[b]	Eta	Stats[c]	Seb	Stats
Antiorthostatic	3.3 ± 0.3[d]	($P = 0.10$)	2.9 ± 0.3	($P < 0.10$)
Orthostatic	2.5 ± 0.2		2.5 ± 0.3	
None	2.9 ± 0.3		2.7 ± 0.3	

[a] Spleen cell proliferation in response to Eta (1 μg/ml), Seb (1 μg/ml), PHA (9 μg/ml), and Con A (2 μg/ml), was determined by measuring incorporation of ^{3}H-thymidine in a 48 hour assay.

[b] Mice were suspended or caged normally for 11 days prior to sacrifice.

[c] Significant differences between antiorthostatic and orthostatic as determined by two-tailed matched T-test analysis.

togens. Furthermore, there are reports that CS can significantly inhibit T cell mitogenesis (Stevenson et al., 1989). In our experiments we studied the effects of AOS on splenic lymphocyte proliferative responses to the mitogens phytohemagglutinin (PHA), Con A, and the staphylococcal exotoxins [often referred to as "superantigens" (Herman et al., 1991)] Seb and Eta. Table 6 shows that both the mitogen- and toxin-stimulated T cell responses were increased by AOS. These results are not consistent with studies in which decreased proliferative responses were observed after exposure to glucocorticoids (Stevenson et al., 1989). However, several investigators have found enhanced proliferative responses of lymphocytes taken from stressed animals (Lysle et al., 1990). Some have suggested that the molecules that regulate spleen lymphocytes are different from peripheral blood lymphocytes which can be regulated by CS (Cunnick et al., 1990). Future investigations will attempt to determine if spleen and peripheral blood lymphocytes that are activated with mitogens or staphylococcal toxins are regulated differently by CS.

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Table 6. Extended.

Stimulation index[a]			
PHA	Stats	Con	AStats
9.3 ± 1.6	($P < 0.01$)	21.6 ± 2.4	($P < 0.05$)
3.2 ± 0.7		12.5 ± 2.0	
4.5 ± 0.7		13.7 ± 1.8	

[a] Numbers represent mean ± S.E.M. Spleen cells used from: N = 9 mice (per treatment group) stimulated with Eta, N = 12 mice (per treatment group) stimulated with Seb, N = 16 mice (per treatment group) stimulated with PHA and Con A.

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Differential RNA regulation by staphylococcal enterotoxins A and B in murine macrophages

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Abstract: Staphylococcal enterotoxin A (SEA) is significantly better than enterotoxin B (SEB) in activating tumor necrosis factor (TNF) secretion by B6MP102 cells. Both toxins bound to B6MP102 cells; however, SEB competed less effectively with SEA than SEA competed with SEB. This suggested that receptors unique to SEA were present on B6MP102 cells. Signal transduction occurred in response to both toxins. Within 30 s after addition, SEA and SEB significantly increased the F-actin concentration in B6MP102 cells. However, only SEA induced increased TNF mRNA levels. B6MP102 cells incubated with interferon-γ and SEB secreted TNF. However, enhanced mRNA expression was delayed and the concentration of TNF secreted was less than that of B6MP102 cells stimulated with SEA. Although these data suggest that receptors unique to SEA are present on B6MP102 cells, they also indicate that staphylococcal enterotoxins differentially regulate TNF at the RNA level, perhaps because of differences in binding to the plasma membrane. *J. Leukoc. Biol.* 55: 523-529; 1994.

Key Words: *staphylococcal enterotoxins • tumor necrosis factor • signal transduction*

INTRODUCTION

The secreted proteins of *Staphylococcus aureus* were recognized for their "mitogenic" properties in 1975 [1, 2]. Since then, a tremendous increase has occurred in the understanding of the effects of staphylococcal exoproteins on white blood cells [3-5]. Often called "superantigens," staphylococcal exoproteins stimulate specific T cell subpopulations based on their interaction with the variable region of the T cell receptor β chain [3-5]. T cell stimulation by superantigens was originally believed to require the presentation of the superantigen on nonpolymorphic regions of class II major histocompatibility complex (MHC II) molecules [6]. However, recent studies suggest that T cells may be activated in the absence of accessory cells or MHC II molecules [7-9], although activation was not as efficient as in the presence of MHC II molecules.

Macrophages are also activated by staphylococcal superantigens. Parsonnet et al. found that human mononuclear phagocytes secreted interleukin-1 (IL-1) in response to toxic shock syndrome toxin 1 (TSST) [10, 11]. Other studies corroborate these findings and also demonstrate that the enterotoxins and exfoliative toxins can activate human, rat, and mouse macrophages [12-15]. Macrophages also release IL-1 in response to alpha toxin [16].

Our group has studied the mouse macrophage response to several staphylococcal extracellular proteins [15]. There are distinct secretion patterns that are toxin and mouse strain dependent. In particular, we found that staphylococcal enterotoxin A (SEA) induced tumor necrosis factor (TNF) secretion by C3HeB/FeJ macrophages (H-2^k, *lps*^{n}) but enterotoxin B (SEB) did not. However, using monocytes of human origin, others have found that SEB could induce the transcription and/or secretion of TNF [13, 17]. Furthermore, some have suggested that macrophages require the presence of T cells to respond to staphylococcal exotoxins [18]. Therefore, it was important to determine (1) whether the differential TNF response to SEA and SEB was unique to C3H mouse macrophages, (2) whether there was a requirement for T cells, and (3) the level of activation at which differential toxin effects occurred (pre- or posttranscriptionally). To answer these questions, we used the macrophage cell line B6MP102 (H-2^b). We assessed whether SEA and SEB differed in their ability to activate TNF secretion, to bind to B6MP102 cells, and to transduce signals across the membrane and/or up-regulate TNF RNA levels.

MATERIALS AND METHODS

Cells

The colony stimulating factor 1-dependent (CSF-dependent) cell line B6MP102 (H-2^b) was described by our group previously [19, 20]. These cells begin to secrete TNF in response to 10 ng/ml lipopolysaccharide (LPS) and secrete maximal concentrations of TNF in response to 1-10 μg/ml LPS; they therefore offered a good experimental system in which to examine superantigen-induced TNF production. Cells were passaged two to three times weekly in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 0.3% L-glutamine, and 15% LM-929 cell-conditioned medium (as a source of CSF-1). B6MP102 do not secrete detectable concentrations of IL-1, IL-6, and TNF in response to CSF-1 under normal growth conditions.

Cells were seeded at the concentration of 1-3 × 10^6 cells per 60-mm culture dish or 3-5 × 10^6 cells per 100-mm tissue culture dish for secretion assays or RNA isolation. LM929 cells (American Type Culture Collection CCL 1.2) were used as a source of CSF-1 and to detect TNF. LM929 cells were passaged similarly to the B6MP102 cells. Peritoneal macrophages were obtained from C3HeB/FeJ mice as described previously [15].

Abbreviations: CSF-1, colony-stimulating factor 1; ETA, exfoliative toxin A; IFN-γ, interferon-γ; IL-1, interleukin-1; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PMB, polymyxin B; SDS, sodium dodecyl sulfate; SEA, staphylococcal enterotoxin A; TNF, tumor necrosis factor.

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Toxin

Purified preparations of SEA and SEB were obtained from Toxin Technologies (Sarasota, FL). Alternate preparations of SEB were obtained from the U.S. Army Medical Research and Development Command (Ft. Detrick, MD). Both SEB preparations gave similar results. Exfoliative toxins A and B (ETA and ETB) were prepared in our laboratory using previously described methodology [15]. The enterotoxins had less than 0.2 ng/ml endotoxin and the exfoliative toxins less than 2.0 ng/ml when diluted to a concentration of 1.0 μg/ml as determined by the *Limulus* amebocyte lysate assay (Sigma, St. Louis, MO). Our assay was sensitive to a concentration of 0.02 ng/ml. B6MP102 cells are not activated by LPS at these concentrations.

RNA isolation and Northern blot analysis

Tumor necrosis factor RNA was detected using a linearized 300-base-pair insert of the murine TNF-α gene. This gene fragment was originally described by Pennica et al. [21] and it was obtained from Genentech. The fragment was obtained by a *PstI*/*Eco*RI double digestion of the pSP64 plasmid containing the gene insert. The Chinese hamster ovary S14 ribosomal protein gene was obtained from Dr. Donald Roufa (Kansas State University). The plasmid containing the 568-base-pair fragment was linearized by *PstI* digestion of pSC14-8 [22]. The detection of S14 served as an internal control for the amount of RNA loaded for Northern blot analysis. Each linearized probe was randomly primed and labeled with digoxigenin-11-dUTP for 20 h using the Genius kit (Boehringer-Mannheim). Hybridization was carried out using nonisotypic methods as described below.

Total cellular RNA was obtained using a modified guanidine thiocyanate, phenol extraction technique (RNAzol B, Cinna/Biotecx Laboratories, Triendswood, TX). Northern hybridization was carried out using standardized concentrations of total cellular RNA that had been estimated by the staining of 18S and 28S ribosomal RNA with Nuclistain (National Diagnostics, Manville, NJ). RNA was prepared for electrophoresis by heating at 50°C for 1 h in the presence of 0.9 M deionized glyoxal, 50% v/v dimethyl sulfoxide, and 0.01 M sodium phosphate. RNA samples were electrophoresed in 1.2% agarose (containing 10 mM sodium iodoacetic acid) with constant buffer circulation. The RNA was transferred to nylon membranes, cross-linked with 2600 Å ultraviolet light, and probed for murine TNF-α and the S14 ribosomal protein housekeeping gene. Northern blots were prehybridized for 1 h at 60°C in 10 ml of buffer containing 10% dextran sulfate, 1 M NaCl, and 1% sodium dodecyl sulfate (SDS). Blots were hybridized for 20 h at 60°C in the same solution containing denatured probe and 100 μg/ml salmon sperm DNA. The membrane was washed twice with 2 × standard saline citrate (SSC) at room temperature, twice with 2 × SSC with 1% SDS at 60°C, and twice with 0.1 × SSC at room temperature. The membrane was washed twice in I-Light blocking solution (Tropix, Bedford, MA) and blocked in fresh I-Light solution for 30 min at room temperature. The blot was incubated with anti-digoxigenin-alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) for 1 h and washed. The blot was incubated with AMPPD (disodium 3-(4-methoxyspirol[1,2-dioxetane-3,2'-tricyclo[3.3.1]decane]-4-yl)phenyl phosphate) substrate as recommended by the manufacturer (Tropix) and chemiluminescence was detected on X-ray film.

Determination of F-actin

We used a modification of the methodology of Howard and Meyer [23] to measure the toxin-induced change in polymerized actin in B6MP102 cells. Peritoneal macrophages or B6MP102 cells were suspended 1×10^6 cells per 100 μl of phosphate-buffered saline (PBS) in sterile, 1.5-ml Eppendorf tubes. Ten microliters of toxin (10 μg/ml) or the stimulatory peptide f-Met-Leu-Phe [28] (1×10^{-6} M, Sigma) were added to cells and incubated for 30 s. The reaction was stopped by adding 11 μl of phosphate-buffered formalin (3.7% final concentration) to each treatment group, including the controls, and incubating the cells at 37°C for 5 min. Five microliters of the fluorescent, actin-binding compound NBD-phallacidin (according to the manufacturer, Molecular Probes, Eugene, OR, excitation maximum 465 nm, emission maximum = 530 nm, K_d = 18 nM for actin, reconstituted according to the manufacturer's directions) were added to each preparation and incubated for 10 min at 37°C. The cells were washed twice in PBS, resuspended in PBS, and analyzed on a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA) as previously described [24].

TNF quantification

Culture supernatants were assayed for TNF essentially as described by Fleming et al. [15], except that MTT (1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used to quantify cell death. Briefly, 50 μl of MTT (2 mg/ml) was added to each assay well. The samples were incubated for 3 h at 37°C. The medium was removed from each sample and 150 μl of ISO:PBS (100 ml of isopropyl alcohol, 40 μl of 5 N HCl, 50 ml of PBS) was added. The samples were read on a plate reader (Cambridge Technologies, Watertown, MA) at 570 nm.

Competitive binding assays

Exotoxins were iodinated with ^{125}I (NEN/Dupont, Boston, MA) using the chloramine-T method. The labeled toxins were aliquoted, stored at −70°C, and used within 2 weeks of iodination. For competitive binding studies, 2×10^5 to 1×10^6 B6MP102 cells were added per well of a round-bottom 96-well plate and incubated on ice with 0–12.8 μg of unlabeled toxin. A minimum of approximately 3000 cpm of ^{125}I-labeled exotoxin was added (calculated from standard dose-response curves; between 0.8 and 3.2 μg). The assay was incubated a minimum of 60 min on ice in a total volume of 200 μl of medium, which allowed 80–100% of the maximum toxin binding possible under these conditions. Cells were pelleted by centrifugation at 325*g*, and the supernatant was removed and counted on a gamma counter. Cells were washed four times, resuspended in 200 μl of medium, and counted. All treatments were done in triplicate and the experiment was repeated a minimum of four times. The reversible nature of the toxin-cell interaction [25] allowed us to calculate dissociation constants (K_d) and receptor numbers by Scatchard analysis.

Miscellaneous reagents

Recombinant murine interferon-γ and TNF-α were obtained from Genzyme (Cambridge, MA). Restriction enzymes were obtained from Promega (Madison, WI). *Escherichia coli* LPS 055:B5 was obtained from Difco (Detroit, MI).

of two receptor populations: one with a high (nM) affinity for toxin and one with a 1000-fold lower (μM) affinity (**Fig. 1A–D**). Both toxins would compete with one another in a highly reproducible manner (Fig. 1A–D). However, as determined by half-maximal inhibitory concentrations and percent maximal inhibition (**Tables 2 and 3**), SEA was a more efficient competitor of ^{125}I-SEB than SEB was of ^{125}I-SEA. These data suggest that SEA may bind to receptors not bound by SEB.

Actin polymerization in response to toxin

The cytoskeleton is important for signal transduction processes mediated through class II MHC molecules [26]. Therefore, we measured F-actin concentration changes in response to several staphylococcal exotoxins. It was possible that SEB could bind to B6MP102 cells and induce a cellular response that was independent of the TNF response. When B6MP102 cells were incubated in PBS there was a continuum of low level F-actin expression in the cells (**Fig. 2**). However, in response to the biologically active tripeptide f-Met-Leu-Phe [27], which is commonly used to activate phagocytes, there was a dramatic shift in B6MP102 actin content (Fig. 2). When the B6MP102 cells were stimulated with the exfoliative toxins, a significant increase in F-actin, similar in magnitude to the positive control, was also observed (Fig. 2). The homogeneous nature of the B6MP102 cell population is exemplified by the cytometric histograms of F-actin distribution in thioglycolate-induced peritoneal macrophages activated with several exoproteins (**Fig. 3**). In particular, two distinct populations were identified when peritoneal macrophages were stimulated with SEA or SEB. These data suggest that both SEA and SEB are capable of inducing intracellular responses in inflammatory macrophages as well as B6MP102 cells.

Northern blot of B6MP102 cell TNF RNA

Because SEA and SEB both appeared to bind to and induce actin polymerization in B6MP102 cells, it was important to determine whether the differential secretion of TNF was regulated at the RNA level or was a secretion anomaly. Northern blots indicate that cells stimulated with SEA for 3 h had increased TNF RNA levels (**Fig. 4**). Only low constitutive levels of RNA were detected in cells stimulated with SEB (Fig. 4). Therefore, SEA appeared to regulate TNF RNA differently than SEB.

Effects of interferon-γ on SEB-induced TNF production

Fast et al. [14] found that only macrophages stimulated with SEB and IFN-γ secreted nitric oxide; cells stimulated with 100 μg/ml SEB alone did not. We previously demonstrated that suboptimal concentrations of IFN-γ can enhance toxin-induced macrophage cytotoxicity [15]. Therefore, to determine whether SEB was capable of providing a "triggering signal" [28] in association with IFN-γ, we incubated B6MP102 cells with SEB and suboptimal concentrations of IFN-γ. Figure 4 shows that after stimulation of B6MP102 cells for 3 h only SEA induced increased TNF RNA levels. Basal RNA levels were detected in B6MP102 cells incubated with medium, SEB, IFN-γ, or SEB + IFN-γ. When macrophages were stimulated with SEB and IFN-γ for 18 h B6MP102 cells did secrete TNF (**Table 4**), but the detectable concentration was lower than that assayed in response to SEA. These data and the fact that IFN-γ did not enhance SEA-induced TNF production (Fig. 4) emphasize the potent nature of SEA as a macrophage stimulant.

TABLE 2. Half-Maximal Inhibitory Concentrations of SEA and SEB

	Cold competitor (μg)	
Ligand	SEA	SEB
^{125}I-SEA	4.8 ± 1.0[a]	12.4 ± 0.6[b]
^{125}I-SEB	6.3 ± 1.4	4.8 ± 1.0

[a] Numbers represent mean ± SEM of > four experiments.
[b] Statistically different from all other treatment groups, $P < .01$.

TABLE 3. Percent Maximal Inhibition of SEA and SEB

	Cold competitor (μg)	
Ligand	SEA	SEB
^{125}I-SEA	66 ± 6[a]	45 ± 7[b]
^{125}I-SEB	56 ± 2	53 ± 3

[a] Numbers represent mean ± SEM of > four experiments.
[b] Statistically different from cold SEA competition of ^{125}I-SEA, $P < .05$.

DISCUSSION

This investigation demonstrated that macrophages respond to several staphylococcal exoproteins in the absence of T

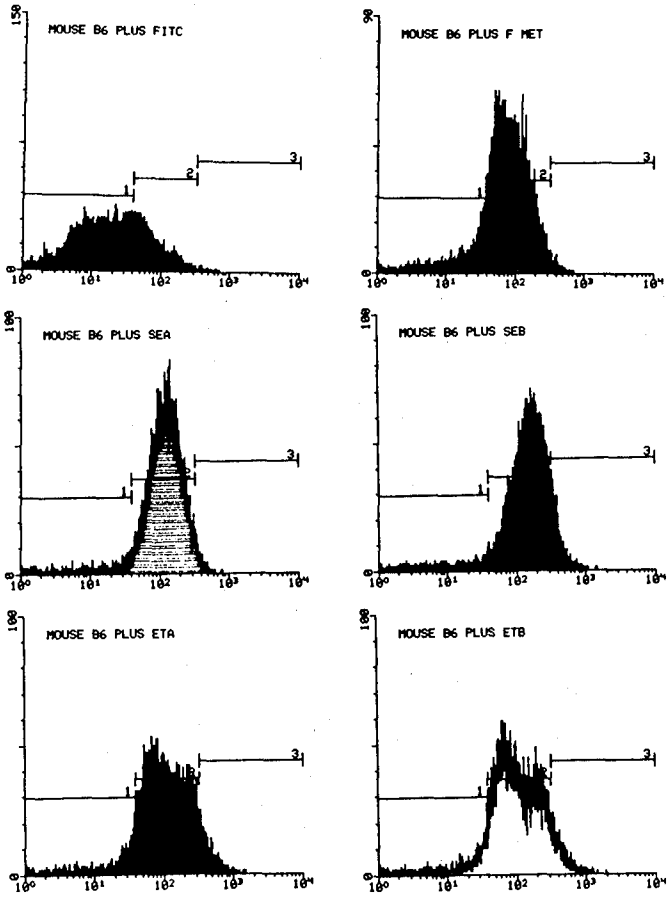

Fig. 2. Polymerized actin in B6MP102 cells detected with NBD-phallacidin and flow cytometry. B6MP102 cells were incubated in PBS (panel labeled plus FITC) or in PBS containing f-Met-Leu-Phe, SEA, SEB, ETA, or ETB (panels labeled as such). Cells were stimulated for 30 s.

RESULTS

B6MP102 cell secretion of TNF

Staphylococcal enterotoxin B does not activate C3HeB/FeJ mouse peritoneal macrophages to secrete TNF to the same extent as the related toxin SEA [15]. Furthermore, others have suggested that macrophages will not secrete TNF in the absence of T cells when stimulated with other staphylococcal exoproteins [18]. We used the bone marrow-derived macrophage cell line B6MP102 to determine whether a requirement for T cells existed in the macrophage response to staphylococcal exotoxins and whether the differential induction of TNF by SEA and SEB was unique to C3HeB/FeJ macrophages. **Table 1** shows that B6MP102 cells secreted TNF in response to several toxins in the absence of T cells. B6MP102 secreted TNF in response to ETA, ETB, and SEA as well as it responds to LPS. In contrast, SEB did not induce the secretion of TNF. Even though TNF secretion could be detected within 3 h after the addition of SEA (see Fig. 1 legend), TNF secretion could not be detected with SEB when B6MP102 cells were stimulated for as long as 18 h. (Table 1). To rule out endotoxin effects, macrophages were stimulated with 10 μg/ml SEA or SEB for 18 h in the presence of 10 μg/ml polymyxin B (PMB), which also caused differential TNF stimulation. SEA induced >13 U TNF/ml in the presence or absence of PMB, whereas SEB induced minimal quantities of TNF with or without PMB treatment. In contrast, 10 μg of PMB inhibited LPS-induced (10 μg/ml) TNF secretion by over 78%. Ten micrograms of PMB inhibited LPS-induced TNF secretion by over 95% when lower concentrations of LPS were used to stimulate the B6MP102 cells. Therefore, TNF secretion by SEA-treated B6MP102 cells does not appear to be due to endotoxin contamination.

TABLE 1. B6MP102 Secretion of TNF in Response to Staphylococcal Exotoxins

Treatment[a]	% Killing of LM929 1:2	% Killing of LM929 1:4	[TNF][b] (U/ml)
ETA (1 μg/ml)	48 ± 1	32 ± 2	76
ETB (1 μg/ml)	40 ± 2	17 ± 2	40
SEA (1 μg/ml)	33 ± 4	11 ± 2	23
SEB (1 μg/ml)	8 ± 2	0 ± 2	<1
LPS (13 μg/ml)	36 ± 4	16 ± 2	34
Medium	15 ± 3	7 ± 2	4

[a] B6MP102 cells were stimulated for 18 h.
[b] [TNF] determined by linear regression of rTNF standards with unknown samples.

It was possible that SEB was a more effective stimulus of TNF receptor shedding than SEA. Furthermore, those receptors would interfere with the quantitation of TNF in our bioassays. Therefore, we examined supernatants from SEB-activated B6MP102 cells for their ability to interfere with the quantitation of known concentrations of murine rTNF. We found that 32 U of rTNF had 68 ± 2 and 69 ± 5% specific killing of LM929 cells in the presence and absence, respectively, of supernatants from B6MP102 cells that had been stimulated with 10 μg of SEB for 18 h. It did not appear that SEB induced TNF receptor shedding.

B6MP102 binding of SEA and SEB

We determined whether the response to SEA and SEB was due to differences in binding to B6MP102 cells. We conducted cold competitor binding assays with both toxins. The biphasic nature of the Scatchard plot indicated the presence

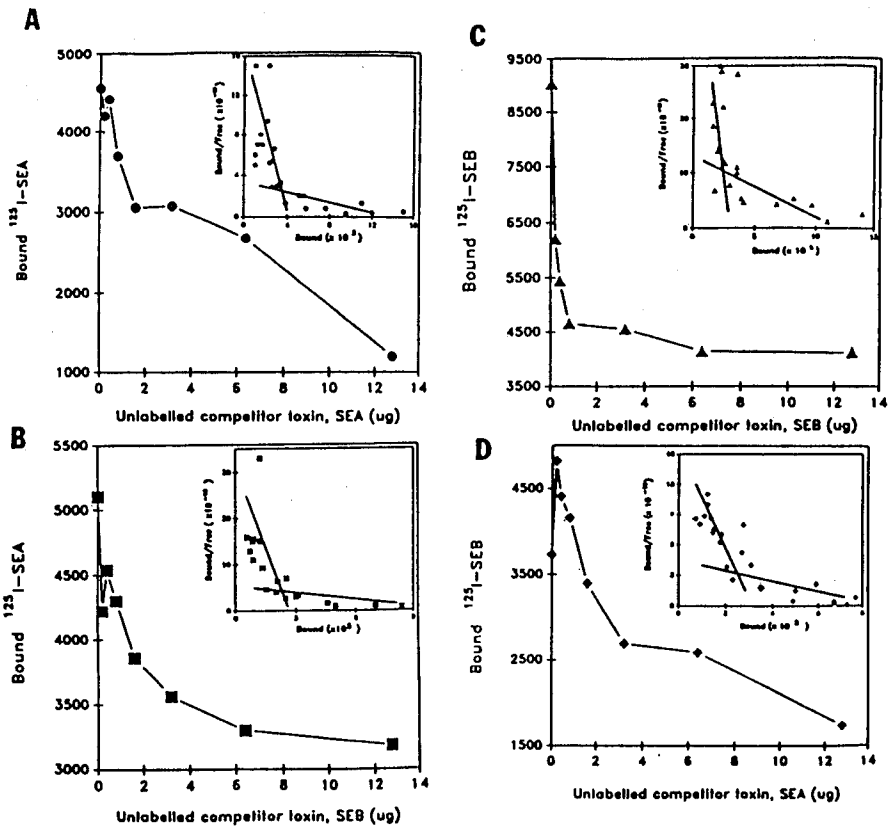

Fig. 1. Competitive inhibition of ^{125}I-SEA (A and B) and ^{125}I-SEB (C and D) binding to B6MP102 cells by cold SEA (A and D) or cold SEB (B and C). Scatchard plots (inserts) of these data were used to determine apparent K_d values and valence (receptors/cell). These graphs are representative of four to six experiments.

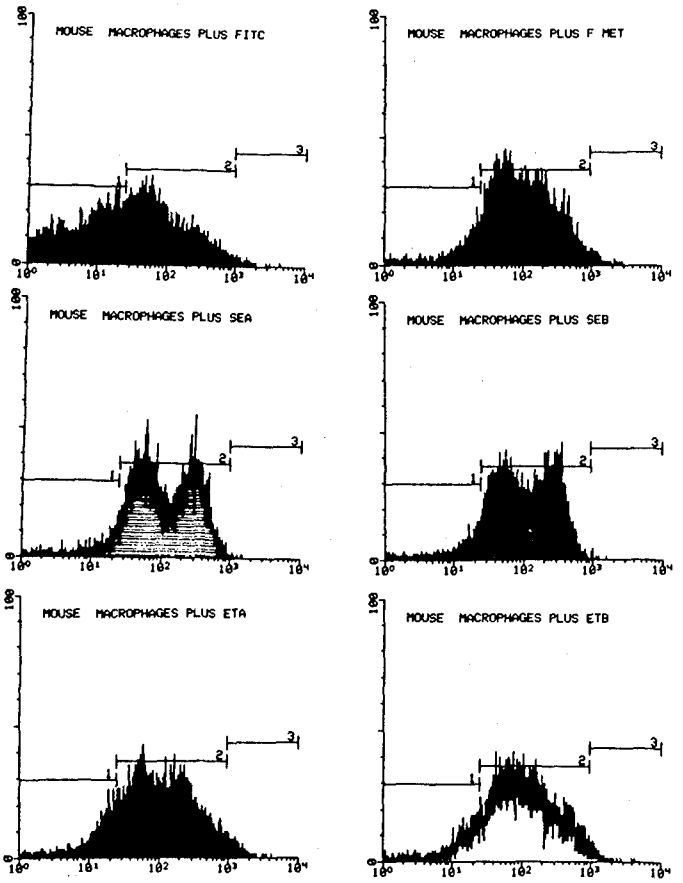

Fig. 3. Polymerized actin in peritoneal macrophages was detected using NBD-phallacidin and flow cytometry. Macrophages were incubated in PBS (panel labeled plus FITC) or in PBS containing f-Met-Leu-Phe, SEA, SEB, ETA, or ETB (panels labeled as such). Cells were stimulated for 30 s.

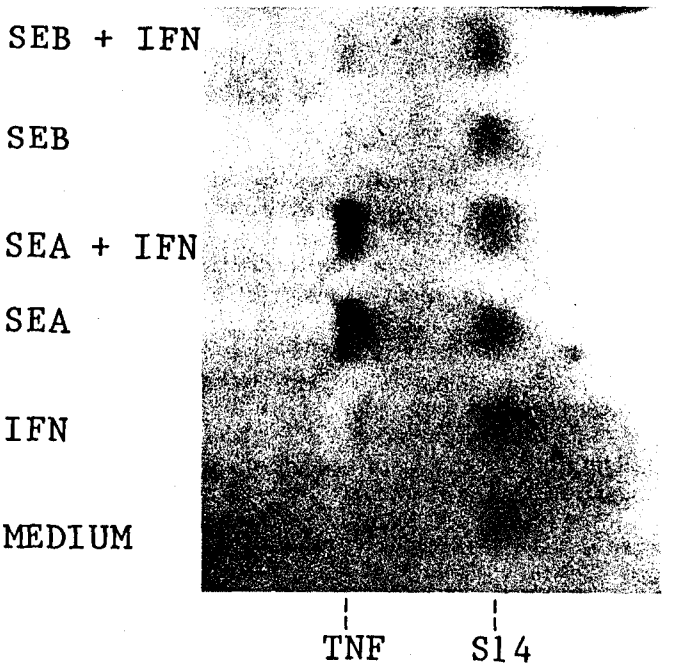

Fig. 4. Northern blot of B6MP102 RNA. Probes were specific for murine TNF-α and the ribosomal protein gene S14. B6MP102 cells were incubated for 3 h in medium or medium containing 10 μg/ml SEA or SEB ± 10 U/ml recombinant, murine IFN-γ or IFN-γ. The amount of TNF secreted by these cells was as follows: medium, <5 U/ml; IFN-γ, <5 U/ml; SEB, <5 U/ml; SEB + IFN-γ, <5 U/ml; SEA, 17 U/ml; SEA+IFN-γ, 18 U/ml.

cells. B6MP102 macrophages responded to SEA, SEB, ETA, and ETB within 30 s of toxin exposure by polymerization of actin. Within 3 h, signal transduction was followed by a burst of transcription, translation, and TNF secretion. B6MP102 cells secreted TNF in response to SEA, ETA, and ETB. Because B6MP102 cells were maintained as a continuous cell line, there was no T cell contamination. See et al. [18] found that staphylococcal exoprotein-induced TNF and IL-1 secretion by monocytes required the presence of T cells. Therefore, T cells are not necessary under some circumstances for toxin-mediated macrophage activation. The signals transduced by staphylococcal exoproteins across the macrophage membrane are sometimes inefficient and require second signals to induce particular responses. The observation, reported in this paper, of IFN-γ-enhanced SEB-induced TNF secretion and previous reports that IFN-γ enhanced macrophage tumoricidal activity induced by staphylococcal exoproteins [14, 15] would support this hypothesis. This idea also follows the general consensus that macrophage activation requires multiple signals [27-29].

These experiments indicate that SEA more efficiently induced TNF production by B6MP102 macrophages than SEB. This is consistent with previous observations using C3H mice [15] and indicates that the differential response is not limited to inflammatory, peritoneal macrophages bearing the H-2^k haplotype. Busam et al. [30] also found that SEB did not induce bone marrow macrophages to secrete TNF even though SEB did induce NF-*k*B within 1 h after exposure to SEB. It was not determined in that study or in ours whether the induction of NF-*k*B is associated with transcription of TNF. There are several binding sites for NF-*k*B in the TNF promoter and it is believed to be an important regulatory element in TNF transcription [31]. Therefore, this will be a focus of our future studies. Others have found that SEB can directly induce the secretion or transcription of TNF by human monocytes [13, 17, 32] but Grossman et al. [32] also demonstrated that SEA was a more potent stimulant of human monocytes than SEB. Exoprotein differences between human and mouse should naturally be expected. Enterotoxin B is probably a more effective stimulant of human macrophages than mouse macrophages because of differences in the toxin-MHC molecule interaction. Staphylococcal exoproteins have varying affinities for different allelic (H-2b> d > k) and species forms (human > mouse) of class II molecules [33, 34] which regulates the ability of class II-expressing cells to activate T cell proliferation [35-37].

TABLE 4. Effect of Interferon-γ on SEB-Induced TNF Secretion by B6MP102 Cells

	TNF (U/ml)[a]	
Treatment[b]	Exp. 1	Exp. 2
SEA	18	32
SEB	4	8
SEB + IFN-γ	14	14
IFN-γ	<2	6
Medium	<2	7

[a] [TNF] determined by linear regression of rTNF standards with unknown samples.

[b] B6MP102 cells were stimulated for 18 h ± 10 U/ml IFN-γ and/or 10 μg/ml SEA or SEB.

We explored mechanisms to explain why SEA but not SEB induced B6MP102 cells to produce TNF. Both toxins bound to B6MP102 cells in a concentration-dependent manner. However, SEA was a more effective competitor of SEB in competitive binding assays than SEB was of SEA. This result was similar to reports of studies with human cells in which SEB also did not compete as effectively with SEA [38]. Buxser et al [39], using spleen cells from B6 mice, found that SEB was a less effective competitor of SEA than SEE. Interestingly, their analyses were conducted with unfractionated spleen cells, many of which do not express class II molecules, whereas we worked with a pure population of macrophages. Nevertheless, we have confirmed their studies.

Our findings suggest interesting structure-function relationships between these enterotoxins. It appears that at least one toxin-induced function, TNF production, may be controlled by differential toxin binding, perhaps caused by differences in SEA and SEB structure. The amino termini of SEA and SEB appear to be important for binding to human class II MHC molecules [40–43]. Furthermore, it appears that both toxins bind a common receptor on B6MP102 cells and at the same time SEA binds a receptor not shared with SEB. It is possible that the F-actin polymerization and the activation of other macrophage functions by SEA and SEB [15] occur through the common receptor, whereas activation of TNF production is mediated through the receptor bound only by SEA. The differential binding of these two toxins to B6MP102 cells is easily explained because of differences in the structures of these molecules. Overall, there is 34% amino acid sequence homology between SEA and SEB [44]. Furthermore, in SEB regions believed to be important for MHC binding—amino acids 13–17, 39–52, and 113–166 [1]—there is 29, 42, and 42% homology, respectively, between optimized, aligned sequences. Therefore, sequence differences make it likely that the toxins bind at least some distinct sites. This hypothesis is also supported by the fact that staphylococcal enterotoxins D and E have 53 and 81% amino acid homology to SEA [44], respectively, and activate macrophages to secrete TNF (S.K. Chapes and J.J. Iandolo, unpublished observation). However, enterotoxin C_1, which is more closely related to SEB (66% amino acid homology to SEB and 29% amino acid homology to SEA and SEE [44]) does not (S.K. Chapes and J.J. Iandolo, unpublished observation).

The binding data presented here suggest that a low-affinity receptor capable of binding SEA and SEB is also present on B6MP102 macrophages. The biphasic Scatchard plot is not due to nonspecific binding because a receptor with similar properties (μM affinity and approximately 10^6 receptors) that binds staphylococcal exotoxins is also present on macrophages from class II MHC-deficient transgenic mice [45]. How this alternate toxin-binding receptor interacts with the higher (nM affinity) toxin-binding receptors is unclear. These receptors are the focus of other investigations in our laboratory [45].

Lastly, this paper demonstrates that the differential activation of TNF production by SEA and SEB is due to differences in regulation at the RNA level. It appears that SEA affects expression or stability of TNF mRNA differently than SEB. This is most likely a transcriptional effect, because others have found that staphylococcal exoproteins enhance transcription of TNF mRNA [17]. Nevertheless, it is possible that the up-regulated levels of TNF mRNA may be due not to increased transcription but to increased stability of the mRNA. There is a UA-rich nucleotide sequence in the 3'-untranslated region of TNF mRNA that controls its stability in response to stimulants [46, 47]. It is possible that SEA stabilizes the low mRNA levels we detect in our Northern blots, whereas SEB does not. SEA and SEB have intriguing differences in their ability to regulate TNF in murine macrophages. Future reports will clarify differences in the signals transduced by these two toxins and resolve the issue of mRNA levels.

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Binding and Activation of Major Histocompatibility Complex Class II-Deficient Macrophages by Staphylococcal Exotoxins†

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Macrophages from C2D transgenic mice deficient in the expression of major histocompatibility complex (MHC) class II proteins were used to identify binding sites for superantigens distinct from the MHC class II molecule. Iodinated staphylococcal enterotoxins A and B (SEA and SEB) and exfoliative toxins A and B (ETA and ETB) bound to C2D macrophages in a concentration-dependent and competitive manner. All four toxins increased F-actin concentration within 30 s of their addition to C2D macrophages, indicating that signal transduction occurred in response to toxin in the absence of class II MHC. Furthermore, ETA, ETB, SEA, and, to a lesser extent, SEB induced C2D macrophages to produce interleukin 6. Several molecular species on C2D macrophages with molecular masses of 140, 97, 61, 52, 43, and 37 kDa bound SEA in immunoprecipitation experiments. These data indicate the presence of novel, functionally active toxin binding sites on murine macrophages distinct from MHC class II molecules.

The gram-positive bacterium *Staphylococcus aureus* secretes up to 30 different exotoxins into its environment. Among these are staphylococcal enterotoxin A (SEA), the major causative agent of food poisoning, staphylococcal enterotoxin B (SEB), toxic shock syndrome toxin 1, and exfoliative toxin A (ETA) and exfoliative toxin B (ETB), which induce scalded skin syndrome (3, 26, 27). These staphylococcal exotoxins are potent T-cell stimulants and have been called superantigens (44). Enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 activate human, rat, and mouse macrophages to secrete tumor necrosis factor (TNF), NO^-, interleukin 1 (IL-1), and IL-6 (2, 13–15, 28, 35, 36). Furthermore, macrophages become cytolytic in the presence of these exotoxins and gamma interferon (13, 15).

The major histocompatibility complex class II (MHC II) molecule is a high-affinity receptor for staphylococcal superantigens. Immunoprecipitation and autoradiography studies demonstrated a direct interaction between staphylococcal exotoxins and the MHC II molecule (16, 33, 39). This interaction is unusual because unprocessed toxin binds to the nonpolymorphic regions of the MHC II molecule, away from the traditional antigen presenting site (10, 23, 38). The toxin-MHC molecule interaction occurs because toxins do not require processing and because the interaction satisfies the simple thermodynamic rules of receptor-ligand interactions. Therefore, the possibility exists that other molecules may be present on cells that have similar structural properties that allow interaction with toxin.

A superantigen binding site, distinct from the MHC II molecule, has been inferred from several studies (4, 24, 34, 43). In particular, in our laboratory, Scatchard analysis of competitive binding data from experiments using B6MP102 bone marrow-derived macrophages indicated the presence of two staphylococcal exotoxin binding receptors. However, in spite of this compelling evidence for the presence of non-MHC II superantigen binding receptors, little is known about these molecules.

Transgenic mice deficient in MHC II molecules (GenPharm International, Mountain View, Calif.) provide an excellent way to study non-MHC II toxin-binding molecules. C2D mice lack cell surface expression of MHC II molecules because of a null mutation in the MHC II Aβ gene (18). Therefore, experiments using cells from C2D mice are not subject to the criticisms made of other methods used to study alternate toxin-binding molecules. There are no concerns over whether the monoclonal antibodies used to inhibit toxin binding completely block the MHC II binding receptor or over whether MHC II molecules are present on cells at concentrations below the sensitivity of the detection assays. These experiments are also not subject to criticisms that MHC II-negative tumor cell lines are not representative of normal cells. Therefore, we used C2D transgenic mice to investigate whether staphylococcal superantigens bind and activate macrophages through a receptor distinct from the MHC II molecules.

MATERIALS AND METHODS

Mice. Transgenic mice, negative for MHC class I (MHC I) (C1D, H-2^b mice) or MHC II (C2D, H-2^b mice), were purchased from GenPharm, Int. The C1D mice were used as a syngeneic, MHC II-positive control that had undergone a genetic manipulation at a locus distinct from MHC II. We confirmed earlier findings (18) that C1D and C2D mice lacked MHC I and II molecules, respectively, by using flow cytometric analysis of thymocytes or spleen cells (1). Additionally, same-age C57BL/6 (B6, H-2^b) mice (Jackson Laboratory, Bar Harbor, Maine) were used as unmanipulated, syngeneic, MHC II-positive controls.

Cells. C1D, C2D, and B6 murine, peritoneal macrophages were obtained by peritoneal lavage as previously described (15). Bone marrow cells were recovered from long bones and were grown into macrophage monolayers on 60- or 100-mm-diameter tissue culture dishes in Dulbecco's modified Eagle medium (DMEM) (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 0.3% L-glutamine, and 15% LM-929 conditioned cell supernatant (as a source of colony-stimulating factor 1). Bone marrow cells were allowed to remain in culture for a minimum of 2 weeks before use to

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allow for the differentiation into macrophages. The TNF-sensitive cell line LM-929 (American Type Culture Collection CCL 1.2) was used as a source of colony-stimulating factor 1 and to detect TNF. LM-929 cells were passaged 2 to 3 times weekly in antibiotic-free DMEM supplemented with 2% fetal bovine serum. IL-6 was quantified with the IL-6-dependent, murine B-cell hybridoma subclone B9 (15). It was cultured in DMEM supplemented with 50 μM 2-mercaptoethanol, 5% fetal bovine serum, and 10 pg of recombinant IL-6.

Toxin. Purified preparations of SEA and SEB were obtained from Toxin Technologies (Sarasota, Fla.). SEA and SEB are a minimum of 96% pure as determined by the supplier. Alternate preparations of SEB were obtained from the U.S. Army Medical Research and Development command (Ft. Detrick, Md.). Furthermore, every new preparation of endotoxin was run through a sodium dodecyl sulfate (SDS) gel and was stained with silver (Bio-Rad Silver Stain Plus, with a sensitivity of approximately 10 ng) to check for impurities. SEA consistently stained as a single band, and both SEB preparations stained as a doublet with a molecular mass of 25 kDa. ETA and ETB were prepared in our laboratory by using strains of *Staphylococcus aureus* and the purification procedure previously described (15). ETA and ETB preparations gave single bands on gels stained with silver. The enterotoxins had ≤0.4 ng of endotoxin-like reactive material per μg of toxin, and the exfoliative toxins had less than 2.0 ng per μg, as determined by the Limulus amebocyte lysate assay (Sigma, St. Louis, Mo.). Our assay was sensitive to a concentration of 0.02 ng/ml.

Saturation and competitive binding assays and determination of K_d. Exotoxins were iodinated with ^{125}I (NEN/Dupont, Boston, Mass.) by using the chloramine-T method. Iodination of toxins achieved a level of at least 8 μCi/μg. The labeled toxins were aliquoted, stored at −86°C, and used within 2 weeks of iodination. To determine if toxin bound cells in a saturable manner, 10^6 peritoneal macrophages were added per well of a round-bottomed 96-well plate and were incubated with 1 μg of ^{125}I-labeled toxin and 0-100 μg of unlabeled toxin. Specific binding was determined by subtracting the signal obtained from ^{125}I-labeled toxin binding in the presence of a 100-fold excess of unlabeled toxin from the signal obtained with labeled toxin alone.

For competitive binding studies, 2×10^5 to 1×10^6 peritoneal or bone marrow-derived macrophages were added per well of a round-bottomed 96-well plate and were incubated on ice with 0 to 64 μg of unlabeled toxin. Between 0.5 μg and 3 μg of ^{125}I-labeled exotoxin was added. The assay was incubated a minimum of 60 min on ice in a total volume of 200 μl of medium which allowed 80 to 100% of the maximum toxin binding possible under these conditions, as determined by previous experiments in our laboratory. Assays were conducted at 4°C to avoid activation and cell attachment to the wells of the microtiter plate. Cells were pelleted by centrifugation at $325 \times g$, and the supernatant was removed and counted on a gamma counter. Pelleted cells were washed four times, resuspended in 200 μl of medium, and counted. All treatments were done in triplicate, and the experiment was repeated a minimum of three times. Dissociation constants (K_ds) and receptor numbers were determined by Scatchard analysis because of the reversible nature of the toxin-cell interaction (12).

Determination of F-actin. We used a modification of the methodology of Howard and Meyer (25) to measure the toxin-induced change in polymerized actin in macrophages. Macrophages (10^6 cells per 100 μl of phosphate-buffered saline [PBS]) were suspended in sterile, 1.5-ml Eppendorf tubes. Ten microliters of toxin (10 μg/ml) or, as a positive control, F-Met-Leu-Phe (10^{-6} M, Sigma) (25) was added to cells and incubated for 30 s. The reaction was stopped by adding 10 μl of phosphate-buffered formalin (final concentration, 3.7%) to each treatment group, including the controls, followed by incubation at 37°C for 5 min. Five μl of the fluorescent, actin-binding compound NBD [*N*-(7-nitrobenz-2 oxa-1, 3 diazol 4)]-phallacidin (Molecular Probes, Eugene, Ore.) (according to the manufacturer, having an excitation maximum of 465 nM, an emission maximum of 530 nM, and a K_d of 18 nM for actin and reconstituted according to the manufacturer's directions) was added to each preparation and was incubated for 10 min at 37°C. The cells were washed twice in PBS, resuspended in PBS, and analyzed on a FACScan flow cytometer (Becton Dickinson, Sunnyvale, Calif.) as previously described (25).

Macrophage stimulation. Macrophages were plated at a density of 10^6 cells per 60-mm-diameter tissue culture plate and were allowed to adhere 1 h, and then the medium was removed. Three milliliters of DMEM containing the appropriate stimulus (10 μg of toxin per ml or 12.5 μg of lipopolysaccharide per ml [used as a positive control]) or medium alone (control) was added for 16 h. After this time, the supernatants were collected, clarified by centrifugation, aliquoted, and used immediately or stored at −86°C until assayed for cytokine secretion.

Cytokine and nitrite quantitation. The macrophage supernatants were assayed for IL-6 content by using the B9 bioassay (21). The B9 cells were washed three times in IL-6-free RPMI medium to remove residual IL-6. Four thousand B9 cells were added to serially diluted, triplicate samples of culture supernatant and were allowed to incubate for 3 days at 37°C. Cell death was quantified by using MTT (1-[4,5-dimethylthiazol-2-*Y*1]-2,5-diphenyltetrazolium bromide). Fifty μl of MTT (2 mg/ml) was added to each assay well and was incubated for 3 h at 37°C. The medium was removed, and 150 μl of Iso-PBS (100 ml of isopropyl alcohol, 40 ml of 5 N HCl, and 50 ml of PBS) was added to dissolve crystals. A microtiter plate reader (Cambridge Technologies, Watertown, Mass.) was used to read A_{570}.

TNF was quantified as described previously (15) except that MTT was used (see description above). Nitrite concentration was determined by use of the Griess reagent (1% sulfanilamide, 0.1% naphylethylene diamine dihydrochloride, and 2% H_3PO_4) as previously described (41). Cytokine and nitrite quantification was based on linear regression of standard curves of recombinant TNF, recombinant IL-6, or $NaNO_2$. Recombinant murine TNF was obtained from Genzyme (Cambridge, Mass.), and recombinant murine IL-6 was obtained from R and D Systems (Minneapolis, Minn.).

Immunoprecipitation. B6MP102 cells and C2D peritoneal macrophages (10^8) were surface labeled with ^{125}I (NEN/Dupont, Boston, Mass.) with lactoperoxidase (Calbiochem) and were incubated with or without 50 μg of unlabeled SEA per ml for 2 h at 4°C. Washed cells were lysed in 1 ml of ice-cold lysis buffer (2% Nonidet P-40, 15 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, and aprotinin). Lysates were precleared for 1 h at 4°C with normal rabbit serum followed by the addition of fixed *S. aureus* bacteria (Pansorbin; Calbiochem). Aliquots were immunoprecipitated with rabbit anti-SEA serum and were immobilized on Pansorbin. Precipitates were washed three times with ice-cold lysis buffer and then boiled in SDS sample buffer, centrifuged, and run on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. ^{125}I-labeled receptors that bound toxin were imaged by exposing dried gels to X-ray film.

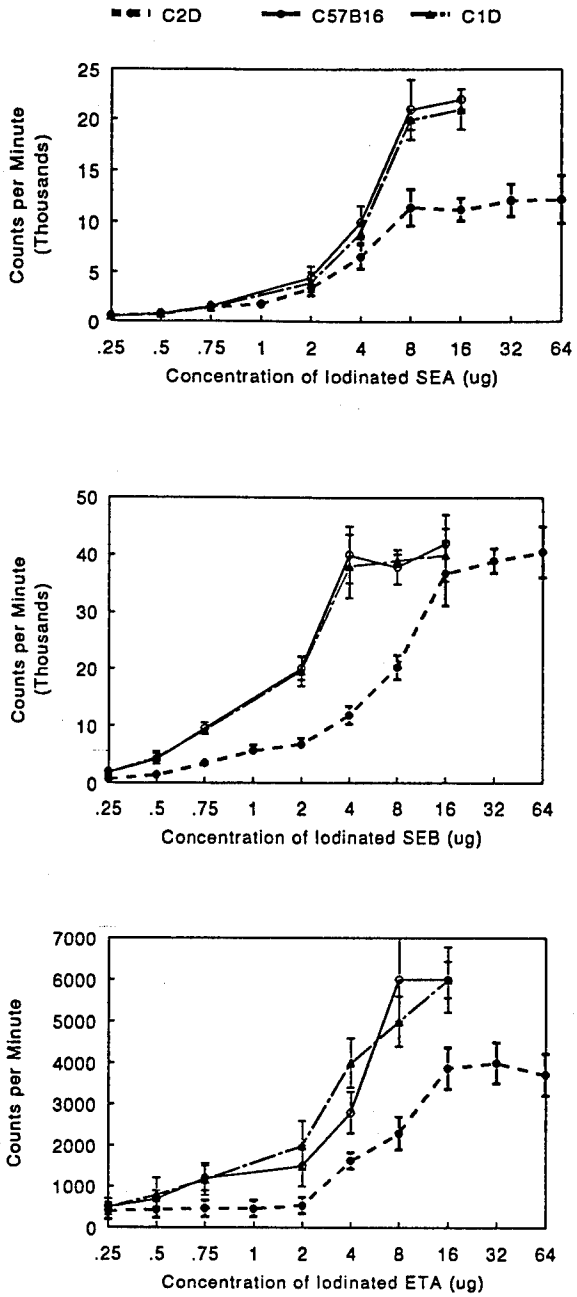

FIG. 1. Dose response curves demonstrating specific and saturable binding of ^{125}I-SEA, ^{125}I-SEB, and ^{125}I-ETA to 10^6 C2D, C1D, or C57BL/6 peritoneal macrophages. Specific binding is the difference between the signal obtained with labeled toxin alone and the signal obtained in the presence of a 100-fold excess of unlabeled toxin. These graphs represent the results of at least three experiments.

RESULTS

Macrophage binding by staphylococcal exotoxins. We determined if exotoxins could bind to non-MHC II receptors on C2D macrophages. Additionally, we calculated the affinity of the interaction between superantigen and non-MHC II receptors. Iodinated SEA, SEB, ETA (Fig. 1), and ETB (data not shown) specifically bound to MHC II-positive and -negative peritoneal and bone marrow-derived macrophages in a concentration-dependent manner, and the binding was saturable for SEA, SEB, and ETA (Fig. 1) (ETB was not tested in these assays). The kinetics of toxin binding to C2D peritoneal macrophages was similar to that to control macrophages, but higher toxin concentrations were necessary to achieve binding comparable to that of MHC II-positive controls (Fig. 1). Iodinated SEA and SEB also bound C2D cells isolated from lymph nodes, in a similar concentration-dependent way (data not shown).

Competitive binding assays, in which 0.5 to 3 μg of iodinated SEA or SEB was inhibited with increasing concentrations of cold SEA or SEB, respectively, indicated that competition occurred for a limited number of binding sites on both MHC II-positive (Fig. 2) and -negative cells. However, the kinetics of the inhibition differed for the MHC II-positive and -negative cells. For the MHC II-positive cells, it usually took less than 6 μg of cold toxin to inhibit binding of the iodinated toxin by 50%. At least 12 μg of cold competitor toxin was needed to achieve 50% inhibition when C2D macrophages were used. Scatchard analysis of C2D macrophage superantigen binding data indicated that a linear line best fit the data indicative of a single binding site class (Slide Write Plus, revision 4; Advanced Graphics Software, Sunnyvale, Calif.) (Fig. 2). The affinity for superantigen of the non-MHC II receptor from both bone marrow and peritoneal macrophages was lower than the affinity measured for the MHC II-positive cells (Table 1). SEA tended to bind C2D macrophages with higher affinity than did SEB, but the difference was not significant. No trend was evident when the numbers of receptors per cell for MHC II-positive and -negative macrophages were compared (Table 1). In contrast to the linear curves found for C2D macrophages, biphasic Scatchard curves indicative of two receptor sites (Fig. 3) were found for B6 peritoneal macrophages.

Actin polymerization in response to toxin. Exotoxin binding to MHC II-positive macrophages leads to activation (9). The cytoskeleton is important to early signal transduction processes. These events can be measured through changes in cellular F-actin concentration. Therefore, to determine if staphylococcal superantigens induce early signal transduction events, we measured changes in F-actin concentration. Exposing MHC II-positive macrophages to all four toxins induces actin polymerization (9). Similarly, unstimulated peritoneal macrophages from MHC II-negative C2D mice responded to the biologically active tripeptide F-Met-Leu-Phe (used as a positive control on the basis of previous work) (9), as well as SEA, ETA, and ETB (Fig. 4). A smaller increase in F-actin concentration occurred when C2D macrophages were stimulated with SEB. However, the changes were not as consistent as those seen with the other toxins, because some of the macrophages did not respond (Fig. 4D). Therefore, superantigens activated rapid intracellular responses in inflammatory C2D macrophages.

Effects of toxin on cytokine secretion. Normal macrophages release cytokines in response to staphylococcal exotoxins (13–15, 28, 35, 36). The alternative toxin binding molecule may play a role in macrophage activation. Therefore, we investigated the potential of exotoxins to activate C2D macrophages to secrete nitrite, TNF, and IL-6.

The results of these experiments were positive. Bone marrow macrophages from C2D and B6 mice secreted IL-6 when they were stimulated with staphylococcal exotoxins (Fig. 5). The amount of IL-6 secreted varied with each toxin stimulant. IL-6 secretion was especially high for SEA-stimulated C2D macrophages, while SEB induced the secretion of low concentrations of IL-6. LPS also induced the secretion of IL-6 by C2D and control B6 macrophages. However, the majority of the toxins were more potent stimulants than was LPS. Addition-

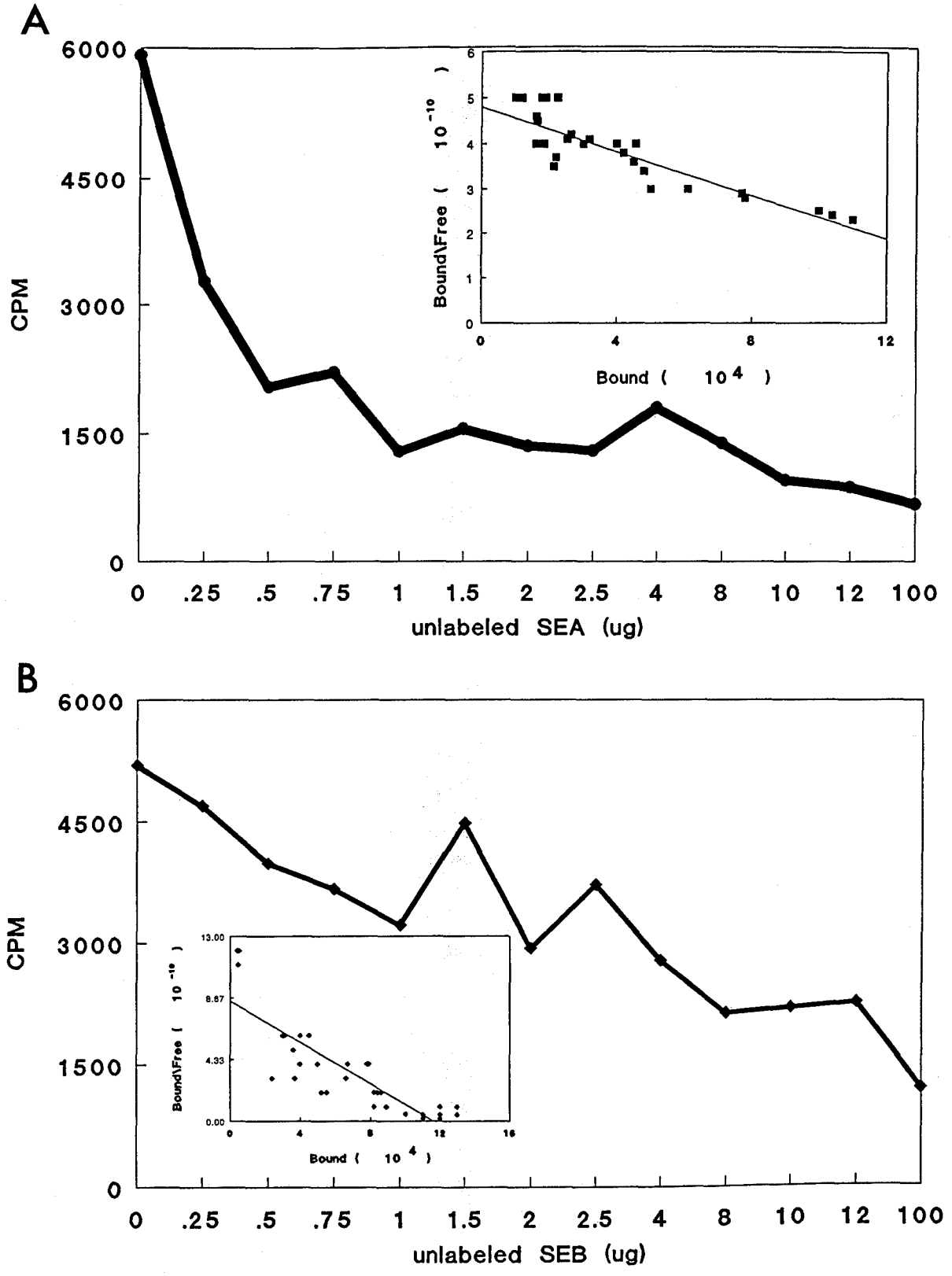

FIG. 2. Competitive inhibition of ^{125}I-SEA (A) and ^{125}I-SEB (B) binding to C2D macrophages by cold SEA or cold SEB. Scatchard plots (inserts) of these data were used to determine apparent K_ds and valences (receptors per cell). These graphs are representative of the results of at least three experiments.

TABLE 1. Binding properties of SEA and SEB for C1D, C2D (C2D-P), and C57BL/6 (B6) peritoneal and C2D (C2D-B) bone marrow macrophages[a]

Cell source	Binding of:			
	^{125}I-SEA vs SEA		^{125}I-SEB vs SEB	
	K_d	No. of receptors/cell	K_d	No. of receptors/cell
B6	1.9×10^{-7}	2.3×10^{6}	1.3×10^{-7}	2.0×10^{6}
C1D	1.4×10^{-7}	2.9×10^{6}	1.1×10^{-7}	2.6×10^{6}
C2D-P	2.0×10^{-6}	3.1×10^{6}	7.3×10^{-5}	3.9×10^{6}
C2D-B	6.6×10^{-5}	3.3×10^{6}	3.8×10^{-5}	5.1×10^{6}

[a] Values are means of results of at least three experiments in which K_d and number of receptors per cell (valence) were determined by Scatchard analysis of competitive inhibition of ^{125}I-SEA and ^{125}I-SEB binding to macrophages by cold SEA or cold SEB (^{125}I-SEA versus SEA and ^{125}I-SEB versus SEB, respectively).

ally, SEA, ETA, and ETB stimulated IL-6 secretion by peritoneal macrophages from C2D and B6 mice (Table 2). However, the level of secretion was lower than that of bone marrow macrophages.

C2D peritoneal, but not bone marrow, macrophages stimulated with SEA, ETA, and ETB also secreted TNF (Fig. 6 and Table 2). However, the TNF response was considerably lower than that seen with MHC II-positive macrophages (Table 2). C2D peritoneal macrophages also secreted nitric oxide in response to several of the exotoxins (Table 2). Again, the secretion of nitric oxide by C2D macrophages was less than that by MHC II-positive macrophages.

To rule out endotoxin effects, macrophages were stimulated with 10 μg of exotoxin per ml for 18 h in the presence of 10 μg of polymyxin B (PMB) per ml. Secretion of IL-6 and TNF by C2D macrophages in response to exotoxins did not significantly change (Table 2). In contrast, 10 μg of PMB inhibited LPS-induced TNF and IL-6 secretion by over 70% (Table 2). Therefore, cytokine secretion by toxin-treated C2D macrophages did not appear to be due to endotoxin contamination.

Immunoprecipitation. We used cell surface labeled C2D macrophages and B6 macrophages (MHC II-positive) incubated with unlabeled SEA to identify the alternative binding sites. Rabbit anti-SEA-serum precipitated bands with molecular masses of approximately 140, 97, 61, 52, 43, and 37 kDa from C2D and B6 macrophages preincubated with SEA (Fig. 7). Additionally, a very light band with a molecular mass of approximately 28 kDa was precipitated from B6 macrophages. In the absence of SEA, no cell surface molecules were precipitated (Fig. 7).

DISCUSSION

We have presented strong evidence that one or more alternative receptors exist on macrophages for staphylococcal superantigens. Our unique findings are supported by the observations of other investigators. Komisar et al. (30) reported that SEB could stimulate rat and mouse mast cells to release serotonin, but the binding site for SEB was not an MHC II molecule. Herrman et al. (24) reported that both human and mouse MHC II-negative target cells could be recognized (i.e., lysed) in a staphylococcal exotoxin-dependent fashion by cytotoxic T-lymphocyte (CTL) clones. Different staphylococcal enterotoxins were recognized preferentially by CTL on MHC II-positive and -negative targets. However, not all MHC II-negative cells could present superantigen to CTL, whereas all MHC II-positive lines tested could. Dohlsten et al. (11) reported that SEB and SEC, but not SEA and SED, could be effectively presented by MHC II-negative colon carcinoma cells. Recently, Bhardwaj et al. (4) reported that MHC II-negative, Ket/BLS1 cells showed a specific binding of ^{125}I-labeled SEA, indicating a low-affinity SEA binding site.

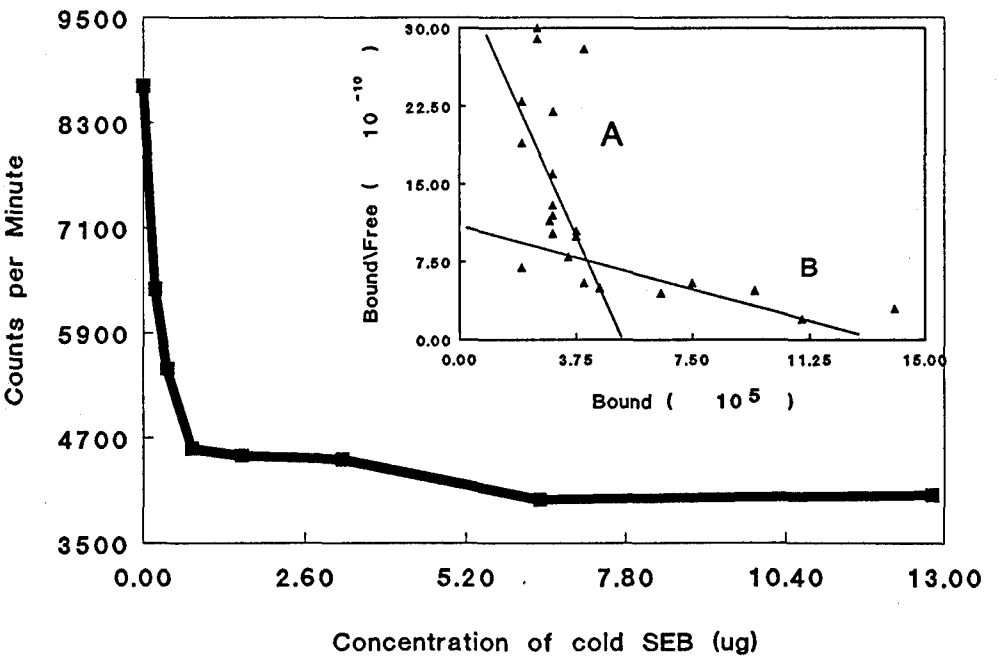

FIG. 3. Competitive inhibition of ^{125}I-SEB binding to C57BL/6 peritoneal macrophages by cold SEB. Scatchard plots (inserts) of these data were used to determine apparent K_ds and valences (receptors per cell). The line labeled A represents a K_d of 7.0×10^{-8} M and 5×10^{5} binding sites. The line labeled B represents a K_d of 3×10^{-5} M and 1.3×10^{6} binding sites.

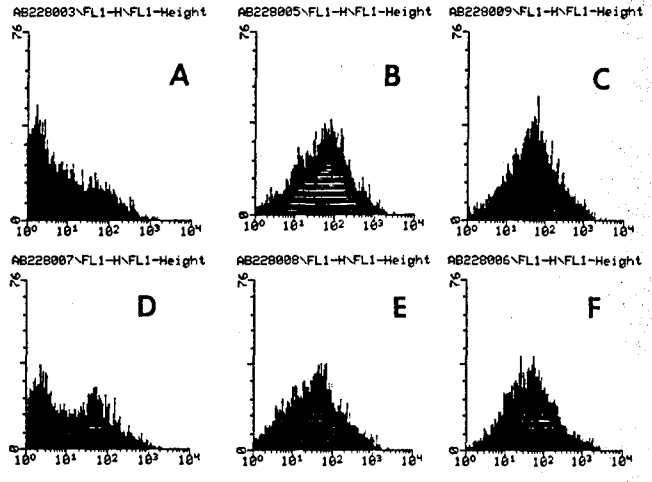

FIG. 4. Polymerized actin in C2D peritoneal macrophages was detected by using NBD-phallacidin and flow cytometry. Macrophages were incubated in PBS (A) in PBS containing F-Met-Leu-Phe (10^{-6} M) (B), or in 10 μg of SEA, SEB, ETA, or ETB per ml (C, D, E, and F, respectively). Macrophages were stimulated for 30 s and then fixed.

Viewed as a whole, the evidence indicates that molecules other than MHC II can bind staphylococcal superantigens.

Several staphylococcal exotoxins, including members from the enterotoxin and exfoliative toxin groups, bound to C2D macrophages. These data suggest that there are conserved structural features of these toxins that allow them to bind to a common receptor. It appears that the amino terminus (17, 29, 32, 37) of the enterotoxin is required for interaction with the MHC II molecule. However, no such data are available for the exfoliative toxins, nor is it known what portion of the toxin from either of these groups binds to the non-MHC II receptors. Therefore, it is not clear what these conserved toxin features are. The complex folding of the enterotoxins (42) and the suggestion that carboxyl portions of the enterotoxins (17, 20) also are necessary for MHC binding indicate that tertiary structure influences toxin-receptor interactions and does not allow for simple linear amino acid comparisons between these groups.

SEA and SEB bound to peritoneal and bone marrow-derived macrophages from C2D mice with a much lower affinity (10^{-5} M) than macrophages from either C1D or B6 control mice (10^{-8} M). This is consistent with reports of Hermann et al. (24) that found that the concentration of enterotoxin needed to lyse MHC II-negative cells was 10- to 100-fold higher than the concentration required to lyse MHC II-positive cells. Additionally, Bhardwaj et al. (4) noted that SEA binding to MHC-negative cells could be detected only at very high concentrations of toxin. In contrast, Lee et al. (31) found that TSST-1 bound to MHC II-negative porcine aortic endothelial cells with a K_d of 5×10^{-7} M. This could reflect a species difference. It has been established that human cells have a higher affinity for toxin than do mouse cells (22). Additionally, we found that toxic shock syndrome toxin 1 was, by far, the most biologically active superantigen (15); this may reflect a basic difference in toxin affinity for the non-MHC II receptor. It is also possible that different alternative receptors

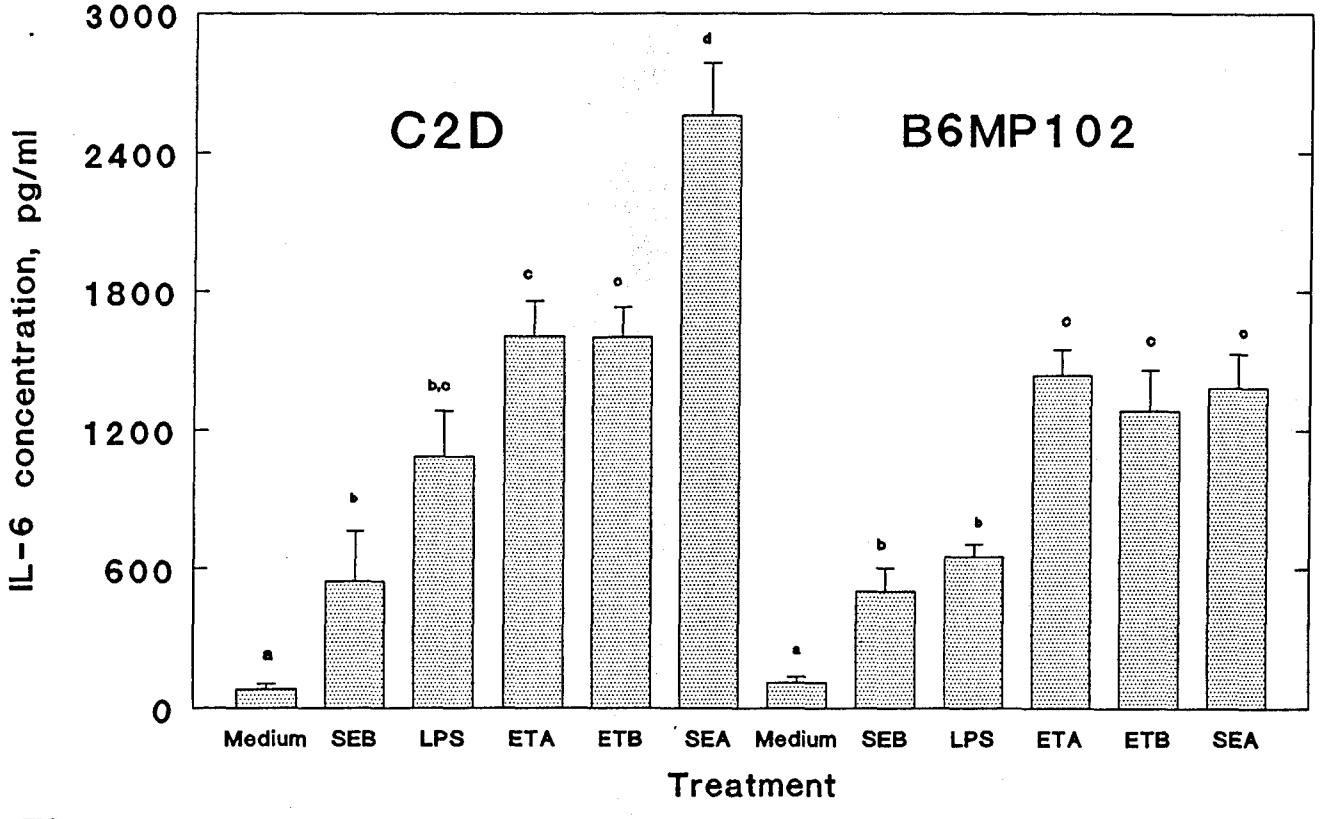

FIG. 5. Secretion of IL-6 by C2D and C57BL/6 bone marrow-derived macrophages in response to staphylococcal exotoxins (10 μg/ml) or LPS (12.5 μg/ml). Cells were stimulated for 18 h. IL-6 concentration was determined by linear regression of recombinant IL-6 standards with unknown samples. Columns with different superscripts differ ($P < 0.05$).

TABLE 2. Summary of peritoneal macrophage activation[a]

Macrophage source and treatment	Activity in C2D cells			Activity in B6 cells		
	TNF (U/ml)	IL-6 (pg/ml)	NO_2^- (nM)	TNF (U/ml)	IL-6 (pg/ml)	NO_2^- (nM)
Medium alone	4 ± 3	296 ± 122	1 ± 1	4 ± 2	175 ± 54	8 ± 4
SEA	14 ± 5	3,100 ± 235[b]	6 ± 2	52 ± 9[b]	2,003 ± 312[b]	30 ± 12[b]
SEA + PMB	13 ± 4	3,000 ± 245[b]	ND[c]	49 ± 6[b]	1,987 ± 269[b]	ND
SEB	10 ± 2	622 ± 109[b]	2 ± 1	6 ± 5	1,222 ± 99[b]	22 ± 9[b]
SEB + PMB	9 ± 2	655 ± 100[b]	ND	6 ± 4	1,234 ± 103[b]	ND
ETA	11 ± 3	1,523 ± 264[b]	4 ± 1	24 ± 10[b]	1,600 ± 119[b]	20 ± 9[b]
ETA + PMB	10 ± 2	1,224 ± 287[b]	ND	21 ± 5[b]	1,523 ± 123[b]	ND
ETB	16 ± 1[b]	2,342 ± 387[b]	6 ± 2	23 ± 8[b]	1,700 ± 298[b]	22 ± 11[b]
ETB + PMB	14 ± 3	2,007 ± 284[b]	ND	21 ± 9[b]	1,593 ± 356[b]	ND
LPS	50 ± 5[b]	1,467 ± 236[b]	12 ± 3[b]	76 ± 4[b]	1,434 ± 216[b]	95 ± 12[b]
LPS + PMB	15 ± 7	675 ± 87[b]	ND	20 ± 3[b]	621 ± 132[b]	ND

[a] Mean activity ± standard deviation from culture supernatants of peritoneal macrophages incubated with and without exotoxin- or endotoxin-containing medium with and without polymyxin B (PMB) for 18 h.
[b] Different from medium ($P < 0.05$).
[c] ND, not determined.

have been identified. Further characterization of the toxin receptor on C2D macrophages should resolve this issue.

The toxin binding affinity reported for normal B6 peritoneal macrophages in this study was similar to those reported for other MHC II-positive cells (7). However, if the alternative binding receptor is present on normal cells, as indicated by the biphasic nature of the Scatchard analysis, this value represents the binding of both receptors. We hypothetically divided the two receptor classes presumed to be found on normal macrophages and calculated the dissociation constants (Table 3). The high-affinity receptor probably represents MHC II. Interestingly, the low-affinity receptor has a binding constant that resembles the toxin receptor on C2D macrophages. For example, the low-affinity toxin receptor on normal macrophages had an affinity of 9×10^{-5} M for SEB, compared with an affinity of 5×10^{-5} for the SEB toxin receptor on C2D macrophages. It should be noted that the amount of toxin (measured in nanomoles) often used in receptor analysis was usually below the amount of toxin (measured in micromoles) needed to detect the alternative receptors which we have characterized in our laboratory. Therefore, it is likely that these alternative molecules are simply overshadowed by the higher-affinity MHC II molecule.

Binding of staphylococcal exotoxins to C2D macrophages leads to cellular activation in much the same way normal macrophages respond (14, 15). After toxin binding, signal transduction occurred rapidly (within 30 s) and was subsequently followed by macrophage secretory activity. Interestingly, toxin-induced IL-6 secretion in C2D macrophages equalled or exceeded that seen with normal B6 macrophages. This was not true for the TNF or nitric oxide secretory responses, suggesting that the low-affinity toxin-binding mole-

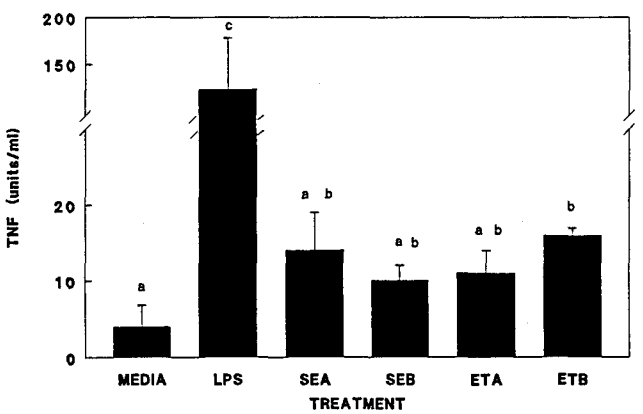

FIG. 6. Secretion of TNF by C2D peritoneal macrophages in response to staphylococcal exotoxins (10 μg/ml) or LPS (12.5 μg/ml). Cells were stimulated for 18 h. TNF concentration was determined by linear regression of recombinant murine TNF standards with unknown samples. Columns with different superscripts differ ($P < 0.05$).

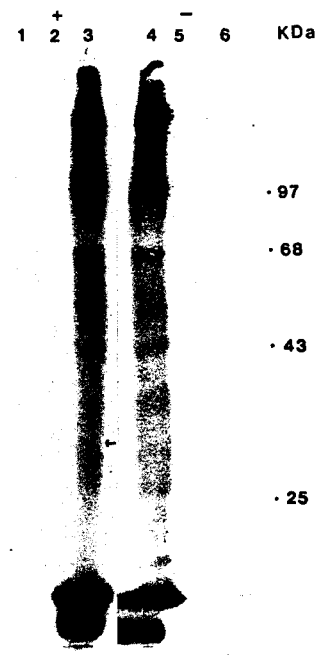

FIG. 7. Cell surface ^{125}I-labeled B6MP102 and C2D macrophages alone (lanes 1 and 6, respectively) or incubated with unlabeled SEA (lanes 3 and 4, respectively) were immunoprecipitated with rabbit anti-SEA-serum. Additionally, labeled cells were incubated with SEA and immunoprecipitated with Pansorbin (lanes 2 and 5, respectively). The arrow points to the 28-kDa band.

TABLE 3. Comparison of the toxin binding the affinities of receptors on normal and C2D macrophages[a]

Toxin	Binding affinity of receptors on:					
	Normal macrophages				C2D macrophages[b]	
	MHC II receptor		Alternative receptor			
	K_d	No. of binding sites	K_d	No. of binding sites	K_d	No. of binding sites
SEA	8×10^{-8}M	4×10^5	8×10^{-5}M	1×10^6	3×10^{-5}M	3×10^6
SEB	7×10^{-8}M	3×10^5	9×10^{-5}M	1×10^6	5×10^{-5}M	4×10^6

[a] Values were obtained by Scatchard analysis of at least three experiments.
[b] Values are averages from peritoneal and bone marrow C2D macrophages.

cules control responses different from those controlled by MHC II molecules. Our laboratory and others have found that SEB, compared with other enterotoxins, does not induce TNF very effectively (7, 13). A similar observation was made during this study. Interestingly, SEB was less effective than the other toxins in inducing IL-6 secretion by C2D macrophages, in contrast to the IL-6 secretory response induced by SEB stimulation of MHC II-positive macrophages with the H-2^k genotype (9). It appears that the IL-6 response is less dependent on the presence of MHC II than are other cytokine responses. Furthermore, the SEB-induced IL-6 response, through the alternative receptor(s), may be poorer because of lower affinity (Table 1) of SEB for the alternative binding sites. Thus, it is becoming evident that the macrophage response to staphylococcal superantigens is regulated by at least two distinct receptors, which display some polymorphism in the ways they bind toxin and in their signal complex secretory responses.

By immunoprecipitation, we identified a heterogeneous array of molecules on C2D peritoneal macrophages capable of binding SEA. These same proteins were present on MHC II-positive cells. Only the faint 28-kDa band, presumably MHC II, was unique to B6MP102 cells. That level of its expression would be consistent with the fact that B6MP102 does not express high concentrations of MHC II on its surface. The nature of the other proteins is still unresolved, but there are some candidate molecules. Cantor et al. (8) have suggested that VLA-4-like molecules may be capable of binding enterotoxins C1 and E but not other toxins. The two chains of this integrin have molecular masses of 110 and 150 kDa. It is possible that the 140- and 97-kDa molecules which we identified are VLA-4. Differences in gel conditions could explain the slight variations in size. Alternatively, C2D mice could have integrin molecules slightly different from those of normal mice. The 43-kDa molecule that was imaged could be MHC I. This hypothesis and observation are consistent with previous observations. Ezepchuk et al. (12) first identified a 43-kDa molecule capable of binding SEA in 1983. Fraser, in 1989 (16), suggested that MHC I would bind nonspecifically, if high concentrations of SEA were incubated with GM4672A cells. Stiles et al. (40) suggested that transgenic MHC I-deficient mice were less sensitive to exotoxin-induced lethality than were wild-type mice, even though the former expressed normal MHC II molecules. The recent crystallization of MHC I and II molecules (5, 6) indicates comparatively similar tertiary structures in these two molecules, especially in the nonpolymorphic regions (5, 6). Furthermore, Hansen et al. (19) have suggested that MHC I engagement cooperates with cytokine receptors and contributes to IL-2- and IL-4-induced proliferation. Therefore, exotoxin binding to MHC I would be capable of contributing to cellular (19) and physiological (36) responses. Additional work will be needed to confirm the nature of the 140-, 97-, and 43-kDa molecules as well as the 61-, 52-, and 37-kDa molecules that we have presented. Now that we have proven the existence of alternative toxin receptor(s), we are working to fully define these non-MHC II molecules.

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Quantitative Spectrophotometric Assay for Staphylococcal Lipase

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We report the development of a specific spectrophotometric assay for the quantitative determination of lipase activity in *Staphylococcus aureus*. The assay is based on the rate of clearance of a tributyrin emulsion, and it can detect as little as 1.0 μg of purified *Pseudomonas* lipase per ml. By comparison with the reaction rates obtained with *Pseudomonas* lipase, we calculated that *S. aureus* PS54C and S6C produce approximately 15 and 60 μg of extracellular lipase per ml, respectively. Neither PS54, which is lysogenized with the converting bacteriophage L54a and is consequently lipase negative (Lip$^-$), nor KSI905, a Lip$^-$ transpositional mutant of strain S6C, was positive in our spectrophotometric assay. The specificity of the spectrophotometric tributyrin assay was confirmed with a triolein plate assay; supernatants from S6C and PS54C hydrolyzed triolein, while supernatants from PS54 and KSI905 did not. In contrast to the results of the spectrophotometric tributyrin assay, all enzyme preparations tested (including commercially purified esterase) were positive when examined by a tributyrin plate assay. The lack of specificity in the tributyrin plate assay emphasizes the need to interpret the results of tributyrin lipolysis kinetically for assessing lipase activity in *S. aureus*.

Staphylococcus aureus produces a wide variety of extracellular proteins (9), several of which are lipolytic (1, 21, 25). A true lipase (glycerol ester hydrolase; EC 3.1.1.3) is the primary lipolytic enzyme (1, 3); however, *S. aureus* produces several other exoproteins that exhibit lesser degrees of lipolytic activity (3, 14, 21). Most notable among the other lipolytic enzymes is an esterase that can be distinguished from lipase by its activity on water-soluble substrates (21, 25).

Interest in the lipolytic enzymes of *S. aureus* stems from the correlation between lipolytic activity and pathogenesis (3). Although its precise role in disease is poorly understood, lipase appears to contribute to the localization of infection, with lipase-positive (Lip$^+$) strains being more common among isolates from deep infections and lipase-negative (Lip$^-$) strains being more common among isolates from superficial infections (8, 17). Staphylococcal lipase also affects immune function. For example, Rollof et al. (15) demonstrated that 12 μg of purified staphylococcal lipase per ml eliminates granulocyte chemotaxis and drastically reduces phagocytic killing.

Using transpositional mutagenesis, we identified a region of the staphylococcal chromosome that is distinct from the lipase structural gene (*geh*) but is required for the production of extracellular lipase (24). To characterize the regulatory effects of this region, a quantitative assay for lipase activity was needed, particularly in light of the additional lipolytic enzymes present in the extracellular milieu of *S. aureus*. Because molecular characterization of the regulatory region required the examination of large numbers of clones, it was also important that the assay be amenable to the screening of large numbers of samples.

The enzymology of lipolytic enzymes is complicated by a variety of factors, most notably the difficulties associated with measuring the activity of a water-soluble enzyme on an insoluble substrate (4). As a result, quantitative lipase assays are generally complicated procedures, requiring specialized instrumentation (4, 20). The most widely used technique involves the determination of fatty acid release from triglycerides by titration in hot ethanol to a phenolphthalein endpoint (4). A modification of this method employs a pH-stat to measure fatty acid hydrolysis (4, 22). Although the modified assay is relatively simple and has been used to quantify lipase activity in *S. aureus* (22), it requires the use of a complicated instrument not readily available in many laboratories. In this communication, we describe a simple spectrophotometric tributyrin assay for the quantitative determination of lipase activity in the culture supernatant of *S. aureus*.

MATERIALS AND METHODS

The strains of *S. aureus* used in this study were as follows: S6C, a hyperproducer of several extracellular proteins including lipase (7); KSI905, a derivative of S6C which carries a Tn*551* chromosomal insertion that inactivates a locus required for expression of the lipase structural gene (*geh*) (24); PS54, which is lysogenized with the converting bacteriophage L54a and is consequently Lip$^-$; and PS54C, which has been cured of L54a and is Lip$^+$ (11, 12). L54a-mediated lysogenic conversion occurs because the *att* site for L54a lies within the lipase structural gene (*geh*) (11, 12); hence, lysogenization with L54a does not affect the expression of lipolytic enzymes other than lipase. For that reason, PS54 and PS54C were used as negative and positive controls, respectively.

S6C, PS54, and PS54C were grown in tryptic soy broth. KSI905, which carries a Tn*551*-encoded erythromycin resistance determinant (23), was grown in tryptic soy broth containing 15 μg of erythromycin per ml. Lipase activity in cell-free culture supernatants was measured. Because lipase activity is highest during post-exponential-phase growth (2, 13), supernatants from stationary-phase cultures were used for all comparative assays. Incubations were at 37°C, with constant rotary aeration. Aeration was used to maximize

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bacterial growth; lipase production is not influenced by the degree of aeration (2). Supernatants were prepared for analysis by centrifugation and filtration with 0.45-μm-pore-size nylon filters. Supernatants were either assayed immediately or stored frozen at −20°C. For agarose plate assays, supernatants were standardized by being diluted with fresh tryptic soy broth to the optical density of the least dense culture.

The specificity of the spectrophotometric assay was examined with esterase purified from porcine liver (EST) (Sigma Chemical Co., St. Louis, Mo.), lipase purified from *Pseudomonas* spp. (PsL) (Sigma), and lipase purified from porcine pancreas (PcL) (Worthington Biochemical Corp., Freehold, N.J.). Commercial enzymes were diluted to a final concentration of 0.05 U/μl in 50 mM Tris (pH 8.0)–7.5 mM $CaCl_2$. Unit definitions for purified enzymes were as follows: for EST, the amount required to hydrolyze 1.0 μmol of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25°C; for PsL, the amount required to produce 1.0 μmol of glycerol from a triglyceride per min at pH 7.0 at 37°C; and for PcL, the amount required to release 1.0 μmol of fatty acid from emulsified olive oil per min at pH 8.0 at 25°C. To compensate for differences in unit definitions, both equal activities (0.5 U) and equal amounts (10 μg) of each purified enzyme were used for comparative assays.

Tributyrin (Eastman Kodak Co., Rochester, N.Y.) and triolein (Sigma) were used as substrates. A 0.5% suspension of each triglyceride in 100 mM Tris (pH 8.0)–25 mM $CaCl_2$ was prepared. The suspensions were emulsified by sonication (40 W for 3 min). Emulsions were stabilized by adding an equal volume of 2.0% standard agarose (plate assays) or 0.8% low-gelling-temperature agarose (spectrophotometric assays). Spectrophotometric assays were initiated by adding 1.0 ml of the prewarmed (50°C) tributyrin emulsion to 100 μl of test solution dispensed into spectrophotometric cuvettes. The reaction was monitored at room temperature (21 to 23°C) by measuring the optical density of the emulsion at 450 nm. Plate assays were done by adding standard amounts of each test solution to wells cut into the appropriate triglyceride agar. Plate assays were incubated at room temperature and evaluated by measuring zones of clearance (tributyrin) or precipitation (triolein).

RESULTS

PsL caused a gradual clearing of the tributyrin emulsion that could be monitored spectrophotometrically at 450 nm (Fig. 1). The initial decrease in absorbance was a linear function of time at a tributyrin concentration of 2.13 mM (0.125%). At higher concentrations, a lag phase was observed, during which the reaction rate was not directly reflected by the decrease in optical density (Fig. 1A). The duration of the lag phase increased with increasing substrate concentration. However, at tributyrin concentrations of up to 8.5 mM, a linear reaction rate (decrease in absorbance versus time) was observed as the optical density of the emulsion decreased from 1.0 to 0.3 (Fig. 1B). At substrate concentrations above 8.5 mM, the time required to clear the emulsion to an optical density within that range was impractical (Fig. 1A).

The initial lag phase was eliminated when PsL was assayed with 2.13 mM tributyrin as the substrate (Fig. 2A). Although the initial reaction rate was linear over a relatively wide range of PsL concentrations (Fig. 2B), culture supernatants from *S. aureus* S6C cleared a 2.13 mM tributyrin emulsion almost immediately (data not shown). Additionally, 2.13 mM tributyrin emulsions were less stable than higher-concentration emulsions. On the basis of these factors, we used 8.5 mM tributyrin for all subsequent studies. Although PsL concentrations below 5.7 μg/ml (0.25 U/ml) exhibited a lag phase when assayed with 8.5 mM tributyrin (Fig. 3A), the linear correlation between reaction rate and enzyme concentration was evident when reaction rates were determined according to the parameters outlined above (Fig. 3B).

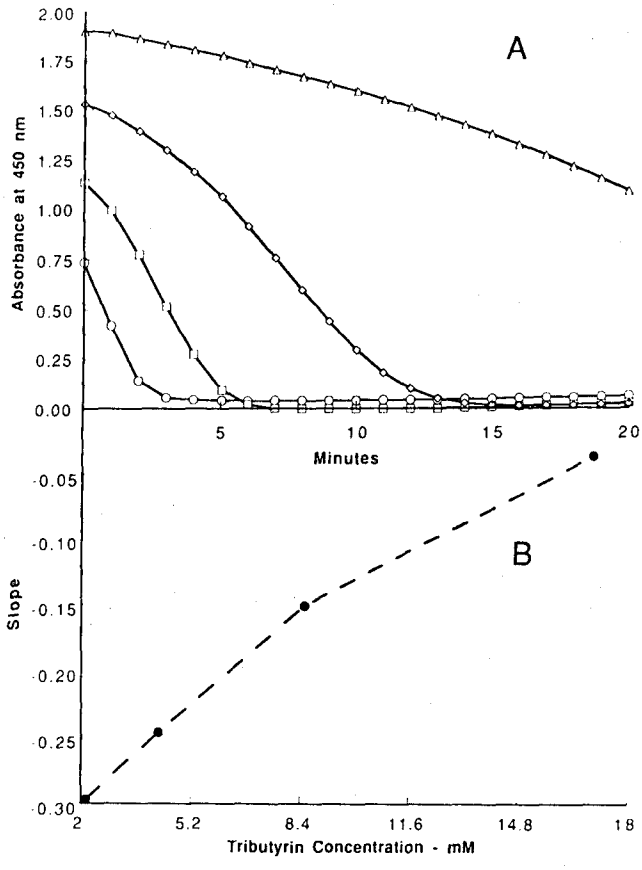

FIG. 1. Reaction rate as a function of substrate concentration. PsL (0.05 U) was assayed with tributyrin concentrations of 17 (△), 8.5 (◇), 4.25 (□), and 2.13 (○) mM. (A) Decrease in absorbance over time; (B) relationship between reaction rate and substrate concentration, with reaction rate defined as the time required to reduce the optical density of the emulsion from 1.0 to 0.3.

When equal activities (0.5 U) of enzyme were used, neither PcL nor EST yielded reaction rates comparable to those observed with PsL (Table 1). EST was active when equal quantities (10 μg) were compared; however, the reaction rate observed with EST was only 25% of that observed with PsL despite the fact that the EST preparation contained more than five times as many units per milligram as the PsL preparation (Table 1). No activity was observed when PcL was examined by using the spectrophotometric tributyrin assay (Table 1). This lack of reactivity presumably arises from the positional specificity of PcL, which does not hydrolyze secondary esters (4). However, all enzyme preparations tested, including PcL, were positive when 0.5 U was assayed with tributyrin plates (Fig. 4A). In contrast, only PsL yielded positive results when assayed with triolein plates (Fig. 4B).

The lack of activity observed with EST in the spectropho-

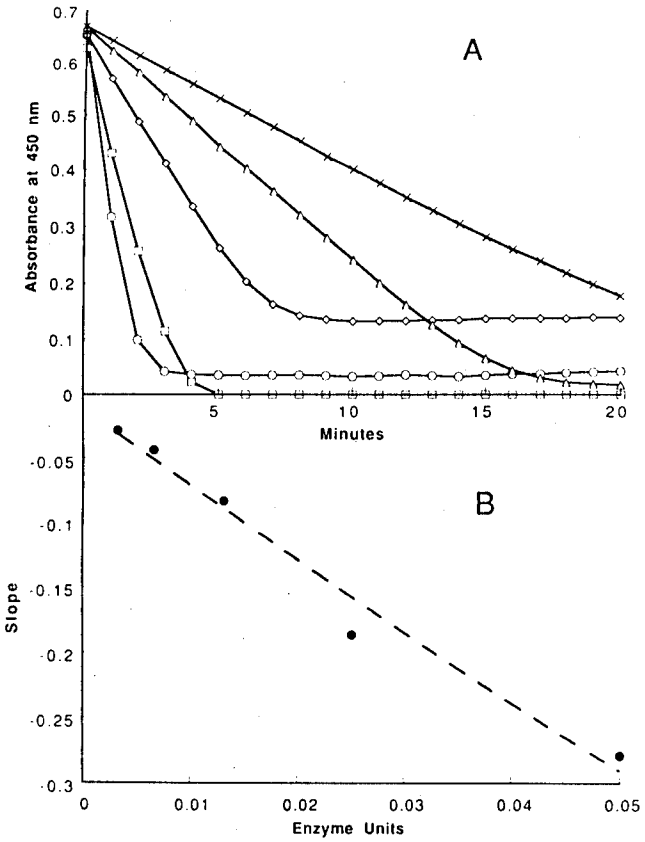

FIG. 2. Reaction rate as a function of enzyme concentration. The substrate was 2.13 mM tributyrin. (A) Decrease in absorbance over time with PsL concentrations of 0.05 (×), 0.025 (△), 0.0125 (◇), 0.00625 (□), and 0.00313 (○) U/ml. (B) Linear relationship between reaction rate (as defined in the legend to Fig. 1) and enzyme concentration.

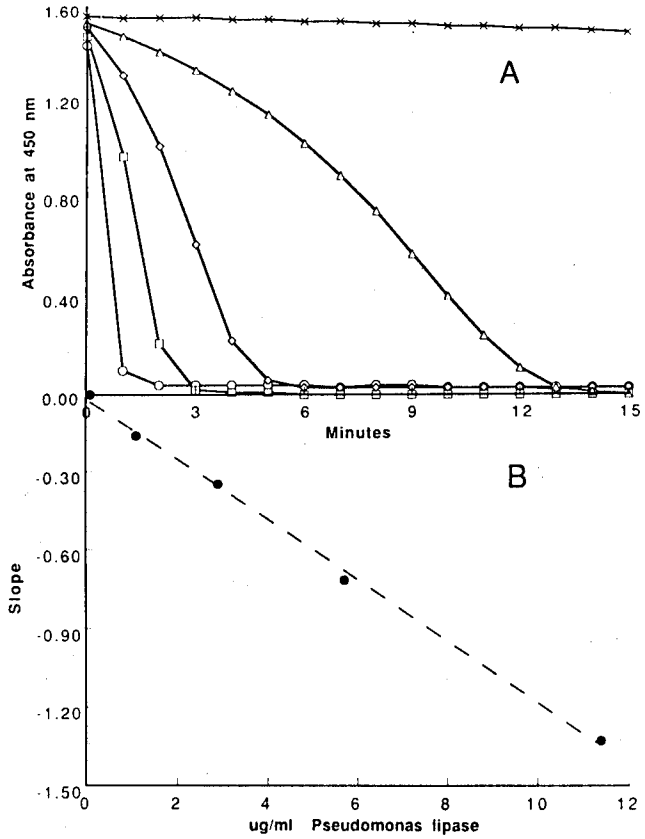

FIG. 3. Construction of a standard curve by using PsL. The substrate was 8.5 mM tributyrin. Reaction rates were defined as described in the legend to Fig. 1. (A) Decrease in absorbance over time. PsL concentrations were 11.4 (×), 5.7 (△), 2.85 (◇), 1.14 (□), and 0.114 (○) μg/ml. (B) Reaction rate as a function of enzyme concentration.

tometric tributyrin assay and the triolein plate assay suggested that the EST activity observed on tributyrin plates did not contribute significantly to the spectrophotometric assay. The results obtained when *S. aureus* culture supernatants were examined by using the spectrophotometric tributyrin assay support that conclusion. As shown in Fig. 5, only culture supernatants from S6C and PS54C were positive in the spectrophotometric assay. Since L54a-mediated lysogenic conversion is lipase specific (11, 12), the failure of PS54 to clear the tributyrin emulsion supports the conclusion that other lipolytic enzymes expressed by *S. aureus* do not contribute to clearing under the assay conditions described here. The specificity of the spectrophotometric tributyrin assay was confirmed by using triolein plates: as shown in Fig. 4B, only PS54C and S6C yielded positive results when examined by the triolein plate assay. In contrast, all *S. aureus* strains yielded various degrees of clearing around the wells of a tributyrin plate (Fig. 4A). The lack of specificity observed with the tributyrin plate assay emphasizes the need to interpret tributyrin assays kinetically for assessing *S. aureus* lipase production.

Finally, we used the spectrophotometric tributyrin assay to quantitate lipase activity in culture supernatants from all Lip^+ *S. aureus* strains. Using PsL as a standard (Fig. 3), we determined that overnight cultures of PS54C and S6C contained approximately 15.5 and 58.5 μg of extracellular lipase per ml, respectively. These values correspond to 0.35 and 1.33 U of PsL lipase per ml, respectively.

DISCUSSION

Lipase is an important lipolytic enzyme of *S. aureus* that contributes significantly to the pathogenesis of staphylococcal infection (4). The fact that isolates from deep infections are generally Lip^+ suggests that lipase plays an important role in tissue invasion (8, 17). However, the production of additional lipolytic enzymes (21, 25) together with the procedural difficulties associated with accurately measuring

TABLE 1. Tributyrinase activities of commercially purified enzymes[a]

Enzyme	Activity (U/mg)[b]	Decrease in optical density at 450 nm obtained with:	
		0.5 U	10 μg
PsL	44	1.25	1.20
PcL	100	0.04	0.03
EST	230	0.06	0.32

[a] Activity is reported as the reaction rate (decrease in absorbance over time) obtained when purified preparations of each enzyme were assayed spectrophotometrically.
[b] See text for unit definitions.

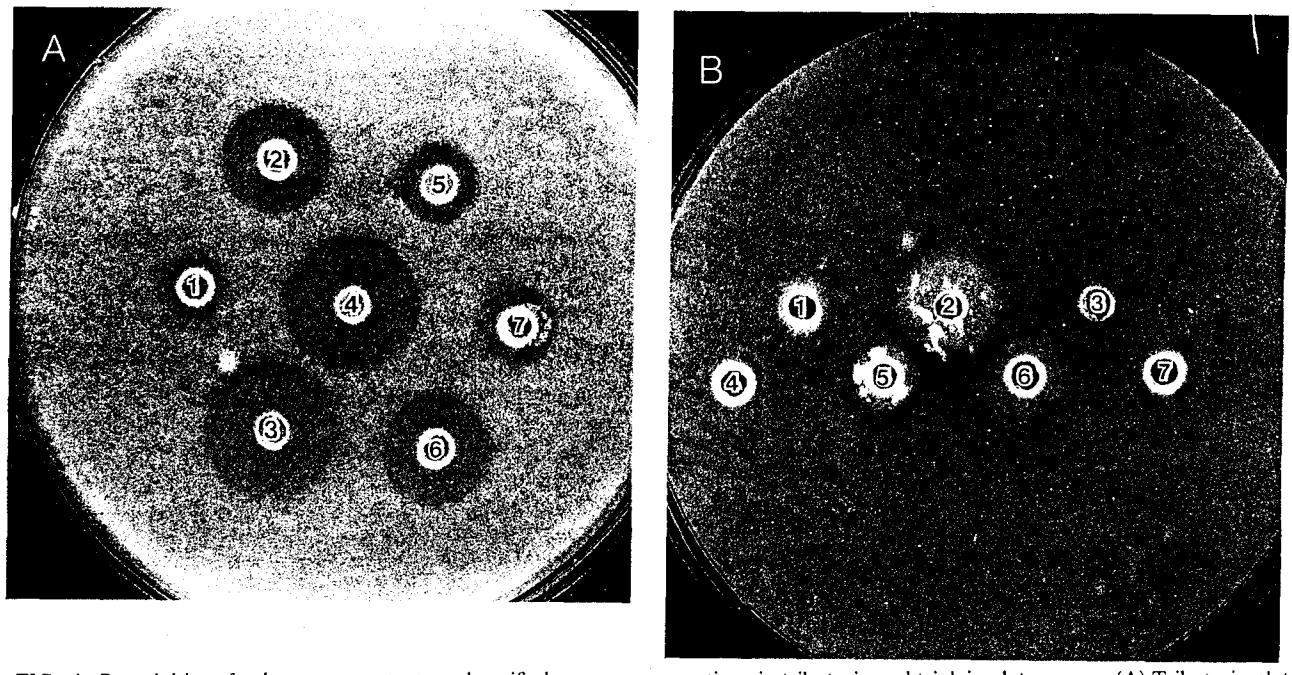

FIG. 4. Reactivities of culture supernatants and purified enzyme preparations in tributyrin and triolein plate assays. (A) Tributyrin plate. 1 and 2, culture supernatants from PS54 and PS54C, respectively; 3, EST; 4, PsL; 5, PcL; 6 and 7, culture supernatants from S6C and KSI905, respectively. (B) Triolein plate. 1, EST; 2, PsL; 3, PcL; 4 through 7, culture supernatants from PS54, PS54C, S6C, and KSI905, respectively.

lipase activity (4) complicates studies aimed at assessing the contribution of lipase to staphylococcal disease (1, 3).

A variety of assays have been used to assess lipase activity in *S. aureus* (1, 3, 16, 17, 19, 22). The most commonly used assays utilize agar plates containing either an egg yolk emulsion or Tween 80 (3, 19). While egg yolk agar provides a convenient method for screening colonies (11), reactions on egg yolk agar are not lipase specific (3, 6). Similarly, the ability to split Tween compounds is not directly correlated with lipase activity (19).

We developed the spectrophotometric tributyrin assay described here as part of a study aimed at characterizing a transpositional mutant of *S. aureus* S6C which exhibits little or no lipolytic activity despite the presence of an intact *geh* structural gene (24). A turbidimetric assay based on the clearance of a lipid emulsion has been described elsewhere (16); however, the assay is not quantitative and, in our experience, is relatively insensitive (data not shown). Since

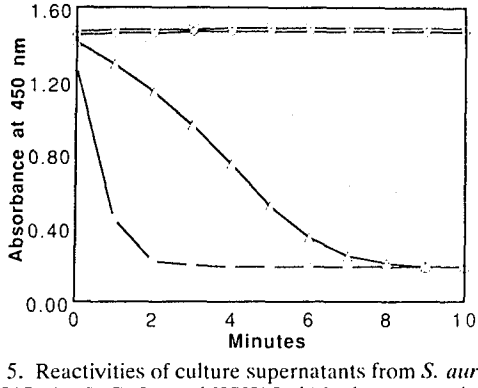

FIG. 5. Reactivities of culture supernatants from *S. aureus* PS54 (□), PS54C (△), S6C (○), and KSI905 (◇) in the spectrophotometric tributyrin assay.

complementation of regulatory mutations in *S. aureus* does not always lead to restoration of 100% activity (10), it was important that the assay used for characterization of this mutant be both sensitive and quantitative.

Tributyrin was chosen as a substrate because it is inexpensive and yields water-soluble products upon hydrolysis. Staphylococcal lipase exhibits little or no positional specificity (18). As a result, tributyrin emulsions clear relatively quickly in the presence of staphylococcal lipase. However, tributyrin is not a lipase-specific substrate (4), and there are reports in the literature suggesting that the use of tributyrin leads to false-positive reactions in studies of lipase expression among the staphylococci (6). These false-positive reactions occur because *S. aureus* also produces an esterase (21); however, as shown in this report, esterase activity does not contribute to the hydrolysis of tributyrin under the spectrophotometric assay conditions described here. Tributyrinase activity has also been associated with staphylococcal coagulase (5). Both of the Lip$^-$ strains used in this study (KSI905 and PS54) produce coagulase in amounts similar to those of their respective parent strains (S6C and PS54C) (data not shown). Hence, the failure of KSI905 and PS54 to yield positive results provides additional evidence that lipolytic enzymes other than lipase present in the extracellular environment of *S. aureus* do not contribute to tributyrin hydrolysis under the spectrophotometric assay conditions described here.

It is important, however, that positive reactions were observed with all *S. aureus* strains when lipase activity was determined by using the tributyrin plate assay. In contrast, only those strains that were positive in the spectrophotometric tributyrin assay were capable of reducing triolein. Triolein meets the strict definition of a lipase substrate in that it contains only long-chain fatty acids (4); hence, triolein can be used to distinguish lipase activity from the activity of nonlipolytic esterases and other enzymes that "fall into the

gray area between esterases and lipases" (4). The inability of PS54 and KSI905 to reduce triolein clearly indicates that the reactions observed on tributyrin plates were false positive. On the basis of direct correlation with the triolein plate assay, we conclude that the spectrophotometric tributyrin assay eliminates these reactions and provides a convenient, specific, and quantitative means of assessing lipase activity in *S. aureus*.

ACKNOWLEDGMENTS

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Phenotypic Characterization of *xpr*, a Global Regulator of Extracellular Virulence Factors in *Staphylococcus aureus*

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We recently described a Tn*551* insertion in the chromosome of *Staphylococcus aureus* S6C that resulted in drastically reduced expression of extracellular lipase (M. S. Smeltzer, S. R. Gill, and J. J. Iandolo, J. Bacteriol. 174:4000–4006, 1992). The insertion was localized to a chromosomal site (designated Ω1058) distinct from the lipase structural gene (*geh*) and the accessory gene regulator (*agr*), both of which were structurally intact in the lipase-negative (Lip$^-$) mutants. In this report, we describe a phenotypic comparison between strains S6C, a hyperproducer of enterotoxin B; KSI9051, a derivative of S6C carrying the Tn*551* insertion at Ω1058; ISP546, an 8325-4 strain that carries a Tn*551* insertion in the *agr* locus; and ISP479C, the parent strain of ISP546 cured of the Tn*551* delivery plasmid pI258*rep*A36. Compared with their respective parent strains, ISP546 and KSI9051 produced greatly reduced amounts of lipase, alpha-toxin, delta-toxin, protease, and nuclease. KSI9051 also produced reduced amounts of staphylococcal enterotoxin B. Coagulase production was increased in ISP546 but not in KSI9051. Using a mouse model, we also demonstrated that ISP546 and KSI9051 were far less virulent than ISP479C and S6C. We have designated the genetic element defined by the Tn*551* insertion at Ω1058 *xpr* to denote its role as a regulator of extracellular protein synthesis. We conclude that *xpr* and *agr* are similar and possibly interactive regulatory genes that play an important role in the pathogenesis of staphylococcal disease.

Much of the pathology of *Staphylococcus aureus* infections can be attributed to the production of extracellular proteins (8, 28). Many of these exoproteins are toxins that are directly responsible for specific pathological effects, while others play less obvious roles in establishing and maintaining infection (21, 22, 28).

In vitro, most exoproteins are preferentially produced at defined points in the growth cycle, generally as the culture enters the post-exponential growth phase (1, 2, 27). Such coordinate production implies a high degree of regulation, the primary mediator of which appears to be a polycistronic locus known as the accessory gene regulator (*agr*) (5, 9, 13, 18–20). The *agr* locus encodes three transcripts, one of which (RNAIII) functions as the effector molecule in a two-component signal transduction system (9, 13).

A number of important questions about the *agr* locus remain, particularly with regard to the signals that influence its expression and the mechanism by which it exerts both positive and negative regulatory effects on protein synthesis. Several lines of evidence suggest that *agr* may function through interactions with other regulatory loci (5, 20, 27). Interestingly, Cheung et al. (4) described a mutation in a regulatory locus (designated *sar* for staphylococcal accessory regulator) that caused decreased expression of delta-toxin, which is encoded within the RNAIII transcript, but increased expression of other proteins that are characteristically downregulated in *agr* mutants. In contrast, the reduced synthesis of delta-toxin seen in *agr* mutants is directly associated with reduced exoprotein production (9, 13).

We recently described a Tn*551* chromosomal insertion in *S. aureus* S6C that results in drastically reduced expression of extracellular lipase (25). The Tn*551* insertion was mapped to a chromosomal site (designated Ω1058) that is distinct from the lipase structural gene (*geh*) and the *agr* locus, both of which were structurally intact in all lipase-negative mutants (25). These results suggested that the Tn*551* insertion at Ω1058 defined a regulatory element required for the synthesis of extracellular lipase (25). In this report, we demonstrate that S6C strains carrying the Tn*551* insertion at Ω1058 also produce less alpha-toxin, delta-toxin, staphylococcal enterotoxin B (SEB), protease, and nuclease. Since the genes encoding each of these exoproteins were intact, we conclude that the Ω1058 insertion defines a global regulator of exoprotein synthesis in *S. aureus*. We also show that mutation of such global regulatory elements causes a drastic reduction in the virulence of *S. aureus* in mice.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains S6C and ISP479C were grown in tryptic soy broth (TSB). KSI9051 and ISP546, both of which carry chromosomal insertions of Tn*551* (17, 25), were grown in TSB containing 15 μg of erythromycin per ml. The presence of erythromycin did not affect the results of any phenotypic assay (data not shown). S6C, KSI9051, and ISP546 have been described before (17, 23, 25). ISP479C was generated by curing the temperature-sensitive transposon delivery plasmid pI258*rep*A36 from ISP479 (kindly provided by Peter A. Pattee, Iowa State University, Ames). ISP479 is the 8325-4 strain used to generate the *agr* mutant ISP546 by transpositional mutagenesis (17). The plasmid was cured by growing ISP479 in nonselective medium at 30°C. After overnight incubation, cells were plated on tryptic soy agar (TSA) and then replica plated to TSA containing 15 μg of erythromycin per ml and to TSA containing 5 μg of penicillin per ml. The strain designation ISP479C represents clones that did not grow under selection with either antibiotic.

Phenotypic assays. Lipase, protease, nuclease, alpha-

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TABLE 1. Oligonucleotide primers used for gene amplification

Gene	Sequence[a]		Reference
	Primer 1	Primer 2	
Nuclease	GTCTGCAACGATTCATGTTGTAG	AAGCAACTTTAGCCAAGCCTTGACG	24
Alpha-toxin	ATTTGATATGTCTCAACTGC	GCTCTAATTTTTAAGTCAGG	7
Coagulase	AGCAGTAGCAAACGCTGACG	GGCTTGTTTTGTGTTGGGCGA	12
Protease	CAAGTTGAAGCACCTACTGG	TAGAGTGTGAATCGGCTTTGG	3
SEB	TTAGTTATTTCTACACCCAACG	CATCATGTCATACCAAAAGC	11
RNAIII	AGATCTATCAAGGATGTGATGTT	GTCATTATACGATTTAGTACAATC	25

[a] Primers 1 and 2 correspond to opposite DNA strands. Sequences are written 5' to 3'.

toxin, delta-toxin, and SEB were assayed in supernatants from overnight (15-h) cultures. Coagulase production was determined in whole cultures. For each experiment, cultures were diluted with TSB to reflect the optical density (550 nm) of the least-dense culture at the time of harvest. Culture supernatants were harvested by centrifugation at 8,000 × *g* and either assayed immediately or stored at −20°C. Assays for coagulase were done at the time of harvest. SEB, alpha-toxin, and delta-toxin production were analyzed by Western immunoblot. For SEB and alpha-toxin assays, supernatants were electrophoresed in sodium dodecyl sulfate-polyacrylamide gels. Proteins were transferred to neutral nylon membranes for analysis. SEB was detected by using antisera produced in rabbits (23). Alpha-toxin was detected by using rabbit antisera produced by inoculation with purified alpha-toxin hexamer (kindly provided by Sidney Harshman, Vanderbilt University, Memphis, Tenn.). Delta-toxin assays were done by dot blot with the immunoglobulin G fraction of rabbit antisera (kindly provided by Frank Kapral, The Ohio State University, Columbus). Delta-toxin blots were done by spotting twofold dilutions of supernatants on neutral nylon membranes. The membranes were air dried and processed for antibody detection with a protein A-alkaline phosphatase conjugate (Boehringer Mannheim, Indianapolis, Ind.). Alkaline phosphatase conjugates were detected by using AAMPD as described by the manufacturer (Tropix, Inc., Bedford, Mass.).

Lipase activity was assessed by a tributyrin assay developed in our laboratory (26). Nuclease was assayed by mixing salmon sperm DNA in a buffer consisting of 25 mM boric acid (pH 8.0) and 12.5 mM $CaCl_2$ with 25 to 100 μl of culture supernatant. After 30 min at 37°C, the DNA was precipitated by adding trichloroacetic acid to a final concentration of 25%. Samples were held on ice for 10 min and then centrifuged to pellet the precipitated DNA. After dilution (1:10) with distilled water, the amount of acid-soluble nucleotides present in the supernatant was determined by measuring the optical density at 260 nm. Protease was assayed by mixing 300 μl of culture supernatant with 800 μl of an azocasein solution (3 mg/ml) in Tris-buffered saline (pH 7.5). After 16 h at 37°C, undegraded azocasein was precipitated by the addition of 400 μl of 50% trichloroacetic acid. The precipitate was removed by centrifugation, and the amount of acid-soluble azocasein in the supernatant was determined by measuring the optical density at 340 nm. Coagulase was assayed by mixing 100 μl of serially diluted culture with 500 μl of rabbit coagulase plasma (Difco Laboratories, Detroit, Mich.). Samples were incubated at 37°C for 2 h. All assays were repeated at least three times. The results of quantitative assays are reported as the activity observed in each mutant relative to that in the appropriate parent strain.

