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Preface

This volume contains the contributions presented at the Fourth European Workshop on Bacterial Protein Toxins, which was held at the Sogesta Centre in Urbino (Italy), from July 3rd to July 6th 1989. The European Workshop on Bacterial Protein Toxins, previously held in Seillac (France) in 1983, and then in Wepion (Belgium) and in Ueberlingen (Federal Republic of Germany) in 1985 and 1987, respectively, is now an established event, where the progress made in the field of bacterial toxinology is presented and discussed. The meetings are organized every two years, each time in a different European country, by an organizing committee composed of experts in the different disciplines. Although the main purpose of these workshops is to bring together the European scientists working on bacterial protein toxins and to provide young European scientists an opportunity to discuss their work, the interest in the meeting of non Europeans has grown exponentially. This confirms the good quality of the meetings and encourages us to do our best in keeping successful the next workshops which will be organized by B. Witholt (The Netherlands) in 1991 and by J. Freer (United Kingdom) in 1993.

The Fourth European Workshop on Bacterial Protein Toxins covered the following topics:
Molecular Architecture of Toxins, Toxin Binding and Internalization, Mechanisms of Cell Intoxication, Regulation of Toxin Expression, Toxins as Virulence Factors, Interaction of Toxins with the Immune System, Application of Toxins.

The wide variety of topics discussed reflects the multidisciplinary nature of bacterial toxinology. The session on the interaction of toxins with the immune system, introduced for the first time at this meeting, reflects both the growing interest in bacterial toxins of immunologists, who have discovered that some of the toxins are "superantigens", and on the other hand the interest of toxinologists in the mechanism of cell-mediated immunity against toxins.

This book is dedicated to Tsuyoshi Uchida, who died few weeks before the meeting where he was due to participate as a speaker.

The organizers of the Fourth European Workshop on Bacterial Protein Toxins 1989 wish to acknowledge the financial

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The organization of the meeting was possible thanks to Sclavo S.p.A. which, in addition to financial support, has provided all technical and secretarial assistance. We are especially indepted to L. Sprugnoli, L. Filippeschi, F. Gambassi and O. Cantoni.

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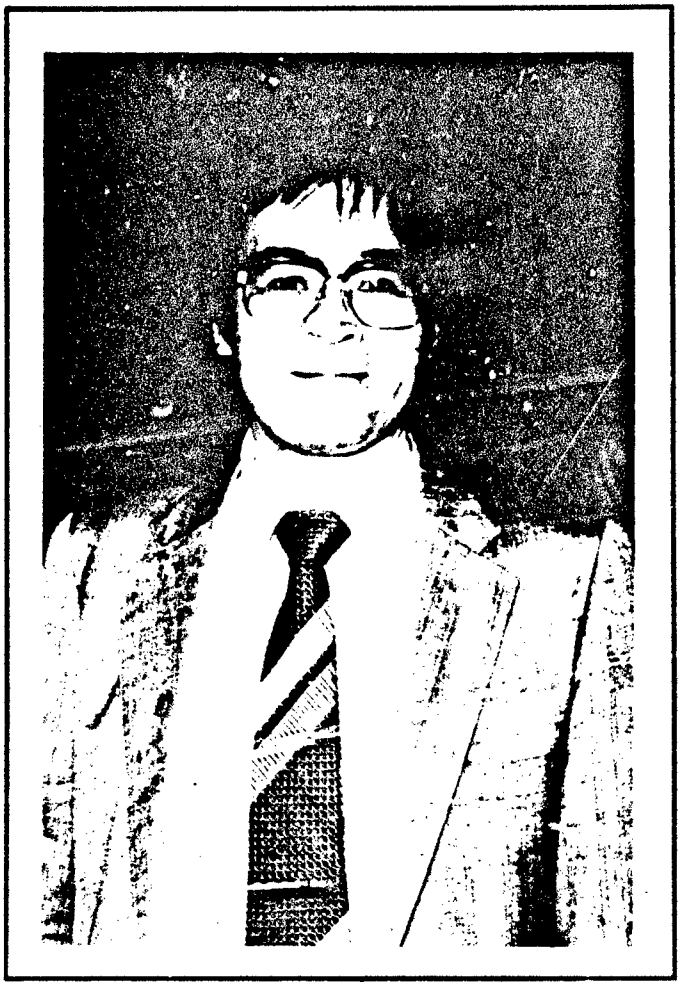
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Tsuyoshi Uchida: 1937–1989

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Tsuyoshi Uchida died on May 3d of this year in the 53d year of his age. He had many friends and admirers among those of us who are gathered here. His contributions to our field were many and almost all of them bore his own special stamp of originality and thoroughness. I first met Tsuyoshi in Dr. Masahiko Yoneda's laboratory in Osaka during the summer of 1967, just before a meeting on microbial toxins that was held in Hakone, Japan. Although Tsuyoshi spoke very little English at that time, he impressed me as a person of unusual intelligence and imagination. I asked Yoneda if he thought Tsuyoshi might be interested in spending a year or two in our laboratory. It took nearly three years to obtain the funds that brought him and his family to the United States. Within a very short time, he not only had endeared himself to everyone in our laboratory, but he had quickly proved that the diphtheria toxin structural gene was not a bacterial gene as I had thought, but was located on the ß-phage genome. His isolation of CRM mutants of the toxin gene opened up the path leading to understanding the molecular genetics of the toxin molecule, its structure-function relationships and its regulation.

After returning to Japan, he soon established himself as a leader in field of microbial toxins. He developed an elegant method for introducing a known amount of a given protein into mammalian cells by first encapsulating them within red cell ghosts which were then fused with the target cell in the presence of Sendai virus. In this way he showed that a single molecule of fragment A of

diphtheria toxin sufficed to kill a mammalian cell even of a species such as the mouse that is resistant to the toxin. He showed that the method could be used to assay the stability of a variety of foreign proteins introduced into the cytoplasm of mammalian cells. He produced antibodies against rat ADP-ribosylated elongation factor 2, affinity-purified by passage through a column containing immobilized fragment A. The antibody precipitated ADPR-EF-2 from species as diverse as mouse, yeast, wheat germ and man. He then proceeded to clone and sequence the hamster EF-2 gene, and demonstrated its homology with other G-proteins and with bacterial EF-G. Recently, his group has identified a 14.5 kD Vero cell membrane-protein as the diphtheria toxin receptor and showed that the toxin molecule also binds to a non-protein cell surface component. Both surface components appear to be involved in transport of toxin across the cell membrane. Finally, in an elegant paper that appeared in Science only a few months ago, it was shown that antibodies to a simple synthetic peptide can inhibit transport of nuclear proteins into the nucleus of mammalian cells. These are but a few of the many exciting findings that have come out of Tsuyoshi's laboratory over the past fifteen years.

Tsuyoshi Uchida had many affectionate friends and admirers throughout the world. He set an example to his students by continuing to work at the bench among them until almost the end. He took a personal interest in all those that worked with him and he was a generous man. No wonder that they loved him. He will be sorely missed by all of us, both as a person and as a leader in our field. It is a fitting tribute, that the published proceedings of this meeting be dedicated to his memory.

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Honorary Lecture

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Microbial Pathogenicity: Reminiscences, Past and Present

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During the past few years, there has been a good deal of interest in proteins produced by bacteria under conditions of "stress". Of particular interest have been proteins that are only produced when the iron concentration in the culture medium becomes limiting. Most of these stress proteins are located in the outer bacterial membrane and have not only been shown to be involved in iron transport into the cell, but also may serve as receptors for bacteriocins and bacteriophages. In many bacterial species their biosynthesis is regulated by the product of the highly conserved *fur* gene. In the case of pathogenic bacteria, they are frequently involved in virulence.

The first "stress" protein of this type that I encountered was diphtheria toxin, some 55 years ago. What I wish to do this morning is to tell you about that encounter, to mention briefly some of the erroneous but sometimes rather interesting speculations it led to and finally review some of the recent work, from other laboratories, that has answered questions that puzzled us a long time ago.

In 1935, when I began working with the diphtheria bacillus and its toxin, the field of intermediary metabolism was beginning to open up. The ubiquity of biochemistry was being realized for the first time. The fact that bacteria needed the same vitamins and amino acids as eucaryotes and used them for biosynthesis of enzymes with functions similar to those present in the cells of higher animals had at last become recognized. In 1935, most pathogenic bacteria were thought to require complex media for their growth containing large amounts of proteins or their breakdown products. Methods for purification of biologically active proteins were primitive and laborious. It seemed to me that if one could grow the bacteria

on a simple medium containing only components of low molecular weight, purification of a secreted protein toxin would become relatively easy. J. Howard Mueller suggested that I try to isolate diphtheria toxin, since he had succeeded in working out conditions for massive growth of the diphtheria bacillus on a medium of known composition except for traces of unknown growth factors present in yeast extract.

I had no difficulty obtaining heavy growth of the diphtheria bacillus on Mueller's medium. The trouble was that only traces of toxin were produced. For many weeks I vainly searched for something containing a "toxin-forming factor" and finally decided to try adding partially purified toxin itself (made on peptone medium of course) to the medium. Then I had a stroke of luck. Since all the Pyrex Fernbach flasks in the laboratory happened to be in use, it was necessary to grow the bacteria in some soft glass Fernbachs stored in the attic. When the toxin was finally harvested, the yield was more than twice as high as that routinely prepared in Pyrex. When powdered glass from one of these soft glass flasks was titrated in Pyrex only about 0.5mg of glass added to 25ml peptone medium increased the yield of toxin significantly, but addition of 5-10mgs inhibited the production of toxin almost completely. There was no effect on bacterial growth. The effect of soft glass could be mimicked by addition of ferrous salts. A mere 0.1ug/ml of Fe stimulated toxin production whereas 0.5ug/ml caused almost complete inhibition. When iron was added in the form of heme or crystalline hemoglobin, less than 1 nanogram/ml increased toxin production significantly, but even relatively large amounts of hemin failed to inhibit. The significance of these latter observations was not realized at the time. All that remained then was to show that removal of excess free iron from Mueller's medium enabled one to obtain high yields of toxin in a peptone-free medium and isolation of highly purified toxin became an easy matter (21). It turned out that we were not the first to observe the inhibitory effect of iron on toxin production (11, 24, 28), but we apparently were the first to recognize its prime importance.

Further study showed that toxin is synthesized de novo and is secreted during the terminal stages of linear growth only after the iron content of the medium has become exhausted. In contrast to most wild type diphtherial strains, e.g. C7(ß), which depend on a classical cytochrome system for their growth, the high toxin-producing Park-Williams #8 strain is able to increase 5-6 fold in mass, at a linear rate, while its contents of iron and of iron enzymes decrease to 15-20% of their normal value. PW#8 contains a defective cytochrome system and all its NAD-linked metabolic electron transport passes through flavoprotein as terminal oxidase. In contrast to C7, O_2-uptake and growth rate are directly proportional to the partial pressure of oxygen and during the final stage of growth, coproporphyrin and toxin are secreted into the medium in direct proportion to the drop in cellular iron content. In the PW#8 strain, succinate is the only known substrate that passes through an autoxidizable cytochrome 558mu as terminal oxidase. Some of the differences between the C7 and PW#8 strains are summarized in Table 1 (18, 20).

Table 1

C. diphtheriae Strains P.W.#8 and C7(ß) compared.

Property		P.W. #8	C7(ß)
Generation time, 35°C		150 min.	50 min.
Major cytochrome bands -196C		558mμ	600, 562, 549mμ
Catalase (Kat.F.)	+ Fe	80	20
	- Fe	15	ND
Succinate oxidation (QO_2)	+ Fe	16-20	90-100
	- Fe	3-5	ND
NADH oxidase	+ Fe	10-15	80-90
alcohol dehydrogenase	+ Fe	40	150
phage yield after U.V./10^8 bacteria		ca. 5	ca. 10^9
burst size		1-2	30-50
corynebactin		-	+
ß-phage particles adsorbed/bacterium		0	ca. 250
toxin yield ug/mg dry wt. bacteria	- Fe	10-12	1.5-2

These striking apparent relationships between iron metabolism and toxin production led us to speculate that diphtheria toxin might be the protein moiety of a bacterial cytochrome and might act on sensitive mammalian cells by interfering with their oxidative metabolism or perhaps by blocking biosynthesis of an essential cytochrome. With Carroll Williams, we soon obtained circumstantial evidence that seemed to favor the latter hypothesis. Williams had found that adult development of the saturnid moth, Platysamia cecropia, was associated with a massive synthesis of cytochromes, not present during insect diapause. Together, we found that small amounts of diphtheria toxin blocked adult development and cytochrome biosynthesis within a few hours although the animals survived many days. If kept in a humid atmosphere, diapausing pupae survived almost indefinitely (22). Of course we now know that all protein synthesis (6,19), not only cytochrome biosynthesis, is blocked by the toxin which inactivates eucaryotic elongation factor 2 by ADP-ribosylation of a modified histidine residue - diphthamide(30).

Diphtheria toxin is apparently an outer membrane protein from which a short leader sequence is split off upon secretion. This was first suggested nearly 25 years ago by the elegant experiments of Hirai et al (10) who showed that when PW#8 cells

are suspended in an iron-free medium in which succinate is the sole energy source, only membrane proteins are synthesized. Toxin constitutes about 35% of the total newly synthesized protein and close to 100% of all the secreted protein. Under these conditions high yields of internally labelled toxin may easily be prepared. For example, the toxin molecule contains 8 methionine residues equally distributed between fragments A and B. On the other hand, of the 16 histidine residues, all but one are on fragment B. Thus it has not been difficult to obtain highly purified toxin doubly or singly labelled with ^{35}S-methionine and/or ^{3}H-histidine. I would like to suggest that such labelled toxin molecules could prove useful in studying the internalization of toxin by sensitive mammalian cells.

Diphtheria toxin is not the only bacterial toxin whose biosynthesis requires iron starvation. The same is true for tetanus toxin (15) and for Shigella dysenteriae toxin (9). Until the last few years, however, virtually nothing was known about the genes involved and about the control of proteins whose biosynthesis was regulated by iron. In the interim of about thirty years, however, a good deal was learned

Table 2

Bacterial "stress" proteins related to virulence induced by Fe starvation

Diphtheria toxin	1936	(21)
Tetanus toxin	1943	(15)
Shiga toxin	1946	(9)
Pseudomonas A toxin, elastase, alkaline protease	1979	(3)
Yersinia proteins related to virulence	1987	(5)
Serratia marcescens hemolysin	1988	(23)
Actinobacillus (Haemophilus) pleuroneumoniae	1989	(8)
Vibrio parahemolyticus	1989	(12)
Vibrio cholerae hemolysin	1988	(27)

about the biochemistry of iron transport, iron-binding proteins and the structure of siderophores, low molecular weight molecules with high affinity for ferric ions. For reviews see (2,7,31). In 1974, Jack Murphy showed that E. coli extracts, programmed with β^{tox+} DNA synthesized toxin *in vitro* and that its synthesis was not inhibited by iron unless a small amount of C7(-) extract was added (16). This was in keeping with earlier suggestions that although the tox structural gene is carried by a β-phage gene, its expression is controlled by a host bacterial protein. Murphy's group went on to show that repression of tox gene expression takes place at the level of transcription and provided evidence that the repressor is a bacterial protein which, in the presence of iron as a corepressor, binds to the tox operon in the promoter region. The repressor is released from the promotor region of the tox operon when iron becomes limiting (17).