Southern blot analysis. Structural genes were analyzed by Southern blot under previously described conditions (25). The 2.9-kb probe used to examine the lipase structural gene (*geh*) was generated by simultaneous *Eco*RI and *Hin*dIII digestion of pLI210 (14). Probes for the protease, nuclease (*nuc*), coagulase (*coa*), SEB (*seb*), alpha-toxin (*hla*), and delta-toxin (*hld*) genes were generated by polymerase chain reaction amplification of S6C chromosomal DNA under previously described conditions (25). The 1.75-kb *hld* probe spanned the entire region encoding RNAIII (25). All other probes represented internal fragments of each gene. The primers used for each amplification are listed in Table 1. The chromosomal region containing the Ω1058 insertion site was examined by using a 26-kb *Eco*RI fragment from pIG995 (25). The 26-kb fragment was gel purified and digested with *Cla*I for use as a probe. All probes were labeled with digoxygenin-11-dUTP and detected with AAMPD as described previously (25).

Virulence studies. Overnight (15 to 18 h) cultures were harvested by centrifugation. Cell pellets were washed twice in sterile phosphate-buffered saline (PBS) and then resuspended in PBS to an optical density (550 nm) of approximately 50. BALB/c mice were inoculated in the peritoneal cavity with 10^{10} CFU in a volume no larger than 100 μl. Six mice were inoculated with each strain. Lethality was assessed after 24 h.

RESULTS

Effect of the Ω1058 insertion on exoprotein production. S6C produced relatively large amounts of lipase (Fig. 1), SEB

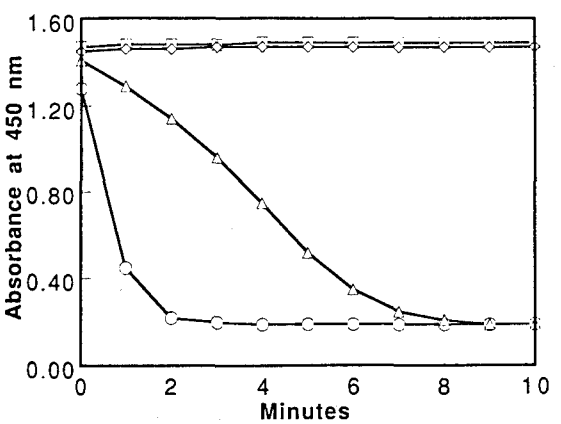

FIG. 1. Lipase activity in *xpr* and *agr* mutants. Rate of decrease in optical density of a tributyrin emulsion mixed with standardized supernatants from overnight cultures of S6C (○), KSI9051 (□), ISP479C (△), and ISP546 (◇).

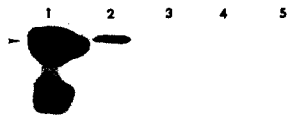

FIG. 2. Production of SEB in *xpr* and *agr* mutants. Culture supernatants from S6C (lane 2), KSI9051 (lane 3), ISP479C (lane 4), and ISP546 (lane 5) were electrophoresed, transferred to neutral nylon membranes, and probed with antibody against SEB. Lane 1 contains purified SEB (arrowhead).

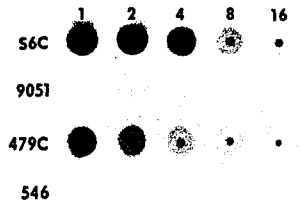

FIG. 4. Production of delta-toxin in *xpr* and *agr* mutants. Culture supernatants were diluted with TSB and spotted onto neutral nylon membranes. The reciprocal of each dilution is marked across the top. Strains are marked along the left. Blots were probed with antibody against delta-toxin as described in the text.

(Fig. 2), nuclease (Table 2), alpha-toxin (Fig. 3), and delta-toxin (Fig. 4). KSI9051, an S6C derivative that carries the Tn*551* insertion at Ω1058, failed to produce detectable levels of lipase (Fig. 1), SEB (Fig. 2), alpha-toxin (Fig. 3), or delta-toxin (Fig. 4). Production of nuclease and protease in KSI9051 was reduced to 20% of the levels observed in S6C (Table 2). Coagulase production in KSI9051 was comparable to that observed in S6C (Table 2). With the exception of coagulase production (Table 2), the phenotype of KSI9051 was identical to that of the *agr* mutant ISP546. Compared with its parent strain (ISP479C), ISP546 produced reduced amounts of lipase (Fig. 1), alpha-toxin (Fig. 3), delta-toxin (Fig. 4), nuclease (Table 2), and protease (Table 2). Production of SEB by ISP546 could not be directly assessed for reasons detailed below.

Genomic analysis. When chromosomal DNA from each strain was digested with *Eco*RI and hybridized with DNA probes specific for the lipase (*geh*), alpha-toxin (*hla*), delta-toxin (*hld*), and coagulase (*coa*) genes, a single, identical fragment was observed in all cases (Fig. 5A through D). A single, identical fragment was also observed when *Eco*RI-digested chromosomal DNA from S6C and KSI9051 was examined with a probe specific for SEB (*seb*) (Fig. 5E, lanes 1 and 2). Neither ISP479C nor ISP546 carried the *seb* gene (Fig. 5E, lanes 3 and 4). Two identical fragments were observed when *Eco*RI-digested chromosomal DNA was examined with a probe specific for the V8 serine protease gene (Fig. 5F) and when *Hin*dIII-digested chromosomal DNA was examined with a DNA probe specific for the nuclease (*nuc*) gene (Fig. 5G).

When chromosomal DNA was digested with *Eco*RI and probed with the 26-kb fragment containing the Ω1058 insertion site in S6C, a 26-kb fragment was observed in all strains except KSI9051 (Fig. 6A, lanes 2 to 4). A 12.6-kb DNA fragment was observed in KSI9051 (Fig. 6A, lane 1). The 12.6-kb fragment observed in KSI9051 reflects the deletion of approximately 18.5 kb of chromosomal DNA that occurred during transposition (25). When *Cla*I-digested chromosomal DNA was examined with the same probe, S6C contained 5.8- and 8.0-kb bands that were absent in both ISP479C and ISP546 (Fig. 6C, lanes 2 to 4). Both bands were also present in *Eco*RI plus *Cla*I double digests of S6C chromosomal DNA (Fig. 6B, lane 2); hence, these fragments must be derived from *Cla*I sites located between the *Eco*RI sites used to clone the 26-kb fragment from S6C.

A 12.0-kb DNA fragment was observed in *Cla*I digests of chromosomal DNA from all strains (Fig. 6C, lanes 1 to 4). A 7.1-kb DNA fragment was observed in *Cla*I digests of chromosomal DNA from S6C, ISP479C, and ISP546 (Fig. 6C, lanes 2 to 4). Because the 7.1- and 12.0-kb DNA fragments were not observed in *Eco*RI-*Cla*I digests of chromosomal DNA from S6C (Fig. 6B, lane 2), these fragments must represent the termini of the cloned region. Similarly, all strains except KSI9051 contained 2.9- and 5.1-kb *Eco*RI-*Cla*I fragments (Fig. 6B, lanes 2 to 4) that were not present in *Cla*I digests (Fig. 6C, lanes 2 to 4). The 2.9-kb *Eco*RI-*Cla*I fragment was also present in KSI9051 (Fig. 6B, lane 1). These results are consistent with the map of the Ω1058 insertion site in KSI9051 published previously (25) and demonstrate that the 2.9- and 5.1-kb DNA fragments represent the termini of the 26-kb fragment used as a probe. For reasons detailed below, the 12.0-kb fragment observed in *Eco*RI-*Cla*I digests of DNA from ISP479C and ISP546 probably represents an internal *Cla*I fragment. All strains except KSI9051 contained six additional DNA fragments that hybridized with the 26-kb probe (data not shown). All six fragments were smaller than 1.0 kb and were present in *Cla*I and *Eco*RI plus *Cla*I digests of chromosomal DNA.

Virulence studies. All six mice injected in the peritoneal cavity with 10^9 CFU of ISP479C died within 24 h (data not shown). Although all six mice injected with 10^9 CFU of S6C were noticeably distressed (exhibited huddling behavior and were lethargic, with staring coats), no other strain was lethal at that dose. However, when the dose was increased to 10^{10} CFU, all six mice injected with ISP479C and four of six mice injected with S6C died within 24 h (Table 3). Pure cultures of the inoculated strains were recovered from the spleens of all dead mice. All six mice injected with 10^{10} CFU of KSI9051

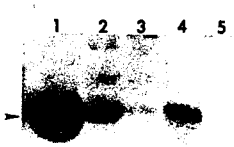

FIG. 3. Production of alpha-toxin in *xpr* and *agr* mutants. Culture supernatants from S6C (lane 2), KSI9051 (lane 3), ISP479C (lane 4), and ISP546 (lane 5) were electrophoresed, transferred to neutral nylon, and probed with antibody against alpha-toxin. Lane 1 contains purified alpha-toxin (arrowhead).

TABLE 2. Protease, nuclease, and coagulase activity in *xpr* and *agr* mutants

Strain	% of activity in parent[a]		
	Protease	Nuclease	Coagulase
KSI9051	19.7	20.3	100
ISP546	3.2	23.1	1,000

[a] Protease activity was approximately fourfold higher in ISP479C than in S6C. Nuclease activity was approximately sixfold higher in S6C than in ISP479C. Coagulase production was comparable in ISP479C and S6C.

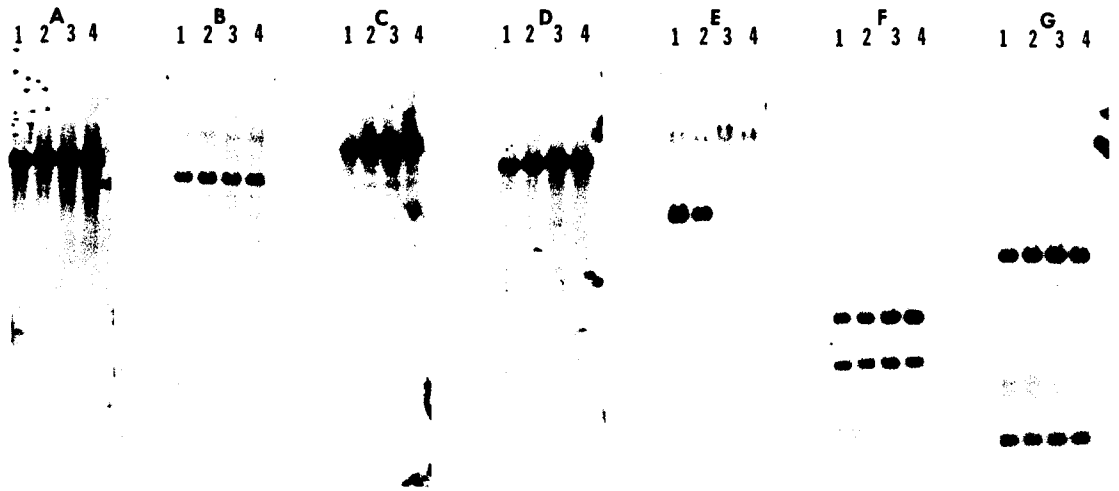

FIG. 5. Analysis of structural genes. Chromosomal DNA was digested with *Eco*RI (panels A through F) or *Hin*dIII (panel G). DNA was transferred to neutral nylon membranes and probed with DNA fragments from the structural genes for lipase (A), alpha-toxin (B), delta-toxin (RNAIII) (C), coagulase (D), SEB (E), protease (F), and nuclease (G). Lanes: 1, S6C; 2, KSI9051; 3, ISP479C; 4, ISP546.

or ISP546 not only survived but exhibited no ill effects. These results were confirmed with both FeJ (5a) (data not shown) and BALB/c mice (Table 3).

DISCUSSION

S. aureus S6C is a hyperproducer of several exoproteins, including SEB (6) and lipase (25). In an effort to define the regulatory events associated with production of these proteins, we generated a series of mutants carrying chromosomal insertions of Tn*551*. One of these (KSI905) produced drastically reduced amounts of lipase even though the lipase structural gene (*geh*) was intact (25). The insertion was transduced back into the S6C parent strain and shown to cause reduced expression of lipase (25). Subsequent analysis localized the insertion to the 670-kb *Sma*I chromosomal fragment generally recognized as fragment A (25). Pattee (16) independently mapped the insertion, which he designated Ω1058, to a site adjacent to the *thy* locus of the staphylococcal chromosome.

In this report, we demonstrate that the Tn*551* insertion at Ω1058 causes reduced expression of a wide variety of exoproteins. With the exception of SEB, which was not produced by ISP479C, and protease (data not shown), all exoproteins examined were produced in relatively large amounts in S6C. In contrast, KSI9051 failed to produce detectable levels of SEB, alpha-toxin, delta-toxin, or lipase. Although less striking, protease and nuclease production was also reduced in KSI9051. The genes encoding each of these exoproteins were intact. We conclude that the Tn*551*

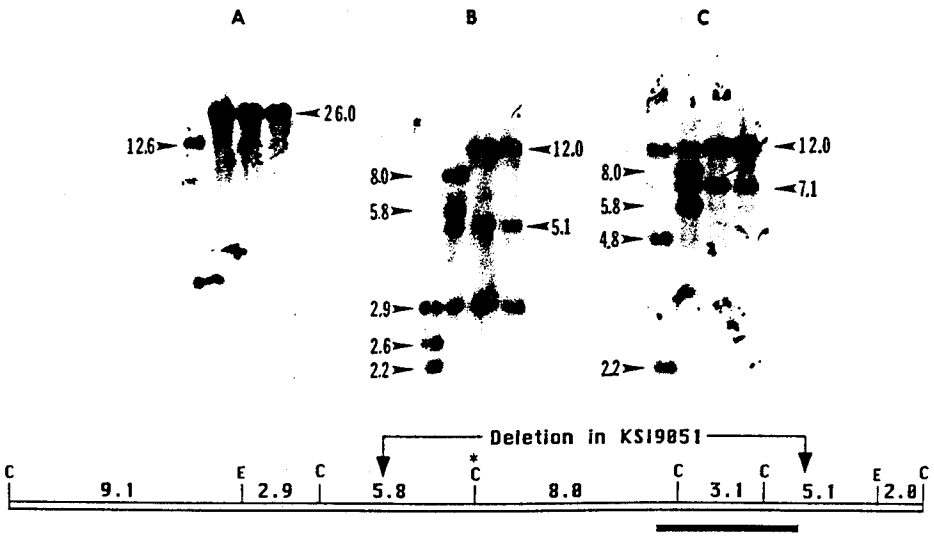

FIG. 6. Analysis of the region encoding *xpr*. Chromosomal DNA was digested with *Eco*RI (A), *Cla*I (C), or both enzymes simultaneously (B). DNA was probed with the 26-kb *Eco*RI fragment containing the *xpr* locus in S6C. The four lanes in each panel contained (from left to right) KSI9051, S6C, ISP479C, and ISP546 DNA. Fragment sizes are shown in kilobases. The schematic is a composite of the mapping data. The polymorphic *Cla*I site is marked by an asterisk. The heavy bar marks a region containing six small *Cla*I fragments. The region deleted in KSI9051 is shown between arrowheads.

TABLE 3. Virulence of *xpr* and *agr* mutants

Strain	Genotype	No. dead/6 mice injected
S6C	*xpr*$^+$ *agr*$^+$	4
KSI9051	*xpr agr*$^+$	0
ISP479C	*xpr*$^+$ *agr*$^+$	6[a]
ISP546	*xpr*$^+$ *agr*	0

[a] Results obtained when mice were injected in the peritoneal cavity with 10^{10} CFU; however, 100% lethality was also observed when mice were injected with 10^9 CFU.

insertion at Ω1058 defines a genetic element involved in the production of extracellular proteins. We have designated this element *xpr* to denote its role as an extracellular protein regulatory element in *S. aureus*.

Regulation of extracellular protein synthesis in *S. aureus* is a complicated process involving a number of physiological and genetic factors (5, 6, 9, 13, 18–20, 27, 29). The best-understood regulatory element is *agr*, which is composed of three transcription units that appear to encode a two-component signal transduction system (13, 17). One of these transcripts (RNAIII) encodes the structural gene for delta-toxin (10); however, it is the RNAIII transcript itself that accounts for *agr*-mediated regulation of exoprotein synthesis (9). While the mechanism of *agr*-mediated regulation is poorly understood, a recent report demonstrated that RNAIII affects the transcription of exfoliative toxin A and protein A in a promoter-dependent manner (15).

Despite the strong evidence that *agr* plays a fundamental and perhaps central role, there is an accumulating body of evidence that additional regulatory elements operate either independently of or in conjunction with *agr* to regulate protein synthesis in *S. aureus*. For example, although both alkaline pH and glucose cause reduced *agr* transcription and reduced synthesis of *agr*-regulated exoproteins, the repression associated with each growth factor appears to be distinct, since the pH effect requires an intact *agr* locus but the glucose effect does not (18, 20). Vandenesch et al. (27) demonstrated that RNAIII was necessary but not sufficient for the temporal regulation of alpha-toxin production. Finally, Compagnone-Post et al. (5) demonstrated that strains producing different amounts of RNAIII carry single and apparently identical copies of the *agr* locus, which suggests that undefined host factors contribute to the transcriptional regulation of *agr*.

Cheung et al. (4) recently described a Tn*551* insertion in a chromosomal locus (*sar*, or staphylococcal accessory regulator) that caused reduced expression of delta-toxin but increased expression of serine protease, lipase, and alpha-toxin. In contrast, expression of all three of these exoproteins is reduced to undetectable levels in *agr* mutants (13). The mechanism by which *sar* affects exoprotein synthesis is unknown. Since strains carrying the *sar* mutation exhibit a phenotype essentially opposite to that observed in *agr* mutants, it was suggested that *sar* may function as a counterregulatory element to *agr*. However, both *sar* and *agr* mutants exhibit decreased expression of delta-toxin (4, 13), which is interesting because delta-toxin is encoded within the RNAIII transcript and RNAIII is the regulatory product of the *agr* locus (9).

Taken together, the results discussed above clearly suggest that *agr*-mediated regulation involves unidentified regulatory elements that either influence *agr* transcription or are affected secondarily through a primary effect mediated by *agr*. In either case, mutation of such a regulatory element should result in an *agr*-like phenotype. With respect to exoprotein production, that is the case with the *xpr* mutation described here. Specifically, all extracellular proteins examined were expressed in greatly reduced amounts in KSI9051 and ISP546. Of particular note is the observation that both *xpr* and *agr* mutants produce greatly reduced amounts of delta-toxin.

The single phenotypic difference between KSI9051 and ISP546 was coagulase production, which was increased in ISP546 but not in KSI9051. In *agr* mutants, synthesis of most exoproteins is decreased while synthesis of coagulase and protein A is increased (13). This differential regulation suggests that *agr* may exert its global effects through distinct interactions with specific sets of regulatory or structural genes. That hypothesis is supported by the results of Vandenesch et al. (27), showing that the temporal regulation of *hla* requires an *agr*-independent signal but the regulation of *spa* (protein A) does not. It is therefore possible that *xpr* represents a genetic element involved in the regulation of some but not all *agr*-regulated proteins.

It must be noted that the chromosomal region encoding *xpr* in ISP479C and ISP546 is polymorphic with respect to the sequence in S6C. When chromosomal DNA was digested with *Eco*RI and probed with the 26-kb fragment containing the *xpr* locus in S6C, a 26-kb fragment was observed in all three strains. However, when *Cla*I-digested DNA was examined with the same probe, S6C contained two fragments that were absent in both ISP479C and ISP546. Since both fragments were also present in *Eco*RI-plus-*Cla*I double digests of S6C chromosomal DNA, they must be derived from *Cla*I sites that occur between the *Eco*RI sites used to clone the 26-kb fragment from S6C. Since the 7.1- and 12.0-kb fragments present in *Cla*I digests of chromosomal DNA from all three strains were absent in *Eco*RI-*Cla*I digests, they must represent the termini of the cloned region together with flanking DNA extending outside the *Eco*RI sites to a *Cla*I site. Similarly, 2.9- and 5.1-kb fragments present in *Eco*RI-plus-*Cla*I-digested DNA were not present when DNA was digested with *Cla*I alone. The 2.9- and 5.1-kb fragments must therefore represent each end of the cloned region. These results clearly indicate that both ends of the 26-kb region encoding *xpr* are identical in all three strains. However, a 12.0-kb DNA fragment was also present when chromosomal DNA from ISP479C and ISP546 was digested with *Eco*RI and *Cla*I.

While these results suggest the presence of a relatively large structural polymorphism within the region encoding *xpr*, we conclude that the 12.0-kb fragment present in *Eco* RI-plus-*Cla*I digests of ISP479C and ISP546 chromosomal DNA merely reflects the absence of an internal *Cla*I site. Several observations support that conclusion. First, hybridization with the 26-kb DNA fragment should yield the same number of fragments in both *Eco*RI-*Cla*I and *Cla*I digests, since the former would merely eliminate the flanking sequences that have no homology with the gene probe. Blots made with S6C chromosomal DNA did contain the same number of hybridizing fragments; however, blots made with *Cla*I-digested chromosomal DNA from ISP479C and ISP546 had fewer hybridizing fragments than blots done with DNA digested with both *Eco*RI and *Cla*I. Also, although all strains carry *xpr* on an *Eco*RI fragment of similar size, the *Cla*I fragments present in S6C contain considerably more DNA than was observed in ISP479C and ISP546. Finally, the two internal *Cla*I fragments present in S6C together are roughly

the same size as the 12.0-kb *Eco*RI-*Cla*I fragment present in ISP479C and ISP546.

From these results, we conclude that the 12.0-kb DNA fragment observed in *Cla*I digests of ISP479C and ISP546 chromosomal DNA represents a doublet, one fragment of which contains an *Eco*RI site. The fact that the intensity of the signal obtained with the 12.0-kb *Cla*I fragment was consistently greater in ISP479C and ISP546 than in S6C supports that hypothesis. The six small *Cla*I fragments present in all strains other than KSI9051 must represent internal *Cla*I sites that were not detected in our original study (25). Their conservation among all strains supports our contention that the region encoding *xpr* is basically intact in the 8325-4 strains and that the polymorphism observed between S6C and the ISP479 strains merely reflects a restriction site polymorphism.

Experiments are under way to determine whether *xpr* affects the transcription of *agr* or vice versa. While the phenotypes of *xpr* and *agr* mutants are very similar, the fact that coagulase production is not upregulated in *xpr* mutants suggests that *xpr* is not required for *agr* function. Whether *xpr* transcription is altered by mutation of *agr* must await definitive characterization of the *xpr* locus.

Finally, it is important to emphasize that mutation of *xpr* or *agr* resulted in a dramatic reduction in virulence. These results clearly emphasize the importance of exoproteins in the pathogenesis of staphylococcal disease. Also, all mice injected with 10^9 CFU of ISP479C died within 24 h, while one-third of the mice injected with 10^{10} CFU of S6C survived. The only qualitative difference between ISP479C and S6C was beta-toxin, which is not produced by S6C. This fact, together with the observation that S6C produces relatively large amounts of alpha-toxin, suggests that beta-toxin is an important virulence factor in the mouse intraperitoneal infection model.

ACKNOWLEDGMENTS

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The Extracellular Protein Regulator (*xpr*) Affects Exoprotein and *agr* mRNA Levels in *Staphylococcus aureus*

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***xpr*, a regulatory element of exoprotein synthesis in *Staphylococcus aureus*, defined by an insertion of Tn*551* into the chromosome of strain S6C, affects the expression of several exoproteins at the mRNA level. Drastic reduction in transcript levels for staphylococcal enterotoxin B (*seb*), lipase (*geh*), alpha-toxin (*hla*), and delta-toxin (*hld*) were detected, while mRNA levels for coagulase (*coa*) and protein A (*spa*) were elevated. Because the delta-toxin gene resides within the RNAIII transcript of the exoprotein regulator, *agr*, the reduction in *hld* message in the mutant strain of S6C is indicative of additional regulatory events in exoprotein gene expression. Northern (RNA) analysis of total cellular RNA hybridized with probes specific for RNAII and RNAIII (the two major transcripts of the *agr* operon) showed that both transcripts were reduced 16- to 32-fold at 3 h (late exponential phase) and 8- to 16-fold at 12 h (postexponential phase). These data confirm our original findings (M. S. Smeltzer, M. E. Hart, and J. J. Iandolo, Infect. Immun. 61:919–925, 1993) that two regulatory loci, *agr* and *xpr*, are interactive at the genotypic level.**

The pathogenesis of *Staphylococcus aureus* is due to a number of virulence factors in the form of toxins, enzymes, and cell wall-associated proteins that concertedly bring about disease (6, 25). Some of these, most notably alpha-toxin, staphylococcal enterotoxin B (SEB), and toxic shock syndrome toxin, are coordinatedly expressed in a temporal fashion. They accumulate, in vitro, during the postexponential phase of growth, while cell surface proteins such as coagulase and protein A are preferentially made during exponential growth (7, 10). This coordinate expression of exoproteins is regulated by the accessory gene regulator (*agr*) (12–14). The *agr* locus is composed of two major divergent transcripts, RNAII and RNAIII (8, 10). The RNAII transcript contains at least two open reading frames that have deduced amino acid similarities with two-component signal transduction systems (8, 10, 22). The RNAIII transcript is directly involved with the regulation of exoprotein synthesis (8, 10).

Cheung and coworkers (2) described a Tn*917* insertion in the *S. aureus* chromosome that also resulted in a pleiotropic effect on several extracellular and cell wall-associated proteins. The insertion site was localized to a region distinct from the *agr* locus and thus has been designated *sar*, for staphylococcal accessory regulator. A phenotypic comparison between the parent and the corresponding mutant revealed an effect contrary to what was observed for both *agr* and *xpr* (21). Alpha-toxin, protease, and lipase were elevated, while coagulase, fibrinogen-binding protein, and protein A were reduced. However, in a more recent study reevaluating the *sar* mutation in different staphylococcal strains, Cheung et al. (3) showed that the expression of alpha- and beta-toxin was, in fact, reduced, and therefore the mutation appears to be *agr*- and *xpr*-like.

Recently, we described a Tn*551* insertion within the *S. aureus* chromosome that resulted in the reduced expression of the extracellular protein, lipase (20). The transposon was localized to a 12.6-kbp *Eco*RI fragment of the staphylococcal chromosome (20). Upon further analysis, the insertion site was determined to be within a 26-kbp *Eco*RI fragment of the wild-type strain, the difference being accounted for by a large deletion that occurred when the transposon inserted into the chromosome. Genetic and physical mapping proved this region to be distinct from that of the *agr* locus (20, 21). Analysis of several extracellular proteins produced by the mutant strain revealed a pleiotropic phenotype similar to that of the *agr* locus. SEB, alpha-toxin, delta-toxin, protease, and nuclease were greatly reduced; thus, we designated this genetic element *xpr*, for extracellular protein regulator (21).

In this study, we found that transposon insertion into the *xpr* region resulted in drastic reduction of alpha-toxin, delta-toxin, lipase, and SEB message, while the levels of protein A and coagulase message were elevated. The reduction in delta-toxin message indicates that *xpr* not only affects expression of delta-toxin but also affects expression of RNAIII. These data suggest for the first time that at least two of these extracellular protein regulators are interactive at the genetic level.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strains used in this study have been described elsewhere (20, 21). Briefly, strains KSI9051 and ISP546 are Tn*551*-induced mutants of S6C and ISP479C, respectively. The transposon insertion in KSI9051 results in reduced levels of several extracellular proteins and has been previously characterized (20, 21). Strain ISP546, like KSI9051, exhibits reduced levels of several extracellular proteins; however Tn*551* resides in the *agr*A locus and, as determined by us, is genotypically distinct from *xpr* (20).

Media and culture conditions. Overnight (15- to 18-h) cultures of either S6C or ISP479C grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) were used to inoculate 350 ml of TSB to an initial optical density of approximately 0.05 at 550 nm. Portions of 50 ml each were aseptically transferred to six 125-ml screw-cap flasks (flask/volume ratio of 2.5) and incubated at 37°C with rotary aeration at 180 rpm. Growth was monitored spectrophotometrically, and samples for RNA isolation were taken every 2 h. Growth conditions for

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KSI9051 and ISP479C were identical to those described above except that erythromycin (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 15 μg/ml was added to TSB prior to inoculation.

RNA isolation. The procedure used for the isolation of RNA was a modification of the methods of Kornblum et al. (11) and Khan (9). At various times during growth, 10-ml samples were aseptically pipetted into 25-ml screw-cap Corex tubes containing 10 ml of an ice-cold acetone-ethanol mixture (1:1). Samples were stored at −20°C until sampling was completed. Cells were harvested by centrifugation at 10,000 × *g* for 15 min. The supernatants were decanted, and cell pellets were suspended in 10 ml of TES (150 mM NaCl, 78 mM disodium EDTA, 100 mM Tris [pH 7.5]) and centrifuged as before. Cell pellets were suspended in 1 ml of TES plus NaCl (2.5 M), and recombinant lysostaphin (Applied Microbiology Inc., New York, N.Y.) was added to a final concentration of 100 μg/ml. Cell suspensions were incubated at 37°C for 30 min or until suspensions became viscous. To each protoplast suspension, 5 ml of RNAzol B (Tel-Test, Inc., Friendswood, Tex.) was added, and the entire mixture was rocked slowly until lysis was complete. To each lysate, 0.6 ml of chloroform was added, after which the lysates were vigorously agitated for 15 s. Each suspension was allowed to incubate on ice for 15 min and then centrifuged (10,000 × *g*, 15 min) to facilitate phase separation. Portions (0.6 ml) were pipetted into sterile microcentrifuge tubes, and an equal amount of isopropanol was added. Each sample was mixed by inversion and stored at −85°C for 45 min. Precipitated RNA was pelleted by centrifugation at 4°C for 15 min. Supernatants were discarded, and each RNA pellet was washed twice with 1 ml of 70% ethanol. Tubes containing RNA pellets were inverted and allowed to stand for approximately 30 min to facilitate alcohol evaporation. Finally, each pellet was suspended in 100 μl of deionized, glass-distilled water pretreated with diethylpyrocarbonate (Sigma). Samples were stored at −85°C until used.

Northern (RNA) analysis. Northern analysis was performed essentially as described by Selden (19). High-quality RNA (A_{260}/A_{280} = 1.9 to 2.0) was standardized by appropriate dilution in diethylpyrocarbonate-treated water to a final concentration of 1 μg/μl. Portions (5 μl) were electrophoresed at 6 V/cm through 1.0% LE agarose (FMC BioProducts, Rockland, Maine) and stained with NucliStain (National Diagnostics, Manville, N.J.). The intensities of the rRNA bands between standardized preparations were densitometrically compared to verify that all samples contained equal amounts of total RNA.

The standardized RNA samples were serially diluted in diethylpyrocarbonate-treated water and denatured at 50°C for 1 h in the presence of glyoxal (Eastman Kodak Co., Rochester, N.Y.) and dimethyl sulfoxide (Fisher Scientific, Fairlawn, N.J.). Samples were electrophoresed at 4 V/cm through 1.2% GTG agarose (FMC) gels prepared in phosphate buffer (10 mM NaH_2PO_4 [pH 7]) containing 10 mM sodium iodoacetic acid (Sigma). RNA was transferred by passive diffusion onto neutral nylon (MagnaGraph; Micron Separations Inc., Westborough, Mass.) membranes and hybridized overnight (18 to 24 h) at 65°C with various gene-specific probes. Membranes were washed as previously described (20).

The probe used for detection of *spa* was a 1.2-kbp fragment generated by *Cla*I digestion of pRIT5 (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). The remaining probes were generated by amplifying gene fragments from S6C chromosomal DNA with the polymerase chain reaction (PCR), using primers and reaction conditions previously described (20, 21). The *sea* and RNAII probes were likewise generated by PCR.

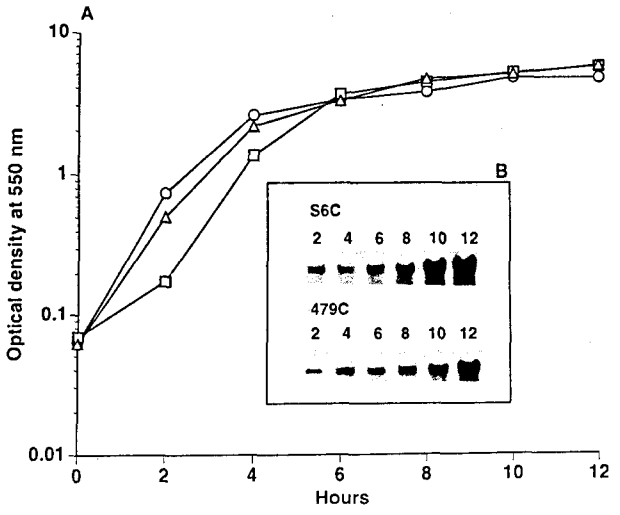

FIG. 1. (A) Representative growth curves of *S. aureus* strains S6C (○), ISP479C (△), and KSI9051 (□). S6C and ISP479C were grown in TSB, and KSI9051 was grown in TSB plus erythromycin (15 μg/ml). (B) Northern analysis of total cellular RNA isolated from S6C and ISP479C at 2, 4, 6, 8, 10, and 12 h of growth and hybridized with a *geh*-specific probe.

The primer pair used for *sea* had the sequences 5′-ACTTG TAAATGGTAGCGAGAAAAGC-3′ and 5′-TATTTGAAT ACTGTCCTTGAGCACC-3′. The primer pair used for RNAII had the sequences 5′-CGAAATGCGCAAGTTCCG TCA-3′ and 5′-CCAACTGGGTCATGCTTACGA-3′. The RNAII primer pair generated an 860-bp fragment encompassing the junction between open reading frames A and B of the *agr* operon (10, 13). PCR products were gel purified by using the Magic PCR system (Promega Corp., Madison, Wis.). All DNA fragments were labelled with digoxygenin-11-UTP as previously described (20). Hybridized probes were detected by autoradiography with alkaline phosphatase-conjugated, anti-digoxygenin $F(ab')_2$ antibody fragments (Boehringer Mannheim) and the chemiluminescent substrate AMPPD (Tropix Inc., Bedford, Mass.).

RESULTS

The effect of erythromycin and the presence of the transposon on growth were determined by comparing growth of KSI9051 in medium containing the antibiotic with growth of S6C in medium lacking the antibiotic. Although a slight lag was observed for the first 2 h, growth of KSI9051 recovered quickly and was equivalent to that of S6C and ISP479C for the remaining time points examined (Fig. 1A). Lipase was chosen as an indicator of time-dependent expression of extracellular proteins because *S. aureus* S6C produces the protein in high quantities during postexponential growth (20). To determine the optimal point during growth to assess the effect of *xpr* on expression of exoproteins, total cellular RNA was isolated from S6C and ISP479C at 2-h intervals (Fig. 1A). Standardized RNA preparations were hybridized with a *geh*-specific probe, and the results indicate that message is expressed throughout the growth of both strains but occurs at elevated levels during the postexponential phase (Fig. 1B). Therefore, in the remaining experiments in this study, RNA was isolated at 12 h of growth unless otherwise noted. In addition, levels of *geh* message were more abundant for S6C than for ISP479C. Strain S6C is known to be a hyperproducer of several extracellular

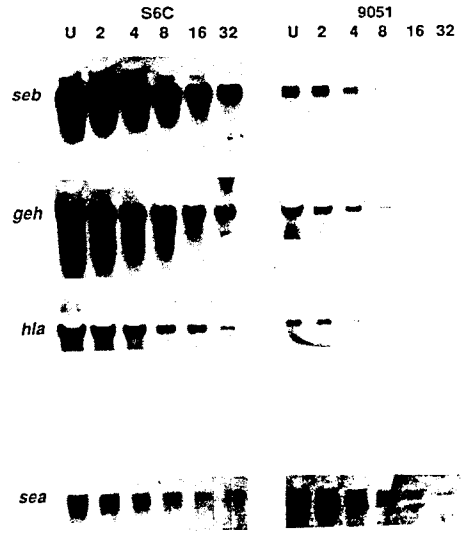

FIG. 2. Total cellular RNA isolated from S6C and 9051 at 12 h of growth and hybridized with either an *seb*- a *geh*-, an *hla*-, or an *sea*-specific probe. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values) samples.

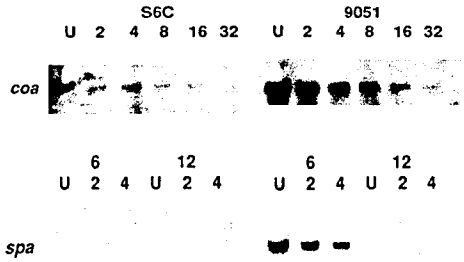

FIG. 3. Total cellular RNA isolated from S6C and 9051 at 12 h of growth for the *coa*-specific probe and at 6 and 12 h of growth for the *spa*-specific probe. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values) samples.

proteins, including lipase (20) and SEB (5). These data are consistent with our earlier report (20) that extracellular lipase activity is produced at elevated levels postexponentially.

***xpr* effect on the expression of extracellular proteins.** Total cellular RNA extracted from S6C and 9051 was probed with DNA fragments specific for the *seb*, *geh*, and *hla* genes. As shown in Fig. 2, these data indicate that the amount of message produced by strain 9051 was greatly reduced in comparison with the parental strain S6C. Message levels were at least 32-fold lower for all three genes. In contrast, the expression of *sea* was unaffected. *sea* transcript levels for S6C and 9051 were similar even at a dilution of 1/32 (Fig. 2). A similar effect in an *agr* mutant background has been reported by Tremaine and coworkers (23). Western blot (immunoblot) analysis revealed no significant differences in SEA levels produced by three different strains of *S. aureus* and their corresponding *agr* mutant strains (23). These data suggest that neither *xpr* nor *agr* regulates the expression of the *sea* gene.

***xpr* effect on the expression of cell wall-associated proteins.** In our earlier report, we determined that the relative clot-forming activity of coagulase was unchanged in the mutant strain 9051 (21). However, in this same mutant strain, *coa* mRNA levels were elevated by 16- to 32-fold (Fig. 3). We attribute this discrepancy to the rather insensitive means (i.e., formation of a fibrin clot in rabbit plasma by serial twofold dilutions of culture filtrate) of assessing coagulase activity. In addition to determining *coa* expression in the *xpr* mutant, we also examined the expression of protein A. Because *spa* message is made during the exponential phase of growth, samples were taken at 6 and 12 h. Expression of *spa* was increased in the mutant strain at least fourfold at 6 h; however, no appreciable difference in expression was observed at 12 h (Fig. 3). Expression of *spa* message in strain 9051 occurred throughout exponential phase rather than being induced when cells entered the postexponential phase of growth (data not shown). These results are identical to those observed for *spa* in an *agr*-null mutant (24).

***xpr* effect on the expression of *agr*.** mRNA levels of the *agr* operon in strains S6C and 9051 were assayed at 2-h intervals by Northern analysis. When an RNAIII-specific probe was used, expression was observed as early as 2 h (early exponential phase; Fig. 1) in S6C but was not detected (data not shown) until 6 h of growth (late exponential phase; Fig. 1) in 9051. Further analysis of RNA samples taken at 3 and 12 h of growth and probed with RNAII- and RNAIII-specific probes demonstrated that transcript levels in 9051 were reduced in comparison with S6C (Fig. 4A and B). At 3 h (Fig. 4A), a 16- to 32-fold reduction was noted, while levels at 12 h (Fig. 4B) were reduced only 8- to 16-fold. The narrowing of the differences between 3 and 12 h is most likely due to the accumulation of message over time.

The *agr* parent and mutant strains, ISP479C and ISP546, were included in this study as a control for the RNAII and RNAIII transcripts. Total cellular RNA was isolated from these strains and hybridized simultaneously with the RNAII- and RNAIII-specific probes (Fig. 4B). *agr* transcript levels in ISP479C were identical to those of S6C. However, little expression of either *agr* transcript was observed in the Agr mutant ISP546. Both RNAII and RNAIII were found in the undiluted lane, and as expected, the size of the RNAII species was smaller due to the insertion of Tn*551*, which results in a truncated message.

DISCUSSION

The disease processes of the staphylococci involve the participation of a number of virulence factors produced by the organism (6). Indeed, the list of factors has risen to greater than 30, ranging from none to many that any one strain can produce (6). The roles that these factors play in the overall process of disease are not clear. However, it is becoming increasingly clear that the coordinate regulatory mechanisms responsible for expression of these virulence factors are complex and multifactorial.

To date, three loci, *agr*, *sar*, and *xpr*, have been identified, and they apparently provide a similar regulatory role for several known *S. aureus* virulence factors (1–3, 10, 20, 21). The genes have all been mapped to distinct loci on the staphylococcal chromosome. All have been identified as regulatory elements on the basis of their similar pleiotropic effects on exoprotein production; that is, exoproteins are positively regulated, while cell wall-associated proteins are negatively regulated. The effect has been shown to occur at the mRNA level for *agr* and *sar* and, as a result of this study, for *xpr*.

Transpositional mutagenesis of *xpr* resulted in reduced levels of message for *hld*, *geh*, *seb*, and *hla*, while *spa* and *coa* message levels were increased. The fact that *hld* was affected was

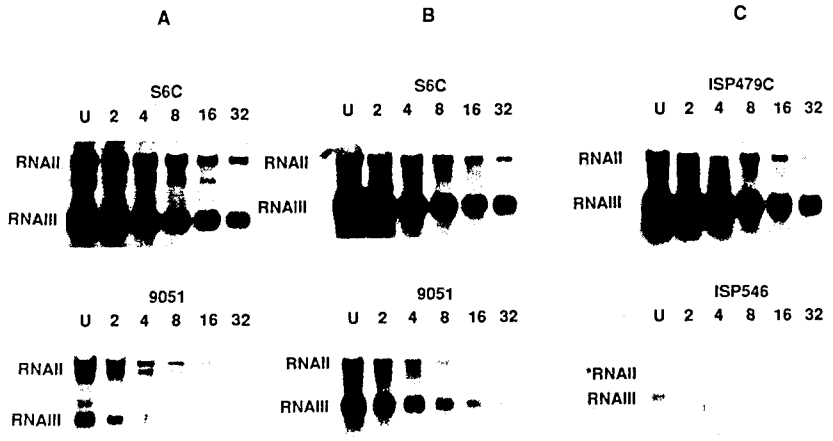

FIG. 4. Northern analysis of total cellular RNA isolated from S6C and 9051 at 3 h (A) and 12 h (B) of growth and hybridized simultaneously with probes specific for RNAII and RNAIII. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values) samples. The RNA species are labelled accordingly. For comparison, total cellular RNA was isolated from ISP479C and ISP546 at 12 h of growth (C) and also hybridized simultaneously with probes specific for RNAII and RNAIII. The asterisk denotes the truncated RNAII molecule in ISP546.

somewhat surprising since the *hld* gene resides within the RNAIII transcript, which is the regulatory component of the *agr* operon essential for exoprotein production in *S. aureus*. Further analysis of the effect of *xpr* on RNAII and RNAIII demonstrated that both transcripts were reduced in the *xpr* mutant. These data indicate, for the first time, that two regulatory components of exoprotein production in *S. aureus* are interactive. However, this finding is not unprecedented. In a recent review, Wanner (28) describes cross-regulation "as the control of a response regulator of one two-component regulatory system by a different regulatory system." Evidence for such a phenomenon is observed in the phosphate (*pho*) regulon of *E. coli* (26, 27). When P_i is limited, the *pho* regulon is induced. Induction involves the *phoR* sensor, which acts as a histidine protein kinase and is thought to phosphorylate the regulator, *phoB*. The activated *phoB*, in turn, transcriptionally activates a number of genes involved with transport and degradation of extracellular phosphorus sources. In addition, control of the *pho* regulon in *pho*R mutants has been shown, depending on which carbon source is metabolized, to involve either CreC or the Pta-AckA system. CreC is the sensor component of the CreC-CreB two-component signal transduction system involved with catabolite regulation, and the Pta-AckA system is associated with acetyl phosphate synthesis. Additional evidence for cross-regulation exists for other two-component signal transduction systems as well.

Recently, studies involving the expression of the exoprotein SEC in *S. aureus* have demonstrated that complex circuits that respond to several environmental signals are operative. Both *sec* and *agr* expression are reduced by the presence of glucose at acidic or nonmaintained pH (17, 18). However, the effect on *sec* does not require a functional *agr* (17). Likewise, expression of *sec* and *agr* is reduced when the organism is grown at alkaline pH, but unlike the glucose effect, the reduction in *sec* message requires an intact *agr* locus (15). Furthermore, this same group has also shown that expression of *sec* is reduced in media of high osmotic strength and that the reduction also does not require an intact *agr* locus (16). The sensory proteins of two-component signal transduction systems respond to some signal either from the environment or from within the cell which initiates the events of signal transduction (22). Although in *S. aureus* these signals include pH, glucose, and osmolarity, others have suggested that involvement of additional factors within the organism also modifies the expression of exoproteins (4, 24). Indeed, this work is supportive of at least two factors being interactive.

How *xpr* and *agr* and perhaps *sar* interact to coordinately regulate extracellular protein expression in *S. aureus* is unknown at present. What appears to be unfolding is a network of multiple regulators responding to numerous external and internal signals that result in a specific response to some environmental stimulus. Evidence has been presented to indicate that the *agr* system responds to pH and glucose. Whether or not *xpr* responds to the same stimuli is not known. Perhaps a more important question to ask is, at what level does the interaction between *xpr* and *agr* occur? It appears that Xpr (to date, the coding capacity of the *xpr* region is unknown) is *trans* acting; if this is so, it may also act as a sensory protein responding to some stimulus (same or different) that results in an activated form capable of interacting with other components resulting in the expression of extracellular proteins. These questions are currently being pursued in this laboratory.

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The effect of lysogeny on the genomic organization of *Staphylococcus aureus*

(Lysogenic conversion; bacteriophage; ϕ11; ϕ12; ϕ13; ϕ15; ϕ42; 42E; phage *att* site; staphylokinase; enterotoxin A; genetic map; restriction-fragment length polymorphism)

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SUMMARY

The genome of *Staphylococcus aureus* strain S6C was shown to contain a prophage inserted within the β-toxin (BT)-encoding structural gene (*hlb*). The phage *att* site was identical to that reported for the BT-converting phages ϕ13 and ϕ42. The prophage carried the genes encoding staphylokinase (*sak*) and enterotoxin A (*sea*), which suggests that it is similar to ϕ42. However, it was not induced in the presence of mitomycin C (MC) and appears to be defective. Mapping studies revealed that the genomes of the BT-converting phages present in strains S6C and PS42D (a ϕ42 lysogen) encode at least one *Sma*I restriction site. Moreover, the PS42D chromosome contained a second prophage that also had at least one *Sma*I site, carried both *sak* and *sea*, and hybridized with DNA probes that also hybridize with the BT-converting phages. The second phage in strain PS42D was mapped to a *Sma*I fragment corresponding to fragment A of the *S. aureus* strain 8325 genomic map. Although the BT-converting phage present in strain S6C could not be induced, a phage was induced from strain S6C using MC. Southern blots suggest that it is similar to ϕ11; however, the restriction patterns of DNA from the induced phage and ϕ11 were clearly distinct. We have designated the inducible phage present in strain S6C as ϕ15, to denote that distinction. Relatively weak hybridization signals were also observed when ϕ15 DNA was used to probe genomic DNA from *S. aureus* strains lysogenized with the BT-converting phages, ϕ13, ϕ42 and 42E. Taken together, our results demonstrate that all of the *Sma*I-defined restriction-fragment length polymorphisms observed among the genomes of the strains examined can be accounted for by the presence of prophage DNA within the staphylococcal genome.

INTRODUCTION

The genetics of *Staphylococcus aureus* are complicated by the occurrence of several forms of variable genetic elements (VGE). Included among these VGE are a wide variety of bacteriophages (Novick, 1990), many of which are used as epidemiological markers (Parker, 1983). At least four staphylococcal bacteriophages are responsible for the lysogenic conversion of specific genomic determinants. The negative conversion of lipase-encoding (*geh*),

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Abbreviations: *att*, phage insertion site; bp, base pair(s); BT, β-toxin; CHEF, clamped homogenous electric field; *hlb*, gene encoding staphylococcal BT; kb, kilobase(s) or 1000 bp; MC, mitomycin C; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; RFLP, restriction-fragment length polymorphism; *S.*, *Staphylococcus*; *sak*, gene encoding staphylokinase; SEA, staphylococcal enterotoxin A; *sea*, gene encoding SEA; VGE, variable genetic element(s).

for example, is due to the site- and orientation-specific integration of the L54a phage genome into the lipase structural gene (Lee and Iandolo, 1985). The negative-conversion of BT (*hlb*) occurs by an identical mechanism, but involves at least three phages (ϕ13, ϕ42 and 42E) (Coleman et al., 1986; 1989), two of which have been shown to utilize the same 14-bp *att* site within *hlb* (Coleman et al., 1991; Carroll et al., 1993). The main distinguishing characteristic among the BT-converting phages involves the simultaneous positive-conversion of other genetic elements. For example, the genome of the serogroup F phage ϕ13 includes the staphylokinase gene (*sak*) (Coleman et al., 1986), while the serogroup F converting phage ϕ42 carries both *sak* and the gene for enterotoxin A (*sea*) (Coleman et al., 1989). Because it has not been possible to induce phage from all Sea$^+$ strains, Betley and Mekalanos have suggested that *sea* sometimes resides on defective prophage DNA. Finally, gene *sea* appears to occupy alternative chromosomal sites sometimes existing in a truncated form (Betley et al., 1984, Betley and Mekalanos, 1985).

The aims of the present studies with *S. aureus* strain S6C were (*i*) identification of an alternative insertion site for the BT-converting bacteriophages; (*ii*) mapping of an alternative chromosomal locus for both *sak* and *sea*; (*iii*) identification of a phage (designated ϕ15) that bears some similarity to ϕ11; and (*iv*) characterization of phage-defined RFLPs in the genome of *S. aureus*.

RESULTS AND DISCUSSION

(a) Evidence for a structural disruption in the *hlb* gene of strain S6C

S. aureus strain S6 hyperproduces a number of extracellular proteins (Smeltzer et al., 1993), but does not produce detectable amounts of BT. The hemolytic activity observed when S6 is grown on sheep blood agar is due to the hyperproduction of α-toxin (data not shown).

The BT clone used in these experiments was kindly provided by Dr. Timothy J. Foster, Trinity College, Dublin, Ireland, and contains the entire *hlb* gene and flanking DNA on a 2.6-kb *Hin*dIII DNA fragment cloned into pUC19 (pM60hlb). It is 0.4 kb larger than functional *Hin*dIII clones from other strains of *S. aureus* (Coleman et al., 1986; 1989). However, the *hlb* DNA fragment on file in GenBank (file, sahlb.Gb Ba; Accession No. X13404) contains two additional *Hin*dIII sites separated by 399 bp and immediately upstream from the *hlb* structural gene. Therefore, we conclude that the additional 0.4 kb present in the M60 fragment represents an RFLP in the DNA flanking *hlb*.

When genomic DNA from *S. aureus* strains ISP479C and ISP546 (Table I) was digested with *Hin*dIII and probed with the 2.6-kb *Hin*dIII DNA fragment from *S. aureus* M60, a single 2.2-kb hybridizing fragment was observed (Fig. 1A, lanes 3 and 4). The single fragment is consistent with the data of Coleman et al. (1986) and confirms that the *hlb* locus is intact in these strains. In contrast, when genomic DNA from strains S6C and KSI9051 was similarly digested and probed, two hybridizing fragments were observed (Fig. 1A, lanes 1 and 2). The sizes of the two fragments (3.8 and 1.8 kb) are similar to those reported in ϕ13 lysogens (Coleman et al. 1989) and suggested that the *Hin*dIII polymorphisms observed in the S6C strains were due to lysogeny.

To further examine the region encoding the *hlb* gene in the S6C strains, we digested the pM60hlb clone with *Hae*III to produce two fragments of 1.3 and 1.6 kb con-

TABLE I

Bacterial strains and their relevant characteristics

Strain[a]	Parent	Lysogenic status[b]	BT phenotype
S6C	S6	ϕ42d, ϕ15	Negative
KSI9051	S6	ϕ42d, ϕ15	Negative
ISP479C	8325-4	None	Positive
ISP546	8325-4	None	Negative
8325	–	ϕ11, ϕ12, ϕ13	Negative
8325-4	–	None	Positive
PS42D	Unknown	ϕ42	Negative
KSI1201	8325-4	ϕ13	Negative
KSI1202	8325-4	ϕ42E	Negative

[a]Strains S6C, KSI9051, ISP479C and ISP546 have been described (Smeltzer et al., 1992; 1993). ISP546 is a derivative of ISP479C that carries a Tn*551* insertion in the *agr* regulatory locus resulting in reduced BT production. PS42D is the propagating strain for phage 42D. Its genetic background has not been determined.

[b]ϕ42d is our designation for the defective phage present in S6C. ϕ15 designates the viable phage cured from the same strain. 'None' refers only to the absence of *hlb*-converting phage in these strains.

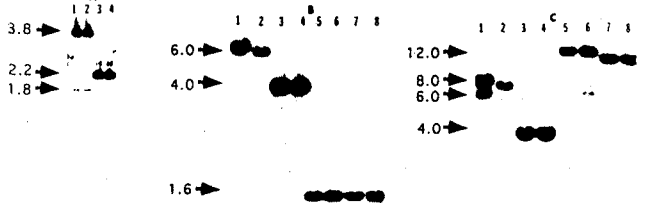

Fig. 1. Structural disruption of the *hlb* gene in S6C. Chromosomal DNA from S6C (lanes 1 and 5), KSI9051 (lanes 2 and 6), ISP479C (lanes 3 and 7) and ISP546 (lanes 4 and 8) was digested with *Hin*dIII (panel **A**), *Cla*I (panels **B** and **C**, lanes 1–4) or *Hae*III (panels **B** and **C**, lanes 5–8), separated on 1% agarose, and probed with the entire *hlb* gene (panel **A**), the region 3′ to the single *Hae*III site present in *hlb* (panel **B**) or the region 5′ to the *Hae*III site in *hlb* (panel **C**). Strains are described in Table I. All Southern blots were done at high stringency using digoxygenin-labelled DNA as previously described (Smeltzer et al., 1992).

taining the 5′ and 3′ ends of the *hlb* gene, respectively. Because the second *Hae*III sites were located within pUC19, these fragments contained a small amount of pUC19 flanking one end of the probes. These fragments were gel purified and used as gene probes as described.

A single 4.0-kb DNA fragment was observed when *Cla*I-digested genomic DNA from ISP479C and ISP546 was probed with either the 3′ (Fig. 1B, lanes 3 and 4) or the 5′ (Fig. 1C, lanes 3 and 4) *hlb* probes. These results are consistent with the data of Projan et al. (1989). On the other hand, the S6C strains contained a single 6.0-kb *Cla*I fragment that hybridized with the 3′ probe (Fig. 1B, lanes 1 and 2) and two fragments that hybridized with the 5′ probe (Fig. 1C, lanes 1 and 2). The single 6.0-kb fragment that hybridized with the 3′ probe is indicative of a *Cla*I-defined RFLP in the S6C strains, while the presence of two fragments that hybridized with the 5′ probe indicate that the RFLP is due to an additional *Cla*I site in or near the 5′ end of the *hlb* gene.

With all strains, when genomic DNA was digested with *Hae*III and probed with the 3′ fragment, a 1.6-kb DNA fragment was observed (Fig. 1B, lanes 5–8). These results demonstrate that the 3′ end of *hlb* is identical in all four strains. However, when the DNA from the S6C strains was probed with the 5′ fragment, two hybridizing DNA bands were observed (Fig. 1C, lanes 5 and 6). In contrast, ISP479C and ISP546 contained a single *Hae*III fragment that hybridized with the 5′ *hlb* probe (Fig. 1C, lanes 7 and 8). Since the *attB* site for the *hlb* converting phages ϕ13 and ϕ42 is located in the 5′ end of the *hlb* gene (Coleman et al., 1991; 1993), these results further support our contention that the restriction site polymorphisms observed in the S6C strains are the result of lysogeny.

(b) Evidence that the disruption in *hlb* is due to lysogeny

When *Hin*dIII-digested genomic DNA from *S. aureus* strains S6C, 8325 (a ϕ11, ϕ12 and ϕ13 lysogen), PS42D (a ϕ42 lysogen), 8325-4 (8325 cured of all phage), KSI1201 (a ϕ13 lysogen) and KSI1202 (a ϕ42E lysogen) was hybridized with the 2.6-kb probe containing *hlb*, three fragments were observed in all strains except PS42D and 8325-4 (Fig. 2A, lanes 3 and 4). The 0.4-kb hybridizing DNA fragment present in 5 of 6 strains examined (Fig. 2A) suggests that these strains have the additional *Hin*dIII site. Like strain M60 (from which the probe was derived), PS42D apparently lacks the site, and therefore does not contain this fragment (Fig. 2A, lane 3).

The two fragments observed on S6C were distinct from those seen in strains lysogenized with ϕ13, ϕ42 or ϕ42E. To verify that these differences were due to the insertion of phage DNA, we synthesized oligo primers as described by Coleman et al. (1991). Two of these originate within *hlb* and two originate within ϕ13 DNA. These primers were used in a PCR amplification using S6C DNA as template according to the parameters used by Coleman et al. (1991). Both primer pairs amplified DNA fragments of the expected size (data not shown). The amplified DNA was gel purified and cloned into the pT7 Blue T-vector (Novagen, Madison, WI, USA). The amplified fragments were then sequenced using T7 and U19 primers. In both cases, the amplified DNA contained regions of both *hlb* and phage DNA (Fig. 2D). Moreover, the 14-bp *att* site present in strain S6C was identical to that reported for ϕ13, and ϕ42 (Fig. 2D).

With the exception of the 0.4-kb fragment, the junction fragment probes produced the same pattern of *Hin*dIII fragments as the 2.6-kb probe (Fig. 2B and 2C). In all strains lysogenized with *hlb*-converting phage, the larger of the two *Hin*dIII fragments hybridized with the right junction probe (Fig. 2C), while the smaller fragment hybridized with the left junction probe (Fig. 2B). These results confirm the presence of phage DNA in the *hlb* gene of strain S6C and are also consistent with the data of Coleman et al. (1991).

Interestingly, PS42D contained two DNA fragments that hybridized with the left junction fragment probe (Fig. 2B, lane 3). Based on the observations that (*1*) the left junction probe contains primarily phage DNA (Coleman et al., 1991); and (*2*) the additional fragment was not observed in blots using *hlb* as a gene probe, these results suggest the existence of a second phage in PS42D at a site other than the *hlb* locus.

(c) The effect of lysogeny on the *S. aureus* map

In previous experiments we examined the *agr* regulatory locus in strain S6C and in the *agr* reference strains ISP479C and ISP546 (Smeltzer et al., 1992). We clearly distinguished a new regulatory locus, *xpr* (Smeltzer et al., 1992; 1993) from *agr*, but we could not conclusively place the *agr* locus on the map of strain S6C. The *Sma*I fragment that contained the *agr* locus in S6C was not present in strain ISP546 or any of the other reference strains examined. As a result, we carried out experiments aimed at defining the map of *S. aureus* strain S6C along with additional *S. aureus* strains described below.

When *Sma*I-digested genomic DNA was examined by CHEF electrophoresis, the patterns observed for strains S6C and PS42D were distinct with respect to each other and with respect to the other strains examined (Fig. 3A). The differences observed among other strains can be accounted for by the presence of ϕ11, ϕ12 and ϕ13 in 8325 (Fig. 3A, lane 3), the absence of ϕ11, ϕ12 and ϕ13 in 8325-4 (Fig. 3A, lane 5), the presence of ϕ13 in KSI1201 (Fig. 3A, lane 6) and the presence of 42E in KSI1202 (Fig. 3A, lane 7). The *att* sites for these phages reside either on *Sma*I fragment A (ϕ12) or F (ϕ11, ϕ13 and 42E)

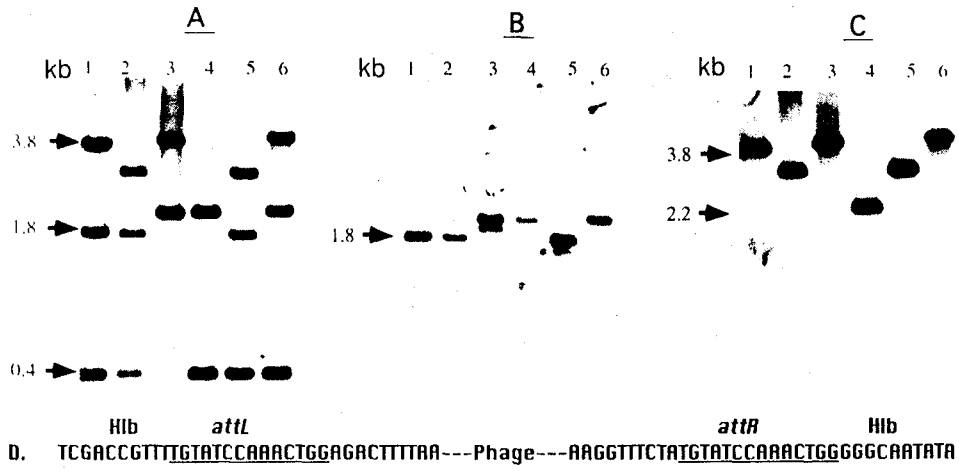

Fig. 2. Characterization of the prophage-*hlb* junction fragments. Genomic DNA from strains S6C (lane 1), 8325 (lane 2), PS42D (lane 3), 8325-4 (lane 4), KSI1201 (lane 5) and KSI1202 (lane 6) was digested with *Hin*dIII, separated on 1% agarose, blotted as described in Fig. 1. The blot was probed with the entire *hlb* gene (panel **A**), the left junction fragment cloned from S6C (panel **B**) or the right junction fragment cloned from S6C (panel **C**). Strain 8325 is lysogenized with ϕ11, ϕ12 and ϕ13 (Novick, 1990). 8325-4 lacks all three of these phages (Novick, 1990). KSI1201 is monolysogenic for ϕ13. ϕ13 inserts into *hlb* and positively-converts for staphylokinase (*sak*) (Coleman et al., 1989). PS42D is the propagating strain for phage 42D. PS42D also contains a second prophage designated ϕ42 (Coleman et al., 1989). ϕ42 inserts into *hlb* (Carroll et al., 1993) and carries both *sak* and the gene for enterotoxin A (*sea*) (Coleman et al., 1989). KSI1202 is lysogenized with 42E, which inserts into *hlb* but does not carry *sak* or *sea* (Coleman et al., 1986). The nt sequence of each junction fragment is indicated in panel **D**. Oligo primers and the reaction conditions used for the PCR amplification of each junction fragment have been described elsewhere (Coleman et al., 1991).

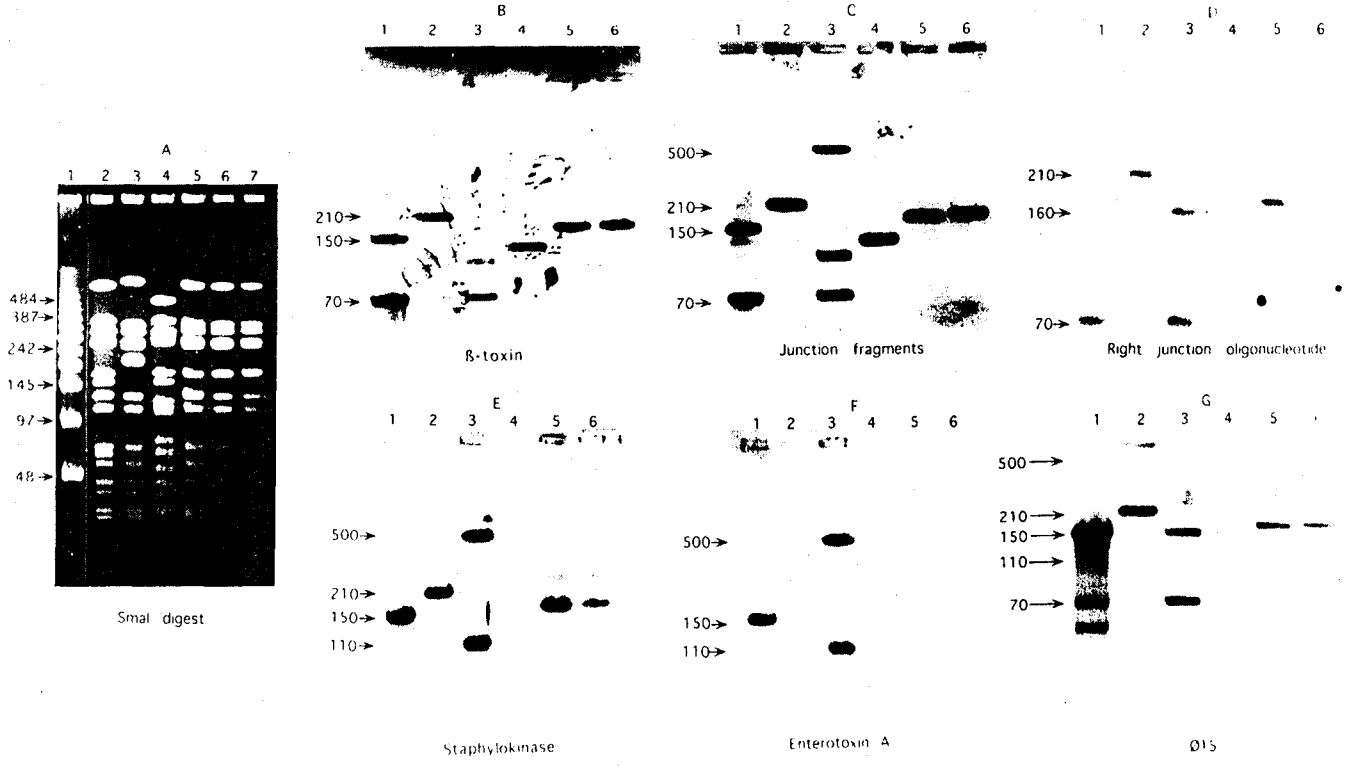

Fig. 3. The effect of lysogeny on the map of *S. aureus*. Whole cells were embedded in 1% agarose and processed for pulsed-field electrophoretic analysis as previously described (Smeltzer et al., 1992). Electrophoresis was carried out as described, except that the voltage was increased to 185 V and the run times were increased to 14 h for each cycle. Lane assignments in Panel **A** are S6C (lane 2), 8325 (lane 3), PS42D (lane 4), 8325-4 (lane 5), KSI1201 (lane 6) and KSI1202 (lane 7). Lane 1 contains phage λ DNA with each rung representing a 48.4-kb increment. Lane assignments in the remaining panels are S6C (lane 1), 8325 (lane 2), PS42D (lane 3), 8325-4 (lane 4), KSI1201 (lane 5) and KSI1202 (lane 6). Panels: **A**, ethidium bromide stained gel; panel **B**, gel probed with the entire *hlb* gene; panel **C**, gel probed with both junction fragments from S6C; panel **D**, gel probed with the right junction oligo synthesized according to the sequence reported by Coleman et al. (1991); panel **E**, gel probed with *sak*; panel **F**, gel probed with *sea*; panel **G**, gel probed with ϕ15 DNA. Probes for *sak* and *sea* were generated by PCR as previously described (Smeltzer et al., 1993). Primers used for amplification of *sak* were 5′-attatagatggttggtgtcg and 5′-tcattctaactgatttctcccca. Primers used for amplification of *sea* were 5′-acttgtaaatggtagcgagaaaagc and 5′-tatttgaatactgtccttgagcacc.

(Pattee et al., 1990). The absence of ϕ12 in S6C, 8325-4, KSI1201 and KSI1202 is evident by the reduced size of fragment A in those strains (Fig. 3A, lanes 2 and 5–7). Similarly, the absence of ϕ11 and ϕ13 in strain 8325-4 reduces the size of fragment F (208 kb, Fig. 3A, lane 3) so that it comigrates with fragment H (135 kb, Fig. 3A, lane 5). Insertion of ϕ13 or 42E into the nonlysogenized form of *Sma*I fragment F results in a 175-kb DNA fragment that co-migrates with fragment G (Fig. 3A, lanes 6 and 7). These results were confirmed by Southern blot (see below) and clearly indicate that all of the RFLPs observed between strains 8325, 8325-4, KSI1201 and KSI1202 can be attributed to the presence or absence of phage DNA. However, both S6C and PS42D contained *Sma*I fragments that were not present in any of the other strains examined (Fig. 3A, lanes 2 and 4). Most notable were the 70 and 150-kb fragments present in S6C (Fig. 3A, lane 2) and the 70, 110, 160 and 500-kb fragments present in PS42D (Fig. 3A, lane 4).

When hybridized with the *hlb* probe, a single fragment was observed in all strains except S6C and PS42D (Fig. 3B). These results demonstrate that ϕ11, ϕ13 and 42E do not contain *Sma*I restriction sites. In contrast, two *Sma*I fragments hybridized with the *hlb* probe in strains S6C and PS42D (Fig. 3B, lanes 1 and 3). None of the four fragments that hybridized with *hlb* in S6C or PS42D were present in any of the other strains examined. Moreover, because the *Sma*I fragments that hybridized with the *hlb* probe also hybridized with probes representing the junction fragments cloned from strain S6C (Fig. 3C, lanes 1 and 3), we conclude that the *hlb* polymorphisms observed in S6C and PS42D are due to the insertion of phage DNA and that the genome of the BT-converting phages present in both strains contains at least one *Sma*I restriction site.

With the exception of strain 8325-4, all *S. aureus* strains examined were shown to contain the *sak* gene on a *Hin*dIII fragment of slightly variable size (data not shown). Only strains S6C and PS42D carried the *sea* gene (data not shown), which suggests that they are the only strains lysogenized with triple-converting phages. When *Sma*I-digested chromosomal DNA was probed with *sak*, all strains were found to carry *sak* on a *Sma*I fragment that also hybridized with *hlb* and with the junction fragment probes (Fig. 3E). A weak signal was also observed with KSI1202, which carries 42E but does not produce staphylokinase. Similarly, S6C and PS42D both carried *sea* on *Sma*I fragments that hybridized with *hlb* and the junction fragment probes (Fig. 3F, lanes 1 and 3). The fact that the *sak* and *sea* probes hybridized to the same *Sma*I fragments that hybridized with the *hlb* and junction fragment probes strongly implies that *sak* and *sea* are associated with the BT-converting phages present in S6C and PS42D.

Interestingly, the junction fragment probes (Fig. 3C, lane 3) and the probes for *sak* (Fig. 3E, lane 3) and *sea* (Fig. 3F, lane 3) all hybridized to a 500-kb *Sma*I fragment that was present only in strain PS42D (Fig. 3A, lane 4). These results are consistent with the presence of the second fragment observed when PS42D genomic DNA was hybridized with the left junction fragment probe (Fig. 2B, lane 3) and provide further support for the hypothesis that PS42D contains more than one insertion of the same or a very similar phage. The single distinguishing characteristic was the apparent lack of a *Sma*I restriction site in the second phage. However, because the PCR product representing the right junction fragment in S6C consists almost entirely of *hlb* DNA (Coleman et al., 1991), it was possible that a fourth *Sma*I fragment was not detected in junction fragment blots of PS42D genomic DNA because of competitive hybridization with the *Sma*I fragments containing the *hlb* gene. To address that possibility, we used the phage-derived oligo used in the PCR amplification of the right junction fragment as a probe. A single right junction fragment was observed in strains S6C, 8325 and KSI1201 (Fig. 3D, lanes 1, 2 and 5). Little or no signal was observed in KSI1202 (Fig. 3D, lane 6), which suggests that the 42E genome is either truncated in that region or is distinct enough that it does not hybridize with the right junction fragment oligo characteristic of the other BT-converting phages. No hybridization signal was observed in the nonlysogen 8325-4 (Fig. 3D, lane 4). Most importantly, two fragments were observed in PS42D (Fig. 3D, lane 3), one of which (160 kb) was distinct from any fragment observed in previous blots using *hlb, sak, sea* or the junction fragments as probes. Taken together, these results demonstrate that a second insertion of a similar, if not identical, phage exists in the chromosome of strain PS42D. The presence of this second insertion is important in that it confirms an alternative genomic location for both *sak* and *sea*. The presence of a *Sma*I site in the second prophage together with the similarity of *Sma*I fragments observed between strains suggests that the fragment A RFLP observed in strain PS42D (Fig. 3A, lane 4) is due to lysogeny. Therefore, the *att* site for the second prophage can be localized to fragment A of the standard *S. aureus* strain 8325 map.

Finally, all of the *Sma*I fragments that hybridized to the *hlb, sak, sea* or junction fragment probes in strains S6C and PS42D are unique to those strains. We conclude that the RFLPs observed in S6C and PS42D arise from the fact that both strains are lysogenized with phages that contain at least one *Sma*I site in their genomes. Moreover, since *hlb* and *agr* both map to *Sma*I fragment F in *S. aureus* strain 8325 (Pattee et al., 1990), and the *agr* locus in S6C maps to the same 70-kb *Sma*I fragment shown

here to contain *hlb* (Smeltzer et al., 1992), we also conclude that the polymorphism we described with regard to the *agr* locus in strain S6C (Smeltzer et al., 1992) is due to lysogeny and does not reflect a fundamental difference in the genetic organization of the S6C genome.

(d) Characterization of the phage induced from S6C

We used MC to induce the *hlb*-converting phage present in strain S6C. The lysate contained viable phage able to plaque on all strains except S6C and PS42D (data not shown). Two plaque types (clear and fuzzy) were observed, however, when each type of plaque was purified and DNA from each examined, they were identical with respect to the restriction patterns obtained with *Eco*RI and *Hin*dIII (data not shown). Surprisingly, the *sak*, *sea* and junction fragment probes did not hybridize with the purified phage DNA. These results demonstrate that the viable phage induced from strain S6C is not responsible for the lysogenic conversion of *hlb*. We are assuming that the prophage present within the *hlb* locus of strain S6C cannot be induced or rescued by complementation, however, it remains possible that some other induction condition might be more appropriate.

When DNA from the phage induced from S6C was used as a probe, all strains, except KSI1201 (8325-4), were found to contain varying amounts of hybridizing DNA (Fig. 4B). The pattern of hybridizing fragments was highly variable. A trend existed, however, in that 8325 contained the most DNA that hybridized with purified DNA from the induced phage, while strains PS42D, KSI1201 and KSI1202 contained reduced amounts of hybridizing DNA (Fig. 4B). These results, together with the mapping data presented below, suggest that the phage induced from S6C is similar to ϕ11. However, the restriction patterns observed when DNA from the phage induced from S6C and DNA from ϕ11 was cut with *Eco*RI

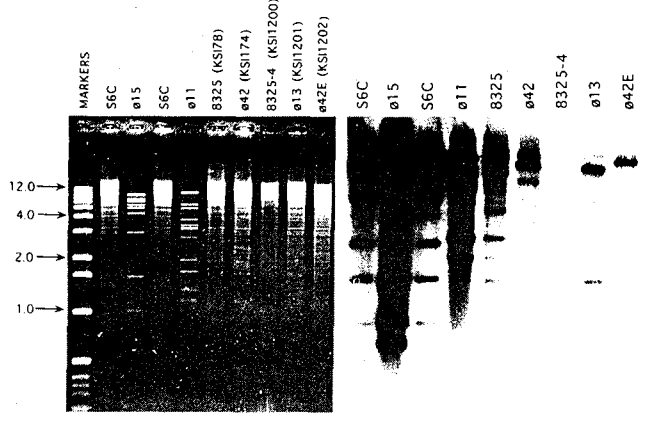

Fig. 4. Relationships among ϕ15, ϕ11 and the BT-converting phages. Chromosomal DNA from S6C, ϕ15, ϕ11, 8325, PS42D, 8325-4, KSI1201 and KSI1202 was digested with *Eco*RI and stained with ethidium bromide (A) or probed with the DNA purified from ϕ15 (B).

were clearly distinct (Fig. 4A, lanes 2 and 4). We conclude that the inducible phage present in strain S6C is distinct from any known staphylococcal bacteriophage and suggest that it be designated ϕ15 to denote that distinction.

In strain S6C, the total size of the *Sma*I fragments that hybridized to the *hlb* probe was approx. 230 kb. Since fragment F is 135 kb in the absence of phage DNA (Pattee et al., 1990) and the typical genome size of staphylococcal phages is 40–45 kb (Pariza and Iandolo, 1974), we hypothesized that the additional 95 kb of DNA might be due to the insertion of two phages into the S6C genome. Consistent with that hypothesis is the observation that DNA from ϕ15 hybridized with the larger of the *Sma*I fragments previously shown to contain part of the *hlb* locus (Fig. 3G, lane 1). These results are also consistent with the observation that the ϕ11 *att* site is located within the same *Sma*I fragment that contains *hlb* (Pattee et al., 1990) and support the conclusion that the inducible phage present in S6C is similar to ϕ11. However, we do not yet have any direct evidence to demonstrate whether ϕ11 and ϕ15 share a common *att* site within the staphylococcal genome.

Finally, the similarity between phage ϕ15 and the BT-converting phages of *S. aureus* was also observed when *Sma*I-digested chromosomal DNA was probed with DNA from ϕ15. Specifically, *Sma*I fragments that hybridized with either *hlb* (Fig. 3B) or the right junction oligo (Fig. 3D) also hybridized with ϕ15 DNA (Fig. 3G). These results, and the relative intensity of the signals observed in strains S6C and PS42D indicate that the similarities observed between ϕ15 and the BT-converting phages are primarily confined to the region near the right junction of ϕ13, ϕ42 and 42E lysogens.

(e) Conclusions

(*1*) *S. aureus* strain S6C carries two prophages, one of which is apparently identical to the triple-converting bacteriophage ϕ42, except that it is defective.

(*2*) The inducible phage present in S6C is similar to ϕ11 and may share a common *att* site, however, the two are clearly distinct with respect to the organization of their genomes. We have designated the inducible phage ϕ15 to denote that distinction.

(*3*) ϕ15 shares some sequence similarity with the BT-converting phages of *S. aureus*. That similarity is confined primarily to the region near the right junction of ϕ13, ϕ42 and 42E lysogens.

(*4*) *S. aureus* strain PS42D carries two insertions of an apparently identical phage. One insertion occurred within *hlb*, while the other resides in *Sma*I fragment A. The second insertion establishes an alternative map position for both *sak* and *sea*.

(*5*) All of the *Sma*I-defined RFLPs observed among

the strains examined in this report are the direct result of the presence of prophage DNA within the staphylococcal genome.

(6) The staphylococcal chromosome exhibits a consistent genetic organization at least within phage groups.

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The Extracellular Protein Regulator (*xpr*) Affects Exoprotein and *agr* mRNA Levels in *Staphylococcus aureus*

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***xpr*, a regulatory element of exoprotein synthesis in *Staphylococcus aureus*, defined by an insertion of Tn*551* into the chromosome of strain S6C, affects the expression of several exoproteins at the mRNA level. Drastic reduction in transcript levels for staphylococcal enterotoxin B (*seb*), lipase (*geh*), alpha-toxin (*hla*), and delta-toxin (*hld*) were detected, while mRNA levels for coagulase (*coa*) and protein A (*spa*) were elevated. Because the delta-toxin gene resides within the RNAIII transcript of the exoprotein regulator, *agr*, the reduction in *hld* message in the mutant strain of S6C is indicative of additional regulatory events in exoprotein gene expression. Northern (RNA) analysis of total cellular RNA hybridized with probes specific for RNAII and RNAIII (the two major transcripts of the *agr* operon) showed that both transcripts were reduced 16- to 32-fold at 3 h (late exponential phase) and 8- to 16-fold at 12 h (postexponential phase). These data confirm our original findings (M. S. Smeltzer, M. E. Hart, and J. J. Iandolo, Infect. Immun. 61:919–925, 1993) that two regulatory loci, *agr* and *xpr*, are interactive at the genotypic level.**

The pathogenesis of *Staphylococcus aureus* is due to a number of virulence factors in the form of toxins, enzymes, and cell wall-associated proteins that concertedly bring about disease (6, 25). Some of these, most notably alpha-toxin, staphylococcal enterotoxin B (SEB), and toxic shock syndrome toxin, are coordinatedly expressed in a temporal fashion. They accumulate, in vitro, during the postexponential phase of growth, while cell surface proteins such as coagulase and protein A are preferentially made during exponential growth (7, 10). This coordinate expression of exoproteins is regulated by the accessory gene regulator (*agr*) (12–14). The *agr* locus is composed of two major divergent transcripts, RNAII and RNAIII (8, 10). The RNAII transcript contains at least two open reading frames that have deduced amino acid similarities with two-component signal transduction systems (8, 10, 22). The RNAIII transcript is directly involved with the regulation of exoprotein synthesis (8, 10).

Cheung and coworkers (2) described a Tn*917* insertion in the *S. aureus* chromosome that also resulted in a pleiotropic effect on several extracellular and cell wall-associated proteins. The insertion site was localized to a region distinct from the *agr* locus and thus has been designated *sar*, for staphylococcal accessory regulator. A phenotypic comparison between the parent and the corresponding mutant revealed an effect contrary to what was observed for both *agr* and *xpr* (21). Alpha-toxin, protease, and lipase were elevated, while coagulase, fibrinogen-binding protein, and protein A were reduced. However, in a more recent study reevaluating the *sar* mutation in different staphylococcal strains, Cheung et al. (3) showed that the expression of alpha- and beta-toxin was, in fact, reduced, and therefore the mutation appears to be *agr*- and *xpr*-like.

Recently, we described a Tn*551* insertion within the *S. aureus* chromosome that resulted in the reduced expression of the extracellular protein, lipase (20). The transposon was localized to a 12.6-kbp *Eco*RI fragment of the staphylococcal chromosome (20). Upon further analysis, the insertion site was determined to be within a 26-kbp *Eco*RI fragment of the wild-type strain, the difference being accounted for by a large deletion that occurred when the transposon inserted into the chromosome. Genetic and physical mapping proved this region to be distinct from that of the *agr* locus (20, 21). Analysis of several extracellular proteins produced by the mutant strain revealed a pleiotropic phenotype similar to that of the *agr* locus. SEB, alpha-toxin, delta-toxin, protease, and nuclease were greatly reduced; thus, we designated this genetic element *xpr*, for extracellular protein regulator (21).

In this study, we found that transposon insertion into the *xpr* region resulted in drastic reduction of alpha-toxin, delta-toxin, lipase, and SEB message, while the levels of protein A and coagulase message were elevated. The reduction in delta-toxin message indicates that *xpr* not only affects expression of delta-toxin but also affects expression of RNAIII. These data suggest for the first time that at least two of these extracellular protein regulators are interactive at the genetic level.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strains used in this study have been described elsewhere (20, 21). Briefly, strains KSI9051 and ISP546 are Tn*551*-induced mutants of S6C and ISP479C, respectively. The transposon insertion in KSI9051 results in reduced levels of several extracellular proteins and has been previously characterized (20, 21). Strain ISP546, like KSI9051, exhibits reduced levels of several extracellular proteins; however Tn*551* resides in the *agr*A locus and, as determined by us, is genotypically distinct from *xpr* (20).

Media and culture conditions. Overnight (15- to 18-h) cultures of either S6C or ISP479C grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) were used to inoculate 350 ml of TSB to an initial optical density of approximately 0.05 at 550 nm. Portions of 50 ml each were aseptically transferred to six 125-ml screw-cap flasks (flask/volume ratio of 2.5) and incubated at 37°C with rotary aeration at 180 rpm. Growth was monitored spectrophotometrically, and samples for RNA isolation were taken every 2 h. Growth conditions for

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KSI9051 and ISP479C were identical to those described above except that erythromycin (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 15 μg/ml was added to TSB prior to inoculation.

RNA isolation. The procedure used for the isolation of RNA was a modification of the methods of Kornblum et al. (11) and Khan (9). At various times during growth, 10-ml samples were aseptically pipetted into 25-ml screw-cap Corex tubes containing 10 ml of an ice-cold acetone-ethanol mixture (1:1). Samples were stored at −20°C until sampling was completed. Cells were harvested by centrifugation at 10,000 × *g* for 15 min. The supernatants were decanted, and cell pellets were suspended in 10 ml of TES (150 mM NaCl, 78 mM disodium EDTA, 100 mM Tris [pH 7.5]) and centrifuged as before. Cell pellets were suspended in 1 ml of TES plus NaCl (2.5 M), and recombinant lysostaphin (Applied Microbiology Inc., New York, N.Y.) was added to a final concentration of 100 μg/ml. Cell suspensions were incubated at 37°C for 30 min or until suspensions became viscous. To each protoplast suspension, 5 ml of RNAzol B (Tel-Test, Inc., Friendswood, Tex.) was added, and the entire mixture was rocked slowly until lysis was complete. To each lysate, 0.6 ml of chloroform was added, after which the lysates were vigorously agitated for 15 s. Each suspension was allowed to incubate on ice for 15 min and then centrifuged (10,000 × *g*, 15 min) to facilitate phase separation. Portions (0.6 ml) were pipetted into sterile microcentrifuge tubes, and an equal amount of isopropanol was added. Each sample was mixed by inversion and stored at −85°C for 45 min. Precipitated RNA was pelleted by centrifugation at 4°C for 15 min. Supernatants were discarded, and each RNA pellet was washed twice with 1 ml of 70% ethanol. Tubes containing RNA pellets were inverted and allowed to stand for approximately 30 min to facilitate alcohol evaporation. Finally, each pellet was suspended in 100 μl of deionized, glass-distilled water pretreated with diethylpyrocarbonate (Sigma). Samples were stored at −85°C until used.

Northern (RNA) analysis. Northern analysis was performed essentially as described by Selden (19). High-quality RNA (A_{260}/A_{280} = 1.9 to 2.0) was standardized by appropriate dilution in diethylpyrocarbonate-treated water to a final concentration of 1 μg/μl. Portions (5 μl) were electrophoresed at 6 V/cm through 1.0% LE agarose (FMC BioProducts, Rockland, Maine) and stained with NucliStain (National Diagnostics, Manville, N.J.). The intensities of the rRNA bands between standardized preparations were densitometrically compared to verify that all samples contained equal amounts of total RNA.

The standardized RNA samples were serially diluted in diethylpyrocarbonate-treated water and denatured at 50°C for 1 h in the presence of glyoxal (Eastman Kodak Co., Rochester, N.Y.) and dimethyl sulfoxide (Fisher Scientific, Fairlawn, N.J.). Samples were electrophoresed at 4 V/cm through 1.2% GTG agarose (FMC) gels prepared in phosphate buffer (10 mM NaH_2PO_4 [pH 7]) containing 10 mM sodium iodoacetic acid (Sigma). RNA was transferred by passive diffusion onto neutral nylon (MagnaGraph; Micron Separations Inc., Westborough, Mass.) membranes and hybridized overnight (18 to 24 h) at 65°C with various gene-specific probes. Membranes were washed as previously described (20).

The probe used for detection of *spa* was a 1.2-kbp fragment generated by *Cla*I digestion of pRIT5 (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). The remaining probes were generated by amplifying gene fragments from S6C chromosomal DNA with the polymerase chain reaction (PCR), using primers and reaction conditions previously described (20, 21). The *sea* and RNAII probes were likewise generated by PCR.

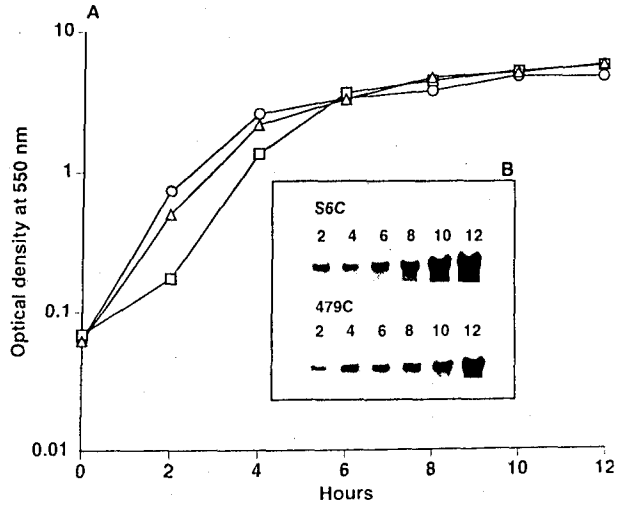

FIG. 1. (A) Representative growth curves of *S. aureus* strains S6C (○), ISP479C (△), and KSI9051 (□). S6C and ISP479C were grown in TSB, and KSI9051 was grown in TSB plus erythromycin (15 μg/ml). (B) Northern analysis of total cellular RNA isolated from S6C and ISP479C at 2, 4, 6, 8, 10, and 12 h of growth and hybridized with a *geh*-specific probe.

The primer pair used for *sea* had the sequences 5′-ACTTG TAAATGGTAGCGAGAAAAGC-3′ and 5′-TATTTGAAT ACTGTCCTTGAGCACC-3′. The primer pair used for RNAII had the sequences 5′-CGAAATGCGCAAGTTCCG TCA-3′ and 5′-CCAACTGGGTCATGCTTACGA-3′. The RNAII primer pair generated an 860-bp fragment encompassing the junction between open reading frames A and B of the *agr* operon (10, 13). PCR products were gel purified by using the Magic PCR system (Promega Corp., Madison, Wis.). All DNA fragments were labelled with digoxygenin-11-UTP as previously described (20). Hybridized probes were detected by autoradiography with alkaline phosphatase-conjugated, anti-digoxygenin $F(ab')_2$ antibody fragments (Boehringer Mannheim) and the chemiluminescent substrate AMPPD (Tropix Inc., Bedford, Mass.).

RESULTS

The effect of erythromycin and the presence of the transposon on growth were determined by comparing growth of KSI9051 in medium containing the antibiotic with growth of S6C in medium lacking the antibiotic. Although a slight lag was observed for the first 2 h, growth of KSI9051 recovered quickly and was equivalent to that of S6C and ISP479C for the remaining time points examined (Fig. 1A). Lipase was chosen as an indicator of time-dependent expression of extracellular proteins because *S. aureus* S6C produces the protein in high quantities during postexponential growth (20). To determine the optimal point during growth to assess the effect of *xpr* on expression of exoproteins, total cellular RNA was isolated from S6C and ISP479C at 2-h intervals (Fig. 1A). Standardized RNA preparations were hybridized with a *geh*-specific probe, and the results indicate that message is expressed throughout the growth of both strains but occurs at elevated levels during the postexponential phase (Fig. 1B). Therefore, in the remaining experiments in this study, RNA was isolated at 12 h of growth unless otherwise noted. In addition, levels of *geh* message were more abundant for S6C than for ISP479C. Strain S6C is known to be a hyperproducer of several extracellular

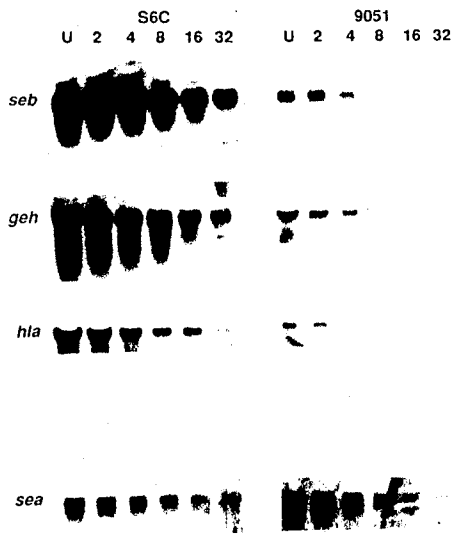

FIG. 2. Total cellular RNA isolated from S6C and 9051 at 12 h of growth and hybridized with either an *seb*- a *geh*-, an *hla*-, or an *sea*-specific probe. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values) samples.

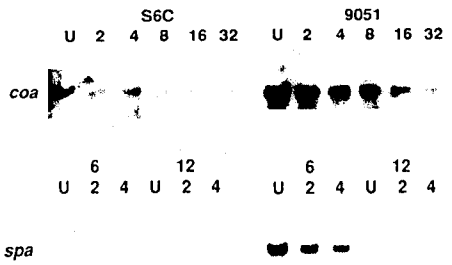

FIG. 3. Total cellular RNA isolated from S6C and 9051 at 12 h of growth for the *coa*-specific probe and at 6 and 12 h of growth for the *spa*-specific probe. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values) samples.

proteins, including lipase (20) and SEB (5). These data are consistent with our earlier report (20) that extracellular lipase activity is produced at elevated levels postexponentially.

***xpr* effect on the expression of extracellular proteins.** Total cellular RNA extracted from S6C and 9051 was probed with DNA fragments specific for the *seb*, *geh*, and *hla* genes. As shown in Fig. 2, these data indicate that the amount of message produced by strain 9051 was greatly reduced in comparison with the parental strain S6C. Message levels were at least 32-fold lower for all three genes. In contrast, the expression of *sea* was unaffected. *sea* transcript levels for S6C and 9051 were similar even at a dilution of 1/32 (Fig. 2). A similar effect in an *agr* mutant background has been reported by Tremaine and coworkers (23). Western blot (immunoblot) analysis revealed no significant differences in SEA levels produced by three different strains of *S. aureus* and their corresponding *agr* mutant strains (23). These data suggest that neither *xpr* nor *agr* regulates the expression of the *sea* gene.

***xpr* effect on the expression of cell wall-associated proteins.** In our earlier report, we determined that the relative clot-forming activity of coagulase was unchanged in the mutant strain 9051 (21). However, in this same mutant strain, *coa* mRNA levels were elevated by 16- to 32-fold (Fig. 3). We attribute this discrepancy to the rather insensitive means (i.e., formation of a fibrin clot in rabbit plasma by serial twofold dilutions of culture filtrate) of assessing coagulase activity. In addition to determining *coa* expression in the *xpr* mutant, we also examined the expression of protein A. Because *spa* message is made during the exponential phase of growth, samples were taken at 6 and 12 h. Expression of *spa* was increased in the mutant strain at least fourfold at 6 h; however, no appreciable difference in expression was observed at 12 h (Fig. 3). Expression of *spa* message in strain 9051 occurred throughout exponential phase rather than being induced when cells entered the postexponential phase of growth (data not shown). These results are identical to those observed for *spa* in an *agr*-null mutant (24).

***xpr* effect on the expression of *agr*.** mRNA levels of the *agr* operon in strains S6C and 9051 were assayed at 2-h intervals by Northern analysis. When an RNAIII-specific probe was used, expression was observed as early as 2 h (early exponential phase; Fig. 1) in S6C but was not detected (data not shown) until 6 h of growth (late exponential phase; Fig. 1) in 9051. Further analysis of RNA samples taken at 3 and 12 h of growth and probed with RNAII- and RNAIII-specific probes demonstrated that transcript levels in 9051 were reduced in comparison with S6C (Fig. 4A and B). At 3 h (Fig. 4A), a 16- to 32-fold reduction was noted, while levels at 12 h (Fig. 4B) were reduced only 8- to 16-fold. The narrowing of the differences between 3 and 12 h is most likely due to the accumulation of message over time.

The *agr* parent and mutant strains, ISP479C and ISP546, were included in this study as a control for the RNAII and RNAIII transcripts. Total cellular RNA was isolated from these strains and hybridized simultaneously with the RNAII- and RNAIII-specific probes (Fig. 4B). *agr* transcript levels in ISP479C were identical to those of S6C. However, little expression of either *agr* transcript was observed in the Agr mutant ISP546. Both RNAII and RNAIII were found in the undiluted lane, and as expected, the size of the RNAII species was smaller due to the insertion of Tn*551*, which results in a truncated message.

DISCUSSION

The disease processes of the staphylococci involve the participation of a number of virulence factors produced by the organism (6). Indeed, the list of factors has risen to greater than 30, ranging from none to many that any one strain can produce (6). The roles that these factors play in the overall process of disease are not clear. However, it is becoming increasingly clear that the coordinate regulatory mechanisms responsible for expression of these virulence factors are complex and multifactorial.

To date, three loci, *agr*, *sar*, and *xpr*, have been identified, and they apparently provide a similar regulatory role for several known *S. aureus* virulence factors (1–3, 10, 20, 21). The genes have all been mapped to distinct loci on the staphylococcal chromosome. All have been identified as regulatory elements on the basis of their similar pleiotropic effects on exoprotein production; that is, exoproteins are positively regulated, while cell wall-associated proteins are negatively regulated. The effect has been shown to occur at the mRNA level for *agr* and *sar* and, as a result of this study, for *xpr*.

Transpositional mutagenesis of *xpr* resulted in reduced levels of message for *hld*, *geh*, *seb*, and *hla*, while *spa* and *coa* message levels were increased. The fact that *hld* was affected was

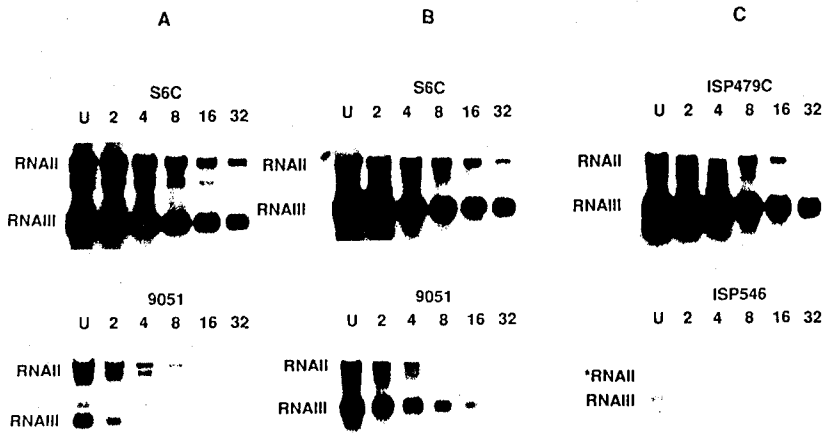

FIG. 4. Northern analysis of total cellular RNA isolated from S6C and 9051 at 3 h (A) and 12 h (B) of growth and hybridized simultaneously with probes specific for RNAII and RNAIII. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values) samples. The RNA species are labelled accordingly. For comparison, total cellular RNA was isolated from ISP479C and ISP546 at 12 h of growth (C) and also hybridized simultaneously with probes specific for RNAII and RNAIII. The asterisk denotes the truncated RNAII molecule in ISP546.

somewhat surprising since the *hld* gene resides within the RNAIII transcript, which is the regulatory component of the *agr* operon essential for exoprotein production in *S. aureus*. Further analysis of the effect of *xpr* on RNAII and RNAIII demonstrated that both transcripts were reduced in the *xpr* mutant. These data indicate, for the first time, that two regulatory components of exoprotein production in *S. aureus* are interactive. However, this finding is not unprecedented. In a recent review, Wanner (28) describes cross-regulation "as the control of a response regulator of one two-component regulatory system by a different regulatory system." Evidence for such a phenomenon is observed in the phosphate (*pho*) regulon of *E. coli* (26, 27). When P_i is limited, the *pho* regulon is induced. Induction involves the *phoR* sensor, which acts as a histidine protein kinase and is thought to phosphorylate the regulator, *phoB*. The activated *phoB*, in turn, transcriptionally activates a number of genes involved with transport and degradation of extracellular phosphorus sources. In addition, control of the *pho* regulon in *pho*R mutants has been shown, depending on which carbon source is metabolized, to involve either CreC or the Pta-AckA system. CreC is the sensor component of the CreC-CreB two-component signal transduction system involved with catabolite regulation, and the Pta-AckA system is associated with acetyl phosphate synthesis. Additional evidence for cross-regulation exists for other two-component signal transduction systems as well.

Recently, studies involving the expression of the exoprotein SEC in *S. aureus* have demonstrated that complex circuits that respond to several environmental signals are operative. Both *sec* and *agr* expression are reduced by the presence of glucose at acidic or nonmaintained pH (17, 18). However, the effect on *sec* does not require a functional *agr* (17). Likewise, expression of *sec* and *agr* is reduced when the organism is grown at alkaline pH, but unlike the glucose effect, the reduction in *sec* message requires an intact *agr* locus (15). Furthermore, this same group has also shown that expression of *sec* is reduced in media of high osmotic strength and that the reduction also does not require an intact *agr* locus (16). The sensory proteins of two-component signal transduction systems respond to some signal either from the environment or from within the cell which initiates the events of signal transduction (22). Although in *S. aureus* these signals include pH, glucose, and osmolarity, others have suggested that involvement of additional factors within the organism also modifies the expression of exoproteins (4, 24). Indeed, this work is supportive of at least two factors being interactive.

How *xpr* and *agr* and perhaps *sar* interact to coordinately regulate extracellular protein expression in *S. aureus* is unknown at present. What appears to be unfolding is a network of multiple regulators responding to numerous external and internal signals that result in a specific response to some environmental stimulus. Evidence has been presented to indicate that the *agr* system responds to pH and glucose. Whether or not *xpr* responds to the same stimuli is not known. Perhaps a more important question to ask is, at what level does the interaction between *xpr* and *agr* occur? It appears that Xpr (to date, the coding capacity of the *xpr* region is unknown) is *trans* acting; if this is so, it may also act as a sensory protein responding to some stimulus (same or different) that results in an activated form capable of interacting with other components resulting in the expression of extracellular proteins. These questions are currently being pursued in this laboratory.

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Murine Macrophage Activation by Staphylococcal Exotoxins†

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We investigated the ability of staphylococcal enterotoxins A and B, exfoliative toxins A and B, and toxic shock syndrome toxin 1 to activate macrophages. All of the toxins tested had the potential to stimulate tumoricidal activity in peritoneal macrophages from lipopolysaccharide-responsive C3HeB/FeJ mice. In contrast, none of the toxins activated cytotoxicity in lipopolysaccharide-unresponsive macrophages from C3H/HeJ mice. We also studied toxin stimulation of monokine secretion. Staphylococcal enterotoxin A, toxic shock syndrome toxin 1, and both exfoliative toxins triggered C3HeB/FeJ macrophages to secrete tumor necrosis factor alpha, but enterotoxin B induced only marginal amounts of tumor necrosis factor. All of the toxins used stimulated interleukin-6 production by macrophages from both strains of mice. Nitric oxide is produced in response to the exfoliative toxins only by the lipopolysaccharide-responsive macrophages. These results suggest that macrophages respond differently to several staphylococcal exotoxins.

Exotoxins from *Staphylococcus aureus* cause diseases such as food poisoning, scalded skin syndrome in infants, and the multisystem disease toxic shock syndrome. The toxins are serologically distinct, single-polypeptide chains, with sizes ranging from 22 kDa to approximately 35 kDa (20).

The toxins can also induce an immune response and have been termed superantigens (37). The toxins were originally described to be mitogenic (25, 36), but recent work shows that the superantigens initiate T-cell proliferation by the T-cell receptor interacting with exotoxins which are bound to class II molecules of accessory cells (24, 26, 35). Kappler et al. (22) found that T cells which express specific V_β sequences as part of their $\alpha\beta$ T-cell receptor are stimulated specifically by staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), and toxic shock syndrome toxin 1 (TSST-1).

Macrophages are also activated by these toxins. Human monocytes stimulated with SEB and TSST-1 for 1 to 6 days secreted tumor necrosis factor (TNF) (11). Beezhold et al. (2) used 20 ng of TSST-1 per ml to stimulate rat macrophages to produce interleukin-1 (IL-1). NK cells also become cytotoxic after stimulation with SEB (1).

We studied the ability of SEA, SEB, TSST-1, exfoliative toxin A (ETA), and exfoliative toxin B (ETB) to activate LPS^n (responsive) and LPS^d (unresponsive) murine macrophages. We showed activation of contact-dependent cytotoxicity, TNF and IL-6 secretion, and nitric oxide production (as measurable by the stable end product of nitrite) (NO_2^-). We also compared the kinetics of cytokine and NO_2^- production after stimulation by different toxins.

MATERIALS AND METHODS

Mice. C3HeB/FeJ (LPS^n) and C3H/HeJ (LPS^d) mice were bred in the animal facilities in the Division of Biology at Kansas State University.

Tissue culture cells. The TNF-sensitive cell line LM929 was obtained from American Type Culture Collection (Rockville, Md.). The simian virus 40-transformed cell line F5b which was cloned from cells derived from C3H.OL embryo fibroblasts ($H\text{-}2^{01}$ K^dD^k) is killed by macrophages by a contact-dependent process and has been described previously (7, 28). LM929 and F5b were cultured three times weekly in antibiotic-free Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, Md.) supplemented with 2% fetal bovine serum, 0.3% L-glutamine (Sigma, St. Louis, Mo.), and 10% Opti-MEM 1 Reduced Serum medium (GIBCO). IL-6 was quantitated with the IL-6-dependent, murine B-cell hybridoma subclone B9 which was obtained with L. Aarden's permission from R. Nordan (National Cancer Institute, Bethesda, Md.). It was cultured in Dulbecco's modified Eagle's medium supplemented with 50 μM 2-mercaptoethanol, 5% fetal bovine serum, and 10 pg of recombinant IL-6 per ml. The hybridoma did not proliferate in response to recombinant TNF, recombinant IL-1, combinations of TNF and IL-1, or any toxin used.

Reagents. Enterotoxins A and B, purified by the procedures of Bergdoll et al. (3), were obtained from Anna Johnson-Winegar (U.S. Army Medical Research and Development Command, Ft. Detrick, Md.) or Toxin Technology (Madison, Wis.). TSST-1 was obtained from Peter Bonventre (Dept. of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio). ETA and ETB were purified in our laboratories from culture supernatants of *S. aureus* UT0003 (ETA) and UT0007 (ETB). Extracellular ETA and ETB were precipitated with saturated ammonium sulfate and dialyzed against water. ETA and ETB were then purified by two cycles of preparative isoelectric focusing. All toxin preparations used in these experiments were biologically active at 100 ng/ml as determined by spleen cell proliferation assay. The exotoxins used in this study were endotoxin free as determined by the *Limulus* amebocyte lysate assay (Sigma). Our assay was sensitive to an endotoxin concentration of 0.02 ng/ml. The presence of lipoteichoic acid was determined by Isaac Ginsburg (Hebrew University Hadassah School of Dental Medicine, Jerusalem, Isreal) using hemagglutination of lipoteichoic acid-sensitized human erythrocytes (14). The toxin preparations used in this study were lipoteichoic acid negative by this method, which is sensitive to a concentration of 0.2 μg/ml. Lipid A-associated protein containing lipopolysaccharide (LPS) was obtained from David Morrison (Dept.

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of Microbiology, Kansas University Medical Center, Kansas City). Recombinant murine TNF and gamma interferon (IFN-γ) were obtained from Genzyme (Cambridge, Mass.). Recombinant murine IL-6 was obtained from R & D Systems (Minneapolis, Minn.). Experiments were conducted in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and 50 μg of gentamycin sulfate per ml (DME).

Cytotoxicity assay. The macrophage cytotoxicity assay was performed as described previously (8). C3HeB/FeJ and C3H/HeJ peritoneal exudate cells were obtained 4 or 5 days after injection of 1.5 ml of sterile thioglycolate broth (Difco, Detroit, Mich.). After peritoneal lavage, the peritoneal exudate cells were washed and pipetted into flat-bottom, 96-well plates at 1×10^5 to 4×10^5 cells per well. After 1 to 2 h the medium was removed and replaced with the appropriate toxin-containing medium (with or without 0.1 U of IFN-γ per ml). Approximately 10^4 chromium-51-labelled F5b cells were added to each well, and the assay mixture was incubated for 16 to 18 h. The microtiter plates were then centrifuged, and 90-μl aliquots from each well were counted in a gamma counter. The percent specific release was calculated as follows: specific release = [experimental release − spontaneous release/(maximal release − spontaneous release)]/× 100. The maximal release and spontaneous release were determined by incubating 10^4 F5b cells in 1 N HCl (maximal) or medium (spontaneous). The spontaneous release was generally less than 40%. As positive controls, macrophages from C3HeB/FeJ mice were stimulated with *Escherichia coli* O55:B5 LPS (12.5 μg/ml) (Difco) and IFN-γ (0.1 U/ml); C3H/HeJ macrophages were stimulated with lipid A-associated protein containing LPS (10 μg/ml) and with IFN-γ (10 U/ml).

Macrophage supernatants. C3HeB/FeJ and C3H/HeJ peritoneal exudate cells were obtained by peritoneal lavage as described above. The cells were plated at a density of 10^7 cells per 60-mm-diameter tissue culture plate, allowed to adhere for 1 to 2 h, and washed with DME. The appropriate toxin in 4 ml of DME was added for 30 min. After 30 min, the plates were washed three times with 1 to 2 ml of medium and 4 ml of fresh medium with or without toxin was added. At 3 and 6 h after the initial 30-min incubation period, the supernatants were collected and 4 ml fresh DME with or without toxin was added to the culture plates.

The supernatants were clarified by centrifugation, aliquoted, and used immediately or stored at −100°C until assayed. Supernatants collected from cultures pulsed with toxin-containing medium are designated (+) toxin. The supernatants designated (−) toxin came from cultures that received only DME after the initial 30-min pulse. As a positive control, C3HeB/FeJ macrophages were pulsed with LPS (12.5 μg/ml). Unresponsive C3H/HeJ macrophages were activated with lipid A-associated protein containing LPS (10 μg/ml) and IFN-γ (10 U/ml) as described previously (9). Cytokine and nitrite quantification was based on linear regression of standard curves of recombinant TNF, recombinant IL-6, or $NaNO_2$.

TNF quantification. Triplicate samples of culture supernatants were serially diluted in DME in 96-well, flat-bottom plates. To each well, 10^4 ^{51}Cr-labelled LM929 cells were added and incubated for 16 to 18 h. After centrifugation, 90-μl aliquots were quantitated for chromium release as described.

Nitrite determination. Nitrite concentration was determined by use of the Griess reagent (1% sulfanilamide, 0.1% naphylethylene diamine dihydrochloride, and 2% H_3PO_4) as described previously (30). Briefly, triplicate 100-μl supernatant samples were placed in microtiter plates. To each well 100 μl of Griess reagent was added and incubated for 10 min at 25°C. A microtiter plate reader (Cambridge Technology, Cambridge, Mass.) was used to read the A_{550}.

IL-6 quantification. The macrophage supernatants were assayed for IL-6 content by the B9 bioassay (17). The B9 cells were washed three times in IL-6-free DME to remove residual IL-6. Two thousand B9 cells were added to serially diluted, triplicate samples of culture supernatant and allowed to incubate for 3 days at 37°C. [^{3}H]thymidine (0.5 μCi per well) was added for 8 h prior to harvesting with a PHD Cell Harvester (Cambridge Technology) and liquid scintillation counting.

TABLE 1. Cytotoxicity of F5b tumor cells by exotoxin-activated macrophages

Activator[a]	% ($\bar{x}$ ± SEM)[b] specific release at:			
	10:1[c]	20:1	30:1	40:1
SEA	13 ± 0	12 ± 0	15 ± 2	29 ± 1
SEB	9 ± 0	8 ± 1	9 ± 1	18 ± 2
ETA	11 ± 1	18 ± 1	21 ± 0	21 ± 2
ETB	22 ± 0	25 ± 1	27 ± 0	19 ± 1
LPS + IFN-γ	34 ± 2	53 ± 1	58 ± 0	44 ± 1
Medium	8 ± 3	3 ± 1	2 ± 2	6 ± 0

[a] Thioglycolate-elicited macrophages were activated with toxin (10 μg/ml) or LPS and IFN-γ as described in the Materials and Methods.
[b] From triplicate samples.
[c] Macrophage-to-target-cell ratio.

RESULTS

Toxin induction of macrophage cytotoxicity. The ability of the toxins to induce cytotoxic macrophages was measured by killing of the cell line F5b, previously characterized by our laboratory (7, 28). The cytotoxicity of C3HeB/FeJ macrophages incubated with the exotoxins was variable, ranging from 0 to 30%. Table 1 is a representative experiment which shows the levels of tumoricidal activity that can be induced by each toxin. None of the toxins stimulated cytotoxicity to the same extent as LPS and IFN-γ. Table 2 shows that the toxins alone did not always induce cytotoxicity (SEA and SEB) or induced very low levels of killing (ETA and ETB). However, when this occurred with either toxin type, cytotoxicity could be induced when used in combination with suboptimal levels of IFN-γ (Table 2). In contrast, no toxin, under any condition, was able to induce C3H/HeJ macrophages to become cytotoxic (data not shown).

Toxin induction of NO_2^-, IL-6, and TNF secretion. Nitric oxide is released by activated macrophages (4, 30). To determine whether arginine metabolism was involved in toxin-induced activation, we measured the extracellular NO_2^- released in response to the toxins. SEA, SEB, or TSST-1 did not stimulate significant quantities of NO_2^- (Table 3). However, responsive macrophages incubated continuously with ETA or ETB released significant quantities of NO_2^- during an 18-h exposure (Table 3). Nitrite was not found in measurable quantities in any other supernatants (data not shown). The LPS[d] macrophages were not stimulated to release measurable nitrite by any of the staphylococcal exotoxins (data not shown).

To determine whether TNF is secreted independently of macrophage cytolytic activity, we incubated macrophages from C3HeB/FeJ mice with SEA. Within the first 3 h TNF

TABLE 2. IFN-γ enhancement of tumoricidal activity

Expt no.	Activator[a]	% ($\bar{x}$ ± SEM)[b] specific release at:		
		10:1[c]	20:1	30:1
1	ETA	6 ± 2	15 ± 3	10 ± 1
	ETA + IFN-γ	14 ± 0	48 ± 3	40 ± 6
	ETB	20 ± 2	19 ± 1	29 ± 0
	ETB + IFN-γ	28 ± 2	33 ± 1	47 ± 2
	Medium	9 ± 2	5 ± 0	6 ± 3
	IFN-γ	5 ± 2	2 ± 2	12 ± 2
	LPS + IFN-γ	26 ± 4	37 ± 3	42 ± 5
2	SEA	9 ± 4	6 ± 1	6 ± 1
	SEA + IFN-γ	11 ± 2	18 ± 7	18 ± 2
	SEB	0 ± 1	0 ± 1	0 ± 1
	SEB + IFN-γ	29 ± 2	24 ± 2	21 ± 17
	Medium	0 ± 0	1 ± 3	6 ± 1
	IFN-γ	3 ± 5	0 ± 4	0 ± 2
	LPS + IFN-γ	19 ± 4	22 ± 3	27 ± 0

[a] Thioglycolate-elicited macrophages were activated with toxin or LPS in the presence or absence of IFN-γ as described in Materials and Methods. Reagents were used in the following concentrations: ETA, ETB, SEA, and SEB, 10 μg/ml (each); IFN-γ, 0.1 U/ml.
[b] From triplicate samples.
[c] Macrophage-to-target-cell ratio.