Table 3

Some Fe-regulated receptor proteins

Organism	Siderophore	Bacterial Membrane Gene	Receptor
E. coli	ferrichrome		phages T1, T2, T5, & 80
	"	fhuA	colicin M
E. coli	enterobactin	feb, cbr	colicin B
"	"	cir	colicin I, V
"	"	slt	phage
Enter. cloaca	aerobactin		cloacin
C. diphtheriae	corynebactin	tox	ß- phage ?
V. anguillarum	anguibactin		receptor OM

In biological fluids, the concentration of inorganic iron, Fe^{+++}, is vanishingly small, $<10^{-18}$ M. The concentration of iron in most aerobic bacteria is between 10^{-3} and 10^{-4}M. In order to multiply in body fluids, therefore, pathogenic bacteria must either find a way to extract iron from iron-binding proteins involved in transport to host tissue cells, or must turn on genes that produce toxic proteins that damage cells and cause them to release iron in a utilizable form. During the last few years a number of pathogens have been shown to utilize one or the other of these methods. For example, Neisseria meningitis synthesize some 2900 surface receptors per cell, specific for human transferrin, from which they can extract the iron which they require for growth (29). In a similar way, several other pathogenic species are able to scavenge Fe from lactoferrin for which they possess specific receptors. Neisseria gonorrhoeae can utilize iron from both transferrin and lactoferrin(13).

Iron transport into most bacterial species, however, is mediated by siderophores. *E. coli*, for example, synthesizes no fewer than five chemically distinct siderophores, each of which binds to a different outer or cytoplasmic membrane receptor. About 30 genes are involved in the iron transport sytem of *E. coli* all of which respond to regulation by the *fur* gene. It has been suggested that the 17 kd histidine-rich *Fur* protein is a repressor that binds ferrous iron as a corepressor (1), as had previously been suggested by Murphy et al (17) to explain the inhibition of diphtheria toxin production by iron. It is important to note that in pathogenic bacteria, the genes that code for *Fur* or *Fur*-like repressor proteins (4) are present on the bacterial chromosome, but the Fe-regulated genes that they repress are usually located on mobile elements such as plasmids, bacteriocins or temperate

bacteriophages (see Tables 3 and 4). The *fur* genes, of course, also control the expression of genes involved in the bacterial biosynthesis of siderophores. Proteins similar to the *Fur* protein have now been found in a number of other bacterial species including C. diphtheriae (25) and have been shown to bind to conserved nucleotide sequences in the promotor regions of iron-regulated genes (see Table 4).

Table 4

Some *Fur* binding sites

Species	Gene	Nucleotide sequence
E. coli	Consensus	GATAATGAT A ATCATTATC
	fur	CTATAATGA A TACGCATTATCT
	slt A	TGAATATGAT T ATCATTTTCA
C. diphtheriae	*tox*	TTATAATTAGG A TAGCTTTACCTAATTAT
S. marcescens	*shl*	GATAATGAT A ATTTCCCCC

Russell et al (25) have recently demonstrated and partially purified a siderophore from the C7 strain of C. diphtheriae which they have named corynebactin. They have shown that the PW#8 strain does not produce corynebactin and suggest that this defect "may be an important factor in determining its capacity to make large amounts of toxin" (26). However, as we have already pointed out (see Table 1) PW#8 differs from C7 in that even after all the external Fe supply has been exhausted, it continues growth and synthesize protein until its cellular Fe content has decreased more than 5-fold. It seems highly probable, therefore, that the diphtheria bacillus, like E. coli produces several siderophores (see Table 3). Moreover, as seen from Table 3, most siderophores have a dual function. When bound to the cell surface, they become become receptor sites for bacteriophages or bacteriocins. It was shown some 25 years ago that whereas C7 is capable of binding some 250 ß phages per bacterial cell, PW#8 binds none (14), even though its prophage carries the tox gene and when induced is almost indistinguishable from ß both morphologically and by its DNA sequence. It is tempting to predict that the corynebactin membrane binding site will prove to be the entry site for ß-phage.

can stimulate diphtheria toxin production, even high concentrations of iron in this form do not repress its biosynthesis(21). Very recently, Stoebner and Payne (27) have shown that V. cholerae can use heme and hemoglobin as sole sources of Fe and produce hemolysin. As they point out, since the Fe^{+++} concentration in body fluids is vanishingly small, any bacterial product capable of cytotoxic action causing release of heme-containing proteins will be advantageous to an invading organism. Indeed most invasive bacteria do produce hemolysins and cytotoxins as outer membrane proteins under conditions of iron starvation.

In conclusion, it seems to me that the work of the last few years suggests that in order to survive in host tissues, a bacterium must learn how provide itself with the iron required for its growth. Only then can it multiply and invade.

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Molecular Architecture of Toxins

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A Genetic Approach to Studying pH-dependent Membrane Interactions of Diphtheria Toxin

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INTRODUCTION

Diphtheria toxin (DT, Mr = 58,342) kills mammalian cells by inhibiting protein synthesis. Delivery of the toxin's enzymic moiety to the cytosol is achieved by a process beginning with receptor-mediated endocytosis. After binding to cell surface receptors, the toxin is conveyed to intracellular acidic vesicles. The low pH causes a conformational change in the toxin exposing hydrophobic regions in fragment B (DTB) leading to insertion of the toxin into the adjacent membrane. Subsequently, fragment A (DTA) translocates across the endosomal membrane and enters the cytosol, where it catalyzes the ADP-ribosylation of elongation factor-2 (EF-2).

The molecular mechanisms responsible for DT intoxication are slowly being revealed. Amino acids participating in catalysis and receptor binding have been identified (1,5). Conversely, residues involved in membrane translocation are still unknown. In vitro DT and DTB form ion-conducting channels in artificial membranes at acidic pH (3,6). Recent findings suggest that these channels are formed during toxin translocation in mammalian cells (12,13). Despite this correlation, a comprehensive understanding of the molecular mechanisms involved in DT channel formation and membrane translocation is lacking. Insight into these mechanisms, and their relationship to one another, would be facilitated by studying toxin mutants deficient in pH-dependent membrane interaction.

In this report we describe a system for isolating mutant forms of DT that are defective in membrane interaction at acidic pH. E. coli cells secreting DT to the periplasm are killed at acidic pH. (An enzymically attenuated toxin, DT-E148S, was used in these experiments to

comply with NIH recombinant DNA guidelines). Utilizing this system as a genetic selection we have isolated pH-resistant variants of DT. One of these mutants, CRM503, appears to be less cytotoxic to mammalian cells due to a defect in pH-dependent membrane translocation.

MATERIALS AND METHODS

E. coli JM103 was used in all bacterial studies. All plasmids are isogenic derivatives of pBR322 containing toxin sequences downstream from the tac promoter (10). Nitrous acid mutagenesis was performed on single-stranded gene fragments of DTB cloned into M13 (8). DNA sequencing was by the dideoxy nucleotide method (14). Toxin-related proteins were purified by FPLC (11). Experimental procedures are outlined in the figure legends, and more specific details have been published elsewhere (10,11).

RESULTS

DT-E148S is an enzymically attenuated form of DT containing serine in place of glutamate-148, a crucial active-site residue. DT-E148S expressed in E. coli is secreted to the periplasm. When exposed to a low pH, in the presence of a lipid bilayer, DT inserts into the bilayer and forms ion-conducting channels (3,6). This suggested that DT-E148S in the periplasm might insert into the bacterial membrane, and adversely affect the cell, if the periplasmic space were acidified. To investigate this possibility, we took E. coli producing DT-E148S and plated them on acidic agar plates. The results in Figure 1 showed that cells synthesizing DT-E148S were killed in a pH-dependent manner; approximately 1 in 10^7 cells survives at pH 5. Cells producing DTA-E148S were unaffected at any pH, demonstrating that amino acid sequences in DTB are responsible for the acid-induced death.