TABLE 4. Kinetics of TNF production by exotoxin-stimulated macrophages

Expt no.	Stimulant	Stimulation conditions[a]	U of TNF secreted[b] at:		
			3 h	6 h	18 h
1	ETA	Continuous (+)	30	4	4
		30 min (−)	10	4	4
	ETB	Continuous (+)	25	40	24
		30 min (−)	6	4	4
	LPS	Continuous (+)	>200	180	>200
	Medium		4	4	4
2	SEA	Continuous (+)	148	26	2
		30 min (−)	34	2	2
	SEB	Continuous (+)	12	2	2
		30 min (−)	2	2	2
	LPS	Continuous (+)	>200	40	20
		30 min (−)	>200	27	2
	Medium		2	2	2

[a] Macrophages were incubated in the continuous presence of toxin (+) or were pulsed for only 30 min (−) with 10 μg of SEA or SEB per ml, 1 μg of ETA or ETB per ml, or 12.5 μg of LPS per ml.
[b] TNF concentrations were determined by linear regression of standard curves with recombinant murine TNF. Peritoneal macrophages were from C3HeB/FeJ mice. Supernatants were collected and assayed for TNF at the indicated times as described in the Materials and Methods. Values represent the amount of TNF produced in one representative experiment of two to four independent trials.

appeared in the culture supernatants (Table 4). After that time, TNF secretion decreased to background levels. In contrast, responsive macrophages incubated continuously with SEB secreted only marginal amounts of TNF. There appears to be a threshold exposure time, because we found that C3HeB/FeJ macrophages pulsed with SEA or SEB for only 30 min did not secrete TNF.

TNF was secreted from C3HeB/FeJ macrophages in response to both ETA and ETB stimulation. The continuous exposure to ETA induced approximately 30 U of TNF per ml over the first 3 h (Table 4). As observed with SEA, the TNF levels dropped to background for the remainder of the 18-h experiment. In contrast, ETB induced 30 to 40 U/ml during each collection period. A 30-min pulse of either exfoliative toxin was not enough to induce significant TNF secretion (Table 4).

At a concentration of 2.5 μg/ml, TSST-1 stimulated TNF secretion when C3HeB/FeJ macrophages were pulsed for 30 min or incubated continuously with toxin (Fig. 1). When responsive macrophages were continuously incubated with suboptimal concentrations (20 ng/ml) of TSST-1, the macrophages also produced 10 U of TNF per ml. C3H/HeJ macrophages were stimulated to produce a small amount of TNF (18 U/ml) only when incubated continuously with 2 μg of TSST-1 per ml (data not shown). SEA, SEB, ETA, and ETB did not stimulate LPS[d] macrophage TNF production (data not shown).

IL-6 was induced by all of the exotoxins tested. Either a 30-min pulse or the continuous exposure of SEA stimulated the LPS-responsive macrophages to secrete increasing amounts of IL-6 over time, with the greatest accumulation occurring during the last 12 h (Table 5). IL-6 was secreted into the culture supernatant in response to SEB, though the quantities were significantly lower and secretion appeared to peak earlier (Table 5). The exfoliative toxins induced much higher levels of IL-6 than the enterotoxins (Table 5). The continued presence of ETA and ETB induced 2 or more ng of IL-6 per ml throughout the 18 h. The kinetics of cytokine secretion induced by the exfoliative toxins also were different from those of that induced by the enterotoxins. Table 5 shows that a 30-min pulse of toxin stimulated the most IL-6 to be secreted within the first 3 h, with decreasing quantities being secreted after this time point. This is the opposite of the kinetics of a 30-min SEA pulse (Table 5).

We found that macrophages from C3H/HeJ mice could be induced to secrete only IL-6 in response to toxin stimulation. Tables 5 and 6 indicate that the continuous presence of SEA stimulated much higher concentrations of IL-6 than did SEB. However, SEB induced significantly more than background levels of IL-6. As found with the LPS[n] macrophages, the exfoliative toxins also induced IL-6 secretion by LPS[d] macrophages. ETA and ETB induced the secretion of 24 and 55 pg, respectively (Table 6) of IL-6 per ml, which compared favorably with the levels induced by the enterotoxins. However, the macrophages were stimulated with only 1 μg of exfoliative toxin per ml as compared with 10 μg of enterotoxin per ml.

The accumulation of IL-6 in the culture supernatant over an 18-h incubation was also measured (Table 6). Macro-

TABLE 3. Nitric oxide produced by toxin-stimulated C3HeB/FeJ macrophages

Treatment[a]	($\bar{x}$ ± SEM) μM, NO_2 [b]
SEA	1.2 ± 1.3
SEB	1.7 ± 1.5
ETA	26.0 ± 2.6
ETB	7.9 ± 0.6
TSST-1	1.9 ± 2.8
LPS	152.0 ± 43.3
Medium	0.9 ± 0.7

[a] The stimulant-containing medium was added to adhered macrophages for 18 h. The supernatant was removed and frozen at −100°C until assayed with Griess reagent.
[b] Values were calculated from the results of two to six experiments by linear regression from a standard of known quantities of nitrite.

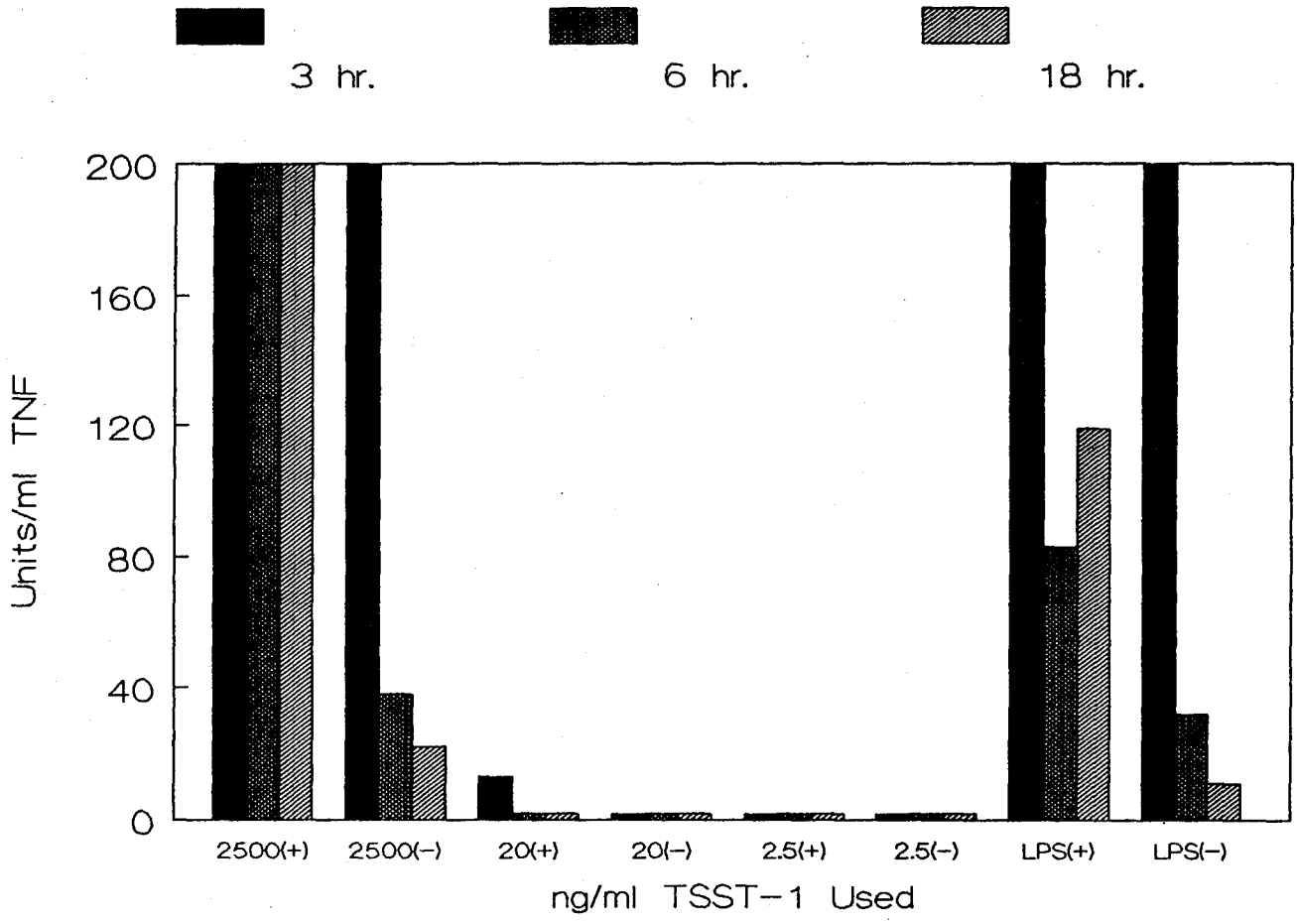

FIG. 1. Kinetics of TNF production by C3HeB/FeJ macrophages stimulated with TSST-1. C3HeB/FeJ macrophages were incubated in the presence (+) or absence (−) of TSST-1 after an initial 30-min pulse of TSST-1. Supernatants were collected and assayed for TNF at 3, 6, and 18 h as described in Materials and Methods.

TABLE 5. Kinetics of IL-6 production by exotoxin-stimulated macrophages

Stimulant	Stimulation conditions[a]	IL-6 secreted (pg/ml)[b] in: C3Heb/FeJ cells at: 3 h	6 h	18 h	C3H/HeJ cells at: 3 h	6 h	18 h
ETA	Continuous (+)	2,170	>2,400	>2,400	7	4	6
	30 min (−)	1,460	1,173	776	4	1	4
ETB	Continuous (+)	1,995	2,311	>2,400	7	9	19
	30 min (−)	1,121	558	524	4	2	6
SEA	Continuous (+)	329	718	>2,400	2	6	32
	30 min (−)	212	218	1,146	4	5	8
SEB	Continuous (+)	758	994	993	6	3	8
	30 min (−)	169	323	150	2	2	5
Medium		42	55	191	2	1	6

[a] Macrophages were incubated in the continuous presence of toxin (+) or were pulsed for only 30 min (−) with 10 μg of SEA or SEB per ml or 1 μg of ETA or ETB per ml.

[b] IL-6 concentrations were determined by linear regression of standard curves with recombinant murine IL-6. Peritoneal macrophages were from the indicated mouse line. Supernatants were collected and assayed for IL-6 at the indicated times as described in Materials and Methods. Values represent the amount of IL-6 produced in one representative experiment of two to five independent trials.

phages from C3HeB/FeJ mice exposed to a 30-min pulse or incubated continuously with ETA, ETB, or SEA secreted large quantities of IL-6. In contrast, the continued presence of SEB was necessary to stimulate greater than background levels of IL-6. The quantities of IL-6 secreted by the LPS[d] macrophages were significantly lower (Table 6). The continued presence of each toxin induced IL-6 secretion, but macrophages from C3H/HeJ mice also secreted IL-6 following a 30-min pulse of ETB or SEA. However, a pulse of SEB or ETA did not yield IL-6 in the culture supernatant (Table 6).

DISCUSSION

Staphylococcal exotoxins induce dramatic pathophysiological changes in vivo. We investigated the potential of these toxins to activate murine peritoneal macrophages, as determined by cytotoxicity and production of nitrite, TNF, and IL-6. Previous studies found that exotoxins secreted by *S. aureus* stimulated T-cell proliferation (6, 25, 26, 35, 36). Spleen cells from both C3H/HeJ and C3HeB/FeJ mice proliferated in response to 100 ng of each of our toxin preparations per ml (data not shown), confirming the biological activity of our reagents. SEA, ETA, ETB, and TSST-1 induced C3HeB/FeJ macrophages to produce substantial amounts of TNF. SEB differed from those toxins by induction of only marginal amounts of TNF. The inability of SEB to induce high levels of TNF secretion contrasts with results

TABLE 6. IL-6 secreted in response to toxin stimulation

Mouse strain	Toxin (μg/ml)	IL-6 (pg/ml)[a]	
		+Toxin	−Toxin
C3HeB/FeJ	ETA (1)	>1,600	>1,600
	ETB (1)	>1,600	>1,600
	SEA (10)	1,318	1,280
	SEB (10)	>1,600	790
	Medium		864
C3H/HeJ	ETA (1)	24	10
	ETB (1)	56	21
	SEA (10)	33	24
	SEB (10)	19	11
	Medium		14

[a] +Toxin, adhered thioglycolate-elicited macrophages were stimulated with toxin for 30 min and washed three times, 4 ml of toxin-containing medium was added, and the mixture was incubated for 18 h. The supernatant was collected, and IL-6 secretion was determined as described in Materials and Methods. −Toxin, treatment is the same as for +Toxin, except that after the wash, Dulbecco's modified Eagle's medium was added and the mixture was incubated for 18 h. The data are representative of two to five experiments, depending on the toxin.

of other recent studies. SEB and TSST-1 have recently been shown to induce human monocytes to transcribe mRNA for IL-1β and TNF (32). Fast et al. (11) also showed that TSST-1 and SEB stimulated human monocytes to produce TNF over a period of 1 to 6 days. It is possible that murine macrophages are not stimulated by SEB to the same extent as human monocytes. Murine macrophages may bind toxins differently than human macrophages because of the toxin's structure and differences in major histocompatability complex class II molecules.

Previous investigations have found that nitric oxide production and TNF secretion are concomitantly induced by the calcium ionophore A23187 (5). Macrophage activation with various toxins resulted in differential secretion of nitric oxide, TNF, and IL-6. For example, ETA and ETB were the only toxins we tested that stimulated nitrite secretion by C3HeB/FeJ macrophages. Although the amounts of nitrite produced were less than that induced by an LPS pulse, these data suggest that ETA and ETB and SEA, SEB, and TSST-1 regulate arginine metabolism differently.

We found that all toxins but SEB induced LPS-responsive macrophages to secrete greater than 15 U of TNF per ml and that only IL-6 is secreted by C3H/HeJ macrophages in response to toxin stimulation. The kinetics of nitrite, IL-6, and TNF secretion after stimulation with various toxins also differs from the response induced by the other toxins. For example, nitrite was measurable only after accumulating for 18 h in the continuous presence of exfoliative toxins, while IL-6 was measurable within 3 h and was continuously made during the 18-h incubation. In contrast, SEA induced TNF secretion within the first 3 h of stimulation, while IL-6 was not secreted in large quantities until after 6 h of exposure. Therefore, these data indicate independent regulation of nitrite, TNF, and IL-6 by these exotoxins.

The finding that some toxins can activate macrophages independently of nitrite secretion is consistent with previous studies by Green et al. (15). They found that TNF but not the macrophage activators C5 and C5a induced nitric oxide production. The production of one monokine in the absence of another or the independent regulation of monokines is consistent with a number of previous studies. For example, Riessenfeld-Orn et al. (27) stimulated human monocytes with *Streptococcus pneumoniae* to induce IL-1 but not TNF production. Lonnemann et al. (23) found that TNF and IL-1 secretion peaked at separate times when human monocytes were stimulated with LPS. Zuckerman et al. (38) also found that serum TNF peaked prior to IL-1 concentrations after in vivo stimulation. The observations that these staphylococcal exotoxins have such distinct effects on macrophages (see Table 7 for a general summary) make them interesting tools for further investigations of macrophage activation.

Tumoricidal activity induced by the toxins was variable. However, at times when no cytotoxicity was induced by the toxins, tumoricidal activity could be obtained by the same toxin preparations in conjunction with suboptimal concentrations of IFN-γ. The irregular induction of cytolytic activity may be a result of differences in the induction of IFN-α and subsequent autocrine responses. This would be consistant with multiple signals being required for macrophage cytolysis (16, 33). The finding that SEA, SEB, and TSST-1 induce cytotoxicity without NO_2^- contrasts with the results of Takema et al. (31). This difference may be attributed to the cells used in analyzing cytotoxicity. Our assays measured the cytotoxicity of F5b cells, and Takema et al. measured the killing of P815 cells. Data from our laboratory indicate that macrophage interactions with P815 cells are different from their interactions with F5b (28) and that F5b is not killed by a nitric oxide-dependent process (37a).

Macrophages from C3H/HeJ mice were not cytotoxic when stimulated with toxins. The lack of activity was not due to toxin breakdown, because the same toxin preparations stimulated spleen cells from unresponsive mice to proliferate. Furthermore, when stimulated with lipid A-associated protein containing LPS and IFN-γ, the C3H/HeJ macrophages responded as expected by becoming cytotoxic or secreting monokines.

The finding that LPS^d macrophages were not activated with the exotoxins is consistent with reports of others. C3H/HeJ macrophages do not respond to the calcium ionophore A23187 (29). They also cannot be made cytotoxic by

TABLE 7. Summary of exotoxin activation of macrophages

Macrophage source and exotoxin	Activity in C3HeB/FeJ cells (LPS responders)				Activity in C3H/HeJ (LPS nonresponders)			
	Tumoricidal activity	TNF (U/ml)[a]	IL-6 (pg/ml)[a]	NO_2^- production	Tumoricidal activity	TNF (U/ml)[a]	IL-6 (pg/ml)[a]	NO_2^- production
SEA	+	150	1,318	−	−	0	36	−
SEB	+	12	1,600	−	−	0	19	−
ETA	+	30	1,600	+	−	0	24	−
ETB	+	40	1,600	+	−	0	55	−
TSST-1	+	200	224	−	−	18	28	−

[a] In culture supernatants of macrophages incubated with toxin-containing medium for 18 h.

Propionibacterium acnes (21) or by the lipid A moiety of gram-negative bacterial LPS (10). Therefore, the genetic defect of C3H/HeJ mice that causes unresponsiveness to other stimuli extends to staphylococcal exotoxins. However, all of the toxins induced the LPS^d macrophages to secrete low levels of IL-6, and TSST-1 induced TNF secretion. The finding that unresponsive macrophages can be induced to secrete monokines without becoming cytotoxic is consistent with the results of previous studies (19). In addition, Flebbe et al. (13) found that C3H/HeJ macrophages could be stimulated by LPS isolated from rough mutants to secrete TNF and IL-1 but required an additional signal of IFN-γ to become cytotoxic. The hypothesis that distinct signals may be provided to macrophages by different toxins is not unprecedented. Smooth and rough LPS activate cells from C3H/HeJ mice differently (12, 13). The presence of protein in LPS also stimulates macrophages in a manner different from that of protein-free preparations (9, 18). We also add that though the C3H/HeJ mouse serves as an interesting model to study how these toxins work, it may not necessarily be one that reflects human disease well.

It is possible that the exotoxins stimulate different macrophage functions because they trigger autocrine responses. Vogel and Fertsch (33) found that macrophages produce IFN in response to priming signals. Macrophages require multiple signals to become fully activated (16), and each staphylococcal exotoxin may stimulate IFN differently to provide those signals. Macrophages respond differently to the various exotoxins tested (Table 7). The kinetics of TNF and nitric oxide secretion in the presence or absence of toxin also indicate that the signals transduced by each exotoxin may be distinct. Alternatively, all of the toxins may stimulate the various responses. However, structural differences may make some more potent stimulants. The conditions we chose to measure stimulation may have failed to detect the poorer activators. Additional studies will be required to elucidate whether signal transduction pathways for these toxins are indeed different.

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Skeletal unloading causes organ-specific changes in immune cell responses

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ARMSTRONG, JASON W., KRISTA A. NELSON, STEVE J. SIMSKE, MARVIN W. LUTTGES, JOHN J. IANDOLO, AND STEPHEN K. CHAPES. *Skeletal unloading causes organ-specific changes in immune cell responses.* J. Appl. Physiol. 75(6): 2734–2739, 1993.—The effects of skeletal unloading using antiorthostatic tail suspension on the mouse immune system are tissue specific. This phenomenon was demonstrated by analyzing cells from the lymph nodes, spleen, and bone marrow. Phytohemagglutinin-induced T-cell proliferation was depressed in lymph nodes after 11 days of antiorthostatic suspension. In contrast, splenic T-cell proliferation in response to phytohemagglutinin was enhanced. Splenic natural killer cell cytotoxicity was unchanged after suspension, which demonstrated the organ- and cell-specific effects of skeletal unloading. Whereas antiorthostatic suspension induced minimal changes in bone, there was a significant depression in the number of macrophage precursors in the bone marrow. Overall, skeletally unloaded animals had slightly higher blood corticosterone levels than did control animals; however, it did not appear to be responsible for the observed changes. In conclusion, skeletal unloading produces organ- and cell-specific changes in the murine immune system rather than a generalized immunosuppression.

phytohemagglutinin; T-cell; antiorthostatic tail suspension; macrophage differentiation

SKELETAL UNLOADING, using the antiorthostatic suspension model, results in bone resorption similar to that seen after extended bed rest, osteoporosis, and spaceflight (12). Antiorthostatic suspension involves the suspension of a rodent in a harness or by its tail so that the animal is maintained with a head-down tilt without its bearing weight on its hindlimbs. Other physiological changes associated with skeletal unloading include muscle atrophy (20), anterior fluid shifts (6), changes in neutrophil activation (4), and increases in stress hormone levels (4). This model can also mimic several immunologic changes induced by spaceflight, which makes it a useful tool for immunologic studies (2).

Previous studies in our laboratory found that skeletal unloading affected the secretion of superoxide by inflammatory neutrophils (4). However, similar treatment did not affect inflammatory macrophages (2, 10). We also found that splenic T-cell proliferation is enhanced by skeletal unloading (10). Nash et al. (13, 14) have shown that rats that have been skeletally unloaded by either antiorthostatic suspension or spaceflight have lymphoid organ-dependent proliferative responses that do not necessarily parallel each other. Furthermore, Sonnenfeld et al. (18) have shown an increase in the percentage of T-cells in rat bone marrow after skeletal unloading in a 14-day spaceflight. This mounting body of evidence suggests that skeletal unloading has differential effects on the immune system.

The purpose of this study was to determine the effects of skeletal unloading on the development, distribution, and/or function of immune cells in mouse bone marrow, lymph nodes, and spleen. These tissues were analyzed because they were altered by skeletal unloading incurred during extended spaceflight on US space shuttle and Soviet COSMOS missions (14, 18). Our results suggest that skeletal unloading does not exert a generalized effect on immunologic processes.

MATERIALS AND METHODS

Animals. Male adult (8- to 11-wk-old) C3HeB/FeJ mice, bred in the animal facility at Kansas State University, were used in these studies. The use of these animals was approved by the Animal Care and Facilities Use Committee at Kansas State University, which complies with National Institutes of Health animal care standards.

Suspension technique. Animals were antiorthostatically suspended using the Wronski-Morey-Holton tail-suspension cage as reviewed by Chapes et al. (2). Briefly, mice were tail suspended at an angle of 22° such that their hindlimbs were skeletally unloaded. Cables connected to the tails of mice were attached to a low-resistance pully system above the cages, which allowed the animals complete movement in any direction within the enclosure. Each suspension experiment involved three suspension categories: antiorthostatic suspension (experimental, as described above), orthostatic control suspension (tail was attached to the pully system; however, the mice bore full weight on both their hindlimbs and forelimbs), and nonsuspended (normal cage control). Mice were weighed before suspension and before being killed. In all experiments, mice were killed between 8:00 and 10:00 A.M. to eliminate differences caused by circadian rhythms. To remain consistent with previous studies, the suspension period was chosen to be 11 days (4, 10).

Blood collection, preparation, and corticosterone assay.

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Immediately before each animal was killed, blood was collected from the retroorbital sinus with use of a Pasteur pipette. The blood was dispensed into microfuge tubes, and serum was collected after coagulation and stored at −20°C. Samples were assayed for corticosterone as described previously (4).

Splenic and lymph node lymphocyte proliferation assay. Proliferation was assayed for all treatment groups simulatneously as previously described by our laboratory (10). Briefly, lymphocytes were obtained by expression of the cells from the spleen or inguinal lymph nodes through a wire sieve. Red blood cells in spleen cell preparations were lysed with ice-cold 0.17 M NH_4Cl for 5 min on ice. Lymphocytes were washed twice with Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) and 5 μg/ml of gentamycin sulfate [supplemented DMEM (DME)]. Lymph node cells (5×10^5/well) or spleen cells (1×10^6/well) were added in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). Wells received 100 μl of medium: DME supplemented with 5.6×10^{-5} M 2-mercaptoethanol with or without phytohemagglutinin (PHA, 9 μg/ml; Wellcome Biotechnology, Research Triangle Park, NC) or exfoliative toxin A (ETA, 20 μg/ml; purified as described in Ref. 10).

Natural killer (NK) cell cytotoxicity. NK cells were obtained from the spleen cell preparations used for T-cell proliferation assays. NK cell cytotoxicity was measured by determining the amount of lysis of YAC-1 tumor cells [American Type Culture Collection (ATCC), Rockville, MD] in a 5-h ^{51}Cr-release assay. The assay was performed in 96-well round-bottomed plates as described (10).

Bone marrow cells. Bone marrow cells were obtained from the femora and tibiae of mice from the three treatment groups. The ends of the femora and tibiae were removed, and the cells were flushed from the bone with the use of DME and a 26-gauge needle. The cells were passed three times through a 19-gauge needle to break up cell clumps, pelleted at 325 *g*, and resuspended in ice-cold 0.17 M NH_4Cl for 5 min to lyse red blood cells. Cells were pelleted again and resuspended in the appropriate media for flow cytometric analysis or cell culture.

Flow cytometric analysis. All steps in this procedure were performed on ice. Bone marrow cells (1×10^6/well), suspended in DME, were added to a 96-well round-bottomed plate. The plate was centrifuged at 400 *g* for 2 min, and the supernatants were discarded. Cells were incubated in 50 μl of FBS per well to inhibit nonspecific Fc receptor binding, washed twice with sorter buffer (Hanks' balanced salt solution containing 2% FBS and 0.2% bovine serum albumin), and incubated with 50 μl of the appropriate primary antibody per well. In all cases except the $F(ab')_2$ secondary antibody, monoclonal hybridoma-derived antibodies were used. After the primary antibody incubation, cells were washed twice in sorter buffer and incubated with secondary antibody [fluoresceinated goat anti-mouse $F(ab')_2$; Cappel, Durham, NC] for 30 min. Cells were washed twice in sorter buffer, resuspended in 300 μl of phosphate-buffered saline containing 1% paraformaldehyde, and stored at 4°C until flow cytometric analysis (FACScan, Becton-Dickinson).

Target cells and antibodies. B-cells were labeled with goat anti-mouse immunoglobulin [$F(ab')_2$]. ATCC hybridoma-derived antibodies used to label cells were as follows: macrophages, TIB 166-derived anti-MAC-2; polymorphonuclear leukocytes, TIB 183; T-cells, HB 23-derived anti-Thy 1; T-helper cells, TIB 207-derived anti-L3T4 (CD4); T-cytotoxic cells, TIB 211-derived anti-Lyt 2 (CD8); and major histocompatibility complex II-positive cells, TIB 94-derived anti-$I\text{-}A^k$.

Bone marrow macrophage colony assay. Bone marrow cells were suspended at a concentration of 1×10^5 cells/1.5 ml of DMEM containing 0.3% agar, 10% FBS, and 15% LM929 fibroblast-conditioned medium [colony-stimulating factor (CSF) I source]. Every 24 h, five microscope view fields were scored for macrophage colonies (a group of >25 cells).

Bone analysis. One femur from each mouse was left intact for bone analysis. These femora were cleaned of nonosseous tissue and were allowed to dry at room temperature (25°C). The bones were rehydrated (0.15 N NaCl, 25°C) for 3 h before mechanical testing. This protocol has been shown to have minimal effects on flexural strength and stiffness (1). The bones were tested to failure under three-point flexure with use of an Instron 1331 servo-hydraulic testing system (16). A deflection rate of 5 mm/min was used, and the force-deflection properties of "stiffness" (the slope of the force-deflection curve during elastic loading) and maximum force were evaluated.

The samples were then sectioned within 0.5 mm of the fracture (at middiaphysis) with the use of an Isomet low-speed diamond saw (Buehler). The middiaphyses were evaluated under a light microscope (×100) using a 3.5-μm resolution micrometer to determine medullary cavity area, cortical area, and the cortical thickness on the ventral and dorsal sides of the cross section (12). Subsequent to the microscopy, the bone pieces were dried at 75°C, weighed, decalcified in Trizma base (Sigma Chemical)-buffered EDTA (pH = 7.0) for 6 days, dried at 75°C, and weighed a second time. The ratio of the decalcified weight to the fully calcified weight was designated the collagen fraction (F_c), and thus the mineral percentage of the bone sample was calculated as $(1 - F_c)100\%$.

Statistical analysis. The Number Cruncher Statistical Package (J. L. Hintze, Kaysvill, UT) was used to perform Student's *t* tests to determine statistical significance of the leukocyte-related experiments. Because of the nongaussian distribution of the bone data, all femoral data were evaluated using the Kruskal-Wallis nonparametric test followed by paired Wilcoxon rank-sum tests where appropriate. A 95% level of significance was used for each test.

RESULTS

Stress measurements. To determine the amount of stress incurred by animals used in this study, we made three measurements. We determined the percentage of the initial body weight lost, the spleen weight as a percentage of final body weight, and the serum corticosterone concentration. There was considerable variation between animals within treatment groups. Table 1 illustrates the overall patterns of stress incurred for the 72 animals used in this study. Not all of these animals were

TABLE 1. *Comparison of stress measurements in skeletally unloaded and control mice*

Suspension	Initial Body Wt Lost, %	P	Spleen Wt, %Final Body Wt	P	Corticosterone Concn, ng/ml	P
Antiorthostatic	8.6±0.9	<0.01	0.3037±0.0092	<0.05	122±14	<0.04
Orthostatic	4.6±0.8		0.3281±0.0080		87±12	
None	−0.8±2.0	<0.05	0.3486±0.0138	<0.1	50±10	<0.01

Values for initial body wt loss, spleen wt, and corticosterone concn are means ± SE; $n = 24$ mice per group. Mice were suspended or caged normally for 11 days before they were killed. Percentage of initial body wt lost was calculated as 100[(wt of mouse presuspension − wt of mouse postsuspension)/wt of mouse presuspension], percentage of spleen wt was calculated as 100(wt of spleen postsuspension/wt of mouse postsuspension), and corticosterone concn in serum was determined by competitive radioimmunoassay. P values were determined by comparison of orthostatic (control) vs. antiorthostatic or no treatment as determined by 2-tailed matched t test analysis.

used for every experiment described in this report. Therefore, we have also reported two of the measurements of stress in each table. As a group, animals subjected to antiorthostatic suspension lost ~8.6% of their initial body weight compared with 4.6% and −0.8% lost by orthostatic and normal control animals, respectively. Antiorthostatically suspended animals averaged ~122 ng/ml corticosterone compared with 87 ng/ml by animals in the two control groups. Therefore, antiorthostatic suspension was slightly more stressful than the other treatments.

Effects of antiorthostatic suspension on T-cell proliferation. We previously found that antiorthostatic suspension enhanced splenic T-cell proliferative responses induced by mitogens and superantigens (10). To determine whether other T-cell populations were similarly affected, we assayed inguinal T-cell proliferation induced by PHA and ETA. Table 2 summarizes the results of four independent experiments. The PHA-induced splenic T-cell proliferation of cells obtained from antiorthostatically suspended animals was significantly greater than that of cells from control animals. Lymph node T-cells obtained from the same antiorthostatically suspended mice exhibited significantly depressed PHA-induced proliferation. ETA-induced proliferation was not significantly affected by antiorthostatic suspension for either lymph node or splenic T-cells. Therefore, antiorthostatic suspension had an organ- and T-cell subpopulation-dependent effect on mice.

Effects of antiorthostatic suspension on NK cell cytotoxicity. To determine whether antiorthostatic suspension would affect other splenic lymphocyte populations, we assayed NK cell-mediated cytotoxicity of YAC-1 tumor cells. Table 3 presents data showing that NK cell-mediated cytotoxicity was not significantly affected by antiorthostatic suspension. Table 4 emphasizes that even when T-cells and NK cells are isolated from the same animals, significantly different responses to antiorthostatic suspension occur. This finding reiterates that antiorthostatic suspension induces organ- and lymphocyte subpopulation-specific effects.

Effects of antiorthostatic suspension on bone. The measured effects of the suspension protocol on bone parameters in our mice were less than those observed for skeletally immature mice (16, 17). The bones of antiorthostatically suspended mice did not demonstrate lower stiffness or strength compared with either control group (Table 5). In addition, the cortical area was not significantly reduced in the femora of the suspended mice. The medullary cavity area of the suspended femora was significantly ($P < 0.05$) larger than that of the control mice only when the data for orthostatic and normal control groups were pooled. The bones of normal control mice had significantly thicker diaphyseal cortices at both the dorsal (3.4%) and ventral (12.6%) measurement locations. In addition to these thickness differences, the femora of normal control mice were more mineralized (69.5%) than those of suspended mice (66.5%). For the thickness and mineralization measurements, the values for orthostatic control mice were intermediate to those of suspended and normal control mice.

TABLE 2. *Effects of skeletal unloading on T-cell proliferation*

Suspension	Medium cpm	PHA cpm	PHA SI	ETA cpm	ETA SI
		Spleen			
Antiorthostatic	5,571±977	126,416±22,619*	25.6±6.2†	66,291±6,154	12.7±1.7
Orthostatic	5,142±833	78,638±20,434	18.0±6.1	72,689±8,147	15.5±3.3
None	5,730±1,175	60,291±18,316	12.6±5.5	82,119±15,283	15.1±3.0
		Lymph node			
Antiorthostatic	2,036±881	71,282±21,097‡	43.9±18.6†	32,125±13,645	24.7±9.1
Orthostatic	1,657±480	111,669±14,152	84.6±23.2	30,612±8,743	26.3±10.0
None	2,146±1,023	114,532±7,029	93.5±31.8	21,364±2,122	19.4±7.9

Values are means ± SE of counts per minute (cpm) and stimulation index (SI) calculated for 4 independent experiments; $n = 4$ per group except for lymph node cells from animals caged normally (no suspension) and stimulated with exfoliative toxin A (ETA; $n = 3$). Lymphocytes were pooled from 3 animals per treatment group per experiment. Splenic and inguinal lymph node lymphocytes were stimulated with 5 μg/ml of phytohemagglutinin (PHA) or 5–10 μg/ml of ETA for 48 h. C3HeB/FeJ mice were suspended antiorthostatically (AOS) or orthostatically (OS) or were caged normally (NOR) for 11 days. Corticosterone concns for animals used in these experiments were AOS, 107±10 ng/ml; OS, 72±19 ng/ml; and NOR, 44±10 ng/ml (AOS vs. OS, $P > 0.10$ and OS vs. NOR, $P < 0.10$ as determined by a 2-tailed paired Student's t test). Statistically significant from orthostatic control by 2-tailed paired Student's t test: * $P < 0.01$; † $P < 0.02$; ‡ $P < 0.09$.

TABLE 3. *Effect of skeletal unloading on splenic natural killer cell cytotoxicity of YAC-1 tumor cells*

Suspension	Specific ^{51}Cr Release 300:1	200:1	100:1	Corticosterone Concn, ng/ml
Antiorthostatic	22.3±7.4	22.0±7.0	10.0±2.0	153±21
Orthostatic	25.3±6.8	24.3±5.8	13.8±2.8	107±16
None	22.7±7.2	25.0±6.1	18.8±4.5	52±13

Values are means ± SE of %specific ^{51}Cr release of 3 (effector-to-target ratio 300:1) or 4 (200:1 and 100:1) independent experiments or of corticosterone concn. Lymphocytes were pooled from 3 animals per treatment group per experiment. Splenic lymphocytes were recovered from C3HeB/FeJ AOS, OS, and NOR mice treated for 11 days. No significant differences in cytotoxicity between any treatment groups at any effector-to-target ratio were observed: AOS vs. OS, $P < 0.10$ and OS vs. NOR, $P < 0.02$ for corticosterone concn determinations by 2-tailed paired Student's t test.

TABLE 4. *Differential effects of skeletal unloading on lymphocyte subpopulations*

Suspension	Lymphocyte Population: Spleen cell proliferation	Spleen NK cell cytotoxicity	Lymph node cell proliferation	Corticosterone Concn, ng/ml
Antiorthostatic	8.9±0.6	41±0	11.9±0.3	100±3
Orthostatic	4.3±0.4	41±0	48.5±5.5	108±42
None	4.6±0.7	40±0	52.6±5.9	34±8

Values for cell proliferation are means ± SE of SI of quadruplicates from 1 representative experiment; these cells were stimulated for 48 h with 5 μg/ml of PHA. Values for natural killer (NK) cell cytotoxity are means ± SE of specific killing of quadruplicates from 1 representative experiment; these cells were assayed for cytotoxicity of YAC-1 tumor cells (effector-to-target ratio 200:1). C3HeB/FeJ AOS, OS, and NOR mice were treated for 11 days. Cells were pooled from 3 animals per treatment group. Average corticosterone concns for 3 animals used in each treatment group were AOS vs. OS, $P > 0.10$ and OS vs. NOR, $P < 0.10$ by 2-tailed Student's t test.

Bone marrow-derived macrophage development. Bone marrow cells from antiorthostatically suspended mice had a significantly reduced capacity to form macrophage colonies in the presence of CSF I compared with both control groups (Fig. 1). Bone marrow cells from skeletally unloaded animals formed only 5.7 ± 1.6 colonies on *day 7* of culture compared with 13.5 ± 1.0 and 13.2 ± 2.9 colonies by cells from orthostatic and normal cage control animals, respectively. This experiment was performed on a total of 15 individual animals for each treatment group in five separate experiments. Similar data were recorded for each experiment.

Effect of antiorthostatic suspension on cell subpopulations of bone marrow. We assayed whether skeletal unloading would affect the cellular composition of the bone marrow. Table 6 lists the percentage of cells expressing phenotypic markers including Thy 1, CD4, CD8, MAC-2, and immunoglobulin. After 11 days of suspension, we observed no differences between the cell subpopulations of the bone marrow cells from antiorthostatic, orthostatic, or normal treatment groups.

DISCUSSION

Skeletal unloading, using antiorthostatic tail suspension, affects the murine immune system. The effects are organ, cell subpopulation, and stimulus dependent. Organ specificity was shown when inguinal lymph node T-cell proliferative responses were depressed after antiorthostatic suspension, whereas splenic T-cell responses were enhanced in response to PHA. Nash et al. (13) have previously shown skeletal unloading to have organ-dependent effects on the rat immune system. They demonstrated a decrease in peripheral blood and lymph node lymphocyte proliferation in response to concanavalin A and no effect on splenocyte proliferation. Postspaceflight analysis of rats found a similar organ-specific effect (14). However, neither study investigated bone marrow, the compartment from which all immune cells arise, so we have extended what is known about compartmentalization to include mice and bone marrow.

Cell subpopulation specificity was illustrated by the differential effects of skeletal unloading on splenic T-cells and NK cells. Whereas antiorthostatic suspension enhanced splenic T-cell proliferative responses to PHA, there was no effect on NK cell cytolytic activity. Similar discrepancies between lymphocyte subpopulations have also been observed in skeletally unloaded rats (14, 15).

TABLE 5. *Femora measurements*

Measurement	Group: Suspended	Orthostatic control	Normal control
Stiffness, N/mm	100.0±19.0	104.3±17.0	99.8±17.5
Maximal force, N	21.0±3.4	21.2±3.4	20.7±2.8
Medullary cavity area, mm^2	0.496±0.090	0.451±0.096	0.458±0.050
Cortical area, mm^2	0.889±0.090	0.897±0.103	0.911±0.108
CT dorsal, μm	207±21	208±22	214±19*
CT ventral, μm	269±46	285±44	303±38*
%Mineral	66.5±2.7	67.1±3.4	69.5±2.1*

Values are means ± SD; n = 15, 14, and 13 mice in suspended, orthostatic control, and normal control groups, respectively. CT, cortical thickness. * Significantly different from suspended group ($P < 0.05$).

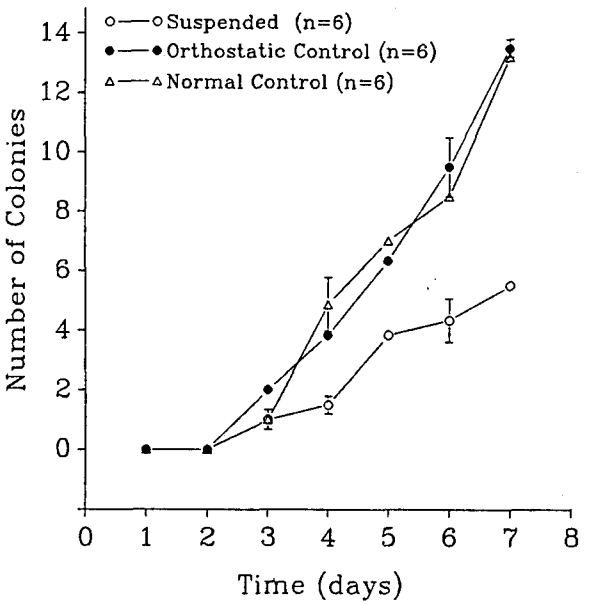

FIG. 1. Effect of skeletal unloading on macrophage development. Absence of error bars indicates SE smaller than set symbol size. Analysis of plasma corticosterone concentrations from mice used in this experiment revealed no significant difference between 3 groups ($P > 0.1$).

TABLE 6. *Flow cytometric analysis of bone marrow cell subpopulations*

Antibody Treatment	Predominant Cell Type or Epitope	%Antibody Positive		
		Antiorthostatic suspension	Orthostatic suspension	Normal control
Anti-Ig	B-cell	21.2±4.0	19.4±4.8	18.9±4.2
TIB 166	Macrophage	4.7±1.2	3.5±0.6	3.3±0.8
TIB 183	PMN	59.7±5.5	58±4.2	57.4±3.8
TIB 94	MHC II	21.7±1.8	22.4±2.2	22.9±3.3
HB 23	T-cell	5.7±0.5	5.2±0.2	5.7±0.9
TIB 207	T_h-cell	3.3±0.9	3.3±0.8	4.4±0.9
TIB 211	T_c-cell	5.2±0.8	4.2±0.6	4.9±0.7

Values are means ± SE of cells from 9 individual mice except experiments involving HB 23-derived antibody (n = 6). Bone marrow cells were recovered from C3HeB/FeJ AOS, OS, and NOR mice treated for 11 days. Ig, immunoglobulin; PMN, polymorphonuclear leukocyte; MHC II, major histocompatibility complex II; T_h-cell, T-helper cell; T_c-cell, T-cytotoxic cell.

Rykova et al. (15) found NK cytolytic activity toward YAC-1 cells to be unaffected by antiorthostatic suspension. Therefore, there appear to be some similarities between the rat and mouse systems. Both species also exhibit a depressed lymph node proliferative response after skeletal unloading, whereas the splenic compartment appears to be refractory to any inhibitory effects of antiorthostatic suspension (10, 13).

Our observed stimulus-dependent effects of antiorthostatic suspension in the mouse are consistent with observations made in the rat (13). Whereas PHA-induced splenic T-cell responses were augmented by skeletal unloading, no such enhancement was seen when the superantigen ETA was used. ETA stimulates a different subpopulation of T-cells than PHA does, which may explain the difference. Cortisone-resistant T-cells are stimulated by PHA, whereas ETA stimulates a smaller subpopulation of T-cells determined by the V_β region of the T-cell receptor (3, 8). We previously reported that antiorthostatic suspension enhanced PHA-induced splenic T-cell proliferation. We have confirmed that observation. However, we also reported the same effect with staphylococcal exotoxins B and ETA. The data presented in this study do not support the latter finding. The discrepancy may be attributed to a change in suspension techniques used by our laboratory. The corticosterone concentrations of mice subjected to the older technique were considerably higher (2) and may have affected the ETA-responsive cells more than the PHA-responsive cells. Corticosterone can affect T-cell subpopulations differently (3).

We found that animals subjected to antiorthostatic suspension or orthostatic suspension exhibited symptoms of stress compared with normally caged mice. Previous studies that subjected mice to those treatments found weight loss, reduced spleen weights as a percentage of final body weight, and increased corticosterone concentrations (4, 10). The use of the Wronski-Morey-Holton tail-suspension cage with mice significantly reduced the symptoms of stress (2). However, there was considerable variation between animals. Therefore, it was important to look at the amount of stress incurred by animals used in any particular experiment. Such an analysis indicates that corticosterone is not responsible for the differential effects of antiorthostatic suspension on lymphocyte proliferation. We conclude this for three reasons. First, if corticosterone were responsible for changes in T-cell proliferation, then one would have expected to see a unidirectional and systemic effect on all PHA-responsive T-cell populations. Second, in a previous study, we found stress hormone levels in orthostatically suspended mice to be in the range of 135 ng/ml (10). In this study, corticosterone concentrations averaged 122 ng/ml in mice subjected to antiorthostatic suspension. Only T-cells from the latter group exhibited enhanced PHA-induced proliferation. Finally, the corticosterone concentrations of the orthostatically and antiorthostatically suspended mice used for the experiments presented in Tables 2 and 3 were not significantly different ($P > 0.10$) despite contrasting T-cell proliferative responses. Some investigators have suggested that corticosterone differentially affects the cells in spleen and peripheral blood (3). This suggestion may offer one reason for the different responses of splenic and lymph node T-cells. We also cannot rule out the contribution of other stress hormones such as prolactin, which can affect lymphocyte responses (21).

Relatively minor changes in bone were observed. Age, sex, genotype, and suspension protocol differences between this and former studies (16, 17) may explain this discrepancy. Subjective observations that C3H mouse bones are harder to cut than B6 mouse bones would suggest that there are strain differences and that C3H mouse bone is more resistant to the effects of skeletal unloading. The differences in cortical thickness between suspended and normal control mice suggest that antiorthostatic suspension was beginning to suppress diaphyseal bone deposition. Direct measurements of bone deposition with use of tetracycline double labels were not implemented to avoid possible antibiotic and/or stress interactions. These measurements would have been useful for validating the suppressed growth hypothesis. Changes in bone thickness and mineralization may be related to changes in the femur marrow milieu. Because the activity of diaphyseal osteoblast cells, derived from mesenchymal cells that line the periosteum and endosteum, is dependent on local circulatory conditions, it is possible that the alteration in bone growth is also affected by altered circulatory and hematopoietic conditions in the femur marrow. The apparently larger effects of suspension on the intramembranous rather than periosteal surface of the diaphysis support this interpretation.

The differential effects of skeletal unloading were also seen in the bone marrow. Antiorthostatic suspension induced a severe depression in bone marrow cell development into macrophages in response to CSF I. In contrast, the distribution of mature cells was unchanged. Similar observations were made for the rat system after antiorthostatic suspension or spaceflight (17, 18). In these studies, depressed bone marrow cell colony formation was seen in response to both CSF I and granulocyte-macrophage CSF. Because skeletal unloading has been shown to affect bone structure (12), it is conceivable that changes in bone affect the microenvironment of the bone

marrow. Changes in bone marrow cytokine levels may be responsible for the impaired colony formation observed after suspension. Candidate cytokines would include CSF I, interleukin-6, and transforming growth factor-β (7, 9, 11). Alternatively, decreased colony formation may result from changes in a given cell population not screened with our repertoire of monoclonal antibodies. For instance, changes in bone marrow stromal cells would be expected to alter hematopoietic cytokine levels and consequently affect macrophage precursors (5). In pursuit of a mechanistic explanation for decreased macrophage colony precursors, we are investigating changes in hematopoietic growth factor production within the bone marrow microenvironment.

Antiorthostatic suspension did not effect bone marrow cell subpopulations. This may seem paradoxical in light of the lower number of detectable macrophage precursors. Eleven days of skeletal unloading may not be long enough to observe changes in mature bone marrow cell subpopulations. Longer unloading periods may be necessary to ascertain whether changes in cell subpopulations within the bone marrow begin to occur. Sonnenfeld et al. (18) have found changes in rat bone marrow T-cell numbers after 14 days of spaceflight and in B-cell numbers after 14 days of suspension. An impaired macrophage hematopoietic process for an extended period should be accompanied by a decrease in peripheral blood monocytes. Taylor et al. (19) have shown a depression in peripheral blood monocyte numbers in astronauts after space shuttle missions. A possible link between changes in peripheral immune cell numbers and hematopoietic activities could be investigated by longer-duration suspensions in which peripheral immune cell populations are monitored along with bone marrow cell populations.

In conclusion, it appears that skeletal unloading produces organ- and cell-specific changes in the murine immune system rather than a generalized immunosuppression. We are currently involved in studies aimed at elucidating the mechanistic phenomena behind these localized effects of skeletal unloading.

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Effects of antiorthostatic suspension and corticosterone on macrophage and spleen cell function

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Abstract: **The purpose of this study was to determine whether antiorthostatic suspension of C3HeB/FeJ mice for a period of 11 days affected macrophage and spleen cell function. We found that antiorthostatic suspension did not alter macrophage secretion of prostaglandin E_2, tumor necrosis factor α, and interleukin-1. Antiorthostatic suspension also did not affect macrophage-mediated contact-dependent cytotoxicity, TNF-mediated cytotoxicity, expression of class II histocompatibility molecules, or concanavalin A and *Bandeiraea simplicifolia* lectin binding sites. The proliferative response of splenic T cells in response to mitogens and staphylococcal exotoxins was significantly enhanced in antiorthostatically suspended mice. We detected significantly higher concentrations of corticosterone in the plasma of antiorthostatically suspended mice. Therefore, there did not appear to be any direct immunosuppressive effects of corticosterone on the parameters tested.** ***J. Leukoc. Biol.*** **52: 202-208; 1992.**

Key Words: *antiorthostatic suspension • corticosterone • stress*

INTRODUCTION

Antiorthostatic suspension is a ground-based stress model developed by Morey [1] that closely stimulates several physiological changes associated with space travel [1-5]. Although some studies have investigated the effect of antiorthostatic suspension on the immune system [4, 6-11] little work has been done to examine the effects of antiorthostatic suspension and the associated rise in corticosterone on inflammatory murine macrophage and spleen cell functions. Therefore, we examined the effects of antiorthostatic suspension on macrophage production of secretory products, cytotoxic function, and expression of cell surface receptors as well as proliferative responses of lymphocytes. Although elevated plasma corticosterone levels were detected in antiorthostatically suspended mice, none of the parameters we studied appeared to be negatively affected by the corticosterone. Furthermore, the mitogen- and toxin-induced proliferation was enhanced for spleen cells isolated from antiorthostatically suspended mice.

MATERIALS AND METHODS

Mice

C3HeB/FeJ mice were bred in the animal facilities in the Division of Biology at Kansas State University. Only mice that had attained a minimum weight of 22 g were used in these experiments.

Reagents

The mitogens phytohemagglutinin (PHA) and concanavalin A (Con A) were purchased from Wellcome Biotechnology (Research Triangle Park, NC) and Sigma (St. Louis, MO), respectively. Exfoliative toxin A (Eta) was purified by isoelectric focusing from ammonium sulfate-precipitated culture supernatants of *Staphylococcus aureus* strain UT0003 [12]. Enterotoxin B (Seb), purified by the procedures of Bergdoll et al. [13], was obtained from Dr. Anna Johnson-Winegar (U.S. Army Medical Research and Development Command, Ft. Detrick, MD). *Escherichia coli* lipopolysaccharide (LPS) 055:B5 was purchased from Difco (Detroit, MI). Recombinant murine tumor necrosis factor (TNF) and interferon-γ (IFN-γ) were obtained from Genzyme (Cambridge, MA). Fluorescein isothiocyanate (FITC)-conjugated Con A (C-7642) and BSI-B_4 (#L-2895) were purchased from Sigma. Goat antimouse immunoglobulin G (IgG) conjugated to FITC was obtained from Hyclone (Logan, UT). Protein A-purified monoclonal antibodies specific for I-A^k (IgG2b) were produced by the hybridoma TIB-94, which was purchased from the American Type Culture Collection (ATCC, Rockville, MD). *Propionibacterium acnes* was obtained from Wellcome Biotechnology.

Tissue Culture Cells

Cell line LM929, a TNF-sensitive fibroblast, BALB/c 3T3 fibroblasts, and VERO cells were obtained from the ATCC. The simian virus 40 (SV40)-transformed cell line F5b is killed by macrophages through a contact-dependent process and has been described previously [14, 15]. LM929, VERO, 3T3, and F5b were cultured three times weekly in antibiotic-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) supplemented with 2% fetal bovine serum (FBS), 0.3% L-glutamine (Sigma), and 10% Opti-MEM 1 reduced serum medium (Gibco).

Suspension Technique

We used the antiorthostatic suspension technique that has been described previously [8]. Each suspension experiment

Abbreviations: ATCC, American Type Culture Collection; Con A, concanavalin A; DMEM, Dulbecco's modified Eagle's medium; Eta, exfoliative toxin A; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL-1, interleukin-1; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; PEC, peritoneal exudate cell; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; PMN, polymorphonuclear leukocyte; Seb, enterotoxin B; SI, stimulation index.

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involved three suspension categories: antiorthostatic suspension (experimental), orthostatic suspension (control), and nonsuspended (normal control). Mice were age, weight, and gender matched among these three categories. The mice that were antiorthostatically suspended were taped to allow the hindlimbs to be unloaded and unrestrained, while the forelimbs supported approximately 30% of their body weight. Orthostatic suspension involved taping the tail of the mice, yet allowing 100% of their body weight to be supported by all four limbs. Normal or nonsuspended mice were caged alone and unrestrained during the suspension period of 11 days. Four days before sacrifice, an inflammatory response was induced in all mice by an intraperitoneal injection of 700 μg of *P. acnes*. In all experiments, mice were sacrificed between 8:00 and 10:00 AM to eliminate differences caused by circadian rhythms.

Blood Collection and Preparation

Immediately before animal sacrifice, blood was collected from the retro-orbital sinus using a Pasteur pipet treated with a disodium ethylenediaminetetraacetate (EDTA)-indomethacin solution (28 nM and 14 μM, respectively). This treatment prevented coagulation and arrested arachidonic acid metabolism. The blood was dispensed into microfuge tubes containing 100 μl of the EDTA-indomethacin solution and then mixed well. Immediately thereafter, the blood was centrifuged at 2500*g* for 15 min at 4°C. A portion of the supernatant (platelet-poor plasma) was collected and stored at −100°C for corticosterone analysis and a portion was acid stabilized for subsequent prostaglandin E_2 (PGE_2) analysis.

Spleen Cell Proliferation Assay

Spleens were removed from euthanized mice, weighed, and homogenized. Red blood cells were lysed with ice-cold 0.17 M NH_4Cl for 5 min on ice. The spleen cells were then washed twice with DMEM containing 2% FBS and 5.0 μg/ml gentamicin sulfate (DME). Cells were plated at 1×10^6 cells/well (100 μl) in Costar 96-well, flat-bottom microtiter plates. Wells then received 100 μl of medium: DMEM supplemented with 2% FBS and 5.6×10^{-5} M 2-mercaptoethanol (2-ME) with or without one of the following stimulants at the concentration indicated: PHA (9 μg/ml), Con A (2 μg/ml), Eta (1 μg/ml), and Seb (1 μg/ml). Cells were incubated for 48 h at 37°C in 8% CO_2. Six hours prior to harvest, 0.5–1.0 μCi of [^{3}H]thymidine was added to each well. Cells were harvested on glass fiber discs and added to vials with 1 ml of Scintiverse BD cocktail (Fisher). Incorporation of [^{3}H]thymidine by the cells was determined by a Tri-Carb 1500 liquid scintillation analyzer (Packard). The stimulation index (SI) was calculated from the following formula:

$$SI = \frac{\text{cpm of experimental}}{\text{cpm of background}}$$

Experimental represents spleen cells incubated in medium plus stimulant. Background represents spleen cells incubated in medium alone. Background proliferation levels of unstimulated spleen cells were not different between treatment groups.

Peritoneal Cells

Peritoneal exudate cells (PECs) were harvested from euthanized mice by washing the peritoneal cavity twice with 10–12 ml of ice-cold phosphate-buffered saline (PBS). Cytosmears were made with 5×10^5 cells and stained with a modified Wright's stain. Three fields of at least 100 cells were scored for macrophages, neutrophils, and lymphocytes.

Peritoneal Macrophage Supernatants

PECs were recovered as described above. Cells were plated at a concentration of 5×10^6 cells per 60-mm tissue culture plate. The cells were allowed to adhere for 1.5–2.0 h before medium containing nonadherent cells was removed. After washing the cells, 3 ml of DME alone or LPS and IFN-γ (12.5 μg/ml and 10 U/ml, respectively). The plates were incubated at 37°C at 8% CO_2 and supernatants were collected after 30 min or 24 h of incubation. The supernatants were clarified by centrifugation, frozen, and stored at −100°C for TNF or interleukin-1 (IL-1) analysis.

Infection of BALB/c 3T3 Target Cells with Vaccinia Virus

The WR strain of vaccinia virus, obtained from ATCC, was propagated and titered using VERO cells. BALB/c 3T3 cells were plated at a concentration of 1×10^6 cells per 60-mm tissue culture plate and then labeled overnight with 100 μCi of ^{51}Cr in 3 ml of DME. One hour before addition to the cytotoxicity assay, the BALB/c 3T3 cells were infected at a multiplicity of infection of 2–3 in 1 ml of DME. The plates were rocked at 37°C; then the cells were washed, dispersed, and added to the assay at a concentration of 1×10^4 cells/well.

TNF Assay

Triplicate samples of peritoneal macrophage culture supernatants were assayed for TNF as described previously using the TNF-sensitive cell line LM929 [12].

IL-1 Assay

IL-1 secretion into culture supernatants was assayed by the previously described comitogenic murine thymocyte assay [16]. It is possible that some of the proliferation measured is due to the additional presence of IL-6. We have reported our measurements of proliferation as IL-1 with the explicit understanding that IL-6 may be a factor.

Cell Surface Labeling for Flow Cytometric Analysis

Peritoneal cells suspended in DME were plated at a concentration of 1×10^6 cells/well in a 96-well, U-bottom microtiter plate. The plate was centrifuged at 400*g* for 2 min and supernatants were removed. Cells were incubated 30 min on ice with 50 μl/well of fetal bovine serum to inhibit Fc receptor binding, which increased specific binding 80 to 160% (data not shown). Cells were washed with 100 μl/well of sorter buffer (Hanks' balanced salt solution containing 0.1% bovine serum albumin and 0.1% sodium azide). Plates were centrifuged and supernatants were removed. Labeling with fluoresceinated lectins: A volume of 20 μl of 0.5 mg/ml FITC-Con A or 0.5 mg/ml FITC-BSI-B_4 was added per well. Cells and labeled lectins were pipetted up and down several times to mix and then incubated for 30 min at 4°C. After centrifugation at 400*g* for 2 min, supernatants were removed and cells were washed twice with 100 μl/well of PBS. Cells were resuspended in 2 ml of PBS containing 1% paraformaldehyde and stored at 4°C. Labeling with primary and secondary antibody: To the appropriate wells, 20 μl of 0.4 mg/ml anti-I-A^k TIB-94 was added. The cells and primary antibody were mixed well and incubated for 30 min

at 4°C. Plates were centrifuged at 400*g* for 2 min and supernatants were removed. Cells were washed twice with PBS at 100 μl/well. A secondary fluoresceinated antibody, goat antimouse IgG (1:4 dilution) was added in a volume of 20 μl/well and mixed. A 30-min incubation at 4°C followed before washing twice with PBS. Cells were resuspended in PBS containing 1% paraformaldehyde and stored at 4°C until flow cytometric analysis. As a background control, PECs were labeled with only the FITC-goat antimouse IgG.

Flow Cytometric Analysis

The proportion of fluorescently labeled PECs was determined using a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA) equipped with an air-cooled, 15-mW argon ion laser that emitted at 488 nm. Parameters analyzed include forward and right-angle light scatter as well as fluorescence properties of the cell. CONSORT 30 software was utilized for data acquisition and analysis of 5×10^3 cells per sample.

Corticosterone Assay

Plasma samples were assayed for corticosterone as described previously [8] except that anticorticosterone antibody was diluted 1:30 to achieve approximately 30% binding.

Statistical Analysis

The Number Cruncher Statistical Package (J.L. Hintze, Kaysvill, UT) was used to perform Student's *t*-test to determine statistical significance on various tests. Chi-square analysis was used to determine statistical significance for PEC differential counts.

RESULTS

Analysis of Peritoneal Exudate Cell Populations of Suspension Mice

Peritoneal exudate cells elicited 4 days after an intraperitoneal injection of *P. acnes* were examined for three cell types—macrophages, lymphocytes, and neutrophils—to determine whether they were altered by antiorthostatic suspension. No significant differences in inflammatory cells were observed between the treatment groups. The macrophages were present in the highest proportion: 68, 69, and 66% for antiorthostatic, orthostatic, and nonsuspended treatment groups, respectively. Percentages of lymphocytes and neutrophils ranged from 14 to 19% for all treatment groups. We also compared the total peritoneal cell numbers washed from individual mice in the three treatment groups and found no significant differences.

Effects of Antiorthostatic Suspension on Body Weight, Spleen Weight, Spleen Cell Number, and Plasma Corticosterone Levels

Exposure to stress increases serum glucocorticoid levels [17]. In turn, glucocorticoids are responsible for causing the depletion of lymphoid organs and the redistribution of cell populations within those organs [18-22]. To assess the amount of stress incurred from antiorthostatic suspension, we compared body weights, spleen weights, total spleen cell numbers, and plasma corticosterone levels of mice from each of the three treatment groups (**Table 1**). In order to determine how antiorthostatic suspension altered body weight, we calculated body weight as a percentage of initial body weight. Nonsuspended mice demonstrated the lowest reduction in body weight (99% of their initial weight), and antiorthostatically suspended mice demonstrated the highest reduction in body weight (89% of initial weight). Spleen weights are presented as a percentage of final body weight. Once again, spleen weights were most significantly reduced in antiorthostatically suspended mice compared to the mice from orthostatic and nonsuspended treatment groups (Table 1). Total spleen cell numbers of 1.6, 1.8, and 2.0×10^3 cells per spleen were determined for antiorthostatic, orthostatic, and nonsuspended treatment groups, respectively (Table 1). Because corticosterone is used as an indicator of stress [23], we measured plasma corticosterone concentrations in the treatment mice. The highest concentration of corticosterone (273 ng/ml) was detected in the plasma of antiorthostatically suspended mice; orthostatically and nonsuspended mice had 135 and 94 ng/ml, respectively (Table 1). Significant differences were determined for body weights ($P < .01$), spleen weights ($P < .01$), spleen cell numbers ($P < .05$), and corticosterone concentrations ($P < .01$) between all treatment groups. We also measured the amount of PGE_2 in the plasma using high-performance liquid chromatography. The concentrations of PGE_2 in the plasma of mice from the three treatment groups did not differ significantly (data not shown).

Cytokine Production

To determine whether the increased amounts of glucocorticoids of antiorthostatically suspended mice affected cytokine production, we measured TNF and IL-1 production. The production of TNF almost doubled in concentration from 30 min to 24 h; however, no significant differences in TNF production were observed between treatment groups (**Table 2**). The ability of activated macrophages to produce IL-1 and PGE_2 was also evaluated. Stimulation with LPS doubled IL-1 production but it did not differ significantly between treatment groups (**Fig. 1**). There also was no difference ($P > .1$ for all comparisons) in PGE_2 secretion between

Table 1. Effect of Antiorthostatic Suspension on Body Weight, Spleen Weight, Spleen Cell Numbers, and Plasma Corticosterone Levels

Suspension[a]	Body weight as % of initial body weight[b]	Spleen weight as % of final body weight[c]	Total spleen cell count (10^3)	Corticosterone[d] (ng/ml)
Antiorthostatic	89 ± 0.6[e,f]	0.67 ± 0.02	1.6 ± 0.1	273 ± 26
Orthostatic	93 ± 0.8	0.85 ± 0.02	1.8 ± 0.1	135 ± 16
None	99 ± 0.6	0.91 ± 0.03	2.0 ± 0.1	94 ± 18

[a] Mice were suspended or caged normally for 11 days prior to sacrifice.
[b] Percentage is calculated as 100 × (weight of mouse postsuspension/weight of mouse presuspension).
[c] Percentage is calculated as 100 × (weight of spleen postsuspension/weight of mouse postsuspension).
[d] Corticosterone levels in plasma were determined by radioimmunoassay.
[e] Numbers represent mean ± SEM; n = 43-51 mice per treatment group.
[f] Significant differences between antiorthostatic vs. orthostatic vs. none (normal control) as determined by two-tailed matched *t*-test analysis. $P < .01$ for body weight, spleen weight, and corticosterone; $P < .05$ for spleen cell count.

Table 2. Tumor Necrosis Factor α Production by Peritoneal Macrophages of Antiorthostatically Suspended Mice

Suspension[a,b]	TNF (U/ml)[c] (± LPS/IFN-γ) stimulation period[d]			
	(−) 30 min	(+) 30 min	(−) 24 hour	(+) 24 hour
Antiorthostatic	0.0	2.5 ± 0.4[e,f]	0.0	4.7 ± 0.6
Orthostatic	0.0	2.7 ± 0.3	0.0	5.4 ± 0.4
None	0.0	2.8 ± 0.2	0.0	5.5 ± 0.4

[a] Peritoneal macrophages from C3HeB/FeJ mice were elicited by i.p. injection of 700 μg *P. acnes* 4 days prior to sacrifice.

[b] Mice were suspended or caged normally for 11 days prior to sacrifice.

[c] TNF determined by multiple regression analysis of unknowns compared to recombinant TNF standard cytotoxicity of ^{51}Cr-labeled LM 929 target cells.

[d] Peritoneal macrophages were cultured in medium or LPS and IFN-γ (10 μg/ml, 10 U/ml, respectively) for 30 min or 24 h.

[e] Numbers represent mean ± SEM; n = 12 per treatment group.

[f] No significant differences between any treatment group as determined by two-tailed matched t-test analysis.

treatment groups. Macrophages secreted 3.5 ± 0.8, 4.0 ± 1.1, and 4.0 ± 0.9 nmol/ml when isolated from antiorthostatically or orthostatically suspended animals or normally housed animals, respectively.

Cell Surface Molecule Expression

Cell surface molecules are essential for the proper regulation and effector functions of the macrophage. We wanted to determine whether antiorthostatic suspension would affect expression of class II molecules, Con A binding sites (mannose and, to a lesser extent, galactose specific), and BSI-B_4 binding sites (galactose specific) on peritoneal exudate cells elicited by *P. acnes*. Each probe detected different proportions of the total population screened during this analysis. On average, monoclonal antibody specific for I-A^k bound 51% of the total population, and Con A bound 97% and BSI-B_4 bound 59% of the total cell population. The percentage of cells staining positive for the various probes was not significantly different between any treatment groups.

Macrophage Cytotoxicity of Tumor Cells and Virus-Infected Cells

Macrophage cytotoxicity can be mediated by a direct contact mechanism or by soluble macrophage products. We have shown that the SV40-transformed cell line F5b is killed by macrophages via a contact-dependent process [14, 15]. Alternatively, virus-infected 3T3 cells, unlike normal 3T3 cells, were found to be susceptible to TNF-mediated cytotoxicity. Because glucocorticoids have been known to inhibit tumoricidal activity [24], we wanted to determine whether antiorthostatic suspension affected either contact-dependent or TNF-mediated cytotoxicity of *P. acnes*-elicited peritoneal macrophages. The results indicate that neither contact-dependent cytotoxicity nor TNF-mediated cytotoxicity was affected by antiorthostatic suspension or elevated corticosterone concentrations (**Table 3**).

Lymphocyte Proliferative Response to Mitogens and Toxins

Little is known about the effects of antiorthostatic suspension on murine T cell blastogenesis. We studied the effects of antiorthostatic suspension on splenic lymphocyte proliferation stimulated by the mitogens PHA and Con A and staphylococcal exotoxins Seb and Eta. Because these molecules stimulate different subpopulations [25–28], the use of these four probes provided a comprehensive evaluation of the proliferative capacity of spleen cells from suspended mice. **Table 4** illustrates these experiments. The toxins, Eta and Seb, did not stimulate the T lymphocytes to the same extent as the mitogens, PHA and Con A. This may be indicative of the small population that is normally stimulated by these toxins (i.e., only T cells with specific Vβ regions: 1, 3, 10, 11, 12, and 17 respond to Sea and 10, 11, and 15 to Eta) [29]. Interestingly, both the toxin- and mitogen-induced lymphocyte responsiveness was increased in antiorthostatic compared to orthostatic and nonsuspended treatment groups, although the difference was more apparent with the mitogen-stimulated lymphocytes (Table 4). For instance, Con A–stimulated lymphocytes had a stimulation index (SI) of 13.7 for nonsuspended and 21.6 for antiorthostatic, and Seb-stimulated lymphocytes had a SI of 2.7 for nonsuspended and 2.9 for antiorthostatic (Table 4). Even though there was considerable variation between mice, statistical analysis of matched animals determined that there was a significant difference in SI between the treatment groups: $P \leq .10$ for toxin-stimulated lymphocytes, $P < .01$ for PHA, and $P < .05$ for Con A (Table 4).

DISCUSSION

Experiments were done to determine the impact of antiorthostatic suspension on the macrophage production of TNF, IL-1, superoxide (data not shown), and PGE_2. None of these mediators were significantly affected by antiorthostatic suspension. To date, few studies have investigated the effect of antiorthostatic suspension on the production of cytokines. Those studies found decreased production of IFN-α/β by mice when challenged with an intravenous injection of polyriboinosinic-polyribocytidylic acid [10, 11]. The reduction of interferon production correlated with a loss of resistance to the diabetogenic strain of encephalomyocarditis virus [11, 30]. Our laboratory previously found impaired superoxide production by peritoneal polymorphonuclear leukocytes (PMNs) of antiorthostatically suspended mice [8].

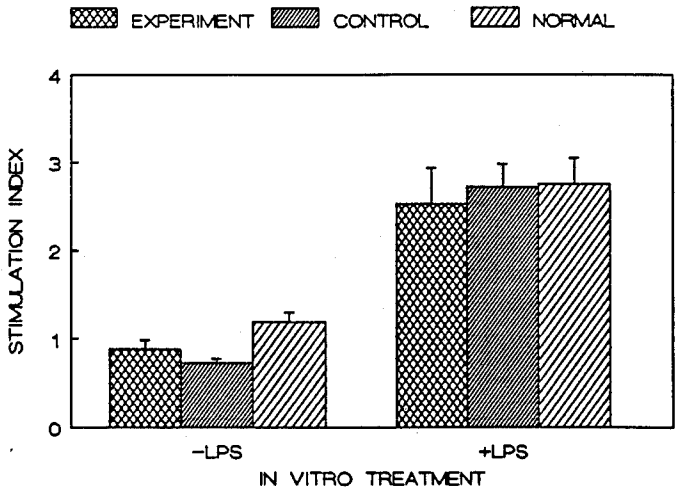

Fig. 1. Interleukin-1 production of *P. acnes*-elicited peritoneal macrophages of antiorthostatically suspended (experiment), orthostatically suspended (control), and nonsuspended (normal) C3HeB/FeJ mice. Macrophages were incubated for 18 h in DME with or without LPS (12.5 μg/ml).

Table 3. Suspension Mouse Macrophage Cytotoxicity of Virus-Infected 3T3 Cells and F5b Tumor Cells

	% specific release[b]		
Suspension[a]	3T3	3T3v[c]	F5b
Antiorthostatic	8 ± 2[d,e]	32 ± 1	37 ± 2
Orthostatic	5 ± 1	30 ± 1	38 ± 3
None	5 ± 1	29 ± 1	32 ± 3

[a]Mice were suspended or caged normally for 11 days prior to sacrifice.
[b]Cytotoxicity was determined in a 16-h assay using a macrophage/target ratio of 30:1.
[c]3T3 cells were infected at a multiplicity of infection of 2.
[d]Numbers represent mean ± SEM; n = 12 mice per treatment group where 3T3 or 3T3$_v$ were target cells and n = 9–11 mice per treatment group where F5b were target cells.
[e]No significant differences between any treatment group with any target as determined by two-tailed matched t-test analysis.

Because those studies focused on the PMNs, whereas macrophages were the subject of the current study, the results may not be directly comparable. To our knowledge, no other studies have investigated the effects of antiorthostatic suspension on the production of any eicosanoid, TNF or IL-1. However, cold water stress has been found to inhibit macrophage production of both TNF and IL-1 [31, 32].

In the course of these studies, vaccinia-infected 3T3 cells, as opposed to normal 3T3 cells, were found to be sensitive to TNF-mediated killing. This is the first report, to our knowledge, that demonstrates that infection of cells with vaccinia virus makes them susceptible to TNF-mediated cytotoxicity. However, this is consistent with studies showing that other viruses [33, 34] and intracellular pathogens [35] can alter cellular sensitivity to TNF. In spite of this, it appears that the physiological changes that occur in response to antiorthostatic suspension do not alter macrophage cytotoxicity when mediated by TNF or by contact.

Consistent with our studies of macrophage secretion and cytotoxicity, the expression of class II histocompatibility molecules and Con A and BSI-B_4 binding sites was not significantly altered by suspension treatments. No direct comparisons to our results can be found in the literature. However, in various stress studies, Ia expression may or may not be affected by elevated corticosterone [36, 37]. Zwilling et al. [37] suggest that factors, other than corticosterone, associated with stress contribute to Ia antigen expression. Interestingly, Steffen and Mussachia [4] reported increased expression of glucocorticoid receptors on muscle tissue in antiorthostatically suspended rats. Macrophages from cold water-stressed animals also were reported to have increased amounts of prothrombinase complex enzymes on the cell surface compared to controls [3]. However, the different experimental systems make comparisons difficult.

Despite numerous studies demonstrating the suppressive effects of glucocorticoids on macrophage secretion [38–46], cytotoxicity [24], or membrane molecule expression [47–51], our results did not show any correlation between elevated corticosterone concentration and any of these parameters. It is possible that we did not see a depression in secretory responses because the *P. acnes*-activated macrophages were less susceptible to corticosteroid-mediated inhibition. Others have found that glucocorticoid-induced inhibition of TNF production by porcine alveolar macrophages and human monocytes could be reversed if the cells were incubated with IFN-γ [45, 52]. Arachidonic acid release by A23187- or zymosan-treated alveolar macrophages also was not inhibited by glucocorticoids if the cells were simultaneously incubated with other stimulants [53]. It is possible that the conditions under which corticosteroids are exposed to cells can influence whether they are inhibitory, stimulatory, or ineffective. In many studies in which glucocorticoids were inhibitory, the effects were seen inyitro using concentrations [53] or incubation times [45] that are not physiological. Another explanation for the lack of macrophage suppression by elevated corticosterone in our experiments could be that during the 11-day suspension period the cells became desensitized or habituated [54]. Chiara and Sobrino [40] found that murine macrophage respiratory bursts were inhibited by dexamethasone if the drug was given only hours before macrophage recovery but not if the cells were tested 1 or 2 days after the dexamethasone was administered.

The increased mitogen- and toxin-induced proliferation of splenic lymphocytes from antiorthostatically suspended mice with significantly elevated corticosterone is not consistent with studies in which decreased proliferative responses were observed after exposure to glucocorticoids. Vischer [55] found that after administration of hydrocortisone to mice, splenic lymphocytes exhibited a decreased proliferative responses to PHA, pokeweed mitogen, and allogeneic cells. Stevenson et al. [56] also reported that the T lymphocytes demonstrated significantly reduced proliferative responses to mitogens when corticosterone was administered in vivo. Blomgren and Andersson [57] reported that exposure to prednisone in vitro had the same effect. Interestingly, the increased glucocorticoid receptor expression on mitogen-stimulated lymphocytes did not increase glucocorticoid-induced inhibition of lymphocyte proliferative responsiveness [58].

Although our lymphocyte response data differ from the results of several groups, the findings of others provide possible explanations for the differences. Gillis et al. [59] found that glucocorticoid-induced inhibition could be ameliorated by IL-2. Almawi et al. [60] found that IL-1, IL-6, and IFN-γ together could counteract the suppressive effects of dexamethasone. Therefore, it may be possible that the in vivo exposure to cytokines counteracted the suppressive effects of corticosterone in our experiments. Nakano et al. [61] reported that glucocorticoid secretion may be mediated by histamine and under certain conditions enhance mitogenesis. They found that one type of histamine agonist prevented

Table 4. Effects of Antiorthostatic Suspension on T Cell Proliferation in Response to Mitogens and Staphylococcal Exotoxins

	Stimulation index[a]		
Treatment (Suspension)[b]:	None	Orthostatic	Antiorthostatic
Eta	2.9 ± 0.3[c]	2.5 ± 0.2	3.3 ± 0.3#
Seb	2.7 ± 0.3	2.5 ± 0.3	2.9 ± 0.3†
PHA	4.5 ± 0.7	3.2 ± 0.4	9.3 ± 1.6*
Con A	13.7 ± 1.8	12.5 ± 2.0	21.6 ± 2.4‡

[a]Spleen cell proliferation in response to Eta (1 μg/ml), Seb (1 μg/ml), PHA (9 μg/ml), and Con A (2 μg/ml) was determined by measuring incorporation of [^{3}H]thymidine in a 48-h assay.
[b]Mice were suspended or caged normally for 11 days prior to sacrifice.
[c]Numbers represent mean ± SEM. Spleen cells used from: n = 9 mice (per treatment group) stimulated with Eta, n = 12 mice (per treatment group) stimulated with Seb, n = 16 mice (per treatment group) stimulated with PHA and Con A. Significant difference between antiorthostatic and orthostatic as determined by two-tailed matched t-test analysis. #P = .01; †P < .10; ‡P < .05; *P < .01.

Con A–induced lymphocyte proliferation, whereas one other histamine agonist and one antagonist enhanced the proliferative response [61]. In addition, investigators using various stress models, specifically electric shock and cold water immersion, have shown enhanced proliferative responses of lymphocytes after mice were subjected to the stressor [32, 62]. Enhanced mitogenesis was dependent on the intensity and frequency of the stress [32, 62]. Interestingly, Cunnick et al. [63], using a shock model, found that glucocorticoids may be involved in suppressing peripheral blood T cell responses but that adrenal gland hormones, like corticosterone, are not involved in suppressing spleen cell mitogenesis. These studies suggest that multiple variables contribute to an altered immune response, such as the type of stressor, the duration of exposure to the stressor, the frequency of treatment with the stressor, the strain of the mouse subject, and the lymphocyte subpopulation.

Because antiorthostatic suspension is often used to simulate many of the physiological changes associated with space flight, it is of interest to compare how each affects immunological functions. Almost all space-related investigations including postflight evaluations [7, 64–66], in vitro space studies [2, 7, 67], and clinostat experiments [68] reported reduced mitogen-induced proliferative responses. Only one study, in which rats were flown on board Kosmos 782, reported a postflight increase in mitogen-induced blastogenic responsiveness [69]. Interestingly, that study was done with splenic lymphocytes. Others have suggested that glucocorticoids and stress affect splenic T cells differently than blood T cells [54, 63]. Nash et al. have found that after stimulation with mitogens rat peripheral blood lymphocyte proliferation is more dramatically depressed after hindlimb suspension than spleen cell proliferation [9]. Therefore, it is possible that murine splenic lymphocytes do not mimic the responses of peripheral blood lymphocytes. This concept is also indirectly supported by the findings that rat spleen lymphocyte membrane molecule changes do not completely parallel changes in human blood lymphocytes in response to space flight [66, 70]. Alternatively, antiorthostatic suspension of mice may not accurately duplicate the effects of space flight on lymphocytes.

In conclusion, glucocorticoids, when elevated to three times normal levels by antiorthostatic suspension, are not inhibitory to macrophage inflammation, secretion, cytotoxicity, or membrane molecule expression. Therefore, elevation of these adrenal hormones does not always result in immunosuppression. Furthermore, although antiorthostatic suspension closely mimics some physiological changes associated with space flight, more comparative mouse work must be done to determine whether immunological functions completely correlate.

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Staphylococcus-Mediated T-Cell Activation and Spontaneous Natural Killer Cell Activity in the Absence of Major Histocompatibility Complex Class II Molecules†

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We used major histocompatibility complex class II antigen-deficient transgenic mice to show that in vitro natural killer cell cytotoxicity and T-cell activation by staphylococcal exotoxins (superantigens) are not dependent upon the presence of major histocompatibility complex class II molecules. T cells can be activated by exotoxins in the presence of exogenously added interleukin 1 or 2 or in the presence of specific antibody without exogenously added cytokines.

Staphylococcal exotoxins, often referred to as superantigens, stimulate T cells expressing specific Vβ region-containing T-cell receptors (9). This occurs through a mechanism thought to be dependent on toxin binding to nonpolymorphic regions of major histocompatibility complex (MHC) class II molecules on accessory cells (7, 15, 23). Recent work suggests that non-MHC receptors may be present on cells that allow the toxin to bind and activate cells (1, 8, 22, 25). However, the nature of these toxin-binding ligands has yet to be fully elucidated.

Our laboratory used C2D (*H-2*^{b}) transgenic mice (GenPharm, Int., Mountain View, Calif.), deficient in the expression of MHC class II molecules, to investigate the activation and function of various lymphocyte subpopulations. We used cells from C1D (*H-2*^{b}; GenPharm) MHC class I-deficient mice, normal B6 (*H-2*^{b}) mice, and/or C3HeB/FeJ (*H-2*^{k}) mice (Jackson Laboratory, Bar Harbor, Maine) as controls. C3HeB/FeJ mouse cells were used as controls because of our familiarity with the responses of the T cells and macrophages of this strain (11, 18). Normal B6 cells were used as an unmanipulated, syngeneic control. C1D mice served as an additional syngeneic control but were subjected to genetic manipulation of a knockout distinct from MHC class II molecules. We confirmed that C1D and C2D mice lacked MHC class I or class II molecules, respectively, by using flow cytometric analysis of thymocytes or spleen cells. A concomitant decrease occurred in CD8- or CD4-expressing cells from C1D or C2D mice, respectively.

Animals used in these experiments were handled in accordance with procedures approved by the Animal Care and Use Committee at Kansas State University.

We evaluated whether splenic natural killer (NK) cells needed to express class II molecules to lyse Yac-1 tumor cells. ^{51}Cr-labelled Yac-1 tumor cells (10^4) were incubated with splenic cells from C1D, C2D, and normal B6 mice in a 5-h cytotoxicity assay (6). The data in Table 1 confirm that MHC class I-deficient mice lack NK cell activity, as has been shown previously (19). However, there was no apparent difference between C2D mice and B6 mice in NK cell activity. Therefore, although the level of MHC class I expression is important on both effectors and targets (4, 16) for NK cell-mediated killing, there does not appear to be a requirement for MHC class II molecules on NK effector cells.

Several recent studies have questioned the absolute role in and contribution to the toxin-mediated T-cell activation process of MHC class II molecules (8, 12, 22, 25). Salamon et al. have also suggested that cytokines are important for the T-cell response to toxin (24). Because T cells may not necessarily require MHC class II molecules to respond to toxin, and cytokines may contribute to activation, we tested the capacity of T cells to respond to toxin in the absence of MHC class II molecules. Table 2 shows experiments confirming that the activation of T cells by staphylococcal enterotoxin B (SEB) and exfoliative toxin A (ETA) is dependent upon the expression of class II molecules by spleen cells when cultures are not supplemented with cytokines. We also demonstrate that exfoliative toxin B (ETB) exhibits stimulatory properties similar to those of the more extensively studied toxins SEB and ETA.

Although it appears that MHC class II molecules are required under normal, in vitro activation conditions, preliminary experiments indicated that exogenous cytokines could induce C2D T cells to proliferate in response to staphylococcal superantigens. When interleukin 1 (IL-1) and IL-2 were added together, stimulation indices of 3.8 and 4.2 were observed when C2D T cells were stimulated by ETA and SEB, respectively (Table 3). This proliferative response

TABLE 1. In vitro NK cell activity is normal in MHC class II transgenic animals

Spleen cells	% Specific ^{51}Cr release[a] at effector/target cell ratio of:		
	300:1	200:1	100:1
B6	31 ± 2	27 ± 1	21 ± 1
C1D	8 ± 2[b]	6 ± 1[b]	8 ± 2[b]
C2D	29 ± 2	32 ± 3	26 ± 0

[a] Values represent $\bar{x}$ ± standard errors of the means of triplicate determinations; data are representative of two experiments. Yac-1 target cells (10^4 per well of microtiter plate) were assayed in a 5-h assay.

[b] Significantly different from B6 and C2D mice as determined by Student's *t* test ($P < 0.01$).

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TABLE 2. MHC class II-deficient murine spleen cells do not proliferate in response to staphylococcal exotoxins without additives

Expt	Mouse strain	Proliferation in response to[a]: No toxin (cpm)	ETA cpm	ETA SI	ETB cpm	ETB SI	SEB cpm	SEB SI
1	C1D	14,919 ± 789	ND[b]	ND	65,312 ± 7,314[c]	4.4 ± 0.5	92,085 ± 5,197[c]	6.2 ± 0.3
	C2D	7,006 ± 1,008	ND	ND	13,209 ± 2,063	1.9 ± 0.3	8,199 ± 878	1.2 ± 0.1
	C3H/FeJ	12,600 ± 2,091	ND	ND	143,059 ± 13,236[c]	11.4 ± 1.1	164,744 ± 4,795[c]	13.1 ± 0.4
2	C1D	5,985 ± 45	27,662 ± 1,287[c]	4.6 ± 0.2	13,131 ± 1,295[c]	2.2 ± 0.2	38,153 ± 894[c]	6.4 ± 0.1
	C2D	6,378 ± 1,163	6,787 ± 631	1.1 ± 0.1	10,783 ± 645[c]	1.7 ± 0.1	7,041 ± 223	1.1 ± 0
	B6	14,329 ± 663	27,101 ± 1,488[c]	1.9 ± 0.1	18,262 ± 852[c]	1.3 ± 0.1	40,821 ± 1,268[c]	2.8 ± 0.1
	C3H/FeJ	13,597 ± 760	95,725 ± 1,198[c]	7.0 ± 0.1	57,847 ± 2,127[c]	4.3 ± 0.2	75,941 ± 1,491[c]	5.9 ± 0.1

[a] Toxin concentration was 10 μg/ml; toxins were prepared as described previously (11). Absolute counts (cpm) and stimulation indices (SI) were obtained in 60-h assays done as described previously (18). Stimulation indices were calculated from cells incubated in medium alone.
[b] ND, not determined.
[c] Significantly different from unstimulated control as determined by Student's t test ($P < 0.05$).

was significantly better than that of cells incubated in the absence of toxin ($P < 0.05$). Splenic T cells from C1D and B6 mice had qualitatively similar proliferative responses to ETA and SEB (Tables 2 and 3). T cells from C1D and B6 mice proliferated well in the presence of IL-1 alone and had much better responses in the presence of both IL-1 and IL-2 (Table 3). Therefore, it appeared that exogenous cytokines might play a role in toxin-induced T-cell proliferation for all three T-cell types (B6, C1D, and C2D). Furthermore, the absence of MHC class I molecules did not affect the proliferative response of T cells in response to superantigens. We next determined the contributions of IL-1 and IL-2 to T-cell proliferation in more closely controlled experiments. Both IL-1 and IL-2 significantly enhanced ($P < 0.05$) class II-positive (B6) and -negative (C2D) mouse spleen cell proliferation in the absence of toxin (Table 4). ETA and SEB induced minimal proliferative responses by T cells in the absence of exogenous IL-1 and IL-2. However, both IL-1 and IL-2 significantly enhanced the toxin-induced proliferative response of C2D and B6 mouse T cells. Proliferation was significantly greater in these cells than in cells stimulated by toxin alone or by cells incubated in cytokines without toxin (Table 4; $P < 0.05$). Furthermore, there appeared to be an additive effect between IL-1 and IL-2 on C2D mouse T-cell proliferation (Table 4). Therefore, in the absence of MHC class II molecules, either IL-1 or IL-2 could promote superantigen-induced T-cell responses. Other studies in our laboratory indicate that peritoneal macrophages from C2D mice are able to bind both ETA and SEB (1). It is possible that the added cytokines worked in concert with toxin that was presented by this alternate receptor. However, it is possible that ETA and SEB bound directly to the T cells, as has been suggested by others (25). Nevertheless, the lack of MHC class II molecules does not preclude the activation of T cells by staphylococcal superantigens. However, the amount of proliferation is significantly lower than that of cells that express MHC class II molecules incubated under similar culture conditions (Tables 2 to 4).

One possible explanation for the greater efficiency of toxin-induced T-cell proliferation in the presence of class II molecules could be that class II molecules have a higher affinity for toxins than the alternative receptor (1). The higher-affinity toxin-MHC binding possibly enhances toxin–T-cell receptor interactions and augments T-cell proliferation. If this hypothesis is true, other molecules which bind toxin without interfering with the T-cell epitope should substitute for MHC-expressing cells to activate T cells. Table 5 illustrates that antibody, complexed to polystyrene in microtiter plate wells, achieved such a result in an antibody- and toxin-specific fashion. Neither SEB nor polyclonal antibody, specific for SEB, was able to stimulate high levels of C2D thymic T-cell proliferation. Anti-SEB antibody and ETA together also induced modest proliferation of C2D T cells. Soluble SEB, in the presence of anti-SEB antibody complexed to plastic, induced very high levels of proliferation (significantly higher than those of various controls, $P <$

TABLE 3. Exogenous cytokine and toxins induce T-cell proliferation in the absence of MHC class II-positive cells

Expt	Mouse strain	Cytokine treatment[a] IL-1	IL-2	Proliferation in response to[b]: No toxin (cpm)	ETA cpm	ETA SI	SEB cpm	SEB SI
1	B6	+	−	5,811 ± 615	19,974 ± 350[c]	3.4 ± 0.1	20,859 ± 1,140[c]	3.6 ± 0.2
	C1D	+	−	16,263 ± 1,680	44,409 ± 560[c]	2.7 ± 0.1	41,434 ± 3,394[c]	2.5 ± 0.2
	C2D	+	−	13,244 ± 397	11,737 ± 567	0.9 ± 0	12,623 ± 1,120	1.0 ± 0.1
2	B6	+	+	22,241 ± 674	134,647 ± 13,220[c]	6.1 ± 0.6	215,837 ± 10,998[c]	9.7 ± 0.5
	C1D	+	+	19,338 ± 424	143,943 ± 14,603[c]	7.4 ± 0.8	145,543 ± 14,297[c]	7.5 ± 0.7
	C2D	+	+	16,878 ± 2,262	64,937 ± 2,265	3.8 ± 0.2	70,698 ± 2,665	4.2 ± 0.1

[a] Splenic T cells were stimulated with 7 pg of recombinant murine IL-1 per ml and 10 U of recombinant murine IL-2 per ml.
[b] Cells were stimulated with 20 μg of ETA or SEB per ml. Numbers represent $\bar{x}$ ± standard errors of the means of triplicate determinations. Stimulation indices (SI) were calculated from cells incubated without toxin.
[c] Significantly different from unstimulated control as determined by Student's t test ($P < 0.05$).

TABLE 4. Effects of IL-1 and -2 on C2D mouse T-cell proliferation

Mouse strain	Cytokine treatment[a]		Proliferation in response to[b]:				
			No toxin (cpm)	ETA		SEB	
	IL-1	IL-2		cpm	SI	cpm	SI
B6	−	−	2,046 ± 52	41,237 ± 3,527[c]	20.2 ± 1.7	21,100 ± 1,262[c]	10.3 ± 0.6
B6	+	−	34,747 ± 3,474[d]	228,686 ± 15,902[c,d]	111.8 ± 7.8	284,794 ± 21,612[c,d]	139.2 ± 10.6
B6	−	+	24,812 ± 2,006[d]	163,702 ± 14,168[c,d]	80.8 ± 6.9	200,859 ± 22,760[c,d]	98.2 ± 11.1
B6	+	+	40,235 ± 5,208[d]	196,158 ± 12,250[c,d]	95.9 ± 6.0	254,104 ± 22,078[c,d]	124.2 ± 10.8
C2D	−	−	2,784 ± 463	6,787 ± 224[c]	2.4 ± 0.1	3,099 ± 244	1.1 ± 0.1
C2D	+	−	20,079 ± 2,264[d]	33,144 ± 1,059[c,d]	11.9 ± 0.4	46,972 ± 1,812[c,d]	16.9 ± 0.7
C2D	−	+	18,725 ± 736[d]	29,586 ± 1,632[c,d]	10.6 ± 0.6	34,544 ± 3,872[c,d]	12.4 ± 1.4
C2D	+	+	43,682 ± 2,857[d]	55,142 ± 966[c,d]	19.8 ± 0.3	60,717 ± 873[c,d]	21.8 ± 0.3

[a] Splenic T cells were stimulated with 10 pg of recombinant murine IL-1 per ml and 10 U of recombinant murine IL-2 per ml.
[b] Cells were stimulated with 20 μg of ETA or SEB per ml. Numbers represent $\bar{x}$ ± standard errors of the means of triplicate determinations. Stimulation indices (SI) were calculated from cells incubated in medium alone. One representative experiment of two is presented.
[c] Significantly different from unstimulated controls incubated in the same cytokine as determined by Student's t test ($P < 0.05$).
[d] Significantly different from toxin-stimulated cells incubated without exogenous IL-1 or IL-2 as determined by Student's t test ($P < 0.05$).

0.05), with stimulation indices of 7.0 and 5.8 recorded in two experiments (Table 5). Interestingly, polyclonal antibodies raised against ETA did not have a similar ability to present toxin, suggesting that some antibodies may interfere with the epitope recognized by the T cell. When epitopes important for T-cell recognition are blocked by toxin-specific antibody, T-cell responses are abrogated (5). However, additional experiments with C2D mouse T cells will be needed to confirm this hypothesis. The importance of the immobilization of the antibody is also exemplified in Table 5. When anti-SEB and SEB were added to thymic T cells in solution, T-cell proliferation was minimal. These data suggest that the conditions under which molecules that present toxin to T cells are very specific but that other molecules may substitute for MHC class II antigens.

TABLE 5. Antibody and SEB induce T-cell proliferation in the absence of MHC class II-positive cells

Expt	Antibody treatment[a]	Toxin	Proliferation	
			cpm	Stimulation index
1	None	None	1,041 ± 363	
	None	ETA	966 ± 180	0.9 ± 0.2
	None	SEB	1,229 ± 367	1.2 ± 0.4
	Anti-SEB(C)	None	1,663 ± 119	1.6 ± 0.1
	Anti-SEB(C)	ETA	2,058 ± 89.9[b]	2.0 ± 0.1
	Anti-SEB(C)	SEB	7,251 ± 1,669[b,c,d]	7.0 ± 1.6
	Anti-ETA(C)	None	715 ± 75.7	0.7 ± 0.1
	Anti-ETA(C)	ETA	1,145 ± 324	1.1 ± 0.1
	Anti-ETA(C)	SEB	1,427 ± 295	1.4 ± 0.3
2	None	None	677 ± 55	
	None	ETA	925 ± 250	1.4 ± 0.4
	None	SEB	1,002 ± 298	1.5 ± 0.4
	Anti-SEB(C)	None	1,627 ± 274[c]	2.4 ± 0.4
	Anti-SEB(C)	ETA	1,375 ± 167[c]	2.0 ± 0.2
	Anti-SEB(C)	SEB	3,939 ± 916[b,c,d]	5.8 ± 1.4
	Anti-ETA(C)	None	738 ± 57	1.1 ± 0.1
	Anti-ETA(C)	ETA	784 ± 142	1.2 ± 0.2
	Anti-ETA(C)	SEB	785 ± 117	1.2 ± 0.2
	Anti-SEB(S)	None	950 ± 215	1.4 ± 0.3
	Anti-SEB(S)	SEB	1,574 ± 1,051	2.3 ± 1.6

[a] Corning 96-well flat-bottom ELISA plates were complexed (C) with polyclonal rabbit (anti-ETA) or goat (anti-SEB) serum at a 1:80 or 1:20 dilution, respectively, and diluted in sterile phosphate-buffered saline (PBS), and 200 μl was added per well. Plates were incubated overnight at 4°C. Untreated wells were blocked with 1% bovine serum albumin-PBS overnight at 4°C. Prior to the addition of cells, wells were washed twice with sterile PBS and toxin was added to appropriate wells (20-μg/ml final concentration). Soluble (S) antibody was added at similar concentrations without the overnight complexing step, blocking step, or washes. Antibody and toxin remained in the wells for the entire proliferation assay under S and C conditions. Thymocytes were from C2D mice.
[b] Significantly different from treatment with toxin alone as determined by Student's t test ($P < 0.05$).
[c] Significantly different from untreated cells as determined by Student's t test ($P < 0.05$).
[d] Significantly different from cells treated with antibody alone as determined by Student's t test ($P < 0.08$).

We have demonstrated that toxin-mediated T-cell proliferation can be induced in the absence of one of the members of the trimolecular complex thought to be important for the activation of T cells by staphylococcal superantigens, class II molecules. Because T cells could be activated in the presence of exogenous IL-1 or IL-2 or when toxin was presented by immobilized antibody, we hypothesize that class II molecules are necessary only to immobilize and efficiently mediate toxin binding to T cells via the T-cell receptor. The observations that correlate toxin binding to MHC class II molecules with the ability of the toxin to activate T cells (14) would support this hypothesis. Others have reported that IL-1 and IL-2 act synergistically to induce T-cell proliferation in the presence of suboptimal concentrations of mitogen (2, 20). Green et al. found that purified human T cells needed both toxin and a costimulant anti-CD28 antibody to activate the T cells in the absence of accessory cells (13). Together, these findings suggest that toxin presentation to T cells in the absence of class II-positive cells is less efficient and supports our proposed role for MHC class II molecules. The hypothesis is further supported by our data indicating that cytokines are not needed to activate T cells in response to toxin when the toxin molecules are presented by appropriate antibodies. Perhaps there are two classes of toxin-binding molecules: (i) those that can bind toxin but require accessory cytokines or signals to complete T-cell activation, perhaps by up-regulating IL-2 receptors (21) and IL-2 secretion (5), and (ii) receptors like MHC class II molecules on cells or immobilized antibody that bind toxin with relatively high affinity, do not interfere with the T-cell epitope, and do not require the presence of exogenous cytokines.

Because thymocytes responded to SEB presented by anti-SEB, it is clear that the cell population responding is the T cell. However, we did not have reagents available to us

during this study to determine whether anti-SEB antibody–SEB complexes induced T-cell proliferation in a Vβ-specific manner. Such an experiment would confirm whether the Vβ-specific nature of the T-cell expansion induced by superantigens is determined at the T-cell receptor level (17) or is MHC molecule dependent as suggested by others (3, 10).

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Three distinct cell phenotypes of induced-TNF cytotoxicity and their relationship to apoptosis

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Abstract: We have identified three distinct cell phenotypes with respect to the conditions under which cells became susceptible to TNF-mediated lysis. These conditions include: *1)* treatment with the protein synthesis inhibitor, cycloheximide; *2)* contact with activated macrophages, and *3)* infection with vaccinia virus. Whereas vaccinia virus-infected 3T3 cells became sensitive to soluble TNF, F5b cells required contact with activated macrophages. We showed that the "macrophage-resistant" F5m cells did not become sensitive to TNF or to killing by activated macrophages after infection with vaccinia virus. Therefore, vaccinia infection does not sensitize all cells to TNF. We also determined the pathways of lysis for cells after sensitization. Whereas 3T3, LM929, and F5b cells were killed by the process of necrosis, F5m cells lysis was characterized by the release of low mol wt DNA fragments (apoptosis). *J. Leukoc. Biol.* 53: 37-44; 1993.

Key Words: *vaccinia virus* • *macrophage* • *nitric oxide*

INTRODUCTION

Tumor necrosis factor-α (TNF), named for its ability to induce the necrosis of tumors, is produced by activated macrophages. Extensive study has revealed that this cytokine possesses many biological activities besides being cytotoxic [1]. The precise mechanism of cytotoxicity of tumor necrosis factor is not known but involves at least two pathways of cell death—apoptosis and necrosis [2, 3]. Apoptosis is characterized by nuclear condensation and degradation of the DNA into nucleosome-sized fragments by an endogenous endonuclease, whereas the cytoplasm and cell membrane remain relatively intact. Necrosis is characterized morphologically by cytoplasmic boiling, and the nucleus remains intact.

The cytolytic pathway appears to be activated in TNF-resistant cells whenever the cell experiences inhibitory conditions. These conditions include the presence of metabolic inhibitors of protein synthesis such as cycloheximide or mRNA synthesis such as actinomycin D. These observations have led some to suggest that TNF induces the expression of a specific protein (or proteins) that renders the cell resistant to TNF-mediated cytotoxicity [4-6]. It is also becoming apparent that viral [7-9] or intracellular bacterial [10] infections may be a biologically relevant means of sensitizing cells to TNF-mediated cytolysis.

For several years our laboratory has been interested in determining how macrophages recognize and kill virus-transformed and virus-infected target cells. Because TNF plays an important role in macrophage-mediated killing, we are continuing to determine its role in this process. To date, we have described an SV40-transformed cell, F5b, which is resistant to killing by soluble TNF and other monokines but is killed by macrophages in a contact-dependent process [11, 12]. We have also described a clonally derived "sister" of F5b, F5m, which is resistant to killing by activated macrophages. Resistance may be due to an inability of the macrophage to bind the target cell [11, 12] because of the release of inhibitory molecules [13]. Other studies by our laboratory show that binding defects do not explain why some targets, such as Balb/c 3T3, are not killed by activated macrophages [12]. Resistance in this case may be a function of other cellular properties such as being a nontransformed cell.

This study describes the mechanism of lysis of F5b cells and additional phenotypic differences between F5b and F5m. We report that F5b lysis, though dependent on contact with macrophages, involves TNF. Furthermore, we investigated the role of TNF in the lysis of vaccinia virus-infected cells as well as the effects of vaccinia infection on macrophage-mediated killing of the different cell types. Vaccinia virus infection induces TNF sensitivity in Balb/c 3T3 cells but not in F5m or F5b cells. We show that the pathway by which TNF induces cytolysis differs between these cell types as do the conditions that induce them to become sensitive.

MATERIALS AND METHODS

Tissue Culture Cells

The Balb/c 3T3 cell line (3T3) and the TNF-sensitive LM929 cell line were obtained from the American Type Culture Collection (ATCC). F5m and F5b cell lines have been described previously [11, 12]. The rabbit kidney cell line RK13, obtained from ATCC, was used to propagate vaccinia virus. All cell lines were continuously passaged three times weekly in antibiotic-free Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% Opti-MEM (Gibco), 2% fetal bovine serum (FBS; J.R. Hazleton, Lenexa, KS), and 0.3% glutamine (Sigma, St. Louis, MO). The assays were done in DMEM supplemented only with 2% FBS.

Anti-Vaccinia Virus Antiserum

Rabbit anti-vaccinia antiserum was generated by immunizing a New Zealand white rabbit with dorsal subcutaneous injections of 1×10^6 live virions. The rabbit was immunized four times and the antiserum was compared to preimmune

Abbreviations: ATCC, American Type Culture Collection; CA, cytosine arabinoside; CHS, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediamine tetraacetate; FBS, fetal bovine serum; MO, macrophage; mTNF, membrane-associated TNF; NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; 3-OMA, 3'-O-methyl adenosine; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; SR, specific ^{51}Cr release; 3T3, Balb/c 3T3 cell line.

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serum to determine specificity. This antiserum detects vaccinia virus-specific antigens at a dilution of 1:800 and is specific for structural proteins, including an 85-kDa hemaglutination protein, the 62-kDa major core protein, and 58- and 33-kDa envelope proteins.

Cytotoxicity Assay

Macrophage cytotoxicity assays were performed as follows. C3HeB/FeJ murine peritoneal exudate cells (PEC) were obtained by peritoneal lavage using cold phosphate-buffered saline (PBS) 4 to 5 days after the mice had been injected with 1.5 ml sterile thioglycollate (Difco, Detroit, MI). The PEC were seeded into a microtiter plate at 1–4 × 10^5 cells per well. The cells were allowed to adhere 1.5–2 h before the medium was removed and replaced with 0.1 ml of fresh medium with or without 12.5 μg/ml *Escherichia coli* lipopolysaccharide (LPS; 055:B5, Difco) and 10 U/ml γ-interferon (IFN; Genzyme, Cambridge, MA). (1 unit activity is defined by the supplier as being approximately 140 ng protein.) Approximately 1 × 10^4 ^{51}chromium-labeled (NEN, Boston, MA) target cells were added to each well and the plate was incubated for 16–18 h. The microtiter plates were centrifuged at 325 × *g* for 3 min; 90 μl aliquots were then taken from each well and counted on a gamma counter. Specific ^{51}Cr release (SR) was calculated as follows:

$$SR = \frac{\text{experimental release - spontaneous release}}{\text{maximal release - spontaneous release}} \times 100$$

The maximal release and the spontaneous release were determined by incubating 1 × 10^4 target cells in either 1 N HCl for maximal release or medium for spontaneous release. Unless specifically stated, spontaneous release ranged from 30 to 40% for the assays described in this paper. For cytotoxicity assays using N^Gmonomethyl-L-arginine (NMMA; Calbiochem, San Diego, CA), macrophages were refed with 100 μl of medium containing 1 mM NMMA, LPS (5 μg/ml), and IFN (20 U/ml) before the addition of the target cells (100 μl), resulting in final concentrations of 0.5 mM NMMA, 12.5 μg/ml LPS, and 10 U/ml IFN. For cytotoxicity assays using purified anti-murine TNF antibodies (Genzyme, Cambridge, MA), macrophages were refed with 100 μl of medium containing 12.5 μg/ml LPS and 10 U/ml γ-IFN. The macrophages were activated for 4 h before removing the medium and refeeding with 100 μl medium containing a 1:50 dilution of the purified anti-TNF antibody (4 mg/ml; Genzyme) or purified rabbit antibody (4 mg/ml; NRS) with 12.5 μg/ml LPS and 10 U/ml γ-IFN. The targets were then added and incubated for 16 h. Apoptosis was determined by specific release of low mol wt DNA labeled with [^{3}H]thymidine, as was previously described [2]. Target cells were prepared as was done for ^{51}Cr release assays except that cells were prelabeled overnight with 5 μCi [^{3}H]thymidine in 3 ml of medium. After 16 h of incubation, 50 μl of medium containing 1.5% Triton-X 100 (final concentration 0.3%) was added per well and incubated an additional hour at 37°C before centrifuging the plate at 625 × *g* for 3 min. A 130 μl aliquot was removed from each well and added to 1 ml of Scintiverse BD (Fisher), then counted on a Packard scintillation counter. For cytotoxicity assays requiring formalin-fixed macrophages, macrophages were seeded in microtiter plates as previously described. They were refed after adherence with 100 μl medium with or without LPS and IFN. The medium was removed and the cells were washed with PBS before adding 100 μl of a 1% formalin solution in PBS. The cells were then incubated at 37°C for 20 min before the formalin was removed and the cells extensively washed with serum-containing medium. Fresh medium was added to the fixed macrophages before adding the target cells.

TNF Assays

Recombinant murine TNF (Genzyme) was serially diluted in triplicate in a 96-well microtiter plate. The resulting concentrations were 64, 32, and 16 U/ml in a volume of 100 μl/well (1 unit activity is defined by the supplier as being 25 ng protein). After adding 100 μl of medium containing 1 × 10^4 target cells, the final concentrations were 32, 16, and 8 U/ml rTNF. Four μl of a 500 μg/ml solution of cycloheximide (CHX) (Sigma) in PBS or PBS alone was added to the appropriate wells. The assay was incubated for 16 h before removing a 90 μl aliquot from each well and quantitation on a gamma counter. Specific ^{51}chromium release was calculated as described. For TNF assays using cytosine arabinoside (CA) (Sigma), similar concentrations of rTNF (as described previously) were serially diluted in medium containing 50 μg/ml of CA and 20 μg/ml CHX. After 100 μl of medium containing 1 × 10^4 target cells was added per well, the resultant concentrations of CA was either 25 or 50 μg/ml with 10 μg/ml CHX.

Vaccinia Virus

The WR strain of vaccinia virus was obtained from ATCC. ^{51}Chromium-labeled target cells were infected with the virus at an MOI of 13 for 1–2 h, while rocking at 37°C. After infection cells were washed, dispersed, and added to the assays similarly to other target cells.

Western Blot Analysis

3–5 × 10^6 3T3 cells were infected with vaccinia virus at an MOI of 2–3 for 1 h in 1 ml medium with or without inhibitors (31.25 μg/ml cytosine arabinoside or 62.5 μM 3'-O-methyl adenosine). The infected cells were incubated overnight (18 h) at 37°C. The cells were dispersed with 0.02% ethylenediamine tetraacetate (EDTA) in 0.8% NaCl, 0.04% KCl, 0.006% Na_2HPO_4, 0.01% dextrose, washed once in PBS, and solubilized with 1 ml 1% NP-40/saline lysing buffer (20 mM Tris, 0.15 M NaCl, pH 8) while incubating on ice for 30 min. Sixty μg (determined by Bicinchoninic Acid method [14]) of protein from each lysate was added per well of a 10% SDS/PAGE gel. The protein was transferred to nitrocellulose, blocked with 10% milk proteins, and probed with a 1:50 dilution of antiserum in TNE buffer (0.6% Tris, 2.9% NaCl, and 0.19% EDTA pH 7.5).

RESULTS

Cycloheximide Induces Sensitivity to TNF-Mediated Cytotoxicity

Metabolic inhibitors can affect cellular sensitivity to TNF. Cells resistant to TNF can become sensitive by inhibiting mRNA or protein synthesis. To determine whether F5b, F5m, or 3T3 cells could respond to TNF, we treated them with CHX. The results in **Table 1** show that F5b, F5m, and 3T3 cells are lysed by TNF in the presence of cycloheximide at concentrations (10 μg/ml) that inhibit protein synthesis by greater than 90% (data not shown). These results indicate that all the cells express TNF receptors and can respond to TNF under the appropriate inhibitory conditions.

TABLE 1. TNF Sensitivity of Vaccinia-Infected and Cycloheximide-Treated Cells

Target[a,b]	Treatment	Specific ^{51}Cr release U/ml rTNF 32	16	8
F5b	None (37)	0 ± 0[c]	0 ± 0	0 ± 0
F5b	Cycloheximide[d] (44)	41 ± 4	43 ± 2	20 ± 5
F5b	Vaccinia[e] (32)	8 ± 4	4 ± 2	2 ± 2
F5m	None (36)	1 ± 1	2 ± 2	7 ± 1
F5m	Cycloheximide (37)	31 ± 5	26 ± 1	21 ± 3
F5m	Vaccinia (28)	3 ± 1	2 ± 3	5 ± 1
3T3	None (29)	5 ± 0	3 ± 1	4 ± 0
3T3	Cycloheximide (29)	60 ± 2	62 ± 0	62 ± 1
3T3	Vaccinia (24)	84 ± 0	80 ± 0	84 ± 1
LM929	None (30)	87 ± 0	77 ± 0	72 ± 0

[a]One × 10^4 targets added per well of a 96-well microtiter plate. Value in parenthesis represent spontaneous ^{51}Cr release for each respective treatment in the absence of rTNF.

[b]Maximum ^{51}Cr release values (CPM) are: F5b, 2757; F5b-inf, 2836; F5m, 4579; F5m-inf, 2150; 3T3 (± CHX), 9783; 3T3-inf, 11491; LM, 9581.

[c]Numbers represent $\bar{X}$ ± SEM done in triplicate of a single experiment representative of four experiments.

[d]Final concentration, 10 μg/ml.

[e]MOI of 13.

F5b Responds to Membrane-Associated TNF

The previous experiment indicated that F5b was lysed by TNF under certain inhibitory conditions. F5b cells are susceptible to macrophage-mediated cytotoxicity in a contact-dependent manner. Activated macrophages have also been shown to express a membrane-associated form of TNF (mTNF) [15–20]. Peck et al. [20] have presented evidence to suggest that mTNF may exhibit cytotoxic activity not found in the soluble form. Therefore, we explored whether F5b cells could be lysed by mTNF. The data in **Table 2** show that F5b can also be lysed by mTNF, as expressed on formalin-fixed, activated macrophages, in the presence of cycloheximide. Cytolysis was inhibited by anti-murine TNF antibodies. Therefore, TNF was responsible for cytotoxicity. Furthermore, mTNF activity was present only on activated macrophages. This mimics contact-mediated killing of F5b cells by activated macrophages and the absence of killing by nonactivated macrophages.

Role of TNF in Macrophage-Mediated Killing of F5b

The observation that F5b cells were responsive to mTNF under inhibitory conditions suggested that TNF might have a role in contact-dependent killing of F5b cells. We tested this hypothesis by adding anti-TNF antibodies directly to a macrophage cytotoxicity assay. We found that in the presence of anti-murine TNF antibodies, macrophage-mediated killing of F5b cells could be inhibited by greater than 80% (**Table 3**). The efficacy of anti-TNF antibodies to inhibit TNF-mediated killing by activated macrophages is clearly shown with the TNF-sensitive cell line LM929, where killing was inhibited by 90% (Table 3). It was possible that the LPS and IFN, used to activate macrophages, may have been responsible for F5b cells becoming sensitive to TNF. However, we found that F5b cells exhibited a negligible sensitivity (2 ± 1 specific ^{51}Cr release) to TNF in the presence of LPS (12.5 μg/ml) and/or IFN (10 U/ml). These results indicate that TNF is involved in the killing of F5b by activated macrophages.

TABLE 2. F5b Sensitivity to Membrane-Associated TNF

Target[a]	Mϕ Treatment[b]	Target treatment	Antibody (40 μg/ml)	Specific ^{51}Cr release Mϕ:T 30:1
F5b	Medium	Cycloheximide	None	0 ± 0[c]
F5b	LPS/IFN	None	None	3 ± 2
F5b	LPS/IFN	Cycloheximide	None	21 ± 1
F5b	LPS/IFN	Cycloheximide	αTNF	0 ± 0

[a]One × 10^4 target cells were added per well of a 96-well microtiter plate.

[b]Murine peritoneal macrophages were incubated with fresh medium with or without a final concentration of 12.5 μg/ml *E. coli* LPS and 10 U/ml γ-IFN for 2 h before being fixed by 1% formalin.

[c]Numbers represent $\bar{X}$ ± SEM done in triplicate.