The F2 protein is similar to CRM45, a chain termination mutant of DT that retains a substantial portion of the toxins hydrophobic sequences and forms channels in artificial bilayers at acidic pH (6). Cells producing F2-E148S also showed a marked decrease in viability at pH 5 but were unaffected at higher pH values. F2-E148S is produced at a level approximately 100-fold lower than that of DT-E148S, which may account for the increased viability of these bacteria at moderately acidic pH. Cells producing wild-type F2 (E148) showed an identical loss of viability to that seen with F2-E148S, demonstrating that the serine substitution at the active site does not play a role in death of E. coli under acidic conditions.

To determine why DT-E148S producing cells were dying at low pH we examined the physiological consequences of

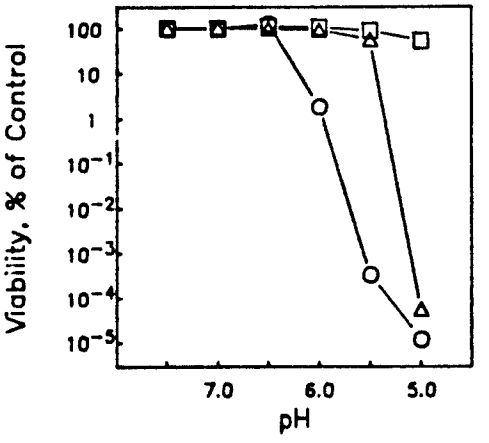


Figure 1. Bacterial viability at different pH. Exponentially growing E. coli, containing plasmids, were plated on L-agar at different pH. The next day viability was assessed and expressed relative to cells plated at pH 7.5. Plasmids and the proteins they encode are as follows: o, pDO1 (DT-E148S); □, pDO3 (DTA-E148S); Δ, ptacF2-E148S (F2-E148S).

exposing these cells and cells producing DTA-E148S to acidic pH. We found that cells synthesizing DT-E148S lost their membrane potential, their ability to retain intracellular ^{86}Rb and their capacity to actively transport proline (10). In each case, cells producing DTA-E148S were unaffected. This demonstrates that it is sequences in DTB that are responsible for the lethal-membrane damage observed in E. coli at acidic pH. Sequences within DTB are known to interact with membranes at low pH.

The loss of viability at acidic pH of cells producing DT-E148S suggested to us that this system could be used as a genetic selection for mutant toxin molecules that do not interact with the bacterial membrane at acidic pH. Gene fragments encoding regions of DTB were cloned into M13, and the single-stranded DNA was mutagenized with nitrous acid. Mutagenized restriction fragments were then cloned into the wild-type vector pDO1, which contains the gene encoding for DT-E148S (10). The ligated DNA was used to transform E. coli JM103, and the cells were plated on acidic agar plates. In this report we present a partial characterization of three of the full-length mutants we have isolated containing amino acid substitutions in their B fragments.

During initial screening we observed that surviving cells producing full-length mutant toxins contained much less DT-immunoreactive material in their periplasm (Table I). Since cells producing F2-E148S contain less toxin-related protein in the periplasm and are more resistant to acid treatment we speculated that the cells producing mutant

TABLE I
Decreased periplasmic levels of toxin mutants contribute to E. coli survival at acidic pH

	Levels of DT-related proteins in the periplasm.		Growth of E. coli producing DT-related proteins after an acidic pH pulse.	
Toxin	- IPTG	+ IPTG	- IPTG	+ IPTG
DT-E148S	100	ND	11	ND
CRM501	2	140	100	26
CRM502	2	82	94	33
CRM503	4	102	102	50
DTA	90	ND	100	ND

Exponentially growing cells (with or without 1 mM IPTG) were harvested and resuspended in growth medium. Aliquots of each culture were adjusted to pH 6.1 or 7.0. After ten minutes, the cultures were brought to pH 8.3, and 2 h later growth was monitored spectrophotometrically. Cell growth after the acid pulse is expressed as a percentage of growth following the pulse at pH 7. In lieu of the acid pulse, a portion of the cells were used to determine the amount of DT-immunoreactive material in periplasmic extracts which is expressed as fmole DT/mU β-lactamase activity.
ND = not determined.

toxins may be surviving the acid selection because of the lower levels of the mutants in the periplasm. To investigate this possibility we increased the levels of the mutant toxins in the periplasm by inducing the cells with 1 mM IPTG. Induction increased the amount of toxin-related material in the periplasm to levels comparable to that found for uninduced cells producing DT-E148S. Even with this increase, the cells still displayed a mutant phenotype as judged by inhibition of growth after an acid pulse relative to that seen in cells containing comparable levels of DT-E148S.

The amino acid changes in the mutant toxins were determined from the DNA sequence of the restriction fragment subjected to mutagenesis. All three mutant toxins contained multiple point mutations in their fragment B moieties. CRM501 had two changes, G268E and V283A; CRM502 also contained two changes, M230I and E232K; and CRM503 had three changes, E349K, N399D, and V443I.

If this genetic selection in E. coli selects toxin mutants defective in pH-dependent membrane insertion/translocation then these mutants might be less cytotoxic to mammalian cells. The experiment in Figure 2 shows that only CRM503 was less cytotoxic than DT-E148S to BS-C-1 cells in a 24 h assay. The reduced cytotoxicity of this mutant could in theory be due to a defect in either receptor binding, membrane translocation or catalysis. We examined each of these functions to determine which is defective in CRM503.

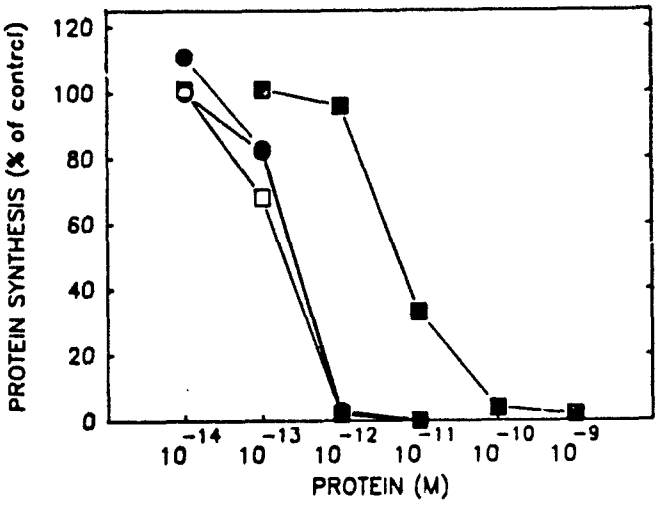


Figure 2. Inhibition of protein synthesis by DT-E148S and mutant toxins. BS-C-1 cells (monkey fibroblasts) were incubated with different concentrations of the nicked toxins for 24 h. Protein synthesis (incorporation of ^{3}H-leucine into acid-insoluble material) is expressed as a percentage of radioactivity incorporated into cells incubated without toxin. o, DT-E148S; ●, CRM501; □, CRM502; and ■, CRM503.

ADP-ribosylation of EF-2 in vitro was unaltered in CRM503 as compared to DT-E148S when both toxins were nicked and reduced (data not shown). CRM503 was also able to block the binding and uptake of radiolabeled DT by BS-C-1 cells as efficiently as DT-E148S (data not shown). These data suggest that the reduced cytotoxicity of CRM503 is not due to either a defect in receptor binding or catalysis.

To examine membrane translocation by CRM503 and DT-E148S we utilized a procedure employed by others (2,9). Treating mammalian cells with NH_4Cl raises the pH in endocytic vesicles and blocks the translocation of DT across endosomal membranes. If toxin-treated cells, incubated in the presence of NH_4Cl, are simultaneously exposed to acidic culture medium the low pH induces surface-bound toxin to translocate across the plasma membrane. When NH_4Cl-treated BS-C-1 cells were incubated with either DT-E148S or CRM503 and then exposed to media at different pH, CRM503 required a much lower pH to inhibit protein synthesis to levels comparable to that of DT-E148S (figure 3). This result suggests that CRM503 is less cytotoxic due to a defect in a pH-dependent step.