Role of Nitric Oxide in Macrophage-Mediated Killing of F5b

We established that F5b cells were killed by activated macrophages via TNF. Moreover, F5b cells were lysed by soluble TNF or mTNF only in the presence of inhibitors like CHX. Therefore, it appears that viable activated macrophages must use some means to sensitize F5b to TNF-mediated lysis. Recently, activated macrophages have been shown to exert cytostatic or cytotoxic effects through the production of nitric oxide (NO) [21–25]. The effects mediated by NO are believed to be the result of inhibition of mitochondrial respiration in the target cell [26, 27]. We wanted to determine whether NO played a role in macrophage-mediated killing of F5b cells. Nitric oxide production can be effectively inhibited in the presence of the arginine derivative NMMA [28]. We found that NMMA, at a concentration sufficient to inhibit nitric oxide production by activated macrophages by greater than 90% (data not shown), had no significant effect on macrophage cytotoxicity of F5b (**Table 4**). This indicates that nitric oxide does not directly contribute to the cytotoxicity of F5b cells.

Vaccinia Virus Effect on TNF Sensitivity of F5b, F5m, and 3T3

Viruses such as adenovirus and vesicular stomatitis virus (VSV) and other intracellular pathogens induce sensitivity to TNF-mediated cytotoxicity. We were interested in knowing whether this was a property of vaccinia virus. F5b, F5m, and 3T3 cells were infected with vaccinia virus; however, only infected 3T3 cells were lysed by TNF (Table 1). **Figure 1** shows similar expression of vaccinia-specific proteins (M_r 109-kDa

TABLE 3. Inhibition of Macrophage-Mediated Killing of F5b by anti-TNF Antibodies

Target[a]	Antibody[b]	Specific ^{51}Cr release
F5b	None[c]	25 ± 2
F5b	αTNF	4 ± 1
F5b	NRS	24 ± 1
LM929	None	62 ± 5
LM929	αTNF	6 ± 3
LM929	NRS	59 ± 2

[a]One × 10^4 target cells were added per well of a 96-well microtiter plate. Macrophage:target ratio was 30:1.

[b]Final concentration of 40 μg/ml of purified αTNF or normal rabbit serum (NRS) added to assay.

[c]Numbers represent $\bar{X}$ ± SEM done in triplicate of a single experiment representative of four experiments.

TABLE 4. Effect of Inhibiting Nitric Oxide Production in Activated Macrophages upon the Killing of F5b Cells

Target[a]	Medium	N^GMMA[b]	Specific ^{51}Cr release Mϕ:T 10:1	20:1	30:1	40:1
F5b (43)	MEM + arg	–	12 ± 3[c]	20 ± 3	39 ± 4	39 ± 6
F5b (44)	MEM + arg	+	12 ± 3	26 ± 5	26 ± 4	30 ± 2
F5b (54)	MEM – arg	–	40 ± 4	50 ± 4	48 ± 4	47 ± 5
F5b (49)	MEM – arg	+	30 ± 4	45 ± 5	42 ± 4	36 ± 3

[a]One × 10^4 target cells seeded per well in 96 well microtiter plate.
[b]Targets incubated with a final concentration of 0.5 mM N^G-monomethyl-arginine (N^GMMA).
[c]Numbers represent $\bar{X}$ ± SEM of six experiments.

envelope protein, 89-kDa hemaglutination protein, 64-kDa major late protein not associated with the virion, 62-kDa major core protein, and 32-kDa protein) in infected 3T3 cells (lane D), F5m cells (lane E), and F5b cells (lane F). Therefore, differences in virus-specific protein expression cannot explain the diminished sensitivity to TNF of infected F5b and F5m cells.

TNF Sensitivity in 3T3 Cells is a Function of Early Viral Protein Expression

Vaccinia virus is a complex virus containing a genome of approximately 185 kb that has the capacity to encode up to an estimated 269 polypeptides [29]. We wanted to determine whether vaccinia-induced TNF sensitivity of 3T3 cells required the expression of virus-specific proteins. To do this, we incubated vaccinia-infected 3T3 cells with 3-O-methyladenosine (3-OMA). This adenosine analog inhibits plaque formation of vaccinia-infected cells by selectively inhibiting vaccinia virus-specific mRNA polymerase without affecting mammalian mRNA polymerases [30]. The effects of inhibiting viral protein expression by 3-OMA resulted in a greater than 90% inhibition of TNF cytotoxicity of infected cells (**Table 5**). **Figure 2** shows that 3-OMA inhibited vaccinia virus-induced TNF sensitivity of 3T3 cells in a dose-dependent manner. Western blot analysis showed that 62.5 μM 3-OMA greatly diminished the expression of viral proteins in infected 3T3 cells (**Fig. 3**; lane D) as compared to untreated infected 3T3 cells using a vaccinia-specific antiserum (Fig. 3, lane A). The inhibitory effect of 3-OMA on viral protein expression also was confirmed by SDS/PAGE. We found that 3-OMA inhibited vaccinia virus-induced proteins of 109, 64, 62, and 32 kDa (Fig. 1, lane A). Only the 85-kDa protein, which also was identified in the Western blot (Fig. 3), was not inhibited. Although 3-OMA was an effective inhibitor for most viral proteins, the expression of the 85-kDa protein and the minor expression of the 33-kDa envelope protein prove that 3-OMA is selectively inhibitory (Fig. 3, lane D). Furthermore, the same concentration of 3-OMA that inhibited virus-induced sensitivity to TNF showed no effect on the resistance of uninfected cells to TNF (Table 5), indicating that the host cell mRNA polymerase was not inhibited. Moreover, infected cells incubated with 3-OMA exhibited minimal characteristics of cytopathology such as cell rounding normally associated with infection. These results show that virus protein expression is required for infected 3T3 cells to become sensitive to TNF.

Vaccinia virus-specific proteins are temporally expressed in infected cells and are classified either as early or late proteins, depending on whether they are expressed before (early) or after (late) the onset of viral DNA synthesis [31, 32]. To determine whether the sensitizing protein (or proteins) was expressed as an early or late protein, we wanted to know what effects inhibiting viral DNA synthesis and subsequent expression of late proteins had on infected the sensitivity of 3T3 cells to TNF. CA has been shown to effectively inhibit the expression of the late vaccinia proteins by inhibiting viral DNA synthesis [32]. The results shown in Table 5 illustrate that inhibition of vaccinia DNA synthesis by greater than 90% (with 31.25 μg/ml CA) had no effect on vaccinia-infected cells becoming sensitive to TNF. We confirmed that CA limited viral protein expression by Western blot using anti-vaccinia antiserum. Only the 33-kDa envelope protein was expressed after CA treatment (Fig. 3, lane C). Cytosine arabinoside treatment inhibited the expression of all viral proteins identifiable by SDS/PAGE (Fig. 1, lane B; note arrows). Therefore, the expression of an early vaccinia protein appears to be responsible for inducing TNF sensitivity in 3T3 cells.

Vaccinia-infected 3T3 Cells Respond to mTNF

We knew that vaccinia-infected 3T3 cells became sensitive to soluble recombinant TNF and became susceptible to killing by activated macrophages (**Table 6**). We were interested in

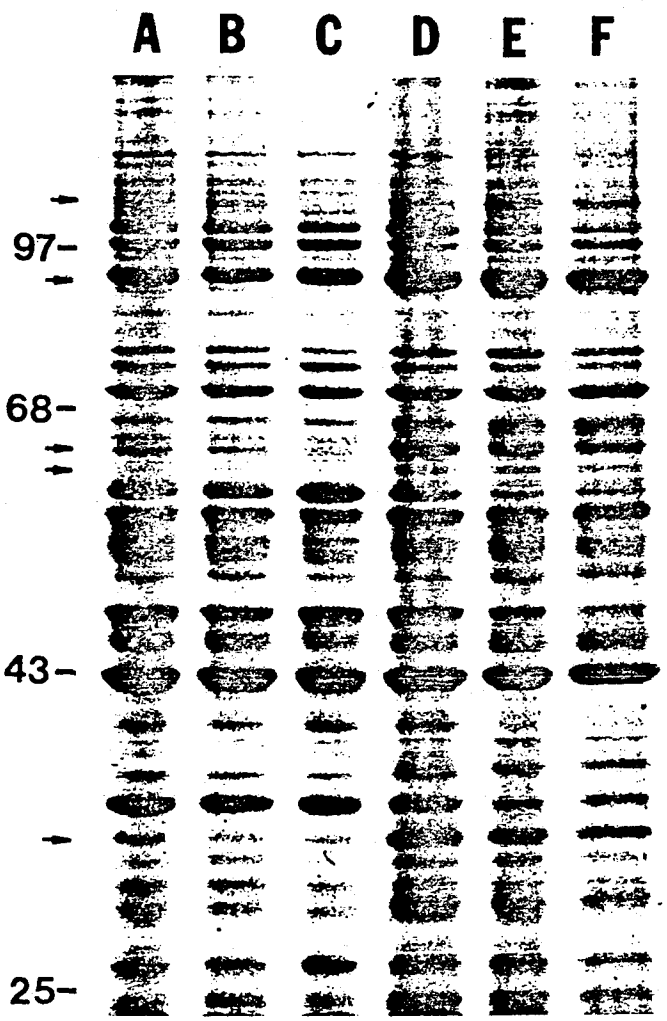

Fig. 1. Expression of vaccinia-specific proteins in infected cells. Lane A, infected 3T3 cells treated with 62.5 μM 3-OMA; lane B, infected 3T3 cells treated with 31.25 μg/ml CA; lane C, uninfected 3T3 cells; lane D, infected 3T3 cells; lane E, infected F5m cells and lane F, infected F5b cells. Sixty μg of protein was added to each lane of a 10% polyacrylamide gel; stained with coomassie blue. Arrows indicate major virus-induced proteins.

TABLE 5. Effects of DNA and mRNA Synthesis Inhibitors on Vaccinia Virus-Induced TNF Sensitivity of 3T3 Cells

Target[a]	31.35 μg/ml cytosine arabinoside	62.5 μM 3'-O-Methyl adenosine	Specific ^{51}Cr release U/ml rTNF 32	16	8
3T3 (28)	–	–	0 ± 1[b]	0 ± 0	1 ± 1
3T3 (29)	–	+	3 ± 2	0 ± 1	1 ± 1
3T3 (30)	+	–	14 ± 1	11 ± 1	ND[c]
3T3-inf[d] (37)	–	–	70 ± 3	70 ± 4	67 ± 3
3T3-inf (32)	–	+	4 ± 2	7 ± 2	7 ± 1
3T3-inf (45)	+	–	72 ± 5	74 ± 4	70 ± 5

[a]One × 10^4 target cells added per well of a 96-well microtiter plate. Values in parenthesis represent spontaneous ^{51}Cr release for each respective treatment in the absence of TNF.

[b]Maximum ^{51}Cr release values (CPM) are: 3T3, 11,463; 3T3-inf, 10,478.

[c]Values represent $\bar{X}$ ± SEM of triplicate samples of a single experiment representative of three experiments; ND, not determined.

[d]One × 10^6 target cells were infected with virus for 45 min at an MOI of 2–3. Cells were infected in the presence of each respective inhibitor at the concentration stated.

whether macrophages could facilitate the killing of infected 3T3 cells through contact using mTNF. Table 6 shows that infected 3T3 cells were able to directly respond to membrane-associated TNF on fixed, activated macrophages. TNF-sensitive LM929 cells similarly show the presence of mTNF activity on activated macrophages but not on nonactivated, thioglycollate-elicited macrophages (Table 6).

Effect of Vaccinia Infection on Macrophage-Mediated Killing of F5m and F5b

Because F5m cells are normally resistant to killing by activated macrophages, we wanted to know how vaccinia virus infection might affect the resistant phenotype of these cells. When F5m cells were infected with vaccinia virus, they retained their resistance to macrophage-mediated killing (**Table 7**). However, the susceptibility of infected F5b cells to killing by activated macrophages was not affected.

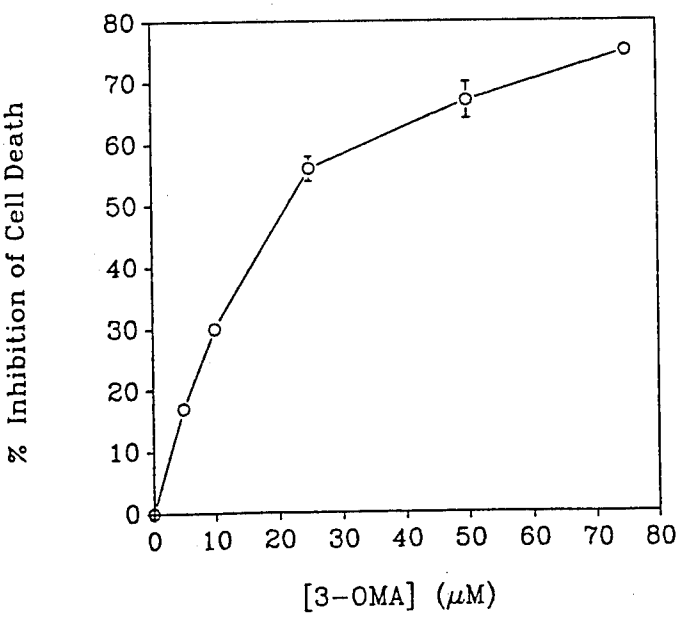

Fig. 2. Dose response of 3-OMA inhibition of TNF-mediated cytotoxicity in vaccinia virus-infected 3T3 cells.

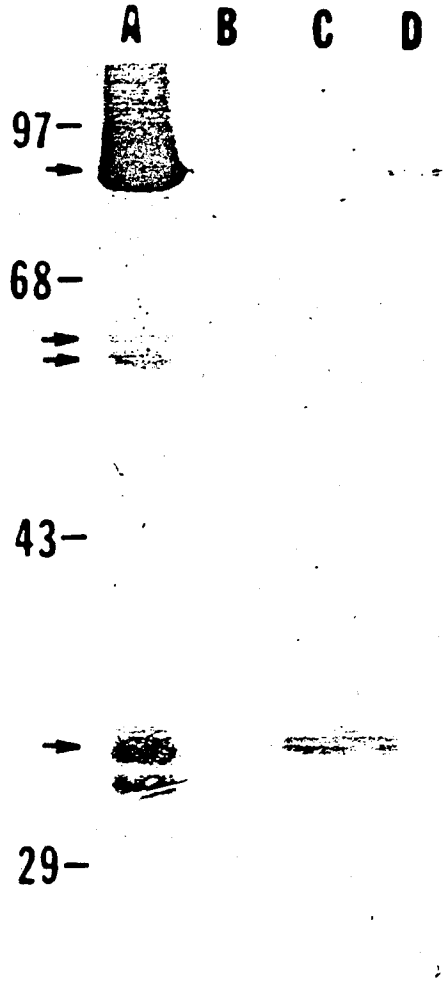

Fig. 3. Western blot of vaccinia-specific protein expression detected by rabbit anti-vaccinia antiserum. Lane A, infected 3T3 cells; lane B, uninfected 3T3 cells; lane C, infected 3T3 cells treated with 31.25 μg/ml CA; lane D, infected 3T3 cells treated with 62.5 μM 3-OMA. Sixty μg of protein was loaded per lane. Arrows indicate the 85-kDa hemaglutination protein, 62-kDa major core protein, and 58- and 33-kDa envelope proteins.

TNF Activates Both Apoptosis and Necrosis

The conditions under which cells became sensitive to TNF-mediated killing varied for each cell line we used in this study. Because of these differences, we wanted to determine if the mechanism by which TNF-induced cytolysis also varied for each of these cell lines. We found that when 3T3 cells were treated with CHX or were infected with vaccinia virus there was no release of low mol wt DNA (**Table 8**). Thus, it appeared that 3T3 cells died by necrotic mechanisms. Similarly, F5b cells also did not release low mol wt DNA in response to TNF after they were treated with CHX (Table 8). In contrast, F5m cells treated with CHX released significant amounts of low mol wt DNA in response to TNF (Table 8). The kinetics analysis of [^{3}H]thymidine showed that [^{3}H]thymidine release was initially detected 6 h after TNF was added and averaged 7% of the final amount released. Chromium release was not detectable until 8 h after the addition of TNF and averaged 11% of the final amount released. Thymidine release averaged 21% of the final release at that same 8 h sampling time. This trend continued at 10 h when ^{3}H release was 57% and ^{51}Cr release was

TABLE 6. Vaccinia-infected Balb/c 3T3 Cell Sensitivity to Membrane-Associated TNF

Target[a]	Mϕ Treatment[b]	Target treatment[c]	Specific ^{51}Cr release
3T3	Medium	Vaccinia	5 ± 2[d]
3T3	LPS/IFN	Vaccinia	24 ± 0
LM929	Medium	None	0 ± 0
LM929	LPS/IFN	None	41 ± 2

[a]One × 10^4 target cells were seeded per well in a 96-well microtiter plate.
[b]Macrophages were incubated with medium with or without 12.5 μg/ml LPS and 10 U/ml IFN for 2 h before fixation with 1% formalin in PBS. Mϕ:T was 30:1.
[c]Balb/c 3T3 infected for 1-2 h at an MOI of 13.
[d]Numbers represent $\bar{X}$ ± SEM done in triplicate.

TABLE 7. Cytotoxicity of Virus-Infected and Virus-Transformed Target Cells by Activated Macrophages

Target[a]	Vaccinia virus[b]	Specific ^{51}Cr release (Mϕ:T) 20:1	30:1
F5b	–	26 ± 1[c]	27 ± 2
F5b	+	24 ± 1	34 ± 1
F5m	–	0 ± 0	0 ± 0
F5m	+	7 ± 0	4 ± 0
3T3	–	9 ± 0	11 ± 1
3T3	+	76 ± 2	85 ± 2

[a]One × 10^4 targets were added per well of a 96-well microtiter plate.
[b]Target cells were infected with vaccinia virus at an MOI of 13 for 1-2 h before adding them to the assay.
[c]Numbers represent $\bar{X}$ ± SEM done in triplicate of one representative experiment of six experiments with F5b, two experiments with F5m, and four experiments with 3T3.

32% of final released amounts. However, at the 13 h sampling time point, both ^{3}H and ^{51}Cr releases reached 67% of their respective final maximal release (at 16 h). Therefore, TNF induces apoptosis in F5m cells.

DISCUSSION

The data presented here identify three different induced TNF-sensitive cell phenotypes. The first phenotype, as exemplified by F5m, is characterized by cells that are lysed by TNF only after treatment with inhibitors like CHX. Phenotype two, represented by 3T3 cells, is characterized by cells that become sensitive to TNF-mediated cytotoxicity after infection with vaccinia virus. The third phenotype is seen with F5b cells. These cells are not sensitive to soluble TNF unless treated with CHX, but apparently become sensitive to TNF after interactions with activated macrophages without CHX treatment. These phenotypes demonstrate the multiple ways that TNF can interact with target cells.

The finding that vaccinia infection makes 3T3 cells sensitive to soluble, TNF-mediated cytotoxicity is consistent with phenomena from other viruses and intracellular bacteria. LeBlanc et al. [9] have shown that VSV infection sensitized 3T3 cells to TNF killing. Others made similar observations using adenovirus or adenovirus gene products [8, 9]. Klimpel et al. [10] showed that TNF sensitivity could be induced in L929 cells infected intracellularly with *Shigella flexneri, Salmonella tryphimurium,* or *Listeria monocytogenes.* Therefore, TNF may be an additional mechanism by which a host can destroy vaccinia-infected cells. Our data suggest that early proteins expressed by vaccinia virus may be responsible for the induction of TNF sensitivity. Although we did not identify which viral protein was actually responsible for inducing sensitivity, it is interesting that the expression of two early proteins of 16-17 kDa and 25-27 kDa was sufficient to allow for CTL recognition and killing of vaccinia-infected cells [33]. Alternately, it is possible that vaccinia infection inhibits host protein synthesis [34] that confers resistance to TNF and that the induction of sensitivity is nonspecific. In either case, not all cells became sensitive to TNF after vaccinia infection even though virus replicated normally in cells that did not become sensitive. Therefore, the mechanism by which vaccinia confers sensitivity is dependent on factors not common to all cells.

We found that activated macrophages utilize TNF to kill the (soluble) TNF-resistant target cell, F5b. Because F5b cells respond to TNF in the presence of inhibitors, it is possible that the role of the macrophage during contact-dependent killing may be to alter the cellular metabolism that induces sensitivity in F5b. However, our data suggest

TABLE 8. Induction of Apoptosis in Target Cells by TNF in the Presence of Cycloheximide or Vaccinia Virus

Target[a]	Treatment[b]	Specific ^{51}Cr release, U/ml rTNF 32	16	Specific ^{3}H release, U/ml rTNF 32	16
LM929 (30/10)[c]	None	83 ± 1[d]	90 ± 0	0 ± 0	0 ± 0
F5b (31/8)	None	0 ± 1	1 ± 2	0 ± 0	0 ± 0
F5b (51/38)	Cycloheximide	46 ± 1	38 ± 4	0 ± 0	2 ± 0
F5m (35/4)	None	3 ± 2	5 ± 0	0 ± 0	0 ± 1
F5m (33/9)	Cycloheximide	30 ± 4	26 ± 6	44 ± 2	28 ± 0
3T3 (24/3)	None	2 ± 0	2 ± 2	0 ± 0	0 ± 0
3T3 (26/6)	Cycloheximide	43 ± 1	41 ± 0	1 ± 0	3 ± 0
3T3 (24/9)	Vaccinia virus[e]	60 ± 3	62 ± 4	0 ± 0	0 ± 0

[a]One × 10^4 cells were seeded per well of a 96-well microtiter plate. Values in parenthesis represent spontaneous ^{51}Cr release (first value) and spontaneous ^{3}H release (second value) in the absence of rTNF.
[b]Cells were incubated during the 16 h assay in the presence of 10 μg/ml cycloheximide.
[c]Maximum release values (CPM; ^{51}Cr release/^{3}H release) are: F5b, 7058/7232; F5m, 3220/4131; 3T3, 10491/4480; LM929, 5299/6310.
[d]Numbers represent $\bar{X}$ ± SEM done in triplicate of one representative experiment from six experiments with F5b, 5 experiments with F5m and two experiments with 3T3.
[e]Cells infected at an MOI of 13 for 1 h before adding them to the assay.

that nitric oxide is not involved in this process. This contrasts with other studies where the cytotoxicity of different cell types is dependent on nitric oxide [22]. Therefore, nitric oxide is not involved in all cytolytic processes. It is possible that other monokines produced by activated macrophages render F5b sensitive to TNF. However, previous results showing that F5b cells are insensitive to the combination of monokines present in activated macrophage supernatants rebut this possibility [11, 12]. The observation that TNF is involved in contact-dependent killing by activated macrophages is consistent with a study by Klostergaard et al. [15], who showed that TNF was responsible for the killing of L929 cells by bacillus Calmette-Guérin-activated macrophages. In addition, Peck et al. [20] have shown that TNF is involved in human monocyte-mediated, contact-dependent killing of K652 cells. Anti-TNF antibodies have also been shown to block macrophage killing of VSV-infected 3T3 cells but not P815 cells [13, 15]. Therefore, data presented here along with that of other investigators [15] have confirmed a previous study by our laboratory [12] that found that the requirements for macrophage cytolysis of P815 cells and F5b cells are different.

We investigated the lytic mechanism of TNF in various cells. As was previously shown by Laster et al. [2], the naturally sensitive cell LM929 did not release low mol wt DNA fragments in the presence of TNF [2]. Therefore, we have confirmed that LM929 are killed by necrosis. 3T3 cells also were killed by necrotic processes after infection with vaccinia virus or treatment with CHX. In contrast, F5m cells did release low mol wt DNA fragments in the presence of TNF and CHX, so we believe that TNF induces apoptosis in these cells. F5b cells treated with CHX do not specifically release low mol wt DNA fragments over background but do release substantial amounts of ^{51}Cr, giving the appearance that they are undergoing necrosis. We note that this latter observation, although highly reproducible, must be interpreted with caution. F5b cells exhibited a much higher sensitivity to CHX than the other cells used in this study, which caused a higher background release of low mol wt DNA. The higher background could have masked any TNF-induced apoptosis as the cause of cell death. Laster et al. [2] have suggested that there may be an intermediate type of lysis that exhibits all the cytoplasmic characteristics of apoptosis without the concurrent nuclear degradation. TNF binding may induce both in F5b cells. The possible differences between F5b and F5m in the way TNF mediates lysis in the presence of CHX is intriguing. These cells arose from the same transformation event [35] but are distinctly different in their susceptibility to macrophage-mediated lysis [11, 35], their expression of membrane glycoproteins, their binding to macrophages [11, 12], and their secretion of inhibitory molecules [13]. We have suggested that F5m may be more resistant to cytotoxicity than F5b because of the difficulty macrophages have in binding to F5m [11, 12]. However, their different responses to TNF in the presence of CHX (necrosis vs. apoptosis; F5b vs. F5m, respectively) or their different sensitivities to the induction of DNA fragmentation in the presence of CHX without TNF (F5b >> F5m) might also contribute to their differences in susceptibility to killing by activated macrophages.

In conclusion, we have made several novel observations that explain how activated macrophages and TNF mediate cytotoxicity. These include: *1)* The (soluble) TNF-resistant F5b cells are killed during contact-dependent, macrophage-mediated cytotoxicity through a process involving TNF; *2)* after infection with vaccinia virus, 3T3 cells are sensitized to soluble TNF-mediated cytotoxicity, and early viral protein expression is sufficient to render the cells susceptible; and *3)* target cells can be sensitized to TNF-mediated killing in distinct ways such as virus infection, contact with macrophages, or treatment with inhibitors. Furthermore, we have confirmed the previous studies that suggested that the mechanism of cell death is cell line dependent.

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PRODUCTION AND ACTION OF CYTOKINES IN SPACE

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ABSTRACT

B6MP102 cells, a continuously cultured murine bone marrow macrophage cell line, were tested for secretion of tumor necrosis factor-α and Interleukin-1 during space flight. We found that B6MP102 cells secreted more tumor necrosis factor-α and interleukin-1 when stimulated in space with lipopolysaccharide than controls similarly stimulated on earth. This compared to increased secretion of interferon-beta and -gamma by lymphocytes that was measured on the same shuttle flights. Although space flight enhanced B6MP102 secretion of tumor necrosis factor-α, an experiment on a subsequent space flight (STS-50) found that cellular cytotoxicity, mediated by tumor necrosis factor-α, was inhibited.

INTRODUCTION

Space flight has complex effects on individuals that occur at several levels. At one level, space flight and/or microgravity directly affect individual cells by interfering or enhancing cellular processes. At a second level, space flight and/or microgravity cause organismic or physiological changes that indirectly affect cells and/or organ systems. Therefore, it is important to understand and discriminate between changes at both levels.

The immune response exemplifies cellular responses inducing systemic or whole organismic changes and make it an appropriate subject for study at both cellular and organismic levels. Host survival is dependent upon the maintenance of the immune system and its coordinated cellular components and cytokines. An interruption in one cellular component can have catastrophic consequences. This is exemplified by acquired immune deficiency syndrome; the loss of $CD4^+$ T cells leads to immunosuppression and death. NASA's long term plans for interplanetary travel are dependent upon the vigor and health of the astronaut crews. Because the immune system is dependent upon cytokines, it is important to determine if space flight affects their production or activity. To this end, we investigated the ability of a bone marrow-derived macrophage cell, B6MP102, to secrete cytokines. We compared B6MP102 secretion simultaneously with lymphocyte secretion of interferon. In addition, we determined whether space flight would affect the function of one cytokine, tumor necrosis factor-α (TNF).

METHODOLOGY

Cells

The bone marrow-derived macrophage cell line, B6MP102, has been described previously /1/. The cells were grown in tissue culture dishes in preparation for shuttle flight /2/. Briefly, cells were dispersed with trypsin plus EDTA, washed, counted and incubated on Cytodex 3 microcarrier beads. Between 2 and 5 x 10^6 B6MP102 cells were incubated with 0.5 ml of a pelleted 2% Cytodex 3 bead solution for Space Transportation System (STS) flights 37 and 43. After attachment, beads-B6MP102 were washed and resuspended in HEPES-containing medium. 2 ml aliquots were aseptically dispensed into 5-ml syringes containing 10-μm filters and were shipped to Kennedy Space Center /2/. The B6MP102 cells were activated using 5-ml syringes mounted to bioprocessing modules (BPMs) /2,3/. The BPMs allowed injection of medium, with or without lipopolysaccharide (LPS) from one 5-ml syringe into the syringe containing the cells. After the incubation was completed, cell-free supernatant was injected into a third syringe where it was stored for return to earth for analysis.

Lymphocytes were obtained from SJL mice or from Ficoll-Hypaque-purified human peripheral blood buffy coats. Their detailed preparation has been described previously /2,4/. Murine lymph node and splenic lymphocytes were cultured for 48 and 24 h for STS-37 and -43, respectively, before being loaded for flight. Thirty-six hours before flight the cells were resuspended in RPMI-1640 medium (supplemented with 28mM HEPES and 10% FBS) and aseptically transferred to "cell syringes" at a concentration of 1 x 10^6 cells /ml, 2.5 ml/syringe. Human peripheral blood lymphocytes were prepared similarly /2/. Lymphocyte activation was done in "cell syringes" /2/; modified double chambered syringes that allowed for cells to be cultured in one chamber and exposed to activator solutions that were contained in a second chamber. Each syringe was activated in orbit to mix the activator solution with the suspended cells. Ground controls were maintained at approximately 22-24°C and flight samples were kept at cabin temperature, which ranged from 20-25°C. After the appropriate incubation time, samples were dispensed from the syringe into a collection vial that contained fixative /2/.

Cytokine Assays

Supernatants from B6MP102 cells were assayed for TNF using bioassay of serially diluted samples as has been described previously /2,5/. Interleukin-1 (IL-1) secretion was assayed using the previously described comitogenic murine thymocyte assay /2,6/. Interferon-α (IFN-α) was assayed using radioimmunoassay as previously described /2,7/. Interferon-γ (IFN-γ) was assayed using enzyme linked immuosorbent assay /2/. Serial dilutions of reference standards were used for all cytokine assays to determine the concentration of cytokine in unknown samples.

Assay of TNF-Mediated Cytotoxicity

Cytotoxicity of the TNF-sensitive cell line, LM929, was assayed on STS-50 as part of the United States Microgravity Laboratory-1 (USML-1) payload. The assay was conducted in fluid processing apparatus (FPA) units; modified glass tubes with bipasses that allowed the mixing of solutions, separated by rubber septa, when that septa crossed the bypass /3/. For these assays, 8.5 x 10^6 LM929 cells were attached to 2.5 ml of a pelleted 2% Cytodex 3 bead solution. These preparations were resuspended in 5.0 ml of Dulbecco's modified Eagles medium supplemented with 2% FBS, 10 mM HEPES and 50 μg/ml gentamycin sulfate ($DMEM_S$). The suspensions were added to 60 mm tissue culture dishes and incubated 8 hours to remove LM929 cells not attached to Cytodex 3 beads. The cells from all 60 mm dishes were pooled into one centrifuge tube, centrifuged at 325 x g and resuspended at a cell concentration of 1.7 x 10^6 cells-beads per 2.5 ml of $DMEM_S$. Cells-beads were resuspended thoroughly and 2.5 ml was added to the first section of each FPA. The second section of the FPA contained 1 ml of DMEM supplemented as above with the addition of 10 μCi [^{3}H]-thymidine and 10 μg of cycloheximide. In FPAs that required LM929 cells to be incubated with TNF, the second chamber also contained 320 units of recombinant, murine TNF. The third chamber contained 1 ml of 1% formalin as a fixative to stop the experiment. In flight, the experiment was initiated by sliding the second septum to the bypass which allowed the mixing of chambers 1 and 2. This exposed the LM929 cells to TNF. Although LM929 cells are naturally sensitive to TNF-mediated lysis, cycloheximide was added to maximize the sensitivity of this experiment. The experiment was designed such that cells lysed by TNF would not incorporate [^{3}H]-thymidine. Therefore, we quantitated cytotoxicity by assaying for the difference in the amount of [^{3}H]-thymidine incorporated into cells, ± TNF, attached to beads, upon the return to earth. The experiment was terminated approximately 29 h after mixing of chambers 1 and 2 by sliding the next septum in the FPA to the bypass and allowing the mixing of chamber 3 (formalin) with cells. This step terminated the experiment and preserved the cells attached to beads until their return to earth. Before quantitating the amount of [^{3}H]-thymidine incorporated into LM929 cells, each cell-bead suspension was washed two times in PBS. The cell-beads were resuspended in 2.0 ml of PBS and an aliquot of the homogeneous cell suspension was added to scintillation cocktail and quantitated on a β-scintillation spectrophotometer. Space experimentals and simultaneous ground controls were prepared in replicates of three, ± TNF.

RESULTS AND DISCUSSION

Secretion of Cytokines by B6MP102 Cells in Space

We measured B6MP102 secretion of TNF and IL-1 after stimulation with LPS on STS-37 and STS-43 space missions. During the former flight, B6MP102 cells were stimulated for 12 and 24 h periods with 12.5 μg LPS. The results in Table 1 illustrate that B6MP102 cells secreted significantly more IL-1 and TNF than simultaneous ground controls during both 12 and 24 h stimulations. We confirmed these results on STS-43. Furthermore, we investigated whether B6MP102 cells would spontaneously secrete cytokines in response to space flight. Like B6MP102 cells incubated on earth, very little spontaneous secretion IL-1 or TNF was detected (Table 1). These data contrast the observations made by Limouse et al. /8/. They found that the THP-1 monocyte cell line produced IL-1 in similar or lower concentrations than ground controls, depending on the culture conditions. However, our results have been confirmed by a subsequent study by Cogoli /9/ who found that human mononuclear cells produced more TNF in space at 0 x g than at 1 x g, over a period of 65 hr, when attached to a growth matrix but did not in the absence of the matrix.

Lymphocyte Secretion of Interferon During Space Flight

Concurrent with our macrophage studies, we investigated whether space flight altered lymphocyte secretion of either IFN-α or IFN-γ. SJL mouse splenic lymphocytes were screened for secretion of IFN-α during the STS-37 mission. Significantly more IFN-α was secreted in space compared to simultaneous ground controls activated with poly I:C for 1 and 14 hours (Table 2). SJL lymph node cells and human peripheral blood mononuclear cells were assayed for secretion of IFN-γ during STS-43. Both lymph node and peripheral blood lymphocytes secreted significantly more IFN-γ after stimulation with concanavalin A than similarly treated, simultaneous controls on earth. Talas et al. had previously found that space flight increased the secretion of IFN-α by lymphocytes /10/. Therefore, we have

Table 1 Production of Cytokines During Space Flight by B6MP102 Cells.

Flight	Condition*	LPS	Cytokine (Total Units) TNFα	IL-1
STS-37	1 x g (12)	+	308 ± 96**	4,660 ± 1,315
STS-37	0 x g (12)	+	1,082 ± 9†	14,287 ± 2,514†
STS-37	1 x g (24)	+	426 ± 76	3,685 ± 474
STS-37	0 x g (24)	+	970 ± 4†	13,750 ± 913†
STS-43	1 x g (24)	-	48 ± 12	517 ± 107
STS-43	0 x g (24)	-	44 ± 16	632 ± 347†
STS-43	1 x g (24)	+	60 ± 8	1,605 ± 238
STS-43	0 x g (24)	+	180 ± 32†	3,331 ± 497†

* B6MP102 cells incubated on earth (1 x g) or in space (0 x g) for 12 or 24 hours in the presence (+) or absence (-) of 12.5 μg/ml lipopolysaccharide (LPS).

** Numbers represent the $\bar{X}$ ± of SEM of duplicate samples.
† Indicates a difference from ground control, $P < 0.05$.

Table 2 Production of Interferon by Lymphocytes During Space Flight.

Flight	Source of Lymphocytes	Condition*	Inducer	Cytokine (Units/ml) IFN-γ	IFN-α
STS-37	murine-spleen	1 x g (.4)	poly I:C	NM**	3.9 ± 1.6**
STS-37	murine-spleen	0 x g (.4)	poly I:C	NM	5.4 ± 1.1
STS-37	murine-spleen	1 x g (1.0)	poly I:C	NM	8.2 ± 1.7
STS-37	murine-spleen	0 x g (1.0)	poly I:C	NM	15.6 ± 2.1†
STS-37	murine-spleen	1 x g (14)	poly I:C	NM	30.1 ± 2.4
STS-37	murine-spleen	0 x g (14)	poly I:C	NM	83.7 ± 6.1†
STS-43	murine-lymph node	1 x g (24)	Con A	7.3 ± 2.2	NM
STS-43	murine-lymph node	0 x g (24)	Con A	17.7 ± 3.0‡	NM
STS-43	murine-lymph node	1 x g (48)	Con A	20.0 ± 3.6	NM
STS-43	murine-lymph node	0 x g (48)	Con A	50.6 ± 4.1‡	NM
STS-43	human-blood	1 x g (24)	Con A	10.8 ± 2.3	NM
STS-43	human-blood	0 x g (24)	Con A	34.8***	NM

* Human or murine lymphocytes incubated for 20 min., 1, 14, 24 or 48 h in the presence of 20 μg/ml polyiosinic-polycytidyic acid or 1.5 μg/ml concanavalin A as indicated.

** Numbers represent $\bar{X}$ ± SEM of duplicate samples; NM indicates not measured.
*** Measured a single sample.
† Indicates a difference from ground control, $P < 0.01$.
‡ Indicates a difference from ground control, $P < 0.05$.

confirmed that observation. Furthermore, we have established that the early kinetics of secretion of IFN-α are similar between earth- and space-stimulated samples (See Table 2). Increased secretion of IFN-γ contrasts with the results of Gould et al. /11/ who reported that rat spleen cell secretion of IFN-γ was inhibited after 1 week of space flight. However, their experiment was carried out ex vivo with spleen cells taken from animals flown in space while our experiment was carried out with cells stimulated in vitro. Therefore, the experiments are not necessarily comparable.

TNF-Mediated Cytotoxicity of LM929 Cells During Space Flight

The data obtained from STS-37 and STS-43 indicated that space flight had a significant effect on the ability of lymphocytes and macrophages to secrete cytokines. We wanted to determine whether the action of these secreted molecules also was affected by space flight. We chose to study the cytokine, TNF, and its ability to cause lysis of LM929 cells. The results of one experiment are outlined in Table 3. We found that the cytolytic activity of TNF was inhibited by space flight, as measured by viable cell incorporation of [^{3}H]-thymidine. Interestingly, the samples flown aboard STS-50 incorporated significantly more [^{3}H]-thymidine than simultaneous ground controls (Table 3).

Table 3 TNF-Mediated Cytotoxicity of LM Cells During Space Flight.

Treatment*	Condition	CPM [^{3}H]-Thymidine Incorporated	% Cytotoxicity***
Medium	1 x g	4,634 ± 210**	----------
TNF	1 x g	2,908 ± 890†	38 ± 19
Medium	0 x g	7,581 ± 1,222†	----------
TNF	0 x g	7,759 ± 384‡	-2 ± 5

* LM929 cells, attached to Cytodex 3 beads, incubated in the presence (TNF) or absence (medium) of 91 μ/ml recombinant, murine TNF for 29 h.

** Numbers represent $\bar{X}$ ± sd of triplicate FPAs per treatment group.

*** [1-(TNF/medium)] x 100.

† Significantly different from medium treatment at 1 x g, $P < 0.04$.

‡ Significantly different from TNF treatment at 1 x g, $P < 0.01$.

Therefore, though space flight appeared to inhibit TNF-mediated cytotoxicity, it also had a dramatic augmenting effect upon the ability of the LM929 cells to incorporate [^{3}H]-thymidine. Whether the diminished cytotoxicity and the enhanced thymidine incorporation are directly related is not known. It is intriguing, however, that others have reported that space flight increased bacterial growth and resistance to radiation and antibiotics but did not affect the growth of non lymphoid cells /12/. Additional experiments will be conducted to confirm and dissect this observation.

CONCLUSION

The experiments presented in this manuscript suggest that space flight has profound, direct effects on a number of cellular functions. Furthermore, the effects are not limited to one cell type or one function. These cellular experiments may not necessarily reflect what will happen in vivo, but they do suggest the need for additional study on the effects of space travel on cytokine secretion and action.

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Immunological Characterization of Pulmonary Intravascular Macrophages

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Pulmonary intravascular macrophages (PIMs) are lung macrophages found apposed to the endothelium of pulmonary capillaries. In many species, they are responsible for the clearance of blood-borne particulates and pathogens; however, little else is known about their roles as immunologic effector cells. We compared PIMs with pulmonary alveolar macrophages (PAMs) to determine the relative immunological activities of these two cell populations. Our results suggested that both populations possess similar phagocytic and bactericidal activities. In assays measuring cytotoxicity, PIMs were more cytotoxic than PAMs against virally infected target cells; however, differences between these macrophage populations were not as marked when noninfected targets were used. LPS-stimulated PIMs produced more T-cell proliferative cytokines than PAMs, and both populations of nonstimulated macrophages produced similar amounts of the cytokines. In contrast, PAMs produced more TNFα and NO_2^- than PIMs when both populations were stimulated with LPS; however, nonstimulated PAMs and PIMs produced similar amounts of TNFα and NO_2. These data suggest that bovine PIMs are immunologically active. Differences between the degrees of activity of PIMs and PAMs indicate that these macrophage populations may have different roles in lung surveillance. Regional Immunology, Vol. 4(4), 236–244, July/August 1992.
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INTRODUCTION

Pulmonary intravascular macrophages (PIMs) are large, irregularly shaped cells that preferentially adhere to the endothelium of pulmonary capillaries (1). They are highly phagocytic (1,2) and are capable of rapid maturation during pulmonary infection (3); however, they also actively metabolize arachidonic acid and may be responsible for lung pathology (2,4). PIMs have been identified in the lungs of animals that are often used as models of human respiratory disease such as pigs, calves, sheep, and goats (1). They also have been identified in the lung capillaries of humans, baboons, rats, dogs, cats, and rabbits (1,5–7), but little is known about the functions of PIMs in these species.

Because pulmonary alveolar macrophages (PAMs) are considered to be the major cells responsible for immune surveillance of the lung, a number of studies have been performed to determine the role of this population in preventing or accentuating bovine respiratory disease (8–14). However, PIMs may also play a very important role in protecting cattle and other mammals that have them against disease. In a study comparing PIM and PAM immune functions in pigs, we found that PIMs were as active as PAMs for many immune parameters and were more active in cytolytic assays (15). Porcine PIMs were phagocytic, bactericidal, cytotoxic, and tumoricidal and produced the cytokines IL-1 and TNFα (15).

Based upon the above information, and particularly the use of calves as models of human respiratory disease, it was our objective to isolate PIMs and PAMs from calves and compare their functional immune activity, including bactericidal and phagocytic activity; non-MHC-restricted (NMRC) and antibody-dependent cellular cytotoxicity (ADCC); and LPS-stimulated and nonstimulated production of T-cell proliferative cytokines, TNFα, and nitrite (NO_2^-). We report that bovine PIMs are immunologically active in all capacities studied. However, differences exist in the degrees of activity expressed by PIMs and PAMs, suggesting different roles for these macrophage populations in lung surveillance.

MATERIALS AND METHODS

Animals. Seven Jersey bull calves, clinically healthy and free of respiratory disease, were purchased from local dairymen for use in these experiments. Calves were housed at the Kansas State University Animal Resource Facility and were

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fed a traditional starter calf diet or milk replacer formula. Calves were 2- to 15-weeks of age and ranged in weight from 29.0 to 84.4 kg, averaging 42.7 ± 7.7 kg.

Isolation of Intravascular Macrophages. Intravascular macrophages were obtained by a modification of previously described techniques (15,16). A whole blood sample was obtained by jugular venipuncture into a vacutainer containing EDTA. Calves were anesthetized with intravenous sodium pentobarbital (Fort Dodge Laboratories, Inc., Fort Dodge, IA) and then were given an intravenous injection containing 71 units heparin/kg body weight and 0.2 ng papaverine HCl (Sigma Chemical Co., St. Louis, MO)/kg body weight, as anticoagulant and vasodilator, respectively. These dosages were increased to 556 units heparin/kg body weight, and 2.2 ng papavarine HCl/kg body weight, after greater than expected blood cell contamination was present in the perfusates of the first two calves. Calves were then euthanized by exsanguination, the trachea was ligated to prevent total pulmonary collapse, and the heart and lungs were removed from the thorax. The left lung was ligated at the level of the primary bronchus to permit collection of alveolar macrophages after the right lung was perfused. An incision was made in the pulmonary artery at the point where it exits from the right ventricle, which allowed a length of sterile silicone tubing to be inserted into the artery. The artery was then ligated into place about the tubing. The opposite end of the tubing was connected to a peristaltic pump. An additional stab incision was made in the base of the left ventricle, and a length of sterile siliconized tubing was inserted into the ventricle and through the atrioventricular valve into the left atrium. The opposite end of this tubing was used to collect the perfusate. Warm perfusates were pumped through the pulmonary vasculature of the right lung at a pressure not exceeding 30 cm H_2O in the following order: 0.1 g Na_2EDTA/500 ml Ca- and Mg-free Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, NY); 1.5 g $NaNO_3$/200 ml phosphate buffered saline (PBS); 0.15 g Na_2EDTA/1.5 l HBSS; 0.275 g $CaCl_2$/500 ml HBSS; 0.1% collagenase (200 units/mg solid, Sigma Chemical Co.); 0.55g $CaCl_2$/1 l HBSS; and 0.5 g Na_2EDTA/2.5 l. The perfusate was collected in sterile, sialinized (Procil-28, SCM Chemicals, Gainesville, FL), 250 ml, centrifuge bottles as the collagenase solution was pumped into the vasculature. The perfusate was kept on ice until centrifugation at 1000 × g for 10 min. The cell pellet was resuspended in Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium (Grand Island Biological Co.) with 5% fetal bovine serum (FBS; Hyclone, Logan, UT) and no antibiotics. Macrophages were obtained by density gradient centrifugation on Ficoll-hypaque (Histopaque 1077, Sigma Chemical Co.). These cells were washed three times, counted, scored for viability with trypan blue exclusion dye, and differentially stained (LeukoStat, Fisher Scientific, Orangesburg, NY) to determine percent macrophages. Cells were resuspended in medium at 5×10^6 macrophages/ml. In all assays, with the exception of the assay for phagocytic activity, macrophages were allowed to adhere to the tissue culture plates for at least 1 hr. After this time, medium and nonadherent cells were removed by aspiration, wells were washed once, and an equal amount of fresh medium was added.

Isolation of Alveolar Macrophages. Alveolar macrophages were obtained by lavaging the left lung with cold, sterile HBSS containing 0.2 g Na_2EDTA/l. The saline was injected into the airways via a catheter attached to a 60 ml syringe. The lavage fluid was flushed in and out of the syringe several times prior to harvesting the macrophage-rich solution. Macrophages were then treated as described for the PIMs.

Collagenase Controls. Pulmonary alveolar macrophages from several of the experimental animals were divided into two groups after isolation. One group of PAMs was treated with the collagenase solution used to obtain the PIMs, and the other was left untreated. The treated PAMs were assayed concurrently with the other PAMs and PIMs to determine the effect of collagenase on cell function.

Tissue Culture Cells. Madin Darby bovine kidney cells (MDBK), porcine kidney fibroblasts (PK-15 cells), and K562 cells were initially obtained from the American Type Culture Collection (ATCC) and were subsequently maintained in our laboratories. E8 is an SV40-transformed murine embryo fibroblast cell line, which has been previously described (17). Monolayers of MDBK cells were grown in 60 mm tissue culture dishes in minimum essential medium (MEM) with 10% FBS (Grand Island Biological Co.). Eight hours before harvesting, cells were infected for 1 hr with 0.5 ml of bovine herpesvirus-1 (BHV-1) at a tissue culture infective $dose_{50}$/ml ($TCID_{50}$/ml) of 10^6. Cells were then washed two times with PBS and reincubated at 37° C, after 5 ml of medium was added. Monolayers of PK-15 cells were grown in 75 cm^2 tissue culture flasks in the RPMI medium described above. Eight hours before harvesting, cells were infected for 1 hr with 20 μl of pseudorabies virus (PRV) at a $TCID_{50}$/ml of 2×10^8. Cells were washed, medium was replaced, and the cells were incubated as described for the MDBK cells. The cells were labelled with 200 μCi of chromium-51 (^{51}Cr, New England Nuclear, Boston, MA) for the last 3 to 5 hr of incubation. Cells were removed from the flasks by trypsinization, washed three times, and resuspended to 1×10^5 cells/ml. E8 cells were grown in 60 mm tissue culture plates. Cells (1×10^6) in 3 ml medium (RPMI with 5% FBS) were allowed to adhere to the culture plates overnight; on the following morning, each plate was labelled with 100 μCi ^{51}Cr. Labelled cells were removed from the plates by trypsinization, washed three times, and resuspended to 1×10^5 cells/ml. K562 cells were grown in the same medium in 75 cm^2 tissue culture flasks. Cells were labelled by adding 200 μCi ^{51}Cr to the flasks for the last 3 to 5 hours of incubation. These cells were washed three times and resuspended to 1×10^5 cells/ml. WEHI 164 (clone 13) cells were a gift from Dr. K.W. Kelley. Cells were grown to monolayers in RPMI 1640 medium supplemented with 10% FBS in 75 cm^2 tissue culture flasks. Cells were removed from the flasks by trypsinization and resuspended in medium at 3×10^5 cells/ml for use in the assay for TNFα.

Bactericidal Activity. A colorimetric assay for quantitating macrophage bactericidal activity was performed essentially as described by Stevens et al. (18). Antiserum was obtained by drawing blood from Holstein dairy cows that had been diagnosed as positive for *Staphylococcus aureus* mastitis. Serum was isolated, and complement was inactivated by incubation in a 56° C water bath for 1 hr. Because PIMs had a higher background OD than PAMs, the OD obtained for control wells containing macrophages but no bacteria was subtracted from the sample OD to determine the corrected OD used in the following calculation:

$$\% \text{ Bacteria Killed} = 1 - \frac{(\text{Corrected OD Sample}) - (\text{OD 90\% Killing})}{(\text{OD 0\% Killing}) - (\text{OD 90\% Killing})} \times 90\%$$

Phagocytosis. Phagocytosis of killed, ^3H-labelled *S. aureus* was assayed as previously described by Roth et al. (19) with some modifications. *Staphylococcus aureus* was grown in trypticase soy broth (TSB; Diffco Laboratories, Detroit, MI) with ^3H-thymidine (ICN Radiochemicals, Irvine, CA). Bacteria were killed by heating the culture at 67° C in a water bath for 1 hr and then increasing the temperature to 75° C for 5 min. Bovine antiserum was obtained as described above. Bacteria were opsonized by adding 50 μl of diluted antiserum

(1:10) to 100 μl of bacteria (1×10^7) in scintillation vials and an additional 300 μl of medium were added. Vials were incubated in a 37° C water bath for 30 min. Fifty microliters of macrophages (2.5×10^5) were added to triplicate tubes and were further incubated for 1.5 hr. Lysostaphin (500 μl, 1 U/ml, ICN Biomedicals Inc., Cleveland, OH) was added, and vials were incubated for an additional 30 min. Macrophage cultures were washed by adding 2 ml of PBS and centrifuging for 10 min at 1250 × g. The supernatant was discarded and the pellet was saved; this procedure was repeated two times. Finally, 1 ml of PBS and 3 ml of scintillation fluid were added, and the tubes were counted on a liquid scintillation counter. Background tubes were prepared by incubating bacteria with lysostaphin only, and standard tubes were prepared by incubating bacteria alone. Percent phagocytosis was calculated as follows:

$$\% \text{ Phagocytosis} = \frac{\text{Experimental Counts per Minute} - \text{Background CPM}}{\text{Standard CPM} - \text{Background CPM}} \times 100$$

Cytotoxicity. Bovine herpesvirus-1-infected and noninfected MDBK cells, as well as PRV-infected and noninfected PK-15 cells, were used as targets in ADCC and NMRC assays. Macrophage cytotoxicity was measured by a ^{51}Cr-release assay as previously described (20). Briefly, labelled target cells (1×10^4/well, 100 μl) were added to triplicate macrophage cultures (5×10^5/well, 100 μl) for each assay. In the NMRC assay, 20 μl of medium were also added to each well; in the ADCC assay, 20 μl of diluted (1:10) porcine PRV antiserum were added to each well. Maximal release of ^{51}Cr was determined by incubating target cells with 3% Triton-X 100 detergent (Sigma Chemical Co.). Control cultures for the NMRC assay consisted of target cells incubated in medium alone, and the value determined for each animal in the NMRC served as its control in the ADCC. Plates were incubated for 12 hr under the conditions described above. ^{51}Cr-release was measured by harvesting the supernatants onto cotton plugs (Skatron Inc., Sterling, VA) and counting on a gamma counter. Percent specific lysis was calculated as follows:

$$\% \text{ Specific Lysis} = \frac{\text{Experimental CPM} - \text{Control CPM}}{\text{Maximum CPM} - \text{Control CPM}} \times 100$$

In addition, assays measuring cytotoxicity against E8 and K562 cells were performed as described above for the NMRC assay.

Cytokine Assays. To stimulate the secretion of T-cell proliferative cytokines (IL-1/IL-6) and TNFα, 5×10^6 macrophages were incubated in 96-well tissue culture plates containing 200 μl of medium with or without 12.5 μg/ml LPS (*E. coli*, 055:B5, Sigma Chemical Co.). Plates were incubated for 2 hr for TNFα and 24 hr for IL-1/6, at 37° C in a humidified atmosphere of 7% CO_2 in air. Supernatants containing cytokines were harvested and stored frozen at −135° C until assay.

Production of T-cell proliferative cytokines was assayed by the previously described comitogenic murine thymocyte assay (21). Proliferation may be induced by both IL-1 and IL-6, however, IL-6 induces proliferation only in concentrations 1000-fold greater than proliferative concentrations of IL-1 (22). Therefore, we feel that the cytokine produced by PIMs and PAMs best measured by this assay is IL-1. Thymocytes were aseptically removed from 4- to 8-week-old, C3H/HeJ mice, teased to release thymocytes, washed in PBS, resuspended at 1×10^6 cells/ml medium (Dulbecco's Modified Eagle's Medium, Grand Island Biological Co.; containing 0.3% L-glutamine, 3×10^{-5} M 2-ME and 50 μg/ml gentamicin sulfate), and distributed into 96-well microtiter plates, 100 μl/well. Doubling dilutions of the test supernatants (100 μl) were added to the wells, which, in turn, were supplemented with 0.45 μg PHA (Burroughs Wellcome, Greenville, NC; 50 μl, 2.25 μg/ml in-well concentration). Background control wells containing thymocytes and PHA only were prepared in triplicate. Plates were incubated for 72 hr under the conditions described above. Tritiated thymidine (1 μCi, 20 μl) was added for the last 8 hr, followed by liquid scintillation counting to determine incorporated radioactivity. Human recombinant IL-1α (Genzyme, Boston, MA) was assayed with the experimental supernatants to serve as a control. Results were expressed as Experimental CPM-Background CPM.

Tumor necrosis factor-α was measured by a colorimetric assay using the WEHI 164 (clone 13) cell line as described previously (15,23,24). Results were calculated as percent specific lysis as follows:

$$\% \text{ Specific Lysis} = 1 - \frac{\text{Sample OD} - \text{Spontaneous Lysis OD}}{\text{100\% Lysis OD} - \text{Spontaneous Lysis OD}} \times 100$$

Nitrite Production. A colorimetric assay, as described by Stuehr et al. (25), was used to determine the amount of NO_2^- present in the LPS-stimulated and nonstimulated macrophage supernatants. An in-lab $NaNO_2$ standard was assayed concurrently with the samples, and medium was used as a negative control. Quantity of NO_2^- present in the samples was determined by regression analysis.

Statistical Analysis. Results of the assays measuring the functional immune activity of PIMs and PAMs were analyzed by paired *t*-test (26). Data are expressed as mean ± SEM using all available data.

Abbreviations. ADCC, antibody-dependent cellular cytotoxicity; BHV-1, bovine herpesvirus-1; CPM, counts per minute; FBS, fetal bovine serum; MDBK, Madin Darby bovine kidney fibroblasts; NMRC, non-MHC-restricted cytotoxicity; NO_2^-, nitrite; PAM, pulmonary alveolar macrophage; PIM, pulmonary intravascular macrophage; PK-15, porcine kidney fibroblasts; PRV, pseudorabies virus; TSB, trypticase soy broth.

RESULTS

Cell Isolations and Identification. Sufficient macrophages from both populations were obtained from most animals to perform all of the assays. Differential counts performed on the cells isolated by density gradient centrifugation indicated that the majority of cells from both lavages, and perfusions were predominantly monocytes/

TABLE 1. Differential Counts and Number of Macrophages Recovered after Purification

Cell Type	Perfusion	Lavage
Macrophages[a]	70 ± 3	93 ± 2
Lymphocytes[a]	28 ± 3	4 ± 1
Granulocytes[a]	2 ± 1	2 ± 1
Total number of macrophages recovered[b]	15 ± 5	13 ± 4

[a]Percent.
[b]Values × 10^7.

macrophages, but the preparations also contained some lymphocytes and granulocytes (Table 1). Differences in the morphology of the two populations were obvious; PIMs were smaller than PAMs and had a larger nucleus to cytoplasm ratio (Fig. 1). In addition, the PIMs were more prone to cluster together on cytospin preparations, whereas PAMs appeared evenly distributed on the slides.

Effect of Collagenase Treatment on Macrophages. We were concerned that the collagenase solution that was used in the PIM isolations may influence macrophage functional activity. Therefore, as in previous experiments (15), we included collagenase-treated PAMs as controls. Results of the assays measuring the effect of collagenase treatment on macrophage function indicated little or no change in macrophage activity with this enzyme (results not shown) as used in our protocol.

Bactericidal and Phagocytic Activity of Pulmonary Macrophages. The capability of PIMs and PAMs to

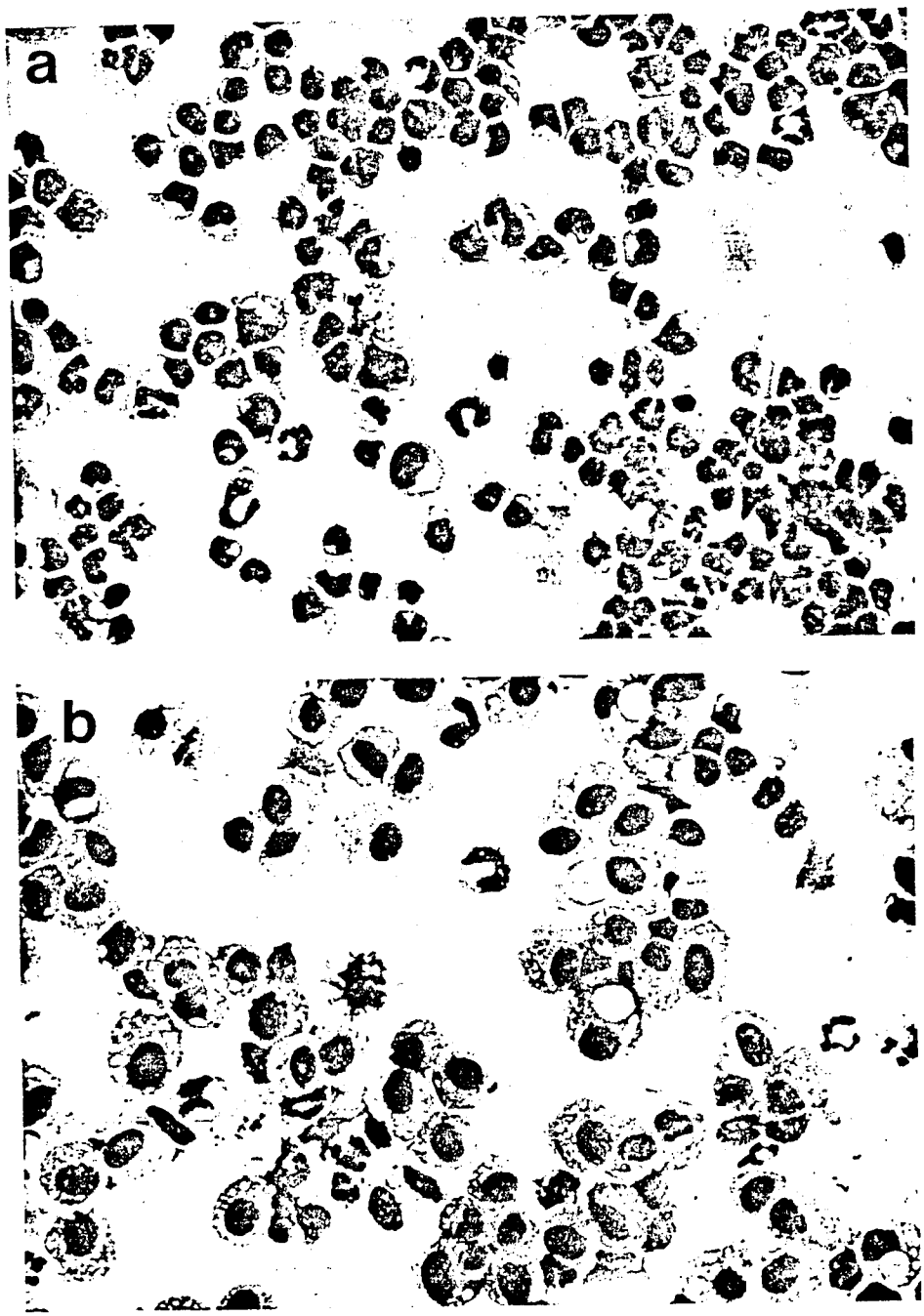

Figure 1. LeukoStat stain of cytocentrifuge preparations of (a) bovine pulmonary intravascular macrophages and (b) pulmonary alveolar macrophages (× 40).

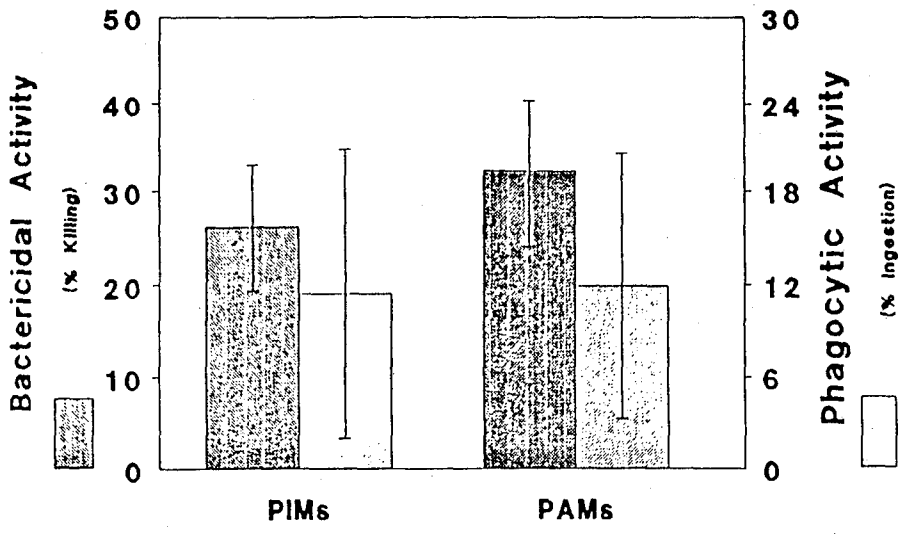

Figure 2. Bovine pulmonary intravascular and alveolar macrophage bactericidal and phagocytic activity presented as mean % killing (n = 7) and % ingestion (n = 5) of *S. aureus* ± SEM, respectively.

phagocytize and kill *S. aureus* was evaluated. Both PIMs and PAMs exhibited similar bactericidal and phagocytic activities (Fig. 2).

Cytotoxic Activity of Pulmonary Macrophages. In the assay measuring NMRC against BHV-1-infected MDBK cells, PIMs lysed more of the targets than PAMs ($P < .01$, Table 2). Results of the ADCC assay against the same target cells followed a similar trend ($P < 0.5$, Table 3). The results of the assay utilizing noninfected MDBK cells as targets indicated that PIMs and PAMs were different for NMRC ($P < .05$), but were similar for ADCC (Tables 2 and 3).

In the four assays measuring cytotoxicity against PK-15 cells, PIMs and PAMs did not differ in NMRC against PRV-infected or noninfected targets, or in their lysis of noninfected PK-15 cells in the ADCC assay (Tables 2 and 3). However, PIMs lysed more PRV-infected targets in the ADCC assay than did PAMs ($P < .05$, Tables 2 and 3).

Although PAMs lysed fewer of the K562 target cells as compared to PIMs, this difference was not significant ($P > .05$, Table 2). Similar results were observed for cytotoxicity against E8 targets ($P > .05$, Table 2).

Cytokine Production of Pulmonary Macrophages. Cytokine production, such as T-cell proliferative cytokines IL-1 and IL-6, and TNFα is an important functional property of macrophages. T-cell proliferative cytokines were produced by both PIMs and PAMs; however, there was a trend for LPS-stimulated PIMs to produce more of these cytokines at the 1/2 dilution. This difference in cytokine production became significant at the 1/4 dilution ($P < .01$, Fig. 3). Without LPS stimulation, both populations of macrophages produced

TABLE 2. Bovine Pulmonary Intravascular and Alveolar Macrophage Non-MHC-restricted Cytotoxicity (% Specific Lysis) of Virus-infected, and Noninfected Target Cells

	Effector Cells	
Target Cells	PIMs	PAMs
MDBK+[b]	39 ± 10[a]	2 ± 1
MDBK−[c]	32 ± 10[a]	1 ± 1
PK-15+[b]	8 ± 3	4 ± 2
PK-15−[c]	16 ± 14	10 ± 10
E8	14 ± 6	0 ± 0
K562	10 ± 5	1 ± 1

[a] $P \leq 0.01$.
[b] + = Infected with virus: BHV-1 for MDBK, PRV for PK-15.
[c] − = Not infected with virus.
n = 4 to 7
Effector to target cell ratio = 50:1.

TABLE 3. Bovine Pulmonary Intravascular and Alveolar Macrophage Antibody-dependent Cellular Cytotoxicity (% Specific Lysis) of Virus-infected, and Noninfected Target Cells

	Effector Cells	
Target Cells	PIMs	PAMs
MDBK+[b]	4 ± 1[a]	1 ± 0
MDBK−[c]	2 ± 1[a]	0 ± 0
PK-15+[b]	19 ± 5	0 ± 0
PK-15−[c]	6 ± 2	1 ± 1

[a] $P \leq 0.05$.
[b] + = Infected with virus: BHV-1 for MDBK, PRV for PK-15.
[c] − = Not infected with virus.
n = 6.
Effector to target cell ratio = 50:1.

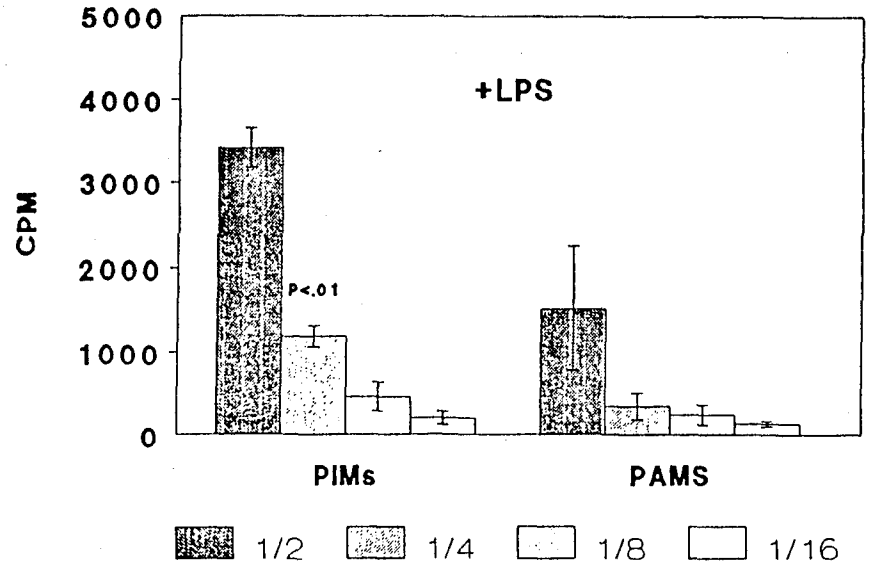

Figure 3. Production of T-cell proliferative cytokines (IL-1/6) by LPS-stimulated bovine pulmonary intravascular and alveolar macrophages at 4 dilutions presented as mean CPM ± SEM, n = 4 to 6. Counts per minute for human recombinant IL-1α assayed concurrently with the bovine supernatants were 687, 399, 562, 434, 158, 0, 96, and 168 CPM for 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, and 0.007 pg/ml, respectively.

similar amounts of T-cell proliferative cytokines (Fig. 4).

Although both populations of macrophages secreted TNFα, PAMs stimulated by LPS lysed nearly three times more WEHI cells than LPS-stimulated PIMs ($P < .01$, Fig. 5). When LPS-stimulated macrophages were compared to their nonstimulated counterparts, only PAMs were affected by LPS ($P < .05$, Fig. 5).

Production of NO_2^- by Pulmonary Macrophages. Nitric oxide is a potent nonspecific defense molecule produced by immunocytes against parasites, tumor cells, and intracellular bacteria. We compared NO_2^- production of PIMs and PAMs and found that the results were similar to those for TNFα, i.e., alveolar macrophages stimulated with LPS produced significantly more NO_2^- than did stimulated PIMs ($P = .05$, Fig. 6); however, nonstimulated macrophages produced similar amounts of this compound ($P > .05$, Fig. 6). Only PAMs appeared to be stimulated by LPS to produce NO_2^- ($P = .01$, Fig. 6).

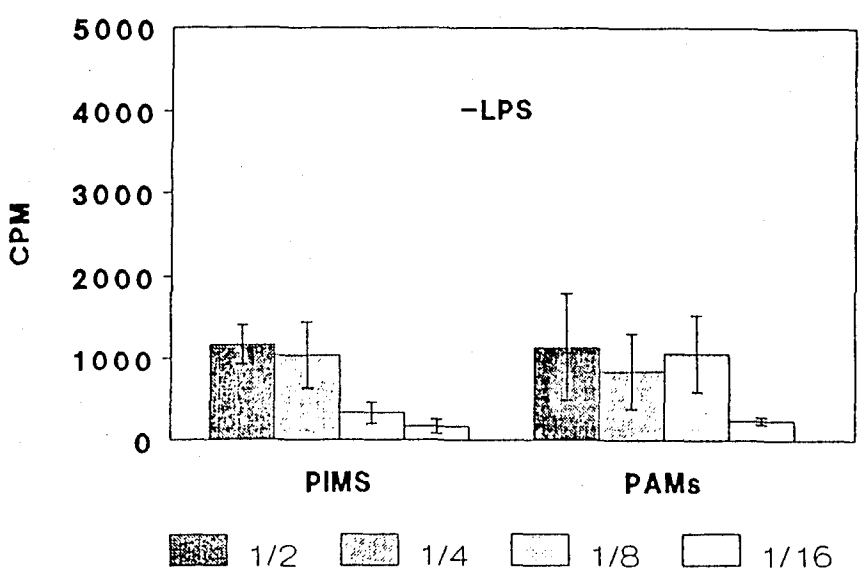

Figure 4. Production of T-cell proliferative cytokines by LPS-nonstimulated bovine pulmonary intravascular and alveolar macrophages at 4 dilutions presented as mean CPM ± SEM, n = 3 to 5.

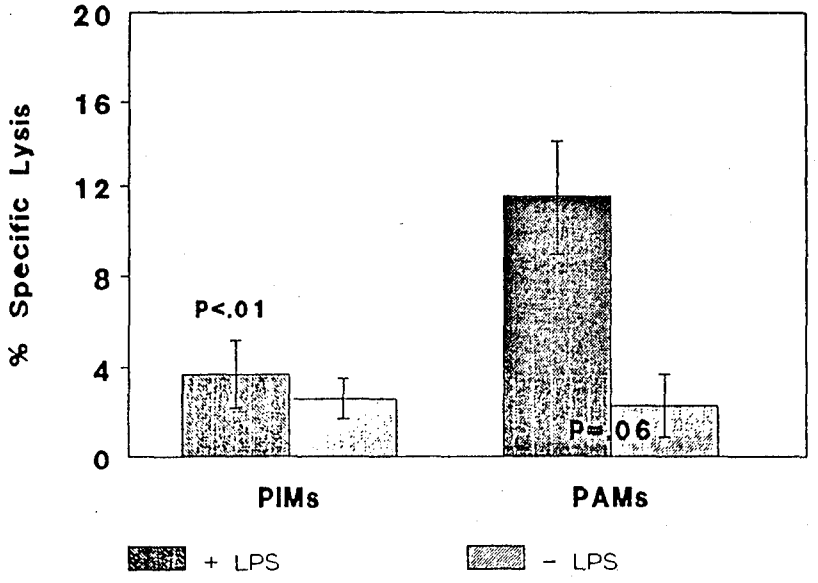

Figure 5. Production of TNFα by LPS-stimulated and nonstimulated bovine pulmonary intravascular and alveolar macrophages presented as mean % specific lysis of WEHI 164 (clone 13) target cells ± SEM, n = 5 to 7. P values above bars indicate differences between PIMs and PAMs. P values within bars indicate differences between +LPS and −LPS.

DISCUSSION

To our knowledge, this study is the first to isolate PIMs from calves. By modifying our isolation technique to maximize collection of a relatively blood-free perfusate, we were able to isolate more PIMs from calves than were obtainable from pigs (15). This may have been due partly to the size, as well as the architecture, of the calf lung. Intravascular macrophages were obtained readily from 2-wk-old calves; however, PIMs do not become prominent in the porcine lung until 7 days of age, and increase in numbers until approximately 30 days (27). When perfusing pig lungs, we found the number of cells isolated to be partially dependent upon the amount of time the connective tissue of a lung would remain unaffected by collagenase treatment (Chitko-McKown et al., unpublished). In virtually every porcine lung perfused, flow of the perfusate would slow over time because of connective tissue breakdown. However, calf lungs did not react to the collagenase in this way; collection of perfusate was never limited by a

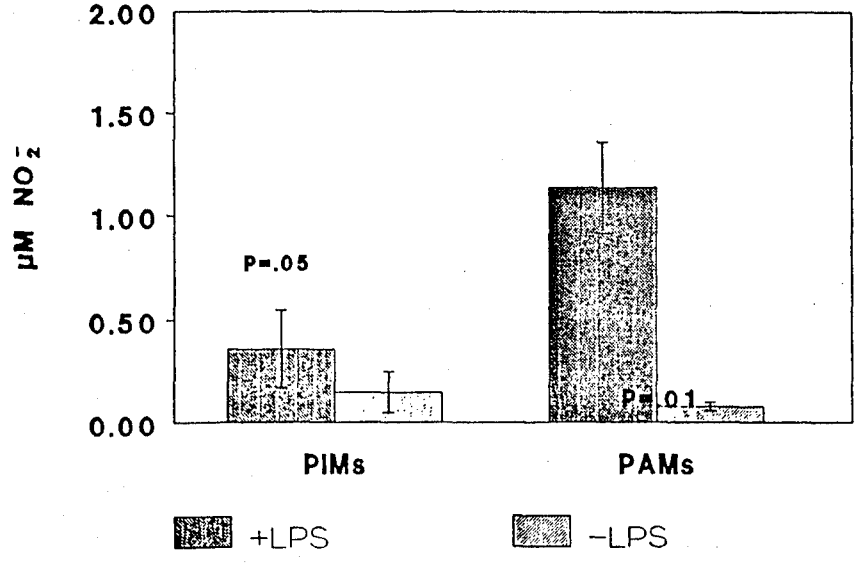

Figure 6. Production of NO_2^- by LPS-stimulated and nonstimulated pulmonary intravascular and alveolar macrophages presented as mean μM NO_2^- ± SEM, n = 4 to 7. P values within bars indicate differences between +LPS and −LPS.

lack of flow. Winkler et al. reported that in the pig lung, increased amounts of collagen are seen in 30- to 60-day-old animals (28). It may be that calf lungs are more differentiated at birth and, therefore, not as susceptible to the effects of collagenase as pig lungs.

Blood clearance of bacteria is one of the major roles of PIMs (1,2); however, only one report documents the bactericidal activity of these macrophages (15). PIMs may play a significant role in the symptoms associated with acute respiratory failure (29–32); ingestion of gram negative bacteria appears to exacerbate this condition more than ingestion of gram positive bacteria, such as *S. aureus*. Our results indicate that pigs and calves were very similar in the efficiency with which they killed live *S. aureus* (15), however, the exact mechanism of killing is unknown. It would be very informative to determine the steps in the pathway from bacterial ingestion and killing to the onset of respiratory distress in susceptible animals.

Both PIMs and PAMs phagocytosed killed *S. aureus*, but the percent ingestion we measured was less than expected, considering the results obtained in the bactericidal assay. Mullan et al. reported that centrifugation was required to bring bacteria in contact with mammary macrophages in their phagocytosis assay (33). Another factor affecting the level of bacterial ingestion may be the bacteria to macrophage ratio. Trigo et al. found that alveolar macrophages were more efficient at ingesting and killing bacteria at a ratio of 10:1 rather than of 1:10 (34). Possibly, our bacteria to macrophage ratio of 40:1 was not optimal for calf PIMs and PAMs. Live bacteria were used in the bactericidal assay, in contrast to the phagocytosis assay; the heat inactivation step used in bacterial preparation might have altered the antigenic determinants on the surface of *S. aureus*, causing a reduction in opsonization by serum antibodies. Alternatively, because considerable variation was observed between younger and older calves, the low phagocytic activity noted might have been related to the age of the animals.

We performed NMRC and ADCC assays against virally infected and noninfected cell targets (MDBK, +/− BHV-1; PK-15, +/− PRV) and NMRC against two tumor cell targets (E8, K562). Because of the difference between PIMs and PAMs in killing BHV-1-infected and noninfected targets, we wanted to determine if this trend held true for other virally infected cells. In all cases, PIMs appeared to be more efficient at lysing the target cells than PAMs, although the only significant differences were against MDBK cells. In all cases, PAMs appeared to have little more than background activity. These results are in agreement with our results obtained with pig PIMs and PAMs (15). In the pig, PIMs killed more than six times the amount of virally infected targets in the NMRC than did the PAMs, which had little or no activity; the same trend was apparent in the ADCC assay. Although in agreement with our earlier work, these results are in contrast to other studies, which have found bovine PAMs to be efficient in killing virally infected targets (9,10,12,35), including those infected with BHV-1. There was also a trend for bovine PIMs to be more cytotoxic than PAMs against tumor targets; in our studies with pigs, we found the opposite to be true (15). We are unsure of the reasons why PAMs were so inactive in all measures of cytotoxicity; however, if age is a significant factor in the cytotoxic performance of PAMs, our data may reflect this.

Because T-cell proliferative cytokines are important components of the immune response of macrophages, we wanted to determine if bovine PIMs produced these cytokines in any quantity. Our results suggest a tendency for PIMs to produce more of these cytokines than PAMs.

The production of TNFα by bovine monocytes has been reported recently by Adams and Czuprynski (36). Our data indicate that both PIMs and PAMs produced TNFα; however, PAMs produced significantly more when stimulated with LPS. This is in contrast with the results of the thymocyte proliferation assay, in which PIMs produced cytokines to a greater degree when stimulated with LPS. This may indicate that various optimal concentrations of LPS exist, which lead to the selective activation of metabolic pathways or the production of specific mediators.

L-arginine-dependent synthesis of nitric oxide, NO_2^-, and nitrate by macrophages correlates with and is required for their execution of nonspecific cytotoxicity towards some tumor cells and microbes (25). Because little is known concerning the means by which PIMs kill their targets, we wished to determine if they produce NO_2^-. Although both PIMs and PAMs produced this metabolite, PAMs produced significantly more NO_2^- when stimulated with LPS. Beasley et al. have shown that IL-1 induces NO_2^- production in rat vascular smooth muscle cells (37). Although LPS does not directly stimulate PIMs to produce NO_2^-, this metabolite may be produced by the capillary smooth muscle cells because of the increased secretion of IL-1 by PIMs.

In summary, numerous factors may affect the degree of activation of PIMs. Because these cells are theorized to be responsible for a great deal of pulmonary pathology associated with gram negative sepsis (2, 29–31), it is surprising that these cells are not stimulated excessively by LPS. This may indicate that additional signals may be required from the surrounding microenvironment for pathological characteristics of PIMs to become apparent. This report is the first to demonstrate that bo-

vine PIMs are capable of a number of immunologic activities and may be as important in the surveillance of the lung as their alveolar counterparts.

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Ground-Based Experiments Complement Microgravity Flight Opportunities in the Investigation of the Effects of Space Flight on the Immune Response: Is Protein Kinase C Gravity Sensitive?

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ABSTRACT

This manuscript briefly reviews ground-based and flight experiments, discusses how those experiments complement each other, and details how those experiments lead us to speculate about the gravity-sensitive nature of protein kinase C.

INTRODUCTION

Post flight studies on astronauts and cosmonauts suggest that space travel induces significant changes in immune cell function and distribution (Taylor and Dardano, 1983; Taylor et al., 1986; Taylor and Janney, 1992). Studies on animals come to similar conclusions (Sonnenfeld et al., 1990). The immune system is important for resistance to microorganisms and the maintenance of health. Therefore, it is important to understand how space flight alters this physiological system.

The effects of space flight are complex. Space flight may directly affect cells by interfering or enhancing cellular processes (Todd, 1989). In addition, space flight and/or microgravity can induce physiological changes that indirectly regulate cell function. The immune response is characterized by cellular responses that protect at the organismic level. Because of this, physiological experiments that look at the immune system as a whole can be complemented by studies that look at changes at the cellular level. We have used physiological experiments and cell biology experiments to address questions about the effects of space flight on the immune system. Though these experiments are not necessarily comparable, their complementarity has focused us on cell and organ systems that may be affected by space flight. Table 1 summarizes many of those experiments and the findings. The following review focuses on our studies of inflammatory cells and their mediators.

Table 1. Summary of complementing ground-based and flight experiments.

Ground-based experiments	Experimental system	Result	Space/microgravity experiments	Flight	Result
PMN secretion of O_2^-	AOS	Depressed secretion from control[a]	PMN secretion of O_2^-	KC-135	enhanced secretion[b]
Macrophage secretion of TNF and IL-1	AOS	No change from control[c]	B6MP102 macrophage secretion of TNF and IL-1	STS-37 STS-43	enhanced secretion[d]
Macrophage secretion of TNF	Inhibition of PKC activation[e]	Inhibited TNF secretion	CSF & PMA-induced tyrosine phosphorylation	KC-135	unchanged[f]
Lymphocyte response to mitogen	AOS	Depressed proliferation (lymph node)[f] enhanced proliferation (spleen)[e]	Lymphocyte response to mitogen or Poly I:C	STS-37 STS-43	enhanced INF-α (spleen)[d] enhanced INF-γ (lymph node)[d]
TNF-mediated cytotoxicity of LM929 cells	Activation of PKC with phorbol ester	Depressed cytotoxicity[f]	TNF-mediated cytotoxicity of LM929 cells	STS-50	depressed cytotoxicity[g]

[a] Fleming et al., 1990; determined after 11 day antiorthostatic suspension (AOS).
[b] Determined in vitro; Fleming et al., 1991
[c] Determined after 11 days of antiothostatic suspension; Kopydlowski et al., 1992.
[d] Chapes et al., 1992*b*.
[e] Chapes et al., 1992*a*.
[f] Armstrong, Woods and Chapes, unpublished. Observations, Kansas State University.
[g] Chapes et al., 1993*b*.

Table 2. Detectable phosphotyrosine in PMN's and macrophages after stimulation.

Treatment[a]	Cells	Gravity conditions[b]	
		1 × g	Microgravity
PMA	PMN	57 ± 8[c]	52 ± 13
CSF-1	Macrophage	185 ± 6	198 ± 18

[a] Peritoneal PMNs or macrophages were stimulated with 100 ng/ml PMA or 1 nM colony stimulating factor-1.

[b] Cells were stimulated at 1 × *g* during level flight or 0 × *g* during parabolic flight on the NASA KC-135 airplane.

[c] Numbers represent the detectable amount of phosphotyrosine detectable using anti-phosphotyrosine monoclonal antibody in an ELISA; $[(A_{405} + Ab) - (A_{405} - Ab)]1000$; $\bar{x} \pm SD$.

Neutrophil Respiratory Bursts

Neutrophils (PMN's) are important inflammatory cells that destroy microorganisms at infected sites. Reactive oxygen species, such as superoxide (O_2^-) are involved in the killing of microbes. Unfortunately, hyper-secretion of these molecules causes detrimental tissue destruction. Changes in cellular secretion of O_2^- during space flight could impair immunity, if there is a diminished O_2^- response, or cause tissue damage, if there is excessive secretion of O_2^-. Therefore, due to the observed neutrophilia seen in astronauts post-flight, we investigated PMN secretion of O_2^-.

We used antiorthostatic suspension (AOS) as a model system because it mimics several of the physiological changes associated with space flight (Musacchia and Steffen, 1983; Wronski and Morey-Holton, 1987; Chapes et al., 1993*a*). Our investigations found significantly depressed O_2^- secretion, by phorbol myristate acetate (PMA)-stimulated inflammatory PMNs isolated from animals subjected to 11 days of antiorthostatic suspension (Fleming et al., 1990). Those results lead us to investigate the effects of microgravity on PMN secretion of O_2^-. We used parabolic flights in the NASA KC-135 airplane to address that issue. Interestingly, PMNs stimulated with the protein kinase C (PKC) activator, PMA, during microgravity secreted higher concentrations of O_2^- than cells stimulated at 1 × *g*. Subsequent studies on inflammatory neutrophils and macrophages suggested that tyrosine kinase activity was unaffected by short periods of microgravity (Table 2) and led us to investigate other signal transduction mechanisms.

Macrophage Secretion of Cytokines

We also began a series of experiments on macrophages that attempted to determine the effects of AOS and microgravity/space flight on processes that took longer than 25 sec to occur; that is, macrophage secretion of the immunoregulatory cytokines, tumor necrosis factor (TNF) and interleukin-1 (IL-1). We discovered that macrophages, isolated from animals subjected

Table 3. Effect of protein kinase C inhibitors or PMA on TNF-mediated cytotoxicity of LM929 cells.

Treatment[a]					
H7		H-8		PMA	
[ ]	% killing	[ ]	% killing	[ ]	% killing
0	84 ± 2[b]	0	84 ± 2	0	84 ± 2
10 μM	78 ± 3	20 μM	76 ± 1	50 ng/ml	6 ± 0
1.25 μM	80 ± 4	2.5 μM	79 ± 1	6.3 ng/ml	29 ± 2
0.31 μM	80 ± 6	0.31 μM	93 ± 4	3.1 ng/ml	43 ± 0

[a] LM929 cells were incubated in the indicated amount of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7); (N-[2-methylamino)-ethyl]-5-isoquinolinesulfonamide). (H8), or phorbol myristate acetate (PMA), 10 μg/ml cycloheximide, and 3 μg/ml tumor necrosis factor for a period of 16 h.

[b] Numbers represent % specific killing of ^{51}Cr labelled cells, $\bar{x}$ ± SEM or triplicates.

to AOS, secreted IL-1 and TNF normally in response to lipopolysaccharide (LPS) (Kopydlowski et al., 1992). Follow-up experiments demonstrated that LPS-induced TNF secretion was protein kinase C-dependent (Chapes et al., 1992*a*). Therefore, after we found that LPS-induced secretion of TNF and IL-1 was enhanced by space flight on two different shuttle flights (Chapes et al., 1992*b*), it appeared that one gravity-sensitive component within the cell might be PKC.

TNF-Mediated Cellular Cytotoxicity

The enhanced secretion of TNF and IL-1 by macrophages during space flight raised the question of how space flight affects the action of cytokines. We investigated that question using an experimental system developed for ground-based investigations. The murine cell line LM929 is naturally sensitive to the cytotoxic effects of TNF. Cytotoxicity does not appear to be dependent upon PKC because several inhibitors of PKC do not affect TNF-mediated killing (Table 3). Interestingly, if PKC is activated in LM929 cells by PMA, the cells become refractory to TNF-mediated lysis (Table 3). We also found that space flight inhibited cytotoxicity by TNF (Table 4; Chapes et al., 1993*b*).

Table 4. TNF-mediated cytotoxicity of LM cells during space flight.

Gravity condition	Percent cytotoxicity[a]
1 × *g*	38 ± 19[b]
0 × *g*	−2 ± 5

[a] LM929 cells attached to Cytodex 3 beads, incubated in the presence of 91 μg/ml recombinant, murine TNF for 29 h.

[b] Numbers represent $\bar{x}$ ± SD of triplicate samples. Adapted from Chapes et al., 1993*b*.

Is Protein Kinase C Gravity Sensitive?

The secretion of O_2^-, in response to a PKC activator, was enhanced during microgravity. Lipopolysaccharide-induced TNF secretion was also enhanced during space flight and this process is PKC-dependent. Does gravity affect PKC? These findings do not fully support the hypothesis that PKC is gravity sensitive. Other cellular processes and/or structures, such as microtubules, or the induction of stress proteins may be affected during microgravity. However, when we consider that the activation of PKC inhibits TNF-mediated cytotoxicity and space flight also inhibits TNF cytotoxicity, the evidence begins to justify the study of PKC in future experiments.

How might gravity affect PKC? Microgravity does not appear to induce the spontaneous secretion of either O_2^-, TNF or IL-1. Therefore, if microgravity activates PKC, other processes, associated with the binding of LPS to its receptor, are necessary to induce secretion. However, the putative activating effect of gravity on PKC might exacerbate the secretion of O_2^-, IL-1 and TNF. PKC activation by microgravity might also explain how space flight abrogates TNF-mediated killing of LM929. The activation of PKC on earth, induces the down regulation of TNF receptors and inhibits TNF-mediated cytolysis (Holtmann and Wallach, 1987).

The idea that PKC is a gravity sensitive component in the cell is supported by other findings. DeGroot et al: (1991) and Limouse et al. (1991) found that PKC-dependent processes or responses stimulated with PMA were significantly affected when induced in microgravity. Therefore, the hypothesis that PKC is affected by microgravity appears to be valid and worthy of future testing.

The Value of Ground-Based Studies

Ground-based experiments have contributed to our overall effort to understand the effects of space flight/microgravity on biological systems. The results of the PMN studies following AOS inspired the KC-135 experiments. The ground-based studies on signal transduction after treatment with TNF have contributed to the development of testable hypotheses on how microgravity affects cells. Lastly, AOS parallels important macrophage and lymphocyte responses that occur in response to space flight (Chapes et al., 1993*a*); substantiating AOS as a valuable ground-based experimental model. Therefore, ground-based studies have proven truly complementary to their flight-based counterparts.

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Effects of murine leukemia virus *env* gene proteins on macrophage-mediated cytotoxicity *in vitro*[1]

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ABSTRACT

F5b Tumor cells were incubated with concentrated culture supernatants taken from cells resistant (F5m) or sensitive (F5b) to contact-dependent macrophage cytotoxicity. Macrophage cell line B6MP102 and murine peritoneal macrophages killed targets incubated with supernatants taken from sensitive cells but poorly killed cells incubated in supernatants isolated from resistant cells. Membranes from cells resistant to macrophage killing, F5m, were fused into F5b cells. The fused F5b cells were killed significantly less than F5b cells fused with F5b cell membranes or untreated F5b cells. The decreased killing of F5b cells corresponded to increased concentrations of gp70^a molecules on F5b cells. Affinity purified gp70^a was added to cytotoxicity assays but failed to inhibit macrophage cytotoxicity. P15E molecules were detectable on both F5b and F5m cells. In addition, a synthetic peptide found to exhibit the inhibitory properties of p15E was added to cytotoxicity assays. P15E synthetic peptide also did not inhibit macrophage cytotoxicity. Therefore, env gene proteins of murine leukemia virus do not appear responsible for inducing tumor cell resistance to activated macrophage contact-dependent cytotoxicity.

INTRODUCTION

The ability to kill tumor cells but not normal cells suggested that macrophages might be involved in tumor immunity (Hibbs 1974, Meltzer 1975). That was substantiated by studies by Russell et al. (1977) and others (Taniyama and Holden 1979, Becker and Haskill 1980) because the number and cytotoxicity of macrophages isolated from regressing tumors was significantly higher than in tumors that progressed.

Macrophages kill tumor cells several ways (Adams and Nathan 1983, Koren et al. 1981, Chapes and Haskill 1983). For instance, macrophages produce tumor necrosis factor alpha (TNF) that, by itself, is either cytotoxic or cytostatic (Old 1975, Sugarman et al. 1985, Kirstein et al. 1986, Feinman et al. 1987, Fletcher et al. 1987, Heicappell et al. 1987). Some targets are not susceptible to the effects of TNF. Yet, they can be killed by macrophages. Killing occurs by a contact-dependent process (Adams and Hamilton

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1984, Chapes et al. 1988a, Rosenkrans and Chapes, 1991).

In recent studies we examined SV40-transformed cells and their susceptibility to killing by macrophages by the contact-dependent process (Chapes et al. 1987, 1988a). The macrophage "susceptible" cell line, F5b, and the "resistant" cell line, F5m, probably arose from the same transformation event (Chapes et al. 1987). These cell lines have served as excellent tools to investigate molecules on the cell surface that might be involved in determining susceptibility to killing. Indeed, using antibody and complement lysis and immunoprecipitation with monoclonal antibodies previously described by Lostrum et al. (1979) we identified that the presence of AKR-specific (or AKR-like) murine leukemia virus (MuLV) gp70^a molecules on tumor cells correlated with resistance to lysis by LPS-activated macrophages (Chapes et al. 1987). More recent experiments demonstrated that incubation of the macrophage-susceptible cell line, F5b, with culture supernatant of F5m cells, causes F5b cells to become more resistant to macrophage binding and killing (Chapes, 1988a).

This study investigated the role of AKR-MuLV env gene proteins on macrophage susceptibility. We found that soluble gp70^a did not inhibit macrophage cytotoxicity. We also found that p15E did not appear to cause tumor cell resistance. Membrane fusion experiments suggest, however, that some molecules in the membrane cause tumor cells to be resistant to contact-dependent cytotoxicity.

MATERIALS AND METHODS

Animals and Cell Lines. C3Heb/FeJ and C3H.OL mice, 8-14 weeks of age were used as the source of peritoneal macrophages in these experiments. Mice were obtained from Jackson Labs (Bar Harbor, ME) or were bred in our animal colony at Kansas State University. B6MP102 is a macrophage cell line found to resemble the cytotoxic ability of peritoneal macrophages (Chapes et al. 1988b) and was used in some experiments. The macrophage-susceptible cell line, F5b, and the macrophage-resistant cell line, F5m, were subcloned from the cell line F5 and have been described previously (Chapes and Gooding 1985, Chapes et al. 1987 and 1988a). The macrophage susceptible cell line E8 has also been described by us previously (Chapes and Gooding 1985, Chapes et al. 1987, Chapes et al. 1988b). All cell lines were maintained in Dulbecco's modified minimal essentials medium (DMEM) supplemented with 5% fetal bovine serum and 0.3% L-glutamine. The IL-2-dependent cell line, CTLL-2, was obtained from Dr. G.W. Fortner, Division of Biology, Kansas State University. It was maintained as described above with 10% Con A supernatants (IL-2), produced as previously described (Chapes et al. 1988b). No antibiotics or antimycotics were used in the continuous culture of cells used in these studies.

Antibodies. The AKR-MuLV gp70-specific antibody, 16-B7 (anti gp70^a) was previously described by Lostrum et al. (1979) and was obtained from Dr. M. Lostrum (Genetic Systems, Seattle, WA). Anti-p15E-specific monoclonal antibodies have been described previously (Lostrum et al. 1979, Cianciolo et al. 1983) and were obtained from Dr. G. Cianciolo (Sphinx Biotechnologies, Durham, NC). Rabbit-anti mouse IgG, IgG fraction was our standard secondary antibody for RIAs. It was purchased from Cappel (Malvern, PA).

Cytotoxicity Assays. The 16-hour ^{51}Cr-release assay of macrophage cytotoxicity has been described previously (Chapes and Gooding 1985). The direct complement-mediated cytotoxicity assay was performed as follows. Target cells were seeded in 60mm tissue culture dishes at a concentration of 8×10^5 cells per plate, the morning before the targets were needed. The cells were labelled with $Na_2{}^{51}CrO_4$ overnight. For the assay, targets were dispersed with EDTA, washed, counted and seeded into microtiter plates at a concentration of 1×10^4 cells per well. Varying dilutions of antibody were added to the wells and cells were incubated with antibody for 1 hour at 37°. Complement (Low-Tox-M rabbit complement, Cedarlane, Hornby, Ontario) at a final dilution of 1:10 was added and the assay incubated for an additional 0.5 hour. Amount of lysis was determined by the amount of released ^{51}Cr as determined previously (Chapes and Gooding 1985).

Radioimmune Assay (RIA). Cell lysates for RIA were prepared by resuspending 5×10^6 cells in NP40-saline (1% NP40, .15M NaCl, 20mM Tris, 1% aprotinin, pH 8) for 30 minutes at 4°C to dissolve cells. The cell suspension was microfuged for 5 minutes and the supernatant kept at -90°C until needed for RIA. The RIA was done as follows.

Nitrocellulose circles were incubated for 45 minutes at a 1:2 dilution of the appropriate cell lysate. The circles were removed, allowed to dry and blocked for 45 minutes using nonfat dry milk (Johnson et al. 1984). Circles were incubated in the appropriate primary antibody for 2 hours. Circles were washed in blocker and incubated in the appropriate secondary antibody for 1 hour. Circles were washed in blocker and were incubated in ^{125}I-protein A dissolved in blocker for 1 hour. The circles were washed in blocker, dried, placed in glass 12 x 75mm tubes and counted on a gamma counter. The numbers presented are the number of counts bound in the absence of specific primary antibody subtracted from the number of counts bound in the presence of specific primary antibody.

Affinity Purification of GP70^a. F5m cells were grown as described above. Cells were dispersed with EDTA, washed and counted. Cells were pelleted and resuspended at a concentration of 1.5 x 10^7 cells/ml of 1% octyl-D-B-glucopyranoside in .15M NaCl, 20mM Tris, 1% aprotinin, pH 8 (C8Glu-saline). After a 0.5 hour incubation, cell lysate was microfuged and stored as described above. 16 ml of cell lysate was used in this purification.

Hybridoma 16-B7 (described above) was grown in DMEM supplemented with 0.3% L-glutamine, 10% FBS, 1% nonessential amino acids, and 1% solution 1, which contained 13.2mg oxylacetic acid, 0.8mg insulin and 5.5mg of sodium pyruvate. Supernatant was collected and concentrated 10x using saturated NH_2SO_4.

Twenty-five ml of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) was washed according to manufacturers directions. Twelve mg of IgG in PBS was diluted in 0.2M $NaHCO_3$, 0.3M NaCl, pH 8. IgG was added to packed Affi-gel so that total final volume was about 50 ml. This mixture was rotated at 4°C for 5 hours. The gel was centrifuged, washed 2X with 0.1M NaHCO3/0.15M NaCl. To packed gel 0.1M ethanolamine-HCL, pH 8 was added. This mixture was rotated at room temperature for 60 minutes. The gel was washed 5 x with equal volumes of PBS. 84% of the IgG added was bound to the Affi-gel.

To purify gp70^a from F5m lysates, 16 ml of lysate was added to an equal volume of packed Affi-gel-16-B7. Antigen was allowed to absorb for 3 hours. Affi-gel-16-B7 was then washed until no more protein could be detected at A_{280} (8 washes). Each wash was considered a fraction and the first two washes contained > 80% of the total protein not absorbed to the column. These fractions were used as controls in experiments. Antigen was allowed to incubate at 4°C overnight. Absorbed antigen was eluted using C8Glu-saline. Ten washes were need to elute all antigen in a final volume of about 50 ml. Fractions 1 and 2 (approximately 25 ml each along with gp70^a were dialyzed against water for 13 days and PBS for 6 days. All three were concentrated to approximately 10-15 ml of total volume using Aquicide I (Calbiochem, LaJolla, CA). After concentration, each sample was dialyzed against PBS for 3 days. Protein determinations by A_{280} were made. Fraction 1, 14.6 mg/ml; Fraction 2, 1.5 mg/ml; gp70^a, 0.14 mg/ml. Various dilutions of each were used in our experiments. This procedure gave us a reasonably pure preparation of gp70^a. The only detectable contaminant on SDS PAGE gels, stained with silver was IgG (See Figure 1).

Synthetic Peptide Synthesis. The p15E synthetic peptide sequence describe by Cianciolo et al. (1985) was synthesized using our modification (Gooden et al. 1985) of the solid state technology originally described by Merrifield (1963) and modified by Gorman (1984). The procedure used sequential attachment of t-Boc-amino acids to a divinylbenzene solid support and cleavage of the finished peptide by triflouoacetic acid/HBR. The synthesis reactions were conducted manually in an apparatus similar to that described by Gorman (1984). The cleaved peptide was purified by HPLC using a C_{18} reverse phase column. The amino acid composition of the purified material was determined using the O-phythalaldehyde agent and HPLC (Lockhart et al. 1975). The peptide was crosslinked to BSA using glutaraldehyde. The final synthesized peptide sequence was LQNRRGLDLLFLKEGGL.

Membrane Isolation and Fusion Techniques. Membranes were isolated from F5b or F5m cells. Cells were grown in roller bottles to a density of 1 x 10^8 cells per bottle. Cells were dispersed with EDTA and resuspended in cold PBS. Cells were pelleted and re-suspended in 0.85% NaCl, 5 x 10^6 cells per ml. Cells were incubated for 30 minutes before being added to isotonic PBS. The cell preparation was centrifuged at 7,500 x g. The pelleted material, which included membranes, was added to a 10 ml solution of polyethylene glycol-dextran solution, "top",

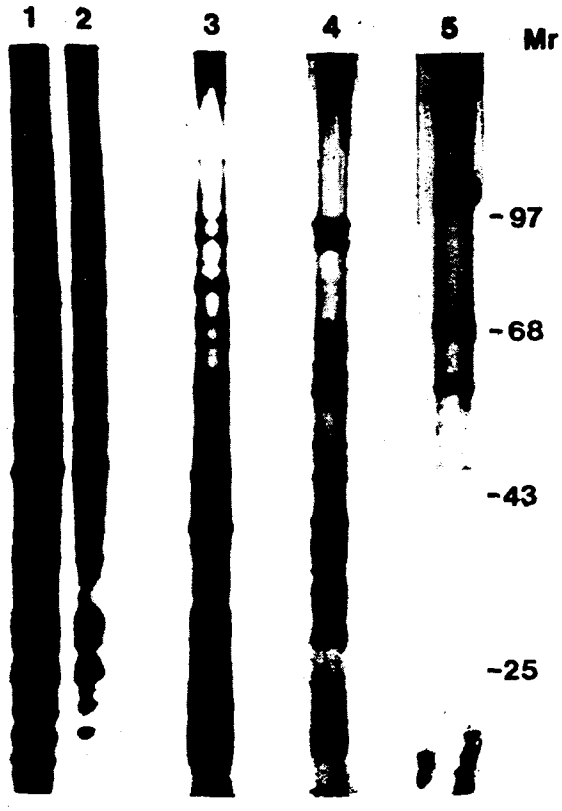

Figure 1. SDS PAGE of Affinity Chromatography-Purified gp70^a. Lanes 1 and 2, whole F5m cell lysate added to affinity matrix; lane 3, first wash of molecules not absorbed to affinity matrix; lane 4, second wash of molecules not absorbed to affintiy matrix; lane 5, gp70^a specifically eluted with 1% octyl-B-D-glucopyranoside, pH 7.

fraction as was described by Brunette and Till (1971). Membranes were isolated using the bi-phasic partitioning procedure described by Brunette and Till (1971).

For fusions, membranes from 1 x 10^7 cells were fused with 1 x 10^6 cells. To fuse membranes with cells, 30% polyethylene glycol 1500 was used. $Na_2{}^{51}CrO_4$-labelled F5b or F5m cells were dispersed with EDTA, washed and resuspended in serum free media containing the appropriate amount of membranes. The membrane-cell preparation was pelleted and 1 ml of 30% polyethylene glycol 1500 was added. Cells were incubated for 1 minute and were centrifuged at 335 x g for 4 minutes at room temperature. The preparation was incubated an additional 2 minutes before media containing 5% FBS was added. The cells were washed and counted on a hemocytometer. Viability after the fusion procedure was always greater than 95% as determined with trypan blue exclusion. The ^{51}Cr release from treated cells was not different from untreated cells. In addition, there was no significant difference between treated and untreated cells 24 hours after fusion. Antibody and complement assay, to detect gp70^a molecules on cells with fused membranes, was done within 2 hours of fusion. Effector macrophages were added to fused targets within 2 hours of the fusion and were incubated with targets for 18 hours.

Growth of F5b Cells in Culture Supernatants of F5m or F5b Cells To grow F5b cells in culture supernatants from F5m cells we used the procedure described previously (Chapes et al. 1988a). The F5m cell line was grown to confluency in 850 cm^2 roller bottles. Approximately 70 ml of cell supernatant was recovered from the cells and was concentrated to a volume of 7 ml using ultrafiltration (Immersible CX-30 ultrafiltration units, Millipore Corp., Bedford, MA). Prior to incubation with the retenate from the ultrafiltration, 5 x 10^5 susceptible F5b cells were plated into 60mm tissue culture dishes and were incubated for 24 hours at 37°C in DMEM containing 8 μg/ml of polybrene (Sigma, St. Louis, MO). Following incubation, cells were incubated with 2 mls of retenate diluted with 1 ml of fresh DMEM containing 24 μg of polybrene. Cells were incubated for 26 hours at 37°C and were dispersed. Cells were then tested for the presence of the AKR virus-specific gp70^a and for their susceptibility to macrophage-mediated cytotoxicity (See Table 1 and Results). As a control for these experiments, supernatant from the macrophage susceptible F5b cell was also concentrated and used in these experiments.

Statistical Analysis. Matched T tests for statistical significance between treatment groups was done using the Number Cruncher Statistical Package, J.L. Hintze, Kaysville, UT.

RESULTS

Macrophage Cytotoxicity of Cells Incubated with F5m Culture Supernatants. The growth of F5b cells in culture supernatants of F5m cells, resulted in cells that were bound

Table 1. Incubation of F5b Cells with Culture Supernatants Derived From F5m Cells

			% Specific ^{51}Cr Release			
			Cell Line			
Effector[b]	Ab dilution	MP:T ratio	F5b	F5m	F5b(bsupt)	F5b(msupt)
Ab + C	1:4		2±2[a]	103±3	10±1	73±4
Ab + C	1:8		0±1	91±6	-0±2	68±3
Ab + C	1:16		4±1	91±3	3±1	57±3
Ab + C	1:32		2±1	68±9	0±4	46±3
B6MP102		10:1	54±2	-3±1	38±9	2±3
Macrophage		30:1	38±9	12±2	44±6	-5±9
Macrophage		40:1	41±9	18±1	47±5	14±2

a. Numbers represent mean ± standard error. Statistical significance between F5b(bsupt) and F5b(msupt) incubated with similar effectors is $p < .05$ for all Ab dilutions and effector:target ratios.

b. Ab is 16-B7 ($\alpha gp70^a$). Macrophages and B6MP102 are described in Materials and Methods.

significantly less than uninfected cells (Chapes et al. 1988a). Similar decreases in macrophage cytotoxicity were observed. To determine whether other kinds of macrophages were negatively affected by F5m culture supernatants, we tested whether F5b cells grown in F5m supernatants would be killed by B6MP102 cells. F5b cells were cultured as described above. B6MP102 cells are a bone marrow-derived cell line that can be activated to be cytotoxic with LPS and other biological response modifiers (Chapes et al. 1988b). We found F5b cells cultured in F5m culture supernatants were not killed well by LPS-activated B6MP102 cells (Table 1). F5b cells incubated in control culture supernatants were killed. These data suggest that B6MP102 cells are affected by F5m culture products similarly to in vivo-derived macrophages.

Effect of Membrane Fusion on Macrophage Cytotoxicity. The expression of $gp70^a$ on cells cultured in F5m culture supernatants correlated with their resistance to killing by peritoneal macrophages and by B6MP102 (Table 1). We previously suggested that only events that depended on contact between tumor cells and macrophages were affected by $gp70^a$ expression. Processes (e.g. macrophage cytostasis) that did not require macrophage-tumor cell contact were unaffected (Chapes et al. 1988a). In addition, there was a correlation between $gp70^a$ expression and resistance to macrophage cytotoxicity (Table 1). Therefore, F5m tumor cell membrane molecules like $gp70^a$ appeared to change sensitivity of F5b tumor cells to macrophage killing by altering the cell's membrane; perhaps by absorbing into the cell membrane. To test that hypothesis, we purified

membranes from macrophage-resistant F5m tumor cells. We fused those membranes into F5b (macrophage-susceptible) cells. We then tested the F5b cell susceptibility to killing. Since the resistant phenotype correlated with the expression of the gp70^a, we measured the amount of membrane integration into F5b cells by measuring the amount of gp70^a on F5b cells after fusion. The data in Figure 2 show the results of two different experiments. In one experiment, the integration of F5m cell membranes into F5b cells caused F5b cells to express more gp70^a than untreated F5b cells (as measured by our ability to lyse F5b with anti-gp70^a and complement). In the second experiment, F5b cells were fused with membranes isolated from F5b cells or F5m cells. In that experiment, only F5b cells, fused with F5m cell mem- branes, had gp70^a detectable by antibody and complement lysis. The amount of gp70^a on F5m membrane-fused F5b cells was significantly less that than normal F5m cells (F5m cells = 100% lysis, as measured by antibody and complement; data not shown on Figure 2). However, the amount of lysis of F5b m- membrane-fused cells was significantly more than normal F5b cells or F5b cells fused with F5b membranes (See Figure 2 legend for a statistical summary).

We also examined macrophage cytotoxicity of F5b cells fused with membranes of F5m or F5b cells. We discovered that the integration of F5m membranes, but not F5b membranes, significantly decreased macrophage cytotoxicity (Figure 3). In one experiment, we found that macrophage cytotoxicity of F5b fused with F5b membranes was 38.2% while cytotoxicity of F5b fused with F5m membranes was 27.9% (40:1 macrophage: target ratio). Although the cytotoxicity of

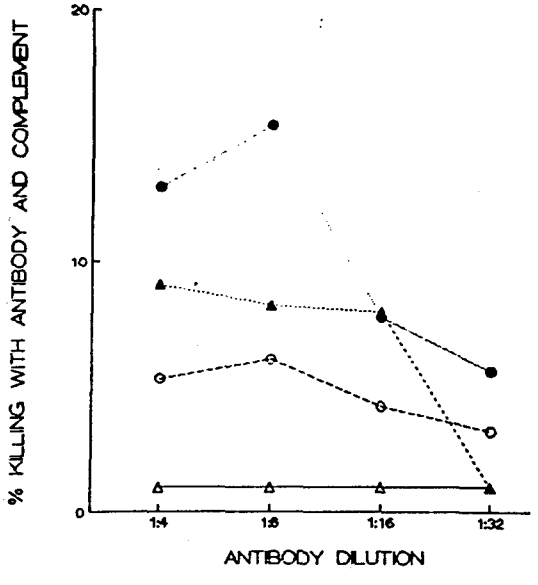

Figure 2. Detection of gp70^a on F5b cells fused with F5m membranes; Experiment 1, ▲; and Experiment 2, ●; or left untreated Experiment 1, △; or fused with F5b membranes, Experiment 2, ○. Gp70^a was detected by incubating cells with anti-gp70^a antibody (16-B7) and complement and measuring % Specific ^{51}Cr-release. Difference between F5b and F5b fused with F5m membranes, in Experiment 1 is significant, P=0.001 (two tailed probability matched t test). Difference between F5b fused with F5b membranes and F5m membranes is significant, P=0.001 (two tailed probability matched t-test).

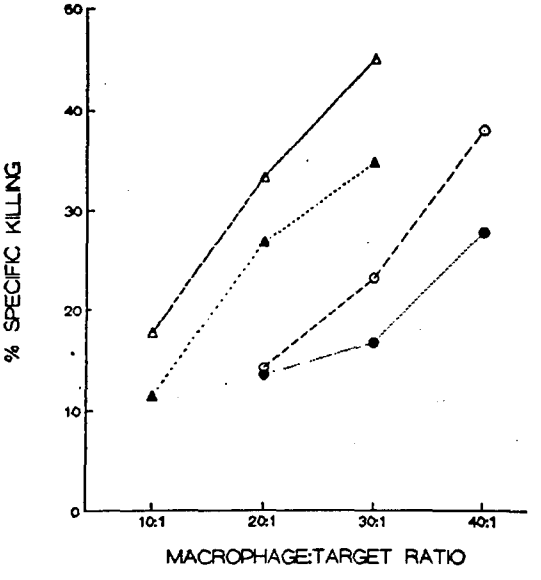

Figure 3. Macrophage cytotoxicity of F5b cells fused with F5m membranes; Experiment 1, ▲; and Experiment 2, ●; or left untreated Experiment 1, △; or fused with F5b membranes, Experiment 2, ○. Killing was detected using Specific ^{51}Cr-release. Difference between F5b and F5b fused with F5m membranes, in Experiment 1 is significant, P=0.005 (two tailed probability matched t test). Difference between F5b fused with F5b membranes and F5m membranes is significant, P=0.01 (two tailed probability matched t test).

F5b cells fused with F5m membranes was higher than normal F5m cells (F5m cytotoxicity was around 0% at all effector:target ratios tested) the decrease in macrophage cytotoxicity was significant (See Figure 3 legend for a statistical summary). In addition, the reduction of killing (about 10% at the highest effector to target ratios) corresponded to the amount of F5m membrane integration as detected by gp70^a, detected with anti-gp70^a antibody. Similar differences in B6MP102 cell cytotoxicity were observed (Table 2). B6MP102 killed F5b cells fused with F5b membranes significantly better than F5b fused with F5m membranes.