DISCUSSION

E. coli secreting a full-length form of DT into their

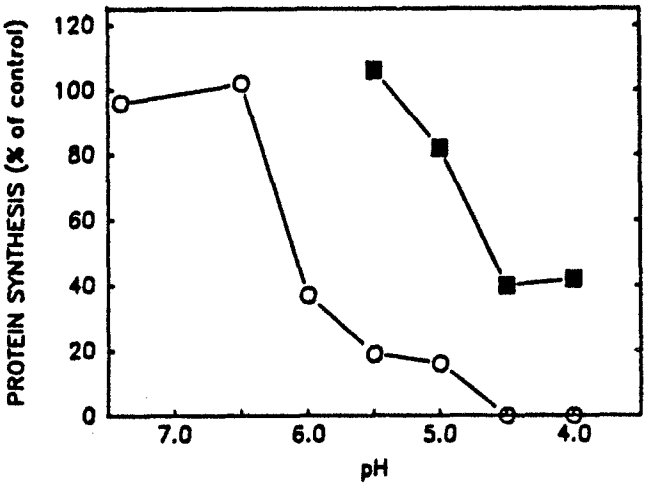


Figure 3. The effect of acidic culture media on DT-E148S and CRM503 intoxication. BS-C-1 cells were incubated in the presence of 20 mM NH_4Cl and 1 nM of the nicked toxins at 37°C for 2 h. All subsequent steps were at 37°C and included 20 mM NH_4Cl. This medium was then exchanged for media at different pH. After ten minutes, the cells were placed in fresh medium at pH 7.4, and protein synthesis was measured 24 h later. Protein synthesis is expressed as a percentage of controls incubated in the absence of toxin. Protein synthesis in controls at pH 4.0 averaged 98% of controls at pH 7.4. o, DT-E148S and ■, CRM503.

periplasm were killed when the bacteria were exposed to an acidic pH. Death was dependent on amino acid sequences present in DTB, a region of the toxin known to insert into membranes at low pH. The low pH caused depolarization of the cells, efflux of intracellular ions and loss of active transport. These effects are sufficient cause for cell death. Similar effects are seen when sensitive bacteria are treated with colicin E1, and colicin E1 has been shown to form ion-conducting channels in artificial membranes. This has led to a model whereby colicin E1 kills sensitive E. coli by forming channels in the bacterial inner membrane (7). We suggest that a similar phenomenon occurs when DT-E148S producing cells are exposed to an acidic pH. The low extracellular pH causes the periplasm to be acidified. This leads to a conformational change in the toxin inducing it to insert into the bacterial inner membrane. The resulting disruption of the permeability barrier leads to cell death.

Utilizing this system as a genetic selection we isolated three full-length mutants containing amino acid changes in their B fragments. All three toxin mutants were present in the periplasm to a lesser extent than DT-E148S. The lower levels of these mutants appeared to contribute to their

survival at low pH. This was shown by the ability of these mutants to inhibit the growth of their host E. coli after an acid pulse when the levels of the mutant toxins were made comparable to that of DT-E148S. Future selections can incorporate induction with IPTG to isolate more pH-resistant mutar s.

One mutant toxin, CRM503, was less cytotoxic to BS-C-1 cells. The reduced cytotoxicty of CRM503 was not due to either a defect in catalysis or receptor binding. When the cytotoxicities of CRM503 and DT-E148S were measured at different pH in the presence of NH_4Cl it was found that a lower pH was required by CRM503 to inhibit protein synthesis. When toxin-treated cells are exposed to media at acidic pH it is thought that this mimics conditions found in endosomes. This suggests that CRM503 is less cytotoxic because it is defective in pH-dependent translocation across endosomal membranes.

The fact that CRM501 and CRM502 were as cytotoxic as DT-E148S to BS-C-1 cells suggests that the low pH genetic selection is capable of selecting at least two classes of mutants. One class, consisting of CRM503, is defective in both mammalian cell membrane translocation and killing of E. coli while the second class is only defective in killing of E. coli. One difference between the bacterial and mammalian systems is the presence of a specific receptor in the latter. The mutations in CRM501 and CRM502 flank a region of the toxin that has been proposed by Falmagne et al as a surface lipid-associating domain (4). It is possible that at acidic pH DT first associates with receptorless membranes through this region. This would be an important interaction in the bacterial periplasm but may not be critical in a mammalian system where specific receptors initially concentrate the toxin at the membrane surface. This is one possible explanation for the differential effects of CRM501 and CRM502 in bacterial and mammalian systems.

The isolation of mutant forms of DT that are less effective in killing E. coli at acidic pH enhances our ability to decipher the molecular mechanisms involved in DT membrane insertion/translocation. It may also be possible to apply this approach to other toxins that interact with membranes at low pH, such as Pseudomonas aeruginosa exotoxin A and anthrax toxin. Also, other conditions, such as ionic strength, may be exploited for isolating other condition-dependent mutants.

ACKNOWLEDGEMENTS

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Protein Structure and Conformational Change

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ABSTRACT

The study of protein structure has seen exciting developments in the past few years, including advances both in experimental techniques for structure determination and in our theoretical understanding of the principles of protein architecture. Here I shall briefly review the general developments and then treat two related topics: (1) the prediction of protein structure from amino acid sequence, and (2) conformational changes in proteins.

1. Introduction

For an overview of the current state of the field, let us follow the course of a molecule as it passes successively through a number of research projects. Let us suppose that the nucleotide sequence of a gene corresponding to a known protein is determined, and translated to an amino acid sequence. Already it is possible to determine whether the protein is closely related to other known proteins, by searching through the data bases for similar sequences. It may emerge from such a calculation that the unknown protein is related to another protein of known function or even of known structure. This result is occurring more and more often, and when it does it provides very useful and reliable information [1].

Meanwhile, back in the laboratory, it may be possible to clone the gene and produce the protein in quantity. Attempts may be made to crystallize it: a process that still depends to a large extent on trial-and-error, but which is usually successful eventually. Crystals of adequate size (at least about 0.2 mm in each dimension) and order will permit the measurement of the X-ray diffraction pattern, from which the structure could be determined. If the protein is small (less than about 125 residues), new techniques of nuclear magnetic resonance may provide a structure.

Once the structure is known, it can be analysed and interpreted by a variety of computational techniques, including molecular graphics. Most structures are recorded in the data base of solved structures (The Protein Data Bank at the Brookhaven National Laboratory in New York). These can then be used as the basis for comparative studies of protein structure and sequence-structure relationships, and for design of "engineered" modifications.

X-ray crystal structure determination

The number of proteins solved to atomic resolution by X-ray crystallography is increasing very quickly: We now know approximately 300 protein structures. This is the result only in part of the growth in population of protein crystallographers; technical advances have occurred both in data collection and in computer analysis of the data [2].

Synchrotrons provide powerful sources, and area detectors permit rapid data collection. As a result, the process requires many fewer crystals, a crucial factor if material is limited. New techniques based on taking advantage of X-rays of variable wavelength from synchrotrons hold much promise also.

The data derived from X-ray diffraction measurements are incomplete: this gives rise to the celebrated "phase problem" [3]. The classical method of solving the phase problem is to make isomorphous derivatives; that is, to introduce heavy metals at particular sites in the protein, without changing the geometry of the crystal packing. By comparing the diffraction pattern of the native crystal with those of the derivatives, it is possible to derive sufficient phasing information to get the process of model-building and refinement started.

Computationally, if the protein is closely related to one of known structure, it is possible to use the known structure as a model, and to try to position and orient it in the unit cell of the crystal of the unknown structure. If the optimal fit can be determined, it can provide phases with which to calculate an electron density, again of sufficient quality to begin the process of model building and refinement.