Inhibitory Effect of Affinity Purified gp70^a on Macrophage Cytotoxicity. The data from the experiments presented in Figures 2 and 3 indicated that F5m membranes could be fused into F5b cells. Interestingly, the decrease of macrophage cytotoxicity of F5b cells appeared to correspond to the amount of F5m membrane integration. Those data supported the hypothesis that products from F5m cells can alter target cell susceptibility. Since the expression of gp70^a correlated with the resistant phenotype, we attempted to determine whether gp70^a might be responsible for resistance. Monoclonal antibody 16-B7 was covalently attached to an Affi-gel matrix (Bio-Rad, Richmond, CA) and gp70^a molecules were isolated from cell extracts of F5m cells as described in materials and methods (See Figure 1). In these experiments, purified gp70^a was added to cytotoxicity assays at various concentrations varying from 35.3 to 8.8 μg per ml. As controls, two fractions of F5m cell material not retained by the Affi-gel-16-B7 matrix were run at different concentrations and compared to gp70^a. As illustrated in Table 3, no concentration of gp70^a significantly inhibited killing of F5b cells, in two different experiments. Concentration of gp70^a was estimated at 35.3 μg/ml (using A_{280}) in one of our experimentals. Therefore, the lack of an effect did not appear to reflect too low a concentration of gp70^a. We estimated there to be at least 8.8 ng gp70^a/cell, based on how much gp70^a we isolated and how many cells we started with.

Determination of the Presence of p15E on macrophage-Susceptible and -Resistant Tumor Cells. Although decreased susceptibility to macrophage killing correlated with gp70^a expression (Chapes et al. 1987 and 1988a) purified gp70^a did not inhibit macrophage cytotoxicity. Gp70^a is an MuLV *env* region gene product. Other investigators presented data to show that another *env* gene product is inhibitory to PMNs and macrophages (Snyderman and Cianciolo 1984). In those studies studies low molecular weight extracts from Friend, Moloney and Rauscher leukemia viruses inhibited macrophage accumulation in vivo. The active inhibitory substance was identified as p15E, an *env* gene coded protein. Because p15E had inhibitory properties, and gp70^{a}a and p15E are products of the *env* gene, we assayed the presence of p15E on the macrophage-resistant and -susceptible cells. Whole cell lysates adsorbed on to nitrocellulose circles were tested in an RIA. Table 4 shows that the susceptible cell, F5b, as well as the resistant cell, F5m, both expressed epitopes of p15E recognized by monoclonal antibodies F45T6, 19F8 and 19VIIIE8 (Lostrum et al. 1979, Cianciolo et al. 1983). However, F5m appeared to express more p15E, based on the dilution of antibody that is needed to detect antigen. Equal numbers of cells were used

Table 2. B6MP102 Cell Cytotoxicity of Membrane-Fused F5b Cells

			% Specific ^{51}Cr Release	
Target	Treatment	(MP:T)	20:1	40:1
F5b	F5b membranes		36[a]	50
F5b	F5m membranes		23	29
F5m	none		-2	-8

a. Numbers represent $\bar{x}$ of triplicate samples rounded to the nearest integer.

Table 3. GP70[a] Inhibition of Macrophage Cytotoxicity of F5b

		% Specific ^{51}Cr Release	
Treatment	Concentration[a]	Exp 1	Exp 2
Medium	--	23 ± 1[b]	23 ± 2
gp70[a]	35 μg/ml	30 ± 3	36 ± 5
gp70[a]	18 μg/ml	23 ± 2	23 ± 2
gp70[a]	9 μg/ml	24 ± 1	23 ± 4
Fraction 1	146 μg/ml	29 ± 4	27 ± 3
Fraction 1	15 μg/ml	16 ± 2	30 ± 1
Fraction 2	148 μg/ml	17 ± 1	29 ± 3
Fraction 2	15 μg/ml	24 ± 2	32 ± 3
Fraction 2	2 μg/ml	NT[c]	24 ± 6

a. Activated macrophages incubated with targets in the presence of gp70[a] or unbound fractions 1 or 2.

b. Numbers represent $\bar{x}$ ± SEM of triplicate samples. F5b target. All macrophages activated with LPS. MP:T, 20:1.

c. Not tested.

and A_{280} readings indicated similar whole cell antigen concentrations were used. Since secreted p15E may be inhibitory to macrophages reacting against targets we also used RIA to detect p15E in the cell supernatant. Interestingly, both cells shed p15E into the supernatant. It appeared that the amount shed by F5m was more than F5b but the amount was not statistically significant. Other MuLV could be detected in both cell types (Chapes et al. 1987). Therefore the presence of p15E on F5b cells was probably due to those MuLV. The presence of p15E on macrophage-susceptible, F5b, cells suggested that it was not inhibitory to macrophage cytotoxicity. However, we tested whether p15E was inhibitory in another experiment. We took advantage of the work of Cianciolo et al. (1985) who characterized the inhibitory portion of the p15E molecule. We used the published sequence of the inhibitory peptide to synthesize a 17 amino acid peptide. Experiments with this peptide demonstrated that it inhibited CTLL-2 cell proliferation, similarly to the peptide described by Cianciolo et al. (1985) (Table 5). However, the p15E peptide did not inhibit macrophage cytotoxicity (Table 6).

Combined Effects of gp70[a] and p15E Peptide on Macrophage Cytotoxicity. Neither purified gp70[a] nor p15E synthetic peptide affected macrophage cytotoxicity. We attempted to abrogate macrophage killing by mixing purified gp70[a] with p15E peptide. The data in Table 7 show that in one experiment the ability of the combined gp70[a] and p15E peptide do not affect the killing of F5b cells. In a second experiment, a different macrophage-susceptible cell, E8, was tested. However, even with the tumor cell E8, we observed no effect of gp70[a] and p15E peptide on macrophage killing.

Table 4. Expression and shedding of MuLV P15E on F5b and F5m Tumor Cells

Experiment 1		CPM Counts Bound			
		Antigen			
Antibody[a]	Dilution	F5b Cell Lysate	F5m Cell Lysate	F5b Supernatant	F5m Supernatant
Goat α Rauscher	1:30	7273[b]	8643	6288	7944
16-B7	1:15	257	1878	246	1052
4F5T6	1:30	1837	3504	1160	1299
	1:60	953	2813	544	595
	1:120	368	1795	200	430
19F8	1:30	728	2860	327	777
	1:60	387	2282	283	460
	1:120	276	1988	106	438
19VIIIE8	1:30	482	2459	227	372
	1:60	284	1987	151	449
	1:120	144	1640	47	215
Experiment 2					
Goat α Rauscher	1:30	9765	11808	6609	6712
16-B7	1:15	47	1666	56	634
4F5T6	1:30	3695	6317	875	1026
	1:60	2422	4235	503	446
	1:120	485	1598	136	39

a. Antibodies = Goat antiRauscher gp70; 16-B7, gp70[a]; 4F5T6, 19F8 and 19VIIIE8, P15E.

b. Numbers represent $\bar{x}$ of triplicate samples rounded to the nearest integer. Standard deviation < 5% for all samples.

Table 5. Inhibition of CTLL-2 Proliferation with P15E Peptide

	CPM Incorporated								
	Exp 1			Exp 2			Exp 3		
Dilution	BSA[a]	Peptide[c]	INH[e]	BSA[a]	Peptide	INH[e]	BSA[b]	Peptide	INH[e]
1:20	90,487[d]	80,995	20	26,834	21,111	21	76,201	53,280	30
1:40	101,550	86,413	15	32,033	20,822	35	78,046	58,618	25
1:80	105,389	104,469	1	34,845	24,589	29	72,307	63,453	12
1:160	107,627	98,978	9	27,233	30,095	0	74,179	70,326	5
Medium alone	105,553			30,184			75,910		

a. BSA mixed to same concentration as added to Peptide in expts 1 and 2 but not treated in coupling reaction. Approximate Final Concentration 3 mg/ml.

b. BSA same as in a but treated like peptide in coupling reaction.

c. Approximate final peptide concentration was 2.2 mM before dilution. BSA concentration was same as in a.

d. Numbers represent $\bar{x}$ of quadruplicate samples.

e. % inhibition of peptide from BSA control.

Table 6. Inhibition of Macrophage Cytotoxicity by P15E Peptide

Experimental Conditions		%Specific ^{51}Cr Release				Control Conditions		% Specific ^{51}Cr Release	
		BSA		Peptide				Media	
Target	Dilution[a]	20:1	40:1	20:1	40:1	Target	Treatment[b]	20:1	40:1
F5b	1:20	23±2	42±4	20±1	37±3	F5m	-LPS	-0±0	-0±2
F5b	1:40	17±2	41±6	19±1	37±1	F5m	+LPS	-2±1	8±1
F5b	1:80	14±2	31±2	18±1	32±2	F5b	-LPS	-1±1	-3±2
F5b	1:160	15±1	36±4	15±1	25±1	F5b	+LPS	19±2	39±4

a. Macrophage cytotoxicity of F5b in the presence of P15E peptide at various dilutions.

b. Macrophage cytotoxicity of F5b or F5m cells in the absence of peptide ± LPS.

Table 7. GP70[a] and P15E Peptide Inhibition of Macrophage Cytotoxicity

	%Specific ^{51}Cr Release	
Treatment[b]	F5b	E8
Medium	18 ± 2[1]	38 ± 1
Fraction 1	21 ± 5	33 ± 4
BSA	12 ± 4	44 ± 4
Peptide	17 ± 1	38 ± 1
GP70[a]	16 ± 3	44 ± 1
Peptide + GP70[a]	23 ± 1	50 ± 3

a. Numbers represent $\bar{x}$ ± SEM of triplicate samples from two different experiments. All macrophages activated with LPS. MP:T, 20:1.

b. Treatment concentrations: BSA, 1:20; Fraction 1, 145 μg/ml; Peptide, 1:40; gp70[a], 35 μg/ml.

DISCUSSION

In a previous investigation we described that incubation of the macrophage-susceptible cell line, F5b, with culture supernatant isolated from F5m cells (resistant) changed F5b to be resistant to macrophage killing. In this paper, we have extended that observation. We found that the macrophage cell line B6MP102 also will not kill F5b incubated in culture supernatants of F5m cells. Therefore, the inhibitory effect of the F5m cell line is not unique to peritoneal macrophages.

We found that membranes from macrophage-resistant F5m cells inhibited macrophage cytotoxicity of F5b cells when fused into F5b cells. These data suggest that inhibitory effects of the F5m cell may be due to molecules expressed in the tumor cell membrane. We previously found that gp70[a] expression was unique to macrophage-resistant cells, like F5m (Chapes et al. 1987 and 1988a). Therefore, we studied MuLV-specific molecules, present in the membrane. We used affinity chromatography to purify gp70[a] and added as much as 35 μg/ml to cytotoxicity assays. We did not see any effect on macrophage cytotoxicity. Therefore, either gp70[a] is not inhibitory to macrophage cytotoxicity or gp70[a] needs to be expressed on or within the membrane of cells to be inhibitory. Our experiments with membrane fusions would support this hypothesis. When F5m membranes were fused into F5b cells we saw a decrease in susceptibility to macrophage cytotoxicity. That decrease was associated with increased expression of gp70[a] within the membrane of the membrane-fused F5b cells. However, other unidentified membrane components and molecules were also carried from the resistant cell membrane during the fusion process. Therefore, it is possible that molecules other than gp70[a] were responsible for suppression. Recent studies by Gooding et al. (1989) suggest that gp70[a] is not responsible for confering target cell resistance to macrophage cytolysis. In those studies gp70[a] expression could be found to be high even on cells sensitive to macrophage-mediated lysis.

The expression of viral gp70 has been addressed in the context of macrophage sensitivity or resistance by others. Nicolson and his colleagues (Miner et al. 1983, Yoshida et al. 1987) found a correlation between macrophage resistance of RAW 117 cells and decrease in the expression of virus envelope gp70. Infection of cells with Moloney or Abelson MuLV and subsequent MuLV gp70 expression was associated with decreased metastatic potential and increased susceptibility to macrophage cytostasis and cytotoxicity. Their studies contrasts ours in that we have described that a type specific gp70 (gp70[a]) is present on resistant cells but not on susceptible cells yet other MuLV gp70 molecules appear to be present on both phenotypes (Chapes et al. 1987). There is considerable diversity in MuLV gp70 molecules (Elder et al. 1977). Perhaps different MuLVs and their gp70 molecules have different effects on cells.

Others investigators found that retrovirus products can be immunosuppressive (Snyderman and Cianciolo 1984). However, in those instances the viral protein responsible for suppression was p15E. In this study we have demonstrated that p15E is present on both susceptible and resistant cell lines. We also performed experiments using a synthetic peptide that is believe to be responsible for the inhibitory effects of p15E (Cianciolo et al. 1985). Those experiments reproduced the inhibitory effect of the p15E peptide on the proliferation of an IL-2 dependent cell line CTLL-2, as was originally detailed by Cianciolo et al. (1985). However, we did not see any diminution of killing of two different macrophage-susceptible cell lines by the peptide. This contrasts with the results of

Harris et al. (1987) and Harrell et al. (1986). Human NK cell cytotoxicity and human monocyte respiratory bursts, respectively, were inhibited by a peptide with the same sequence. However, the discrepancy may be due to species (human vs mouse) or effector cell (NK and monocyte vs macrophage) differences. Furthermore, in the studies of Harrell et al. (1986) respiratory burst activity of human monocytes was assessed by measuring superoxide anion release. There is some question about the role of superoxide in tumor cell killing (Sorrell et al. 1978) so those measurements may not be applicable to our system. Our findings also contrast the findings of Kleinerman et al. (1987). In those studies IL-1-dependent monocyte cytotoxicity of A375 cells was inhibited by p15E. We have found that F5b and E8 target cells are not lysed by soluble IL-1. Therefore, the distinct mechansisms of killing of A375 and F5b or E8 targets probably is reflected by differences in sensitivity to p15E inhibition.

It is possible that the peptide does not affect macrophages similarly to the native p15E molecule. Alternatively, the peptide may not inhibit macrophages as it does other cells. However, we find that p15E is present on F5b and F5m cells (macrophage-"susceptible"and -"resistant") and is shed into the supernatants of both cell lines. Therefore, we suggest that p15E did not directly inhibit macrophage cytotoxicity. This conclusion is indirectly supported by the studies of Schmidt and Snyderman (1988). They found that p15E expression was not associated with tumorigenic potential. We previouly found that resistance of SV40-transformed fibroblasts to macrophage cytotoxicity correlated with in vivo tumor growth (Chapes and Gooding 1985). Nicolson's group has similar data (Miner and Nicolson 1983, Yoshida et al. 1987). Therefore, if p15E is found on both macrophage-susceptible and -resistant cells and macrophages influence tumorigenesis; then p15E probably does not influence macrophage cytotoxicity of the SV40-transformed targets used in our studies.

In conclusion, neither env gene proteins studied, $gp70^a$ and p15E, appeared to be inhibitory to macrophage cytotoxicity (Tables 3 and 4), even though the expression of $gp70^a$ correlated with decreased killing (Table 1, Figures 1 and 2), and binding (Chapes et al. 1988a). It is possible that membrane bound $gp70^a$ has different effects on macrophages than affinity purified $gp70^a$. Fusion of $gp70^a$- containing membrane fragments into F5b cells decreased their killing by macrophages (Figures 2 and 3). However, other membrane molecules, not yet defined, may have been responsible for the effect. These findings are consistent with recent data of Laster et al. (1988). They found that resistant cells related to F5m (Chapes et al. 1987) secrete substances that "incapacitate" macrophages. Therefore, our observations may be explained by those inhibitory molecule(s). Inhibitory molecule(s) may have been absorbed by F5b cells when they were cultured in F5m culture supernatants or were fused with F5m membranes. Studies characterizing the nature of F5m resistance are currently underway in our laboratory to address this hypothesis.

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Macrophage Binding of Cells Resistant and Sensitive to Contact-Dependent Cytotoxicity[1]

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We compared macrophage binding and killing of F5b cells to the binding and killing of P815 mastocytoma cells and to several other nontransformed and transformed cell lines. Formalin fixation of elicited or activated macrophages did not affect binding of F5b or 3T3 cells but did abrogate binding of P815 cells. However, formalin fixation abrogated resident macrophage binding of F5b and 3T3 cells. Therefore, depending on the type of macrophage or target cell, formalin fixation may affect binding. Only the binding of P815 cells was dependent upon activation; macrophage binding of target cells F5b and 3T3 was not. Even though macrophages bound F5b and 3T3 cells, macrophages only mediated contact-dependent cytotoxicity against F5b cells. Macrophages did not kill 3T3 cells. Experiments also compared macrophage binding and killing of the uv-light-induced tumor cell lines 1422, 2237, and 2237a46. Only the cell line 2237a46 was susceptible to contact-dependent killing. Both 1422 and 2237 cells were resistant. In contrast, cell lines 2237a46 and 1422 were bound by activated macrophages while 2237 cells were bound poorly. © 1991 Academic Press, Inc.

INTRODUCTION

Activated macrophages (MPs)[4] kill tumor cells but not normal cells through a contact-dependent process. The interaction is said to involve at least two steps: selective binding of tumor cells to the surface of activated MPs and secretion of cytolytic substances (2). Binding can be subdivided into "strong" and "weak" binding (19). Weak binding has no metabolic requirements, is not temperature sensitive, does not require trypsin-sensitive structures for binding, and does not require cations (18). The strength of weak binding is less than 16 μdyn per cell (2, 19). On the other hand, strong ("specific") binding is temperature sensitive, requires metabolic activity, cations, and trypsin-sensitive structures for binding. Strong binding is characterized by binding

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[4] Abbreviations used: MP, macrophage; PA, *Propionibacterium acnes;* LPS, lipopolysaccharide.

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strengths of greater than 240 μdyn/cell (binding that withstands 1300*g* disruptive forces) (2, 18, 19). Most studies on the binding of MPs to tumor cells have characterized the interaction between activated MPs and nonadherent tumor cells such as P815 (2, 15, 18, 19). Only a few investigations have attempted to establish the characteristics of MP–tumor binding to adherent cells (6, 8, 9, 20).

How MPs discriminate between cells they kill and cells not killed is poorly understood. Adams *et al.* found that strong binding of P815 and EL-4 tumor cells by C3H/HeJ MPs was not followed by cytolysis due to defects in the ability to release cytolytic products (1). Recently, we demonstrated that activated MPs, fixed with 1% formalin, bind at least two adherent tumor cell lines comparably to viable, activated MPs (8). These data, along with other studies (15) have led us to question the role strong binding plays in contact-dependent cytolysis of adherent cells. Molecular interactions other than binding may be necessary to stimulate the MP to become cytotoxic. The objectives of these experiments were to determine if: (i) there was a difference in macrophage binding of adherent F5b and nonadherent P815 tumor cells, (ii) activated or elicited macrophages bind cells differently, and (iii) there is a cause and effect relationship between strong MP binding of a target cell and macrophage killing of said cell.

MATERIALS AND METHODS

Animals. Peritoneal MPs from C3H.OL and C3H/HeJ mice were used. Mice were bred and maintained in our animal colony in the Division of Biology at Kansas State University.

Cell lines. LM929 and 3T3 cells were purchased from the American Type Culture Collection. F5b and F5m are SV40-transformed fibroblast cell lines that have been described previously to be sensitive (F5b) or resistant (F5m) to contact-dependent cytotoxicity (6). P815 was obtained from Drs. Sam Fan and Dolph Adams (Duke University). The ultraviolet-light-induced tumor cells 2237, 2237a46, and 1422 (10) were obtained from Dr. William Fortner (Kansas State University). All cell lines except LM929 were insensitive to lysis by soluble monokines, like tumor necrosis factor-α (TNF), produced by activated MPs ((6, 14, 15) and Table 6). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (Hazelton Research Products, Lenexa, KS), 0.3% L-glutamine, and 10% Opti-MEM (GIBCO, Grand Island, NY). All cell lines were passaged thrice weekly. On the day prior to use, cells were seeded into 60- or 100-mm tissue culture plates at 1 or 2×10^6, respectively. The cells were labeled with 100 or 150 μCi of $Na_2\ ^{51}CrO_4$ for 60- and 100-mm plates, respectively.

Macrophages. Peritoneal MPs were elicited with an ip injection of Brewer's thioglycollate broth. Macrophages were activated *in vivo* by ip injection of thioglycollate along with 700 μg *Propionibacterium acnes.* Macrophages were harvested by peritoneal lavage with phosphate-buffered saline (PBS) 4 to 5 days after injection and used the day of lavage unless stated otherwise. The percentage of MPs was determined by differential staining. Elicitation of MPs with thioglycollate yielded greater than 90% MPs. Injection of *P. acnes* and thioglycollate yielded approximately 80% MPs. Peritoneal lavage of unstimulated mice yielded approximately 45% MPs.

Macrophage assays. The MP cytotoxicity assay has been described in detail previously (7). Briefly, MPs were seeded in 96-well microtiter plates. Following MP attachment (1.5–2.0 hr) the plates were washed twice with buffer to remove nonadherent

cells. After being washed, ^{51}Cr-labeled targets were added and incubated at 37°C for 16 to 18 hr. Following incubation an aliquot of the supernatant was taken and counted in a gamma counter. The percentage of targets lysed in the presence of MPs was determined as follows:

$$\% \text{ Killing} = \frac{(\text{cpm of target} + \text{MP}) - (\text{cpm of target only})}{(\text{total cpm added to each well}) - (\text{cpm of target cell only})} \times 100.$$

The MP binding assay also has been described previously (6, 8). In brief, MPs were pipetted into polyvinyl chloride microtiter plates. Following MP adhesion, the plates were washed twice to remove nonadherent cells. Subsequently, ^{51}Cr-labeled targets were added and then plates were sealed with an adhesive plastic film and incubated at 37°C for either 1.5 or 3 hr. After incubation the plates were inverted and centrifuged at 1300*g*, and then the plates were cut with a hot wire and the individual wells counted on a gamma counter. Centrifugation (1300*g*) was used to denote specific or strong binding of cells as opposed to nonspecific or weak binding (19). The MPs were either used viable or fixed. If used fixed, the MPs were fixed with 1% formalin for 20 min at room temperature. In both assays MPs were seeded at 2 or 3×10^5 cells per well and targets were seeded at 1×10^4 cells per well resulting in 20:1 and 30:1 effector to target cell ratios. The percentage of target cells bound by MPs was determined as follows:

$$\% \text{ Binding} = \frac{(\text{cpm of target} + \text{MP}) - (\text{cpm of target only})}{\text{total cpm added to each cell}} \times 100.$$

This calculation substracted out nonspecific binding of target cells to the plastic which was generally less than 10%.

Experimental rationale. Four experiments were conducted to address our aforementioned objectives. Experiment 1 determined the effect of formalin (1%) fixation and sodium borohydride (0.05%) on macrophage binding of F5b on the day of harvest and 6 days later. MPs were fixed with either 0 or 1% formalin (diluted in PBS) for 20 min. Subsequently, one-half of each group was treated with 0.05% sodium borohydride (diluted in PBS) and the others were treated with PBS. Sodium borohydride reduces free aldehydes (3) and should abrogate nonspecific binding. Cells were treated for 1 hr at 4°C. Following incubation, MPs were washed twice with warm PBS. Two microtiter plates were prepared. One was used immediately and one was stored at 4°C and used 6 days later. The macrophages in the stored plate were incubated in Hepes–Hanks buffer at pH 7.2. The plate was sealed with adhesive film to maintain a neutral buffer pH.

Experiment 2 was conducted to compare the binding and killing of the adherent tumor cell line (F5b) to the nonadherent tumor cell line (P815). Binding was evaluated by using viable and fixed macrophages that were or were not activated with LPS. Killing was evaluated with viable, nonactivated or LPS-activated MPs. Experiment 3 determined the binding and killing potential of many MP types. Macrophages from two strains of mice were used. Macrophages from mice C3H/HeJ are not responsive to LPS and *P. acnes*. Macrophages from C3H.OL mice are capable of being activated both *in vivo* and *in vitro*. We directly compared several viable and fixed macrophage types for binding and killing of F5b and 3T3 cells. In these experiments, we determined whether the divalent cations were required for binding by including EDTA in the

binding assay. The final experiment (4) was designed to confirm whether binding and killing were directly related using uv-light-induced adherent tumor cell lines.

RESULTS

Characteristics of formalin-fixed macrophage binding. Our laboratory demonstrated that formalin-fixed MPs bind viable tumor cells (8). It has been suggested that fixed MPs secrete or release IL-1 and fixation may not completely inactivate MPs (17). Furthermore, fixation may produce free aldehyde groups that cause nonspecific sticking of proteins or cells to fixed MPs. Therefore, to confirm that MPs were physiologically inactive, we studied MP binding of F5b cells. Neither formalin fixation nor sodium borohydride treatment decreased MP binding. However, the storage of viable MPs at 4°C for 6 days did decrease their binding potential, but storage did not affect fixed MP binding of F5b (Table 1). We note that the monolayer of viable MPs remained reasonably intact over the period of 6 days and that the decrease in binding was not due to the detachment of MPs. Therefore, fixed MP binding does not appear to be due to residual physiological function nor to nonspecific sticking caused by formalin-induced changes.

Comparison of macrophage binding of P815 with F5b. Most data on MP binding of tumor cells have dealt with nonadherent targets, specifically the P815 mastocytoma cell line. P815 cells have not been directly compared to F5b cells. Since the binding characteristics of F5b (6, 8) appeared to be different from those described for P815 (2, 18, 19), it was of considerable interest to directly compare MP binding of F5b with P815 (Table 2). The binding of F5b by fixed MPs was not dramatically different from the binding by viable MPs, regardless of whether MPs are activated. When we examined the binding of P815 by MPs we found that only activated, viable MPs would bind P815 cells. The amount of binding was significant but it was comparatively less than

TABLE 1

Effect of Formalin and Sodium Borohydride on Macrophage Binding of F5b Cells

		Percentage targets bound	
		Day of use[a]	
Macrophage[b] treatment	Sodium[c] borohydride	0	6
Viable	–	32 ± 1[d]	4 ± 0
	+	27 ± 1	6 ± 1
1% Formalin	–	33 ± 3	25 ± 1
	+	37 ± 2	24 ± 1

[a] Two × 10^5 thioglycollate-elicited macrophages were plated per well and used viable or fixed with 1% formalin. Macrophages were set up identically on two plates. The first plate was used in a 3-hr binding assay on the day of collection (Day 0) and the other plate was used 6 days later in a 3-hr binding assay. The second plate was kept at 4°C prior to use as described under Materials and Methods. Targets were seeded at 1 × 10^4 for a final macrophage to target ratio of 20:1.

[b] Macrophages fixed with 1% formalin diluted in PBS for 20 min.

[c] 0.05% sodium borohydride in PBS.

[d] Numbers represent $\bar{X}$ ± SEM of quadruplicate samples from a representative experiment.

TABLE 2

Macrophage Binding and Cytotoxicity of F5b and P815 Tumor Cells

Macrophage treatment[a]	Target[b]	Percentage targets bound[c]		Percentage targets killed
		Viable	Fixed	
−LPS	F5b	34 ± 1	31 ± 0	0 ± 6
+LPS	F5b	39 ± 2	38 ± 1	56 ± 13
−LPS	P815	6 ± 1	3 ± 1	0 ± 2
+LPS	P815	22 ± 1	4 ± 1	55 ± 5

[a] Macrophages were incubated ±12.5 μg/ml LPS for 12 hr prior to the addition of target cells.

[b] One × 10^4 target cells were added per well for binding and cytotoxicity assays. Macrophage to target ratio was 20:1.

[c] One and one-half hour binding assay.

[d] Numbers represent $\bar{X}$ ± SEM of quadruplicate (binding assay) or triplicate (cytotoxicity assay) samples from a representative experiment.

F5b. Neither thioglycollate-elicited nor formalin-fixed, activated MPs bound P815 cells. When we assayed the viable MPs for cytotoxicity, only activated MPs would kill F5b and P815 cells (Table 2). Therefore, the interactions between MPs, P815, and F5b are different.

Tumor cell binding and killing by resident, elicited, and activated macrophages. The ability of MPs to discriminate tumorigenic from nontumorigenic cells has been established. Since MP binding of F5b cells was different from P815 cells, even if both cell types were susceptible to contact-dependent cytolysis, we wanted to characterize MP binding of cells that are resistant to MP cytotoxicity. We compared the ability of various types of MPs to bind both F5b tumor cells and 3T3, an immortalized but nontransformed cell line that is not killed by activated MPs (Table 3; Ref. (14)). Viable MPs elicited by thioglycollate or activated by LPS *in vitro* or by *P. acnes in vivo* bound both F5b and 3T3 cells. Formalin fixation of any of these MPs did not appear to inhibit binding. Viable, thioglycollate-elicited or *P. acnes*-activated MPs from C3H/HeJ mice also bound F5b and 3T3 cells, but did not kill F5b cells (Table 3). Therefore, binding of thioglycollate-elicited MPs from C3H.OL mice was not due to contaminating LPS in our assay medium activating the thioglycollate-elicited MPs.

The binding of F5b cells to viable, resident MPs resembled that of the other kinds of MPs (Table 3). However, the binding of 3T3 cells to viable, resident MPs was considerably less (Table 3) than that of viable, thioglycollate-elicited MPs, which also were not cytotoxic (Table 3). Interestingly, when resident MPs were fixed with formalin, we found that binding of both F5b and 3T3 cells was significantly reduced. These data suggest that binding structures on resident MPs may be different than those on inflammatory MPs; they appear to be sensitive to formalin treatment.

Effect of EDTA on macrophage binding of 3T3 cells. Formalin fixation did not affect the binding of F5b or 3T3 cells to either thioglycollate-elicited or activated MPs. Since it has been suggested that divalent cations are required for specific binding (18) and might be the determining factor in triggering cytolysis, we tested whether EDTA would affect the binding of F5b differently from the binding of 3T3 cells. The data in Table 4 indicate that binding of both F5b and 3T3 cells by either thioglycollate-elicited or

TABLE 3

Effect of Macrophage Type on Binding of F5b and 3T3

Type of macrophage[a]	Percentage targets bound[b]		Percentage targets killed[c]	
	F5b	3T3	F5b	3T3
C3H/HeJ				
Thio viable	34 ± 8[c]	34 ± 7	0 ± 2	4 ± 1
Thio + PA viable	37 ± 6	23 ± 6	0 ± 1	8 ± 2
C3H.OL				
Thio viable	34 ± 4	32 ± 6	6 ± 2	8 ± 1
Thio fixed	30 ± 5	30 ± 12	NT[d]	NT
Thio + LPS viable	20 ± 5	24 ± 3	31 ± 4	3 ± 1
Thio + LPS fixed	27 ± 8	38 ± 16	NT	NT
Thio + PA viable	31 ± 5	18 ± 7	19 ± 2	7 ± 1
Thio + PA fixed	25 ± 13	21 ± 5	NT	NT
Resident viable	30 ± 7	13 ± 3	5 ± 2	6 ± 1
Resident fixed	8 ± 1	5 ± 5	NT	NT

[a] Macrophages elicited by intraperitoneal injection of thioglycollate (Thio) broth ± 700 μg *P. acnes* (PA). Macrophages activated *in vitro* with 12.5 μg per milliliter LPS.

[b] One × 10^4 target cells added per well for cytotoxicity and binding assays. Macrophage to target ratio was 20:1.

[c] Numbers represent the $\bar{X}$ ± SEM of triplicate samples from a representative experiment.

[d] Not tested.

P. acnes-activated MPs was disrupted by over 50% in the presence of EDTA. This suggests that divalent cations are required for the binding of both adherent cell types.

Macrophage binding and cytotoxicity of uv-light-induced tumor cells. We screened a panel of three ultraviolet-light-induced tumor cells and found differences in their ability to be killed by activated MPs (Table 5). These cell lines are not susceptible to killing by soluble monokines (Table 6). We find that the uv tumor cell lines 2237 and

TABLE 4

Effect of EDTA on Macrophage Binding of F5b and 3T3 Cells

Target[a]	Macrophage[b]	Percentage No. targets bound	
		Buffer[c]	EDTA[c]
F5b	Thio	28 ± 3[d]	3 ± 2 (88)[e]
F5b	Thio + PA	25 ± 6	6 ± 3 (76)
3T3	Thio	18 ± 2	5 ± 4 (71)
3T3	Thio + PA	24 ± 7	5 ± 4 (81)

[a] One × 10^4 Target cells added per well. Macrophage to target ratio was 20:1.

[b] Macrophages elicited by intraperitoneal injection of thioglycollate (Thio) broth ± 700 μg *P. acnes* (PA).

[c] Binding assay was done in the presence or absence of 2.5 m*M* EDTA.

[d] Numbers represent $\bar{X}$ ± SEM of three experiments.

[e] Number in parentheses is percentage of inhibition. It is calculated 1− (percentage of cells bound in the presence of EDTA ÷ percentage of cells bound in the absence of EDTA) × 100.

TABLE 5

Comparison of LPS-Activated Macrophage Binding and Cytotoxicity between uv-Light-Induced Tumor Cells and SV40-Transformed Tumor Cells

	Percentage targets		
	Bound	Killed	
Target[a]	(MP:T) 20:1	20:1	30:1
F5b	22 ± 2[b]	20 ± 6	35 ± 5
F5m	12 ± 3	3 ± 3	7 ± 7
2237	14 ± 2	5 ± 5	1 ± 2
2237a46	22 ± 7	15 ± 6	29 ± 15
1422	24 ± 3	1 ± 1	1 ± 1

[a] One × 10^4 target cells added per well. Macrophages elicited by intraperitoneal injection of thioglycollate. Macrophages were activated *in vitro* with 12.5 μg *Escherichia coli* LPS (055:B5). Macrophage to target ratio of 20:1 or 30:1.

[b] Numbers represent $\bar{X}$ ± SEM of four experiments.

1422 are resistant to contact-dependent killing (Table 5). They are as resistant to killing as the SV40-transformed cell, F5m, that we have described previously (5, 6, 8). However, a variant of 2237, 2237a46, was almost as susceptible to cytotoxicity as F5b cells (Table 5). When we examined the binding of these cells by activated MPs

TABLE 6

Tumor Necrosis Factor Lysis of uv-Light-Induced Tumor Cells

			Percentage targets killed[a]			
Treatment[a]	Target	Supernatant dilution:	1:2	1:4	1:8	1:16
Medium	LM929		9 ± 2[b]	4 ± 1	3 ± 1	0 ± 3
Medium	F5b		7 ± 3	7 ± 3	3 ± 2	9 ± 1
Medium	F5m		11 ± 3	8 ± 2	11 ± 3	8 ± 1
Medium	1422		8 ± 1	10 ± 1	7 ± 1	8 ± 2
Medium	2237		1 ± 4	0 ± 2	0 ± 2	0 ± 1
Medium	2237a46		8 ± 1	8 ± 1	6 ± 1	8 ± 0
TNF	LM929		53 ± 4	26 ± 2	14 ± 2	2 ± 2
TNF	F5b		10 ± 0	11 ± 1	9 ± 1	10 ± 1
TNF	F5m		1 ± 2	7 ± 0	9 ± 2	3 ± 3
TNF	1422		8 ± 1	9 ± 2	6 ± 1	6 ± 1
TNF	2237		0 ± 0	0 ± 2	0 ± 3	0 ± 1
TNF	2237a46		9 ± 1	8 ± 0	8 ± 0	8 ± 1

[a] Target cells treated with supernatants from LPS-responsive macrophages, incubated overnight in the presence (TNF) or absence (medium) of 12.5 μg/ml *E. coli* (055:B5) LPS.

[b] Numbers represent $\bar{X}$ ± SEM of triplicate samples.

[c] Percentage specific ^{51}Cr release determined in a 16-hr assay as described for activated macrophage cytotoxicity.

we found that 1422 cells were bound as well as 2237a46 even though they were not killed. In contrast, the 2237 cells were bound poorly, showing binding analogous to F5m cells which have been found to be bound poorly by MPs when compared to F5b (6, 8). Therefore, the lack of cytotoxicity of some tumor cells (e.g., 1422) is not due to the lack of strong binding by activated MPs.

DISCUSSION

We compared MP binding of P815 cells to the binding of F5b cells. Previous studies found that MPs must be metabolically active and activated in order to bind P815 cells (19). We obtained similar results. Interestingly, MPs do not have the same requirements for binding F5b cells. Both cell lines are resistant to the effects of soluble monokines, like TNF-α, but both may be killed by activated MPs by a mechanism apparently dependent upon contact. Neither cell is killed by unactivated MPs. Differences between these two cell lines are that P815 cells are nonadherent and have a leukocyte (mastocytoma) origin. F5b cells are fibroblasts in origin and are adherent cells. It does not appear that the ability to adhere affects MP binding since we have found several adherent cell lines that are bound poorly by MPs when compared to F5b cells. These include VERO cells (8), F5m cells (Ref. (8) and Table 5), and the uv-light-induced tumor cell 2237 (Table 5).

An alternative model by which macrophages and tumor cells can bind is an important finding. However, many unknown molecular differences exist between P815 and F5b, and it will take considerable biochemical and molecular analysis to determine how those differences contribute to binding. The F5b cell line was originally identified as a unique clone of the F5 cell line (5). F5b differed from other F5 clones (e.g., F5m and F5f) in the expression of the AKR-specific gp70^a, in sensitivity to contact-dependent MP cytotoxicity, in binding to activated MPs, and in the ability to affect MP migration (5, 6, 8, 13). Therefore, the differences that exist between P815, F5b, and "resistant" clones such as F5m make the F5b cell an excellent model in which to study tumor cell recognition and control.

The experiments presented here establish that the resistance of several cell lines to contact-dependent cytotoxicity is not due to a defect in binding by MPs. We found that 3T3 cells and 1422 tumor cells are not killed yet they are bound by MPs comparably to cells that are susceptible to killing (e.g., F5b, 2237a46).

Our results confirm and tie together the findings of others. Adams *et al.* (1, 16) found that MPs will bind to targets without killing them. The MPs used in those studies had defects in their ability to secrete lytic mediators. Hamilton and Fishman (11, 12) found that activated MPs could recognize both normal and tumor cells. Their studies defined recognition by the ability of normal cells to competitively inhibit killing of tumor cells. They did not establish that binding occurred between resistant targets and MPs. Our studies establish that binding does occur between resistant cells and MPs. Furthermore, our macrophages could secrete lytic mediators yet they still did not kill resistant targets. The data *in toto* support the hypothesis that MP binding of cells occurs normally and is not the sole process that determines whether killing will occur. Apparently, other molecular interactions in addition to binding between the target and the MP must occur. Virus infection of cells appears to provide the necessary requirements (4, 14).

Thioglycollate-elicited MPs were able to bind target cells similarly to activated MPs. However, binding was not universal to all MPs. We found that viable, resident MPs

could bind F5b tumor cells and to a lesser extent, 3T3 cells; however, binding was abrogated by fixation. Fixation also affected the binding of P815 cells. The data suggest that several classes of binding structures may exist on MPs. Some are formalin-resistant. Alternatively, the observation may be due to quantities and/or distribution of binding structures expressed on different macrophages and tumor cells.

Formalin fixation can produce free aldehyde groups that promote nonspecific adherence. However, in experiments where fixed MPs were treated with chemicals that reduce aldehydes, tumor cell binding was still high. We previously found that target cell binding by formalin-fixed MPs was dependent upon trypsin-sensitive structures on fixed MPs (8). Together, the data suggest that fixed MP binding is not due to nonspecific sticking of target cells.

In conclusion, the data presented in this manuscript lead us to question the role strong binding plays in contact-dependent cytolysis of adherent cells by fully activated macrophages. Although strong binding or close contact may be required, molecular interactions between the macrophage and tumor cell in addition to binding appear to contribute to the cytolytic process.

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Effects of Extracellular Matrix Proteins on Macrophage Differentiation, Growth, and Function: Comparison of Liquid and Agar Culture Systems

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ABSTRACT Both spaceflight and skeletal unloading suppress the haematopoietic differentiation of macrophages (Sonnenfeld et al., *Aviat. Space Environ. Med.*, 61:648–653, 1990; Armstrong et al., *J. Appl. Physiol.*, 75:2734–2739, 1993). The mechanism behind this reduction in haematopoiesis has yet to be elucidated. However, changes in bone marrow extracellular matrix (ECM) may be involved. To further understand the role of ECM products in macrophage differentiation, we have performed experiments evaluating the effects of fibronectin, laminin, collagen type I, and collagen type IV on macrophage development and function. Bone marrow–derived macrophages cultured on four different ECM substrates in liquid culture medium showed less growth than those cultured on plastic. Significant morphological differences were seen on each of the substrates used. Phenotypically and functionally, as measured by class II major histocompatibility molecule (MHCII) expression, MAC-2 expression, and the secretion of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), these macrophages were similar. In contrast, bone marrow–derived macrophages cultured in suspension, using agar, showed no difference in growth when exposed to ECM proteins. However, IL-6 and TNF-α secretion was affected by fibronectin, laminin, collagen type I, and collagen type IV in a concentration-dependent manner. We conclude that the ECM products fibronectin, laminin, collagen type I, and collagen type IV have profound effects on macrophage development and function. Additionally, we suggest that an ECM-supplemented agar culture system provides an environment more analogous to in vivo bone marrow than does a traditional liquid culture system. © 1994 Wiley-Liss, Inc.

Spaceflight suppresses haematopoietic differentiation of macrophages (Sonnenfeld et al., '90, '92) and red blood cells (Vacek et al., '83) and decreases circulating blood monocyte numbers (Taylor et al., '86). Furthermore, simulated spaceflight, using antiorthostatic suspension, also inhibits macrophage development (Armstrong et al., '93; Sonnenfeld et al., '92). The mechanism behind this suppressed haematopoiesis has yet to be elucidated. We are currently investigating two potential mechanisms that may contribute to macrophage hypodevelopment during skeletal unloading and spaceflight. First, haematopoiesis may be influenced by skeletal unloading due to changes in cytokine levels within the bone marrow microenvironment. Second, changes in extracellular matrix (ECM) composition of bone marrow following skeletal unloading may alter macrophage development. The ECM has been postulated to be a gravity-sensitive biological component (Spooner, '92; Ingber, '92) and spaceflight may have direct or indirect effect on it and haematopoiesis.

Macrophages develop from haematopoietic progenitor cells following exposure to environmental and chemical signals (Rutherford et al., '93; Koenigsmann et al., '92). ECM products such as collagen, laminin, and fibronectin contribute to progenitor cell development by providing both these influences and are important in the localization of macrophage progenitor cells in bone marrow (Weinstein et al., '89; Koenigsmann et al., '92). Therefore, any changes in ECM levels in bone marrow or the bone environment, such as occurs with collagen following spaceflight (Spengler et al., '83; Turner et al., '83), may alter macrophage differentiation and function (Kaplan, '83; Sporn et al., '90). The experiments presented in this study focus on the role of ECM products in macrophage development using bone marrow from normal animals. Such data are important for understanding

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the relationships between the ECM, haematopoiesis, and spaceflight.

Often macrophage development is studied in vitro using liquid culture systems on a plastic substrate; however, this environment has few similarities with the three-dimensional ECM-rich structure of bone marrow. Therefore, we have compared the effects of the ECM products collagen I, collagen IV, laminin, and fibronectin on macrophage development in both a liquid culture system and a three-dimensional agar culture system. We report ECM-dependent differences in macrophage development and function using these two culture systems.

MATERIALS AND METHODS

Animals

Male and female adult (8–12 weeks), C3HeB/FeJ mice, bred in the animal facility at Kansas State University, were used in these studies. The use of the animals was approved by the Animal Care and Facilities Use Committee at Kansas State University, which complies with NIH Animal Care standards.

Extracellular matrix products

The soluble ECM products, human fibronectin, mouse collagen type IV, rat tail collagen type I, and mouse laminin were obtained from Collaborative Biomedical Products (Bedford, MA). These ECM products were used at concentrations ranging from 1 to 10 μg/ml. The purity, as determined by SDS-PAGE by the manufacturer, was >85%, 98.2%, 90.3%, and >85% for fibronectin, collagen type IV, collagen type I, and laminin, respectively. All tissue culture microplates coated with ECM molecules were obtained from Collaborative Biomedical Products.

Bone marrow cells

Bone marrow cells were obtained from the femora and tibiae of mice. The ends of the femora and tibiae were removed and the cells were flushed from the bone using Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and a 26-gauge needle. Cells were passed three times through a 19-gauge needle to break up cell clumps, pelleted at 325*g*, and resuspended in ice-cold 0.17 M NH_4Cl for 5 minutes to lyse red blood cells. Cells were pelleted again and resuspended in the appropriate media for cell culture.

Proliferation assays for macrophages in liquid cultures

Five × 10^5 bone marrow cells, in 1.5 ml of DMEM supplemented with 10% FBS and 15% LM929 supernatant [macrophage colony stimulating factor (CSF-1) source], were seeded per 30 mm well in six-well microplates (Collaborative Biomedical Products). Following 3 days of culture, 1.5 ml of fresh medium was added and on day 6 of culture, non-adherent cells and medium were removed, and 1.5 ml of fresh medium was added together with 1 ml of 1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan (MTT; Ferrari et al., '90). Following a 3-hour incubation, the medium-MTT solution was removed and MTT formazan crystals were solubilized using a solution of 66% v/v isopropanol, 33% v/v PBS, 7 × 10^{-5}% v/v 5 M HCl. Relative proliferation was then quantified by absorbance at 570 nm.

Proliferation assays for macrophages in agar cultures

Bone marrow cells were suspended at a concentration of 1 × 10^5 cells per 1.5 ml of DMEM containing 0.3% agar, 10% FBS, and 15% LM929 fibroblast-conditioned medium. Every 24 hours, five microscope view fields were scored for macrophage colonies (a group of >25 cells).

Flow cytometric analysis

All steps in this procedure were performed on ice. Bone marrow–derived macrophages (day 6 of culture) were removed from 30 mm wells by a 15-minute EDTA incubation followed by a high-pressure wash with a Pasteur pipet. One × 10^6 cells were added per well to a 96-well, round-bottomed plate. The plate was centrifuged at 400*g* for 2 minutes and supernatants were discarded. Cells were incubated in 50 μl of FBS per well to inhibit nonspecific Fc receptor binding, washed twice with sorter buffer (Hank's balanced salt solution containing 2% FBS and 0.2% BSA), and incubated with 50 μl of the appropriate primary antibody per well [anti-I-A^k {anti-class II major histocompatibility molecules, MHCII} and anti-MAC-2 were derived from the American Type Culture Collection (ATCC, Rockville, MD) hybridoma cells lines TIB94 and TIB166, respectively]. Following the primary antibody incubation, cells were washed twice in sorter buffer and incubated with secondary antibody [fluoresceinated goat anti-mouse $F(ab')_2$; Cappel, Durham, NC] for 30 minutes. Cells were washed twice in sorter buffer,

resuspended in 300 µl of PBS containing 1% paraformaldehyde, and stored at 4°C until flow cytometric analysis (FACScan, Becton-Dickinson, MA).

Cytokine assays

The secretion of interleukin-6 (IL-6) by bone marrow–derived macrophages grown in liquid or agar cultures for 6 days was quantified in triplicate using the IL-6-dependent cell line, B9. Diluted supernatants or rIL-6 standards were added to 3×10^3 B9 cells/well in 96-well plates for 3 days. IL-6 levels were then quantified using the above described MTT procedure. Tumor necrosis factor-α (TNF-α) was quantified using triplicate samples of culture supernatants as described previously using the TNF-α-sensitive cell line LM929 (Fleming et al., '91).

Statistical analysis

Student's *t*-tests were used to determine statistical significance. A 95% level of significance was used for each test.

RESULTS

The effect of ECM molecules on the proliferative rate and morphology of bone marrow–derived macrophages in liquid culture

To determine the effects of ECM molecules on macrophage development bone marrow–derived macrophages were cultured on dishes pre-coated with either collagen type I, collagen type IV, laminin, or fibronectin. All grew significantly ($P < 0.05$) slower than macrophages cultured on plastic substrate (Fig. 1). However, the slower macrophage proliferation was similar on culture dishes treated with collagen type I, collagen type IV, laminin, or fibronectin. Although macrophage growth rates on various ECM were similar, Figure 2 shows that the morphology of those macrophages [as identified by non-specific esterase stain (α-Naphthyl acetate esterase kit, Sigma, St. Louis, MO)] on each of the five substrates varied dramatically. Morphological changes induced by a 24-hour treatment with 10 U/ml interferon-γ, (IFN-γ, Genzyme, Cambridge, MA) and 12.5 µg/ml lipopolysaccharide (LPS; Difco, Detroit, MI) varied also, depending on the culture substrate (Fig. 2). Cells cultured on collagen type IV or laminin showed very little change following stimulation. However, macrophages cultured on either plastic, collagen type I, or fibronectin showed a retraction of cellular processes following IFN-γ and LPS treatment.

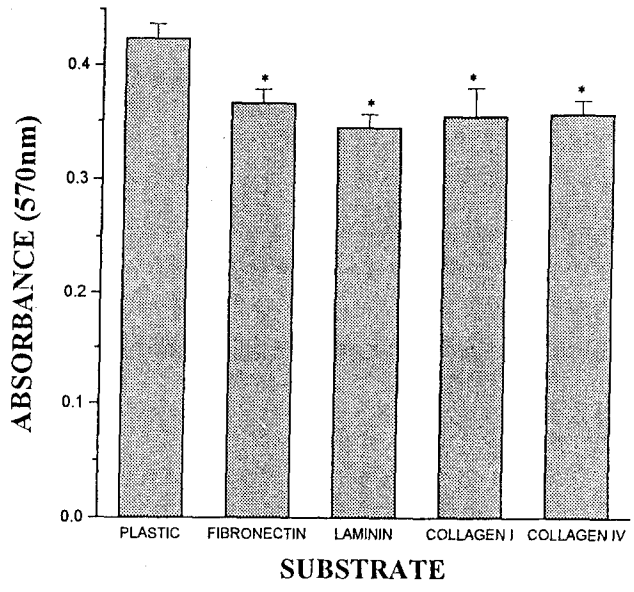


Fig. 1. The effect of fibronectin, laminin, collagen type I, and collagen type IV on bone marrow–derived macrophage proliferation in liquid cultures. Bone marrow cells from four mice were pooled and placed into culture for 6 days in the presence of CSF-1. Measurements were performed in hexad using an MTT assay (*$P < 0.05$ compared to plastic). One representative experiment is shown.

Phenotypic analysis of bone marrow–derived macrophages cultured on ECM supplemented substrates

Bone marrow–derived cells were identified as macrophages by non-specific esterase activity (Fig. 2), MHCII expression, MAC-2 expression, (Fig. 3), adherence and a dependence on macrophage colony stimulating factor. Figure 3 shows MHCII expression and the expression of the macrophage-specific marker MAC-2 on cells cultured on all five substrate types. The level of expression of these molecules was not significantly different between the five culture

Fig. 2. Photomicrographs (×400) of bone marrow–derived macrophages cultured on either fibronectin, laminin, collagen type I, or collagen type IV. Photomicrographs were taken on day 6 of culture following staining for non-specific esterase activity. Cells were either untreated or treated with IFN-γ (10 U/ml) and LPS (12.5 µg/ml) for 24 hours prior to staining and fixation. Photomicrographs were taken using a Zeiss Axiophot microscope (Zeiss Inc., West Germany).

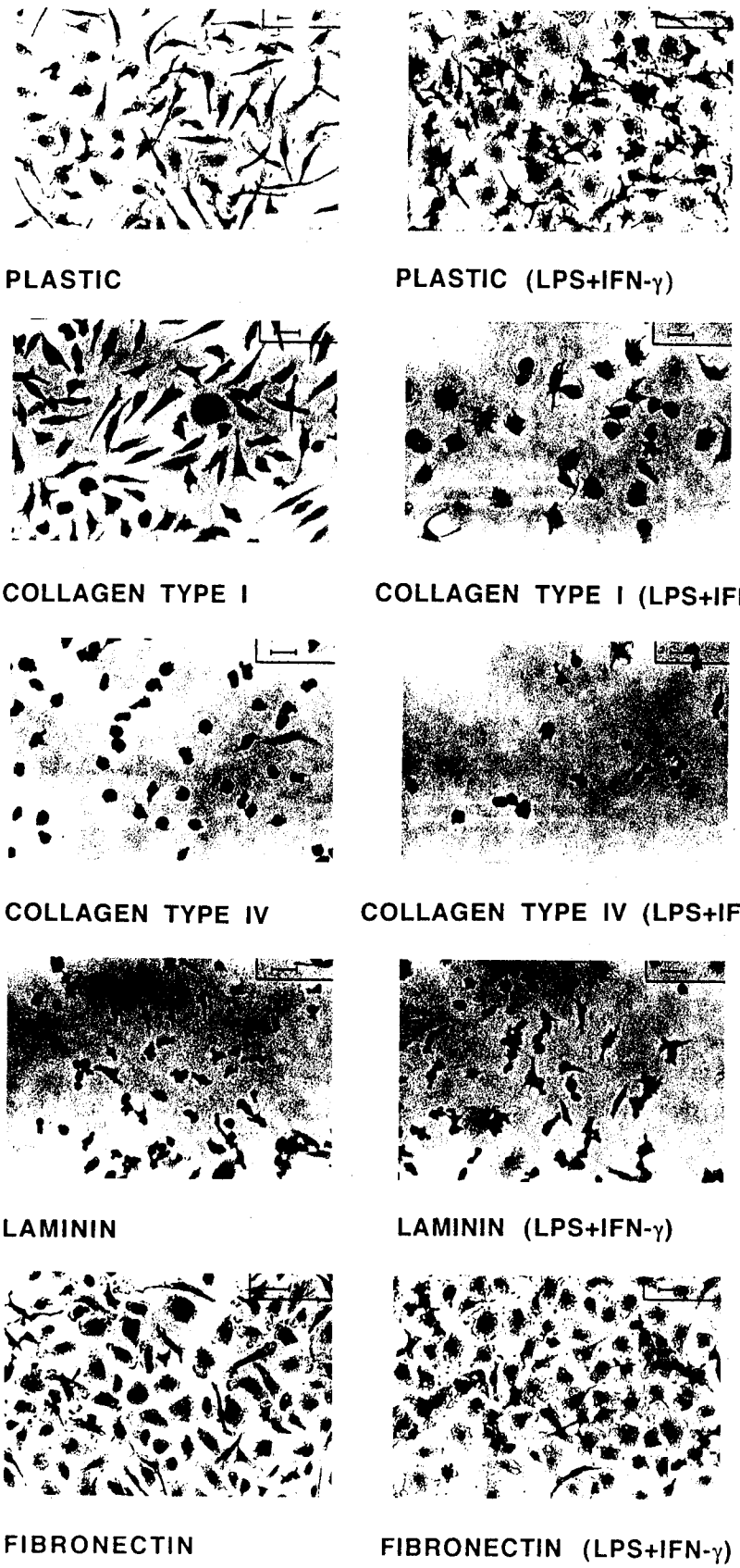

PLASTIC
PLASTIC (LPS+IFN-γ)
COLLAGEN TYPE I
COLLAGEN TYPE I (LPS+IFN-γ)
COLLAGEN TYPE IV
COLLAGEN TYPE IV (LPS+IFN-γ)
LAMININ
LAMININ (LPS+IFN-γ)
FIBRONECTIN
FIBRONECTIN (LPS+IFN-γ)

Figure 2.

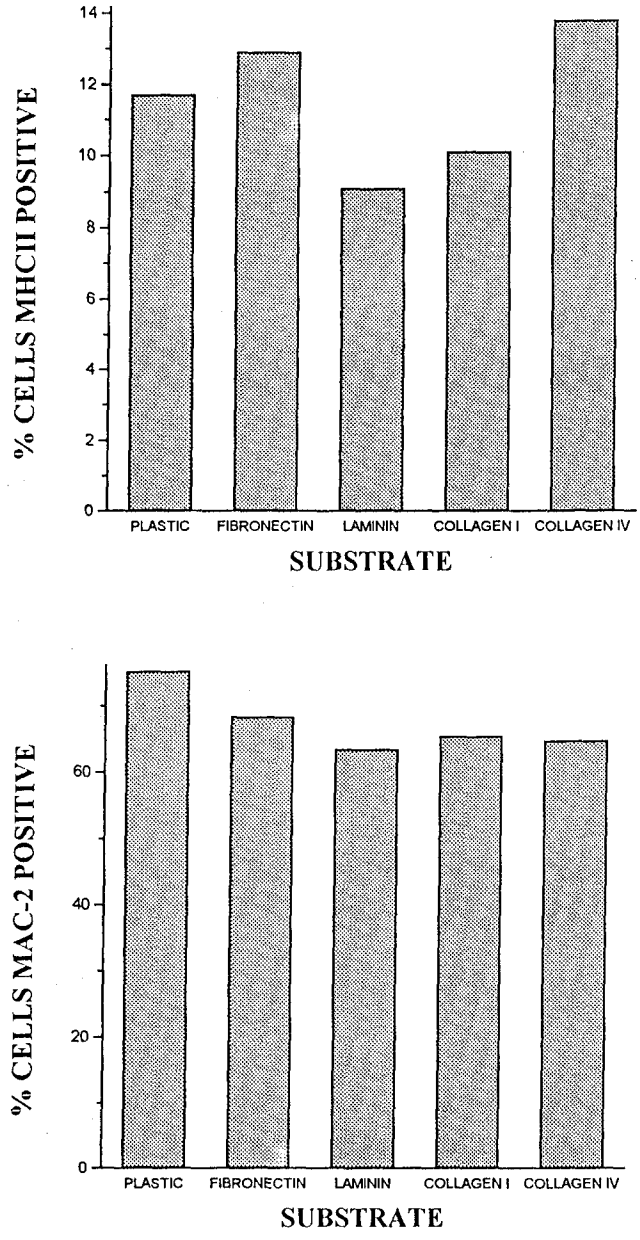


Fig. 3. MHCII and MAC-2 expression by bone marrow–derived macrophages cultured on either a fibronectin, laminin, collagen type I, or collagen type IV substrate. The percentage of cells staining positive for MHCII or MAC-2 on day 6 of culture are given. A representative experiment is shown using pooled bone marrow from three mice (differences between experimental groups were not significant).

groups tested. The phenotypic marker, MAC-2, is not expressed on freshly isolated bone marrow cells due to the low percentage of mature macrophages in bone marrow (Ho et al., '82; Armstrong et al., '93). The presence of MAC-2 following 6 days of culture indicated that bone marrow cells had differentiated into mature macrophages.

IL-6 and TNF-α secretion by bone marrow–derived macrophages cultured on ECM-supplemented substrates

To determine if the observed morphological differences between macrophages cultured on different ECM proteins were accompanied by functional changes, we measured cytokine secretion by bone marrow–derived macrophages grown on ECM proteins. On day 6 of culture, IL-6 was constitutively secreted by macrophages regardless of substrate type (Fig. 4). The amount of IL-6 secreted by macrophages cultured on plastic, collagen type I, collagen type II, fibronectin, or laminin was similar. No TNF secretion was detected for cells in liquid cultures for any given substrate type. Furthermore, TNF secretion could not be induced by a 24-hour treatment with IFN-γ and LPS.

The effect of ECM molecules on the proliferative rate of bone marrow–derived macrophage colonies in agar suspension

Although the liquid culture system can provide valuable insights on the effects of various ECM molecules on macrophage growth and function, the bone marrow is a three-dimensional environment that is poorly reproduced by culture dishes coated with ECM. Therefore, we studied bone marrow macrophage development in agar cultures containing ECM proteins. We wanted to determine if this culture system could more closely simulate the in vivo bone marrow environment.

Figure 5 shows colony growth for bone marrow cells in the presence of CSF-1 and either collagen type I, collagen type IV, laminin, fibronectin, or no additive (control). In contrast to the results obtained using liquid cultures, proliferative rates in three-dimensional agar cultures were not different following the addition of ECM molecules. Three concentrations (10 μg/ml, 5 μg/ml, and 1 μg/ml) of each substrate were tested. Additionally, no difference in colony size or morphology was apparent (data not shown).

IL-6 and TNF-α secretion by bone marrow–derived macrophages cultured in a three-dimensional ECM-supplemented culture system

We determined if macrophages grown in the three-dimensional agar system paralleled the secretory activity of macrophages grown in liquid cultures. Figure 6 shows a significant decrease in IL-6 production by bone marrow–derived macro-

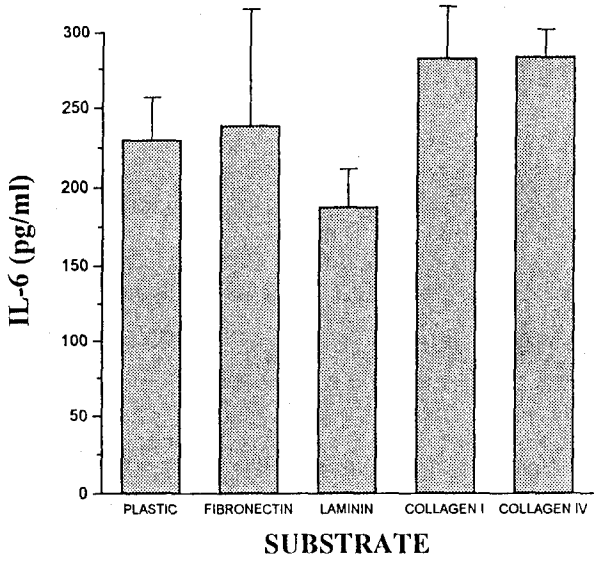


Fig. 4. The effect of substrate type on constitutive IL-6 production by bone marrow–derived macrophages in liquid culture. Supernatants were collected on day 6 of culture. One representative (bone marrow of three mice pooled and measured in triplicate) of two experiments shown; differences between experimental groups were not significant.

phages in agar cultures supplemented with ECM molecules. This effect was concentration-dependent. The quantity of IL-6 secreted by all ECM-supplemented cultures was closer to control values at 1 μg/ml than at 10 μg/ml. TNF-α was constitutively secreted in agar cultures supplemented with either fibronectin, laminin, collagen type I, or collagen type IV (Fig. 7). Again, TNF-α secretion was ECM molecule concentration–dependent. Maximal TNF-α was secreted in the 5–10 μg/ml concentration range depending on the ECM molecule present (contaminating endotoxin levels for each of the ECM products at 10 μg/ml were: fibronectin 2 ng/ml, laminin 0.2 ng/ml, collagen type I < 0.02 ng/ml, and collagen type IV < 0.02 ng/ml. These levels are not sufficient to activate bone marrow macrophages; Chapes et al., '88). Interestingly, collagen type I induced secretion in a reverse dose-dependent fashion in two independent experiments, again illustrating the distinct biological activities of each of the ECM proteins. TNF-α was not detected in agar cultures which had not been supplemented with either fibronectin, laminin, collagen type I, or collagen type IV.

DISCUSSION

Dramatic differences in macrophage morphology were observed when bone marrow cells were cultured on different substrates in the presence of CSF-1. Additionally, growth was slower in cultures supplemented with ECM molecules compared to macrophages cultured on basic tissue culture plastic. These results suggest that ECM products play an influential role in macrophage development. The lower proliferation rates of macrophages on ECM-coated plastics may be due to enhanced differentiation of macrophages in the presence of ECM molecules (Wistell and Schook, '92; Rutherford et al., '93). However, the similarity in phenotypic markers, especially MAC-2, and cytokine secretion between cells grown on plastic and ECM proteins does not support this hypothesis. It is possible that growth was suppressed because ECM proteins can bind CSF-1 (Roberts et al., '88; Suzu et al., '92) and reduce its availability to cells. However, the unaffected growth rates of macrophages in culture in the presence of ECM proteins does not support this hypothesis. The morphological differences caused by various ECM substrates may, to some extent, be attributed to differences in cell binding (Brown and Juliano, '85; Koenigsmann et al., '92; Lanier et al., '88) and subsequent intracellular changes (Sporn et al., '90) to each of the four molecules tested. Fibronectin resulted in higher levels of cell spreading and adhesion than laminin. This result is consistent with a similar study using the monocytic cell line U937 (Bauvois et al., '92).

It should be noted that different methodologies were used to assay cell growth in the agar and liquid culture systems. The nature of the MTT assay makes it inapplicable to an agar culture system. Furthermore, reliable cell counts are difficult to obtain with cells cultured in liquid due to the extremely adherent nature of bone marrow–derived macrophages. As the MTT assay reflects the activity of a cellular mitochondrial enzyme (Ferrari et al., '90), it is possible that differences observed using this assay could be attributed to changes in cellular metabolic activity rather than cellular proliferation. However, we found that ^{3}H-thymidine labeling of bone marrow–derived macrophages, cultured on differing substrates, gave comparable proliferative rates to that of MTT (data not shown). Therefore, it appears that the MTT assay, in this situation, reflected proliferative rates and not changes in metabolic rates.

Macrophage development in agar suspension cultures was not altered by the presence of either fibronectin, laminin, collagen type I, or collagen

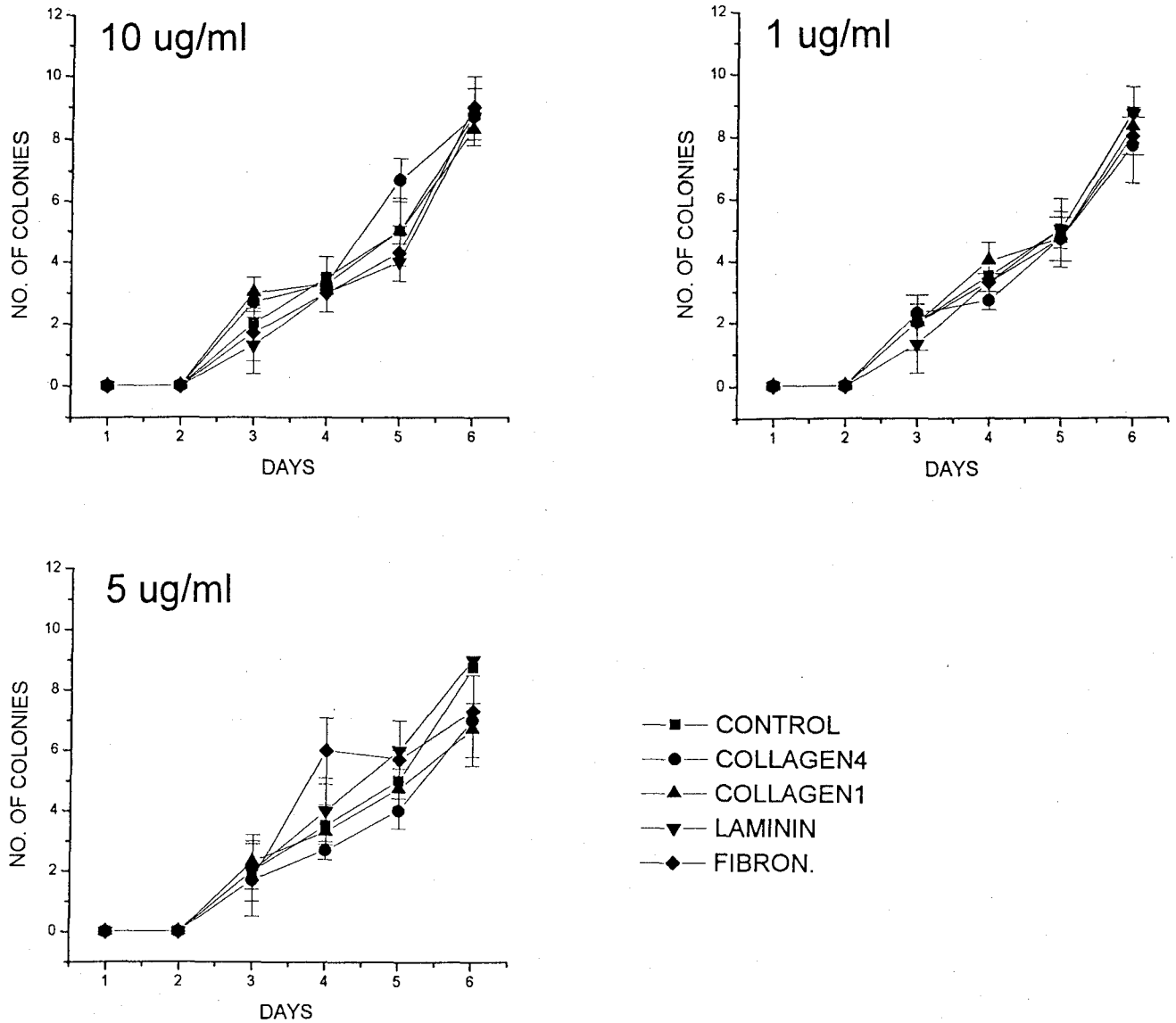
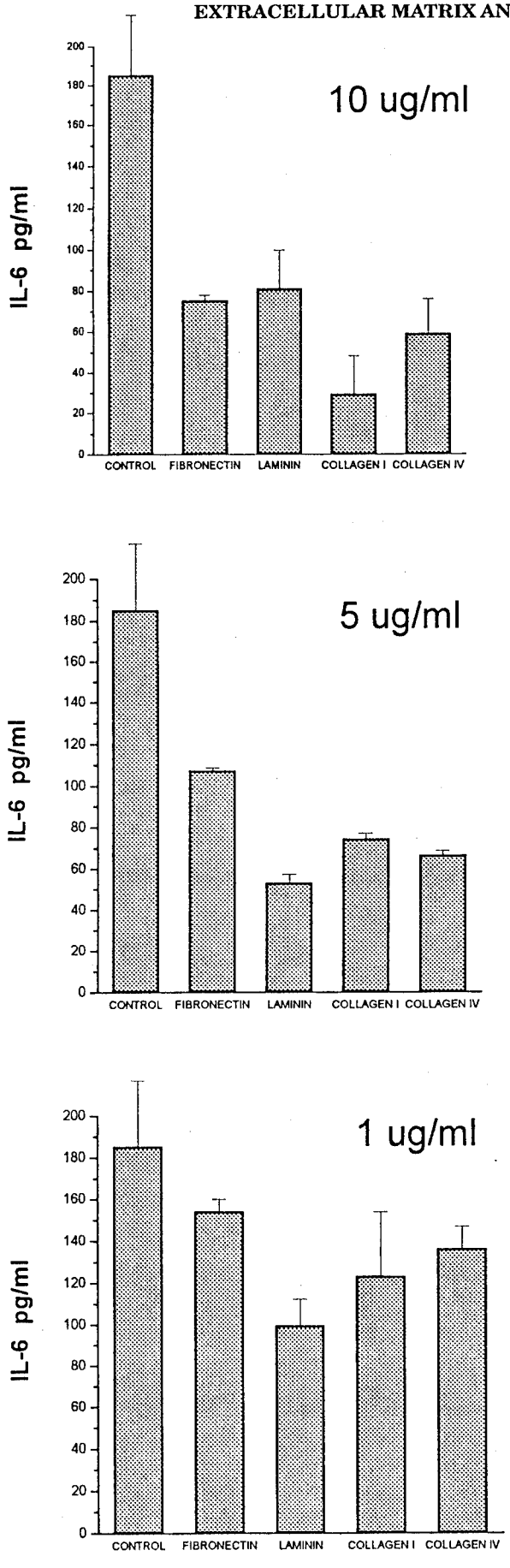


Fig. 5. The effect of ECM molecules on bone marrow–derived colony formation in agar suspension cultures. Soluble fibronectin, laminin, collagen type I, or collagen type IV were added to agar cultures creating a final concentration of either 10 μg/ml, 5 μg/ml, or 1 μg/ml as indicated. One representative (bone marrow of four mice pooled and measured in triplicate) of two experiments shown; differences between experimental groups were not significant.

type IV. This result contrasts the growth of macrophages on ECM proteins in liquid cultures. Although there were no differences in macrophage growth in agar cultures, IL-6 and TNF-α secretion were significantly affected. Therefore, biologically active concentrations of ECM molecules were present in these cultures.

IL-6 is an essential cytokine for normal macrophage proliferation and differentiation (Jansen et al., '92). However, when cells were cultured in agar, significantly less IL-6 was secreted under ECM-supplement conditions. This contrasts with earlier findings by Jansen et al. ('92). They showed an essential role for IL-6 in macrophage development using a methylcellulose suspension culture system and anti-IL-6 antibodies. It is possible that IL-6 may not play a major role in macrophage proliferation in the presence of ECM proteins. Alternatively, enough IL-6 still may have been produced to support macrophage development. In fact, in the presence of high concentrations of IL-6, bone marrow macrophage development can be inhibited (Reidy and Stewart, '92).

Constitutive TNF-α secretion by macrophages



was induced in agar cultures containing either fibronectin, laminin, collagen type I, or collagen type IV but not in agar cultures lacking ECM molecules or liquid cultures with or without an ECM substrate. This is an important finding which demonstrates that an agar suspension culture system differs from conventional liquid systems. Furthermore, this culture system when used with ECM molecules may provide and environment more analogous to the bone marrow environment in vivo. TNF-α is important for the differentiation of macrophage populations (Scheibenbogen et al., '91; Rutherford et al., '93) and the regulation of stromal cell production of colony stimulating factors and IL-6 (Arai et al., '90). Therefore, the agar system and ECM proteins may approximate the natural TNF response in vivo. How this occurs in unclear. It is possible that macrophages cultured in an ECM-supplemented agar system are more mature in 6 days of culture that those in ECM-free conditions. Alternatively, ECM molecules may be activating TNF-α secretion through a direct signaling pathway (Eierman et al., '89; Beezhold and Personius, '92; Hershkoviz et al., '93). More work is needed to resolve this interesting question.

Microgravity and ground-based studies have revealed the importance of cell adhesion for normal cell proliferation and function (Cogoli et al., '93; Gmunder et al., '90). It is now known that the inability of lymphocytes to proliferate in vitro during exposure to microgravity is due to a lack of sedimentation and substrate adherence (Gmunder et al., '90; Cogoli et al., '93). More recent in vitro studies utilized culture systems which allowed cells to remain adherent in microgravity (Chapes et al., '92; Cogoli et al., '93). It is unlikely that the same lack of cell adherence occurs in vivo during exposure to microgravity conditions; however, changes in extracellular matrix in vivo, as a result of microgravity conditions (Spengler et al. '83; Turner et al., '83), may affect cell attachment and ultimately cell function.

In summary, we conclude that two different culture systems, liquid and agar, produce distinct re-

Fig. 6. The effect of ECM molecules on bone marrow–derived colony IL-6 production in agar suspension cultures. Soluble fibronectin, laminin, collagen type I, or collagen type IV were added to agar cultures creating a final concentration of either 10 μg/ml, 5 μg/ml, or 1 μg/ml as indicated. One representative (bone marrow of four mice pooled and measured in triplicate) of two experiments shown. Significant differences were found for all extracellular matrix supplemented cultures (10 μg/ml) compared to control cultures ($P < 0.05$).

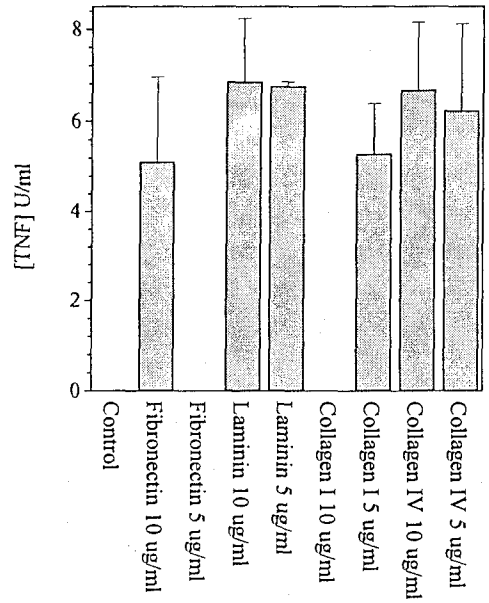


Fig. 7. The effect of ECM molecules on bone marrow–derived colony TNF-α production in agar suspension cultures. Soluble fibronectin, laminin, collagen type I, or collagen type IV were added to agar cultures creating a final concentration of either 5 μg/ml or 10 μg/ml as indicated. One representative (bone marrow of four mice pooled and measured in triplicate) of two experiments shown. The lack of a column for a given sample indicates that no TNF-α was detected. In all conditions where TNF-α was detected, levels were significantly higher than that of the control condition ($P < 0.05$).

sponses by differentiating macrophages to the ECM molecules fibronectin, laminin, collagen type I, and collagen type IV. Macrophage heterogeneity in vivo is often tissue-dependent (Rutherford et al., '93). It is likely that the ECM environment of a tissue regulates the morphological and functional differences seen in macrophages. Additionally, we propose that situations which alter the ECM environment of the bone marrow, such as spaceflight, may have the potential to affect haematopoiesis through an ECM-dependent mechanism.

ACKNOWLEDGMENTS

We thank Wilma Shuman and Signe Balch for their excellent technical and laboratory assistance. We also thank Dr. Brian Spooner for the use of his laboratory's photomicrographic equipment. This work was supported by NASA Specialized Center of Research and Training grant NAGW-2328, NASA grant NAGW-1197 and U.S. Army Medical Research and Development Command Grant DAMD 17-89-Z-9039. This is a Kansas Agricultural Experimental Station Publication 94-11-J.

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Interleukin-2 therapy reverses some immunosuppressive effects of skeletal unloading

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Armstrong, Jason W., Signe Balch, and Stephen K. Chapes. Interleukin-2 therapy reverses some immunosuppressive effects of skeletal unloading. *J. Appl. Physiol.* 77(2): 584–589, 1994.—Using antiorthostatic suspension, we characterized hematopoietic changes that may be responsible for the detrimental effect of skeletal unloading on macrophage development. Skeletally unloaded mice had suppressed macrophage development in unloaded and loaded bones, which indicated a systemic effect. Bone marrow cells from unloaded mice secreted less macrophage colony-stimulating factor and interleukin-6 than control mice. Additionally, T-lymphocyte proliferation was reduced after skeletal unloading. We show that polyethylene glycol-interleukin-2 therapy reversed the effects of skeletal unloading on macrophage development and cell proliferation.

macrophage; T-cell; spaceflight

SKELETAL UNLOADING (by use of the antiorthostatic suspension model) causes a number of physiological changes that mimic spaceflight: bone resorption, muscle atrophy, and fluid shifts (11, 21, 28). Spaceflight and antiorthostatic suspension also inhibit macrophage development from bone marrow cells (3, 25). However, the mechanisms by which skeletal unloading affects the immune system are not clear. Cytokines play an essential role in the hematopoiesis of all white blood cells (1, 18). Interleukin-6 (IL-6) and colony-stimulating factor-1 (CSF-1) are cytokines that are important for macrophage growth and development (14, 18). Therefore it is possible that skeletal unloading affects macrophage development through changes in bone marrow cytokine production. We previously demonstrated that macrophage development is affected by changes in skeletal loading, extracellular matrix, or alterations in histocompatibility molecule expression in the bone marrow microenvironment (2–4). Because changes in the marrow microenvironment can have such dramatic effects on hematopoiesis, we assessed how skeletal unloading affected bone marrow cytokine secretion.

Lymph node T-cell proliferative responses are also inhibited by skeletal unloading mediated through spaceflight or antiorthostatic suspension (3, 22, 23). Interleukin-2 (IL-2) is a well-characterized T-cell growth factor. However, IL-2 can bind and activate monocytes, the precursors of macrophages, to produce CSF-1 (5). Therefore, on the basis of the known functions of IL-2, we used polyethylene glycol-conjugated recombinant IL-2 (PEG-IL-2) as an experimental treatment for the immunosuppressive effects of skeletal unloading.

We report that antiorthostatic suspension reduced the number of macrophage precursor cells systemically. The effect was not restricted to skeletally unloaded bones. Additionally, the secretion of IL-6 and CSF-1 by bone marrow cells from skeletally unloaded mice was reduced compared with control mice. PEG-IL-2 therapy prevented immunosuppression induced by skeletal unloading. Macrophage progenitor cell numbers and lymph node T-cell responses remained normal with PEG-IL-2 preventive therapy.

MATERIALS AND METHODS

Animals. Male adult (8–12 wk) C3HeB/FeJ mice bred in the animal facility at Kansas State University were used in these studies. The use of these animals was approved by the Animal Care Facilities Use Committee at Kansas State University, which complies with National Institutes of Health Animal Care Standards.

PEG-IL-2 therapy. Mice were injected once with PEG-IL-2 (0.5 mg/kg ip; Chiron, Emeryville, CA). In all PEG-IL-2 therapy experiments, normal control and orthostatic control mice were injected intraperitoneally with an equal volume (~300 μl) of pyrogen-free water immediately before they were subjected to suspension. The final dosage protocol was selected after consultation with the supplier and preliminary rodent dose-finding experiments to identify optimal concentrations. The 0.5 mg/kg dose had no apparent pathological effects on lungs and liver.

Antiorthostatic suspension. Animals were antiorthostatically suspended using the Wronski-Morey-Holton tail-suspension cage (6). Briefly, mice were tail suspended at an angle of 22° such that their hindlimbs were skeletally unloaded. Cables connected to the tails of mice were attached to a low-resistance pulley system above the cages, allowing animals complete movement in any direction within the enclosure. Each suspension experiment involved two suspension categories: antiorthostatic suspension (experimental, as described above) and orthostatic control suspension (tail was attached to the pulley system; however, the mice bear full weight on hind- and forelimbs). Sex- and weight-matched normally housed mice were included in initial experiments to provide cells for a "normal control." Mice were weighed before suspension and before they were killed. In all experiments, mice were killed between 8:00 and 10:00 A.M. to eliminate differences caused by circadian rhythms. A suspension period of 11 days was chosen to remain consistent with previous studies (3, 8, 17).

Bone marrow macrophage colony assay. Macrophage development from bone marrow cells was assayed as described previously (2, 3). Briefly, bone marrow cells were obtained from the femora, tibiae, and humeri of mice from the three treatment groups. The ends of femora and tibiae were removed, and the cells were flushed from the bone using Dulbecco's modified Eagle's medium (DMEM) and a 26-gauge needle. Cells were passed three times through a 19-gauge needle to break up clumps, pelleted, and resuspended at a concentration of 1×10^5 cells/1.5 ml of DMEM containing 0.3% agar, 10% fetal bovine serum (FBS), and 30% LM-929 fibroblast-conditioned medium (CSF-1 source). After 6 days of culture, five microscope view fields were scored for macrophage colonies (a group of >25 cells).

Lymph node T-cell proliferation. Proliferation was assayed for all treatment groups simultaneously, as previously described by our laboratory (3). Briefly, lymphocytes were ob-

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TABLE 1. *Effect of skeletal unloading on body weight and spleen weight*

Treatment	n	Body Weight Gained, %initial wt	Spleen Weight, %final body wt
Normal control	3	+3.7±2.4	0.263±0.001
Orthostatic control	9	−5.8±2.3	0.272±0.001
Suspended	11	−3.9±2.8	0.268±0.003
Suspended + rIL-2	8	−7.5±1.7	0.352±0.008*

Values are means ± SE; n, no. of animals. rIL-2, recombinant interleukin-2. * $P < 0.05$, analysis of variance.

tained by expression of the cells from inguinal lymph nodes through a wire sieve. Lymphocytes were washed twice with DMEM containing 2% FBS and gentamycin sulfate (5 μg/ml); 5×10^5 lymph node cells were added per well (100 μl) in Costar (Cambridge, MA) 96-well flat-bottom microtiter plates. Wells received 100 μl of medium: DMEM supplemented with 5.6×10^{-5} M 2-mercaptoethanol, 2% FBS, and gentamycin sulfate (5 μg/ml), with or without phytohemagglutinin (PHA; 9 μg/ml; Wellcome Biotechnology, Research Triangle Park, NC). The cells were incubated for 48 h and pulsed with [^{3}H]thymidine (0.5 μCi/well) for 6–8 h before harvest. The cells from each well were harvested on a Cambridge PHD cell harvester on glass fiber filters, placed in scintillation counting fluid, and counted on a Packard 1500 scintillation counter. Stimulation index (SI) was calculated

$$SI = \left(\frac{\text{cpm samples} + \text{PHA}}{\text{cpm samples} - \text{PHA}}\right) \times 100$$

Cytokine assays. Bone marrow cell cytokine secretion was assayed by incubating freshly isolated bone marrow cells in RPMI 1640 supplemented with 10% FBS for 18 h (5×10^6 cells/3 ml), as described previously (4). Supernatants were assayed for IL-6 content by bioassay with use of the IL-6 growth-sensitive cell line B9. Growth was quantified using a 1-[4,5-dimethylthiazol-2-yl]-3,5 diphenylformazan (MTT) assay, and concentrations were determined by linear regression and a recombinant IL-6 (R & D, Minneapolis, MN) standard curve (sensitivity 625 fg/ml). Supernatants were assayed for transforming growth factor-β (TGF-β) content by bioassay with use of the TGF-β growth-sensitive cell line CCL64 (American Type Culture Collection, Rockville, MD). TGF-β concentrations were determined by an MTT assay used in conjunction with a recombinant TGF-β (R & D) standard curve (sensitivity 7.5 ng/ml). Supernatants were assayed for CSF-1 content by bioassay with use of the CSF-1-dependent cell line B6MP102. CSF-1 concentrations were determined by MTT assay and a recombinant CSF-1 (Chiron) standard curve (sensitivity 60 ng/ml). Experiments using recombinant cytokines and bone marrow cell supernatants demonstrated our bioassays to be unaffected by bone marrow cell supernatant cytokines other than those being assayed. IL-2, interleukin-4, and interferon-γ were assayed by standard-capture enzyme-linked immunosorbent assay (sensitivity 600 pg/ml).

Statistical analysis. The Number Cruncher Statistical Package (J. L. Hintze, Kaysville, UT) was used to perform Student's t tests to determine statistical significance. A 95% level of significance was used for each test. Stress data were interpreted using an analysis of variance at a 95% level of significance. All n values indicate number of animals used in a given experiment.

RESULTS

Stress data. We previously measured and correlated stress and immune parameters after skeletal unloading (3, 6, 8). Although the effects of skeletal unloading on a number of immune parameters are not stress related (3, 8), we monitored changes in body weights and spleen weights, for all animals, as indicators of stress. Changes in these parameters correlated with circulating corticosterone concentration changes (3, 6, 8, 17). Table 1 shows that the percent body weight gained was not significantly different ($P < 0.05$) among all treatment groups used in this study. However, animals not subjected to either suspension technique gained weight, whereas those in the suspension groups lost weight. Therefore restraint had some apparent effect on the mice. Spleen weight as a percentage of final body weight was not different between normal and suspended animals. However, treatment with PEG-IL-2 significantly increased spleen weight as a percentage of final body weight ($P < 0.05$). Overall, our skeletal unloading protocol appeared to im-

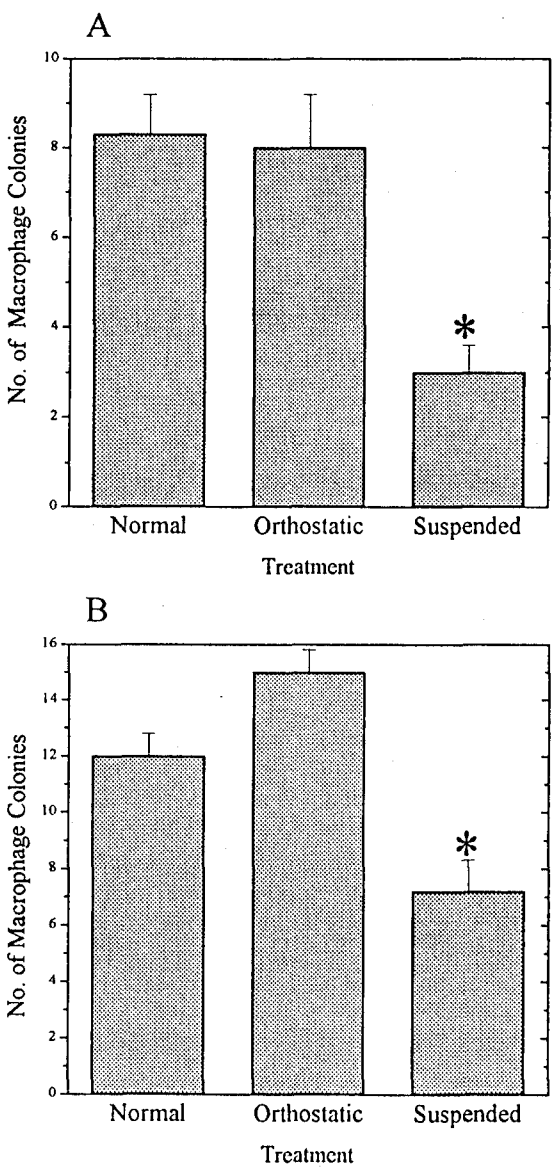

FIG. 1. Effects of skeletal unloading on macrophage development. Macrophage colonies derived from femora and tibiae (*A*) and humeri (*B*) were isolated from mouse bone marrow and cultured for 7 days in colony-stimulating factor-1 (CSF-1). One of 3 independent experiments is shown; $n = 3$ for each condition; results were similar in all experiments. * $P < 0.02$ compared with normal control mice.

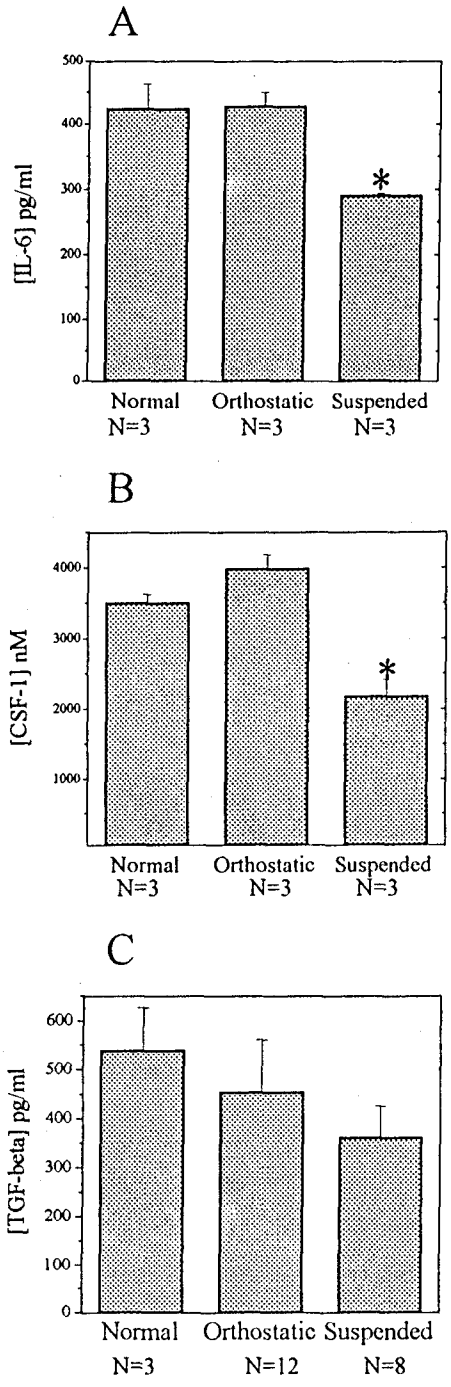

FIG. 2. Effects of skeletal unloading on bone marrow cell cytokine secretion. Bone marrow cell cytokine secretion was determined from supernatants of freshly isolated bone marrow cells cultured for 18 h in interleukin-6 (*A*), CSF-1 (*B*), and transforming growth factor-β (*C*). One of 3 independent experiments is shown; $n = 3$ for each condition; results were similar in all experiments. * $P < 0.05$ compared with normal control mice.

pose minimal stress, as measured by weight gain and spleen weight between normal and suspended animals.

Macrophage development. Skeletal unloading, via antiorthostatic suspension, reduces mouse hindlimb macrophage development (3). The data in Fig. 1*A* confirmed this finding. However, it has not been shown whether this effect is local or systemic. Figure 1*B* shows that macrophage development from bone marrow cells isolated from the weight-bearing forelimbs (humerus) of skeletally unloaded mice was also reduced. These data indicated a systemic effect of skeletal unloading on hematopoiesis that was not localized to bones subjected to unloading.

Hematopoietic cytokines. Macrophage hematopoiesis is primarily controlled by the cytokines CSF-1 and IL-6 (18). Therefore we analyzed the IL-6 and CSF-1 secretory activity of freshly isolated femur bone marrow cells from skeletally loaded and unloaded mice (Fig. 2). TGF-β secretion was also measured because of its known role as an inhibitor of macrophage hematopoiesis (20). Bone marrow cell IL-6 and CSF-1 secretion was significantly lower in mice that had been skeletally unloaded (Fig. 2, *A* and *B*). This was consistent with the observed suppression in macrophage development after skeletal unloading and suggested a possible mechanism behind this phenomenon. TGF-β levels were not significantly different when the three groups were compared. IL-2, interleukin-4, and interferon-γ were not detected in bone marrow cell supernatants.

T-cell proliferation. Skeletal unloading inhibits macrophage development as well as lymph node T-cell proliferation (3). Figure 3 shows that lymph node T-cell proliferation (induced by PHA) was inhibited ex vivo after skeletal unloading. IL-2 is an important cytokine in the in vivo T-cell response (1). Therefore we tested whether PEG-IL-2 was capable of ameliorating the effects of skeletal unloading on T-cell mitogenesis. Figure 3 shows that one prophylactic administration of PEG-IL-2 (0.5 mg/kg) at the start of antiorthostatic suspension reversed the suppressive effects of skeletal unloading.

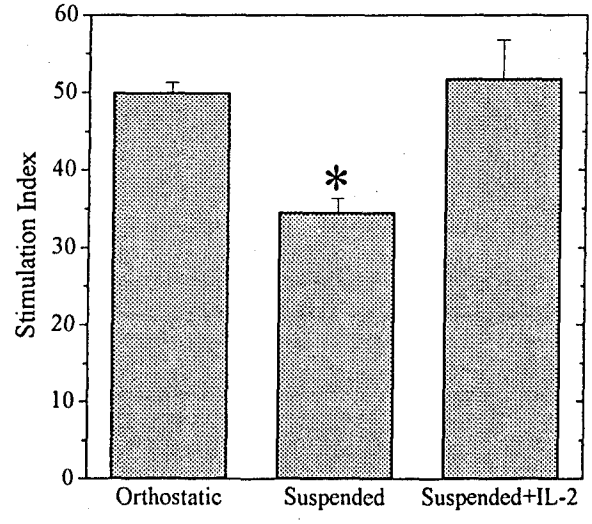

FIG. 3. Effects of polyethylene glycol-recombinant interleukin-2 (PEG-IL-2) therapy on phytohemagglutinin (PHA)-induced lymph node T-cell proliferation in skeletally unloaded mice. Lymph node T-cell proliferation is presented as a stimulation index over lymph node cells that were not exposed to PHA. Mice were injected immediately before suspension with PEG-IL-2 (0.5 mg/kg) or pyrogen-free water. * $P < 0.05$ compared with orthostatic control mice (1 of 3 representative experiments is shown; all experiments had similar results; lymph node T-cells were pooled from 3 animals per treatment group per experiment). Raw data (counts/min): orthostatic + medium, 821 ± 23; orthostatic + PHA, 40,981 ± 2,247; suspended + medium, 1,132 ± 62; suspended + PHA, 39,103 ± 1,195; suspended + IL-2, 677 ± 66; suspended + IL-2-PHA, 35,079 ± 1,232.

PEG-IL-2 therapy and macrophage development. Inasmuch as PEG-IL-2 therapy was effective in maintaining normal PHA-induced T-cell proliferation and because IL-2 receptors are found on cells of the macrophage lineage (5, 9, 27), we tested whether PEG-IL-2 affected macrophage progenitor cell development in skeletally unloaded mice. Figure 4 shows that PEG-IL-2 therapy (0.5 mg/kg) maintained macrophage hematopoiesis at levels comparable to orthostatic controls. Although PEG-IL-2 therapy reversed the effects of skeletal unloading on macrophage development, it did not reverse the reduced bone marrow cell cytokine secretion (Fig. 5). Bone marrow cells from mice that had been suspended and treated with PEG-IL-2 and mice that had been suspended without PEG-IL-2 treatment secreted similar amounts of IL-6, CSF-1, and TGF-β.

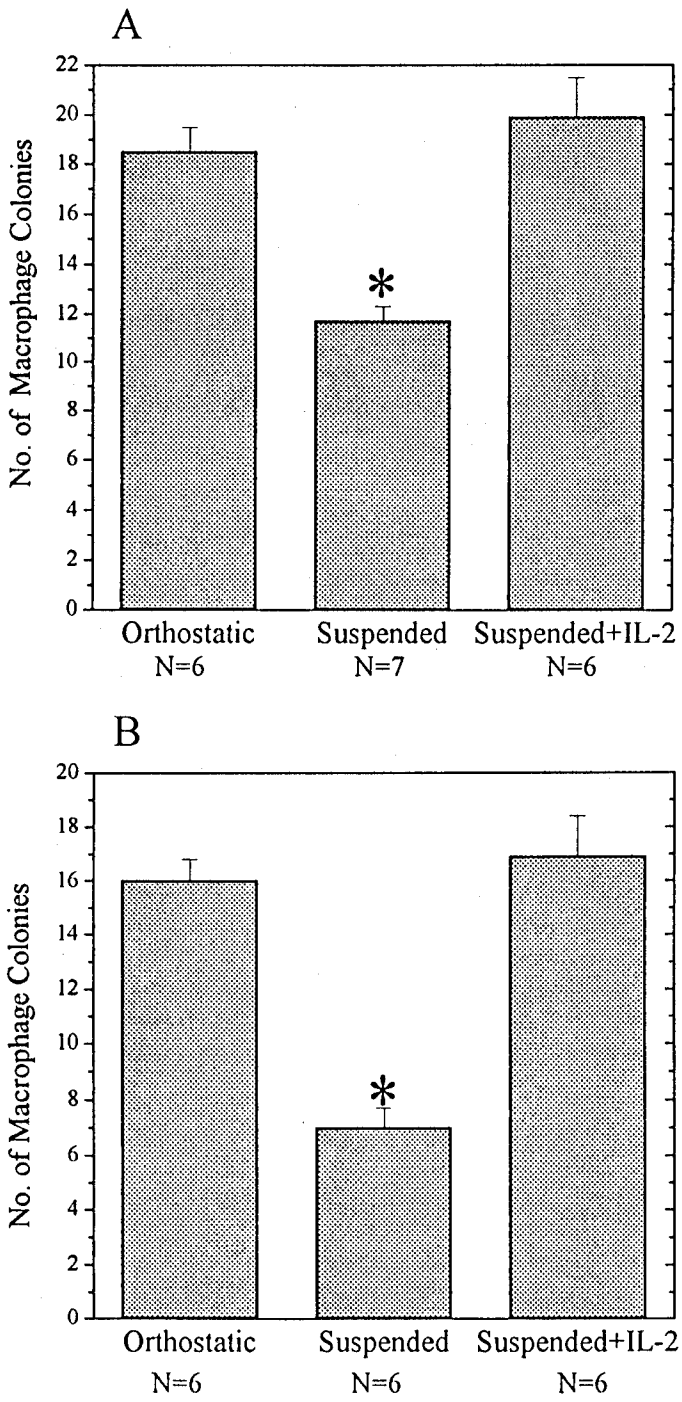

FIG. 4. Effects of PEG-IL-2 therapy on macrophage development in skeletally unloaded mice. Macrophage colonies were derived from mouse bone marrow from femora and tibiae (*A*) and humeri (*B*) that was cultured for 7 days. * $P < 0.03$ compared with normal control mice.

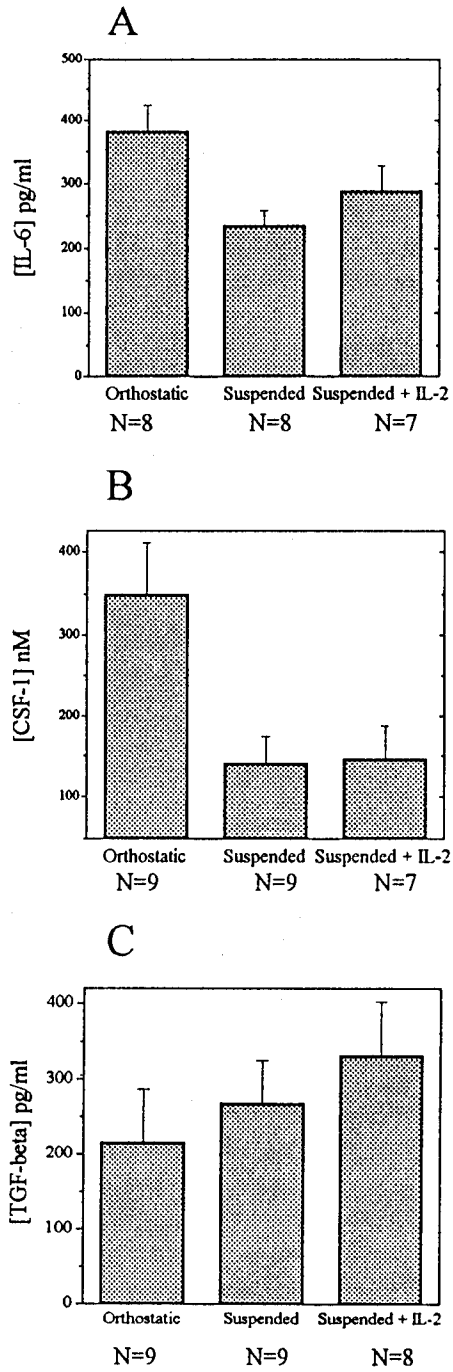

FIG. 5. Effects of PEG-IL-2 therapy on bone marrow cell cytokine secretion in skeletally unloaded mice. Bone marrow cell cytokine secretion was determined from supernatants of freshly isolated bone marrow cells cultured for 18 h in IL-6 (*A*), CSF-1 (*B*), and transforming growth factor-β (*C*).

Effect of recombinant IL-2 (rIL-2) on in vitro bone marrow macrophage colony formation. In vivo rIL-2 therapy enhanced macrophage development in skeletally unloaded mice; however, it had no apparent effect on the ex

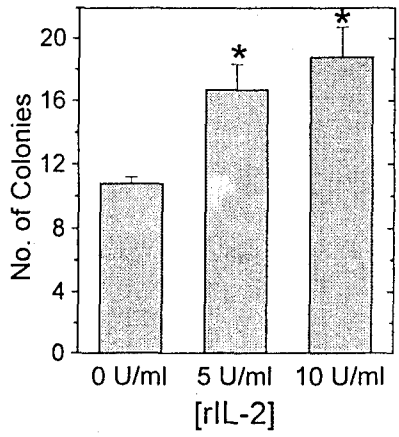

FIG. 6. Effects of recombinant IL-2 (rIL-2) on in vitro macrophage development. Macrophage colonies were isolated from normal mouse bone marrow and cultured for 7 days in CSF-1 and rIL-2. * $P < 0.01$ compared with 0 U/ml control condition (0 U/ml, $n = 5$; 5 U/ml, $n = 5$, 10 U/ml, $n = 6$).

vivo secretion patterns of bone marrow cells. Therefore we determined whether IL-2 directly affected macrophage development in vitro. Figure 6 shows that rIL-2 significantly increased CSF-1-dependent macrophage colony formation in agar cultures.

DISCUSSION

Skeletal unloading significantly reduces femoral bone marrow-derived macrophage development (3, 26, 28). In this report, we show that skeletal unloading also reduced macrophage development when bone marrow cells were isolated from weight-bearing forelimbs. These data show that the effects of skeletal unloading on murine macrophage hematopoiesis are systemic in nature. This observation is consistent with findings that show that hindlimb unloading affects bone mineralization in the hindlimbs and weight-bearing forelimbs of mice (23). The systemic nature of this effect may be explained by the absence of circulating CSF-1, which concomitantly regulates bone mineralization (via osteoclasts) and hematopoiesis (1, 13). However, other hormones or cytokines may also be involved (e.g., TGF-β, IL-6, and/or interleukin-1). It is unlikely that corticosteroids induced the changes in hematopoietic activity. We previously showed that the effect of skeletal unloading on macrophage development is unrelated to corticosterone levels or other indicators of systemic stress (3). In this study, we found no difference in stress levels, as measured by body weight loss and spleen weight between normal and suspended animals. PEG-IL-2 therapy did increase spleen weight as a percentage of body weight. This result was not unexpected (1) and indicated that PEG-IL-2 was biologically active in vivo.

The mechanism(s) that causes suppressed macrophage hematopoiesis during skeletal unloading is unknown. Macrophage hematopoiesis is primarily controlled by the cytokines CSF-1 and IL-6 (18). IL-6 plays an important role in early and late macrophage development, whereas CSF-1 is primarily involved in the latter stages of differentiation and proliferation (1, 14, 16). TGF-β, also found within the bone marrow microenvironment, is a cytokine produced by a variety of cells and has the ability to inhibit macrophage hematopoiesis (20). We found that bone marrow cell secretion of CSF-1 and IL-6 was depressed in mice that had been skeletally unloaded. One interpretation of these results is that skeletal unloading suppresses macrophage development by reducing bone marrow cell secretion of IL-6 and CSF-1. A large proportion of bone marrow IL-6 and CSF-1 is derived from bone marrow stromal and hematopoietic nursing cells (10). Therefore it may be a perturbation in these hematopoietic support cells that causes abnormal macrophage development in skeletally unloaded mice. However, we have found no difference in the percentage of stromal cells, as identified by stromal cell-specific antibody KMI6 (15), in bone marrow of skeletally unloaded and control mice (S. Balch, J. W. Armstrong, and S. K. Chapes, unpublished data).

The effects of skeletal unloading on lymphocytes are compartmentalized (3, 22, 23). However, lymph node T-cell proliferation is also reduced after skeletal unloading. Inasmuch as IL-2 is an important T-cell regulatory molecule (1), we tested whether PEG-IL-2 was capable of abolishing the effects of skeletal unloading on T-cell mitogenic responsiveness. We found that PEG-IL-2 therapy reversed the effects of skeletal unloading on T-cell responsiveness as well as macrophage development. The role of IL-2 in PHA-induced T cell proliferation is open to question. Nash et al. (22, 23) reported depressed responses to mitogens in T-lymphocytes from skeletally unloaded rats, even in the presence of normal concentrations of IL-2. Therefore, why should administration of PEG-IL-2 in vivo reconstitute a subsequent mitogen response ex vivo? One hypothesis is that skeletal unloading does not directly affect T-cell responsiveness but depletes one or more subpopulations of T-cells in the lymph node. Mitogens and superantigens stimulate different populations of T-cells (7, 11, 19). Furthermore we and other investigators showed stimulus- and organ-dependent effects of skeletal unloading on T-cells (3, 17, 22, 23). Therefore in vivo administration of PEG-IL-2 might restore those affected subpopulations in vivo. Then, upon ex vivo assay, apparent responsiveness of these cells may be restored. Restoration could occur through expansion of the T-cell numbers in the lymph node or changes in lymphocyte trafficking. Alternatively, it is possible that PEG-IL-2 administration in vivo augments all T-cell responsiveness ex vivo. However, this does not appear to be the case, inasmuch as splenic T-cell responses, which are not suppressed by skeletal unloading, are not affected by PEG-IL-2 therapy (data not shown). Obviously, proof of our former hypothesis would require additional experiments.

The return of macrophage hematopoiesis to normal levels after skeletal unloading and PEG-IL-2 therapy allows for some speculation. First, PEG-IL-2 may have stimulated T-cells to produce granulocyte-macrophage colony-stimulating factor, which directly enhanced macrophage hematopoiesis (1). Alternatively, PEG-IL-2 may have acted directly on developing macrophage populations, inasmuch as macrophages have functional IL-2 receptors (5, 26). PEG-IL-2 therapy did not reverse the effect of skeletal unloading on bone marrow cell cytokine secretion. It therefore appeared that the effect of PEG-

IL-2 was independent of these pathways and was able to override any detrimental effects of low CSF-1 and IL-6 secretion. We demonstrated that rIL-2 enhanced macrophage development when added to normal CSF-1-supplemented bone marrow cultures. These novel data provide additional support that PEG-IL-2 may have acted directly on macrophages/macrophage precursors to enhance macrophage hematopoiesis. In other studies, IL-2 provided radioprotection and enhanced myeloid colony formation in response to granulocyte-macrophage colony-stimulating factor (9, 26). Our data support those findings that IL-2 can enhance macrophage hematopoiesis in situations of immunosuppression.

One may speculate that normal macrophage hematopoiesis requires bone marrow cell IL-2 production and that skeletal unloading perturbs this IL-2 production and consequently reduces macrophage development. This scenario is not supported by our results, which indicated that freshly isolated bone marrow cells did not secrete detectable amounts of IL-2. However, our assay did not assess in situ IL-2 production, and it is possible that bone marrow cells produce IL-2 within the marrow microenvironment but not in vitro.

In summary, we showed that macrophage hematopoiesis and lymph node T-cell responsiveness were reduced after skeletal unloading. Low secretion of bone marrow CSF-1 and IL-6 may account for the suppression in macrophage development after skeletal unloading. PEG-IL-2 therapy was able to reverse the effects of skeletal unloading on T-cell responsiveness and macrophage hematopoiesis.

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Cytokine secretion by immune cells in space

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Abstract: Cultured, bone marrow-derived macrophages, murine spleen and lymph node cells, and human lymphocytes were tested for their ability to secrete cytokines in space. Lipopolysaccharide-activated bone marrow macrophages were found to secrete significantly more interleukin-1 and tumor necrosis factor when stimulated in space than when stimulated on earth. Murine spleen cells stimulated with poly I:C in space released significantly more interferon-α at 1 and 14 hours after stimulation than cells stimulated on earth. Similarly, murine lymph node T cells and human peripheral blood lymphocytes, stimulated with concanavalin A in space, secreted significantly more interferon-γ than ground controls. These data suggest that space flight has a significant enhancing effect on immune cell release of cytokines *in vitro*. *J. Leukoc. Biol.* 52: 104–110; 1992.

Key Words: *space immunology · cytokines*

Introduction

The induction of antibody and cellular immune responses depends on the successful functioning of macrophages, T cells, and B cells. During the response to invading microorganisms like bacteria and viruses, T cell and B cell (the effectors of cellular and humoral immunity, respectively) activation is dependent on various cytokines. Secretion by macrophages of one of these cytokines, interleukin-1 (IL-1), is required [1, 2]. Macrophages also produce tumor necrosis factor α (TNF), which is involved in the cytotoxicity of virus-infected and transformed cells and in the regulation of lipoprotein lipase, an enzyme involved in lipid storage [1]. Both of these cytokines have pleiotropic effects on a number of host responses. T cells also require a complex interaction with accessory cells that involves antigen presentation and stimulation by IL-1. Once activated, the T cells respond by making cytokines, such as interleukin-2, as well as interferons.

Several experiments on orbiting spacecraft during the past decade have shown significant changes in immune cell function during space flight. During 6 days of microgravity, human lymphocytes secreted four to eight times more α-interferon (IFN-α) than controls in response to several interferon inducers [3]. In contrast, after 7 days of microgravity, the postflight response of rat lymphocytes to an IFN-γ inducer showed that their secretion of IFN-γ was dramatically inhibited [4]. The in vitro blastogenic response of lymphocytes to mitogen stimulation was decreased more than 90% during space flight [5, 6], whereas inflight control experiments at 1*g* showed only a 50–60% reduction in mitogenic response [5, 6] compared to ground controls. The basic mechanisms for these altered cell functions during space flight are not understood.

The purpose of this study was to determine whether space flight conditions affect a bone marrow-derived macrophage cell (B6MP102) in its secretion of T cell-stimulatory cytokines, such as IL-1, or other important molecules, such as TNF. In addition, we determined whether space flight would affect the activation of lymphocytes to secrete IFN-α and IFN-γ.

MATERIALS AND METHODS

Cells

The anchorage-dependent, bone marrow-derived macrophage cell line B6MP102 (C57B16, H-2^b) has been described [7]. B6MP102 growth is dependent on colony-stimulating factor 1, and CSF-1 does not induce secretion of IL-1, IL-6, or TNF. We previously reported that these cells can be activated with a number of biological response modifiers, including lipopolysaccharide (LPS) [7]. B6MP102 cells are passed two to three times weekly in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 6% fetal bovine serum (FBS), 0.3% L-glutamine, 10% Opti-MEM (Gibco), and 15% LM929 (American Type Culture Collection) cell-conditioned medium (as a source of CSF-1).

The lymphocytes used for the interferon experiments were either harvested from SJL (H-2^s) mice or purified, by Ficoll-Hypaque gradient centrifugation, from heparinized human peripheral blood buffy coats. Human cells were frozen in liquid nitrogen until use as described below. Mouse splenic lymphocytes (SLs) were harvested, separated on a Ficoll-Hypaque gradient, and cultured in 25-cm^2 flasks in RPMI-1640 medium supplemented with 10% FBS, according to published methods [8], at 37°C for 2 days before shipment to Kennedy Space Center (KSC) and loading into the cell syringes described below. Lymph node cells (LNCs) were harvested, separated on a Ficoll-Hypaque gradient, and stimulated with concanavalin A (Con A, 1 mg/ml) in RPMI 1640 medium supplemented with 10% FBS. Lymph node cells were cultured for 10 days and passed as previously described [8] before shipment to KSC. Cells were harvested, washed, and resuspended in fresh medium and loaded into the cell syringes as described below.

Abbreviations: BPM, bioprocessing module; ConA, concanavalin A; CSF-1, colony-stimulating factor 1; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IFN, interferon; IgG, immunoglobulin G; IL-1, interleukin-1; KSC, Kennedy Space Center; LNC, lymph node cell; LPS, lipopolysaccharide; PHA, phytohemagglutinin; SL, splenic lymphocyte; STS, Space Transportation System; TNF, tumor necrosis factor.

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Reagents and Animals

Cytodex 3 microcarrier beads (Pharmacia-LKB Biotechnology, Uppsala, Sweden) were prepared according to manufacturer instructions to make approximately a 2% bead solution. Recombinant IL-1 and TNF-α were obtained from Genzyme (Cambridge, MA). Interferon-α (mouse 4.7×10^5 IRU/mg protein), used as the standard for a sandwich-plate radioimmune assay, and the rabbit antimouse immunoglobulin G (IgG), used as the second antibody, were obtained from Sigma (St. Louis, MO). The anti-IFN-α IgG antibodies were obtained from Boehringer Mannheim (Indianapolis, IN). Enzyme-linked immunosorbent assay (ELISA) kits for IFN-γ assays were obtained from Amgen (Thousand Oaks, CA) and Gibco. The Con A, polyinosinic-polycytidylic acid (poly I:C), and HEPES buffer were obtained from Sigma. Phytohemagglutinin (PHA) was obtained from Burroughs Wellcome (Research Triangle Park, NC). *Escherichia coli* (055:B5) LPS was obtained from Difco (Detroit, MI). DEAE dextran was obtained from Pharmacia-LKB. C3H/HeJ mice were bred in the animal facility in the Division of Biology at Kansas State University. SJL mice (7-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME).