Once a preliminary model is available, other computer programs have demonstrated great power in refining it. The first "revolution", now about 15 years old, was the substitution of molecular graphics programs, notably Alwyn Jones' FRODO, for the building of physical models [4]. The use of computer graphics has been extended, still by Jones, to make use of more perspicuous representations of the electron density (the shrinking of the three-dimensional volume of density within a contour level to a linear "skeleton" with the same connectivity) and the use of data bases of well-determined protein structures to construct a model based on the the skeletal representation of the electron density map [5].

The second "revolution" was the use of molecular dynamics, restrained by the experimental diffraction data, to explore the conformational space in the vicinity of the rough model, to seek the best match between the refined structure and the measured diffraction pattern. This procedure, implemented in several computer programs of which the best known is Axel Bruenger's EXPLORE, has greatly sped up the process bringing a model from the rough stage to one that is quite close to the correct structure [6]. The result of EXPLORE is model that must be checked closely by the crystallographer, and generally subjected to one of the classical refinement procedures, to produce the final result. The use of molecular dynamics has transferred to the computer the job of testing many modifications of the models that both fit the experimental data and are also stereochemically reasonable, which used to take crystallographers weeks or months of manual labour.

Protein structure determination by Nuclear Magnetic Resonance

For many years, X-ray crystallography was the only source of detailed structural information about macromolecules. Recent developments in nuclear magnetic resonance have permitted the determination of protein structures directly in solution [7,8]. The measurements produce a set of distances, usually but not always between protons in the molecule, from which the coordinates have to be deduced. This can be done either by molecular dynamics, subject to restraints implied by the distances, or by a geometrical process based on the experimental measurements plus stereochemistry.

One limitation of NMR structure determination is that it is limited to relatively small proteins: the largest structure determined by NMR was lysozyme (128 residues) [9].

2. Prediction of protein structure from amino acid sequence

A frequently-asked question:

> "Here is a new sequence. What can I say about the structure and function of the corresponding protein?"

If the unknown protein is related to one of known structure, inferences based on the known structure and the degree of the relationship can provide the most reliable clues to the nature of the unknown protein. Comparisons of proteins solved to high resolution by X-ray crystallography and well refined show that there is a relationship between the deviation of the amino acid sequence and the deviation of the three-dimensional structure [10-11]. This relationship, shown in Figure 1, is common to all families of protein studied, and serves as a guide to the quality of model-building that can be expected.

In Figure 1, each point corresponds to the comparison of two related proteins. For each pair, a "core" of the structures retains the same qualitative fold; the remainder can show very extensive conformational changes. For example, if one compared from this point of view the capital letters R and B, the common core would correspond to those portions of the letters represented by the letter P. Outside this common core, R has a diagonal stroke and B has a loop.

Once the common core of each pair of structures is found, the sequence divergence is calculated as the fraction of identical residues in the core (the process of determining the core ensures that the same number of residues are specified from each structure, and specifies that they are aligned). The measure of divergence of structure is the root-mean-square deviation of the backbone atoms of the common core residues.

It is clear from the data that as the sequences diverge, the structures diverge. Moreover, as the sequences diverge, the fraction of residues in the core -- the fraction of the structure that retains the same qualititive fold -- may also decrease substantially. For sequences more closely related than 50% residue identity, the core is observed to contain at least 95% of the residues, and the refolding of the remaining 5% will involve only minor surface loops. But for very distantly related structures, with residue identities below 20%, often evolution has had (and has taken) the opportunity to alter far more of the structure -- the core can in such cases amount to as little as 40%

of the structure or as much as 80-90% [11].

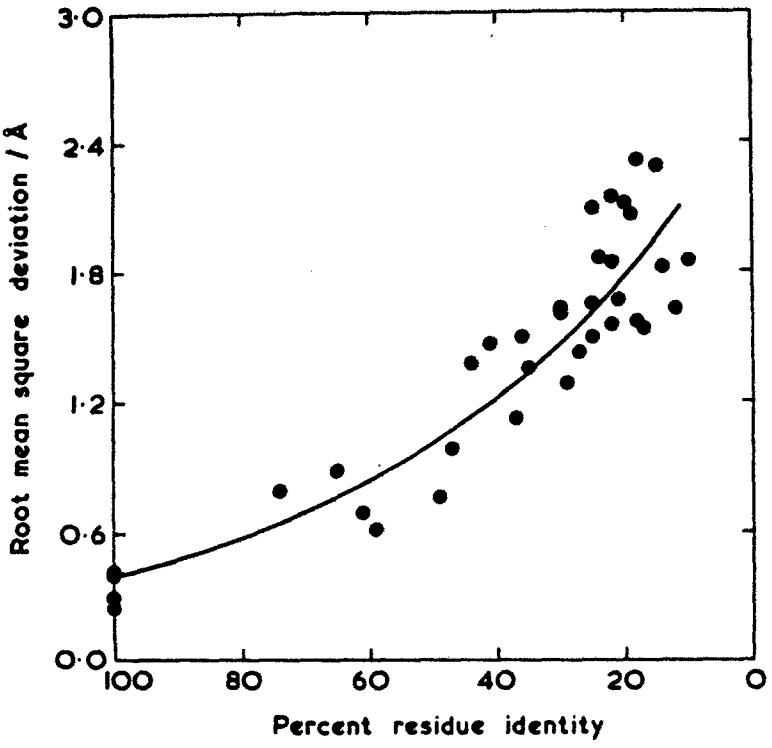


Figure 1. The relation between divergence of amino acid sequence and divergence of structure in homologous proteins.

In comparing homologous structures, it is frequently the case that a portion of the structure retains the same general fold, and the rest changes its fold qualitatively, and may in the cases of very distantly-related proteins be subject to such extensive insertion and deletion of residues that it is not even meaningful to try to align the amino acids sequences in these regions. In order to derive a quantitive relationship between the divergence of sequence and structure, it was necessary to isolate the "common core" of each pair of proteins -- the portion that retains the same fold -- and to measure the percent residue identity (abscissa) and root-mean-square deviation of the mainchain atoms (ordinate) within the common core. (See reference [10] and [11] for details.)

Let us consider three representative points on the curve. First, the points at 100% residue identity represent independent structure determinations of the same protein. The observed differences in structure arise primarily from crystal-packing forces -- the molecules have different environments in the crystal and are subjected to different patterns of intermolecular contacts. The average value of root-mean-square difference in atomic positions in these pairs of structures is 0.33 A. The implication is this: Protein conformation is determined primarily by amino acid sequence, but modified by subsidiary factors, mainly crystal-packing forces but also the conditions of solvent and temperature. The value 0.33 A estimates the magnitude of the effects of these secondary factors. Conformational changes in proteins will be discussed in the next section.

Consider next the pair of sulphydryl proteases papain and actinidin. In this case the core contains 206 residues (out of a total of 212 in papain and 218 in actinidin). Thus the core includes almost all of either molecule. The residue identity is 49%, and the r.m.s. deviation of the core is 0.77 A.

This degree of homology -- or any closer relationship -- provides a useful basis for model building. Thus, suppose one had determined the amino acid sequence of a new sulphydryl protease 49% homologous to either actinidin or papain, and built a model of this protein by taking the backbone of actinidin (or papain, as the case may be) and replacing the mutated sidechains, retaining the sidechain conformation of the parent structure whenever possible. If the structure of the new protein was solved by X-ray crystallography, we should expect, on the basis of this relatively close relationship between the amino acid sequences, that:

(1) A core of over 90% of the residues would retain a common fold. Fewer than 20 residues would be found in loops with radically different conformations.
(2) The backbone atoms could be superposed with a root-mean-square deviation of about 1.0 A or less. The binding site might show even less deviation, as evolution tends to alter binding sites relatively conservatively, provided that one is dealing with a family of proteins in which function is maintained.
(3) The sidechains of 90% of the nonmutated residues, and of 50% of the mutated residues, would have similar conformations.