Preparation of B6MP102 Cells for Space Experimentation

B6MP102 cells were grown in 100-mm-diameter tissue culture dishes. Cells were dispersed with 0.25% trypsin plus 0.2% EDTA, washed, counted, and incubated with Cytodex 3 microcarrier beads for 6-8 h at 37°C in supplemented DMEM, containing 10 mM HEPES (pH 7.3), in 1.5-ml microfuge tubes that were rocked at speed 5 on a Bellco plate rocker (Bellco Glass, Vineland, NJ). Both 5×10^6 and 2.1×10^6 B6MP102 cells, in 1.0 ml, were incubated with 0.5 ml of a pelleted 2% Cytodex 3 bead solution for Space Transportation System (STS) flights 37 and 43, respectively. After attachment, beads-B6MP102 cells were pooled, washed in DMEM, and resuspended in 20 ml of supplemented DMEM containing 10 mM HEPES. Aliquots of 2 ml were aseptically dispensed into 5-ml syringes containing 10-μm filters (**Fig. 1**) and prepared for transportation to KSC. Ten syringes were prepared for each mission. Five syringes were randomly chosen for transport to Cape Canavaral (flight samples) and five syringes (ground controls) were transported around Manhattan, KS. Ground controls were maintained at approximately 22-24°C during the duration of the flight and flight samples were at cabin temperature, approximately 20-25°C. Flight samples for STS-37 were shipped by overnight express mail from Manhattan to Cape Canavaral. The time interval from cell dispersion to launch was 72 h. The experiment was initiated approximately 6 h into flight and appropriate samples were collected 12 and 24 h post activation. Samples for STS-43 were hand-carried on commercial airline flights from Manhattan to Orlando, FL. Cells were driven to Cape Canavaral from Orlando. The time interval from cell dispersion to launch was approximately 96 h. The experiment was initiated approximately 24 h into the flight and samples were collected approximately 24 h later. Based on the analysis of ground controls, 80-90% of the B6MP102 cells were attached and still viable 24 h after activation. For both flights, the samples were stored at cabin temperature for the duration of the flight. STS-37 was a 6-day mission flown at an altitude of 248 nautical miles, an apogee of 247 nautical miles, and a perigee of 239 nautical miles. STS-43 was a 9-day mission flown at an altitude of 174 nautical miles, an apogee of 171 nautical miles, and a perigee of 161 nautical miles. Samples were recovered approximately 3 h after shuttle landing and were placed on ice. Ground control samples were placed on ice at times to coincide with flight samples. Flight samples were returned to Manhattan, KS, 24 and 48 h after recovery for STS-37 and STS-43, respectively. In Manhattan, ground and flight samples were checked for contamination, color, and syringe integrity. All samples were in satisfactory condition and were not contaminated. All samples were filtered through a 0.45-μm filter and stored at −100°C until bioassays for cytokines were performed.

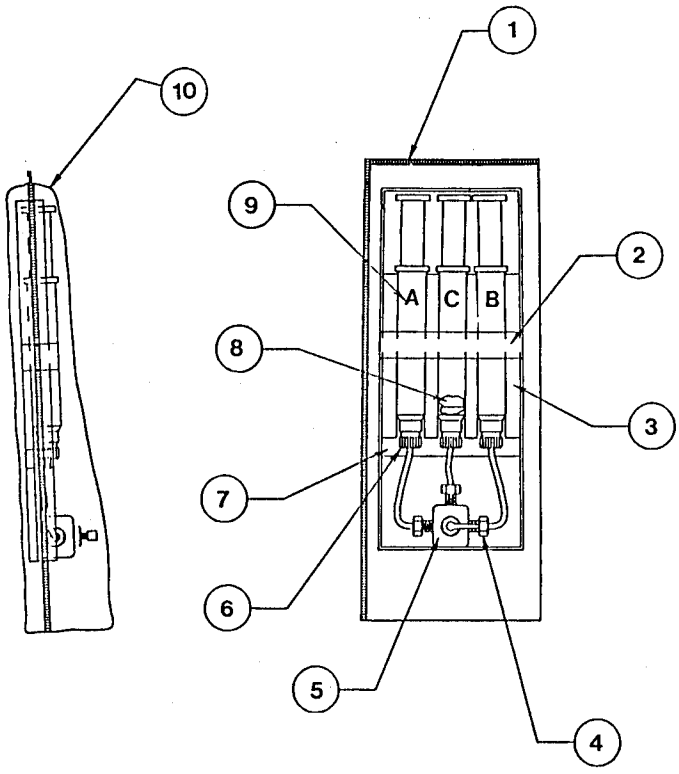

Fig. 1. Schematic diagram of a bioprocessing module. 1, Aluminum tray; 2, Arno adhesive tape; 3, foam inserts; 4, Hamilton plastic screw assembly; 5, aluminum three-way stopcock; 6, Hamilton Luer-Lok screw assembly; 7, foam layer; 8, filter assembly; 9, 5-ml syringes; 10, plastic isolation bag.

Preparation of Lymphocytes for Space Experimentation

Mouse splenic and lymph node lymphocytes were transported to KSC in 25-cm^2 culture flasks completely filled with supplemented RPMI 1640 medium. The cells were shipped by commercial air from Houston and delivered to KSC within 8 h after removal from the incubator in Houston. Upon arrival at KSC, the cells were reseeded into fresh medium, in five flasks, and placed in a 37°C incubator adjusted to 5% CO_2 in air. These cells were cultured for 48 and 24 h for STS-37 and -43, respectively, before being loaded for flight. Thirty-six hours before flight the cells were harvested, centrifuged, and counted, using trypan blue dye to determine the viability and total cell number. For STS-43, human peripheral blood lymphocytes were stored frozen in liquid nitrogen after harvest from Ficoll-Hypaque gradients at the University of Alabama in Huntsville. Cells were transported to KSC on dry ice. They were thawed and suspended in RPMI 1640, supplemented with 10% FBS, 48 h before loading into syringes for flight. Viability was determined by trypan blue exclusion and cell numbers were determined on

a hemacytometer. Human peripheral blood lymphocytes were >84% viable after thawing and at the time of loading. Murine splenic and lymph node cells were >90% viable at the time of loading. The cells were resuspended in fresh RPMI 1640 medium (supplemented with 28 mM HEPES and 10% FBS), then aseptically transferred to the cell syringes at a concentration of 1×10^6 viable cells/ml (2.5 ml/syringe). Chamber B of each cell syringe was also loaded with 1.0 ml of RPMI 1640 with HEPES and 10% FBS, containing either 20 mg/ml of poly I:C and 100 mg/ml of DEAE-dextran (STS-37) or 1.5 mg/ml of Con A (STS-43), which was added to the cells upon activation of the experiment during the flight.

Flight Hardware

The B6MP102 cells were activated using 5-ml syringes mounted to bioprocessing modules (BPMs, Fig. 1). The BPMs were previously flown on STS-7 (cell attachment experiment DSO-0413). The BPMs are aluminum trays with molded foam and a three-way stopcock that allows mounting and connection of three 5-ml syringes (Beckton Dickinson, Rutherford, NJ). The middle syringe had a 10-μm nylon filter mounted in a stainless steel case sealed inside (see Fig. 1) using Silastic 732 RTV adhesive (Dow Corning, Midland, MI). This allowed injection of medium (with or without LPS) from syringe A into syringe C (middle with filter) and injection of cell-free supernatant into syringe B at the appropriate times. Syringe B was detached after flight and returned to Manhattan for recovery of supernatant as described.

Maintenance and activation of the lymphocytes were accomplished using a modified double-chambered syringe, "cell syringe," that was encased in a Lexan plastic housing providing secondary containment. A schematic of such a syringe is shown in **Figure 2.** Each syringe was activated in orbit, by the crew member, by attaching a plunger handle and depressing it to allow the activator solution (poly I:C on STS-37 and Con A on STS-43) to mix with the suspended cells. Samples were dispensed from the syringe into a multidose injection vial through a needle-valve assembly at specified times after activation. The sample vials contained either 0.8 ml of 1% glutaraldehyde or 0.5 ml of 50% ethanol as a cell fixative. The samples were stored at cabin temperature in the orbitor middeck until return to earth. Thereafter,

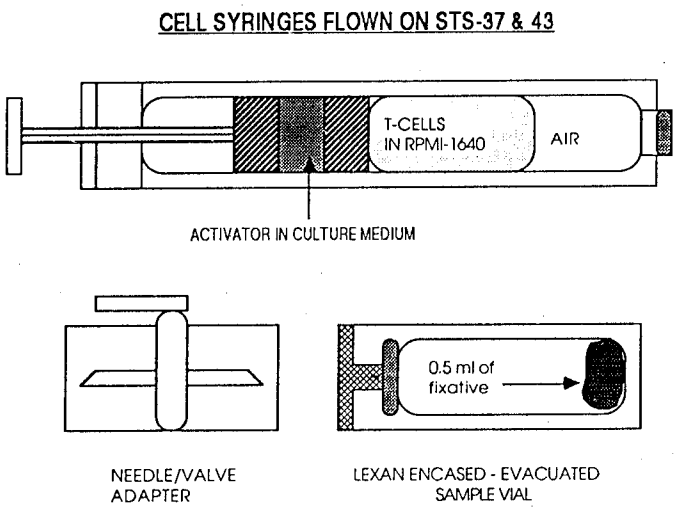

Fig. 2. Schematic diagram of a cell syringe.

the samples were kept at 4°C for transport to Houston and until the assays were performed.

Cytokine Assays

Triplicate samples of the B6MP102 macrophage culture supernatants were serially diluted in supplemented DMEM in 96-well, flat-bottomed microtiter plates. For TNF assay, TNF-sensitive LM929 cells, labeled with ^{51}Cr as previously described [9], were added at a concentration of 1×10^4 cells per well. After an incubation period of 16–18 h at 37°C, the microtiter plates were centrifuged and 90-μl aliquots, harvested from each well, were counted on a gamma counter. Percent specific ^{51}Cr release was calculated as described previously [9].

IL-1 release was assayed by the previously described comitogenic murine thymocyte assay [2]. Thymuses were removed aseptically from 4- to 8-week-old C3H/HeJ mice, teased to release thymocytes, washed, and resuspended in DMEM containing 0.3% glutamine, 3×10^{-5} M 2-mercaptoethanol, and 50 μg/ml gentamicin sulfate. Thymocytes were distributed into 96-well flat-bottomed microtiter plates at a concentration of 1×10^6 cells/well in 100 μl of medium. Doubling dilutions of test supernatants (100 μl) were added to the wells, in addition to 0.45 μg PHA/well. Plates were incubated for 72 h at 37°C and 8% CO_2 in air. Eight hours before harvest, 1 μCi of [^{3}H]thymidine (20 μl) was added to each well. Incorporation of [^{3}H]thymidine was measured on a scintillation counter. Background control wells contained thymocytes and PHA only. Tumor necrosis factor and IL-1 quantification in the supernatant samples was based on linear regression of standard curves of rTNF and rIL-1.

Due to presence of glutaraldehyde (STS-37) or ethanol (STS-43) in the samples, IFN-α (STS-37) secreted into the culture medium was measured using a sandwich-plate radioimmunoassay incorporating ^{125}I-labeled protein A, as previously described [10]. The assays for IFN-γ (STS-43) were conducted using an ELISA in which the chromogenic product was read at 405 nm after a 30- or 45-minute incubation period. Serial dilutions of the reference standards and the unknowns were used to determine the IFN-α or IFN-γ concentration in the supernatant of each flight sample.

Statistical Analysis

Analysis of covariance of the IFN-α radiolabel binding data was conducted using a Fortran parallel line analysis program published by Swank et al. [11]. Analysis of variance (ANOVA) was performed on the IFN-γ data using Statview software (BrainPower, Calabasas, CA) that computed the Scheffe F-test for significance. The Number Cruncher Statistical Package (J.L. Hintze, Kaysville, UT) was used to perform Student's t-tests to determine statistical significance of B6MP102 production of cytokines in space. A sample size (n) of 2 was used for statistical determinations. Averages of replicates of 2 or 3 were used to determine $\bar{x}$ for each n.

RESULTS

Cytokine Secretion by B6MP102 Cells During STS-37

We found that 308 units of TNF were released with a 12-h stimulation on the ground, compared to 1082 units of TNF released in space (space = 3.5× ground, $P < .05$) (**Fig. 3**). Similarly, 426 units of TNF were secreted, with a 24-h stimulation, by ground controls, compared to 970 units in space (space = 2.3× ground, $P < .05$) (Fig. 3). When su-

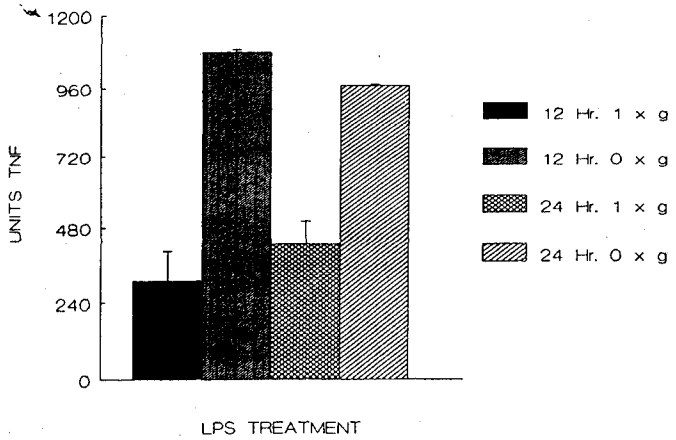

Fig. 3. Production of TNF by B6MP102 during STS-37. B6MP102 cells were pulsed for 12 or 24 h in space or on earth with 12.5 μg of LPS. Each bar represents the results for duplicate samples assayed in triplicate (mean ± SEM).

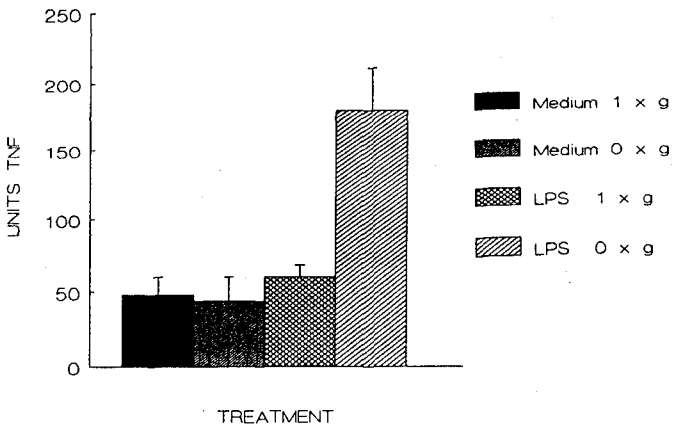

Fig. 5. Production of TNF by B6MP102 during STS-43. B6MP102 cells were incubated for 24 h in space or on earth in medium or medium containing 12.5 μg of LPS. Each bar represents the results for duplicate samples assayed in triplicate (mean ± SEM).

pernatants were assayed for thymocyte proliferation, approximately 4660 units of IL-1 was released by ground controls with a 12-h stimulation, compared to approximately 14,280 in space (space = 3.1× ground, $P < .05$). Approximately 3685 units of IL-1 was secreted with a 24-h stimulation by B6MP102 cells on the ground, compared to approximately 13,750 units secreted in space (space = 3.7× ground, $P < .05$) (**Fig. 4**).

Cytokine Secretion by B6MP102 Cells During STS-43

Because significantly more cytokine was secreted during the STS-37 flight, compared to samples stimulated on earth, we wanted to confirm our results on a subsequent shuttle flight. Since no qualitative difference was observed between the 12- and 24-h incubation periods, we modified the experiment on STS-43 to look for spontaneous activation of B6MP102 cells during space travel. On STS-43, B6MP102 cells were incubated for 24 h in space, or on the ground, in medium with or without 12.5 μg of LPS. B6MP102 cells not stimulated with LPS produced 44–48 units of TNF in space or on the ground, respectively (**Fig. 5**). In contrast, LPS-stimulated B6MP102 ground control cells released approximately 60 units of TNF compared to approximately 180 units of TNF released by B6MP102 cells in space (space = 3× ground, $P < .05$) (Fig. 5). A similar trend was seen for IL-1 production. Medium-incubated B6MP102 cells produced 517 or 632 units of IL-1 on the ground or in space, respectively. LPS-stimulated B6MP102 cells produced approximately 1605 units of IL-1 on earth compared to approximately 3331 units of IL-1 in space (space = 2.1× ground, $P < .05$) (**Fig. 6**).

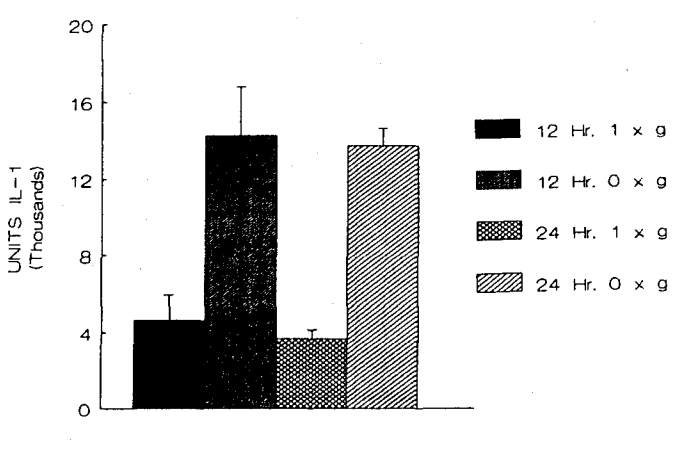

Fig. 4. Production of IL-1 by B6MP102 during STS-37. B6MP102 cells were pulsed for 12 or 24 h in space or on earth with 12.5 μg of LPS. Each bar represents the results for duplicate samples assayed in triplicate (mean ± SEM).

Viability of Lymphocytes

To determine the approximate viability of the lymphocytes in our experiments, we analyzed aliquots of our ground controls, which were kept in syringes and at temperatures similar to those for the flight samples. Based on these samplings, the mean viability of the SL cells was 91% at launch and 86% at the time of completion of the last inflight experiment on STS-37. The mean viability of the LNCs flown on STS-43 was 85.5% at launch and 74% at the time of completion of the 48-h experiment. The mean viability of human lymphocytes was 83% at launch and 71% at completion of the 48-h experiment. In addition, trypan blue viability determinations were done on the lymphocyte cultures upon their return to earth, within 4 h after landing. On STS-37 the splenic lymphocyte viability was 25% (based on five flight syringes) some 4 days after the last sample was taken. On STS-43 the mean viability of the LNCs was 36% and the human lymphocyte viability was 65%, 7 days after the last flight sample was taken.

Interferon-α Secretion by Splenocytes During STS-37

There was only a marginal difference ($P = .15$) between the controls (3.9 IU/ml) and the flight samples (5.4 IU/ml) in the 24-min samples. However, 1 h after stimulation the ground controls secreted 8.2 IU/ml compared to 15.6 IU/ml secreted in space (**Fig. 7**). This was 1.9 times more in space than in the controls ($P = .001$). By 14 h after stimulation 30.1 IU/ml had been secreted by the controls compared to 83.7 IU/ml secreted in space (20 h after reaching orbit). This is 2.8 times more IFN-α secretion ($P < .001$) in space than on earth.

Interferon-γ Secretion by Lymphocytes During STS-43

The mean IFN-γ secretion by ground control mouse T cells was 7.3 IU/ml 24 h after Con A stimulation, compared to

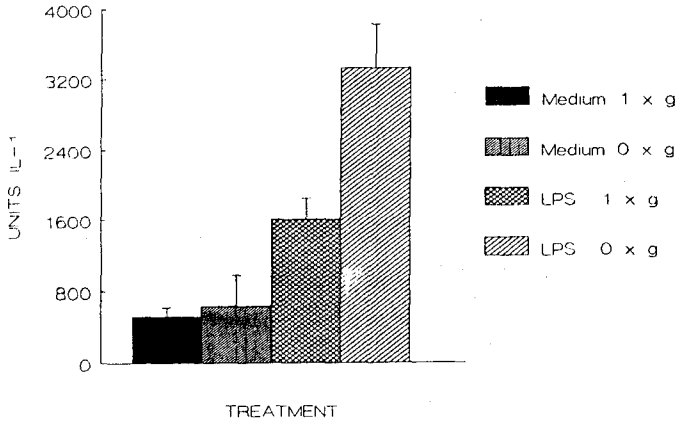

Fig. 6. Production of IL-1 by B6MP102 during STS-43. B6MP102 cells were incubated for 24 h in space or on earth in medium or medium containing 12.5 μg of LPS. Each bar represents the results for duplicate samples assayed in triplicate (mean ± SEM).

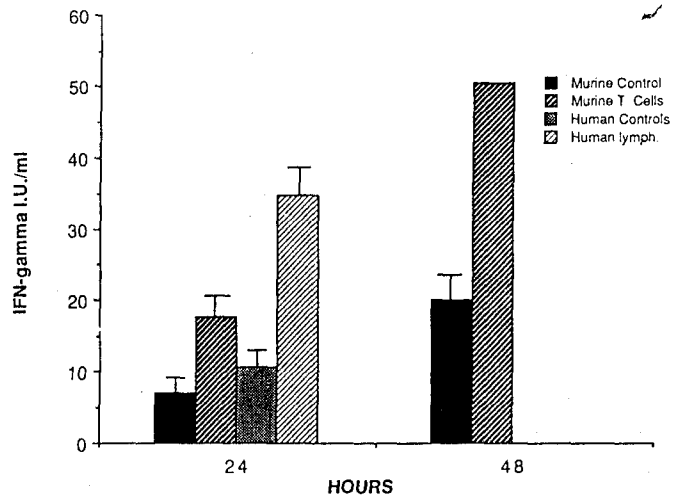

Fig. 8. Production of IFN-γ by T lymphocytes during STS-43. Human and murine T lymphocytes were incubated with 1.5 μg/ml of Con A for 48 h in space or on earth. Flight samples were taken at 24 and 48 h after stimulation. Each bar represents the mean IFN-γ of duplicate samples ± SEM except for murine lymph node T cells at 48 h, where flight constraints allowed the collection of only one sample.

17.7 IU/ml secreted by flight cells (**Fig. 8**). This was 2.6 times more in space than by the ground controls (P = .018). The mean IFN-γ secretion by the control human lymphocytes was 10.8 IU/ml, compared to 34.8 IU/ml secreted in space. This was 3.2 times more in space than by the controls (P = .026). By 48 h after Con A stimulation (~72 h after reaching orbit) 20 IU/ml had been secreted by the control mouse LN cells compared to 50.6 IU/ml secreted by LN cells in space. This is 2.6 times greater secretion in space than on earth. We note that only one replicate was collected for the 48-h murine LN space sample because of flight constraints. However, the trend toward increased secretion by that sample was consistent with the 24-h murine and human samples.

DISCUSSION

Few have had the opportunity to investigate the effects of a space environment on cytokine secretion by macrophages or other immune cells. The data presented here were obtained on two shuttle flights in 1991. Lipopolysaccharide-induced cytokine secretion by B6MP102 cells was significantly more than secretion by control cells stimulated on earth. These observations are in contrast to those of Limouse et al. for samples flown on the Soviet biosatellite Cosmos 2044 [12]. They found that the THP-1 monocyte cell line produced similar or decreased amounts of IL-1, depending on whether the cells were stimulated during the coculture with Jurkat T cells and anti-CD-3 monoclonal antibody or directly with phorbol ester. Obvious explanations for the discrepancy include differences in cell lines and/or experimental conditions. The latter is exemplified by the fact that THP-1 cells variably secreted IL-1 in response to different stimuli [12]. Another explanation for the differences might be that our cells were attached to microcarrier beads during the activation process. It is not clear whether the THP-1 cells were attached to a surface. Macrophage activity may be dependent on anchorage to a matrix and the concomitant changes that occur in the attached cell's cytoskeleton, on which secretion may be dependent [13, 14].

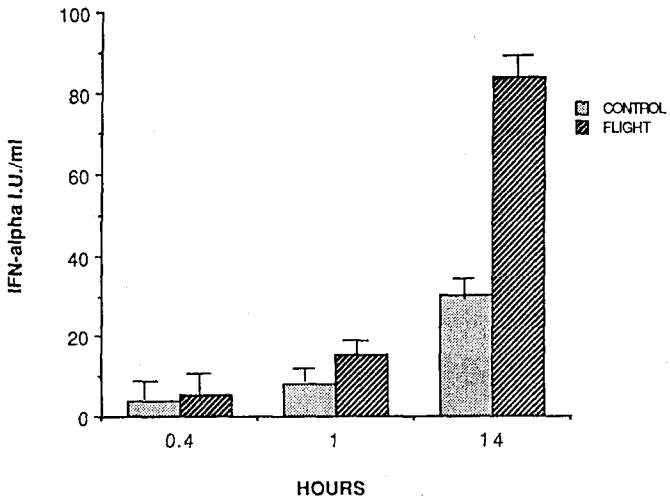

Fig. 7. Production of IFN-α by splenocytes during STS-37. Mouse splenocytes were induced for 14 h with 20 μg/ml of poly I:C during space flight or on earth. Each bar represents the results of duplicate assays for IFN-α in each of three replicate cell syringe experiments where the medium was sampled at the indicated times after addition of the poly I:C (mean ± SEM).

The increase in IFN-α release during STS-37 confirmed the findings of Talas et al. [3] of increased in vitro secretion during 6 days of space flight. However, this was the first time the initial release was studied 6 h after orbital insertion and within 14 h after induction with poly I:C. This is important because all cells exhibit homeostatic responses to dramatic changes in their local environment. Launch vibrations, fluid shear, and rapid temperature changes could have caused significant changes in cell function [15]. In addition, cell stress could have induced dramatic temporary changes in cell physiology [16, 17]. The rapid synthesis of "stress proteins" and temporary cessation of normal protein synthesis during stress responses could have affected our experiments. The fact that detectable levels of IFN-α were found 24 min after induction on STS-37 suggests that secretion of IFN-α by SLs, in response to poly I:C, was not compromised by stress responses to environmental changes during launch.

The increased secretion of IFN-γ from T cells in space contrasts with the results of Gould et al. [4]. They reported that the capacity of rat spleen cells to release IFN-γ was dramatically inhibited after the rats had flown 1 week in space on SL-3. One possible reason for this discrepancy is that T cells may increase their secretion of IFN-γ soon after the

beginning of space flight but may have a different response if they are activated after being in space for longer periods of time. Alternatively, the flight times and other conditions (e.g., apogee or perigee), which usually are not duplicated between flights, may contribute to some of the observed differences. Remarkably, however, the results we obtained on STS-37 and STS-43 showing increased secretion are consistent (with our own data and those obtained in other shuttle flights [3]) in spite of differences in flight times and conditions. Finally, we must exercise caution when comparing in vivo or ex vivo physiological experiments with in vitro cell biology experiments. An appropriate time course study is required to illuminate the IFN-γ response of T cells in vitro during a week-long space flight.

The mechanism(s) underlying enhanced cytokine secretion is unknown. However, the cytoskeleton may play an important part in controlling cytokine secretion. The cytoskeleton is a complex intracellular network that plays a role in cellular attachment, movement, and exocytosis [18] and may be affected by microgravity [19, 20]. We have found that inflammatory cell production of O_2^- is enhanced by microgravity [21]. Because others have suggested that the production of reactive oxygen species may be dependent on the cytoskeleton [22] and preliminary studies suggest that microgravity may also enhance actin polymerization in human polymorphonuclear neutrophils (W. Wiesmann, Walter Reed Army Institute, personal communication), there is a precedent for this hypothesis. Furthermore, treatment of cells with substances that alter the cytoskeleton alters cellular secretion [13, 14]. Interleukin-1β has been associated with microtubules [23]. Therefore, it is not unreasonable to assume that IL-1 and TNF secretion may be altered if microgravity alters the cellular microtubule network.

Nevertheless, it is possible that other cell components are affected by space flight and are responsible for alterations in secretion. Todd has cataloged a number of intracellular structures that theoretically could be affected by gravity if they were suspended in a fluid environment [19, 20], including organelles such as mitochondria. In addition to possible effects on cellular structures that participate directly or indirectly in the exocytosis events of secretion, the reduced-gravity environment could alter secretion by elimination of gravity-driven microconvection currents in the cultures [19]. It is possible that such an environment causes an enhanced autocrine secretory response that is not observed when gravity-dependent processes move secreted substances away from the local microenvironment. In like fashion, increased secretion of TNF and IL-1 may be due to increased production of O_2^- [24], which also can be enhanced by microgravity [21].

Although the results of these cell biology experiments do not necessarily reflect what will happen in vivo, it is important to determine whether similar processes occur in vivo during space travel. They may not be similar. Rose et al. found that antiorthostatic suspension of mice resulted in reduced IFN-α production by the mice [25]. However, murine antiorthostatic suspension has yet to be correlated with space flight results. Interferons-α and -γ are physiological biological response modifiers and may induce increased macrophage activity including increased monokine secretion [26]. The monokines IL-1 and TNF are potent inducers of bone resorption [26, 27]. Therefore, if increased physiological concentrations of interferon, IL-1, or TNF are induced during space flight, it may be detrimental. In fact, bone resorption does occur during space flight [28]; whether this is due to increased cytokine production is unknown. In addition, Manié et al. [29] presented data indicating a trend toward greater IL-1β production after than before flight by phorbol ester-activated human monocytes isolated from the same individuals after and before flight. Therefore, based on the data currently available, the effects of space travel on cellular cytokine production should be given serious consideration.

ACKNOWLEDGMENTS

It is impossible to name all the individuals who helped in the successful completion of these space flight experiments; however, we are thankful for their contributions. In particular, we thank Mr. Steven Hoynowski for helping prepare B6MP102 cells; Dr. Hammad Atassi, Dr. Fatma Shaban, and Ms. Elizabeth Pham, Baylor College of Medicine (BCM), for their assistance in preparing the murine lymphocytes and conducting the interferon assays; Mr. Ken Buyle, Ms. Lou Ann Claassen, and Drs. Marvin Luttges and Louis Stodieck for their contributions to the planning of these experiments; and the astronauts of STS-37 and -43, especially Drs. Jay Apt and Shannon Lucid, who executed the experiments in space. This research was supported by BioServe Space Technologies, NASA contract NAGW-1197, NASA Specialized Center of Research and Training grant NAGW-2328, U.S. Army Medical Research and Development Command grant DAMD17-89-Z-9039, and NASA contract NAS9-18065 to the BCM.

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Antiorthostatic suspension as a model for the effects of spaceflight on the immune system

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Abstract: We describe the use and appropriateness of antiorthostatic suspension in immunological investigations. This manuscript describes the model and discusses how well data obtained by using the model correlate with spaceflight data. This review concludes with some suggestions for future experiments using antiorthostatic suspension. ***J. Leukoc. Biol.*** **54: 227-235; 1993.**

Key Words: *space immunology • gravitational biology*

WHAT IS ANTIORTHOSTATIC SUSPENSION?

Antiorthostatic suspension is a technique developed in response to the need for studying physiological systems that change in a microgravity environment (defined by Todd as "The prolonged free-fall condition . . . 10^{-6}-10^{-4} *g* . . . ." [1]). Because access to space was (and still remains) limited, it was necessary to have a way to induce changes in vivo that reasonably mimicked or paralleled those induced by microgravity. There is ample evidence from postflight studies of human peripheral blood that various cells of the immune system are affected by microgravity. The study of these effects requires a ground-based model system. Parabolic flight and drop towers can be used to study the effects of the downward gravitational vector on objects, animals, or people on Earth. Because parabolic flights and drop towers only offer very short periods of microgravity, the development of models had to be done within the constraints of gravity.

Various models of immobilization, immobilization with head-down tilt, and stress have been used to investigate specific physiological changes, such as muscle disuse or elevated stress hormones [2,3]. However, no model completely satisfied investigators. In 1979 Morey and colleagues [4,5] described a model that simulated an aspect of spaceflight (unloading weight from part of the body). The technique allowed for the suspension of rats at a 30° head-down tilt. The hind limbs of the rat were completely unloaded while the front limbs bore weight and were able to grasp the floor grid. The rats were able to move in a 360° circle at will. In 1980 Musacchia et al. [6] described the unloading of rats with harnesses that allowed movement in an arch of 140°. Ilyin and Novikov [7], in Russia, also were early proponents of rodent antiorthostatic suspension. They used pulleys and harnesses to unload the hind limbs of rats for the study of bone changes [8]. The early techniques were successful because reproducible bone, fluid, and muscle changes could be induced (**Table 1**). However, limited range of movement and stress were drawbacks. The model described by Morey and other variations are often referred to as antiorthostatic (orientation with the head down), hypokinetic, hypodynamic (restricted-movement) suspension models. Other commonly used names include spinal traction, tail traction, harness suspension, and skeletal unloading with head down tilt.

DIFFERENT METHODS OF ANTIORTHOSTATIC SUSPENSION

Antiorthostatic suspension has undergone considerable evolution since its inception in the late 1970s. In the original model described by Morey [4] for rats, room-temperature-vulcanizing silicone rubber glue was used to attach formed, orthopedic casting material contoured to the shaved back of the rat; this material was then used to attach the rat to a free-moving aluminum bar. This technique allowed the rat to have limited, voluntary, 360° movement and permitted some grooming. The head-down tilt caused fluid and organ shifts toward the upper part of the body. Harness suspension eliminated the need for shaving the back of the rat and using of silicone glue but restricted movement somewhat.

Manipulation imposed stress on animals. Stress is often defined physiologically by a significant increase in corticosterone concentration in serum [35, 36], which can negatively affect immune cells and organs [9, 14]. The pulley suspension device appears to overcome the problem of restrictive movement and stress. Wronski and Morey-Holton [14] popularized the use of free-moving pulleys, first used by Ilyin's group [8]. Morey-Holton's group used pulleys that rolled on the top of Plexiglass cages and allowed voluntary movement in two dimensions. Tail suspension also was adapted for use with the pulleys. Together, tail suspension and free movement in two dimensions was found to be superior to back suspension [14]. Tail-suspended animals gained weight and showed a smaller increase in corticosterone concentrations and less lymphoid organ atrophy. Thus, with animals suspended in this manner, the effects of unloading could be selectively studied.

Antiorthostatic suspension also has been adapted for mice by using both the harness [9, 27, 37] and tail methods [16, 17, 31, 38, 39] of unloading. In some studies, mice were tail-suspended from cables in 29 cm x 18 cm x 13 cm cages (**Fig. 1A** and B). The tail-suspension technique allowed mice to have access to the entire cage, with 180° movement to the left and right, and prevented use of the hind limbs for movement

Abbreviations: Con A, concanavalin A; INF-γ, interferon-γ; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; NK cell, natural killer cell; PHA, phytohemagglutinin; SRBC, sheep red blood cell.

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Table 1. Summary of Physiological and Immunological Changes Induced by Antiorthostatic Suspension

Physiological variables	Suspension model (species)	Reference
Muscle		
Soleus and gastrocnemius muscle atrophy[a]	Antiorthostatic/harness (rat)	2, 6, 9, 10, 11
Muscle glucocorticoid receptor expression	Whole body/harness (rat)	12
Change in soleus muscle fiber distribution	Antiorthostatic/harness (rat)	13
Bone		
Diminished periosteal bone formation rate[a]	Antiorthostatic/back suspension (rat)	4, 8
Trabecular bone volume[a]		
Marrow fat[a]	Antiorthostatic/tail (rat)	14, 15
Tibiofibular junction bone formation[a]		
Bone strength decrease[a]	Antiorthostatic/tail (mouse)	5, 16, 17
Blood and plasma		
Neck subcutaneous tissue pressure[a]	Antiorthostatic/tail (rat)	18, 19
Central venous pressure increase	Antiorthostatic/tail (rat)	20, 21
Heart rate, arterial pressure, and volume increase	Antiorthostatic/back (rat)	22
Increased red blood cell count depressed erythroporesis	Antiorthostatic/tail and body harness (rat)	23, 24, 25
Unchanged anti-sheep red blood cell antibody response	Antiorthostatic/back suspension (rat)	26
Depressed interferon α/β response in vivo	Antiorthostatic/harness (mouse)	27, 28
Depressed resistant to encephalomyocarditis virus-D		
Increased interferon-γ response in vivo	Antiorthostatic/ortho-static/harness (rats)	28
Cell-mediated immunity		
Unchanged splenic natural killer cell cytotoxicity	Antiorthostatic/tail suspension (rat, mouse)	30, In press[b]
Depressed PMN respiratory burst	Antiorthostatic/tail suspension (mouse)	31
Unchanged peritoneal Mϕ cytokine secretion & IA antigen expression	Antiorthostatic/tail suspension (mouse)	32
Depressed bone marrow macrophage precursors[a]	Antiorthostatic/tail (rat, mouse)	33, In press[b]
Lymphoid organ specific effects of antiorthostatic suspension[a]	Antiorthostatic/tail (rat, mouse)	32, 34, In press[b]

[a]Indicates a correlation with physiological responses induced by spaceflight.
[b]J.W. Armstrong, K.A. Nelson, S.J. Simske, M. Luttges, J.J. Iandolo, S.K. Chapes (1993), *J. Appl. Physiol.* In press.

or support. Although that method of tail suspension proved quite useful, recent work, using adaptations of the Wronski-Morey-Holton tail suspension cage [14] for mouse suspensions (Fig. 1C and D), suggests that the Wronski-Morey-Holton system is significantly less stressful. Comparisons were made of serum or plasma corticosterone concentrations and percentages of initial body weight lost of mice tail-suspended in 29 cm x 18 cm x 13 cm suspension cages and mice suspended by their tails using the Wronski-Morey-Holton pulley suspension technique (**Table 2** and Fig. 1). Although the comparison was done retrospectively (pulley-suspension mice were compared with mice used in a previous study [32]), the mice were matched for strain, sex, and weight. The antiorthostatic suspension, with pulleys (Fig. 1C and D) and two-dimensional access to the cage, appeared to be the lesser stressful of the two environments.

PHYSIOLOGICAL CHANGES INDUCED BY ANTIORTHOSTATIC SUSPENSION AND THEIR RELATION TO SPACEFLIGHT

One strength of antiorthostatic suspension is that it induces many physiological changes that parallel those induced by spaceflight (Table 1). For example, suspension has been particularly useful for studies on bone structure and composition [3]. Bones taken from mice and rats suspended antiorthostatically have a lower bone mass than do weight-bearing controls. Furthermore, this lower bone mass is accompanied by lower bone strength [16, 17]. Similar phenomena occur in rats and humans during spaceflight [3]. Rats flown in space have lower bone growth rates. Similarly, growth arrest lines have been seen in bones from antiorthostatically suspended rats and bones from rats flown in space [4,5]. Rats flown in space as well as antiorthostatically suspended rats have decreased numbers of osteoblasts, but numbers returned to normal after flight [3].

Antiorthostatic suspension also successfully models the fluid shifts and muscle atrophy associated with spaceflight. Hargens et al. [18] used tail suspension to show that fluids move toward the head in antiorthostatically suspended rats, a response seen in humans during spaceflight and in other simulated forms of microgravity [40]. Tipton et al. [19] suggested that rats suspended with a 30° head-down tilt that bear 50% of their body weight resemble humans flying in space in that they exhibit "head and neck puffiness." Mussachia's group [6] specifically developed their method of antiorthostatic suspension to study changes in unloaded muscles. They and others have shown that changes in the soleus muscle most closely parallel the muscle atrophy associated with spaceflight in mice and rats [9, 10, 41], with the gastrocnemius muscle also being affected in a fiber-dependent manner [9-11]. Other variables such as oxidative capacity, glycolytic enzyme activities, and capillarization also change in response to unloading (for review see ref. 41).

Antiorthostatic suspension also has been used to study variables associated with the blood and circulation with various degrees of success. Shellock et al. [20] found that rat central venous blood pressure may be increased in response to suspension and is suspension-angle dependent. This type of response is also seen in astronauts [21], but the capacity for adaptation may be different. Furthermore, increases in hematocrits, reduction in plasma volume, suppressed erythropoiesis, and reduction in red cell mass are seen in antiorthostatically suspended mice as well as in humans in space [23].

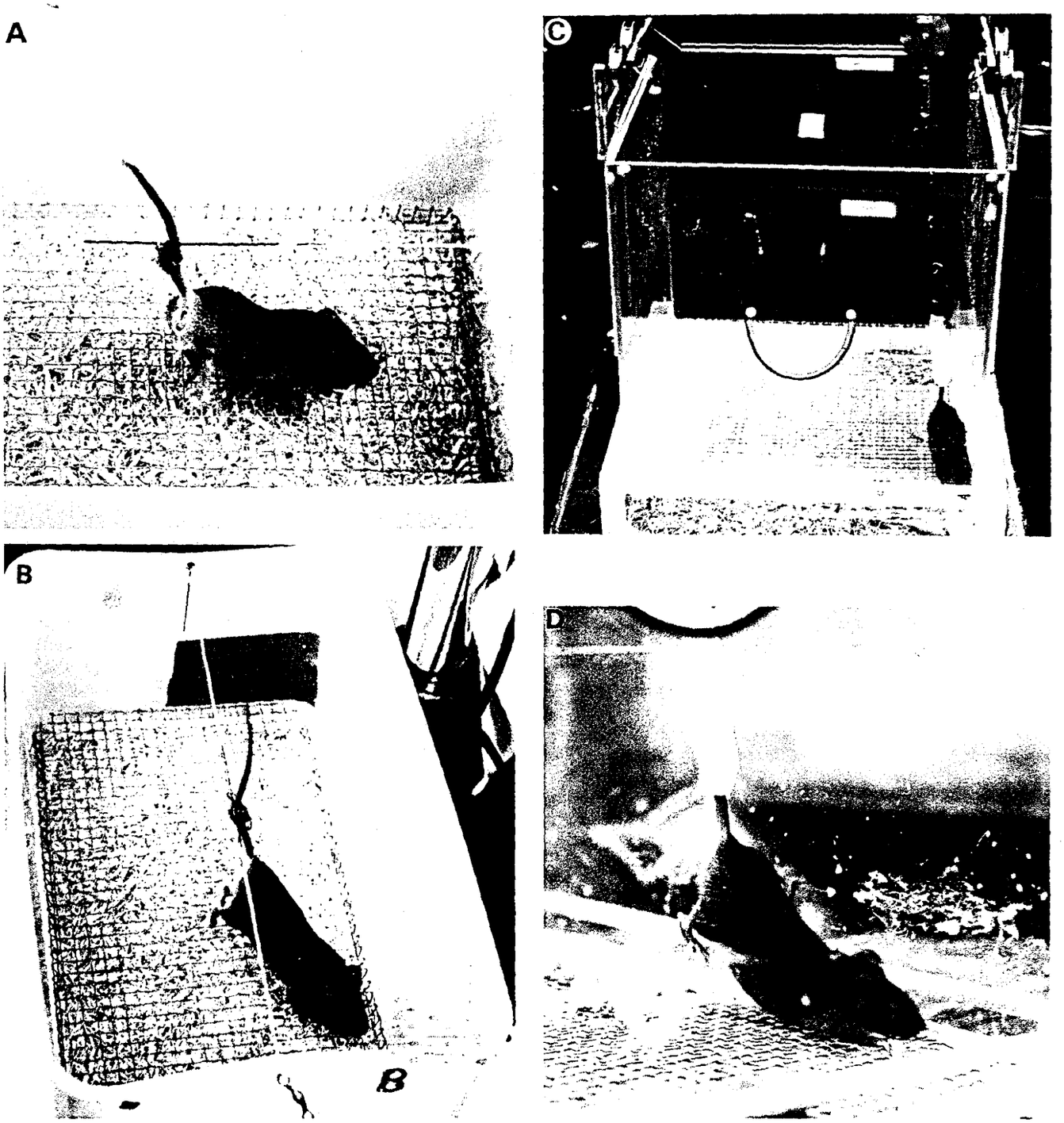

Fig. 1. Mice antiorthostatically tail-suspended in 29 cm × 18 cm × 23 cm cages on nylon cables, A and B; or using pulley suspension, C and D.

THE DISTINCTION OF ANTIORTHOSTATIC SUSPENSION FROM STRESS MODELS

Spaceflight involves more than microgravity. In addition to increased gravitational forces during takeoff and landing, spaceship occupants must adapt to a whole new environment. This adaptation may involve stress, but stress is not the sole variable of spaceflight. Likewise, there is a misconception that placing rodents in antiorthostatic suspension is analogous to subjecting them to models designed solely to induce stress, i.e., high or low temperatures, treadmill exercises, noise, swimming, or sleep deprivation. Furthermore, some people believe that because none of these stress models would be used to mimic microgravity, it is unlikely that antiorthostatic suspension would be any more appropriate. Admittedly, it is impossible to escape the effects of gravity while remaining on Earth. Therefore, regardless of how well physiological models parallel changes seen during spaceflight, no model will be perfect. Because so many diverse systems are affected by antiorthostatic suspension in ways similar or closely related to spaceflight (Table 1), it becomes apparent that this is the most acceptable known ground-based model

Table 2. Comparison of Body Weight and Corticosterone Levels with two Different Suspension Techniques

Technique[a]	Suspension *Treatment*[b]	Initial body weight lost (%)	Corticosterone (ng/ml)
A	Antiorthostatic	10.6 ± 1.1[c]	320 ± 42[d,e]
A	Orthostatic	7.4 ± 1.6[e]	123 ± 14[e]
A	None	1.1 ± 0.8[d]	70 ± 15[d]
B	Antiorthostatic	8.3 ± 1.0[d]	123 ± 16[d,e]
B	Orthostatic	4.0 ± 0.8[e]	87 ± 12[e]
B	None	−0.8 ± 2.3[d]	43 ± 10[d]

[a]Mice were suspended in 29 cm × 18 cm × 13 cm cages (group A; see Fig. 1A and B) or in Wronski-Morey-Holton suspension cages [14] (group B; see Fig. 1C and D). Mice in groups A and B were sex (male) and weight matched. Group A plasma and group B serum corticosterone concentrations were compared.

[b]Mice were antiorthostatically or orthostatically suspended or were normally housed for 11 days.

[c]Numbers represent $\bar{x}$ ± SEM, n = 20 for all comparisons.

[d]$P < .05$, normal cage control or antiorthostatic suspension treatment vs. orthostatic suspension treatment (Student's matched t-test).

[e]$P < .05$, suspension group A vs. suspension group B (Student's matched t-test).

of spaceflight. It is much more appropriate than other models designed solely to stress animals. The fluid shifts toward the upper parts of the body, muscle atrophy, and bone resorption occur in response to unloading. None of these processes is approximated by any of the abovementioned animal stress models. The question is whether or not we can study effects of weightlessness on the immune system apart from the effects of increased corticosteroid, a stress-induced hormone.

The neuroendocrine link to the immune system has not been overlooked in antiorthostatic suspension modeling. Stress hormones significantly influence cellular responsiveness, and several investigators included the measurement of stress hormones in their studies. Fleming [31] et al. and Kopydlowski et al. [32] found that with mice, antiorthostatic suspension was associated with significant increases in corticosterone concentrations and splenic atrophy. Caren et al. [26] found splenic atrophy in rats antiorthostatically suspended for 22 days. Similar atrophy of lymphoid organs was observed when rats were flown in space for 22 days [42]. However, we previously noted that some of the increased corticosterone concentrations may be due to the antiorthostatic technique used (Table 2). Harness suspension of rats results in a transient rise in corticosterone for 1-3 days before it returns to normal [12]. Rats subjected to antiorthostatic suspension by the pulley system [14] tended to have higher corticosterone concentrations than did control rats, but the differences were not statistically significant [34]. Corticosterone concentrations were only slightly elevated in mice subjected to the same suspension technique (Table 2). Corticosterone concentrations of rats flown on Cosmos 1887 did not differ from controls, but adrenal weight data suggested that the flight may have affected glucocorticoid concentrations [43]. The levels of stress induced by antiorthostatic suspension and spaceflight appear to be comparable. Likewise, humans also have been observed to be stressed and have elevated cortisol concentrations after spaceflight [44, 45]. Therefore, the model appears to be appropriate.

ANTIORTHOSTATIC SUSPENSION AND ITS EFFECT ON IMMUNE RESPONSES: COMPARISONS WITH DATA OBTAINED FROM SPACEFLIGHT

The lack of spaceflight opportunities provided the impetus for the development of ground-based physiological models. Spaceflight has validated the antiorthostatic suspension model for a number of physiological variables. There have been fewer opportunities to validate the model for the study of the immune system because these studies have often been difficult to control and execute. Therefore, the results have been mixed. We have attempted to summarize some of the most important comparisons (Table 1).

Antiorthostatic suspension was first used to study changes in the immune system in 1980. Caren et al. [26] investigated whether rats immunized with sheep red blood cells (SRBCs) had altered anti-SRBC responses. Little difference was found between the 21-day antiorthostatically suspended rats and the normal cage controls or orthostatically suspended weight-bearing controls (animals restrained in a horizontal position and allowed to bear weight). Although these data have never been directly correlated with actual spaceflight data, it is interesting that Voss [46] found no measurable differences in total blood immunoglobulin concentrations of astronauts [37].

The phenotypes of cells found in various lymphoid tissues have been studied after antiorthostatic suspension and spaceflight. There does not appear to be a direct correlation between these two treatments. For example, Sonnenfeld et al. [33, 47] found that spaceflight significantly enhanced rat spleen cell expression of $CD4^+$ and $CD8^+$ cells in two different experiments and immunoglobulin-bearing cells (B cells) in one of two experiments. Neither $CD4^+$ nor $CD8^+$ T cells were elevated in rat splenic populations after antiorthostatic suspension [34, 47]. Likewise, mice did not show a significant change in cellular distribution of peripheral blood or peritoneal inflammatory cells after antiorthostatic suspension [31, 32]. This finding with mice was not consistent with the postflight neutrophilia or monocytopenia seen in shuttle crew peripheral blood [44, 48]. Furthermore, rat antiorthostatic suspension did not induce the general increase of $CD4^+$ T cells in rats [34] that was seen in peripheral blood cells of shuttle astronauts postflight [44]. Although these data may appear to be disappointing to advocates of the antiorthostatic suspension model, the humans and animals that flew in space were also subjected to the rigors and conditions of re-entry and readaptation to gravity. No study using antiorthostatic suspension was designed to account for recovery and readaptation. Furthermore, no study was directly matched for times and tissues sampled.

In vitro responses of cells obtained from animals subjected to antiorthostatic suspension have been studied more than have the humoral components of the immune system. Lymphocyte proliferation has become a standard measure of cellular immune responsiveness after spaceflight. Extensive study of peripheral blood lymphocyte responses has been done for both U.S. astronauts and Russian cosmonauts. In general, most workers reported that spaceflight led to a depressed response of peripheral blood cells to mitogens in culture [44, 48, 49]. In contrast, in one experiment, Mandel and Balish [50] found that flight rats showed an enhanced splenic proliferative response to mitogens. However, as they point out, ". . . the small number of rats in each group and

the large standard deviations in the data indicate that caution should be exercised in interpreting the results." Importantly, only peripheral blood lymphocytes from humans in space have been examined.

In more recent experiments with lymphocytes from rats in space, differences in mitogen responsiveness were found to depend on the lymphoid origin of the cells. In the Cosmos 2044 mission, inguinal lymph node cells from rats were tested for proliferation and interleukin-2 (IL-2) production in response to mitogenic lectins or phorbol esters and ionomycin. Proliferation or IL-2 production by lymphocytes from the flight rats was not significantly different from that of the controls [51]. However, when the lymphocytes from the spleens of the same animals were tested for mitogen responsiveness, the lymphocytes from the flight animals gave a depressed proliferative response (A. Lesnyak, Institute for Biomedical Problems, Moscow, Russia, personal communication, 1989). A similar lymphoid organ-specific response was seen after a 4-day flight aboard the U.S. space shuttle STS-41 [51]. In this case, lymph node lymphocytes gave a depressed proliferative response to concanavalin A (Con A) whereas splenocyte proliferation did not differ from that of ground controls. Moreover, this organ-specific variation was seen after antiorthostatic suspension. Nash et al. [34] reported that there were differences in rat lymphocyte responses depending on the lymphoid organ source of the lymphocytes. Peripheral blood and anterior lymph node lymphocyte proliferation, induced by Con A and phytohemagglutinin (PHA), but not splenic lymphocyte proliferation was significantly depressed by antiorthostatic suspension. Kopydlowski et al. [32] reported that antiorthostatic suspension of mice results in the enhanced proliferation of splenic lymphocytes to PHA. Direct comparison of splenic lymphocyte proliferation and lymph node lymphocyte proliferation shows that antiorthostatic suspension of mice also had a lymphoid organ-dependent effect (**Table 3**) as it does in rats [34]. When antiorthostatic suspension stimulated splenic lymphocyte proliferation induced by PHA, a suppressive effect was seen with lymphocytes from the lymph node.

These organ-specific differences are important for several reasons. First, they indicate that suppression of lymphocyte responsiveness is not likely due to increased plasma corticosteriods. If it were, a whole-body effect rather than a tissue-specific effect would be expected. Second, they suggest that fluid shifts caused by microgravity may be very important. Lymphocyte migration and homing are undoubtedly affected by fluid shifts. Finally, because only a small percentage of the total lymphocytes are found in blood at any time, a blood sample may tell only a limited story. In summary, although a complete cross-tabulation has not been made for all species, for all lymphoid organs, under all conditions, there appears to be some consistency between spaceflight and antiorthostatic suspension with regard to organ-specific mitogen-induced cellular proliferation.

T cell activation is associated with increased expression of IL-2 receptors (IL-2R). IL-2R expression of mitogen-stimulated T cells after antiorthostatic suspension has been examined. Nash et al. [34] found that Con A-stimulated spleen cells from antiorthostatically suspended rats expressed a similar number of IL-2R as did mitogen-stimulated cells from control animals. In all cases, as expected, the unstimulated lymphocytes expressed very few IL-2 receptors. This lack of significant IL-2R expression by unstimulated cells contrasts with the IL-2R expression on unstimulated spleen cells isolated from rats that flew on two Cosmos biosatellites. IL-2R expression of spleen cells from animals flown on Cosmos 1887 was significantly enhanced compared with ground control animals [33]. Spleen cells from animals flown on Cosmos 2044 showed a trend toward greater expression than did controls; however, the values were not statistically significant [47]. Because of a lack of statistical significance in the Cosmos 2044 flight data, there was no significant correlation of IL-2R expression between spleen cells isolated from flight rats and control, antiorthostatically suspended rats; however, both show the same trend [47]. The variability in IL-2R expression after spaceflight makes it hard to compare suspension model data with flight data. More studies must be done with IL-2R expression, both in spaceflight and with antiorthostatic suspension models.

In contrast to cytokine receptors, cytokine secretory production has been more extensively studied in rats and mice after antiorthostatic suspension [27-29, 52, 53]. The ability of rat spleen cells, taken from animals suspended for 1 or 2 weeks, to produce interferon-γ (IFN-γ) was enhanced [53]. Interestingly, IL-1 and IL-2 production by these cells remained at the same level as in cells from nonsuspended control animals. Therefore, not all cytokines behaved similarly. Nash et al. [34] found that the ability of mitogen-stimulated lymphocyte secretion of IL-2 was not affected by 7 days of antiorthostatic suspension, which was consistent with the lack of change in IL-2 secretion after spaceflight. The enhanced production of INF-γ was not consistent with the findings of Gould et al. [46] who found that spleen cells taken from rats flown in space for 1 week produced less IFN-

Table 3. Effect of Antiorthostatic Suspension on PHA-Induced Proliferation of Mouse Lymphocytes[a]

Lymphocytes[b]	Suspension	Stimulation index[c]	cpm incorporated	
			+ PHA	- PHA
Spleen	Antiorthostatic	24 ± 2[d]	114,099 ± 16,707[d]	4,799 ± 614
Spleen	Orthostatic	12 ± 1	60,297 ± 13,765	5,121 ± 571
Spleen	None	13 ± 0	82,221 ± 6,604	6,160 ± 498
Lymph node	Antiorthostatic	52 ± 5	88,584 ± 17,732	1,711 ± 188
Lymph node	Orthostatic	116 ± 9	145,000 ± 24,430	1,249 ± 279
Lymph node	None	146 ± 9	121,565 ± 14,906	835 ± 189

[a] Adapted from Armstrong et al. (1993) *J. Appl. Physiol.* In press.

[b] Lymphocytes were isolated from the spleen or inguinal lymph nodes after C3HeB/FeJ mice were suspended or normally housed for 11 days. Animals were killed immediately after removal from the suspension or regular cage.

[c] Stimulation index = [(CPM + PHA)/(CPM - PHA)] × 100.

[d] Numbers represent $\bar{x}$ ± SD of quadruplicate samples. Cells from three animals were pooled and used in each group.

γ. One possible explanation for the discrepancy is that the stress of flight recovery negatively affects the responses of spleen cells. However, the IL-3 secretion of those same cells was not significantly different than that of spleen cells obtained from control rats [29]. Therefore, cytokine regulation appears to be a complex process, subject to subtle physiological perturbations. This complexity is exemplified by the findings that secretion of cytokines by isolated cells is often enhanced significantly by spaceflight when cells are not subject to the complex in vivo physiological environment [54, 55] and the magnitude of the response can be stimulus dependent [56].

Splenic natural killer (NK) cell activities were compared directly after antiorthostatic suspension and spaceflight. Rykova et al. [30] found that rat splenic NK activity against Yac-1 target cells was depressed after spaceflight but not after antiorthostatic suspension . Interestingly, splenic NK activity against the human NK target, K562, was not depressed, whereas bone marrow cell activity was [30]. Antiorthostatic suspension did not affect bone marrow-mediated NK cytotoxicity of K562 [30]. Likewise, mouse splenic NK activity against Yac-1 cells was not affected by antiorthostatic suspension (**Table 4**). The diminished activity of the NK cells recovered from spaceflight rats was apparently not due to defects in their ability to bind targets in vitro [30]. Because NK activity has been studied in such few instances, additional work must be completed to confirm these findings.

Bone marrow macrophage precursors have been investigated in both rat and mouse systems. This variable has been reproducibly validated by spaceflight. Antiorthostatic suspension of rats was done in conjunction with the flight of rats in space in the Cosmos 2044 flight. Both these treatments reduce the number of macrophage precursors found in the bone marrow of weight-bearing bones [47]. This finding confirmed previous flight data [33]. In addition, mice subjected to 11-day antiorthostatic suspension also had diminished numbers of macrophage precursors (**Table 5**). Therefore, the changes in bone, which may be associated with changes in local concentrations of substances such as prostaglandin E_2, 1,25 dihydroxyvitamin D_3 (calcitriol), and transforming growth factor-β, may affect the development of macrophages [57]. Circulating concentrations of calcitriol are significantly depressed in rats after antiorthostatic suspension [58, 59]. Interestingly, the expression of the phenotypic marker associated with rat suppressor T cells, OX-8, was enhanced in bone marrow cells in one of two spaceflights in which it was examined [33, 47], but it was not enhanced by antiorthostatic suspension [47]. Pan T cell (W3/13), helper T cell (W3/25), and IL-2R (OX-39) expression on bone marrow cells was also reproducibly enhanced by spaceflight but not by antiorthostatic suspension [33, 47]. Therefore, cell types other than macrophages possible are affected differently by antiorthostatic suspension and spaceflight. Myriad regulators in the bone marrow could differentially alter macrophages [57]. Alternatively, mature cells, which are measured by these phenotypes, may be affected differently from immature cells. The finding that rat hemopoietic stem cell numbers are also depressed by spaceflight would be consistent with the latter hypothesis [60].

Table 4. Mouse Natural Killer Cell Activity in Response to Antiorthostatic Suspension[a]

	Specific ^{51}Cr Release (%)		
Suspension[b]	(E:T)[c] 200:1	100:1	50:1
Antiorthostatic	24 ± 1	16 ± 1	11 ± 1
Orthostatic	23 ± 0	20 ± 0	11 ± 1
None	25 ± 1	19 ± 1	11 ± 1

[a]Adapted from Armstrong et al. (1993) *J. Appl. Physiol.* In press.
[b]C3HeB/FeJ mice were suspended or normally housed for 11 days before being killed immediately upon removal from the suspension or regular cage.
[c]Spleen cells were incubated with 1 × 10^4 ^{51}Cr-prelabeled Yac-1 cells at the indicated effector target ratio for 4 h. Cells for each suspension treatment were pooled from three mice.

Table 5. Number of Macrophage Colonies Formed in Response to Macrophage Colony Stimulating Factor After Antiorthostatic Suspension of mice[a]

Suspension[b]	Number of colonies[c]
Antiorthostatic	5.5 ± 0.1
Orthostatic	13.5 ± 0.3
None	13.2 ± 0.2

[a]Adapted from Armstrong et al. (1993) *J. Appl. Physiol.* In press.
[b]C3HeB/FeJ mice were suspended or normally housed for 11 days before being killed immediately upon removal from the suspension or regular cage.
[c] $\bar{x}$ ± SEM of colonies after 7 days of culture in soft agar, five view fields per plate, three plates per mouse n = 6 mice per treatment $P < .05$ using Student's t-test, Antiorthostatic vs. Orthostatic control.

ANTIORTHOSTATIC SUSPENSION AND ITS EFFECT ON IMMUNE RESPONSES: EXPERIMENTS THAT NEED TO BE DONE IN SPACE

Some immunological variables have been studied in response to antiorthostatic suspension but have not yet been examined in response to spaceflight. These variables include inflammatory neutrophils, activated macrophages, and cytokines associated with their activation. For example, studies to elucidate the effects of antiorthostatic suspension on murine peritoneal inflammatory cells showed that neutrophils have reduced ability to produce superoxide anion [31]. Follow-up studies showed that the reduced neutrophil response is not due to corticosterone but to factors associated directly with antiorthostatic suspension [61]. Kopydlowski et al. [32] did a comprehensive analysis of the effects of antiorthostatic suspension on macrophage functional, secretory, and membrane molecule expression. They found that neither the secretion of IL-1, tumor necrosis factor, prostaglandin E_2, and O_2^- nor the expression of Ia antigens, Con A, receptors or *Bandeiraea simplicifolia* receptors were affected by antiorthostatic suspension. A similar lack of effect on macrophage-mediated cytotoxicity of tumor or virus-infected cells also was reported [32]. Interestingly, recent data by Manié et al. [62] suggest that IL-1 secretion by human peripheral blood mononuclear cells is unaffected by spaceflight. These findings are important because macrophage secretion of IL-1 and tumor necrosis factor and the expression of class II and other plasma membrane molecules are central to both innate and acquired immune responsiveness.

Sonnenfeld's group has studied the production of murine interferon-α/β by lymphocytes and its concentration in serum [28, 53]. One- and 2-week antiorthostatic suspension significantly reduced the amount of IFN α/β produced by cells in serum [28]. Splenic lymphocytes also produced less IFN-α/β than did controls after 1-week suspensions and more IFN-α/β if the cells were taken from animals that were suspended for 2 weeks [53]. Encephalomyocarditis-D virus was able to infect antiorthostatically suspended animals but

not orthostatically suspended or control animals [27]. Therefore, as suggested by Sonnenfeld et al. [37], reduced IFN concentrations may allow the growth of pathogenic microorganisms in space because of the amelioration of a natural control mechanism.

FUTURE PROSPECTS FOR THE ANTIORTHOSTATIC SUSPENSION MODEL

There are at least two ways to evaluate the utility of the antiorthostatic suspension model for use in immunological studies: (1) immune variables in rodents subjected to spaceflight and antiorthostatic suspension can be compared and (2) changes in response to rodent antiorthostatic suspension can be compared with the space-induced changes seen in humans. However, only a relatively few studies have been done that allow comparisons between antiorthostatic suspension and spaceflight. Mitogen-induced lymphocyte proliferation is a response that is of significant interest because the organ-dependent response of rodent spleen cells, lymph node cells, and peripheral blood cells appears comparable between spaceflight and antiorthostatic suspension. The decline of rodent macrophage precursors in the bone marrow was also seen after antiorthostatic suspension and spaceflight. Although similar studies have not been done in humans, a monocytopenia seen in astronaut peripheral blood after spaceflight may indicate changes in the number of monocyte precursors arriving from the bone marrow [44].

The subpopulations of lymphoid cells isolated from various rodent organs after spaceflight did not match the cells isolated from rodents after antiorthostatic suspension or compare with cells isolated from humans, but few studies have been done. Also differing for rodents were IFN-γ secretion and NK cell cytotoxicity. Again, generalizations cannot be based on only one or two studies. Because of this lack of information, some results may be due to a peculiarity of an individual spaceflight or suspension protocol. There also may be differences between mouse and rat immune responses that have yet to be considered. The differences in rat spleen cell expression of IL-2R on unstimulated cells that were analyzed after Cosmos 1788 and 2044 [33, 47] exemplify this problem. The IL-2R expression was elevated but not statistically different from ground controls on the latter flight but was significantly different on the former. Thus, it becomes difficult to determine what the standard spaceflight response really is and what antiorthostatic suspension data should be compared with. Furthermore, the immunologic effects of microgravity are likely due to the interaction of organismic-level physiological responses with direct effects on immune cells themselves. Antiorthostatic suspension allows modeling of the organism-level effects of microgravity but not the direct effects of microgravity on internal cellular processes [1]. Therefore, differences should be expected between the immunologic effects of microgravity and those observed in suspension modeling.

Because of the relatively few immunological studies that use antiorthostatic modeling, it would be premature to dismiss the model as inappropriate for studying the immune response. In fact, some of the results indicate that the model may be useful in elucidating mechanisms of cellular regulation and development. There are three areas that warrant further investigation: (1) the cause and effect of bone marrow macrophage precursor reduction, (2) the compartmentalization of lymphocyte proliferative responsiveness in different lymphoid organs, and (3) the effect of recovery from antiorthostatic suspension on the immune system.

Antiorthostatic suspension results in fewer macrophage precursors in the bone marrow. Nevertheless, the number of blood monocytes and the activity of inflammatory macrophages in the peritoneum after an 11-day suspension are not different from those of control animals [31, 32]. This is an interesting conundrum that suggests that the short periods of fewer precursor macrophages do not affect the peripheral blood and inflammatory macrophage pool. However, nothing is known about the effects of longer antiorthostatic suspensions. Because macrophages are long-lived cells, there may be a significant lag time before fewer monocytic and inflammatory cells are seen the blood or peritoneum. Also interesting is that bone changes significantly in response to antiorthostatic suspension. What causes the diminution in the bone marrow macrophage precursors? Studies on cytokines and other cell regulators would be of interest.

Studies using antiorthostatic suspension suggest that lymphocyte proliferation is affected in a lymphoid-organ-dependent fashion [34](J.W. Armstrong et al., *J. Appl. Physiol.*, in press; 1993). This is one of the most recent and interesting results to come out of antiorthostatic suspension experiments; it correlates with the results of spaceflight and suggests that a compartmentalization may occur [51, 52]. Suspension, like spaceflight, may affect lymphocyte trafficking. The fluid shifts seen in both also may affect immune cell composition and response. The antiorthostatic suspension technique will be valuable in determining how cell trafficking and fluid shifts affect specific lymphoid cell populations. In particular, the antiorthostatic suspension technique can and should be used to address why different lengths of spaceflight (e.g., Cosmos 2044, 13.8 days, and STS-41, 4 days) or flight conditions (Russian biosatellite vs. U.S. space shuttle) differentially affect the responses seen within different lymphoid organs. Finally, the differential tissue response indicates that stress, in general, is unlikely to account for the depressed proliferation of lymphocytes in select lymphoid organs. Because this phenomenon is seen in other stress models [63], pulley suspension allows for the investigation of lymphoid organ–dependent lymphocyte regulation in the absence of high concentrations of corticosterone.

Although recovery has been looked at in studies of bone, no immunological studies published to date included effects of recovery of animals after antiorthostatic suspension. Readaptation has played a role in virtually every cellular response that has been measured for rats and humans returning from space. Immunologists have not yet investigated this potentially important factor with antiorthostatic suspension and should make it a priority.

In conclusion, spaceflight offers a challenge and an opportunity not only to the engineer but to the life scientist. The living organism is complex, and finding a model to adequately address one aspect of the environment, gravity, on this system is difficult. In addition the limitations of space, both in numbers and kinds of experiments, make imperative the use of ground-based models to generate hypotheses. The antiorthostatic suspension model, when properly validated, offers this possibility.

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BRIEF COMMUNICATION

Class I and class II major histocompatibility molecules play a role in bone marrow-derived macrophage development

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Abstract: Class I and class II major histocompatibility complex (MHC) molecules play significant roles in T cell development and immune function. We show that MHCI- and MHCII-deficient mice have low numbers of macrophage precursors and circulating monocytes, as well as abnormal bone marrow cell colony-stimulating factor type 1 secretion and bone composition. We suggest that MHCI and MHCII molecules play a significant role in macrophage development. *J. Leukoc. Biol.* 55: 658–661; 1994.

Key Words: *MHC · macrophages · colony-stimulating factor 1 · hematopoiesis*

The continual replenishment of macrophages is crucial for a fully functional immune system because of their role in both cell-mediated and humoral immunity [1]. Although the contributions of cytokines [1, 2] and extracellular matrix proteins [3, 4, 4a] to macrophage development have been studied, little information is available on the role of major histocompatibility complex (MHC) molecules in macrophage development. The recent availability of transgenic mice deficient for MHCI or MHCII molecules (Genpharm, Mountain View, CA) enabled us to investigate the process of macrophage development in the absence of these molecules. ment in the absence of these molecules.

The MHCI and MHCII expression on cells from C1D mice (H-2^b, MHCI deficient) and C2D mice (H-2^b, MHCII deficient) was examined to ensure that class I and class II molecules, respectively, were absent. **Figure 1** demonstrates a normal MHCI and MHCII phenotype on cells from normal B6 mice (H-2^b), which were used as controls in these experiments. The flow cytometric histograms for cells from C1D and C2D mice show no staining for MHCI and MHCII molecules, respectively. These results indicate appropriate lack of MHC expression and confirm findings of previous studies [5, 6].

The assessment of macrophage precursors in the bone marrow is often done using an in vitro colony-forming assay [7] containing exogenous macrophage colony-stimulating factor (CSF-1). We used this technique to assay bone marrow macrophage precursors from C1D, C2D, B6, and C3HeB/FeJ mice. Although the haplotype of C3HeB/FeJ mice (H-2^k) does not match that of C1D and C2D mice, this strain was used as an additional control drawing upon our familiarity with C3HeB/FeJ bone marrow-derived macrophages [8]. Little difference was found in spleen weight when expressed as a percentage of body weight (B6 0.350 ± 0.027, n = 8; FeJ 0.386 ± 0.011, n = 2; C1D 0.359 ± 0.030, n = 7; C2D 0.0292 ± 0.014, n = 7; no significance between groups at 95% confidence level, ANOVA) or the number of femur bone marrow cells recovered from each strain of mice (B6, 6.3 ± 0.4 × 10^7, n = 4; FeJ, 3.6 ± 0.3 × 10^7, n = 4; C1D, 7.0 ± 0.6 × 10^7, n = 5; C2D, 5.8 ± 0.6 × 10^7, n = 7). However, marrow cell recovery was subject to some variation due to the methodology used for bone marrow cell removal. In contrast, a significant depression in macrophage colony formation was seen when C1D and C2D bone marrow cells were compared with C3HeB/FeJ and B6 controls (Fig. 2A). The depression suggests that macrophage hematopoiesis is suppressed in mice lacking MHCI or MHCII molecules. Whether the depression in macrophage precursors affected the repertoire of mature macrophages in vivo was evaluated. Mice were injected intraperitoneally with thioglycolate broth and peritoneal macrophage inflammation was measured (Fig. 2B). It was found that the number of inflammatory peritoneal macrophages in C1D and C2D mice was significantly lower than in C3HeB/FeJ and B6 controls. This result supports the findings of the in vitro colony-forming assay and provides strong evidence that a physiological defect exists involving macrophage hematopoiesis in mice lacking MHCI or MHCII molecules. Further evidence for a depressed macrophage hematopoietic process in MHCII-deficient mice was obtained by comparing the number of monocytes in the circulation in C2D and normal mice. B6 mice had significantly more monocytes (3.9 ± 1.1 × 10^4 monocytes/ml, n = 5) than C2D mice (1.84 ± 0.72 × 10^4 monocytes/ml, n = 4; significance determined by a Mann-Whitney nonparametric test).

Macrophage development from bone marrow progenitor cells can be perturbed by the alteration of one or a number of factors [2]. Variables include cytokine concentrations [2, 9], inhibitory endocrine factors [10], and bone structure including extracellular matrix composition [3, 4, 4a]. Therefore, we compared the cytokine secretory activity [interleukin-6 (IL-6), transforming growth factor-β (TGF-β), and CSF-1] of bone marrow cells from C1D, C2D, and normal mice (**Fig. 3**). IL-6 is an essential cytokine for normal macrophage development, for both early macrophage progenitor cell proliferation [9] and later macrophage differentiation [11]. In fact, if IL-6 is present at unusually low or high concentrations, macrophage development may be suppressed [9, 12]. We found that freshly isolated bone marrow cells from C3HeB/FeJ, B6, C1D, and C2D mice all secreted similar amounts of IL-6 in an 18-h incubation assay (Fig. 3A). This suggests that differences in IL-6 secretory activity of bone marrow cells were not responsible for the

Abbreviations: ANOVA, analysis of variance; CSF-1, colony-stimulating factor type 1; IL-6, interleukin-6; MHC, major histocompatibility complex; TGF-β, transforming growth factor β.

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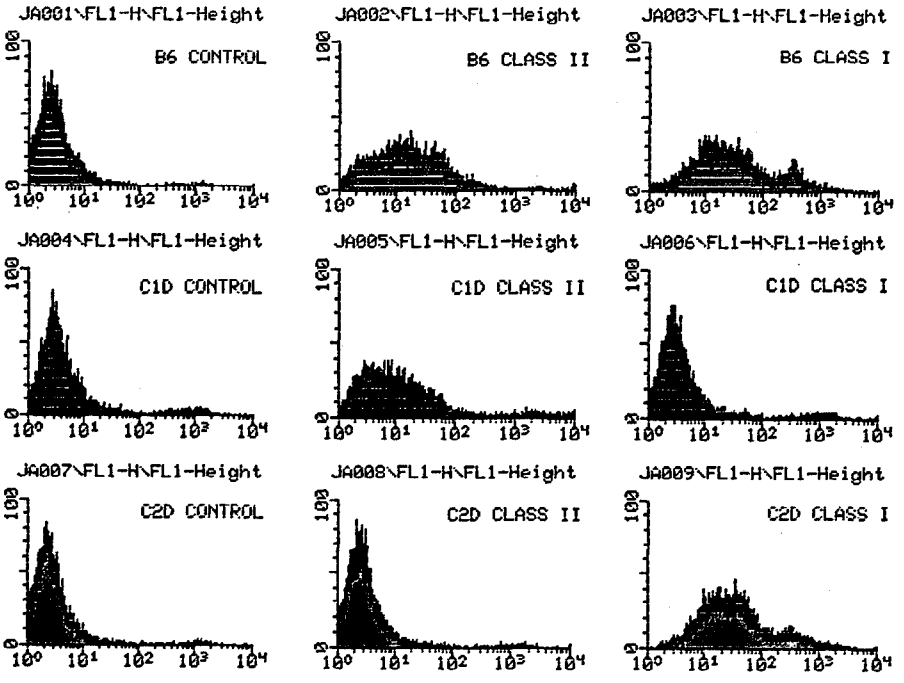

Fig. 1. MHC molecule expression on thymocytes from age-matched C1D, B6, and C2D mice (8–12 weeks of age). Single cell suspensions were formed from three freshly removed thymi and incubated with primary antibody [Y-3 anti-K^b (anti-MHCI; a generous gift from Dr. David Lee, University of Missouri, Columbia) or hybridoma HB-183 (ATCC, Rockville, MD) derived anti I-A (anti MHCII)] and fluoresceinated secondary antibody [$F(ab')_2$; Cappel, Durham, NC] for 30 min, washed three times, and analyzed by flow cytometry (FACScan, Becton-Dickinson, MA). A representative of three experiments is shown. (Left) Control; (middle) stained with anti-class II antibody; (right) stained with anti-class I antibody; (top) B6 cells; (middle) C1D cells; (bottom) C2D cells.

depressed macrophage hematopoiesis. TGF-β has diverse effects on many different cell types including macrophages and macrophage progenitor cells [10, 13]. As the actions of TGF-β on macrophage hematopoiesis are considered to be inhibitory [10, 13] we tested for the secretion of both the active and latent forms of this molecule from freshly isolated bone marrow cells (Fig. 3B and C). Secretion of both the active and latent forms of TGF-β did not differ between all cell types tested. In contrast, CSF-1 concentrations in the same cell supernatants differed ($P < .05$) in both MHCI- and MHCII-deficient cell cultures compared to normal controls (Fig. 3D). Surprisingly, we discovered higher rather than lower concentrations of CSF-1 secreted by both C1D and C2D transgenic compared to control mouse bone marrow cells. One possible interpretation is that macrophage development in MHCI- and MHCII-deficient mice is depressed because of down-regulation of CSF-1 receptors as a result of abnormally high levels of CSF-1 and consequent CSF-1-mediated cellular activation [14]. This hypothesis is at variance with results of studies using different colony-stimulating factors [15]. For example, overproduction of granulocyte-macrophage colony-stimulating factor (GM-CSF) led to enhanced numbers of peritoneal macrophages without changes in circulating monocytes [15]. Our result clearly appears to document the different biological properties of the colony-stimulating factors.

Upon removing femora and tibiae of C1D and C2D mice, we noticed what appeared to be differences between the bones of C3HeB/FeJ, B6, C1D, and C2D mice. Potential differences in bone were quantified with regard to composition and strength (**Table 1**). These tests were considered important because cytokines can affect bone resorption [17] and changes in bone would be expected to affect the bone marrow microenvironment [4a, 18]. We found that the bones of C3HeB/FeJ mice contained more mineral and were stiffer and stronger than the bones of mice of the $H\text{-}2^b$ haplotype (B6, C1D, and C2D; $P < .01$). This difference was not unexpected given the substantially different bone properties observed in different strains of mice [19]. When comparing normal B6 mice with C1D and C2D mice, we found a correlation between absence of MHC molecules and both lower mineral percentage (%Min) and reduced bone microhardness. This was consistent with the difference between B6 and pooled C1D and C2D humerus for microhardness ($P = .09$). Interestingly, the difference in microhardness was not consistent with the trend observed for stiffness, which was nearly identical among the three groups. Strength was generally greater in the bones of C1D and C2D mice than those of B6 mice. However, stiffness and strength tests measure overall characteristics of bone and do not necessarily contradict specific microhardness and mineralization data. When the microhardness was normalized by bone strength, the values for the B6 humeri (13.1 ± 1.7 kg/N mm^2) were significantly different ($P = .02$) from those for the pooled C1D and C2D humeri (11.4 ± 2.0 kg/N mm^2). These differences between B6 compared to C1D and C2D humeri in terms of MHCI and MHCII molecules and bone characteristics may result from differences in bone formation and/or bone remodeling processes. As noted above, the correlated changes in bone characteristics and depressed macrophage haematopoiesis are important for two reasons: (1) colony-stimulating factors can affect bone resorption [17], resulting in altered bone properties, and (2) the interrelated nature of the precursor cells for bone and immune cells [19, 20].

Macrophage development is impaired in mice deficient in MHCI or MHCII molecules. Based on our investigations, the changes in macrophage development may be the result of abnormal levels of CSF-1 and/or changes in bone structure and dynamics. Alternatively, macrophages could require functional MHC molecules during their development. During U937 monocytic differentiation, characteristic peptides are presented at certain stages of the developmental process by MHCI molecules [23]. Absence of MHC could directly affect differentiation. However, macrophage progenitor cell

MHCII expression is low and remains low before cellular activation [1] and does not support this mechanism. Therefore, we predict that in the case of MHCII-deficient mice (and probably MHCI-deficient mice) depressed hematopoiesis is not the result of a lack of MHC expression on macrophages or macrophage progenitor cells. It is more likely that in the absence of MHC molecules, macrophage development is affected by changes in crucial nursing factors such as cytokine expression, local bone constituents, and extracellular matrix and stromal cell populations. This hypothesis would be consistent with the cell and bone relationships described in osteopetrotic mice [24, 25] and mice lacking the proto-oncogene c-*fos* [26]. Shown here, the MHC-deficient mice offer a novel model in which to study both macrophages and bone development as well as the interdependences in their development.

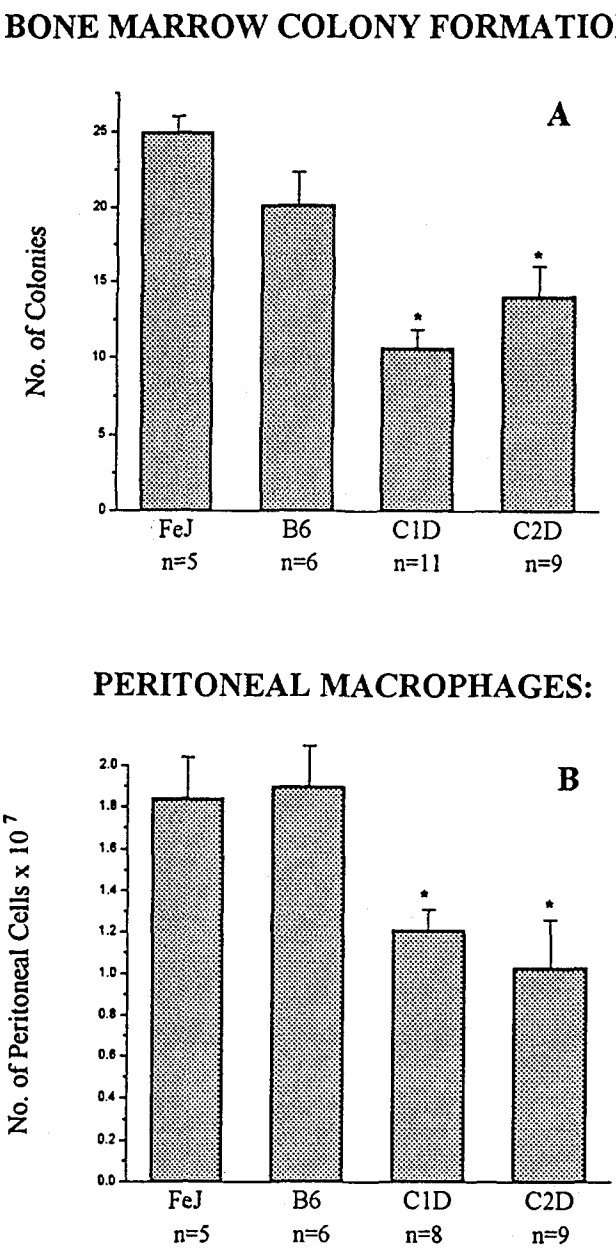

Fig. 2. (A) Bone marrow-derived macrophage colony formation in mice lacking MHCI or MHCII molecules. Bone marrow cells were suspended at a concentration of 1×10^5 cells per 1.5 ml of Dulbecco's modified Eagle's medium (DMEM) containing 0.3% agar, 10% fetal bovine serum (FBS), and 15% LM929 fibroblast-conditioned medium. On day 6 of culture, five microscope view fields were scored for macrophage colonies (a group of > 25 cells). $^*P < .05$ compared to C3HeB/FeJ and B6 control mice (*t*-test). (B) Number of thioglycolate-elicited peritoneal macrophages in mice lacking MHCI or MHCII molecules. Mice were injected with 1.5 ml of thioglycolate 4 days before peritoneal lavage with ice-cold phosphate-buffered saline. The number of cells recovered was determined using a hemocytometer. Total numbers of peritoneal cells recovered: FeJ, $1.84 \pm 0.2 \times 10^7$, B6, $1.9 \pm 0.2 \times 10^7$, C1D, $1.21 \pm 0.1 \times 10^7$, C2D, $1.1 \pm 0.3 \times 10^7$. $^*P < .05$ compared to C3HeB/FeJ and B6 control mice (*t*-test).

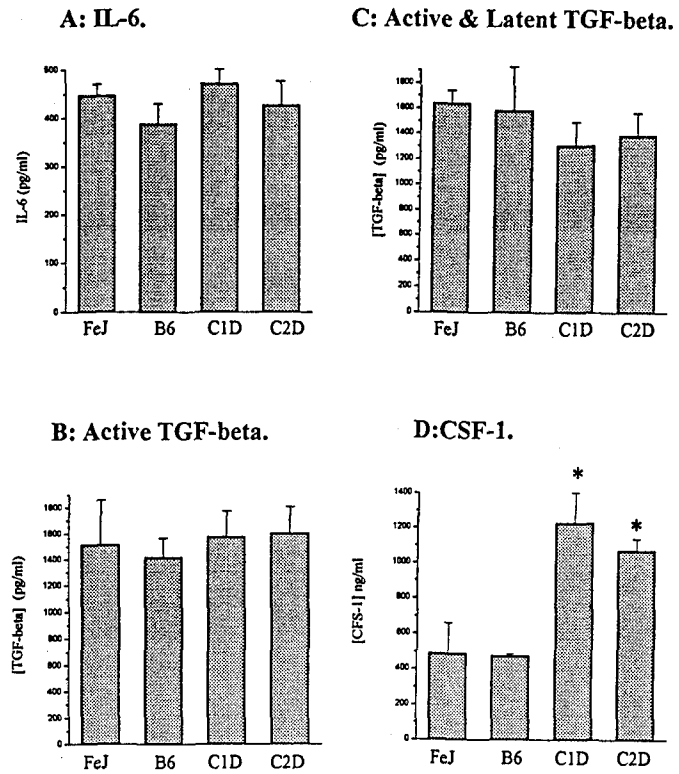

Fig. 3. Interleukin-6, TGF-β, and CSF-1 secretion by bone marrow cells isolated from C3HeB/FeJ, B6, C1D, or C2D mice. Bone marrow cells were obtained from the femora and tibiae of mice. The ends of the femora and tibiae were removed and cells were flushed from the bone using a 26-gauge needle and DMEM supplemented with 2% FBS. Cells were passed three times through a 19-gauge needle to break up cell clumps, pelleted, and then resuspended in RPMI tissue culture medium supplemented with 10% FCS. Cell suspensions were then incubated at 37°C and 5% CO_2. Following an 18-h incubation, the cell suspensions were centrifuged and supernatants were collected and stored at −80°C until analysis. (A) Freshly isolated bone marrow cell IL-6 production (5×10^6 cells/3 ml tissue culture medium). Supernatants were assayed for IL-6 content by bioassay using the IL-6 growth-sensitive cell line B9. Growth was quantified using an MTT assay [4a] and concentrations were determined from an rIL-6 (R&D, Minneapolis, MN) standard curve. (B) Freshly isolated bone marrow cell TGF-β production. Supernatants were assayed for TGF-β content by bioassay using the TGF-β growth-sensitive cell line CCL64 (ATCC, Rockville, MD). TGF-β concentrations were determined by an MTT assay used in conjunction with a rTGF-β (R&D, Minneapolis, MN) standard curve. (C) Freshly isolated bone marrow cell CSF-1 production. Supernatants were assayed for CSF-1 content by bioassay using the CSF-1-dependent cell line B6MP102 [16]. CSF-1 concentrations were determined by bioassay using [^{3}H]thymidine incorporation and an rCSF-1 standard curve. Cell lines used for cytokine bioassays were unaffected by bone marrow cell cytokines other than their specific proliferative cytokine.

TABLE 1. Differences in Bone Mechanical Characteristics for C3HeB/FeJ, B6, C1D, and C2D mice[a]

Mouse type	Femur, %MIN	Humerus		
		Stiffness	Strength	Microhardness
C3HeB/FeJ	69.7 ± 1.9 (11)	65.1 ± 4.7 (2)	11.0 ± 0.1 (2)	111 ± 1 (2)
B6	64.3 ± 1.7[b,c] (10)	45.3 ± 7.1 (12)	8.4 ± 1.2 (12)	107 ± 6[d] (7)
C1D	63.1 ± 1.9[b] (5)	45.7 ± 7.2 (7)	9.7 ± 1.6 (7)	103 ± 5 (7)
C2D	62.7 ± 1.8[b] (9)	46.6 ± 15.7 (13)	8.8 ± 2.1 (13)	104 ± 8 (7)

[a]Data are presented as mean ± SD and the number of samples per group is indicated in parentheses. The diaphyses of the femora were carefully cleaned of nonosseous tissue, dried at 105°C for 24 h, and weighed (Dry-M), then ashed at 800°C for 24 h and reweighed (Min-M). The percent mineral (%Min) of these diaphyseal sections was calculated from the Min-M/Dry-M ratio. Humeri were cleaned of nonosseous tissue and allowed to dry at 25°C. Three hours before mechanical testing humeri were rehydrated (0.15 N NaCl, 25°C) [21]. The bones were then tested to failure under 3-pt flexure using an Instron 1331 servohydraulic testing system [8]. A deflection rate of 5 mm/min was utilized, and the force deflection (P-δ) properties of stiffness (slope of the P-δ curve during elastic loading) and maximum force (P_m), or strength, were evaluated. Humeri were allowed to dehydrate for 48 h at room temperature, and these samples were embedded in a clear epoxy (Epo-Kwick, Buehler) and sectioned within 0.5 mm of the fracture using an Isomet low-speed diamond saw (Buehler). Sections were polished and loaded into a hardness tester for evaluation of mid-diaphyseal longitudinal microhardness [22]. The microhardness value used was the average of four measures each taken from the mid-diaphysis, at mid-cortex of the lateral, medial, ventral, and dorsal locations of bone samples. Statistical analysis was done by analysis of variance (ANOVA), followed by Duncan's multiple range test for comparisons among the four mouse strains.

[b]Significantly less than C3H/FeJ value for this assay ($P \leq .01$).

[c]Significantly greater than pooled (C1D and C2D) value for this assay ($P \leq .05$).

[d]Significantly greater than pooled (C1D and C2D) value for this assay ($P < .01$).

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Effects of Corticosterone and Microgravity on Inflammatory Cell Production of Superoxide

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In this investigation we studied the effects of corticosterone and microgravity on *Propionibacterium acnes*-induced inflammatory cells ability to produce superoxide (O_2^-). We found in vitro and in vivo exposure of murine peritoneal inflammatory cells to corticosterone did not inhibit the O_2^- response. We also found that in microgravity *P. acnes*-induced inflammatory cells were capable of producing four times as much O_2^- as at 1*g*. Therefore, neither corticosterone nor microgravity experienced during parabolic flight prevents an O_2^- response by inflammatory cells.

Key words: inflammation, corticosteroids, respiratory burst

INTRODUCTION

Intraperitoneal stimulation of mice with *Propionibacterium acnes* results in a series of identifiable steps. These are characterized by neutrophil immigration, macrophage phagocytosis of PMNs, and the appearance of macrophages that are cytotoxic [4]. The inflammatory response by neutrophils and macrophages to *P. acnes* is well characterized. It is an ideal model to use to study inflammation and inflammatory cells.

Information on immunological responses during space travel is limited. Previous studies have presented pre- and post-flight data. Taylor et al. [31] studied the crew members of 11 space shuttle flights. They found decreases in the number of lymphocytes, eosinophils, and monocytes accompanied by increases in the number of neutrophils. In addition, they state that the crew members with the "greatest losses were those that were exposed to the most stressful flights." It is unknown whether these changes were actually due to stress or microgravity.

Ground based models have also been used to simulate microgravity as there are limited flight opportunities in which to study immune responses. Two models have been tested. The clinostat has been used to study in vitro T cell mitogenesis with the results appearing to accurately reflect the effects of space travel on T cells [7,9]. Antiorthostatic suspension has been found to be an acceptable ground-based model to simulate the physiological conditions of space travel. It has been found to closely simulate the bone loss, muscle atrophy, and fluid shifts associated with space travel [19,29,32]. Because antiorthostatic suspension does reflect physiological changes associated with space travel it has also been used to determine if the immune response is affected [1,3,11, 23,26].

We previously investigated how antiorthostatic suspension affects inflammation and inflammatory cells induced by *P. acnes* [10]. In those studies we found that cells from antiorthostatically suspended mice had an impaired ability to produce superoxide (O_2^-) compared to control cells. This was accompanied by a decreased ability of those cells to kill phagocytosed bacteria. We also found that antiorthostatically suspended mice were subjected to higher levels of stress as indicated by spleen weight determinations and serum corticosterone levels.

Defects in cellular responsiveness can be a result of physiological regulators (glucocorticoids) or the result of defective cells. The purpose of this investigation was to determine at what level superoxide responses by *P. acnes*-induced inflammatory cells might be affected. Specifically, experiments were performed to determine whether increased corticosterone levels or microgravity conditions affected superoxide responses.

MATERIALS AND METHODS

Animals

Corticosterone and suspension studies. Male and female C3HeB/FeJ mice, 7–14 weeks of age, were bred in the animal facilities at Kansas State University.

KC-135 studies. Twelve-week-old C3H/HEN HSD mice obtained from Harlan Sprague Dawley (Indianapolis, IN) were used in these experiments.

Induction of Inflammatory Cells

Inflammatory cells were induced by intraperitoneal injection of 350–700 μg *P. acnes*. Twenty hours after injection, inflammatory cells were collected by washing the peritoneal cavity with 20 ml ice-cold phosphate-

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buffered saline (PBS). The collected cells were washed and resuspended in normal saline (N. saline; KC-135 experiments) or Hanks' buffered salt solution (HBSS; ground-based experiments).

Superoxide Assay

Corticosterone and suspension studies. Superoxide generation induced in response to phorbol-myristate-13-acetate (PMA) was determined by the superoxide dismutase (SOD) -inhibitable reduction of ferricytochrome C. Nine hundred microliters of *P. acnes*-induced peritoneal cells were placed in 12 × 75 mm tubes at a concentration of 4×10^5–1×10^6 cells/tube depending on the experiment. The cells in HBSS were mixed with 100 μl of 80 μM ferricytochrome C, 100 μl (200 U) SOD, and/or 10 μl (10 μg/ml) PMA. The mixtures were incubated for 15–20 min and O_2^- was quantitated by determining reduced cytochrome C at 550 nm on a spectrophotometer in clarified supernatants (centrifuged at 325*g*). An absorbance coefficient of 21.1 mM cm^{-1} was used for the calculation of O_2^-.

KC-135 assays. The assay procedure described above and equipment were modified for use on the National Aeronautics and Space Administration (NASA) KC-135 airplane (NASA 930) that is used for parabolic flights. Two × 10^6 *P. acnes*-induced peritoneal cells in 1 ml N. saline were drawn into a 3 ml syringe. The syringe was connected to a 3-way stopcock which was attached to another 3 ml syringe (Fig. 1). One milliliter of N. saline containing the following reagents was drawn into the second syringe: 160 μM ferricytochrome C per ml; and depending on the assay, 400 U per ml of superoxide dismutase and/or 200 ng per ml PMA. At the appropriate time during the flight (1*g* or microgravity), the syringe containing the desired reagents was mixed with the syringe containing the cells as illustrated in Figure 1. Immediately after mixing, 1 ml of the syringe contents was injected into a flow-through cuvet. To remove cells and air, two 0.45 μm filters and one 0.22 μm filter were in place (Fig. 2). Therefore, the buffer that was injected into the cuvet was cell-free. The initial amount of O_2^- produced was quantitated by measuring reduced cytochrome C at 550 nm on a spectrophotometer. After the appropriate length of time (10–20 s) the remaining aliquot was injected into the flow-through cuvet and the amount of O_2^- produced was charted on a chart recorder. The difference between initial values of O_2^- produced and final values of O_2^- was used as the amount of O_2^- produced during the 10–20 s period. An absorbance coefficient of 21.1 mM cm^{-1} was used for the calculation of O_2^-. The length of time of assay (10–20 s) was taken into account in calculating final values of O_2^- produced.

Reagents

Ferricytochrome C, PMA, and SOD were obtained from Sigma (St. Louis, MO). *P. acnes* (*C. parvum* CN 6134) was obtained from Wellcome Biotechnology Ltd (Research Triangle Park, NC). [^{3}H]-Corticosterone was purchased from NEN Research Products (Boston, MA). The lyophilized corticosterone antiserum B3-163 was purchased from Endocrine Sciences (Tarzana, CA), reconstituted with distilled water, and stored at −110°C.

Corticosterone Radioimmunoassay

Serum samples were treated as previously described [10] with the exceptions noted below. The serum was not extracted with ethyl acetate. A binding curve which paralleled the standard curve was obtained using 2–50 μl of pooled normal mouse serum. A 1:25 dilution of the reconstituted antibody yielded approximately 33% binding.

Statistical Analysis

Student's t-test was used to determine statistical significance of the difference in means using the Number

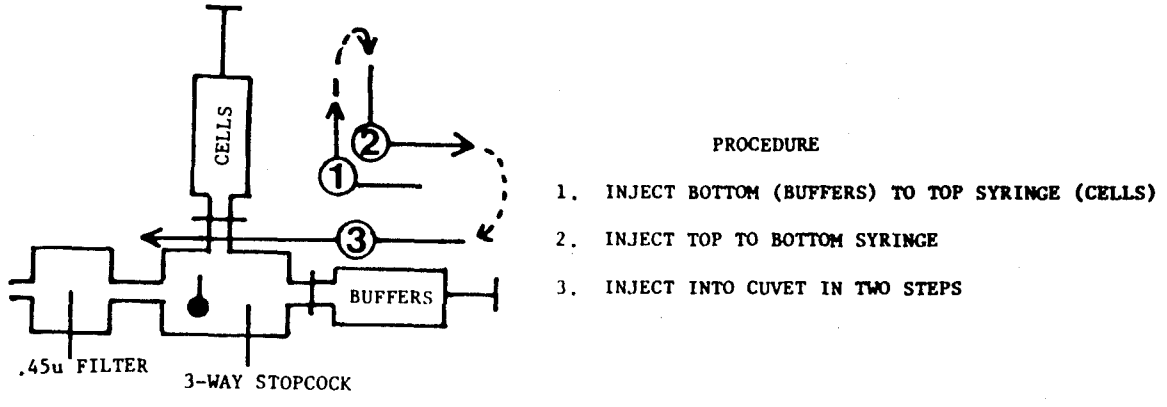

Fig. 1. Schematic diagram of the syringe assemblies used to mix and inject cells into the spectrophotometer for analysis of O_2^- in microgravity and 1*g*.

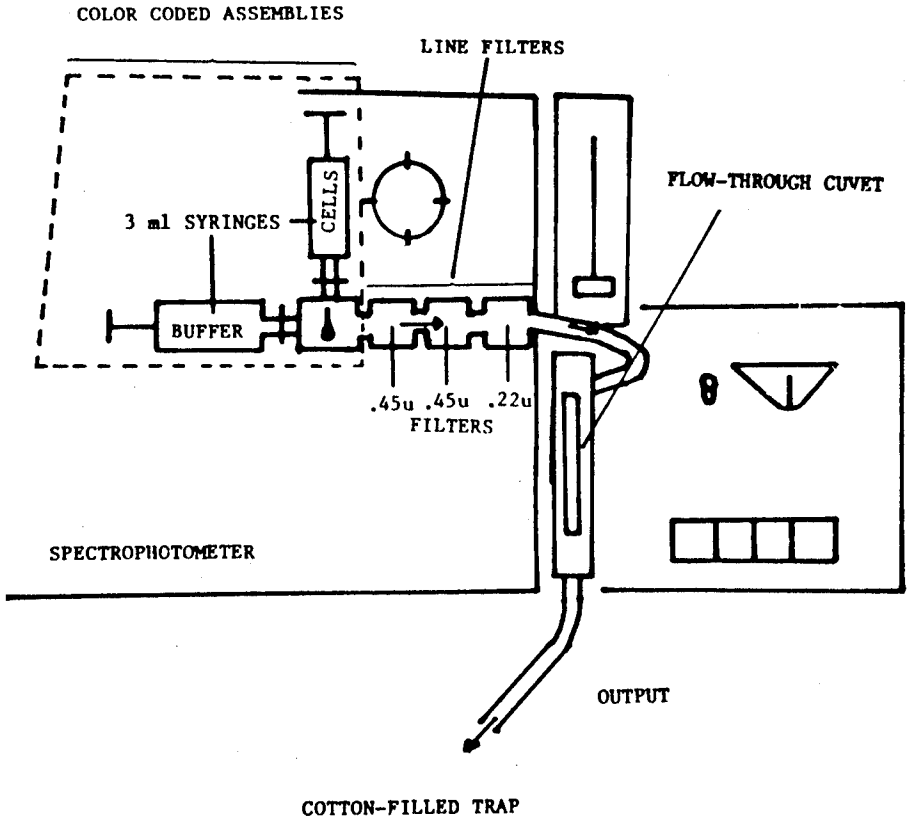

Fig. 2. Schematic diagram of the syringe assembly, filter set-up, and spectrophotometer used for analysis of O_2^- during microgravity and 1*g*.

Cruncher Statistical Package (J.L. Hintze, Kaysville, UT).

RESULTS

Effects of Corticosterone on Superoxide Production

It is well established that glucocorticoids will suppress immune responses including O_2^- production [2,12]. Corticosterone has also been found to inhibit macrophage presentation of antigen in mice [25]. Therefore, in our previous study [10] the depressed superoxide responses of cells isolated from antiorthostatically suspended mice could have been due to higher levels of glucocorticoids, namely corticosterone. To test this hypothesis, we performed a series of experiments to determine the effects of corticosterone on O_2^- production by *P. acnes*-induced murine inflammatory cells.

In the first of these experiments, inflammatory cells were incubated in varying concentrations of corticosterone for a period of 1 hr. We found that cells incubated in concentrations of corticosterone as high as 2,400 ng/ml were unchanged in their ability to produce O_2^- when compared to controls (Table 1). We note that 2,400 ng/ml corticosterone is 4 times as high as the serum corticosterone levels previously reported in any mouse that had been antiorthostatically suspended and about 12 times as high as the group average [10].

It is possible that a 1 hr incubation in corticosterone was not long enough to inhibit an O_2^- response. There-

TABLE 1. Effect of Corticosterone on *P. acnes*-Induced Inflammatory Cell Production of O_2^-

Treatment[a]	nM O_2^- produced
600 ng/ml	0.7 ± 0.2[b]
Control[c]	1.0 ± 0.1
1,200 ng/ml	0.3 ± 0.3
Control[c]	0.4 ± 0.1
2,400 ng/ml	0.4 ± 0.2
Control[c]	0.5 ± 0.2

[a]*P. acnes*-induced inflammatory cells incubated in medium containing various concentrations of corticosterone for 1 hr at 37°C before PMA induction of O_2^-.
[b]Nos. represent $\bar{X}$ ± SEM of triplicate samples.
[c]The corticosterone stock solution was diluted in absolute ethanol. Similar volumes of absolute ethanol were added to medium controls to account for ethanol effects.

fore, *P. acnes*-induced inflammatory cells were incubated in physiological concentrations of corticosterone (600 ng/ml) for up to 3 hr. We attempted longer incubation periods but the viability of neutrophils in vitro was unsatisfactory when they were cultured longer than 3 hr. We found that O_2^- production of cells incubated in corticosterone in vitro for 3 hr was not significantly different from medium-incubated controls (Table 2).

Because O_2^- production by *P. acnes*-induced inflammatory cells might only be affected by long-term, chronic exposure to corticosterone, we attempted additional experiments. In these experiments we fed mice corticosterone for a period of 11–12 days, induced inflammatory cells with *P. acnes*, and tested the cells for their ability to produce O_2^-. We found that those mice fed corticosterone had glucocorticoid-induced splenic atrophy (Tables 3, 5). Furthermore, we found a significant increase in serum corticosterone levels in the mice which had been fed corticosterone (667 ng/ml) as compared with control mice, with an average of 51 ng/ml (Table 3). Although feeding mice corticosterone had a dramatic effect on serum corticosterone levels, inflammatory cells taken from those animals produced O_2^- similar to cells from control animals.

Differential counts were made of the peritoneal exudate cells (Table 4). We found the control mice to have approximately 63% PMNs, 9% lymphocytes, and 19% macrophages. Corticosterone-fed mice had 87% PMNs, 4% lymphocytes, and 8% macrophages. Using chi-square analysis, this was a significant difference ($P < .1$). Table 4 also illustrates a difference in total cells present in the peritoneum 20 hr after ip injection of *P. acnes*. The experimental mice had an average of 1×10^7 cells compared to 3.6×10^6 peritoneal cells from control mice. We previously demonstrated that peritoneal PMNs have a greater capacity to produce O_2^- than macrophages [5]. Therefore, it was possible that PMNs from animals with elevated corticosterone levels had diminished capacity to produce O_2^-. The apparent ability of peritoneal cells from animals with elevated corticosterone levels to produce equivalent amounts of O_2^- to animals not fed corticosterone may have been because there were 28% more PMNs in that population. To test that hypothesis, equal numbers of PMNs from corticosterone-fed and control mice were assayed for the ability to produce O_2^-. The data in Table 5 illustrate that PMNs from mice fed

TABLE 2. Effect of 3-hr In Vitro Corticosterone Incubation on *P. acnes*-Induced Inflammatory Cell Production of O_2^-

Experiment	Treatment[a]	Length	nM O_2^- produced
1	Control[b]	0 hr	1.5 ± 0.3[c]
	Control[b]	3 hr	1.1 ± 0.2
	600 ng/ml	3 hr	1.7 ± 0.5
2	Control[b]	1 hr	0.5 ± 0.1
	600 ng/ml	1 hr	0.4 ± 0.1
	Control[b]	3 hr	0.4 ± 0.2
	600 ng/ml	3 hr	0.5 ± 0.2

[a]*P. acnes*-induced inflammatory cells incubated in medium containing 600 ng/ml corticosterone for the indicated length of time before PMA induction of O_2^-.
[b]The corticosterone stock solution was diluted in absolute ethanol. Similar volumes of absolute ethanol were added to medium controls to account for ethanol effects.
[c]Nos. represent $\bar{X}$ ± SEM of triplicate samples.

TABLE 3. Correlation Between Superoxide Production and Glucocorticoid-Induced Splenic Atrophy

Animal[a]	Approximate amount of corticosterone ingested[a]	Serum corticosterone level	Final spleen weight	Spleen weight as a % of final body weight	nM O_2^- production
(C) 1	0	31 ng/ml	102 mg	.363	3.3 ± 0.1[b]
(C) 2	0	28 ng/ml	94 mg	.348	3.0 ± 0.1
(C) 3	0	34 ng/ml	97 mg	.339	2.4 ± 0.0
(C) 4	0	73 ng/ml	98 mg	.355	2.9 ± 0.3
(C) 5	0	91 ng/ml	109 mg	.356	2.7 ± 0.4
(E) 6	78 mg	215 ng/ml	37 mg	.132	2.8 ± 0.1
(E) 7	62 mg	1,164 ng/ml	28 mg	.097	3.6 ± 0.4
(E) 8	82 mg	839 ng/ml	44 mg	.138	3.0 ± 0.2
(E) 9	64 mg	260 ng/ml	33 mg	.119	2.4 ± 0.2
(E) 10	73 mg	860 ng/ml	26 mg	.091	2.9 ± 0.2
$\bar{X}$ control[c]	0	51 ± 13 ng/ml	100 ± 3 mg	.352 ± .004	2.9 ± 0.2
$\bar{X}$ experimental[c]	72 ± 4 mg	667 ± 185 ng/ml	33 ± 3 mg	.116 ± .009	2.9 ± 0.2

[a]Five mice per group were fed oiled mouse chow ± corticosterone. Animals were fed ad libatum for 11 days.
[b]Nos. represent $\bar{X}$ ± SEM of triplicate samples.
[c]Nos. represent $\bar{X}$ ± SEM of values obtained from 5 mice per group.

TABLE 4. Peritoneal Exudate Cell Differentials of Corticosterone-Fed Mice

Treatment Group[a]	Total cells obtained	Percent				
		PMNs	Lymphocytes	Macrophages	Eosinophils	Mast cells
Control	$3.9 \pm 0.5 \times 10^6$	63 ± 2	9 ± 2	19 ± 2	6 ± 2	4 ± 1
Experimental	$1.0 \pm 0.6 \times 10^7$	87 ± 2	4 ± 1	8 ± 1	0 ± 0	2 ± 1

[a]All mice were injected ip with 700 μg *P. acnes* 20 hr before recovery of PEC.
Nos. represent $\bar{X} \pm$ SEM of 5 animals per group. Chi-square analysis of differential indicates a significant difference ($P < .01$) between treatment groups.

TABLE 5. Superoxide Production by PMNs From Corticosterone-Fed Mice

Mouse	Approximate amount of corticosterone ingested[a]	Final spleen weight	Spleen weight as a % of final body weight	nM O_2^- production
(C) 1	0	116 mg	.530	2.7 ± 0.2[b]
(C) 2	0	121 mg	.600	2.4 ± 0.3
(C) 3	0	110 mg	.543	2.5 ± 0.3
(E) 4	89 mg	32 mg	.145	2.8 ± 0.3
(E) 5	99 mg	33 mg	.148	2.6 ± 0.1
(E) 6	99 mg	42 mg	.181	3.0 ± 0.0
$\bar{X}$ control[c]	0	116 ± 3	$.558 \pm 0.2$	2.5 ± 0.1
$\bar{X}$ experimental[c]	96 ± 3 mg	36 ± 3	$.158 \pm 0.1$	2.8 ± 0.1

[a]Three mice per group were fed oiled mouse chow ± corticosterone. Animals were fed ad libitum for 12 days.
[b]Nos. represent $\bar{X} \pm$ SEM of triplicate samples.
[c]Nos. represent $\bar{X} \pm$ SEM of values obtained from 3 mice per group.

corticosterone produce similar amounts of O_2^- as mice not fed corticosterone. Therefore, elevated corticosterone levels do not appear to reduce the ability of PMNs to produce O_2^-.

Effects of Microgravity on Superoxide Production

Since glucocorticoid levels did not appear to directly affect O_2^-, we wanted to determine whether microgravity conditions might make inflammatory cells dysfunctional. To do this, measurements of inflammatory cell production of O_2^- were made during two different parabolic flights on NASA 930. Values obtained from each flight were similar and data were pooled. We found that the amount of O_2^- produced during microgravity ranged from 93 pM to 434 pM per s (Fig. 3). The amount of O_2^- made by 1*g* controls were generally lower than that made by cells stimulated in microgravity (Fig. 3). We found that PMA induced a mean concentration of 59 pM O_2^- per s when inflammatory cells were stimulated at 1*g* (Table 6). Cells pulsed with PMA in microgravity produced a mean concentration of 241 pM O_2^- per s (Table 6). That amount was statistically different from 1*g* controls ($P < .01$). No O_2^- was produced in the absence of PMA. Therefore, parabolic flight did not induce spontaneous O_2^- production. In addition, if the assays were done in the presence of superoxide dismutase, no reduction of ferricytochrome C was observed. Therefore, the reduction of cytochrome C was due to O_2^-.

DISCUSSION

These studies were initiated to follow-up observations made in a previous investigation reported by our laboratory [10]. In this study, we found that we could not inhibit O_2^- production by exposing cells to corticosterone in vitro or in vivo. Inflammatory cells taken from mice with elevated levels of corticosterone produced O_2^- similarly to controls (as measured in vitro). These results contrast with those of previous studies. Szefler et al. [30] found that glucocorticoid incubation of monocytes in vitro for a period of 24–50 hr resulted in decreased O_2^- production. Schultz and Kleinschmidt also reported that glucocorticoids will inhibit O_2^- [24]. Coates et al. [6] found that human PMNs treated with either dexamethasone or Auranofin had poor O_2^- responses. Furthermore, corticosteroids will inhibit macrophage expression of

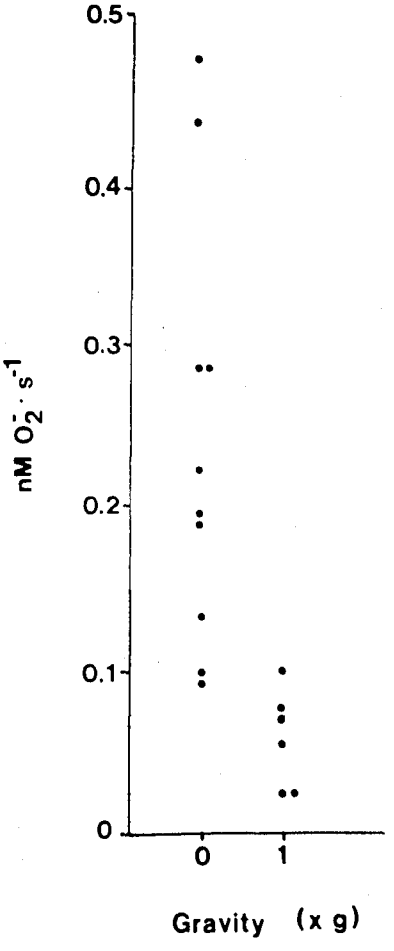

Fig. 3. Superoxide production for each individual assay during microgravity and 1*g*.

TABLE 6. Superoxide Production by *P. acnes*-Induced Peritoneal Exudate Cells at 0g[a]

n	PMA	Treatment	pM $O_2^- \cdot s^{-1}$
4	−	1*g*	0 ± 0[b]
6	+	1*g*	59 ± 10
3	−	0*g*	0 ± 0
10	+	0*g*	241 ± 40*

[a]Peritoneal exudate cells induced by ip injection of 350 μg *P. acnes* 20 hr before cell recovery.
[b]Nos. represent $\bar{X}$ ± SEM of n replicates.
*Significantly different from 1*g* control ($P < .01$) as determined by Student's t test.

major histocompatibility class II molecules, IL-1 production, and tumoricidal activation [14–16,25,35].

There are several possible reasons inflammatory cells from mice with elevated corticosterone levels do not have diminished O_2^- responses. For example, not all cells or cellular responses may be affected by corticosterone. Endothelial cell secretion of IL-1 is unaffected by glucocorticoid treatment [35]. Glucocorticoids also fail to inhibit arachidonic acid metabolism [28]. There may be species or glucocorticoid differences. In the studies of Coates et al. [6] human PMNs were treated with dexamethasone in vitro. Our corticosterone studies were done in vitro and in vivo, using mice. Corticosterone is the major murine corticosteroid [21]. Alternatively, long-term in vivo exposure of PMNs to corticosterone may desensitize the cells to its inhibitory effects. There are putative receptors for glucocorticoids on, and in, cells [2] and these could be down-regulated since glucocorticoids affect PMN membranes [17,20]. However, PMNs are short-lived cells and glucocorticoids are supposed to affect the development of cells from the bone marrow [2]. Thus, it is not clear how receptor down-regulation might occur. Another possibility is that the physiological concentrations of corticosterone reached in the animals used in these experiments (e.g., mean = 667 ng/ml) are not at physiologically inhibitory concentrations. In our experiments, there were considerably more cells recovered from corticosterone-fed animals than controls. Glucocorticoids at physiologically inhibitory concentrations inhibit immigration of inflammatory cells to sites of infection [2]. Therefore, it might appear that the corticosterone concentrations were not high enough. The data of Normann et al. [21] do not support this hypothesis. In their studies, serum corticosterone levels of 162 ng/ml were found to be anti-inflammatory. The pronounced splenic atrophy experienced by our corticosterone-fed mice also argues against this possibility [8]. Regardless of the mechanism, under the conditions we have described, murine PMN production of O_2^- was not affected by corticosterone.

To the best of our knowledge this is the first study to actually measure an inflammatory cell response in microgravity. Our findings of enhanced superoxide responses by cells exposed to short periods of microgravity add to an ongoing, controversial discussion. Although he does not necessarily support this thinking, Todd writes "It is easy to perceive that gravity acts on large systems, but difficult to accept its effects on cells, since the components of cells are rather close to the limit of size and mass influence by the gravitational field . . ." [34]. In support of this concept, human fibroblasts did grow as well in space as they did on earth [18], suggesting that microgravity does not affect cells. Other data, however, do not support that point of view. For example, paramecia and lymphocytes have altered growth or blastogenic responses in microgravity [7,22]. Additionally, *Escherichina coli* have increased resistance to antibiotics in space [33]. Therefore, microgravity can affect at least some individual cellular responses. Our studies differ from previous investigations in that our inflammatory cells were affected by short (10–20 s) exposures to

microgravity as opposed to long exposures. Therefore, it appears that some cells can sense changes in microgravity in very short periods of time. To our knowledge no data are available to confirm or refute our observation because of the difficulty in finding responses that are measurable in 20 s intervals. However, our data point out the importance of measurements that allow the detection of changes in cells in short, discrete time intervals. In our opinion, the faster measurable responses can be detected, the closer we can come to determining the primary level at which microgravity does exert its effects on cells. We also point out that long-term exposure to microgravity for weeks and months might have distinctly different effects on the ability of cells to produce O_2^- or perform other functions.

It is possible that our altered responses were due to changes in fluid mixing during microgravity conditions. However, our experiments were designed so that fluids were vigorously mixed between two syringes connected by a 3-way stopcock (See Fig. 1). Mixing was not dependent upon random motion or on convection currents, which are greatly diminished at reduced gravity. Since studies performed by Henry et al. [13] and Spooner et al. [27] have demonstrated that receptor-ligand binding proceeds normally during short periods of microgravity when such vigorous mixing is used, it appears that the changes were measured can be attributed to microgravity-induced, intracellular alterations. Perhaps the cytoskeletal elements that may be involved in releasing O_2^- are less constrained and allow the cell to release more O_2^- into the extracellular milieu. It is also possible that other regulatory elements that are active within cells at 1*g* are inhibited in microgravity and result in enhanced O_2^- production. A number of intracellular structures might be affected by microgravity [34]. We will need additional experiments to determine the exact mechanism involved.

In conclusion, the depressed O_2^- production by inflammatory cells from antiorthostatically suspended mice observed in previous studies [10] does not appear to be due to elevated levels of the major murine stress glucocorticoid, corticosterone. The average serum corticosterone levels previously reported for antiorthostatically suspended mice was 280 ng/ml [10] compared to 667 ng/ml in corticosterone-fed mice reported in this current investigation. Furthermore, short periods of actual microgravity do not inhibit inflammatory cells from producing O_2^-. Therefore, physiological factors that are encountered during antiorthostatic suspension and may be encountered during space flight appear to be responsible for diminished O_2^- responses. Because of this, other processes and cellular functions that occur during *P. acnes*-induced inflammation should be investigated in antiorthostatically suspended mice to provide some ideas on how space travel might affect immunity.

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Analysis of Exoprotein Gene Expression in an *xpr* Mutant Strain of *Staphylococcus aureus* when RNAIII is Provided in *trans*

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INTRODUCTION

Regulation of exoprotein expression in *Staphylococcus aureus* involves at least three distinct loci on the *Sma*I chromosomal maps of *S. aureus* NCTC 8325 and S6; the accessory gene regulator (*agr*) located on fragment F (14), the extracellular protein regulator (*xpr*) located on fragment A (20), and the staphylococcal accessory gene regulator (*sarA*) located on fragment D (3). Each region has been characterized by restriction endonuclease digestion and Southern analysis and shown to be distinct, yet their effect on exoprotein expression appears to be similar (1, 3-6, 8, 11, 15, 17, 20, 21). The best described is the *agr* locus which consists of two divergent transcripts, RNAII and RNAIII, that function in both positive and negative roles to regulate exoprotein synthesis (6, 8, 11, 15, 17). They are generally required for expression of extracellular proteins while expression of cell surface proteins is generally repressed (6, 8).

The larger transcript, RNAII, contains four open reading frames, two of which have deduced amino acid similarities with two-component signal transduction systems (7, 8, 22), and presumably functions to activate transcription of RNAIII. The effector molecule of the *agr* operon is the RNAIII transcript itself and not any translation product from the RNAIII region (12). When expressed from a non-*agr*-regulated promoter (12, 23) the appearance of RNAIII in an *agr* null mutant (12) results in complete restoration of *agr* activity.

The *xpr* mutation we recently described also results in a pleiotropic phenotype similar to *agr* (20, 21). Moreover, in addition to the loss of multiple extracellular protein phenotypes, this deletion also dramatically

reduces the expression of δ-toxin (whose mRNA is contained within the 5' end of the RNAIII transcript). In fact, the lack of δ-toxin production and the drastic reduction of δ-toxin mRNA in the *xpr* mutant led us to conclude that *xpr* and *agr* are interactive at the genetic level (5, 21).

Because RNAIII provided in *trans* from a non-*agr*-regulated promoter complimented *agr* null mutants, this study examined whether RNAIII in *trans* could also compliment *xpr*. Even though RNAIII was restored to wild-type levels, ~~only a partial~~ no restoration of message for SEB and α-toxin was observed. These data suggest that in addition to RNAIII, gene products within the *xpr* region are required for full expression of exoproteins in S. aureus. ~~In addition,~~ However over expression of RNAIII in an *xpr/agr* double mutant resulted in restoration of Hla and Seb message to wild-type levels and suggests an additional regulatory role for genes within the RNAII transcript of the *agr* operon.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* S6C and KSI9051 are the *xpr* parent and mutant strains, respectively and have been previously described (20, 21). *S. aureus* ISP479C and ISP546 are the *agr* parent and mutant strains, ~~respectively~~ (10, 15) and were kindly provided by Peter A. Pattee(Iowa State University, Ames, IA). *S. aureus* KSI2043 was constructed by ϕ11 mediated *trans*duction of the *agr* deletion cassette (Δ*agr*1057-4546::*tetA(M)*) from *S. aureus* RN6911 (kindly provided by Steve J. Projan, American Cyanamid Co., Lederle Laboratories, Pearl River, N.Y.) into the *xpr* mutant strain, KSI9051. Strain RN6911 was generated by allele replacement of *agr* with a modified copy which contained a 3.0 kbp fragment with the *tetA(M)* resistance marker in place of the 3.4 kbp

*Cla*I/*Hpa*I internal fragment of the *agr* operon (12, 13). Plasmid pSK734 (Fig. 1) was kindly provided by Saleem A. Khan (University of Pittsburgh, Pittsburgh, PA.). It was constructed by cloning a 678 base pair, PCR-generated promoterless RNAIII gene into the ß-lactamase promoter vector pRN6725 (12). Plasmid pSK734 was transformed into *S. aureus* strains by electroporation (9).

Southern analysis. Chromosomal DNA was isolated by the method of Dyer and Iandolo (4). DNA was digested with the restriction endonuclease *Eco*RV and resolved by electrophoresis through 0.8% LE agarose (FMC BioProducts, Rockland, Maine) gels in TBE buffer (18). Processing of DNA for Southern analysis was and the generation of digoxygenin labeled probes for detection of RNAII (agrAB), RNAIII (agr12), *seb* (SEB), *hla* (α-toxin), and *spaA* (protein A) have been described in earlier publications (5, 21). The probe used to detect the *tetA(M)* resistance marker was a 3.0 kbp *Bam*HI/*Eco*RI fragment from pRN6680. This fragment containing the *tetA(M)* resistance marker was gel purified using the Wizard PCR system (Promega Corp., Madison, Wis.). and labelled with digoxygenin-11-UTP.

Hybridized probes were detected using alkaline phosphatase-conjugated, antidigoxygenin $F(ab')_2$ antibody fragments (Boehringer Mannheim Corp., Indianapolis, Ind.) and the chemiluminescent substrate AMPPD (Tropix Inc., Bedford, Mass.) as previously described (5, 20).

RNA isolation and Northern analysis. Tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) or TSB with the appropriate antibiotic was inoculated with an overnight broth culture (15 to 18 h) to an initial optical density of 0.05 at 550 nm and shaken at 180 rpm at 37C. At 3, 6, and 12 h, a 10 ml sample was aseptically removed and pipetted into a 25

ml screw-cap Corex tubes containing 10 ml of an ice-cold acetone-ethanol mixture (1:1). Isolation of total RNA was accomplished using the RNAzol B (Tel-Test, Inc., Friendswood, Tex.) reagent and the procedure of Hart et al. (5). High-quality RNA (A_{260}/A_{280} = 1.9 to 2.0) was standardized by appropriate dilution in diethylpyrocarbonate-treated water to a final concentration of 1 μg/μl and Northern blot analysis was performed according to Hart et al. (5).

RESULTS

Genotypic characterization of the *xpr/agr* mutant. A double mutant (*agr/xpr*) was constructed by phage ϕ11-mediated *trans*duction of the *agr* deletion cassette (Δ*agr*1057-4546::*tetA(M)*) from *S. aureus* strain RN6911 into KSI9051. Several transductants resistant to erythromycin and tetracycline were obtained and one in particular, KSI2043 was chosen for further study.

The lower section of Figure 2 is an autoradiogram of a Southern blot of EcoRV digested chromosomal DNA isolated from strains S6C, KSI9051, KSI2043, and RN6911 probed with agrAB, agr12 and *tetA(M)*. In Panel A, only the Agr+ strains, S6C (lane 1) and KSI9051 (lane 2) posessed a 4.2 kbp band that hybridized to the probe (see upper diagram). The 4.2 kbp fragment observed is specific for the region encompassing the junction between open reading frames A and B of the *agr* operon (5, 8). As described, the remaining 2 strains are deleted in this region and were not expected to hybridize. However, when hybridized against the agr12 (Panel B) probe four fragments characteristic of the *agr* region (8) appeared in DNA of strains S6C and KSI9051. They are represented by bands at 4.2, 0.98, and 0.24 kbp (lanes 1 and 2, respectively). The fourth band (ca. 1.2

kbp) denoted by an asterisk represents a fragment from the *Eco*RV site at position 254 of the *agr* sequence to a site outside of the region (see upper diagram).

When the same DNA was probed with the *tetA(M)* fragment no hybridizing bands were observed for either S6C or KSI9051 (Panel C, lanes 1 and 2). However, DNA from KSI2043 and RN6911posessed two hybridizing bands at 3.0 and 1.8 kbp, which are the expected sizes (8, 12) for *Eco*RV-digested DNA containing the *agr* deletion cassette (lanes 3 and 4, and schematic). DNA from KSI2043 and RN6911 hybridized with agr12 produced identical *Eco*RV restriction pattern indicative of the *agr* deletion cassette (Panel B, lanes 3 and 4, and schematic). Only three hybridizing bands are present due to the absence of the *Eco*RV site at position 1472 of the *agr* sequence (8) shown schematically in figure 2. Taken together, these data indicate that the double mutant, KSI2043 carries mutations in *xpr* and *agr* that are identical to those represented in the individual mutant strains.

Expression of RNAIII in the *xpr* and the *xpr/agr* mutant. Total RNA isolated from 3 h cultures of S6C, KSI9051, KSI2043, and the same strains containing the plasmid pSK734 was hybridized with agr12 which is specific for RNAIII (Fig. 3). As we reported (5), RNAIII in KSI9051 was reduced when compared to S6C (Fig. 3, Panels A and B). In contrast, RNAIII in all three strains containing pSK734 was substantially increased and exceeded even the wild-type levels of the parent S6C. This elevated expression is most likely due to the plasmid copy number and to the constitutively expressed ß-lactamase promoter. A second hybridizing RNA (upper band) was also seen in strains containing the plasmid and presumably represents an unprocessed ß-lactamase/RNAIII fusion

transcript observed in a previous study involving a similar plasmid construct in the *agr*-null mutant strain, RN6911 (23). These data also demonstrate that the double mutant KSI2043, which contains deletions in the *xpr* and *agr* regions, does not make RNAIII (Fig. 3C). On the other hand, the expression of RNAIII in KSI2043 (pSK734) is upregulated and solely due to the activity of the ß-lactamase promoter. This clearly establishes that in the S6C genetic background, the *bla* promoter is not responsive to the regulatory function of either *xpr* or *agr*.

To verify the functionality of the ß-lactamase promoter in the *agr* mutant background (strain 8325-4), total RNA was isolated from ISP479C, ISP546, and the same strains containing pSK734 and hybridized with agr12 (Fig. 4). RNAIII in the *agr* mutant strain (ISP546) was undetectable (Fig. 4, Panel A) but, was restored to wild-type levels in the same strain containing pSK734 (Panel B). The upper band in figure 4 (Panels A and B) represents the ß-lactamase/RNAIII fusion transcript (23).

Expression of *hla*, *seb*, and *spa* in the *xpr* mutant containing pSK734. Total RNA from S6C, KSI9051, and KSI9051(pSK734) was isolated at 6 and 12 h of growth and hybridized with gene-specific probes for *hla*, *seb* and *spa* (Fig. 5, Panels A, B and C, respectively). As we reported (5) and in comparison to the parent strain S6C, message levels for *hla* and *seb* were reduced while *spa* levels were elevated in the *xpr* mutant, KSI9051 (Fig. 5, Panels A, B, and C, respectively). When message levels in KSI9051(pSK734) were compared to KSI9051, *hla* (Fig. 5A) and *seb* (Fig. 5B) message levels were approximately equal. When *spa* message levels in S6C, KSI9051, and KSI9051 containing pSK734 were compared, an inverse effect was observed; that is, message levels in KSI9051 (pSK734) were nondetectable when compared not only to the same strain

lacking the plasmid but to S6C as well (Fig. 5C).

These data indicate that RNAIII expressed from pSK734 does not compliment the *xpr* mutation in KSI9051. Even in the presence of higher than wild type levels of RNAIII, message levels for both *hla* and *seb* in KSI9051 (pSK734) were still considerably less than what was observed for S6C (Fig. 5A and 5B). In contrast, over expression of RNAIII resulted in undetectable *spa* message levels in KSI9051 (pSK734) which when compared to S6C which suggest that the positive control of *spa* expression is due to RNAIII alone.

Expression of *hla*, *seb*, and *spa* in the *xpr/agr* double mutant containing pSK734. As in the previous experiment, total RNA isolated from S6C, KSI9051, KSI2043, and KSI2043(pSK734) was hybridized with *hla*, *seb*, and *spa* specific probes (Fig. 6, Panels A, B, and C, respectively). The *hla* and *seb* message levels in KSI2043 were similar to those in KSI9051, that is, approximately 16- to 32-fold less than S6C (Fig. 6A). Surprisingly, the Hla and Seb message levels were restored to wild-type levels in KSI2043(pSK734) (Fig. 6A). In the case of *spa*, message levels in KSI2043 were approximately 2- to 4-fold higher than what was observed in KSI9051 (Fig. 6C). As observed with KSI9051(pSK734), KSI2043(pSK734) had no detectable *spa* message.

These data suggest the over expression of RNAIII from pSK734 in a strain devoid of any other *agr* component (namely, any of the four putative open reading frames encoded by RNAII) results in complete restoration of *hla* and *seb* expression.

DISCUSSION

It is becoming increasingly clear that the extracellular

regulatory loci of *Staphylococcus aureus* are interactive. Both *xpr* and *sar* affect the expression of *agr*. Cheung and Projan (2) have shown that inactivation of the *sarA* gene results in reduced levels of RNAIII. This mutation ultimately resulted in generalized reduction of exoprotein production, presumably by limiting the amount of RNAIII needed for extracellular protein gene expression. When RNAIII was provided in *trans* from an unregulated promoter, ß-toxin appeared to be restored to wild type levels. However, RNAIII levels were not fully restored suggesting that additional genetic factors may also be required for complementation of *agr*.

Since Hart et al. (5) have shown that a large deletion in the region designated *xpr* also results in reduced levels of both RNAII and RNAIII, this study was initiated to investigate the interaction of *xpr* and *agr*. In order to accomplish this goal, it was necessary to construct a strain that was mutant at both loci. This was accomplished by transduction of the *agr*-deletion cassette into KSI9051 (*xpr*). This double mutant (*agr, xpr*) was verified by restriction analysis to be structurally similar to the individual mutants at these particular loci. Phenotypically, mRNA exoprotein production by the double mutant was more repressed than either parent (data not presented). Furthermore, we have also established that the *bla* promoter-RNAIII fusion vector, pSK734 can function in the *S. aureus* S6 background as efficiently as in the 8325-4 strain. In either genotype, the *bla* promoter is not regulated by functions provided by either *agr* or *xpr*.

When transcription of *hla*, *seb* and *spa* was examined in the single mutant strains alone and with RNAIII provided in trans several interesting observations were apparent. Firstly, RNAIII over expression does not

complement the mutation in *xpr*. Neither Hla message nor Seb message was increased when high levels of RNAIII were present, but message was completely repressed. This latter finding is in agreement with Vandenesch et al. (23) who concluded that *spa* and *hla* are not reciprocally regulated and that RNAIII causes immediate cessation of *spa* transcription. These data also indicate that *xpr* is not involved in the negative regulatory functions of *agr*. A second important finding in this work is that overexpression of RNAIII in the double mutant does, in fact, complement and allow transcription of Hla and Seb message at nearly wild type levels. Genotypically, strains KSI9051(pSK734) and KSI2043(pSK734) are isogenic, differing only in the presence of the RNAII gene. We reported earlier that RNAII was severly repressed, but present in low levels in KSI9051 (5). The data presented in Fig. 5, line 3 indicate that the presence of RNAII represses expression of Hla and Seb, while in its absence (line 5) nearly wild type levels of mRNA are present. These data suggest that *xpr* gene products interact with RNAII or its products to relieve repression. In the absence of either gene (lines 2 and 3) repression occurs, but in the absence of both genes (line 5) it is relieved and RNAIII is able to initiate exoprotein gene transcription. We conclude therefore, that the regulatory role of RNAII is not limited to signal transduction and it or one of its gene products interacts with *xpr* to regulate exoprotein expression.

It has been hypothesized that the AgrA and B genes of the *agr* operon function as a two-component signal transduction system coordinately regulating exoprotein expression in response to environmental stimuli (7, 8, 22). However, the role of the remaining genes, AgrC and AgrD are highly speculative. Since the operon is transcribed at low level throughout growth, these proteins may interact with gene products of *xpr* to repress

agr transcription either early in growth and in a poising role when RNAII transcription is induced in post-exponential phase. In these functions, *xpr* fits many of the criteria cited by Vandenesch et al. (23) for the additional temporal signal required for induction of transcription of exoprotein genes.

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FIGURE LEGENDS

FIG. 1. Plasmid pSK734 is a transcriptional fusion of the RNAIII gene (678 bp) to an 839bp truncated *blaZ* gene. A PCR generated promoterless RNAIII fragment was cloned into the *Eco*RI site of pRN6725 (12) to generate pSK734.

FIG. 2. (Top) Schematic of selected restriction endonuclease sites in the wild-type and deletion/insertion regions of the agr operon as determined from Kornblum *et al.* (8), Novick *et al.* (12), and Nesin *et al.* (13). Probes used for characterization are labelled accordingly and their lengths shown as double-headed arrows. Restriction endonuclease sites are shown parenthetically in base pairs. (Bottom) Southern analysis of chromosomal DNA isolated from S6C (1), KSI9051 (2), KSI2043 (3), and RN6911 (4) and digested with EcoRV. Digested DNA was hybridized with agrAB (Panel A), agr12 (Panel B), or *tetA(M)* (C) digoxygenin-labelled probes. Hybridizing bands are marked by arrows and their sizes in kilobases. The asterisk denotes the *Eco*RV fragment that extends from the *Eco*RV site (254) within the *agr* region to a site outside of the region.

FIG. 3. Total cellular RNA isolated from S6C and S6C containing pSK734 (A), KSI9051 and KSI9051 containing pSK734 (B), and KSI2043 and KSI2043 containing pSK734 (C) at 3 h of growth and hybridized with the agr12 (RNAIII) probe. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U), 4-fold, or 16-fold diluted (numerical values) samples.

FIG. 4. Total cellular RNA isolated from ISP479C and ISP546 (A) and ISP479C and ISP546 containing pSK734 (B) at 12 h of growth and hybridized with the agr12 (RNAIII) probe. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values) samples.

FIG. 5. Total cellular RNA isolated from S6C, KSI9051, KSI2043, and KSI2043 alone and containing pSK734 at either 6 (for *spa* detection) or 12 h (for hla and seb detection) of growth and hybridized with either an *hla*- (A), an *seb*- (B), or an *spa*- (C) specific probe. RNA concentrations were standardized according to A_{260} values and loaded as either undiluted (U) or twofold serially diluted (numerical values) samples.

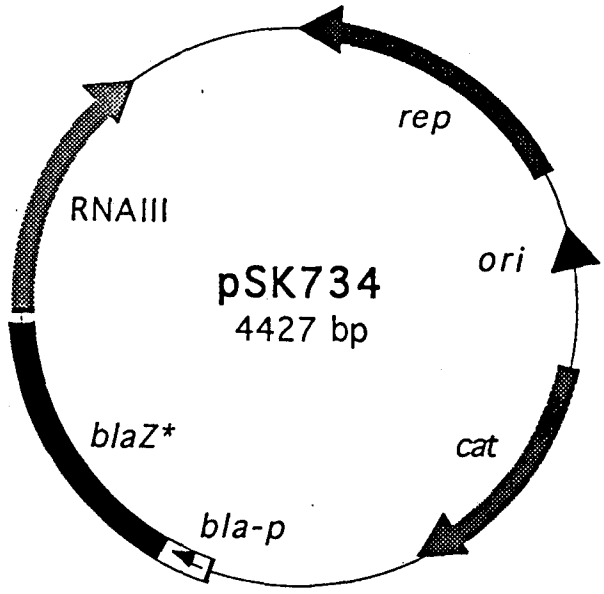
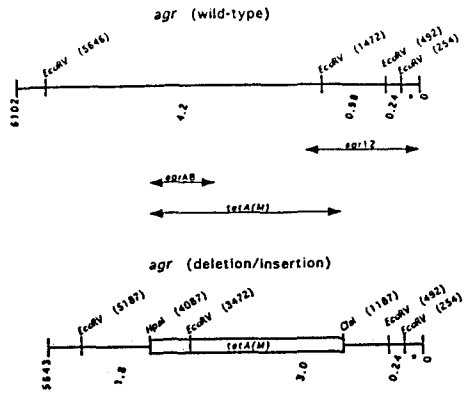


FIG1



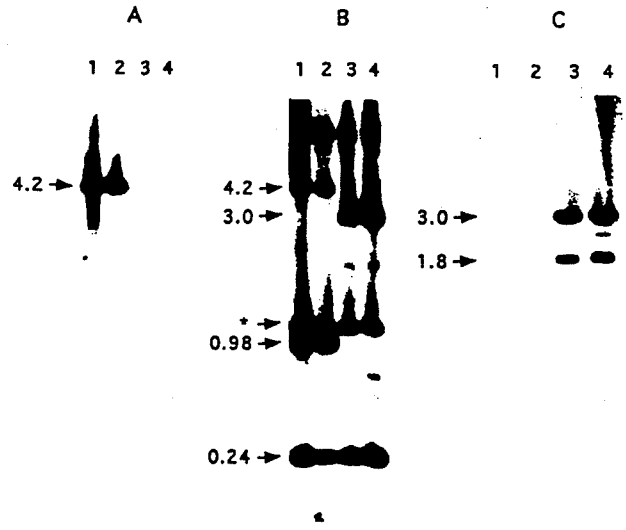


FIG2

FIG3

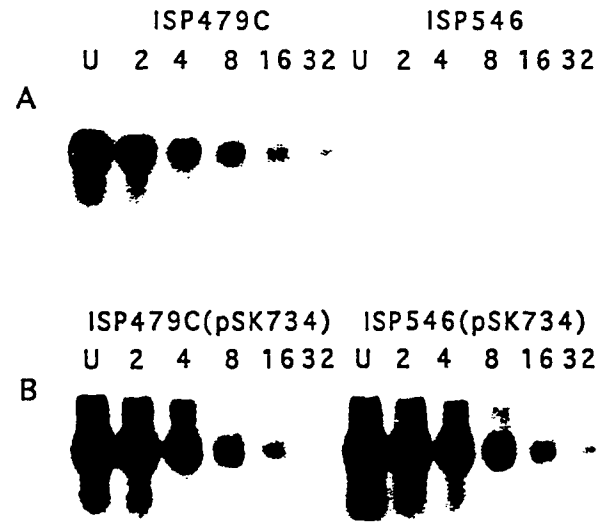
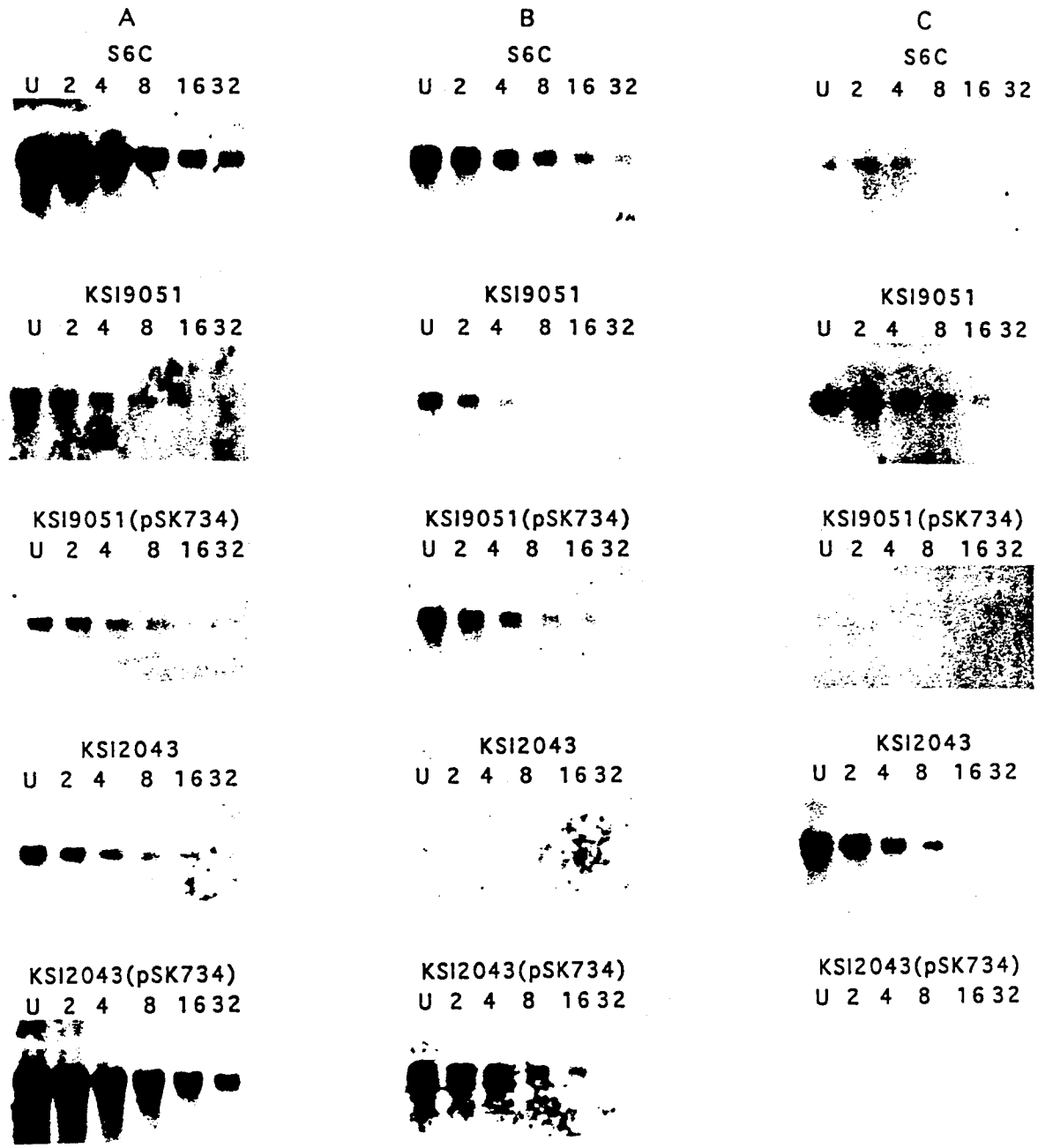


FIG4


A
S6C
U 2 4 8 16 32
KSI9051
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KSI9051(pSK734)
U 2 4 8 16 32
KSI2043
U 2 4 8 16 32
KSI2043(pSK734)
U 2 4 8 16 32
B
S6C
U 2 4 8 16 32
KSI9051
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KSI9051(pSK734)
U 2 4 8 16 32
KSI2043
U 2 4 8 16 32
KSI2043(pSK734)
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C
S6C
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U 2 4 8 16 32

FIG 5

THE EFFECTS OF rM-CSF AND rIL-6 THERAPY ON IMMUNOSUPPRESSED ANTIORTHOSTATICALLY SUSPENDED MICE

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KEY WORDS: macrophage, T cell, M-CSF, IL-6, antiorthostatic suspension, spaceflight.

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ABSTRACT:

Antiorthostatically suspended mice had suppressed macrophage development in both unloaded and loaded bones indicating a systemic effect. Bone marrow cells from those mice secreted less macrophage colony stimulating factor (M-CSF) and interleukin-6 (IL-6) than control mice. Because M-CSF and IL-6 are important to bone marrow macrophage maturation, we formulated the hypothesis that suppressed macrophage development occurred as a result of the depressed levels of either M-CSF or IL-6. To test the hypothesis, mice were admistered recombinant M-CSF or IL-6 intraperitoneally. We showed that rM-CSF therapy, but not rIL-6 therapy, reversed the suppressive effects of antiorthostatic suspension on macrophage development. These data suggest that bone marrow cells that produce M-CSF are affected by antiorthostatic suspension and may contribute to the inhibited maturation of bone marrow macrophage progenitors.

Abbreviations used in this chapter:

PHA, phytohemagglutinin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte\macrophage colony stimulating factor; IL-6, interleukin-6; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; rhM-CSF, recombinant human macrophage colony stimulating factor; rhIL-6, recombinant human interleukin-6; rIL-2, recombinant interleukin-2; PEG-IL-2, polyethylene-glycol conjugated recombinant human interleukin-2.

INTRODUCTION:

Skeletal unloading induced by spaceflight or antiorthostatic suspension inhibits macrophage development from bone marrow cells (4, 6, 37, 38). Bone marrow cells from antiorthostatically suspended mice secrete low levels of interleukin-6 (IL-6) and macrophage-colony stimulating factor (M-CSF) compared to control animals (6). Both these cytokines strongly influence macrophage hematopoiesis (2, 24). Therefore, we proposed the hypothesis that their depression contributed to suppressed macrophage development (2, 6, 17, 24). Depressed T cell responsiveness and macrophage hematopoiesis in antiorthostatically suspended mice was ameliorated by polyethylene-glycol-conjugated recombinant IL-2 (PEG-IL-2) therapy (6). Because of our success with the PEG-IL-2 therapy protocol, and the key roles of M-CSF and IL-6 have in hematopoiesis, we treated antiorthostatically suspended mice with recombinant M-CSF and IL-6 to attempt to reverse immunosuppression induced by antiorthostatic suspension (3-6).

We report that antiorthostatic suspension reduced the number of macrophage precursor cells systemically and inhibited bone marrow cell M-CSF and IL-6 secretion. Recombinant human M-CSF therapy maintained normal macrophage development. In contrast, rhIL-6 therapy did not augment depressed bone marrow macrophage hematopoiesis in antiorthostatically suspended mice. However, it did reverse depressed lymph node T cell proliferation.

MATERIALS AND METHODS:

ANIMALS.

Male, adult (8-13 weeks) C3Heb/FeJ mice were bred in the animal facility at Kansas State University and were used in these studies. The use of these animals was approved by the Animal Care and Use Committee at Kansas State University which complies with NIH Animal Care Standards.

rM-CSF and rIL-6 THERAPY.

Mice were injected intraperitoneally (i.p.) with rhM-CSF (0.5 mg/kg, Chiron Corp., Emeryville, CA) on days 0, 4 and 8 of antiorthostatic suspension. The dosage protocol for rhM-CSF was based on the $T_{1/2}$ (28 hours) of M-CSF and consultation with the supplier. For rhIL-6 (DNAX, Palo Alto, CA) studies mice were either injected every second day of suspension with 10 μg of rhIL-6 or every day of suspension with 10 or 25 μg of rhIL-6. The dosage protocols for rhIL-6 were selected based on data from previous rIL-6 studies (30, 31) and its $T_{1/2}$ of 6-8 hours. In all cytokine therapy experiments, restraint control and antiorthostatic control mice were injected intraperitoneally with an equal volume (approx. 100 μl) of pyrogen-free saline at times corresponding to cytokine administration.

Animals were antiorthostatically suspended using the Wronski-Morey-Holton tail suspension cage as reviewed by Chapes *et al.* (8) and described by Armstrong *et al.* (4, 6). Briefly, mice were tail suspended at an angle of 22° such that their hindlimbs were skeletally unloaded. Cables connected to the tails of mice were attached to a low resistance pulley system above the cages allowing animals complete movement in any direction within the enclosure. Each suspension experiment involved two categories: (experimental; as described above) and restraint control (control; tail was

attached to the pulley system, however, the mice bear full weight on both their rear and forelimbs). Mice were weighed prior to suspension and prior to sacrifice. In all experiments, mice were sacrificed between 8:00 and 10:00 AM to eliminate differences caused by circadian rhythms. Eleven days was chosen as the suspension period to remain consistent with previous studies (4, 6, 10, 21).

BLOOD COLLECTION, PREPARATION AND CORTICOSTERONE ASSAY.

Immediately before animal sacrifice, blood was collected from the retro-orbital sinus using a Pasteur pipet. The blood was dispensed into microfuge tubes, serum was collected after coagulation and stored at -20° C. Samples were assayed for corticosterone after extraction with ethyl acetate using a competitive radioimmuno assay as described previously (4, 10, 21).

BONE MARROW MACROPHAGE COLONY ASSAY.

Macrophage development from bone marrow cells was assayed as described previously (3, 4, 6). Briefly, bone marrow cells were obtained from the femora, tibiae and humeri of mice from the three treatment groups. The ends of femora, tibiae and humeri were removed and the cells were flushed from the bone using DMEM and a 26-gauge needle. Cells were passed three times through a 19-gauge needle to break up clumps and cells were pelleted and resuspended at a concentration of 1 x 10^5 cells per 1.5 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 0.3% agar, 10% FBS and 15% LM929 fibroblast-conditioned medium (M-CSF source). After 6 days of culture, 5 microscope view fields were scored for macrophage colonies (a group of >25 cells) as macrophages specifically form colonies in respone to M-CSF at the exclusion of other white blood cell types (24).

LYMPH NODE T CELL PROLIFERATION.

Proliferation was assayed for all treatment groups simultaneously as previously described by our laboratory (4, 6). Briefly, lymphocytes were obtained by expression of the cells from inguinal lymph nodes through a wire sieve. Lymphocytes were washed twice with DMEM containing 2% fetal bovine serum (FBS) and 5 μg/ml of gentamycin sulfate. Five x 10^5 lymph node cells were added per well (100 μl) in Costar (Cambridge, MA) 96-well, flat-bottom microtiter plates. Wells received 100 μl of medium: RPMI supplemented with 5.6 x 10^{-5} M 2-mercaptoethanol (2-ME), 5% fetal bovine serum (FBS) and 5 μg/ml of gentamycin sulfate, with or without PHA (10 μg/ml; Wellcome Biotechnology, Research Triangle Park, NC). The cells were incubated for 48 hours and were pulsed with 0.5 μCi per well of [^{3}H]-thymidine for 6 hours prior to harvest. The cells from each well were harvested on a Cambridge PHD cell harvester on glass fiber filters, placed in scintillation counting fluid and counted on a Packard 1500 scintillation counter. Stimulation index (SI) was calculated:

$$SI = \frac{(\text{CPM samples + PHA})}{(\text{CPM samples - PHA})} \times 100$$

CYTOKINE ASSAYS.

Bone marrow cell cytokine secretion was assayed by incubating freshly isolated bone marrow cells in RPMI supplemented with 10% FBS for 18 hours (5 x 10^6 cells/3ml) as described previously (5, 6). Supernatants were assayed for IL-6 content by bioassay using the IL-6 growth-sensitive cell line B9. Growth was quantified using an MTT assay and concentrations were determined by linear regression and a rIL-6 (R&D, Minneapolis, MN) standard curve (sensitivity 625 fg/ml). Supernatants were assayed for TGF-β content by bioassay using the TGF-β growth sensitive cell line CCL64 (ATCC, Rockville, MD). TGF-β concentrations were determined by an MTT assay used in conjunction with a rTGF-β (R&D, Minneapolis, MN) standard curve (sensitivity 7.5 ng/ml). Supernatants were assayed for M-CSF content by bioassay using the M-CSF-dependent cell line B6MP102. M-CSF concentrations were determined by MTT assay and a rh-CSF (Chiron Corporation) standard curve (sensitivity 60 ng/ml). Experiments using recombinant cytokines and bone marrow cell supernatants demonstrated our bioassays to be unaffected by bone marrow cell supernatant cytokines other than those being assayed.

FLOW CYTOMETRIC ANALYSIS OF BONE MARROW CELLS.

Bone marrow cells were analyzed for expression of KMI-6-specific antigen and MAC-1 (CD11b, ATCC antibody TIB 128; ref. 1) using monoclonal antibodies and flow cytometry as described previously (3, 4). Fluorescent-antibody-labelled bone marrow cells were detected using a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA) equipped with an air-cooled, 15 mW argon ion lasar that emitted at 488 nm. Consort 30 software was utilized for data acquisition and analysis of 5-10 x 10^3 cells per sample. Four independent experiments (pooled cells from 4 mice per treatment group per

experiment) were averaged and presented as X ± SEM (n=4) in Figure 6. KMI-6 monoclonal antibody was originally described by Jacobsen *et al.* (16). It stains 2-3% of murine bone marrow cells and identifies a 110 kDa molecule on stromal cells that secrete IL-6 and appear to nurture B cell differention (12, 16). MAC-1 is a macrophage-granulocyte specific marker and was used as a control and comparison to KMI-6-specific molecules. CD11b is a 170 kDa molecule that is not found on bone marrow resident-macrophages (1) but increases in expression as monoblasts mature. Up to 85% of monocytes are positive for CD11b (43).

STATISTICAL ANALYSIS.

Student's *t*-tests or ANOVA were used to determine statistical significance. All n values indicate the number of animals used in a given experiment.

RESULTS:

STRESS DATA

The effects of antiorthostatic suspension on macrophage development and T cell responsiveness have not been related to serum corticosterone concentrations (4, 6, 10, 22). However, as the immune system can be affected by stress (8, 9, 10, 22) and additional animal handling was required in these experiments, we monitored changes in body weights, spleen weights and corticosterone, for all animals, as indicators of stress. Table 1 shows that the percentage of body weight lost was not significantly different ($p>0.05$) among all treatment groups used in rhM-CSF studies. It is interesting to note that animals not subjected to antiorthostatic suspension lost less weight than mice subjected to restraint, however, this difference was not significant. Therefore, head-

down-tilt had some apparent effect on mice. Based on previous observations (4, 8) mice used in this study lost weight in all groups due to the stress of regular i.p. injections. Mice treated with rhIL-6 at a dose of 10μg every second day or every day lost significantly more weight than control animals ($p<0.1$; sham injected and unloaded or loaded). These data indicate that the procedure did cause weight loss. However, for both rhM-CSF and rhIL-6 therapy studies serum corticosterone levels were not significantly different when control and treatment groups were compared (ANOVA; Table 2).

Spleen weights as a percentage of final body weight were not different between normal and non-cytokine treated suspended animals. However, treatment with rhM-CSF significantly increased spleen weight as a percentage of final body weight ($p<0.05$, ANOVA; Table 1), indicating rhM-CSF bioactivity. Spleen weights of animals used in rhIL-6 therapy studies were significantly different when antiorthostatically suspended animals were compared to restraint controls ($p<0.05$, *t*-test; Table 1). However, antiorthostatically suspended mice treated with rhIL-6 did not show a significant difference in spleen weight compared to restraint controls. These data indicated a biological effect of rhIL-6 therapy.

MACROPHAGE DEVELOPMENT.

Spaceflight and antiorthostatic suspension reduce mouse and rat macrophage hematopoiesis (4, 6, 37, 38). The data in Figure 1A and 1B confirmed that macrophage development was suppressed following antiorthostatic suspension and indicated that rhM-CSF therapy reversed the immunosuppressive effect of antiorthostatic suspension on this process. In contrast rhIL-6 therapy, at either 10 μg every second day or 10 μg per day,

did not ameliorate the effects of antiorthostatic suspension on macrophage development (Figures 2A and 2B). We also injected mice with 25 μg every day during the course of an 11 day suspension. In those two experiments, we also did not see restoration of the progenitor macrophage numbers (e.g. hindlimbs: restraint control 8.6 ± 2.2 colonies, n=6; suspended, 4.4 ± 1.6 colonies, n=5 [p = 0.07, compared to orthostatic]; suspended + rhIL-6, 5 ± 3.9, n=6 [p = 0.03, compared to restraint control]. Therefore, it did not appear that rhIL-6 could restore macrophage hematopoieses like rhM-CSF.

HEMATOPOIETIC CYTOKINES.

Bone marrow cell secretion of two important cytokines for macrophage hematopoiesis (M-CSF and IL-6; 24) is depressed following antiorthostatic suspension (6). Therefore, we analyzed freshly isolated bone marrow cell M-CSF and IL-6 secretion from restraint control and antiorthostatically suspended and mice following rhM-CSF and rhIL-6 therapy (Figures 3 and 4). Transforming growth factor-β (TGF-β) secretion was also measured due to its known role as an inhibitor of macrophage hematopoiesis (24). Bone marrow cell IL-6 and M-CSF secretion, but not TGF-β-secretion, were significantly lower in mice which had been antiorthostatically suspended (Figures 3 and 4), confirming previous data (6). Antiorthostatically suspended mice treated once every 4 days with rhM-CSF, beginning at the commencement of suspension, showed normal bone marrow cell TGF-β secretion and IL-6 secretion (Figures 3C and 3A, respectively). However, rhM-CSF therapy did not change the abnormally low levels of M-CSF secretion by bone marrow cells taken from skeletally unloaded mice (Figure 3B). Recombinant-human IL-6 therapy did not overcome the immunosuppressive effects of antiorthostatic suspension on bone marrow cell cytokine secretion (Figure 4).

T CELL PROLIFERATION.

Recombinant-human IL-6 therapy at 10 μg/mouse/48 hours did not return PHA-induced lymph node proliferation to normal levels in mice which had been skeletally unloaded (Figure 5A). However, at a dose of 10 μg/mouse/24 hours *ex vivo* T cell proliferation in response to PHA of skeletally unloaded mice was normal (Figure 5B).

ANTIBODY STAINING OF STROMAL CELLS.

To determine if one population of stromal cells was affected by antiorthostatic suspension, freshly isolated bone marrow cells from mice subjected to antiorthostatic suspension or restraint were stained with antibodies specific for B cell-nurturing stromal cells (16) or CD11b (anti-MAC-1). We found that bone marrow cells from antiorthostatically suspendedy mice had similar numbers of KMI-6 and CD11b-staining cells (Figure 6). Approximately 4% and 22% of bone marrow cells from mice from both treatment groups were detected by KMI-6 and CD11b-specific antibodies, respectively (Figure 6).

DISCUSSION:

Suppression of macrophage hematopoiesis by antiorthostatic suspension is characterized by reduced *ex vivo* colony formation and hematopoietic cytokine secretion (e.g. M-CSF and IL-6; 6). Therefore, we investigated whether the therapeutic administration of these cytokines would restore normal macrophage hematopoiesis. Antiorthostatically suspended mice treated with rhM-CSF had normal *ex vivo* macrophage development. However, rhM-CSF therapy did not return bone marrow cell M-CSF secretion to normal levels. Therefore, rhM-CSF appeared to successfully

reconstitute macrophage progenitor cells in antiorthostatically suspended mice without affecting the endogenous production of M-CSF. These data suggest that macrophage hematopoiesis was affected because antiorthostatic suspension suppressed nursing/stromal cells which normally provide M-CSF (2, 24) but did not affect the developmental potential of the macrophage progenitor cells. When those progenitor cells were exposed to M-CSF they differentiated normally. These data are consistent with observations in osteopetrotic mice which had congenitally absent levels of M-CSF (47) and produced abnormally low numbers of macrophages at early ages (44, 45) but could be restored to normal with injections of recombinant human M-CSF (21). The fact that IL-2 enhanced macrophage colony formation in response to M-CSF and restored normal macrophage hematopoiesis (6) also supports that antiorthostatic suspension affects nursing/stromal cells rather than macrophages or macrophage precursors. Our data are also consistent with the well established activity of M-CSF of enhancing macrophage growth, function and viability (24). In fact, the only time when M-CSF does not appear to help macrophage hematopoiesis is when adoptively transferred progenitors are infused into patients that have undergone chemotherapy (23).

The fact that antiorthostatic suspension affects macrophage progenitors systemically and appears to affect bone demineralization in a systemic fashion (4) is consistent with the hypothesis that antiorthostatic suspension affects the M-CSF producing stromal cell. Macrophage-colony stimulating factor affects bone resorption (15) and hematopoiesis. Moreover, the osteoclasts, which control resorption, share a common origin with macrophages (34, 41). Therefore, these results may have more global application beyond immunosuppression; extending to studies on bone.

Recombinant-human IL-6 therapy failed in ameliorating the immunosuppressive effects of antiorthostatic suspension on macrophage development. The dose-response protocols for rhIL-6 therapy were in and above the ranges of those used in previous studies to enhance platelet hematopoiesis and tumor rejection (30, 31). We did not attempt higher rhIL-6 therapy doses because 25 μg rhIL-6/mouse/day increased lung vascularization and bleeding (as observed by visual examination during dissection) in some treated mice compared to plecebo-injected mice or mice given only 10 μg of rhIL-6 per day. Higher rhIL-6 treatment doses probably would have resulted in cytokine-induced complications and capillary leak syndrome which would negate any beneficial effects gained by higher dose rhIL-6 therapy. Furthermore, rhIL-6 appeared to have a biological effect on treated mice by enhancing depressed lymph node T cell proliferation and affecting spleen weights. Therefore, although stromal cells do produce IL-6 (12, 18) it did not appear that the lowered IL-6 production by stromal cells caused depressed macrophage hematopoiesis. It appears that depressed IL-6 secretion reflected the abnormally low numbers of differentiating macrophages in skeletally unloaded mice. This hypothesis is supported for several reasons. 1. Shirai *et al.* (36) showed that approximately 50% of bone marrow cell IL-6 secretion is produced by bone marrow macrophages which are stimulated by M-CSF (2, 24). In our hands, rhM-CSF therapy restored bone marrow cell secretion of IL-6 concurrently with macrophage progenitors. 2. While IL-6 has been shown to enhance myeloid and macrophage colony formation, it is not known as the primary, or an essential, cytokine for macrophage development (2, 17, 20, 24). 3. Interleukin-6 is the primary cytokine for B cell hematopoiesis (2, 12, 18, 20) and we have shown that antiorthostatic suspension did not diminish B cell numbers in

bone marrow (4), nor did it appear to affect one of the stromal cells (KMI-6-positive) that are thought to nurture B cell development and that produce IL-6 (12, 16). Therefore, stromal cells that produce IL-6 secretion were probably not affected by antiorthostatic suspension. 4. The amount of IL-6 (>200 ng/ml) secreted by bone marrow cells from antiorthostatically suspension mice was significantly reduced but was still produced at relatively high levels (31, 36, 46). Therefore, we conclude that low IL-6 levels in antiorthostatic suspended mice was the result of reduced numbers of macrophages/macrophage precursors rather than the causative agent of depressed macrophage differentiation.

Antiorthostatic suspension affects lymphocyte populations in a compartmentalized manner (4, 32, 33). However, lymph node T cell proliferation was also reduced following antiorthostatic suspension. As PEG-IL-2 therapy reversed the effects of antiorthostatic suspension on T cell proliferation, we tested whether rhM-CSF or rhIL-6 therapy would also ameliorate this immunosuppressive effect of antiorthostatic suspension. Recombinant human IL-6 reversed the effects of antiorthostatic suspension on T cell proliferation. These data are consistent with studies showing stimulatory effects of IL-6 on T cells (19, 20) and are consistent with PEG-IL-2 therapy studies where lymph node responses were also enhanced (6). In contrast, recombinant human M-CSF therapy had inconsistent effects on lymph node T cell proliferation (data not shown). Perhaps these results were obtained because M-CSF primarily acts on macrophages (24). Additional experiments will be needed to resolve the effects of rhM-CSF on lymph node T cells.

The ability of rhM-CSF or rhIL-6 to restore macrophage hematopoiesis or T cell proliferation, respectively, appears to be unrelated to high serum corticosterone

concentrations or stress associated with head down tilt which would cause weight loss. These parameters were similar between animals that were antiorthostatically suspended and were given cytokine or placebo. We have previously found that changes induced by antiorthostatic suspension on macrophage and lymph node activity were unrelated to indicators of stress (i.e. corticosterone concentration, weight loss). Therefore, the factors mediating changes in immune biology that we have measured in our model are more likely to be the result of other physiological changes associated with head-down tilt suspension. These include anterior fluid shifts, changes in bone composition and skeletal muscle atrophy (7, 13, 35). However, we found substantially higher corticosterone concentrations in the animals used in this study compared to our previous work (4, 8). This probably reflects the increased handling of animals and daily or periodic injections required by our protocols. We have already established that small perturbations in this model can affect stress levels (8). Nevertheless, the fact that our restraint controls had extremely high corticosterone concentrations, yet had apparently normal macrophage hematopoiesis and lymph node T cell responses, reaffirms that corticosterone is not responsible for these immunological changes in antiorthostatically suspended mice. Furthermore, high serum corticosterone concentrations did not inhibit the therapeutic value of these cytokines. This may not be so surprising since the physiological changes associated with antiorthostatic suspension have been shown to be beneficial or stimulatory (4, 22, 25, 26).

In summary, we have characterized that rhM-CSF but not rhIL-6 can restore normal macrophage hematopoiesis in antiorthostatically suspended mice. This confirms our previous study using PEG-IL-2 which showed that macrophage hematopoietic precursors

could be therapeutically enhanced with cytokine treatment. It is interesting to note that PEG-IL-2 was able to enhance macrophage progenitor numbers without increasing bone marrow cell secretion of IL-6 whereas rhM-CSF did not. Therefore, the mechanisms of action cannot be the same. Side-by-side comparisons would appear to be the next order of business.

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Table 1: The effect of antiorthostatic suspension on body weight and spleen weight.

Treatment:	Body Weight Lost[+] (% initial weight)	Spleen Weight[#] (% body weight)	n
rhM-CSF Therapy (0.5 mg/kg/96 hours)			
Restraint Control	3.8±1.8††	0.297±0.008	9
Suspended	6.7±1.8	0.290±0.009	9
Suspended + rhM-CSF	6.6±1.7	0.323±0.009*	9
rhIL-6 Therapy (10 μg/mouse/48 hours)			
Restraint Control	7.6±1.3	0.247±0.010	3
Suspended	13.1±1.5	0.298±0.011*	3
Suspended + rhIL-6	10.3±2.7	0.266±0.001	3
rhIL-6 Therapy (10 μg/mouse daily)			
Restraint Control	5.2±2.0	0.254±0.006	6
Suspended	14.5±1.1†	0.292±0.008*	6
Suspended + rhIL-6	10.2±1.5†	0.267±0.002	6

[+] Body weight lost is represented as a percentage of the initial body weight.

[#] Spleen weight is represented as a percentage of final body weight.

* $p < 0.05$, ANOVA.

† $p < 0.1$ when compared to restraint control, *t*-test.

†† numbers represent X ± SEM of measurements from n animals.

Table 2: The effect of antiorthostatic suspension on plasma corticosterone levels.

Treatment:	Corticosterone (ng/ml)	n
rhM-CSF Therapy (0.5 mg/kg/96 hours)		
Restraint Control	363±62*	9
Suspended	268±59	9
Suspended + rhM-CSF	276±43	9
rhIL-6 Therapy (10μg/mouse/48 hours)		
Restraint Control	497±78	3
Suspended	745±223	3
Suspended + rhM-CSF	727±63	3
rhIL-6 Therapy (10μg/mouse/24 hours)		
Restraint Control	547±66	6
Suspended	623±86	6
Suspended + rhM-CSF	425±77	6

* Numbers represent X ± SEM of samples from n animals.

Figure Captions:

Figure 1: The effects of antiorthostatic suspension and rhM-CSF therapy (0.5 mg/kg/4 days) on macrophage development. Macrophage colonies were isolated from mouse bone marrow and cultured for 7 days in M-CSF. A: Macrophage colonies derived from femora and tibiae in restraint control mice, suspended mice and suspended mice treated with rhM-CSF. B: Macrophage colonies derived from humeri in restraint control mice, suspended mice and suspended mice treated with rhM-CSF. * $p<0.05$ compared to restraint control mice, *t*-test. Numbers represent X ± SEM of 9 mice per treatment group.

Figure 2: The effects of antiorthostatic suspension and rhIL-6 therapy on macrophage development. Macrophage colonies were isolated from mouse bone marrow and cultured for 7 days in M-CSF. A: Macrophage colonies derived from femora and tibiae in restraint control mice, suspended mice and suspended mice treated with rhIL-6 at 10 μg/mouse/48 hours or 10 μg/mouse/24 hours. B: Macrophage colonies derived from humeri in restraint control mice, suspended mice and suspended mice treated with rhIL-6 at 10 μg/mouse/48 hours or 10 μg/mouse/24 hours. * $p<0.05$ compared to restraint control mice, *t*-test. Numbers represent X ± SEM of n mice per treatment group.

Figure 3: The effects of antiorthostatic suspension and rhM-CSF therapy (0.5 mg/kg/4 days) on bone marrow cell cytokine secretion. Bone marrow cell cytokine secretion was determined from supernatants of freshly isolated bone marrow cells cultured for 18 hours. * $p<0.05$ compared to restraint control mice. Numbers represent X ± SEM of samples from 9 mice per treatment group.

Figure 4: The effects of antiorthostatic suspension and rhIL-6 therapy (10 μg/mouse/48 hours or 10 μg/mouse/24 hours) on bone marrow cell cytokine secretion. Bone marrow cell cytokine secretion was determined from supernatants of freshly isolated bone marrow cells cultured for 18 hours. * $p<0.05$ compared to restraint control mice. Numbers represent X ± SEM of samples from n mice per treatment group.

Figure 5: The effects of rhIL-6 therapy on PHA induced lymph node T cell proliferation in skeletally unloaded mice. Lymph node T cell proliferation is presented as a stimulation index over lymph node cells which were not exposed to PHA. Numbers represent X ± SEM of replicate samples (4) in one experiment. Mice were treated with one of two dosing protocols as indicated in the figure. * $p<0.05$ compared to restraint control mice (1 of 2 and 1 of 1 experiments are shown for 10 μg/mouse/24 hours and 10

μg/mouse/48 hours, respectively; replicated experiments had similar results) Lymph node T cells were pooled from 3 animals per treatment group per experiment.

Figure 6: Flow cytometric analysis of bone marrow cells from antiorthostatically or orthostatically suspended mice or restraint controls. Freshly isolated cells were stained with MAC-1- or KMI-6-specific monoclonal antibodies (10 μg/well) as described in ref. 16. Numbers represent X ± SEM of 4 experiments where cells from 4 animals in each treatment group were pooled in each experiment and were analyzed by flow cytometric analysis.

The Effect of Space and Parabolic Flight on Macrophage Hematopoiesis and Function

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RUNNING TITLE: The effect of microgravity on macrophages.

KEY WORDS: macrophage, hematopoiesis, microgravity, spaceflight, IL-2, differentiation, proliferation.

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ABSTRACT:

We used weak electric fields to monitor macrophage spreading in microgravity. Using this technique, we demonstrated that bone marrow-derived macrophages responded to microgravity within eight seconds. We also showed that microgravity differentially altered two processes associated with bone marrow-derived macrophage development. Spaceflight enhanced cellular proliferation and inhibited differentiation. These data indicate that the space/microgravity environment significantly affects macrophages.

Abbreviations used in this paper:

BMM, Dulbecco's Modified Eagle's Medium containing 10% FBS and 15% LM929 fibroblast-conditioned medium (CSF-1 source); BMMH, BMM supplemented with 20 mM HEPES; FBS, fetal bovine serum; CGBA, commercial generic bioprocessing apparatus; CSF-1, colony stimulating factor-1; FPA, fluid processing apparatus; GM-CSF, granulocyte/macrophage colony stimulating factor; IL-4, interleukin-4; IL-6, interleukin-6; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MHC-II, class II major histocompatibility molecule; PEG-IL-2, polyethylene-glycol conjugated recombinant human interleukin-2; rIL-2, recombinant interleukin-2; STS, space transportation system; TGF-ß, transforming growth factor-ßb.

INTRODUCTION:

A number of experiments in recent years have characterized immunological changes in both animals and humans exposed to spaceflight (1-5). Additionally, a large body of evidence is accumulating which suggests that spaceflight can alter both immune and non-immune cell functions (6-12). Therefore, the immunological consequences of spaceflight may be a combination of physiological responses and changes in cell behavior. However, research involving the effects of microgravity on single cells is still in its infancy.

Spaceflight and physiological models of spaceflight have shown that skeletal unloading alters macrophage development from bone marrow cells (2, 3, 13, 14). Spaceflight can induce a monocytopenia (1) and some astronauts had abnormal numbers of circulating monocytes (macrophage precursors) following spaceflight (5). Therefore, as longer spaceflight missions become more common it is important to understand how extended exposure to spaceflight will affect hematopoiesis and macrophage function. Spaceflight via direct and/or indirect gravisensing, launch and reentry forces, hydrostatic pressure and radiation all can impact cells and organisms.

Cytokines play an essential role in the hematopoiesis of white blood cells (15, 16). We found that depressed marrow secretion of interleukin-6 (IL-6) and colony stimulating factor-1 (CSF-1) was associated with defective macrophage hematopoiesis in skeletally

unloaded animals (13, 14). These cytokines are important for macrophage growth and development (16, 17) and the data implicate that cells that produce these cytokines may be negatively affected in a space environment (2, 3). Changes in the bone marrow extracellular environment may also be responsible for changes in macrophage hematopoiesis (18). Additionally, macrophage hematopoiesis may be influenced by direct effects of space flight on cell function (19). Therefore, the purpose of this study was to begin to dissect how some aspects of space flight, such as microgravity, affects bone marrow macrophages and hematopoiesis.

In this report we demonstrate that macrophages *in vitro* sensed and responded to microgravity within seconds. Additionally, we show that *in vitro* macrophage development was affected by spaceflight, adherence and interleukin-2 (IL-2). Spaceflight enhanced cellular proliferation and decreased differentiation.

MATERIALS AND METHODS:

Animals.

Male, adult (8-12 weeks) C3Heb/FeJ mice were bred in the animal facility at Kansas State University and were used in these studies. The use of these animals was approved by the Animal Care Facilities Use Committee at Kansas State University which complies with NIH Animal Care Standards.

B6MP102 Bone Marrow-Derived Macrophage Cells.

B6MP102 cells are a CSF-1-dependent, adherent bone marrow-derived macrophage cell line (20) which is capable of cytokine production (Interleukin-1 [IL-1] and tumor necrosis factor-α [TNF-α]) following stimulation with phagocyte agonists (e.g. lipopolysaccharide [LPS]; 7, 20). These cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% FBS and 15% LM929 fibroblast-conditioned medium (CSF-1 source) (BMM).

Microgravity Attained Through KC-135 Parabolic Flight.

The NASA KC-135 aircraft is capable, through parabolic flight, of producing repetitive periods of microgravity approximately 20-25 seconds in duration (21, 22). This aircraft is based at Ellington Airfield (Houston, TX) and performs approximately forty parabolas per flight. Each 20-30 second microgravity period is separated by approximately 30 seconds of two times one unit gravity. This NASA platform provides

access to microgravity without the restrictions (equipment and availability) associated with space shuttle flight. However, unlike shuttle or biosatellite flights, microgravity is achieved for only short periods of time and is interpersed with variable and hypergravity conditions. Therefore, although the KC-135 offers access to microgravity, the experimental conditions are not necessarily comparable to long-term space flights.

Real-Time Cell Spreading Assay During KC-135 Flight.

Macrophage cell spreading was monitored using weak electric fields in a modified version of the assay originally developed by Giaever *et al.* (23-24) and diagrammed and used for macrophages by Kowolenko et al. (25). Briefly, a circuit was constructed which contained a variable impedance induced by B6MP102 cells adhering/spreading to/on a silver electrode which was inserted through the side of a 12 x 75 mm polypropylene snap cap tube (Falcon # 2063). A constant current 4kHz AC signal of approximately 1 mA was passed through the circuit causing a variable voltage proportional to the impedance created by B6MP102 cell adherence/spreading (when the system is treated as a simple resistor capacitor series circuit). The test-tube circuit was wired to an attached multiplexed analog-to-digital converter. The data were acquired and stored in a 286 microcomputer. Four cultures were monitored simultaneously at a rate of one sample per second for each culture. In this system, an increase in voltage represented an increase in cell spreading (due to an increase in electrode impedance i.e. spreading impedes current flow). The electrode area available to cells was approximately 5 x 10^{-4}

cm^2 which allowed the monitoring of groups of approximately 8-24 cells. Cells were added to sterile electrode-containing test tubes and incubated at 37°C for approximately twenty-hours prior to KC-135 flight. Cells were washed into experimental culture media (Dulbecco's Modified Eagle's Medium containing 10% FBS and 15% LM929 fibroblast-conditioned medium [CSF-1 source] and 20 mM HEPES {BMMH}) approximately 4 hours prior to flight. During flight, cultures were maintained at 37°C. All equipment was bolted to the cabin floor according to NASA KC-135 saftey protocols.

Semi-adherent Bone Marrow-Derived Macrophage Cultures Adapted for Spaceflight.

Bone marrow cells were obtained from the femora and tibiae of mice. The ends of femora and tibiae were removed and the cells were flushed from the bone using BMMH and a 26-gauge needle. Cells were passed three times through a 19-gauge needle to break up clumps and cells were pelleted and resuspended at a concentration of 1×10^7 cells per 2.8 ml of BMMH. One $\times 10^7$ cells were loaded into each fluid processing apparatus (FPA) culture vessel (22). During spaceflight all FPA cultures were housed inside the BioServe Space Technologies commercial generic bioprocessing apparatus (CGBA, 22). In brief, this equipment provides a three chamber mixing apparatus in which cells can be cultured under sterile conditions and treated with experimental reagents at pre-determined times during flight (Figure 1). Bone marrow cells were maintained in one chamber and were mixed with the contents of other chambers, which contained fresh medium or formaldehyde, depending on whether not viable cells were to

be maintained (Figure 1). Cell numbers were estimated post-flight using a hemacytometer and cell viability was tested by trypan blue exclusion. For all spaceflight experiments, concurrent ground controls were performed using cells from the same pool of cells used for spaceflight. Both spaceflight and ground control experiments were performed at ambient temperature (approximately 23°C to 27°C). Experiments were flown onboard the space shuttle missions STS-57, STS-60 and STS-62 during June 1993, February 1994 and March 1994 respectively. During STS-57 space shuttle temperatures ranged from 16 to 25°C. For STS-60 and STS-62 the ambient space shuttle temperatures were 22.5$\pm$0.5 and 27.0$\pm$0.5 respectively. Our average laboratory temperature (used for ground controls) was 23.7 $\pm$ 0.1.

Adherent Bone Marrow-Derived Macrophage Cultures.

Bone marrow cells were isolated as described above and seeded in 100-mm culture dishes at a concentration of 5 x 10^6 cells per plate in 6 ml BMM. On day 3 of culture, 3 ml of fresh BMM were added to cultures. Supernatants were collected on day 6 of culture and the cells were assayed for proliferation and the presence of phenotypic markers (described below). Proliferation was quantified using an MTT assay (14, 18). Cultures were maintained at 37°C and 8% CO2 for the duration of culture.

Interleukin-6 Assay.

Ground control and spaceflight supernatants were assayed for IL-6 content by

bioassay using the IL-6 growth-sensitive cell line B9 as previously described (14, 18). Growth was quantified using an MTT assay and concentrations were determined by linear regression and a rIL-6 (R&D, Minneapolis, MN) standard curve (sensitivity 625 fg/ml). Interleukin-6 production was quantified post-flight for space shuttle flights STS-57 and STS-60 from supernatants of viable cells returned to earth.

Flow Cytometric Analysis.

All steps in this procedure were performed on ice. Non-adherent bone marrow-derived macrophages were harvested from FPAs by a thorough wash with culture medium. Adherent bone marrow-derived macrophage colonies were harvested by a high pressure saline wash with a Pasteur pipet which was preceded by a 15 minute EDTA incubation. One x 10^6 bone marrow-derived macrophages, regardless of origin, were added per well of a 96-well, round-bottomed plate. The plate was centrifuged at 400 x g for 2 minutes and supernatants were discarded. Cells were incubated in 50 ml of FBS per well to inhibit non-specific Fc receptor binding, washed twice with sorter buffer (Hanks' balanced salt solution containing 2% FBS and 0.2% BSA) and incubated with 50 ml per well of the appropriate primary antibody (ATCC hybridoma TIB166 derived anti-MAC-2 and ATCC hybridoma HB94 derived anti-MHC-II were used as primary monoclonal antibodies). Following the primary antibody incubation, cells were washed twice in sorter buffer and incubated with secondary antibody (fluoresceinated goat anti-mouse $F(ab')_2$; Cappel, Durham, NC) for 30 minutes. Cells were washed twice in sorter

buffer, resuspended in 300 ml of PBS containing 1% paraformaldehyde and stored at 4°C until flow cytometric analysis (FACScan, Becton Dickinson). MAC-2 and MHC-II expression was quantified post-flight for the space shuttle flights STS-57, STS-60 and STS-62

Statistical Analysis.

Student's *t*-tests were used to determine statistical significance.

RESULTS:

Macrophage Cell spreading during parbolic flight.

Previous studies by our group demonstrated that phagocyte respiratory bursts were significantly enhanced during 10^{-2} x g episodes of parabolic flight (26). We have also suggested that signal transduction processes were affected by space flight (10). Although these data suggest that cellular responsiveness to a stimulus can be affected by the microgravity environment, it was still unclear if microgravity affected resting cells. Therefore, the aim of these experiments was to test whether macrophages would spontaneously respond to a change from 1xg to 10^{-2} x g. Cell spreading was chosen as it is a macrophage property which can change within seconds (27) and can be monitored on a real time basis (25). Figure 2b shows that B6MP102 macrophages responded to the 10^{-2}x g environment attained during parabolic flight by increasing cell spreading. This response was rapid. It occurred within eight to fifteen seconds of the establishment of the 10^{-2} x g environment and ceased shortly after returning to a normal or hypergravity environment. The fluctaions that occurred upon returning to normal (as represented by small spikes at the end of some parabolas (Figure 2) represented electronic noise of our system and did not correspond to any response. Furthermore, we did not see any adaptation to parabolic flight by the B6MP102 macrophages. Macrophages responded to 68.5% of the microgravity episodes they were exposed to (n=62 responses over 3 separate flights). Figure 2a demonstrates that this response was not simply due to a

change in force vectors (movement). Only one spike, due to electronic noise, was observed during level flight (Fig 2A). Therefore, we concluded that B6MP102 cells did not respond to severe flight turbulence. Interestingly, periods of 1.7 x g to 1.8xg also did not significantly affect cell spreading.

Cellular Proliferation During Spaceflight.

The evidence from KC-135 flight experiments indicated that bone marrow-derived macrophages spontaneously responded to microgravity. Because cellular responses during short periods of microgravity may not necessarily reflect the long-term effects of spaceflight on cells (26), we wanted to examine whether long-term exposure to spaceflight would affect normal bone marrow macrophage processes. Because of hardware limitations, macrophage adherence assays could not be performed during space shuttle flights. Furthermore, those assays are more reflective of early, transitory macrophage responses and are not the most suitable assays for measuring long-term space-flight effects. Therefore, we examined the effects of spaceflight on bone marrow-derived macrophage differentiation; an important macrophage process known to be affected *in vivo* by spaceflight (2, 3, 5) and skeletal unloading (3, 13, 14). Spaceflight increased proliferative rates of bone marrow-derived macrophages (Figures 3 and 4). During STS-57 we found more cells in flight cultures fixed on day 6 of flight ($p<0.001$, $n=6$) compared to similarly fixed ground controls (spaceflight $7.31 \pm 0.38 \times 10^6$; ground controls $4.60 \pm 0.29 \times 10^6$; Figure 3A). Viable cell cultures returned to Earth also had

more cells (>95% viability as determined by trypan blue for all sets) when compared to ground controls at 32 hours post-flight (spaceflight 3.25 $\pm$ 0.05 x 10^6; ground controls 2.23 $\pm$ 0.02 x 10^6; $p<0.025$, n= 2; Figure 4A). Similar results were obtained when cultures were fixed in space on day 7 of flight during STS-60 (spaceflight 8.16 $\pm$ 2.31 x 10^6; ground controls 3.29 $\pm$ 1.0 x 10^6; $p=0.11$, n= 4; Figure 3B) and STS-62 (spaceflight 6.61 $\pm$ 0.29 x 10^6; ground controls 4.87 $\pm$ 0.40 x 10^6; $p<0.001$, n=8; Figure 3C). Post-flight analysis of viable cell cultures flown on STS-60 showed an increased number of bone marrow-derived macrophages compared to ground controls (spaceflight 3.62 $\pm$ 0.51 x 10^6; ground controls 2.37 $\pm$ 0.52 x 10^6; $p<0.015$, n= 4; Figure 4B). These data strongly suggest that spaceflight enhanced bone marrow macrophage growth.

Interleukin-6 Secretion during space flight.

Interleukin-6 is an important cytokine for macrophage hematopoiesis and is produced in large amounts by differentiated bone marrow-derived macrophages compared to undifferentiated cells (14, 18). Therefore, IL-6 secretion serves as a good indicator of the degree of cellular differentiation. We found that cells which had been exposed to spaceflight secreted less IL-6 than matched ground controls. For example, bone marrow macrophages secreted approximately 200 pg/ml if they differentiated during spaceflight compared to over 500 pg/ml for matched ground controls (STS-60 $p=0.06$, n=4; Figure 5B). Similar results were obtained form the STS-57 flight ($p=0.01$, n=2; Figure 5A). Therefore, it appeared that spaceflight enhanced bone marrow macrophage

growth at the expense of differentiation.

The Effect of Spaceflight on Macrophage Antigen Expression.

Although it appeared that bone marrow macrophage differentiation was inhibited by spaceflight, it was possible that spaceflight/microgravity altered the secretory capacity of cells. Therefore, we also analyzed other markers of differentiation. The phenotypic expression of MAC-2 and MHC-II is indicative of macrophage activation and differentiation states (18, 27). Therefore, we quantified these molecules on bone marrow derived macrophages to assess differentiation. We found that bone marrow-derived macrophages exposed to spaceflight/microgravity on STS-57 and STS-60 expressed significantly lower numbers of these antigens than ground controls when assayed using flow cytometry (Table 1). Cells that differentiated on STS-62 indicated a similar trend, however, it was not statistically significant compared to ground controls. Using a matched t-test for all data we found that macrophages cultured in space expressed less MAC-2 ($p<0.05$) and MHC-II ($p<0.08$) than ground controls. Therefore, it appeared that differentiation, as measured by antigen expression, agreed with IL-6 secretion data and appeared to be inhibited.

Effects of rIL-2 on Bone Marrow-Derived Macrophage Cultures During Spaceflight.

Interleukin-2 increased bone marrow-derived macrophage proliferation *in vitro* (14). Because IL-2 reversed the inhibitory effects of skeletal unloading *in vivo* (14) we

analyzed effects of rIL-2 and microgravity on macrophage hematopoiesis *in vitro*. We found that rIL-2 (10 U/ml) increased bone marrow-derived macrophage proliferation compared to cultures without rIL-2 ($p<0.02$, $n=4$; Figure 3C). However, rIL-2 did not have a similar enhancing effect in cultures flown in space (Figure 3C). We also assessed MHC-II and MAC-2 expression on cells treated with rIL-2. Recombinant-IL-2 did not significantly change MAC-2 and MHC-II expression when ground cultures were compared to flight cultures (Table 1). However, rIL-2 significantly reduced MAC-2 expression in bone marrow-derived macrophage cultures compared to cells cultured in the absence of rIL-2 ($p<0.03$, $n=4$; Table 1).

The Effect of Temperature and Adherence on Bone Marrow-Derived Macrophage Phenotypic Markers.

Our data suggested that spaceflight augmented bone marrow macrophage proliferation at the expense of differentiation as assessed by IL-6 secretion and MHC-II and MAC-2 antigen expression. In spite of the reproducibility of the data over a number of space flights, we noted that IL-6 secretion and antigen expression of cells incubated in FPAs did not compare to IL-6 secretion and antigen expression of cells kept in normal cultures (18). Of course, part of the difference could be explained by the required ambient temperature of our flight experiments. In addition, FPAs required siliconization (SigmaCote) so that rubber septa could be easily moved within the glass barrel. Siliconization, though not toxic, reduced the adherence of bone marrow macrophages to

glass. Therefore, to help us put our flight data into perspective, we assessed the effects of temperature and adhesion on the bone marrow macrophage differentiation markers MHC-II and MAC-2. We found that bone marrow-derived macrophages cultured at 37°C in a semi-adherent situation (FPAs) expressed significantly less MAC-2 and MHC-II when compared to adherent cultures ($p<0.002$; Table 2). Therefore, FPA culture alone significantly reduced each of those surface molecules. We also examined the effects of temperature. Maintaining semi-adherent cultures at 37°C or ambient (23.7°C) conditions did not significantly affect MAC-2 expression. However, cells cultured in FPAs at ambient temperature expressed significantly higher levels of MHC-II than FPA cultures maintained at 37°C. Therefore, MAC-2 and MHC-II are not regulated by temperature in the same way. More importantly, neither was reduced because of lower temperatures which suggested that the reduction of MAC-2 and MHC-II molecule expression during space flight was not a temperature effect. Interestingly, rIL-2 significantly inhibited MHC-II antigen expression in adherent cultures incubated at 37°C. Just the opposite occurred when cells were cultured in FPAs. Interleukin-2 treatment did not affect MAC-2 expression (Table 2). Therefore, culture conditions [adherent vs. FPA (semi-adherent)], temperature and the presence of IL-2 affected molecular expression on bone marrow macrophages in an antigen-specific fashion.

DISCUSSION:

There is strong evidence that spaceflight/microgravity can change physiological processes within living organisms (29). However, there is controversy over whether individual mammalian cells are affected by the gravity vector. A number of *in vitro* microgravity studies have indicated that microgravity can affect mammalian cell function (7, 9, 11, 30). These spaceflight/microgravity studies have examined the responses of cells to stimuli and not spontaneous cellular responses. In addition, microgravity-induced effects sometimes may be accounted for by gravity-dependent changes in culture conditions rather than direct effects on cell behavior. For example, cultures in microgravity lack thermal convection (19), where autocrine cellular secretions and cellular waste products could accumulate around the surface of a cell rather than disperse throughout the culture vessel. This effect might induce changes in cell behavior. Therefore, we tested the spontaneous change in macrophage spreading, a characteristic which varied rapidly and was quantifiable on a real time basis in a microgravity environment. We found that B6MP102 macrophages responded to a 10^{-2} x g environment extremely rapidly as indicated by increased cell spreading. This is the first indication that mammalian cells extemporaneously alter their physical make-up because of gravity. These data support the hypothesis presented by Todd (19) and corroborate the data which showed that cellular responses were affected in microgravity (7-9, 11, 30).

B6MP102 macrophages secrete higher concentrations of cytokines in microgravity (7). The increased B6MP102 cell spreading during microgravity is consistent with that

data when one considers that macrophage cell spreading is a marker of cellular activation (18, 27). Photomicroscopic techniques allow the visualization of cell spreading at the micrometer level within 25 seconds (27). Electrophysiological techniques, such as the one employed in this study, can assay changes in cell spreading in the nanometer range (23-25) and can therefore detect changes in much shorter time periods. Changes in B6MP102 cell spreading were only observed when microgravity was sustained for more than eight seconds. Therefore, the biochemical reactions that regulate that change must occur within that eight second window. The "perception" of reduced gravity by B6MP102 may be mediated through mechanosensitive stretch-activated ion channels (31-33) or the extracellular matrix (32, 34). Further experiments will be required to accurately identify the signaling pathway(s) associated with gravity sensing in macrophages.

It appears that the biochemical responses that occur within eight seconds of exposure to microgravity translate into long-term changes in bone marrow macrophages. This effect may be the result of a cascade of signals arising from changes in adherence or a combination of biochemical changes of which cell spreading is one of the earliest. All cultures exposed to spaceflight proliferated faster than ground controls. However, those cells were less differentiated, as measured by IL-6 secretion and differentiation antigens. Proliferation and differentiation are two processes which are often antagonistic (18, 35-39). Therefore, it appears that spaceflight/microgravity increased macrophage proliferation at the expense of differentiation. This datum is consistent with the findings of Meehan *et al.* (1992) which showed abnormal mononuclear leukocytes *in vivo*

following spaceflight. They found that astronaut blood monocyte numbers were higher than normal following spaceflight and expressed low levels of insulin-like growth factor-1 receptors, were smaller in size and less granular (i.e. less differentiated). However, one must be cautious in relating *in vitro* cell biology spaceflight experiments to physiological spaceflight observations. Our *in vitro* cultures were not exposed to physiological endocrine factors associated with spaceflight or an *in vivo* matrix environment. Nonetheless, increased *in vitro* cell proliferation in microgravity has been observed on a number of occasions (30, 40). Based on data presented in this manuscript, it is possible that microgravity affects cellular proliferation both directly (effects on cell function) and indirectly (by changing culture conditions). Interleukin-6 has been shown to suppress proliferation and enhance differentiation in both HL-60 and U937 macrophage/granulocyte-like cells (37). Therefore, it is possible that spaceflight cultures exhibited enhanced proliferation and suppressed differentiation as a result of abnormally low IL-6 secretion.

IL-2 can increase *in vitro* macrophage colony formation (14). During STS-62 we investigated whether rIL-2 would affect macrophage differentiation or proliferation during spaceflight. We found that rIL-2 did increase proliferative rates in ground controls but not in spaceflight cultures. There are at least two possible explanations. Firstly, macrophage IL-2-induced signal transduction may be altered during spaceflight causing no rIL-2 effect on macrophage proliferation. Some have found that IL-2 binding activates protein kinase C (15) and protein kinase C is affected by microgravity (8, 10).

However, this scenario seems unlikely since the T cell IL-2 signal transduction pathway appears to be active in microgravity (9). Alternatively, spaceflight FPA cultures could have reached their maximal proliferative capacity which could not be further increased by IL-2 treatment. This could occur through two mechanisms: the cells could have been dividing at their maximal rate in microgravity, or the culture conditions of the FPAs were depleted of culture nutrients (e.g. oxygen, glucose, CSF-1) and additional proliferation was not possible.

The FPA hardware used in these studies allowed for the collection of reproducible data on bone marrow macrophage differentiation. However, there were some disadvantages compared to conventional ground-based culture systems. Macrophage development and function are influenced by adherence (18, 41, 42). Therefore, we were not surprised that bone marrow-derived macrophages cultured at 37°C in FPAs expressed significantly less MAC-2 and MHC-II than macrophages that differentiated in conventional adherent cultures. Lower temperature did not significantly inhibit the expression of MAC-2 when macrophages differentiated in FPAs, however, MHC-II expression was significantly higher in ambient cultures. Therefore, the apparent inhibition of differentiation during space flight as measured by the reduced expression of MAC-2 and MHC-II can not be explained because of diffferences in temperature between flight samples and ground controls.

Additionally, our experiments indicated that MAC-2 and MHC-II antigens were regulated independently as rIL-2 significantly down regulated MHC-II but not MAC-2 in

conventional adherent cultures maintained at 37°C. A similar dichotomy occurred in the experiment done in FPAs. However, rIL-2 upregulated MHC-II expression compared to untreated cells whereas MAC-2 remained unchanged. The fact that spaceflight down regulated both MAC-2 and MHC-II suggests that multiple cellular systems were affected during spaceflight. Moreover, our studies indicate that the regulation of MAC-2 and MHC-II antigens is complex; influenced by temperature, gravity and adherence. Therefore, following the example of Cogoli's group (9, 43), we have been cautious with our interpretations basing them on a good understanding of our culture system. Clearly, gravitational biology studies have influenced important ground-based experiments applicable to the whole field of cell biology and antigen regulation.

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Table 1: The effect of spaceflight on bone marrow-derived macrophage phenotypic markers.

Treatment:	%Cells MHC-II[a]	% Cells MAC-2[a]	n
Flight STS-57	7.9±0.4*	11.6±1.3	2
Ground STS-57	10.5±0	16±1.6	2
Flight STS-60	3.4±1.6+	0.5±0.2	4
Ground STS-60	7.9±1.7	4.1±1.6	4
Flight STS-62	4.4±0.6	5.1±0.9	4
Ground STS-62	5.2±0.3	5.8±0.4	4
Flight STS-62 + rIL-2	4.1±0.9	1.4±0.9++	4
Ground STS-62 + rIL-2	4.2±0.7	3.0±1.0++	4

[a] Represent mean ± SEM of n samples

* $p < 0.05$ compared to ground control, t-test.

+ $p < 0.1$ compared to ground control, t-test.

++ $p < 0.05$ compared to analogous culture without rIL-2, t-test.

Table 2: The effect of temperature, adherence and rIL-2 on bone marrow-derived macrophage phenotypic markers.

Treatment:	%Cells MHC-II[a]	% Cells MAC-2[a]	n
Adherent, 37°C	27.7±1.2	57.7±0.4	3
Adherent + rIL-2, 37°C	0*	53.7±3.9	3
Semi-adherent, 37°C	4.3±1.2*	10.9±3.0*	8
Semi-adherent + rIL-2,37°C	8.4±0.8*[Y]	11.7±1.4*	5
Semi-adherent, ambient	7.5±0.4*[Ξ]	6.2±1.0*	5

[a] Represent means ± SEM of n samples.

* $p < 0.05$ compared to adherent 37°C cultures.

[Y] $p < 0.1$ compared to semi-adherent 37°C cultures not supplemented with rIL-2.

[Ξ] $p < 0.05$ compared to semi-adherent 37°C cultures.

Figure Legends:

Figure 1: Time-line profiles of activities for bone marrow macrophage experiments flown on the space shuttle missions STS-57, STS-60 and STS-62.

Figure 2: The effect of KC-135 microgravity on macrophage cell spreading. Panel A: control recording done during level 1 x g flight and turbulent flight. Panel B: Changes in cell spreading in response to a microgravity environment. The end of the trace, after 240 min, also reflects level flight when parabolas had ended. Top line in each panel represents accelerometer trace scaled on the left. Bottom line in each panel represents impedence (spreading), scaled on the left.

Figure 3: The effect of spaceflight on bone marrow-derived macrophage proliferation. Bone marrow cells were added to FPAs, refed during flight, fixed during flight and counted on a hemacytometer after return to Earth. The scheduling of each event for each respective flight is summarized in Figure 1. Flight and ground samples were prepared at the same time, handled following the same time line and were quantitated simultaneously.

Figure 4: Post-flight quantitation of viable macrophages exposed to spaceflight. Bone marrow cells were added to FPAs, refed during flight and counted on a hemacytometer post flight. Viable cells were distinguished from dead cells by trypan blue exclusion (> 95% viability). The scheduling of each event for each respective flight is summarized in Figure 1.

Figure 5: The effects of spaceflight on bone marrow-derived macrophage IL-6 secretion. FPA cultures (ground and flight) were prepared, supplied with additional medium and viable cell culture supernatants were analyzed at the times indicated in Figure 1. Following spaceflight supernatants were analyzed by bioassay using the IL-6-sensitive cell line B9.

Figure 1

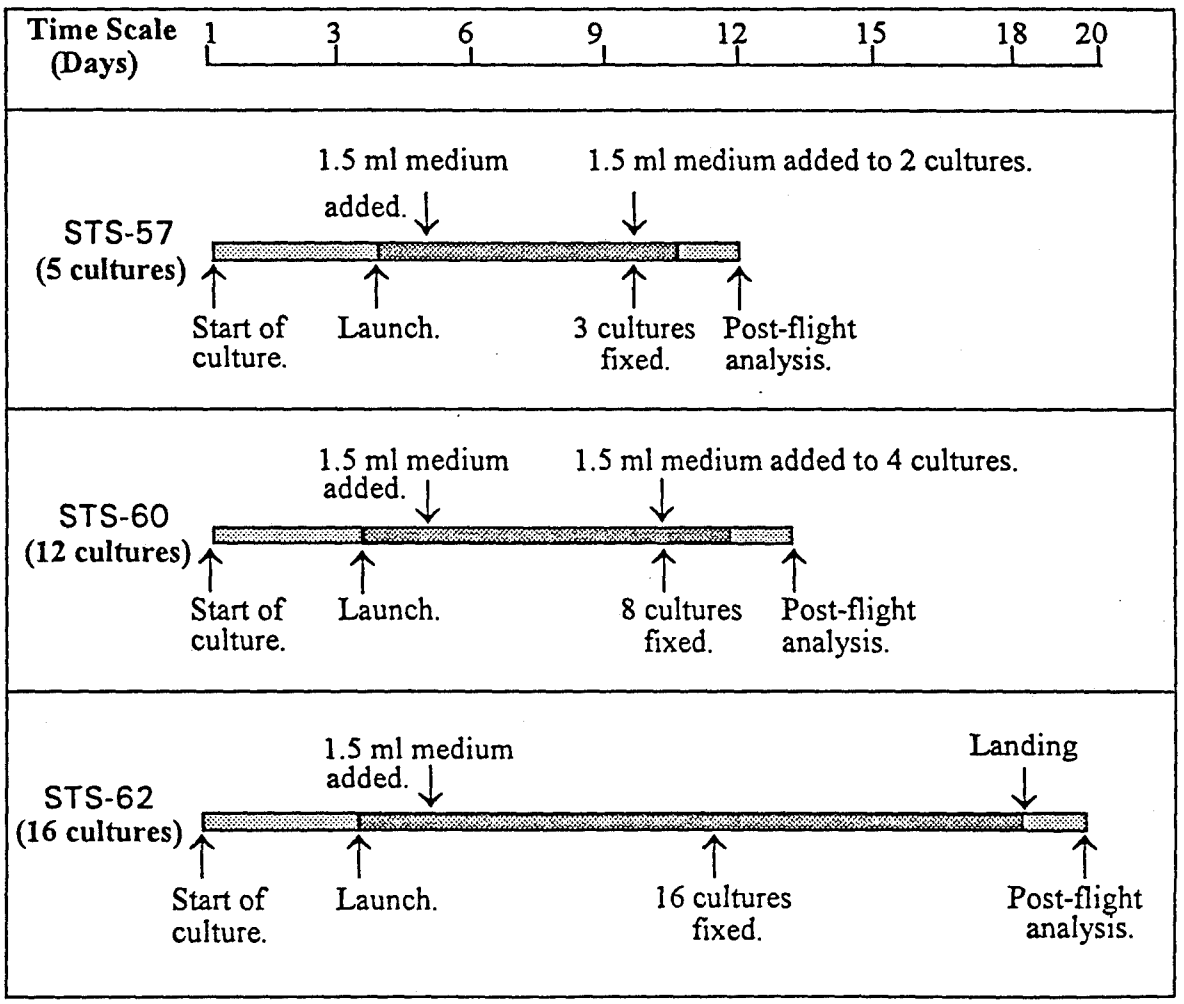

Time Scale (Days)
1
3
6
9
12
15
18
20
STS-57 (5 cultures)
1.5 ml medium added.
1.5 ml medium added to 2 cultures.
Start of culture.
Launch.
3 cultures fixed.
Post-flight analysis.
STS-60 (12 cultures)
1.5 ml medium added.
1.5 ml medium added to 4 cultures.
Start of culture.
Launch.
8 cultures fixed.
Post-flight analysis.
STS-62 (16 cultures)
1.5 ml medium added.
Landing
Start of culture.
Launch.
16 cultures fixed.
Post-flight analysis.

Figure 2

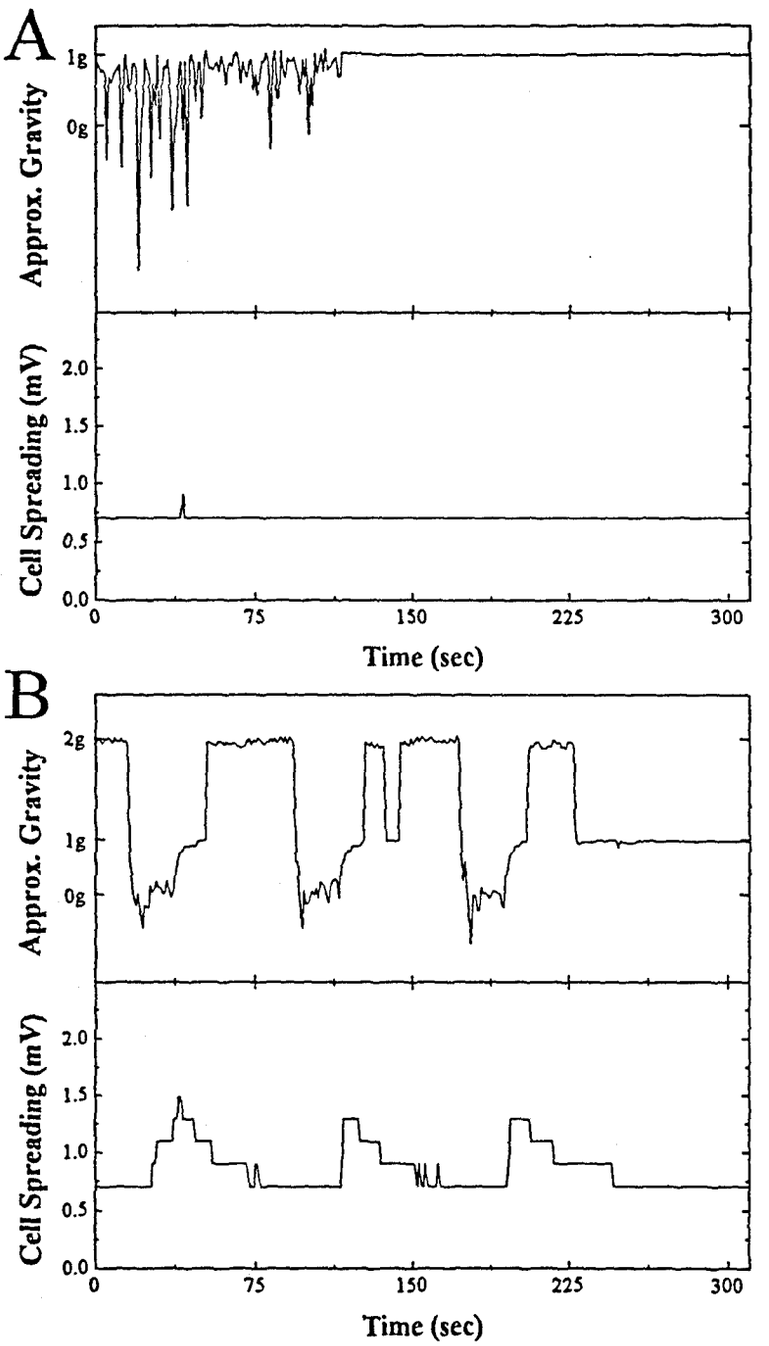
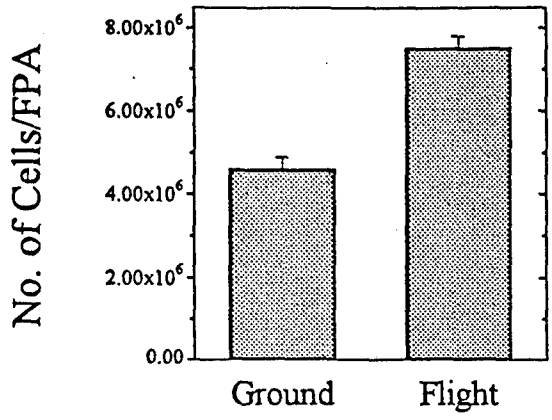
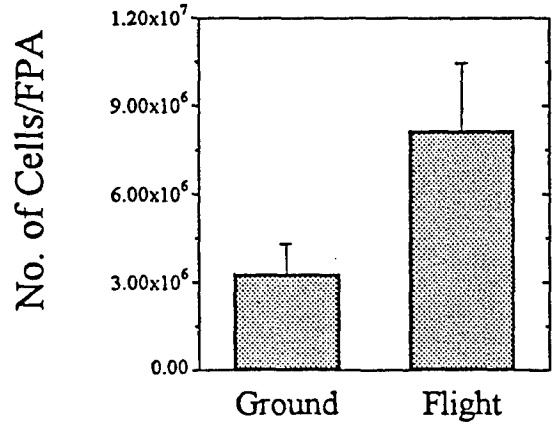

A
Approx. Gravity
1g
0g
Cell Spreading (mV)
2.0
1.5
1.0
0.5
0.0
0
75
150
225
300
Time (sec)
B
Approx. Gravity
2g
1g
0g
Cell Spreading (mV)
2.0
1.5
1.0
0.5
0.0
0
75
150
225
300
Time (sec)

Figure 3

A: STS-57 formaldehyde treated.



B: STS-60 formaldehyde treated.



C: STS-62 formaldehyde treated.

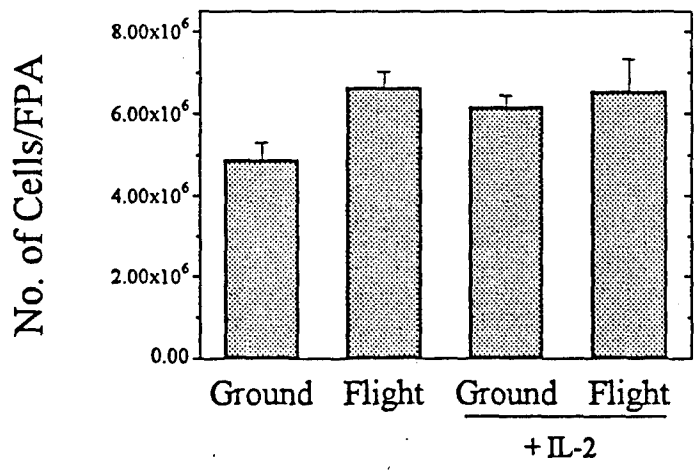
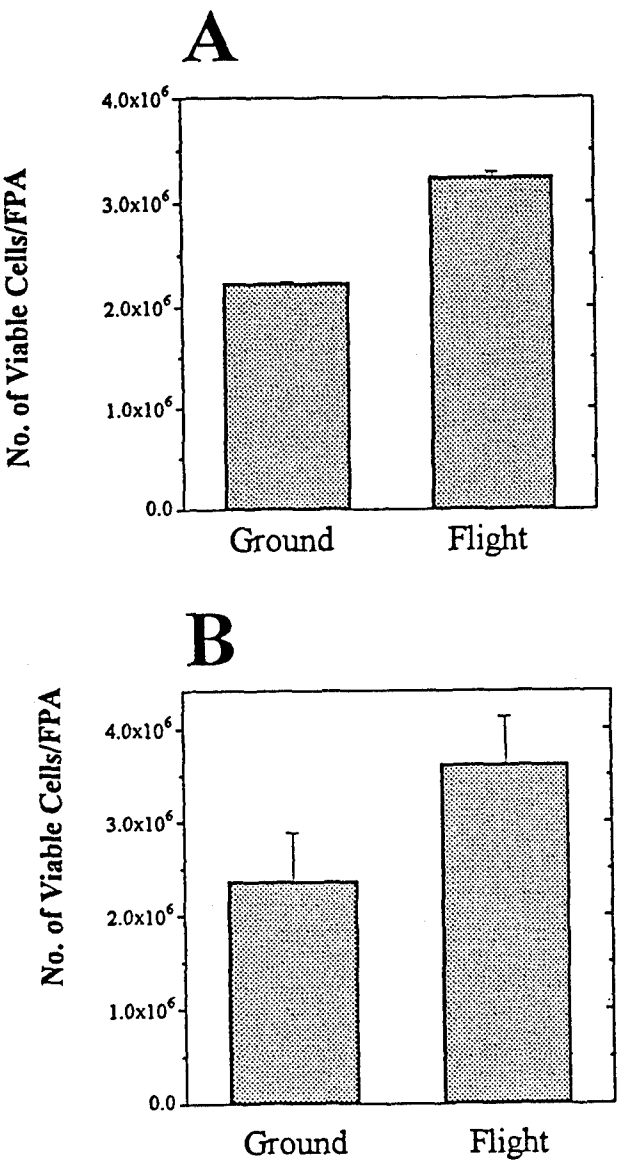


Figure 4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Flight
B
No. of Viable Cells/FPA
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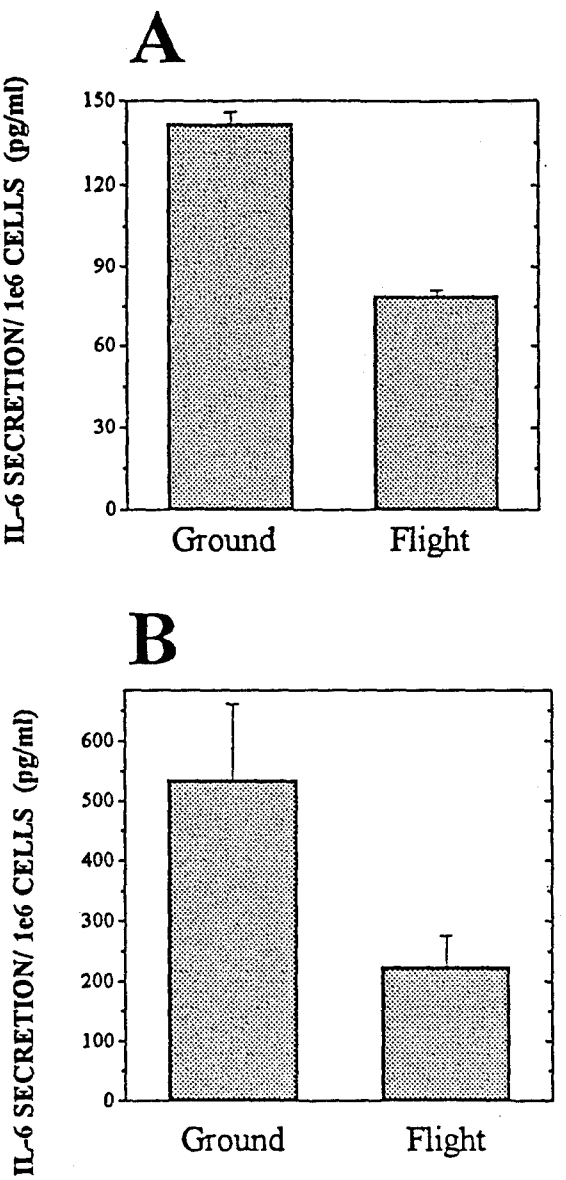
3.0x10^6
2.0x10^6
1.0x10^6
0.0
Ground
Flight

Figure 5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600
500
400
300
200
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Ground
Flight

N-Tosyl-Phenylalanine Chloromethyl Ketone (TPCK) Reverses the Resistance of F5m Tumor Cells to Macrophage-Mediated Cytolysis[1]

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Running Title: Tumor cell resistance to macrophage-mediated cytolysis

Key words: macrophage, tumoricidal, tumor necrosis factor, TPCK, nitric oxide

Footnotes:

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4 Abbreviations: NO, nitric oxide; NMA, N^G-methyl-L-arginine; TNF, tumor necrosis factor-alpha; TPCK, N tosyl-phenylalanine chloromethyl ketone; TLCK, N-p-tosyl-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; Mϕ, macrophage.

ABSTRACT

The SV40-transformed cells F5b and F5m became more sensitive to killing by activated macrophages after pretreatment with tosyl-phenylalaninelchloromethyl ketone (TPCK). This effect was specific for TPCK. Other serine protease inhibitors such as tosyl-lysinechloromethyl ketone (TLCK), phenylmethylsulfonyl flouride (PMSF), or chymotstatin had no effect upon killing. The enhanced macrophage-mediated killing of F5m cells correlated with increased binding to macrophages and was dependent on tumor necrosis factor-α (TNF).

INTRODUCTION

Macrophages become tumoricidal when treated with interferon-γ (IFN) and lipopolysaccharide (LPS) (1). These macrophages discriminate between normal and transformed cells and kill only the transformed cells (2). TNF and nitric oxide (NO) are important mediators of macrophage-mediated tumoricidal activity (3-8). For example, the F5b cell line, isolated and characterized (7,9-11) by our group is killed by activated macrophages through a TNF-dependent process (7). Cytotoxicity is contact-dependent but the processes which occur during binding are poorly understood; especially since binding appears to be a universal interaction between cells killed and not killed by activated macrophages (11). Our group has also described a sister clone of F5b, F5m, which is resistant to macrophage-mediated killing (9,10). However, this sister clone is similar to F5b in that it was sensitized to TNF-mediated cytolysis by a purified, biologically active peptide fragment from a membrane sialoglycoprotein ubiquitiously expressed on cells believed to be involved in contact inhibition (Woods *et al.* manuscript in preparation). The similarity between F5b and F5m cells in those assays suggested that F5m cells were able to protect

themselves from metabolic inhibition naturally induced by activated macrophages with F5b cells.

Other investigators have reported that serine protease inhibitors can alter patterns of TNF cytolysis (12-14). Because F5m cells are resistant to macrophage-mediated killing while F5b cells are killed by macrophages involving TNF, we investigated the possible role of serine proteases in the resistance of F5m cells to macrophage-mediated killing. We report that TPCK, a chymotrypsin-like protease inhibitor specifically sensitized F5m cells to macrophage-mediated killing which was dependent on TNF.

MATERIALS AND METHODS

Cells and Tissue Culture Balb/c 3T3 cells were obtained from the American Tissue Type Culture Collection (ATCC). The SV40-transformed cells, F5b and F5m were previously characterized (9-11). The cells were passaged 3 times weekly in Dulbecco's minimal essential medium (DMEM, Gibco, Grand Island, NY) supplemented with 2% fetal bovine serum (FBS, Gibco), 10% Opti-MEM (Gibco) and 0.3% L-glutamine. All continous tissue culturing was done in the absence of antibiotics and antimycotics.

Protease inhibitors Stock solutions of the protease inhibitors N-tosyl-phenylalanine chloromethyl ketone (TPCK), N-pα-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulfonyl fluoride (PMSF) and Chymostatin (Sigma, St. Louis, MO) were dissolved at a concentration of 4 mM in dimethylsulfoxide (DMSO) and stored at -20°C.

Cytotoxicity assays Murine peritoneal macrophages were harvested by lavage 4-5 days after i.p. injection of 1.5 ml of thioglycollate broth (Difco, Detroit, MI). The cells were suspended at a concentration of 2 x 10^6 cells/ml in fresh DMEM. The macrophages were

seeded in a microtiter plate; 2 x 10^5 macrophages/well (20:1 effector to target ratio) (assays with anti-TNF) or 3 x 10^5 macrophages/well (30:1 effector to target ratio) (assays with or without N^G-methyl-L-arginine; NMA, Sigma, St. Louis, MO). The macrophages were allowed to adhere for 1 hr, the medium was removed and the cells were refed with DMEM containing 12.5 μg/ml lipopolysaccharide (LPS, Difco) and 10 U/ml interferon-γ (IFN, Genzyme, Cambridge, MA). Target cells were radiolabeled 100 μCi of $Na_2{}^{51}CrO_4$ overnight (NEN, Dupont). The targets were trypsinized and resuspended in medium at a concentration of 10^5 cells/ml. A 100 μl aliquot of cells (10^4 cells/well) was added to the wells containing the macrophages. Three wells containing labeled target cells alone were used as a maximal release after 100 μl of 2N HCl was added and three wells containing targets alone with 100 μl medium served as a spontaneous release control. After a 16 hr incubation in 8% CO_2 at 37°C, 90 ul aliquots were removed from each well and the ^{51}Cr released was quantitated in a Packard gamma counter. Specific ^{51}Cr release (SR) was calculated from the following equation:

$$SR = \frac{\text{Experimental release (CPM) - Spontaneous release (CPM)}}{\text{Maximal release (CPM) - Spontaneous release (CPM)}} \times 100$$

In experiments using protease inhibitors, the desired number of target cells were aliquoted in 12 x 75 disposable tubes, centrifuged at 375 x g for 3 minutes, resuspended in 1 ml of medium containing 20 μM of each of the protease inhibitors. The cells were treated with each inhibitor for 10 min. at r.t., then centrifuged. The medium was removed and the cells were resuspended in fresh medium at a concentration of 10^5 cells/ml then added to the macrophages. For assays containing NMA (Sigma); 50 μl of medium with or without 2 mM NMA was added to the wells, to which 50 μl of 2 x 10^5 target cell/ml suspension was added. For assay with anti-TNF antibodies; the macrophages were activated for 4 hrs, then

the medium was removed and the cells were refed with 100 μl of fresh medium containing 500 μg/ml anti-TNF antibodies or normal rabbit antibodies. 100 μl of 10^5 target cell/ml suspension was added to the assay.

Binding assay Peritoneal macrophages were obtained as described for the cytotoxicity assay (also see ref. 11). The cells were seeded in UV-treated (18 hrs) flexible polyvinyl chloride microtiter assay plates (Falcon, Lincoln Park, NJ) and allowed to adhere for 1-2 hrs. The macrophages were washed and refed with 100 μl of fresh medium. Chromium-labelled target cells, pretreated with the inhibitors as described in cytotoxicity assay were removed with 0.2% ethylenediamine tetraacetate (EDTA, Fisher) and centrifuged at 325 x g. After the medium was removed and the cells resuspended at 10^5 cells/ml and 100 μl cell suspension was added per well; three wells without macrophages served as background binding. The plates were sealed with pressure sensitive adhesive film (Falcon) and incubated 1.5-2 hrs at 37°C. The sealed plate was inverted and centrifuged at 1300 x g. The wells were separated with a hot wire and counted in a gamma counter and the % binding was calculated by the following equation:

$$\% \text{ binding} = \frac{[\text{Experimental CPM (+ M}\phi\text{) - Spontaneous CPM (- M}\phi\text{)}]}{\text{Total CPM added}} \times 100$$

Anti-TNF antiserum A New Zealand white rabbit was hyperimmunized with carrier-free recombinant murine TNF (rmuTNF; Genzyme). The first immunization was given into the hind footpads and subcutaneously in the subcapsular region of the back in complete Freund's adjuvant. All subsequent injections were given similarly in incomplete Freund's adjuvant. The rabbit was bled twice monthly. Hyperimmune serum inactivates 64 Units/ml rmuTNF in LM929 bioassay > 90% at a 1:128 dilution. This antiserum also specifically inactivates rat and rabbit TNF in similar bioassays. Preimmune sera had no effect on TNF

bioassays at similar dilution. This antiserum does not interfere with IL-1 and IL-6 biological activities. The antibodies were precipitated (2x) with 40% saturated ammonium sulfate (NH_4SO_4) then dialyzed against normal saline for 24 hours. Protein concentration was determined by the Bicinchoninic Acid method (BCA) (15).

SDS/PAGE Analysis 4 x 10^6 cells were treated for 10 minutes with 20 μM inhibitors, then incubated 1.5 hours at 37°C. The cells were washed with normal saline then lysed using 0.75 ml of NP40/saline (Sigma) [.15 M sodium chloride, 20 mM Tris (Fisher), .12 IU aprotinin/ml (Sigma)]. The protein concentration was quantitated by the BCA method. Sixty µg of proteins in sample buffer with 1 mM dithiothreitol (Sigma) were added to each lane of a 10% polyacrylamide gel. The gel was silver stained using silver stain plus (BioRad, Hercules, CA).

RESULTS

The pretreatment of F5b and F5m cells with TPCK enhances their susceptibility to macrophage-mediated killing It was previously shown that TNF-mediated killing of virus-infected and tumor cells was affected by TPCK, an inhibitor of chymotrypsin-like serine proteases (12-14). We determined whether short treatments of F5b and F5m with TPCK, which did not adversely affect their viability, would affect macrophage-mediated killing of these two cell lines. This was particularly important because macrophages kill F5b cells by a TNF-dependent process (7). In these assays, F5b and F5m cells were pretreated with inhibitors for 10 min., then washed free of the drugs and placed with macrophages. Figure 1 shows that the pretreatment of F5b cells with TPCK for 10 min. at room temperature enhanced their susceptibility to macrophage-mediated killing ($p < 0.01$

compared with medium and TLCK treatments). Similar pretreatment rendered resistant F5m cells susceptible to macrophage-mediated killing ($p < 0.02$ compared with medium and TLCK treatments) while the nontumorigenic 3T3 cells remained relatively resistant to lysis (Figure 1a). Enhanced cytotoxicity was not observed when cells were pretreated with the other serine protease inhibitors TLCK or PMSF (Figure 1b). Moreover, the presence of LPS and IFNγ in the assay did not induce the pretreated cells to become sensitive to TNF (data not shown). The TPCK-pretreatment enhanced macrophage-mediated killing in a dose-dependent fashion (Figure 2). Chymostatin, another inhibitor of chymotrypsin was unable to enhance target cell susceptibility to activated macrophages (Figure 3) nor could it competitively inhibit the effect of TPCK pretreatment (data not shown).

The role of TNF and NO in TPCK-enhanced macrophage-mediated killing The enhancement of macrophage-mediated killing of F5b and F5m cells pretreated with TPCK was intriguing. Furthermore, 3T3 cells were not killed by macrophages after pretreatment with TPCK. Because F5b cells are normally killed via a TNF-dependent process by activated macrophages (7), we determined whether TNF was involved in the enhanced cytotoxicity. The killing of TPCK-pretreated F5b and F5m cells by macrophages was abrogated in the presence of anti-TNF antibodies (Figure 4). While others have found that macrophage-mediated killing SV40-transformed cells required the presence of TNF and nitric oxide (NO) (8) we found that normal in vitro macrophage cytotoxicity of F5b did not require NO (7). Nevertheless, the selectively enhanced killing of F5m and F5b cells after treatment with TPCK could require this reactive nitrogen radical. To test this hypothesis, we used N^G-methyl-L-arginine (NMA), a competitive inhibitor of nitric oxide synthase, to inhibit NO production. This NMA concentration inhibited nitric oxide production by LPS-

activated macrophages in preliminary experiments with a greater than 90% efficiency (data not shown). It caused some inconsistent inhibition of killing of the untreated and TLCK-pretreated F5b cells, however, it had no effect on TPCK-induced killing of F5b or F5m (Figure 5). NMA alone did not sensitize the cells to TNF because F5m cells did not become sensitive to macrophage-mediated killing in the absence of TPCK-pretreatment (Figure 5). These data suggests that TPCK-induced killing of F5m and F5b cells was mediated by TNF and did not involve nitric oxide.

TPCK-pretreatment enhanced macrophage binding of F5m cells Previous studies into the mechanism of resistance of F5m cells to macrophage-mediated killing found that these cells were bound less efficiently by macrophages (10,11). Although binding itself does not cause cytolysis (11), it is an important step during contact-dependent killing of tumor cells by macrophages. Therefore, we tested the hypothesis that TPCK pretreatment reversed the resistance of F5m cells by enhancing binding between F5m cells and macrophages. We found that pretreating F5m cells with TPCK, but not TLCK, significantly enhanced their binding to macrophages (Figure 6). Moreover, binding correlated with the specific effects of TPCK, but not TLCK, on killing. Although TPCK also increased F5b binding by macrophages, the enhancement was not statistically significant (Figure 6). SDS/PAGE analysis of F5m and F5b cell lysates after treatment with the inhibitors TPCK and TLCK did not show any detectable differences in the protein patterns from those of the nontreated lysates (Figure 7).

DISCUSSION

Our results show that macrophage-mediated cytolysis of F5m cells was enhanced by the chymotrypsin inhibitor TPCK. We were unable to reproduce this effect with other serine protease inhibitors which indicates a specificity for TPCK. These data, especially those with chymostatin, a chymotrypsin inhibitor, suggest that the enhancing effect may independent of TPCK's protease inhibitory activity. Alternatively, we may have discovered an assay that finely discriminates the specificity of these inhibitors. This would be quite unique since not all inhibitory activities of TPCK and TLCK are distinguishable. For example, both TPCK and TLCK can inhibit the shedding of TNF receptors from THP-1 cells (16).