Such a model would give a reasonable picture of the unknown structure, at a level useful for analysis of its structural and functional properties. If the sequences were more closely related, the quality of the model would be correspondingly improved.

The level of 50% residue identity provides a useful "rule-of-thumb" in deciding whether model building by homology will be satisfactory. For proteins more distantly-related than this rough threshold, the model-building procedure described would be less successful, and one would be discouraged from trying to build a full-blown three-dimensional model. The core might include no more than 40% of the sequence, with 60% of the chain adopting a qualitatively different fold. The geometry of the core residues would be altered more radically -- the root-mean-square deviation would be much higher. The one consolation is that the active site might be relatively well conserved between the known and unknown structures, permitting some analysis of the effects on function of mutations within the active site itself.

Unfortunately, there is no better way of generating a model of an unknown protein from a very distant relative, nor is there any procedure -- such as those based on conformational energy calculatios or molecular dynamics -- that will reliably improve such a model. This is an active subject of current research.

3. Conformational change in proteins

Many proteins, including bacterial toxins, undergo conformational changes as part of their function. What can we say about the mechanism of such conformational changes?

Although crystal structures provide "snapshots" of proteins in particular states, comparisons of crystal structures can provide information about the nature and mechanism of conformation change. In the previous section we have discussed the conformational changes produced by mutations during evolution; next we take up the mechanism of conformational changes produced by crystal-packing forces and changes in state of ligation. We shall mention the implications of conformational changes to the theoretical problems of (1) predicting protein structure from amino acid sequence, and (2) the engineering of protein-ligand interactions. The latter problem includes both the design of ligands de novo, and the modification of known protein-ligand systems -- for example, to enhance affinity or to alter specificity.

Insulin and citrate synthase illustrate the "helix-interface-shear" mechanism of conformational change, in which packed helices move with respect to one another to limited extents; larger movements can occur as a cumulative effect.

By their nature, sets of atomic coordinates derived from crystal structures do not explicitly reveal the dynamics of proteins in solution. Accordingly you will see that our studies have been based on comparisons of two particular states of proteins, in different crystalline environments or in different states of ligation. In the case of bacterial toxins, only one high-resolution structure is known, that of colicin A [12], but only one form of this molecule has been solved.

What kinds of conformational changes can one see by comparing the solved crystal structures of proteins? The available data arises from the cases in which different sets of coordinates have been determined for a single protein, corresponding to a single amino acid sequence. These may arise either from entirely independent crystal forms, which may have been crystallized from different conditions of solvent and temperature, or from structure determinations of crystals with more than one molecule in the asymmetric unit. In both cases, the environment -- the nature of the crystal-packing contacts -- is in general different.

These data have permitted analysis of the mechanism of conformational change in certain cases of ligand-induced conformational changes. The best data are available for proteins composed largely of helices. In insulin and citrate synthase, conformational changes can occur by a deformation of the interface between packed helices, allowing one helix in such a pair to slide by up to about 1.5 A with respect to the other; 1.5 A seems to be

(roughly) a limit of the "plastic deformation" of helix interfaces. In cases where close packing prevents simple rigid-body or "hinge-bending" changes in tertiary or quaternary structure, large conformational changes (e.g. 10 A shifts) can occur as the cumulative effect of individual shifts helices with respect to each other.

Insulin

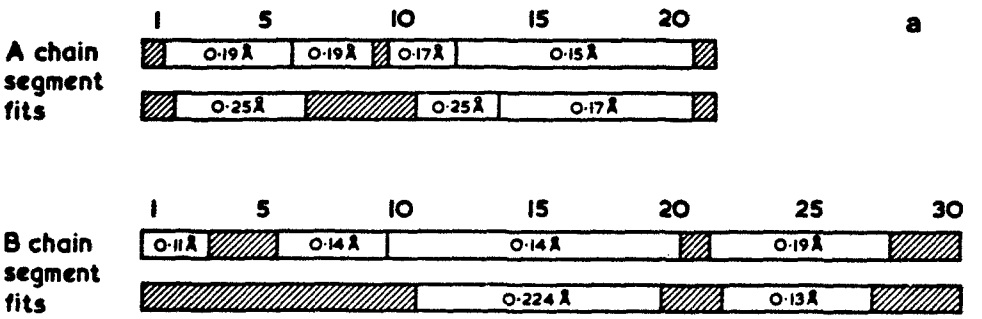
The pig insulin molecule is an interesting system to study in terms of conformational changes, because it illustrates the effects of both crystal-packing forces and changes in state of ligation. Two forms of pig insulin have been solved to high resolution [13-14], and provided the data for our investigations [15].

The pig insulin monomer consists of two chains, A (21 residues) and B (30 residues). The chains are linked by two disulphide bridges. Two monomers form a dimer, held together by hydrogen bonding between strands of beta-sheet and van der Waals contacts. In the presence of Zn++ the dimers assemble into hexamers. Crystals grown at low ionic strength produce the 2Zn form, containing two Zn ions per hexamer; but at high ionic strength an alternative form is produced containing four zinc atoms per hexamer. The asymmetric unit of each crystal contains two monomers -- thus each hexamer can be thought of as a trimer (with perfect, crystallographic three-fold symmetry) of dimers. The two monomers of each dimer have similar but not identical structures.

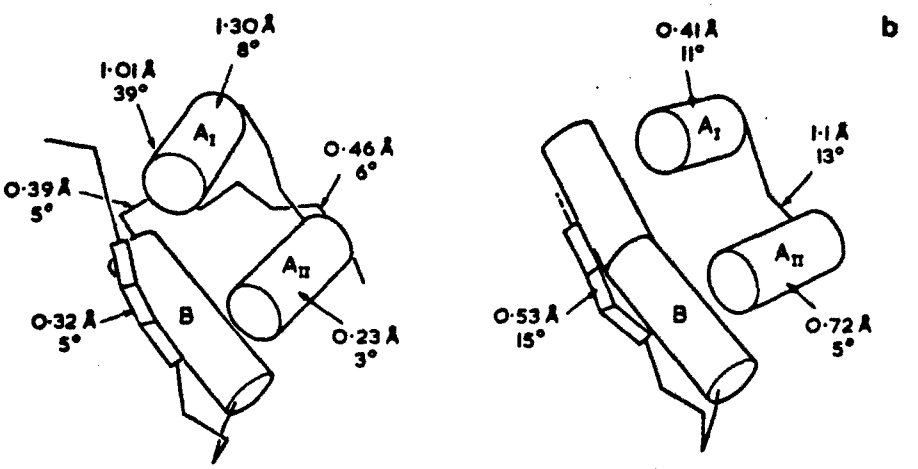
It follows that the 2Zn and 4Zn crystal structures provide us with four independent monomer structures. Of these, one monomer of the 2Zn form is very similar in conformation to the corresponding monomer in the 4Zn form, and can serve as a "reference structure" for analysis of conformational changes and the search for their origin. In the 2Zn form, the two monomers are rather similar; in the 4Zn form the N-terminus of the B chain has changed its conformation entirely in the process of forming the new, off-axial zinc binding site. (Symmetry conditions dictate that the two zinc ions in 2Zn insulin must fall on the three-fold axis; and that at least one of the four zinc ions in 4Zn insulin must be on the axis. In the crystal structures, one of the axial zinc binding sites is very similar in 2Zn and 4Zn forms; the 4Zn form contains three off-axial zinc binding sites related by the threefold symmetry.)

A detailed comparison of the main-chain conformations of the insulin monomers [14] suggests a physical picture of the deformations: One can think of dealing with a succession of nearly-rigid segments of chain (which largely retain their conformations, relative to the reference structure) connected by more flexible regions. The well-fitting regions can be fit to the homologous regions of the reference molecule with r.m.s. deviations of main-chain atoms of 0.11-0.25 A. (See Figure 2.) The only significant segment that exhibits a gross change in topology is the N-terminus of the B chain in one of the molecules of the 4Zn form.