These results are opposite of those reported by others where soluble rTNF-mediated cytolysis of cells was inhibited by TPCK (12-14). Therefore, soluble TNF-mediated lysis appears to be a distinct process from TNF involvement during macrophage-mediated cytotoxicity. One must note, however, that the experiments are not comparable. In soluble TNF assays, target cells were treated with inhibitors for the entire length of the assay. In experiments described in this paper, target cells were only pretreated for 10 minutes. Many cellular processes are probably affected by long-term treatment with TPCK.

The enhancing effect of TPCK was selective for F5m and to a lesser extent F5b cells but was not generally seen with the nontumorigenic 3T3 cells. Therefore, although F5m and 3T3 cells are not killed by activated macrophages they must have different cellular mechanisms of resistance. Since F5b and F5m cells are transformed by SV40 virus this difference is consistent with the regulatory perturbations characteristic of SV40-transformation and T antigen expression (17). Consistent with this is that TPCK-pretreated

F5m cells were killed by activated macrophages in a TNF-dependent manner similar to that previously reported for F5b cells (7). Moreover, both of the SV40-transformed cells, but not the 3T3 cells, were sensitized to TNF-mediated lysis by a purified peptide fragment from a surface sialoglycoprotein (Woods *et al.* manuscript in preparation). Thus, F5m cells have a protective mechanism, inhibitable by TPCK, which prevents their sensitization to TNF by activated macrophages. However, because 3T3 cells are resistant to killing by activated macrophages or membrane molecules which can sensitize cells to TNF, one should not expect 3T3 to express or require the same biochemical protective mechanism as F5m cells.

The previous explanation for F5m resistance was that there were impaired binding interactions between F5m cells and macrophages (10,11). The resistant nature of F5m was illustrated when F5b cells, fused with liposomes made from purified F5m membranes, showed a small but significant decrease in their susceptibility to macrophage-mediated killing (18). Those results indicated that a difference in membrane constituent was responsible for decreased killing and binding. The data presented in this paper support that hypothesis. Additionally, Laster *et al.* (19) reported that supernatants from F5i, a resistant sister clone of F5m, contained an inhibitor of macrophage migration and would be consistent with an active protective process in F5m cells. Although we were not able to identify specifically altered proteins in TPCK-pretreated F5b or F5m cells using SDS/PAGE analysis (Figure 7), it appears that some TPCK-inhibitable biochemical pathway regulates macrophage binding of tumor cells. This makes sense because serine proteases control many important membrane processes such as the activation of cell surface receptors (20) and also helps to facilitate tumor cells invasiveness (21,22). Moreover, it has been shown

that tumor cells which are resistant to macrophage-mediated killing also exhibit increased metastatsis and malignancy (23). This observation may support our results in suggesting that resistance to macrophages and metastases may be associated with expression of surface serine proteases on tumor cells.

In summary, these results contribute to our understanding of macrophage-SV40-transformed tumor cell interactions. F5m cells have a defense mechanism that disrupts the sensitization stage of the killing process. TPCK-pretreatment overcomes this defense and F5m cells are sensitized by macrophages to TNF, a process independent of NO. Therefore, in the absence of the protective mechanism, F5b and F5m are killed in the same manner. This proposed mechanism of macrophage-mediated killling may not necessarily be applicable to other tumor cell systems. For example, macrophages kill P815 cells using a contact-dependent process involving NO not TNF (24). Indeed, in our hands the binding requirements for killing P815 differed from the requirements for F5b cytotoxicity (10). Nevertheless, we have successfully constructed a working model to explain the general observation first made by Chapes *et al.* in 1987 (9). That was, that macrophages kill F5b but not F5m or related subclones of the F5 tumor series (25).

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FIGURE LEGENDS

Figure 1. Pretreatment of target cells with TPCK, TLCK or PMSF for 10 min. at room temperature. A. TPCK-pretreatment specifically sensitized F5b and F5m cells but not 3T3 cells to macrophage-mediated killing. B. Serine protease inhibitors TLCK and PMSF did not sensitize F5b and F5m cells to macrophage-mediated killing. The target cells were treated with 20 μM concentration of the inhibitors for 10 minutes at room temperature. The inhibitor-containing media were removed and the cells were resuspended in fresh medium prior to adding them to the macrophages. Assay 30:1 ratio (macrophage: target cell) and the results recorded 16 hours later. * Two-tailed T test indicated that the the effects of TPCK treatment on F5b and F5m cells susceptibility was significant: medium vs TPCK $p=0.017$; TLCK vs TPCK $p=0.002$. ** $p < 0.02$ TPCK treatment vs medium and TLCK. Error bars represent SD.

Figure 2. Dose reponse of TPCK pretreatment on the enhancement of macrophage-mediated killing. Assay done at 30:1 ratio (macrophage: target cell) Treatment protocol of the target cells is described in Figure 1. The absence of error bars indicate that the SD was smaller that the graphic symbol.

Figure 3. Pretreatment of F5m and F5b cells with TPCK or chymostatin. Assay done at a 30:1 ratio (macrophage: target cell). Treatment protocol was as described in Figure 1.

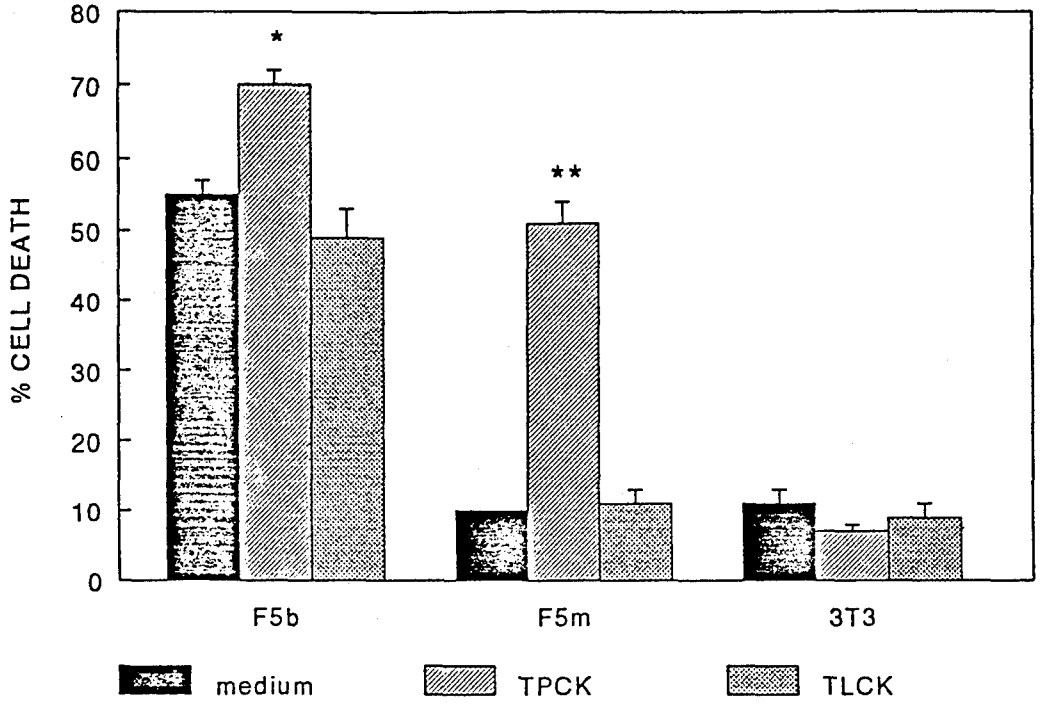
Figure 4. The role of TNF in macrophage-mediated killing of pretreated cells. Rabbit anti-TNF polyclonal antiserum or preimmune serum (250 μg/ml) was added to the assay 4 hours after the macrophages were activated at the time the target cell addition.

Figure 5. The role of nitric oxide in the sensitization of TPCK-pretreated targets to TNF. N^G-methyl-L-arginine was added to the cytotoxicity assay to a final concentration of 500 μM.

Figure 6. TPCK-pretreatment F5m cells enhanced their binding to macrophages. The target cells were pretreated as described in Figure 1. * $p = 0.051$ compared with TLCK treatment.

Figure 7. SDS/PAGE analysis of proteins from cell lysates 1.5 hours after a 10 minute treatment with TPCK and TLCK. Lanes 1-3 are lysates from F5b cells after treatment with (1) medium; (2), 20 μM TPCK; and (3) 20 μM TLCK. Lanes 3-6 are lysates from F5m cells after treatment with (3) medium; (2), 20 μM TPCK; and (3) 20 μM TLCK.

a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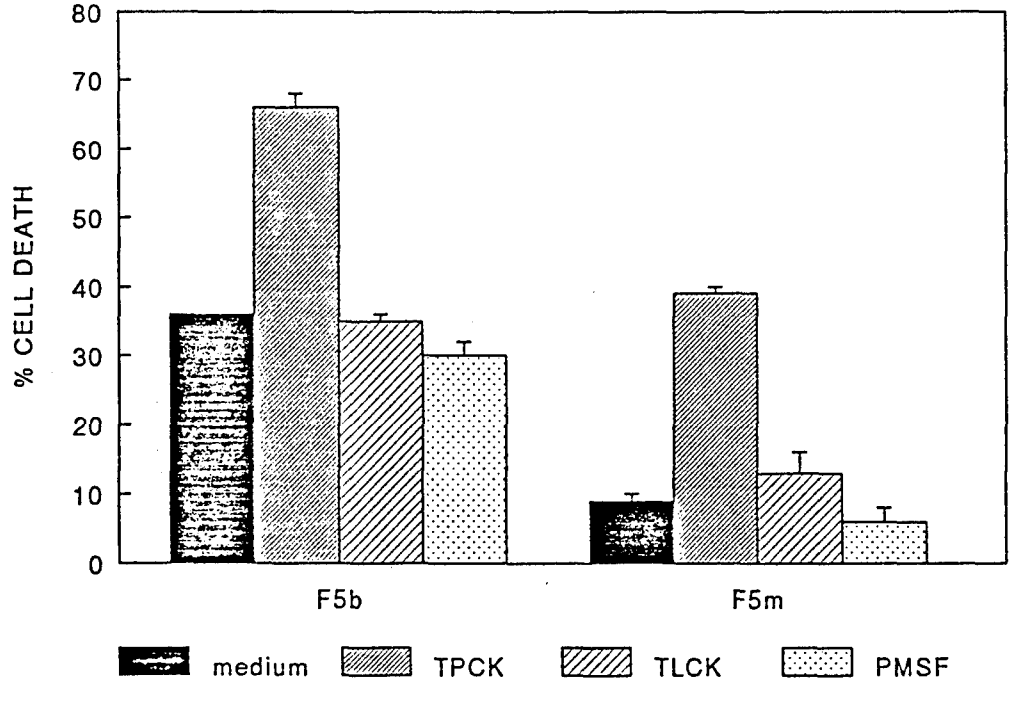
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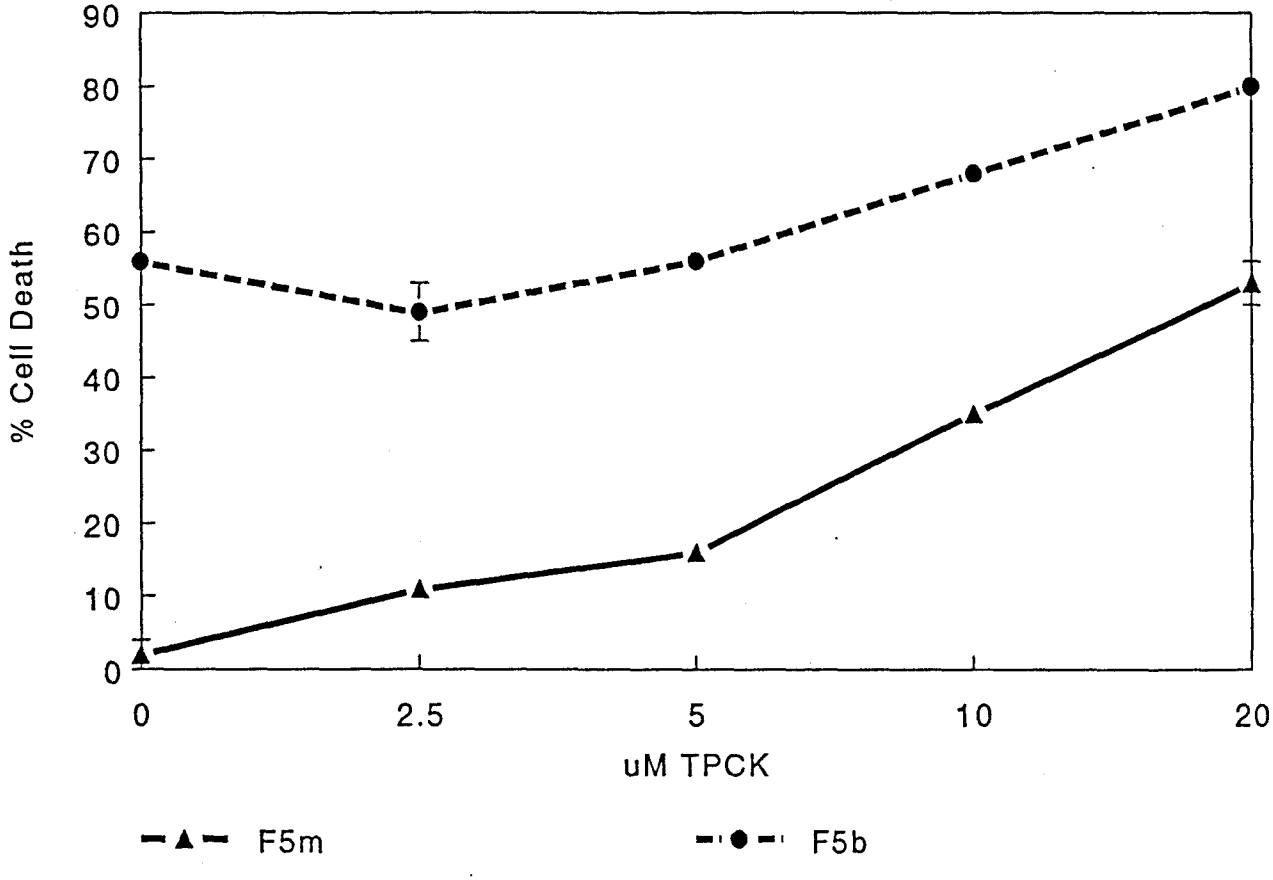
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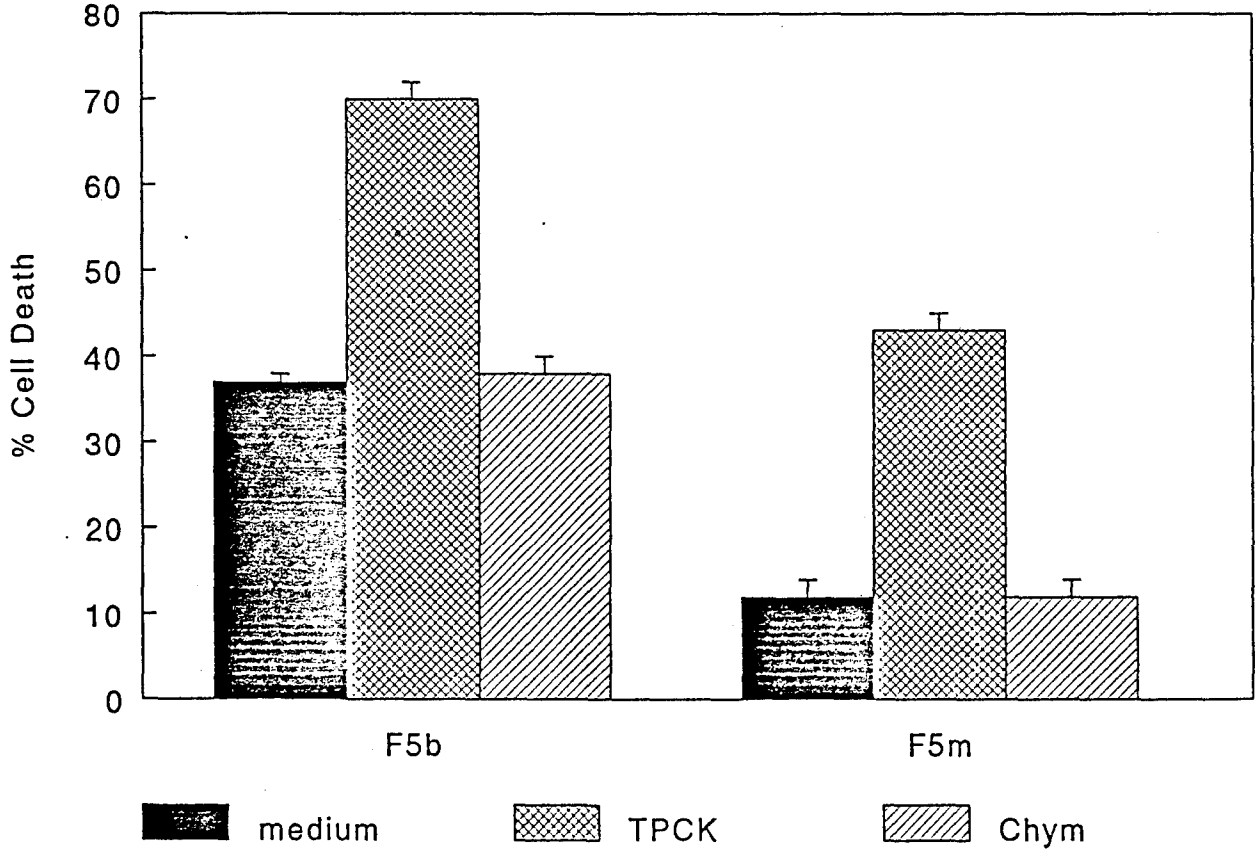
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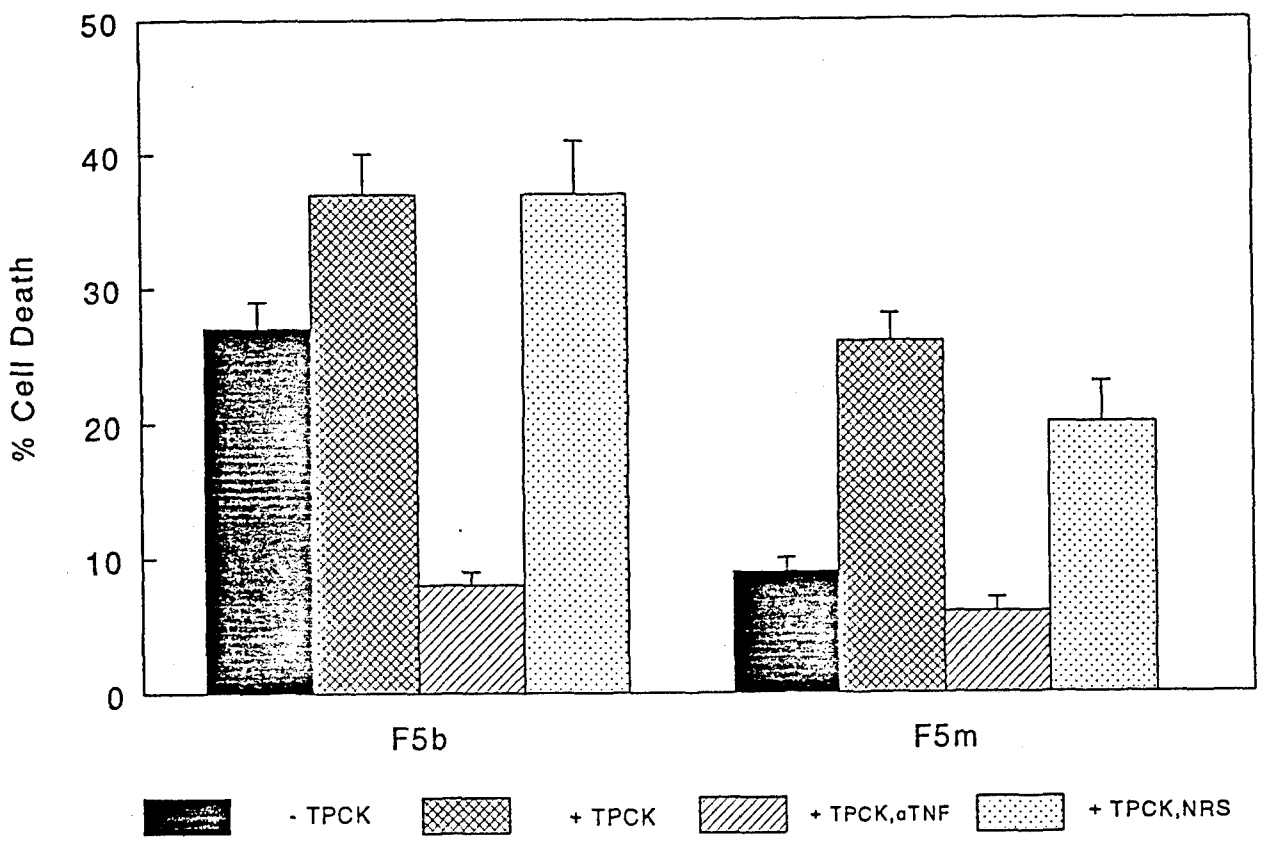
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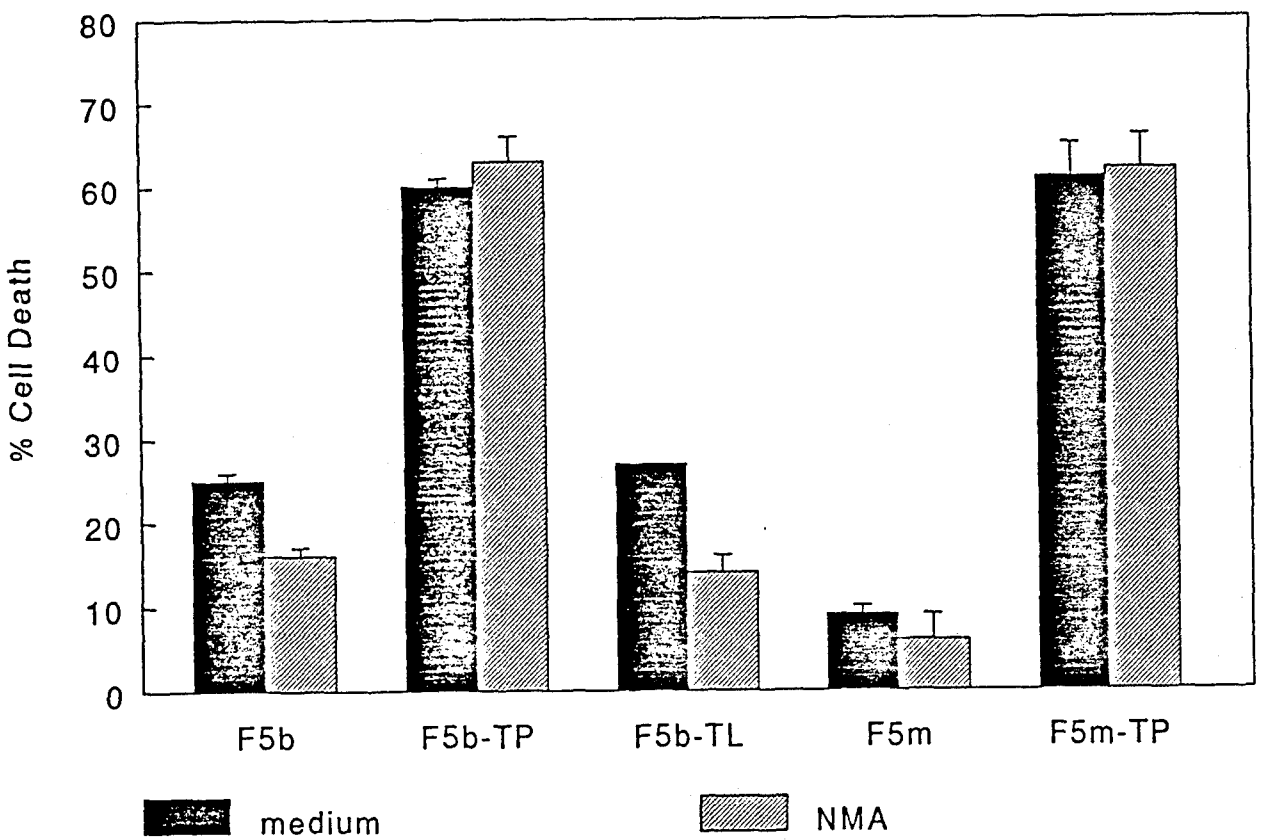
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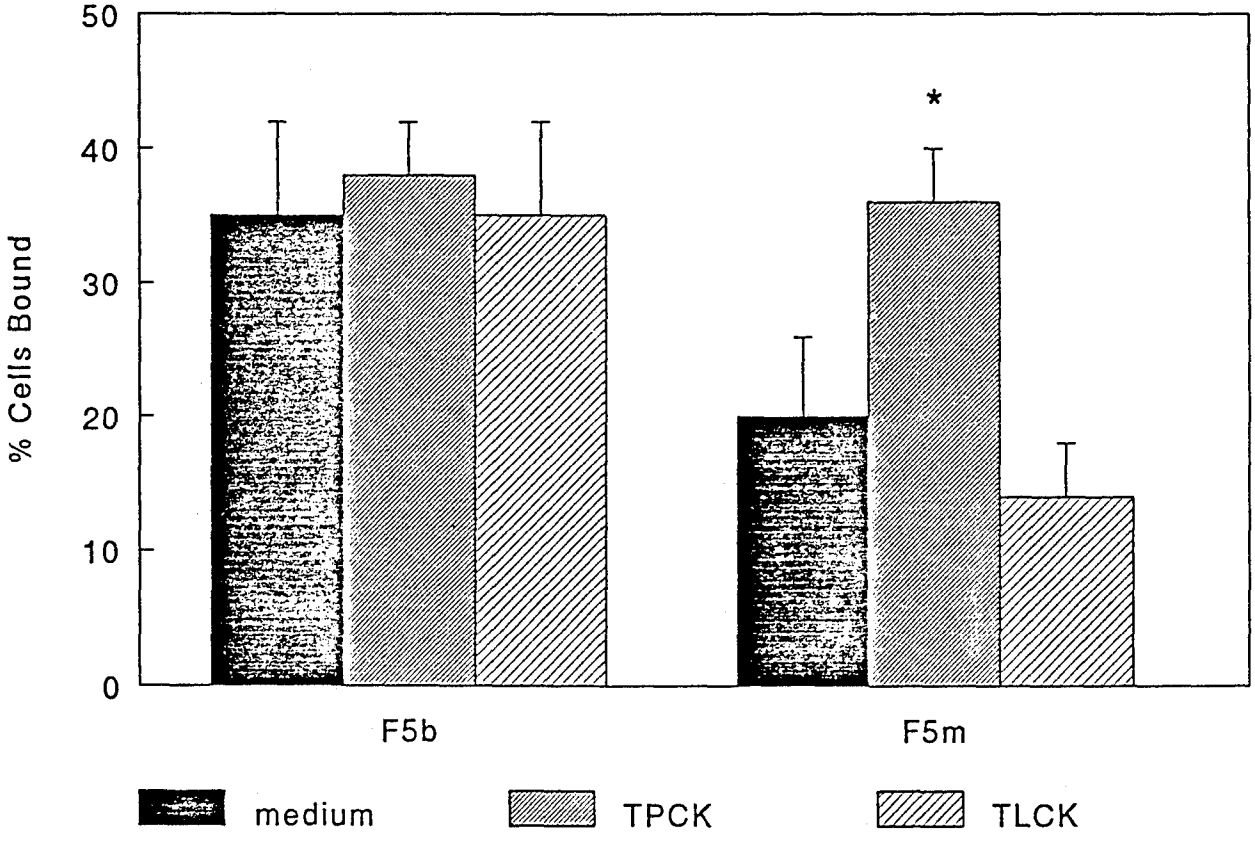
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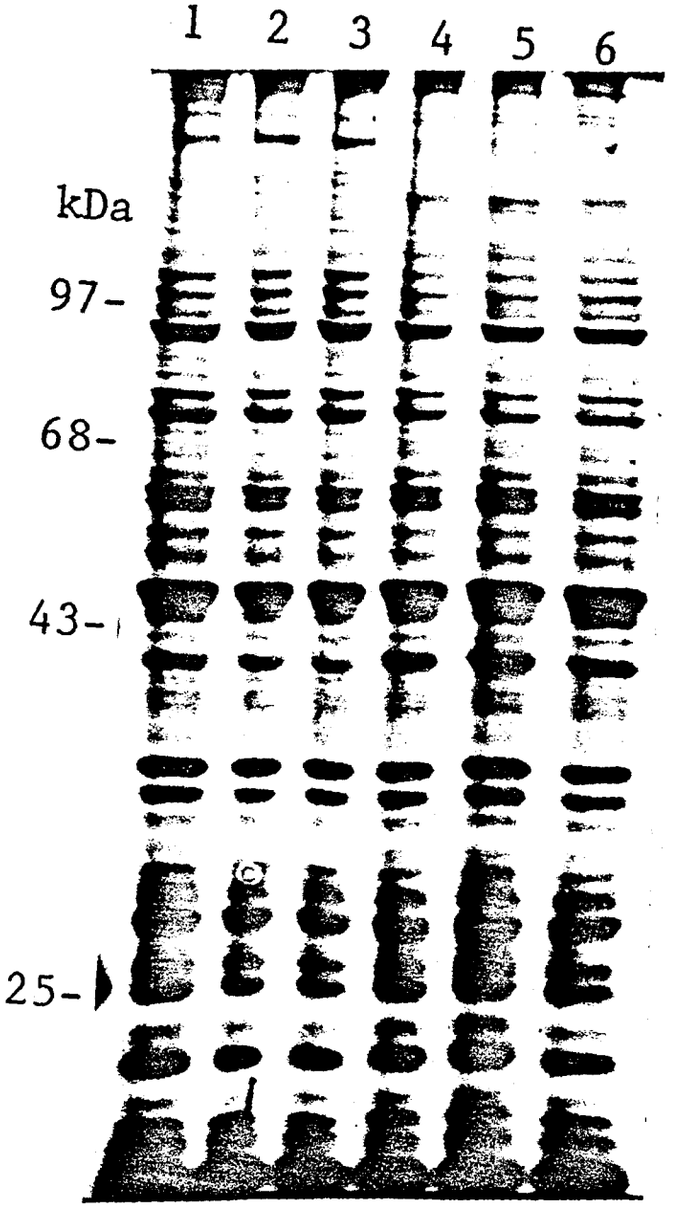
Figure 7



MACROPHAGE CELL LINES C2D AND C2DT DERIVED FROM MHC II KNOCK OUT MICE EXPRESSES ALTERNATIVE SUPERANTIGEN BINDING MOLECULES

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ABSTRACT

We describe the isolation and initial characterization of two bone marrow-derived macrophage cell lines isolated from MHC class II-negative transgenic mice. These cells were similar to B6MP102 macrophages in morphology and growth requirements. They were not spontaneously cytotoxic and were only capable of modest killing of F5b tumor cells when stimulated with LPS and IFN-gamma, but not when stimulated with LPS alone or with staphylococcal exotoxin. C2Dt cell lines phagocytosed labeled Pansorbin similarly to B6MP102 but less than C2D peritoneal macrophages. These cell lines secreted IL-6, but not TNF or NO, in response to LPS or staphylococcal exotoxin. Several molecular species on C2D macrophages bound SEB in immunoprecipitation experiments. This makes the C2D cell lines useful tools for studying staphylococcal exotoxin-macrophage interactions distinct from MHC II binding.

INTRODUCTION

The macrophage functions as a scavenger, an antigen presenting cell and as a cytolytic effector cell. Therefore, it is studied by many groups. Because experiments often require large numbers of homogenous macrophages, several laboratories have developed macrophage cell lines to study problems in macrophage biology (1-3).

Macrophage cell lines have been established by transformation of macrophage cells with retroviruses and oncogenes (4-6). Macrophage cell lines have also been developed by culturing bone marrow cells in the presence of macrophage colony stimulating factor (CSF-1) and maintaining the continuously dividing macrophages (7).

Macrophages are capable of expressing class II major histocompatibility (MHC II) molecules. However, some macrophage cell lines, usually derived from tumor cells, are MHC

II-negative (8). MHC II-negative cells provide unique models to study the importance of the MHC II molecule. However, use of these cells has been limited due to criticisms that include the possibility of low level MHC II expression (below the sensitivity of detection assays) or concerns about the representative nature of the cell population.

Recently a MHCII-negative transgenic mouse was developed (9) by targeted IAß gene disruption. These animals have proven important for studies on T cell development and activation (10) as well as macrophage development (11). Unfortunately their cost and limited availability made the acquisition of large numbers of homogenous macrophages almost impossible. Therefore, we set out to establish MHC II-negative bone marrow derived macrophage cell lines. In this report, we describe the isolation of two C2D-derived bone marrow macrophage cell lines. These macrophages have some properties in common with inflammatory macrophages and B6MP102 cells. Importantly, these cell lines allowed us to identify non MHC II superantigen binding molecules.

MATERIALS AND METHODS

Animals. Transgenic mice, negative for MHC II (C2D, H-2^b) were purchased from GenPharm, Int. (Mountain View, CA) or were bred in isolators in the animal facilities in the Division of Biology at KSU.

Cells and Cell Cultures. SV40-transformed cells F5b and F5m and their susceptibilities to in vitro macrophage cytotoxicity have been described previously (15,16). Balb/c 3T3 cells were obtained from the American Type Culture Collection (ATCC, CCL 163). The bone-marrow derived macrophage cell line, B6MP102 (12-14), was used for comparative analysis in most experiments. B6MP102 was cultured in tissue culture medium consisting of DMEM (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum, 0.3% L-glutamine and 15%

LM-929 conditioned cell supernatant (as a source of CSF-1). The tumor necrosis factor-(TNF) sensitive cell line, LM-929 (ATCC CCL 1.2) was used as a source of CSF-1 and to detect TNF. LM-929 and 3T3 cells were passaged 2-3 times weekly in antibiotic-free DMEM supplemented with 2% fetal bovine serum. IL-6 was quantified with the IL-6-dependent, murine B-cell hybridoma subclone B9 as described previously (14). It was cultured in DMEM supplemented with 50 μM 2-mercaptoethanol, 5% fetal bovine serum and 10 pg of recombinant IL-6. The MHC II-positive macrophage cell line J774A was obtained from Dr. D. Takemoto, Kansas State University and was used as an MHC II-positive control cell line for the immunoprecipitation experiments. J774A was cultured similarly to LM-929 and 3T3 cells.

Reagents. Purified preparations of SEA and SEB were obtained from Toxin Technologies (Sarasota, FL). Alternate preparations of SEB were the generous gift of A. Johnson-Winegar (U.S. Army Medical Research and Development command, Ft. Detrick, MD). Both SEB preparations gave similar results and each had less than 0.4 ng/μg endotoxin as determined by the Limulus amebocyte lysate assay (Sigma, St. Louis, MO). Our assay was sensitive to a concentration of 0.02 ng/ml. Lipopolysaccharide (E. coli)(LPS) was obtained from Difco Laboratories (Detroit, MI). Recombinant murine interferon gamma (IFN-gamma) was purchased from Genzyme (Cambridge, Mass.). C2D mouse, peritoneal macrophages were obtained by peritoneal lavage 4 or 5 days after injection of 1.5 ml of sterile thioglycolate broth (Difco, Detroit, Mich.).

Isolation of a Stable C2D Macrophage Cell Line. Bone marrow cells were recovered from long-bones (17) and cultured into macrophage monolayers on 60- or 100-mm tissue culture dishes in DMEM (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum, 0.3% L-glutamine and 15% LM-929 conditioned cell supernatant (as a source of CSF-1). Cells

were fed 3x a week, and passaged when confluent. Cells grew at a steady rate after 12 weeks in culture and 2 crisis periods when the majority of the cells died. After the second crisis period, the cells were passaged 3x a week in tissue culture medium.

Transformation of C2Dt cells. Cells were transfected with the PSV-3 plasmid (Amp^r and Tet^s : a gift from Dr. Roy Geib, University of Indiana - Terre Haute Center for Medical Education) containing the SV40 promotor and large T antigen. Plasmids were propagated in *Escherichia coli* JS4. Plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide density gradients. Cells in log phase growth were transfected with 10-15 μg of plasmid DNA by the calcium phosphate procedure (18). T Ag expression was confirmed using DOT blot analysis of transformed cell lysates and monoclonal antibody against large T antigen (pab 416, Hybridoma obtained from Dr. Linda Gooding, Emory University, Atlanta, GA). This bone marrow-derived cell line was named C2Dt.

Cytotoxicity assay. The macrophage cytotoxicity assay was performed as described previously (15). Briefly, C2D, C2Dt or B6MP102 cells were added into flat-bottom, 96 well plates at 1 x 10^5 to 4 x 10^5 cells per well. After 2 hours, the medium was removed and replaced with the appropriate stimulus. Treatments included medium only, IFN-gamma (0.1 U/ml), LPS (12.5 μg/ml) ± IFN-gamma, SEA (10μg/ml) ± IFN-gamma. Approximately 10^4 ^{51}Cr-labelled target cells were added to each well and the assay was incubated for 16 h. The microtiter plates were then centrifuged and 100 μl aliquots from each well were counted in a gamma counter. The percent specific release was calculated as follows: specific release = [experimental release - spontaneous release/(maximal release -spontaneous release)]/x100. The maximal release and spontaneous release were determined by incubating 10^4 target cells in 1 N HCL (maximal) or medium (spontaneous). The spontaneous release was generally less

than 25% for all cell lines.

Cell phenotype determination. C2D and C2Dt cells were screened for their expression of nonspecific esterase (α-Naphthyl acetate esterase substrate, Sigma Diagnostics), an enzyme usually present in cells of the monocyte-macrophage linage. Cells were evaluated for the expression of Mac-2, MHC I and MHC II antigens using flow cytometry as described previously (11). Single cell suspensions of C2D, C2Dt or B6MP102 cells were incubated with primary antibody [Y-3, anti-K^b (a generous gift from Dr. David Lee, University of Missouri, Columbia) or hybridoma HB-183 (ATCC) derived anti I-A and fluoresceinated secondary antibody [$F(ab')_2$; Cappel, Durham, NC] for 30 min, washed three times and analyzed on a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA).

Phagocytosis assay. Phagocytosis was quantified using a commercially prepared fixed *Staphylococcus aureus* (Pansorbin, Calbiochem: San Diego, CA) stained with propidium iodide (PI: Calbiochem: San Diego, CA) according to the procedure of Bohmer et al. (19). Briefly, equal volumes of Pansorbin and PI were mixed and allowed to stand for 30 min at room temperature. The bacteria were washed twice and resuspended in Hanks Balanced Salt Solution (HBSS: 5% wt/vol). PI-labeled Pansorbin (50 µl) was added to 1 x 10^6 macrophages preincubated with PBS, LPS (12.5 µg/ml) or LPS + sodium azide (20 mM: negative control) and incubated for 10 min. in a 37° C water bath. After centrifugation the samples were washed twice and resuspended in 500 µl HBSS. Cells were analyzed on a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA).

Macrophage stimulation Macrophages were plated at a density of 10^6 cells per 60-mm-diameter tissue culture plate, allowed to adhere 1 h, and the medium removed. Three ml of DMEM containing the appropriate stimulus (10µg/ml toxin, 12.5 µg/ml LPS, or medium) were

added. After 16 h the supernatants were collected, clarified by centrifugation, aliquoted, and used immediately or stored at -80° C until assayed for cytokine secretion.

Cytokine and nitrite quantitation. The macrophage supernatants were assayed for IL-6 content using the B9 bioassay (20). The B9 cells were washed three times in IL-6-free RPMI to remove residual IL-6. Four thousand B9 cells were added to serially diluted, triplicate samples of culture supernatant and allowed to incubate for 3 days at 37 C. Cell death was quantified by measuring MTT (1-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) uptake. Each assay well received 50 µl of MTT (2 mg/ml) and the plate was incubated for 3 hr at 37° C. The medium was removed and 150 µl of Iso:PBS (100 ml isopropyl alcohol, 40 ml 5 N HCL, 50 ml phosphate buffered saline) was added to dissolve crystals. A microtiter plate reader (Cambridge Technology, Cambridge Technologies, Watertown, MA) was used to read absorbance at 570 nM.

TNF was quantified as described previously (14) except MTT was used (see above). Nitrite concentration was determined with the Griess reagent (1% sulfanilamide, 0.1% naphylethylene diamine dihydrochloride and 2% H_3PO_4) as previously described (21). Cytokine and nitrite quantification was based on linear regression of standard curves of recombinant, murine TNF, rIL-6, or $NaNO_2$. Recombinant, murine TNF was obtained from Genzyme (Cambridge, MA) and rIL-6 was obtained from R and D (Minneapolis, MN).

Immunoprecipitation. C2D macrophages (1×10^8) were surface-labeled with ^{125}I (NEN/Dupont, Boston, MA) using the lactoperoxidase method (Calbiochem) and incubated with or without 100 µg/ml unlabelled SEB for 4 hr at 4° C. The cells were washed in PBS and then lysed in 1 ml ice cold lysis buffer (2% NP40, 15 mM Tris-HCL pH 8.0, .15 M NaCl, 1 mM phenyl methyl sulphonyl fluoride and aprotinin). After centrifugation the lysates were incubated with normal

rabbit serum for 1 h at 4° C , followed by the addition of fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem) for 1 h at 4° C. After centrifugation, supernatants were immunoprecipitated with goat anti-SEB serum, followed by rabbit anti-goat and immobilization on Pansorbin. Precipitates were washed 3 times with ice cold lysis buffer then boiled in SDS sample buffer, centrifuged and electrophoreses on a 10% SDS PAGE gel. Receptors were imaged using audioradiography.

RESULTS

Cell Phenotype. The phenotype of the C2D and C2Dt cells was analyzed with antibodies specific for MHC I and II and Mac 2 molecules by flow cytometry. Both clones expressed MHC class I at levels comparable to B6MP102 (Table 1), but about half that detected on LM-929 cells. Neither C2D or C2Dt expressed substantial amounts of MHC II (Table 1) even after incubation with 50 U/ml recombinant murine IFN-gamma for 24 h (data not shown). When compared to B6MP102 cells (Table 1), very low levels of Mac 2 were expressed on the surface of C2D and C2Dt cells. A dot blot utilizing monoclonal antibody to T antigen demonstrated that T cell antigen was expressed on the C2Dt but not the C2D or B6MP102 cell lines (Data not shown). Histochemical staining indicated that virtually all the C2D and C2Dt cells were non-specific esterase-positive (Figure. 1). C2D and C2Dt and the wild type B6MP102 cell line cells had morphological features typical of macrophages. Also evident in Figure 1, the cells were adherent with multiple processes and both were larger in size than the B6MP102 cells.

In Vitro Culture of C2D and C2Dt. C2D and C2DT cells were relatively easy to grow in culture and manipulate. We have maintained both cell lines in continuous culture as adherent cells for more than one year in the tissue culture medium described. In our hands, C2D and C2Dt

were easily dispersed with 3-5 min treatment of warm trypsin/EDTA (0.25% trypsin, 0.02% EDTA). The cells were washed from the tissue culture dish with gentle jets of medium from a pipet. Generally, 1 x 10^6 cells were harvested from a confluent 100-mm culture plate. Cells were usually reseeded at 1 x 10^5 in 8 ml of medium without extensive washing, however, cells have been reseeded successfully using cell numbers as low as 1 x 10^4. A lag in cell growth occurred after reseeding, but cells began exponential growth within 24 h. C2Dt cells grew faster than C2D cells (Figure 2), but growth rates for both are similar to the rates previously determined for B6MP102 macrophages (13). Both cell lines grew in the absence of CSF-1. However, they grew significantly faster when it was included in the medium (Figure 2). When the media were not changed, growth plateaued at 96 h. However, if cells were re-fed every 48 h they continued growing at approximately the same rate. Unlike B6MP102 cells, C2D and C2DT grew to a higher density and formed a heavy monolayer when fresh medium was continuously supplied.

Cells were frozen in 10% dimethyl sulfoxide (22) in FBS. When thawed, individual cell aliquots from C2D and C2Dt lines yielded, on average, 70% and 55% recovery, respectively, after 6 months at -80° C.

Assessment of C2D cell cytolytic activity. We determined cell-mediated tumorlytic activity of activated and nonactivated C2D cells and compared them to B6MP102 macrophages. F5m 3T3 and F5b cells were used as targets to determine whether C2D and C2Dt cells would maintain the ability to discriminate between transformed (e.g. F5b) and nontransformed (e.g. 3T3) cells (23) or would fail to kill F5m cells as similarly to normal macrophages or B6M102 cells.

C2D and C2Dt were unable to kill F5m and 3T3 (data not shown), and had a low level

of cytolytic activity against F5b (Table 2). C2D cells did not spontaneously kill F5b if cultured in tissue culture medium in the absence of any stimulant (Table 2). Only C2D and C2Dt pulsed with 12.5 μg/ml LPS and IFN-gamma exhibited >10% killing of F5b at more than 1 effector to target ratio (Table 2). The amount of killing was consistently less than that by similarly activated B6MP102 macrophages. Therefore, C2D and C2Dt cells were poor cytolytic effector cells.

Phagocytosis. Both C2D and C2Dt were similar to B6MP102 cells in phagocytosis of labeled *S. aureus* (Figure 3). However, the level of phagocytosis was lower than that observed for C2D peritoneal macrophages (Figure 3). The efficiency of C2D in phagocytosing Pansorbin was not significantly influenced by the addition of LPS or LPS + IFN gamma (Data not shown). Additionally C2D and C2Dt cells behaved similarly in all experiments.

Cytokine secretion. Macrophages produce cytokines in response to LPS and staphylococcal exotoxins (14,24). Therefore, we tested the ability of C2D macrophages to secrete TNF, IL-6 and NO in response to these stimulants. When exposed to enterotoxin A or B or to LPS, C2D and C2Dt secreted IL-6. However, the amount of IL-6 secreted varied with each toxin stimulant. SEA induced >3100 pg/ml while SEB induced the secretion of <700 pg/ml. Although LPS also induced IL-6 secretion the superantigens were more potent stimulants.

B6MP102 secreted >35 units of TNF (Table 3) and 10 μMoles NO (data not shown) in response to SEA or LPS stimulation. In contrast, in response to all stimuli, C2D and C2Dt macrophages secreted very low levels of TNF (Table 3) and little to no NO (data not shown).

Alternative binding site. We have described the presence of non-MHC II binding sites for SEA on C2D peritoneal macrophages (24). Because C2D macrophages did not maintain all of the characteristics of normal macrophages (i.e. cytolytic activity, TNF and NO secretion),

we determined if alternative superantigen receptors were present on the C2D cell line. To identify superantigen binding sites, ^{125}I-surface labeled C2D macrophages were incubated with SEB and anti-SEB antiserum. Proteins with Mr greater than 140,000 (140 kDa) and bands of 97 kDa, 52 kDa, 43 kDa and 37 kDa were consistently precipitated from C2D macrophages (Figure 4). J774A macrophages (25-27) were used as an MHC II positive control in these experiments because of their constitively high level of MHC II expression compared to B6MP102 or thioglycolate-induced peritoneal macrophages. Although J774A expressed the same complement of SEB toxin binding molecules as C2D cells, additionally protein with Mr of approximately 28 kDa and 14 kDa were also precipitated (Figure 4). In the absence of SEB or goat-anti-SEB, no cell surface molecules were precipitated (Figure 4).

Discussion

We have described the isolation and characterization of bone marrow-derived MHC II-negative macrophage cell lines C2D and C2Dt. We believe that these are the first macrophage cells lines derived from C2D knock-out, transgenic mice. We have described the growth characteristics, responsiveness to biological response modifiers known to activate macrophages and a potential use for these cells in identifying and understanding the role(s) of non MHC II staphylococcal exotoxin binding receptors .

Both cell lines appear to be immortalized, C2D through continuous culture in CSF-1 and C2DT through transformation with SV40 large T antigen. Both have been in continuous culture for over one year and can be frozen and successfully recovered. They have been periodically checked for MHC expression and have always been MHC II negative and MHC I positive. C2D and C2DT cell lines stain for nonspecific esterase indicative of a macrophage/monocyte linage. They also have morphology similar to another bone marrow-

derived cell line, B6MP102. C2D and C2Dt expressed low levels of Mac 2 antigen, indicative of differentiated macrophages (28). The B6MP102 macrophage cell line still expressed high levels of Mac 2. Therefore, these new macrophage cell lines were transformed at an earlier stage of differentiation than B6MP102 or transformation has ameliorated Mac 2 expression. The latter is very possible because unlike B6MP102 cells, C2D and C2Dt were not CSF-dependent. Therefore, C2D and C2Dt appear to grow more like the macrophage cell lines J774a and P388D1, which grow quickly and are growth factor independent (1,25).

Like peritoneal macrophages and B6MP102 (13), neither C2D or C2DT exhibited spontaneous killing activity. Furthermore, C2D and C2Dt only could be activated to kill F5b tumor cells in the presence of LPS and IFN-gamma, however, killing was only half that achieved by B6MP102. Additionally, C2D and C2Dt did not kill F5m or 3T3 cells which paralleled the cytotoxicity of B6MP102. The killing pattern exhibited by C2D and C2Dt is not unique. WEHI-3 also demonstrated little cytotoxicity towards susceptible target cells (25,29). Macrophage cell lines may vary in their killing ability due to different levels of activation and/or differentiation (25,30).

The stage of macrophage differentiation, based on the expression of "mature" macrophage markers (i.e. Mac 1 and Mac 2) appears to correlate positively with phagocytotic capacity (31). Because C2D cells were intermediate in their ability to phagocytose and express low levels of Mac 2, they appear to be intermediate in their development; especially compared to B6MP102 which resemble more mature, peritoneal-like macrophages.

IL-6 secretion by C2D and C2Dt was similar to B6MP102 and peritoneal macrophages: all secrete significant quantities of IL-6 in response to stimulation by LPS or staphylococcal exotoxins (14,32). However, the secretion pattern was dependent on the stimulus. C2D and

C2Dt secreted less IL-6 in response to SEB stimulation than did B6MP102. Additionally, C2D and C2Dt appeared to secrete more IL-6 in response to the other stimulants.

C2D and C2Dt secreted little to no TNF or NO in response to all stimulants tried. This was in contrast to B6MP102 and peritoneal macrophages (14) that secrete TNF and NO in response to exotoxins and LPS. However, Wynn (29) demonstrated that TNF secretion varied between macrophage cell lines, and speculated that this functional heterogeneity between macrophages may reflect differences in the maturity, origin or differentiation of the macrophage population. It should be noted that this inability to secrete TNF and NO may be partly responsible for the limited cytotoxic ability exhibited by C2D and C2Dt since F5b is killed by TNF after contact with macrophages (33).

By immunoprecipitation, we identified a heterogeneous array of molecules on C2D macrophages capable of binding SEB. These same proteins were present on the MHC II-positive cells. Only the intense 28 kDa band, presumably class II MHC, and the light 14 kDa band were unique to J774A cells. These molecules were similar to molecules immunoprecipitated by our laboratory using SEA and thioglycollate peritoneal macrophages from C2D mice (24). Therefore, in vitro culture and transformation has not affected this macrophage property. The nature of these proteins is still unresolved, but candidates include VLA-4 (34) for the 140 kDa and 97 kDa bands, and MHC class I for the 43 kDa band (24,35,36). These C2D cell lines will be indispensable in the additional work needed to define the nature of non MHC II superantigen binding molecules. Since this is only an initial characterization of the C2D and C2Dt cells additional studies will be necessary to define how closely they parallel other macrophage functions.

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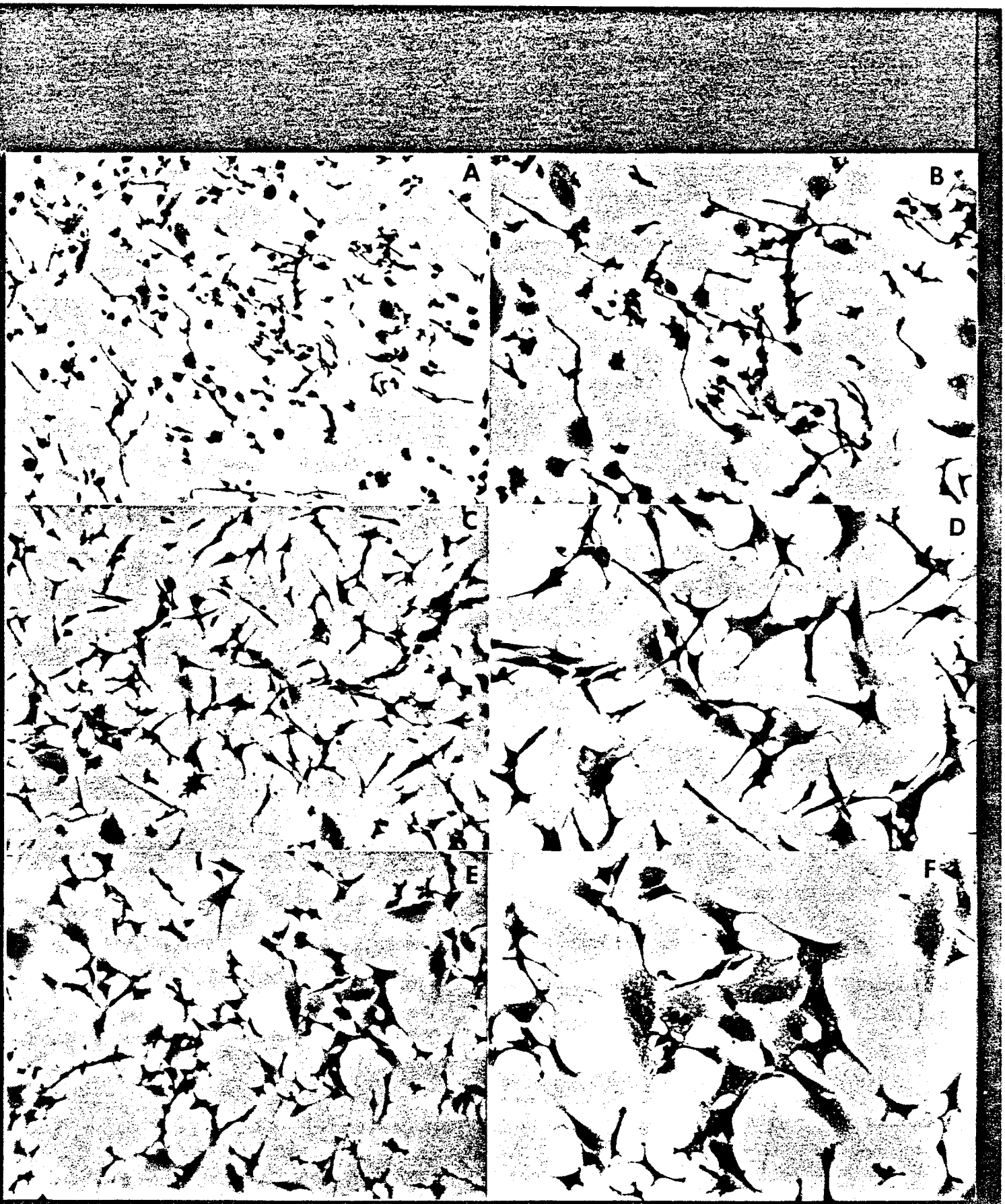
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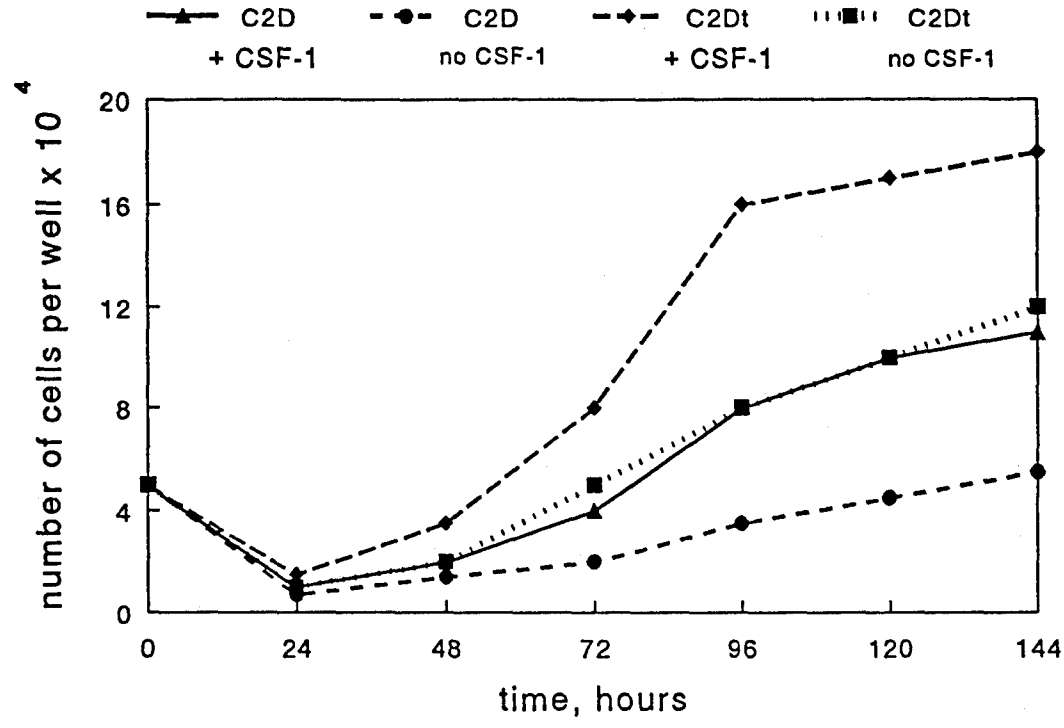
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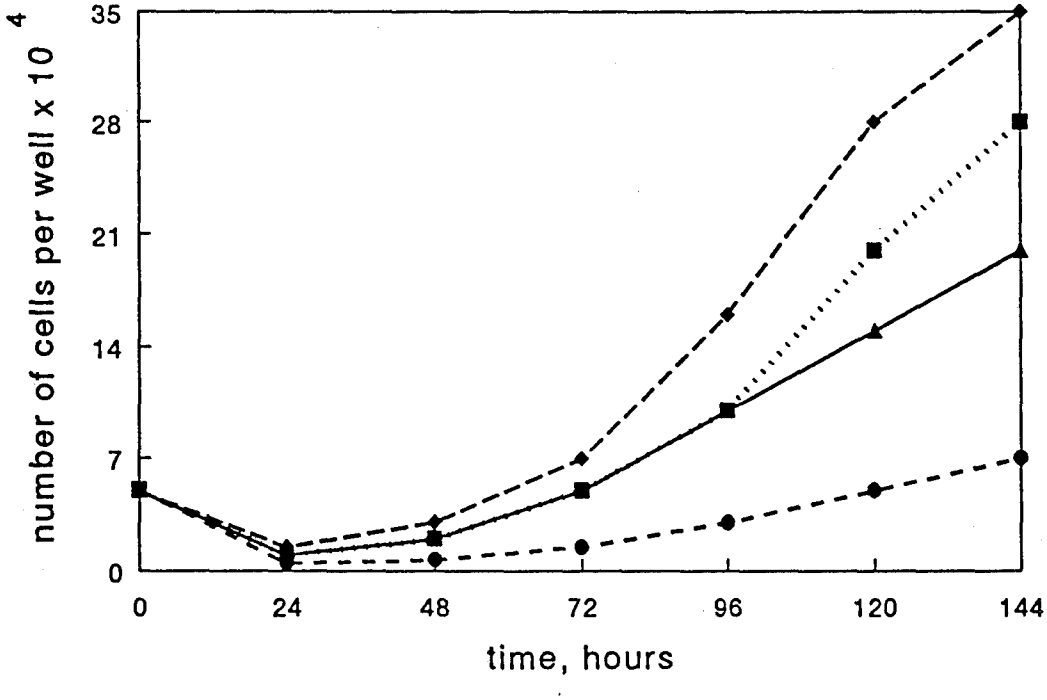
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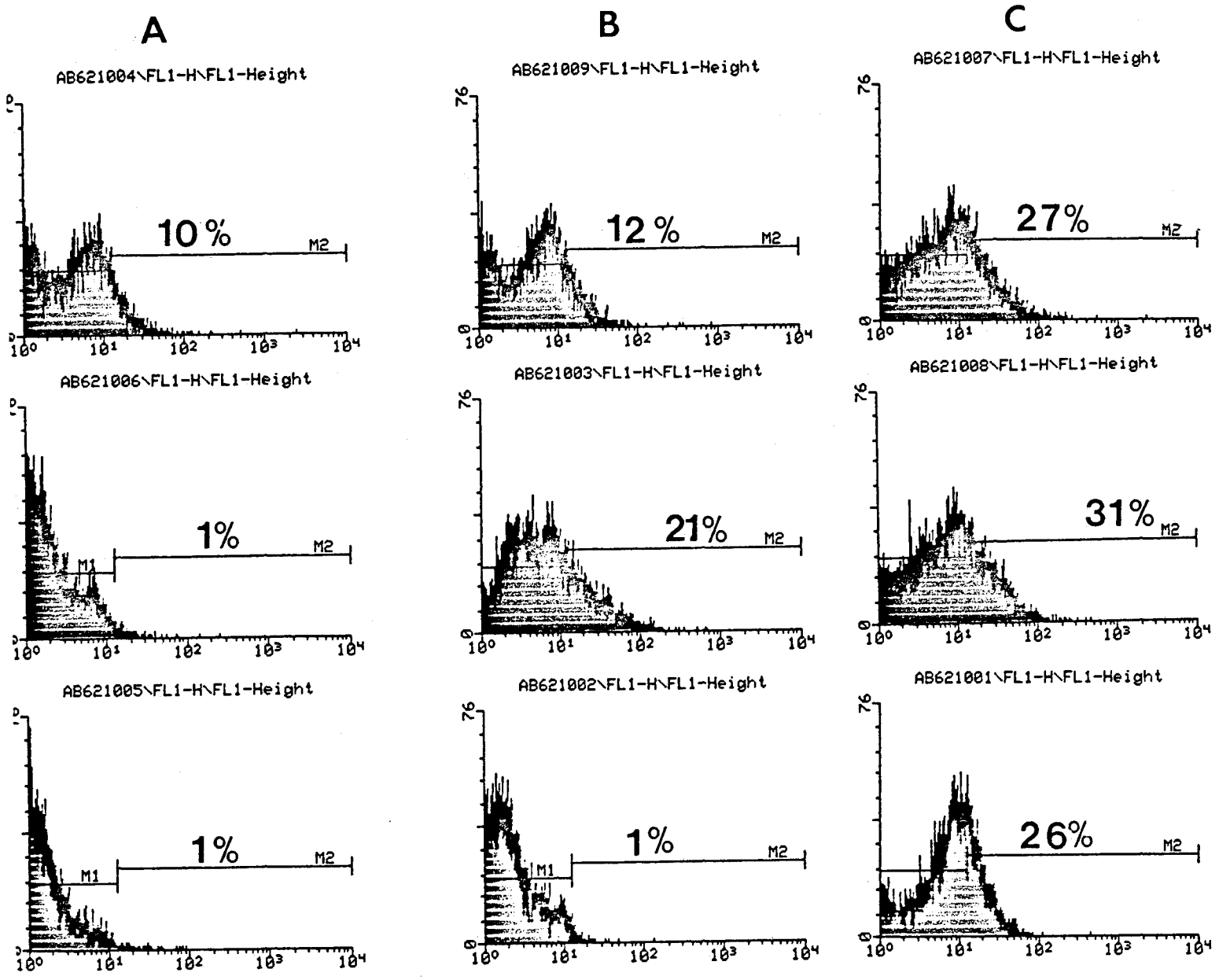
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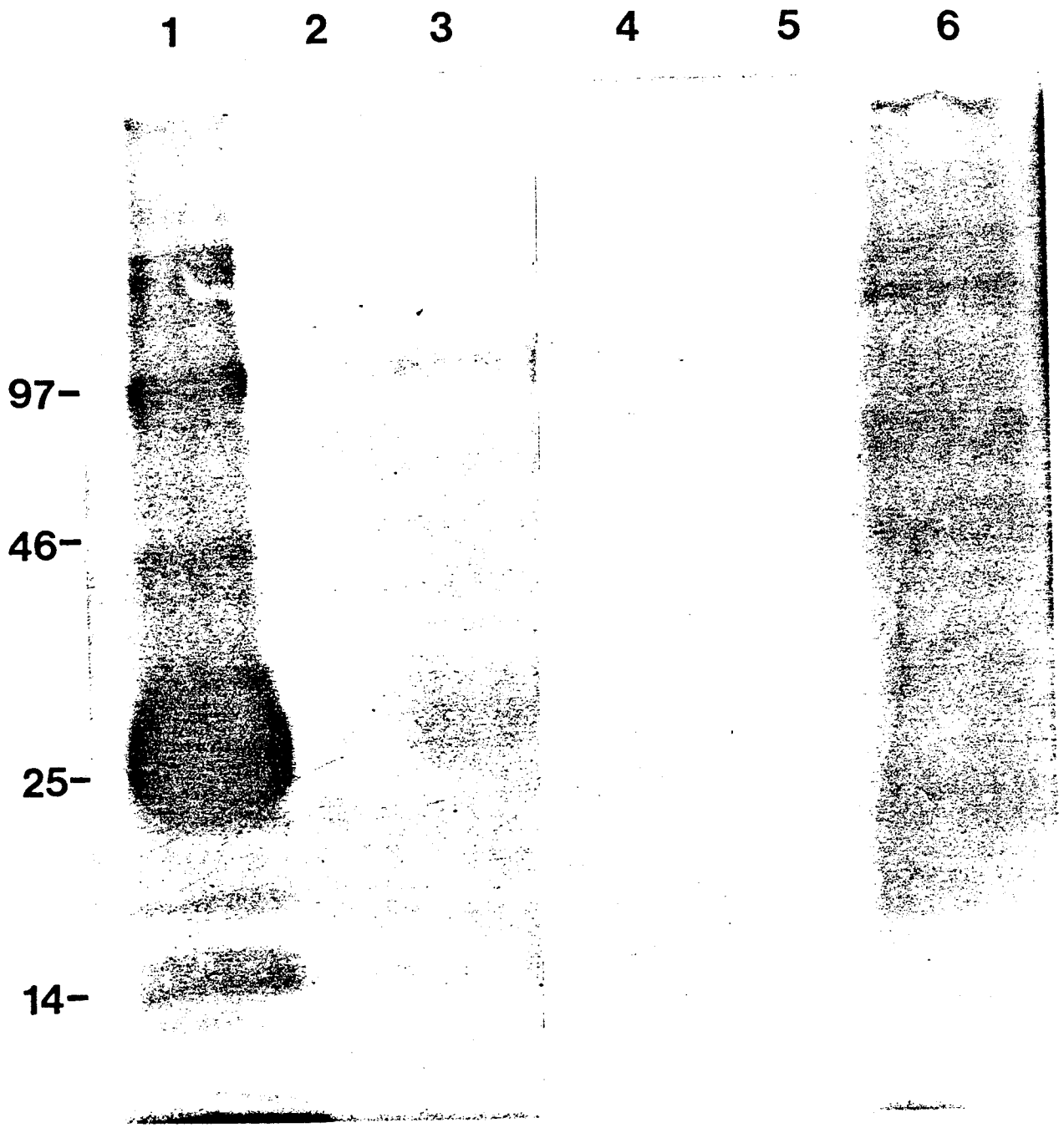
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TABLE 1. FACS analysis of the molecules present on the cell surface of C2D, C2Dt and B6MP102 macrophages[a].

Cell Line	$F(ab')_2$ Control	Cell Surface Molecule		
		MHC class II	MHC class I	Mac-2
		% of cells in the third gate		
C2D	5.5	6.3	29.3	10.5
C2DT	4.5	6.1	25.1	11.8
B6MP102	4.5	51.5	25.6	56.2
LM929	1.0	1.0	64.8	1.0

[a] Molecule expression was determined using a single cell suspension incubated with antibody against MHC class I or II or Mac-2 and fluoresceinated secondary antibody $F(ab')_2$ and then analyzed by flow cytometry. The negative control was obtained using FITC-$F(ab')_2$ alone. Cells were considered positive for a surface molecule when fluorescent intensity of ab-bound cells shifted to the right (as indicated by an increase in cell number in the third gate) when compared to the negative control.

TABLE 2. Comparison of C2D, C2Dt and B6MP102 Cytotoxicity

				% Specific ^{51}Cr release[a]			
Effector line	Activator	Target	(MP:T)	10:1	20:1	30:1	40:1
C2D	MEDIUM	F5b		0.5 ± 3[b]	1.5 ± 1	2.7 ± 1	1.9 ± 1
C2D	LPS	F5b		8.6 ± 2	8.0 ± 1	9.8 ± 1	9.0 ± .5
C2D	SEA	F5b		7.6 ± 2	7.2 ± 2	5.6 ± 2	8.6 ± .5
C2D	LPS + IFN	F5b		9.1 ± 4	8.5 ± 4	10.6 ± .5	14.5 ± 1
C2D	SEA +IFN	F5b		11.1 ± 3	8.1 ± 3	8.5 ± .2	7.3 ± 1
C2D	IFN	F5b		1.1 ± 1	2.7 ± 2	2.1 ± .4	3.8 ± 1
C2Dt	MEDIUM	F5b		-2.1 ± 1	4.4 ± 1	1.6 ± .2	2.9 ± 1
C2Dt	LPS	F5b		2.0 ± 1	3.3 ± 3	3.3 ± .5	3.5 ± 1
C2Dt	SEA	F5b		1.8 ± 2	3.0 ± 2	6.4 ± .4	6.5 ± 1
C2Dt	LPS + IFN	F5b		4.5 ± 2	12.0 ± 2	16.5 ± .4	9.8 ± 1
C2Dt	SEA + IFN	F5b		1.8 ± 2	3.0 ± 1	4.3 ± .3	5.0 ± 1
C2Dt	IFN	F5b		0.7 ± 2	0.8 ± .4	2.0 ± 1	2.0 ± 1
B6MP102	MEDIUM	F5b		-3.7 ± 2	8.3 ± 2	8.5 ± 1	7.6 ± .5
B6MP102	LPS	F5b		17.2 ± 3	26.8 ± 4	28.2 ± 1	28.7 ± 1
B6MP102	SEA	F5b		3.0 ± 2	14.2 ± 3	16.1 ± 1	15.9 ± 1
B6MP102	LPS + IFN	F5b		24.9 ± 4	33.6 ± 3	35.0 ±	34.2 ± 1
B6MP102	SEA + IFN	F5b		17.3 ± 3	26.9 ± 1	27.1 ± 1	27.1 ± 1
B6MP102	IFN	F5b		9.3 ± 4	10.2 ± 2	6.8 ± 1	7.8 ± 1

[a] 16 h cytotoxicity assay.

[b] Values are mean ± standard error of mean of two experiments.

TABLE 3. Cytokine and NO secretion by C2D, C2DT and B6MP102 macrophages in response to stimulation with LPS or staphylococcal exotoxin

Macrophage source & treatment	Activity in C2D cells		Activity in C2DT cells		Activity in B6MP102 cells	
	TNF (U/ml)	IL-6 (pg/ml)	TNF (U/ml)	IL-6 (pg/ml)	TNF (U/ml)	IL-6 (pg/ml)
Medium	1±1[a]	296±122	1±1	312±157	5±3	211±66
SEA	8±2	3100±235*	9±2	3300±321*	63±16*	2833±112*
SEB	1±1	622±109*	2±1	1011±215*	8±3	1553±199*
ETA	6±2	1523±264*	6±1	1442±271*	39±11*	1327±345*
ETB	5±1	2342±387*	5±2	2493±288*	50±29*	1558±236*
LPS	5±1	2845±277*	7±2	2241±338*	80±17*	2159±284*

[a] Mean ± SD activity of culture supernatants from macrophages incubated with medium containing staphylococcal exotoxin (10 μg/ml) or LPS (12.5 μg/ml) for 18 h.

* Different from medium only control ($P<.05$).

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Running title: Superantigen activation of macrophages.

Description of Protein Kinase Activation in Murine Macrophages by Staphylococcus aureus Superantigens[1,2]

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FOOTNOTES

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[2] This work was supported by the U.S. Army Research and Development Command grant #DAMD 17-89-Z-9039, American Heart Association Grant KS-94-GS-33, NIAID grant AI-17474, NASA grants NAGW-1197 and NAGW-2328 and the Kansas Health Foundation.

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[4] Abbreviations used in this paper: Staphylococcal enterotoxin A or B (SEA, SEB), staphylococcal exfoliative toxin A or B (ETA, ETB), major histocompatibility complex class II molecules (MHC II), protein kinase C (PKC).

Abstract

We investigated the involvement of protein kinases in staphylococcal enterotoxin and exfoliative toxin activation of murine macrophages. Early signal transduction, as measured by changes in F-actin concentration, was protein kinase-dependent. The enterotoxins (SEA and SEB) were inhibited differently by a panel of protein kinase inhibitors that included H7, H8 and HA1004 than were the exfoliative toxins (ETA and ETB). TNF and nitric oxide secretion were blocked by inhibitors of protein kinase C regardless of the toxin stimulant. IL-6 secretion was also suppressed by the panel of inhibitors. However, the inhibition pattern was different from that seen in the TNF experiments. ETA, ETB, and SEA, but not SEB induced macrophage protein phosphorylation. One common protein was phosphorylated by SEA, ETA and ETB. Six proteins were uniquely phosphorylated by SEA and four proteins were only phosphorylated following ETA or ETB stimulation. These results suggest that protein kinases are involved in toxin-induced signal transduction in macrophages and that the subsequent responses are dependent on the toxin stimulant.

Introduction

Staphylococcal exotoxins, also known as microbial "superantigens", have been linked to several pathologies including food poisoning, scalded skin syndrome and toxic-shock syndrome (1-4). Unlike conventional antigens, unprocessed staphylococcal exotoxins bind the major histocompatibility complex class II molecule[4] (MHC II) and/or alternative receptors (5-8). The exotoxins are presented to T cells and contribute to their activation (9,10). Additionally, monocytes and macrophages are activated by staphylococcal exotoxins. These molecules stimulate murine and human macrophages to secrete cytokines including interleukin-6 (IL-6), interleukin-1, tumor necrosis factor alpha (TNF) and nitric oxide (NO) (11-

16). This massive production of cytokines is believed to play a key role in staphylococcal pathogenesis (15,17,18).

The exact mechanism(s) used by staphylococcal exotoxins to stimulate macrophage cytokine secretion is unproven. In theory, toxins bind to a cellular receptor(s) which leads to the generation of intracellular signals concluding with some degree of cell activation (19-21). The required signal transduction pathways are not completely elucidated. Staphylococcal toxins induce translocation of PKC to the membrane fraction (22), tyrosine phosphorylation (23), and the activation of NF-kB (24,25). The activation of NF-kB is down regulated by inhibitors of protein kinase C (PKC) (24). However, protein kinases other than PKC appear necessary for TNF and IL-1 production by monocytes activated with toxic shock syndrome toxin-1 (TSST-1) (26). Therefore, different or multiple protein kinases may be important for individual responses. Additionally, these kinases may work in a dose- and time-dependent manner (27).

Since kinases normally phosphorylate specific cellular proteins, and appear necessary for a variety of cell functions (20,21), it is possible that phosphorylation may be stimulus-dependent. Therefore, we examined the phosphoproteins in macrophages after stimulation with staphylococcal enterotoxin A and B (SEA and SEB), and exfoliative toxins A and B (ETA and ETB). Furthermore, we examined which kinases may be important to cellular activation and cytokine secretion by using various protein kinase inhibitors.

Materials and Methods

Mice. C3HeB/FeJ mice were bred in the animal facilities in the Division of Biology at Kansas State University.

Tissue culture cells. The TNF-sensitive cell line LM929 was obtained from the American Type Culture Collection (Rockville, Md). LM929's were cultured in antibiotic-free, Dulbecco's modified Eagle's medium (DME; GIBCO, Gaithersburg, Md.) supplemented with 2 % fetal bovine serum. The murine, B-cell hybridoma subclone B9 was grown as described previously (14).

Reagents. Enterotoxins A and B were obtained from Toxin Technology (Sarasota, FL). ETA and ETB were prepared in our laboratory using the purification procedure previously described (14). All toxins were used at a concentration of 10 μg/ml. The enterotoxins had 0.2 to 0.02 ng/ml of endotoxin and exfoliative toxins less than 2.0 ng/ml as determined by the Limulus amebocyte lysate assay (Sigma, St.Louis MO). Our assay was sensitive to a concentration of 0.02 ng/ml. Additionally, basic activation and exotoxin induced cytokine production was not influenced by polymyxin B (CalBiochem. La Jolla, CA). Lipopolysaccharide (LPS) was purchased from DIFCO Laboratories (Detroit, MI) and used at a concentration of 12.5 μg/ml which we have previously shown to activate macrophages (14).

Protein kinase inhibitors were purchased from CalBiochem (San Diego, Ca.). H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine, di-HCL], H-8 {N-[2-(methylamino)-ethyl]-5-isoquinolinesulfonamide, di-HCL} and HA1004 [N-(2-guanidinoethyl)-5-isoquinolinesulfonamide, HCL] were used the concentrations (Ki of PKC) of 6.0, 15 and 40 μM, respectively. At these concentrations, it is generally agreed that these drugs inhibit PKC

activation by serving as competitive inhibitors of ATP binding to free PKC which prevents active enzyme complex formation (28-30). Specificity for PKC was obtained by also using these inhibitors at lower concentrations (3.0, 1.2 and 2.3 μM H7, H8, and HA1004, respectively, known to inhibit cyclic nucleotide-dependent kinases but not PKC (1,10,20).

Macrophage Cytokine Secretion. C3HeB/FeJ mouse, peritoneal macrophages were obtained by peritoneal lavage 4 or 5 days after injection of 1.5 ml of sterile thioglycolate broth (Difco, Detroit, Mich.). The cells were washed and 10^7 cells were added per 60-mm-diameter tissue culture plate. The cells were adhered for 2 hr and washed with DME. Inhibitors were added in 3 ml of DME for 15 min. before the appropriate toxin was added. After 18 hr, supernatants were collected, clarified by centrifugation, aliquoted, and used immediately or stored at -80° C until assayed.

Macrophage Lysates. C3HeB/FeJ mouse, peritoneal macrophages were obtained and adhered as described above. Cells were washed twice with phosphate-free DME and incubated 2 hr. The appropriate toxin and ^{32}P (H_3PO_4 in H_20; 1 mCi) was added in 3 ml of phosphate-free DME for 1 hr. Subsequently, the cells were washed, collected and resuspended in 100 μl of Tris buffer (pH 7.4, containing 40 μM phenylmethylsulonylfluoride {PMSF}, 10 μg/ml pepstatin A, 100 mM NaF, 4 mM EDTA, 2 mM sodium orthovanadate and 4 mM sodium pyrophosphate), lysed, centrifuged at 13,600 RPM (5000 x g) for 15 min. The supernatant was stored at -80° C until use.

TNF, IL-6 and Nitric Oxide Quantitation. Triplicate samples of macrophage supernatant were assayed for TNF using the LM929 bioassay (11) and for IL-6 content by the B9 bioassay (31), as described by our group previously (14). Nitrite concentration was determined by use of the Griess reagent (1% sulfanilamide, 0.1% naphylethylene diamine dihydrochloride, and 2%

H_3PO_4) as previously described (32). Supernatants were diluted for bioassays and at those concentrations the PK inhibitors did not affect detection of TNF or IL-6.

Two-Dimensional PAGE and Analysis. The lysates made from ^{32}P-labelled cells were electrophoresed in two-dimensions using a modification of the method of O'Farrell (33). In the first dimension, proteins were separated using isoelectric focusing. Seventy-five µl samples and standards were applied to 30% acrylamide/1.8% bisacrylamide tube gels (2.5 mm internal diameter) containing ampholytes pH 3-10 (Fisher Scientific, Pittsburgh, PA). The gels were run for 16 hr at 400 V followed by two hr at 800 V. The tube gels were then overlaid to the top of 10% polyacrylamide slab gels. The gels were stained with Coomassie Blue and dried. Phosphorylated proteins were identified by autoradiography and analyzed using the Molecular Dynamics computing densitometer model 300 A imaging system.

Determination of F-actin. Polymerized actin was measured using a modification of the methodology of Howard and Meyer (34) that we have described previously (11). Briefly, 1 x 10^6 thioglycollate-elicited macrophages (>98% viability as determined by trypan-blue staining) were suspended in PBS or PBS and kinase inhibitors at the described concentrations. Nonadherent macrophages were stimulated with the indicated reagent (F-met-leu-phe or toxin) for 30 sec. and fixed, permeabilized and stained with NBD phallacidin as described (11, 34).

RESULTS

Early Signal Transduction. Staphylococcal exotoxins (SEA, SEB, ETA, and ETB, 10 µg/ml) induced an increase in F-actin concentration in murine macrophages within 30 sec. (11). To assess the kinases involved in early signal transduction, actin polymerization was measured in the presence of H-7, H-8 and HA1004. SEA and SEB-induced actin polymerization was

inhibited by H-7, H-8 and HA1004 at concentrations known to inhibit PKC (10, 15 and 40 μM IC_{50} for H-7, H-8 and HA1004, respectively); (28,29,30); (Table 1). Actin polymerization was not significantly inhibited by H-7, H-8 or HA1004 at lower concentrations known to inhibit cyclic nucleotide-dependent kinases (3, 3, and 10 μM IC_{50} for H-7, H-8 and HA1004, respectively); (28-30). In contrast, F-actin concentration increases induced by ETA and ETB were inhibited by all concentrations of H-7, H-8 and HA1004 tested in our studies (Table 1), suggesting a role for cyclic nucleotide-dependent kinases in exfoliative toxin stimulation. None of the inhibitors had detrimental effects on macrophages when added alone (viability > 95%. In addition, basal [unstimulated] cytokine secretion levels were unchanged in the presence of inhibitors).

Secretion of TNF, IL-6 and nitric oxide. SEA, ETA, and ETB induced macrophages to secrete > 55 units of TNF over a period of 18 h. In contrast, SEB induced less than 20 units of TNF. To determine if PKC was involved in superantigen-mediated TNF secretion, we studied the effects of PKC inhibitors on TNF secretion. SEA-, SEB-, ETA- and ETB-induced TNF secretion was inhibited by 10 μM H-7 (Table 2). Additionally, SEA- and ETB- induced TNF secretion was inhibited by 15 μM H-8. SEB- and ETB- induced TNF secretion was inhibited by 40 μM HA1004 (Table 2). SEA-, SEB-, ETA- and ETB-induced TNF secretion was not significantly inhibited by 3 μM H-7, 3 μM H-8, or 10 μM HA1004 (Table 2).

SEA, ETA and ETB, and to a lesser extent SEB, induce peritoneal macrophages to secrete NO. Similar to the TNF results, SEA-, SEB-, ETA-, and ETB-induced NO secretion was inhibited by 10 μM H-7 (Table 3). SEA-, SEB- and ETB-induced NO secretion was also inhibited by 40 μM HA1004 (the IC_{50} of PKC). H-8 at the IC_{50} of PKC inhibited ETB-induced macrophage NO secretion. In contrast, SEA-, SEB-, ETA- and ETB-induced NO secretion

was not significantly inhibited by 3 μM H-7, 3 μM H-8 or 10 μM HA1004, concentrations known to inhibit cyclic nucleotide-dependent kinases (Table 3).

All the toxins tested induced macrophages to secrete >1400 pg/ml of IL-6 over a period of 18 h. Secretion was inhibited by 10 μM H7 and 15 μM H-8 regardless of the toxin stimulant (Table 4). Interestingly, SEB-induced IL-6 secretion was inhibited by 3 μM H-7 and 3 μM H-8 concentrations. ETA induced IL-6 secretion was inhibited by 3 μM H-8 and 10 μM HA1004. Therefore, except for SEB, the pattern of inhibition mostly suggested that PKC activation was necessary for IL-6 secretion in response to toxins.

Differences in protein phosphorylation. To determine if SEA, SEB, ETA, and ETB induced phosphorylation of different proteins in murine macrophages, we used two-dimensional gel analysis. Cells were stimulated with 10 μg of toxin or 12.5 μg LPS for 1 hr and were compared to cells exposed to LPS or buffer alone. Proteins were considered phosphorylated if they appeared reproducibly in the majority of experiments. A minimum of 3 experiments were run with each toxin stimulant. Phosphorylated proteins that appeared constantly on gels from stimulated and unstimulated cells were considered to be continuously expressed proteins and were used as points of reference. Proteins were not considered unique to the exotoxins if they appeared on the LPS gels. A densitometer was used to more accurately examine protein phosphorylation in a specific region of a gel.

Macrophages incubated with SEA expressed 7 phosphoproteins not expressed by macrophages incubated in medium alone or stimulated with LPS: 66 kDa, 65 kDa, 60 kDa, 60 kDa, 56 kDa, 53 kDa, and 46 kDa, with pI of 5.5, 5.5, 5.5, 9.0, 5.4, 5.1, and 5.0, respectively (Figures 1, 2 and 3: proteins labeled as A,B,C,H,D,E and F, respectively). Six of these proteins were in the 4.5 to 6.0 pH range (Figure 1), and were unique to SEA

stimulation as they were not phosphorylated with buffer only or SEB gel (Figure 1) or the representative ETA/ETB gel (Figure 1). However, in the same pH range, ETA and ETB induced the phosphorylation of a unique 43 kDa protein with a pI of 6.1 (Figure 1: protein labeled as G) not seen on the SEA, medium/SEB or LPS gels (Figure 1).

SEA induced phosphorylation of a 60 kDa protein in the 8.2-9.5 pH range (pI 9.0)(Figure 2: labeled as H). ETA and ETB also induced the 60 kDa phosphoprotein that was seen after SEA stimulation (Figure 2). However, in the same pH range ETA and ETB also induced phosphorylation of two 60 kDa sized proteins with pI of 8.4, and 8.8, respectively (Figure 2: labeled as I and J, respectively) that were not present with SEA stimulation (Figure 2). Additionally, ETA and ETB stimulated the phosphorylation of a protein in the 5.9 to 7.0 pH range (Figure 3: labeled as K). This 12 kDa, 6.1 pI protein was not present on the representative SEA gel (Figure 3). In contrast to the other exotoxins tested, SEB did not upregulate the number of phosphoproteins compared to buffer controls.

DISCUSSION

Staphylococcal exotoxins belong to a group of structurally similar proteins that range 26-30 kDa in size. Staphylococcal exotoxins bind to moieties distinct from the peptide binding groove of the MHC II molecule and induce a variety of cytokines in a receptor-ligand-dependent fashion (12-14,35). We investigated potential second messengers and early biochemical responses that staphylococcal exotoxins stimulate in macrophages.

All the staphylococcal exotoxins required the PKC enzyme for TNF and NO secretion based on the observations that H-7, the most specific inhibitor of PKC, effectively blocked TNF and NO secretion. None of the inhibitors reduced TNF or NO at concentrations known to inhibit cyclic nucleotide-dependent kinases (28-30). This observation supports previous

reports of See and Chow (26) and Matsuyama et al (36). They found that cytokinegene transcription or protein secretion induced by TSST-1- or SEB-activated monocytes were PKC-dependent. Staphylococcal exotoxin-induced macrophage activation also resembles macrophage activation by other stimuli. PKC was involved in the induction of NO synthase (37) and TNF secretion (29,38) by macrophages induced with IFN-gamma or LPS.

In contrast to its poor ability to stimulate TNF secretion, SEB induced murine macrophages to secrete IL-6 like other superantigens. This confirms our earlier report (14) and indicates that IL-6 is regulated distinctly from TNF. This hypothesis is supported by the fact that the inhibition of SEB-induced IL-6 secretion by low concentrations of H7 and H8 implicated a requirement for cyclic nucleotide-dependent kinases. Moreover, this observation is consistent with work showing that fibroblasts, HeLa cells, lymphocytes and astrocytes required both the cAMP-dependent kinases and PKC for IL-6 production (39-41). It is also noteworthy that SEB induced IL-6 secretion without the apparent upregulation of phosphoproteins 1 hr after stimulation, but TNF secretion did not occur. This also suggests distinctive regulatory mechanisms for these two cytokines. However, we have not yet correlated the expression of any specific phosphoprotein with cytokine secretion and the phosphoprotein pattern may reflect the different kinetics of cytokine induction (42).

More than one signal transduction pathway may be activated following toxin exposure because distinct epitopes or receptors exist for microbial superantigens. For example, we have demonstrated that binding of staphylococcal exotoxins to MHC class II-deficient macrophages leads to IL-6 secretion equal to that seen by MHC class II positive macrophages, but poorer TNF secretion (5). Therefore, IL-6 secretion may be controlled by alternate superantigen receptors whereas TNF may be controlled by the MHC II molecule.

Alternatively, differential signalling maybe explained by differences in superantigen binding to the MHC II molecule. SEB and TSST-1 have separate binding sites (43) to MHC II molecules consistent with their somewhat distinct tertiary structures (44,45). SEA also has at least one distinct binding site from SEB (11,43). Therefore, it is not surprising that enterotoxin and exfoliative toxins utilize different signalling pathways. For example, cyclic nucleotide-dependent kinases appeared less important than PKC for SEA- and SEB- induced increases in F-actin concentration, but were more important in the F-actin shifts induced by ETA and ETB.

SEA, ETA and ETB, but not SEB, induced phosphorylation of several murine macrophage proteins unique from those phosphorylated in response to LPS. Eleven proteins were phosphorylated reproducibly in response to toxin. These being 60 kDa, and 44 kDa molecules in response to SEA, ETA or ETB and 5 phosphorylated proteins unique to SEA stimulation and 4 unique to ETA or ETB stimulation. The variability in protein phosphorylation that we observed may have been because we assayed phosphoproteins at only one time point during a dynamic cell response. Alternatively, it is possible that basal protein phosphorylation differences occurred due to in vitro manipulation (46). Interestingly, we did not see an obvious dephosphorylation of any proteins. This contrasts with observations presented by See et al. (27) who reported that TSST-1 activated human monocytes to dephosphorylate several cellular phosphoproteins in a dose and time-dependent manner (27). Those authors did not determine if dephosphorylation was related to cytokine induction.

It is not surprising that SEB was a less potent inducer of phosphoproteins given its lowered ability to induce TNF and nitric oxide secretion (11,14). However, this is the first report, to our knowledge, that showed that the exfoliative toxins induced phosphoproteins not

phosphorylated in response to enterotoxins (60 kDa, 60 kDa, 43 kDa and 12 kDa). Similarly, novel, SEA induced the phosphorylation of proteins, 66 kDa, 65 kDa, 60 kDa, 56 kDa, 53 kDa, and 46 kDa not induced by the exfoliative toxins. These are further indications that the biochemical responses induced by these separate toxin families are not identical and are consistent with our observations that macrophages respond distinctly to different toxins (14). This is also consistent with the fact that the toxin families cause different clinical pathologies (2,3).

In conclusion, we suggest that different staphylococcal exotoxins induce distinct biochemical processes in macrophages. These differences can be observed at various stages of the macrophage response. 1. Enterotoxin induced cytoskeletal changes that occur immediately (within 30 sec.) were inhibited differently by protein kinases inhibitors H7, H8 and HA 1004 than were exfoliative toxin induced cytoskeletal changes. 2. Phosphoproteins that appeared 1 hour after enterotoxin stimulation were different than those present after 1 hour of exfoliative toxin exposure. 3. The cytokines that were secreted by macrophages several hours after toxin exposure were different and were variably inhibited by different kinase inhibitors (e.g. SEB vs. other toxins). Studies linking the identified phosphoproteins with distinct kinase activities are justified based on these findings.

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FIGURE LEGENDS

Figure 1. Protein phosphorylation patterns from macrophages stimulated 1 hr with LPS (12.5 μg/ml), SEA, SEB or ETA (10 μg/ml) were examined using two-dimensional PAGE and autoradiography. Differences between phosphoproteins from pH 4.5 to 6.0 were compared. Cells stimulated with SEB expressed phosphoproteins identical to those from unstimulated macrophages. These phosphoprotein spots were used to aid orientation, one is labeled reference 1 (R1). Cells stimulated with SEA expressed 6 unique phosphoprotein; 66 kDa (labeled as protein A), 65 kDa (B), 60 kDa (C), 56 kDa (D), 53 kDa (E) and 46 kDa (F) with pI of 5.5, 5.5, 5.5, 5.4, 5.1 and 5.0, respectively. Cells stimulated with ETA expressed a unique 43 kDa protein (G).

Figure 2. Protein phosphorylation patterns from unstimulated macrophages and macrophages stimulated 1 hr with SEA, or ETA/ETB 10 μg/ml were examined using two-dimensional PAGE and autoradiography. Differences between phosphoproteins from pH 8.2 to 9.5 were compared. Stimulation of macrophages with ETA/ETB or SEA induced a 60 kDa phosphoprotein (labeled as protein H). ETA/ETB stimulation also induced two 60 kDa proteins (I and J), that were not induced by SEA. A protein that was always phosphorylated was labeled as reference protein 3 (R3) for orientation purposes.

Figure 3. Protein phosphorylation patterns from unstimulated macrophages and macrophages stimulated 1 hr with SEA or ETA/ETB (10 μg/ml) were examined using two-dimensional PAGE and autoradiography. Differences between phosphoproteins from pH 5.5 to 7.0 were compared. ETA or ETB stimulation induced a 12 kDa protein (labeled as protein K), that was not induced by other exotoxins. A protein that was always phosphorylated was labeled as reference protein 4 (R4) for orientation purposes.

TABLE 1. Involvement of protein kinases in staphylococcal exotoxin induced F-actin polymerization

Stimulant[a]	Inhibitor (concentration)					
	H-7 (3 μM)	H-7 (10μM)	H-8 (3μM)	H-8 (15 μM)	H1004 (10μM)	H1004 (40 μM)
	% inhibition[b]					
Medium	1 ± 1[c]	5 ± 2	1 ± 1	3 ± 3	3 ± 3	7 ± 3
SEA	17 ± 2	87 ± 21*	35 ± 5	69 ± 10*	33 ± 11	63 ± 7*
SEB	17 ± 13	60 ± 26*	24 ± 25	74 ± 26*	14 ± 18	72 ± 31*
ETA	51 ± 15*	77 ± 11*	68 ± 9*	60 ± 9*	79 ± 11*	73 ± 17*
ETB	70 ± 20*	75 ± 5*	64 ± 12*	73 ± 12*	54 ± 7*	69 ± 14*

[a] Toxin (10 μg/ml)

[b] % inhibition calculated from the % cells in gates 2 and 3 (cells with polymerized actin), with the exotoxin only treatment used as the maximum and medium only treatment as the minimum.

[c] Numbers represent mean ± SEM of 3-5 independent experiments.

* Different from exotoxin only treatment (maximum) $P<0.1$, based on matched t test.

TABLE 2. Involvement of protein kinases in staphylococcal exotoxin induced TNF secretion

Stimulant[a]	No inhibitor	Inhibitor (concentration) H-7 (3 μM)	H-7 (10 μM)	H-8 (3 μM)	H-8 (15 μM)	H1004 (10 μM)	H1004 (40 μM)
		TNF concentration (units/ml)					
Medium	10±1	9±4[b]	8±6	9±3	8±4	9±3	8±5
SEA	65±2	43±12 (34)[c]	17±3 (74)*	53±5 (19)	28±5 (57)*	57±8 (12)	48±11 (26)
SEB	17±2	14±3 (18)	4±1 (77)*	15±2 (12)	12±3 (29)	15±3 (12)	8±2 (49)*
ETA	55±3	43±6 (22)	13±3 (76)*	51±3 (7)	44±7 (20)	39±12 (29)	48±7 (13)
ETB	56±4	62±4 (0)	2±2 (96)*	39±10 (30)	25±2 (55)*	45±11 (20)	30±2 (46)*
LPS	55±5	ND[d]	ND	ND	ND	ND	ND

[a] Toxin (10 μg/ml) or LPS (12 μg/ml) for 18 h.

[b] Numbers represent mean ± SEM of 3-5 independent experiments.

[c] Average % inhibition from samples without inhibitor (in parenthesis).

[d] Not Determined.

* Different from exotoxin only treatment, $P < 0.1$, based on matched t test.

TABLE 3. Involvement of protein kinases in Staphylococcal exotoxin induced NO secretion

Stimulant[a]	No inhibitor	Inhibitor (concentration)											
		H-7 (3 μM)		H-7 (10 μM)		H-8 (3 μM)		H-8 (15 μM)		H1004 (10 μM)		H1004 (40 μM)	
	NO concentration (μM)												
Medium	1.9±0.5	2.0±0.5[b]		1.0±1.0		2.1±0.3		1.5±0.4		1.8±0.3		1.0±0.5	
SEA	12.5±2.0	9.6±2.0	(23)[c]	1.4±1.0	(89)*	7.9±4.0	(37)	7.6±3.0	(39)	16.8±2.0	(0)	6.0±1.0	(52)*
SEB	3.0±0.4	2.4±0.3	(20)	1.0±0.2	(68)*	2.7±0.5	(10)	2.5±0.3	(17)	2.1±0.5	(30)	1.5±0.2	(50)*
ETA	10.0±2.0	6.0±3.0	(40)	2.0±0.5	(80)*	11.5±1.0	(0)	7.0±2.0	(30)	10.0±1.0	(0)	12.0±2.0	(0)
ETB	10.4±1.0	7.2±2.0	(31)	4.3±1.0	(60)*	12.0±1.0	(0)	3.6±1.0	(65)*	8.0±2.0	(23)	3.6±1.0	(65)*
LPS	27.0±2.0	ND		ND		ND		ND		ND		ND	

[a] Toxin (10 μg/ml) or LPS (12 μg/ml) for 18 h.

[b] Numbers represent mean ± SEM of 3-5 independent experiments.

[c] Average % inhibition from samples without inhibitor (in parenthesis).

[d] Not Determined.

* Different from exotoxin only treatment, $P < 0.1$, bases on matched t test.

TABLE 4. Involvement of protein kinases in staphylococcal exotoxin induced IL-6 secretion

Stimulant[a]	No inhibitor	Inhibitor (concentration) H-7 (3 μM)	H-7 (10 μM)	H-8 (3 μM)	H-8 (15 μM)	H1004 (10 μM)	H1004 (40 μM)
		IL-6 concentration (pg/ml)					
Medium	204±17	200±62[b]	160±62	211±45	179±22	219±36	166±58
SEA	1546±57	1348±65 (13)[c]	616±45 (60)*	1094±125 (29)*	763±23 (51)*	1219±111 (21)	1270±136 (18)
SEB	1801±122	886±87 (51)*	741±59 (59)*	992±100 (45)*	958±173 (47)*	1601±45 (11)	1856±160 (0)
ETA	1788±199	1506±111 (16)	999±45 (44)*	1115±55 (38)*	906±77 (49)*	1224±245 (32)*	1478±167 (17)
ETB	1436±44	1114±56 (22)	1009±112 (30)*	1132±27 (21)	874±45 (39)*	1297±120 (10)	1084±100 (25)
LPS	1429±102	ND[d]	ND	ND	ND	ND	ND

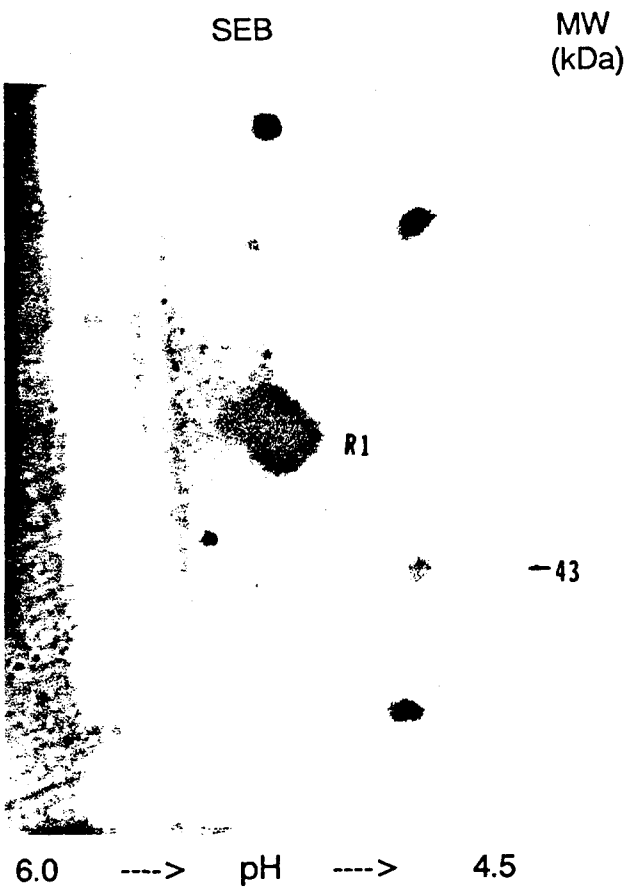
[a] Toxin (10μg/ml) or LPS (12 μg/ml) for 18 h.

[b] Numbers represent mean ± SEM of 3-5 independent experiments.

[c] Average % inhibition from the samples without inhibitor (in parenthesis).

[d] Not Determined.

* Different from exotoxin only treatment, $P < 0.1$, based on matched t test.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(kDa)
R1
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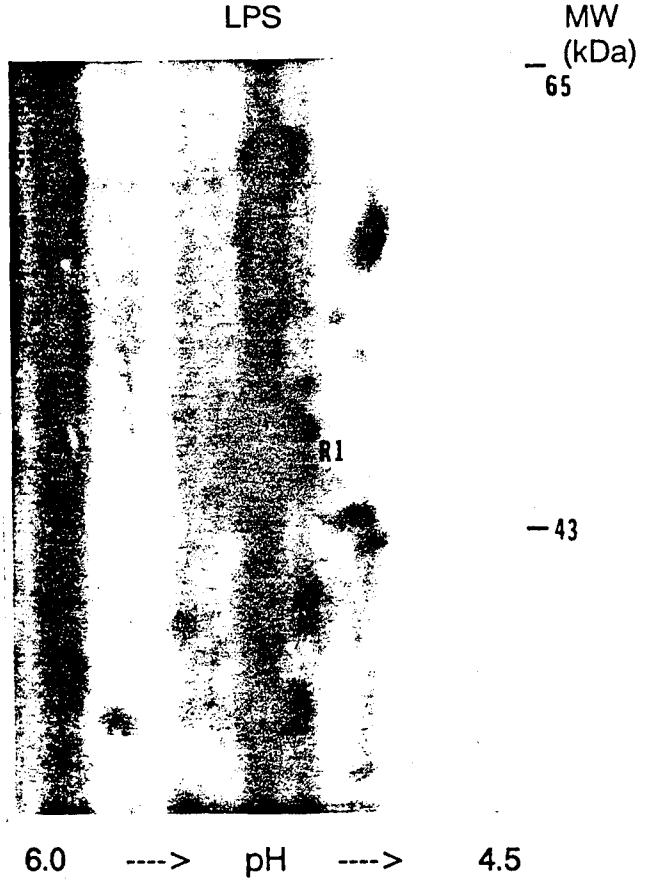
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MW
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65
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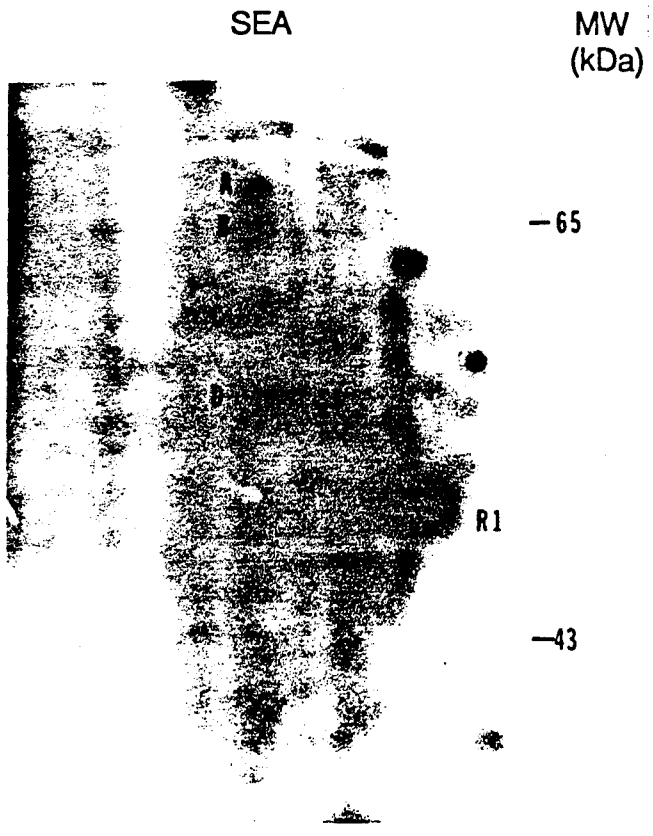
6.0 ----> pH ----> 4.5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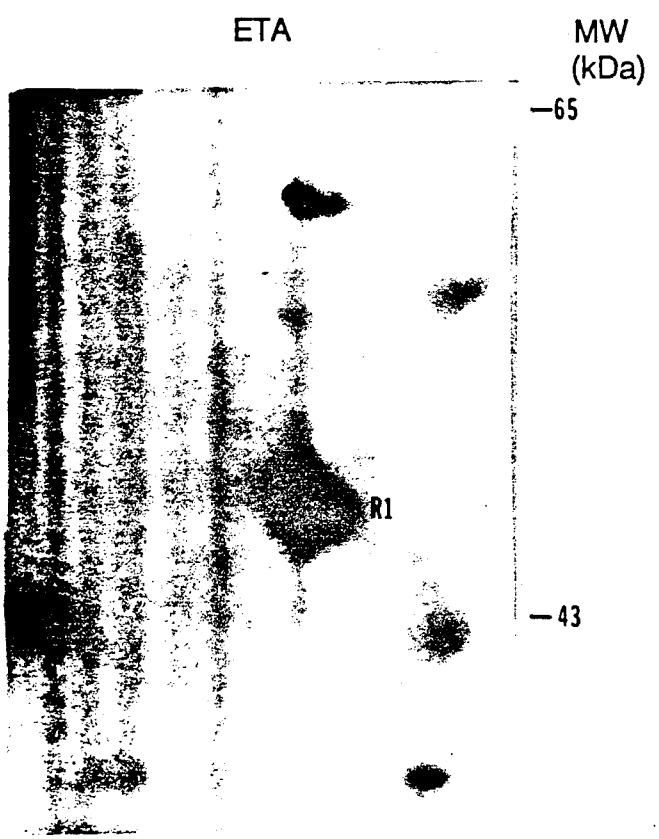
MW
(kDa)
A
B
65
R1
43
6.5 ----> pH ----> 4.5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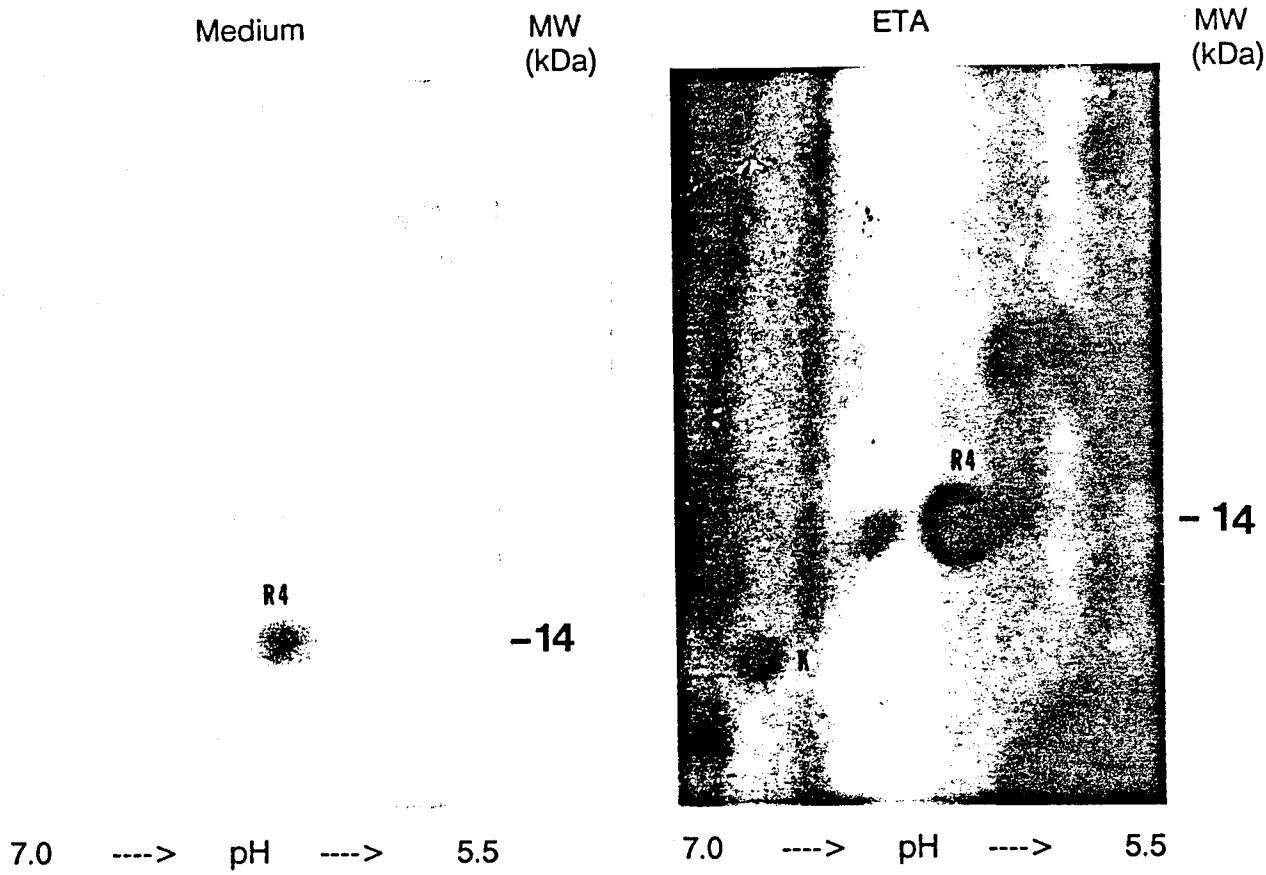
MW
(kDa)
65
R1
43
6.0 ----> pH ----> 4.5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ETA
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SEA
MW
(kDa)
R4
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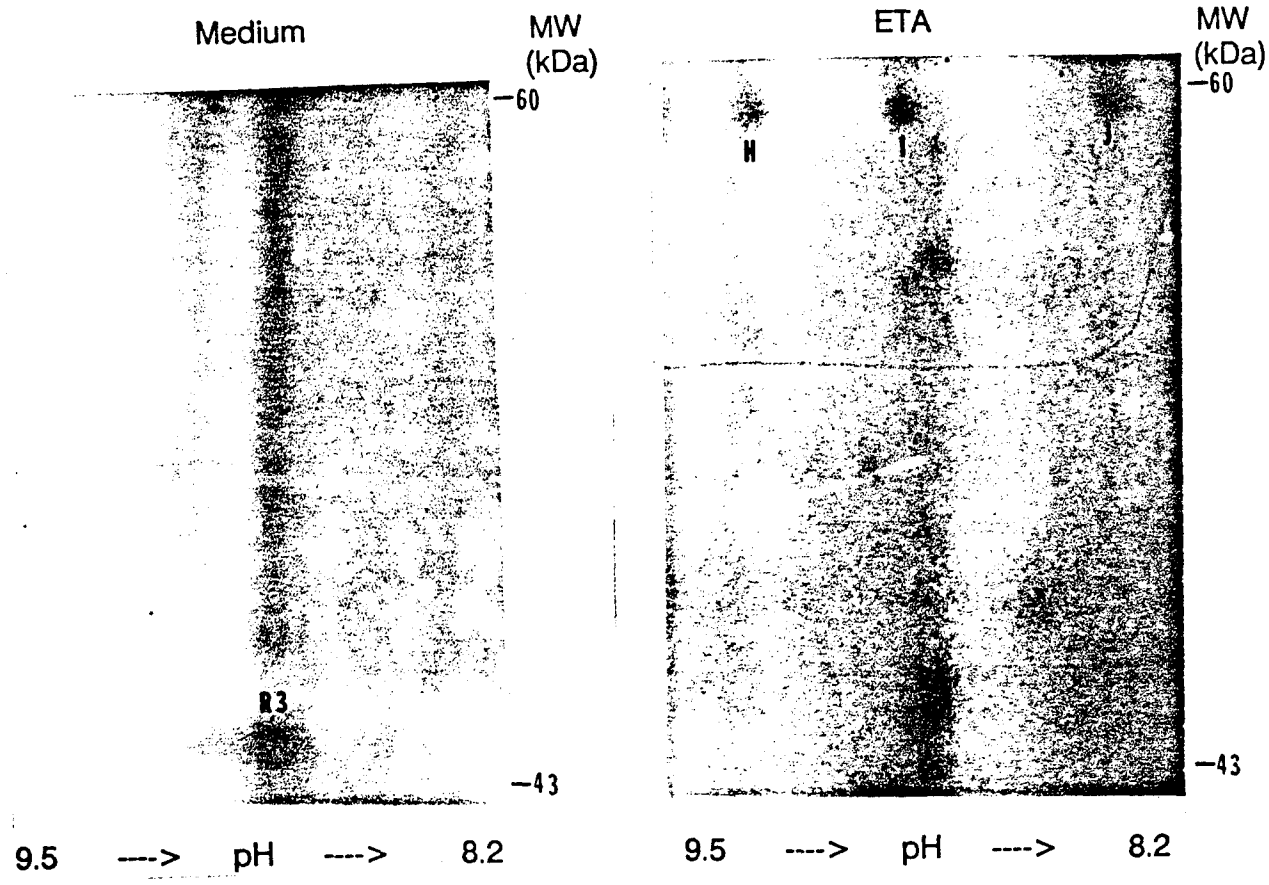
7.0 ----> pH ----> 5.5


Medium
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R3
43
9.5 ----> pH ----> 8.2
ETA
MW (kDa)
60
H
I
43
9.5 ----> pH ----> 8.2
SEA
MW (kDa)
60
H
R3
43
9.5 ----> pH ----> 8.2