The segments that we describe as well-fitting are maximal, in the sense that if one tries to add residues to them, or to superpose two of them together, the deviations increase substantially. This implies that even the nearly-rigid segments are moving with respect to one another. Indeed, we have measured the magnitudes of these movements and find them to be in most cases no more than about 1.5 A.



Fits of Chain Segments of Molecules 1 to Molecule 2 of 2 Zn Insulin



2 Zn Insulin Molecule 2 — 4 Zn Insulin Molecule 2

Shift of Chain Segments of Molecules 1 Relative to Molecules 2

Figure 2. Results of comparison of reference monomer of pig insulin with the monomers of different conformation. (a) The segments the main chain coordinates of which can be well superposed are light and contain numbers indicating the root-mean-square difference in atomic positon; the poorly-fitting segments are shaded. (b) The shifts in the segments relative to those in the reference monomer, after fitting of the monomers of different conformation onto the reference monomer by superposing the main-chain atoms of the B helix. The numbers give the shift and rotation by which the segments have moved relative to those of the reference monomer. (See ref. [15].)

How are these movements accomodated: why does not the close-packing of interfaces between packed helices hinder these displacements? We found that the shifts in packed helices are made possible by small changes in the torsional angles which allow the sidechains in the interface to shift. These changes in angles are usually quite small, not permitting the angles to flip from one local minimum to another. It is this condition of maintaining local minima that seems to impose a limit on the magnitude of the shift of one helix relative to another.

We might summarize the mechanism of conformational change in insulin by suggesting that shifts in packed helices are facilitated by small conformational changes within helix interfaces, which permit finite but limited displacements of the main-chain atoms. We call this the "helix interface shear" mechanism of conformational change, and suggest that the maximum displacement observed in most cases, 1.5 A, represents the limit of plastic deformation of the helix interface.

This mechanism has interesting general implications for the long-range transmission of conformational change. Consider two grossly idealized extremes: If proteins were infinitely "soft" any local conformational change would be dissipated in the immediate vicinity of the perturbation. If proteins were infinitely "hard" any local conformational change could cause only global movements of a rigid unit, and not allow deformations of the type required by "induced fit" of enzymes to substrates, or allosteric changes in tertiary structure. The observation that proteins have a potential for deformation, but only a limited one, shows how conformational change can be transmitted over long distances, and even amplified. In insulin, this mechanism of transmission of conformational change allowed us to understand the reason for the difference in conformation of Phe B25 between the two monomers in both 2Zn and 4Zn insulin. (See Figure 3.) In one molecule (the reference structure) the ring of Phe B25 packs into its own monomer; in the other the ring points out across the dimer interface to pack against the other monomer. We have shown that this occurs because of the pocket occupied by Phe B25 in the reference structure is deformed in the other monomer. This deformation is the result of the transmission of conformational change from a perturbation arising from crystal packing forces, initiated at a site 20 A away at the surface of the hexamer. (See ref. [15] for details.)

Closure of interdomain clefts: Citrate synthase

Many enzymes change conformation in response to the binding of substrates and cofactors. Often the active site occupies a cleft between two domains, and binding of cofactors is accompanied by the cleft's closing over the ligands. Functional reasons for this conformational change may include the necessity to orient catalytic groups around the substrate, the discrimination against unwanted competitive ligands, and the exclusion of water from the active site. (For reviews see references [16-18].)

An notable example of such closure of an interdomain cleft occurs in the enzyme citrate synthase, a large dimeric protein. The crystal structures of two forms of citrate synthase were determined by Remington, Wiegand & Huber [19]. The monomer (of about 440 residues), contains 20 helices that form a two-domain structure. (See Figure 4.)

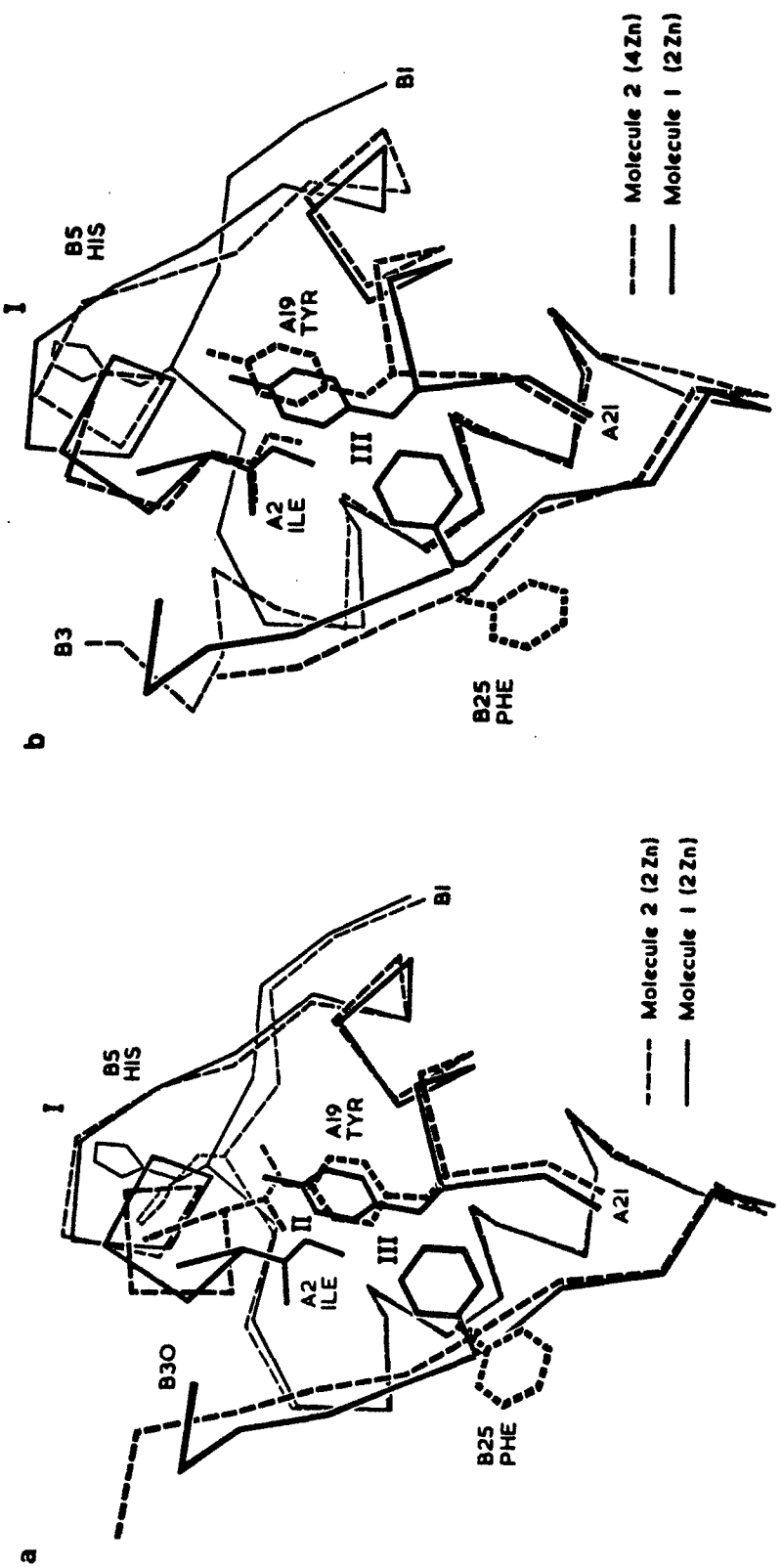


Figure 3. Superpositions of the reference monomer and the monomers of different conformation in 2Zn and 4Zn insulin. It is possible to trace the propagation of conformational change from a crystal packing interaction at the top of the picture (His B5 to Phe B25, 20 A away. (See ref. [15].)

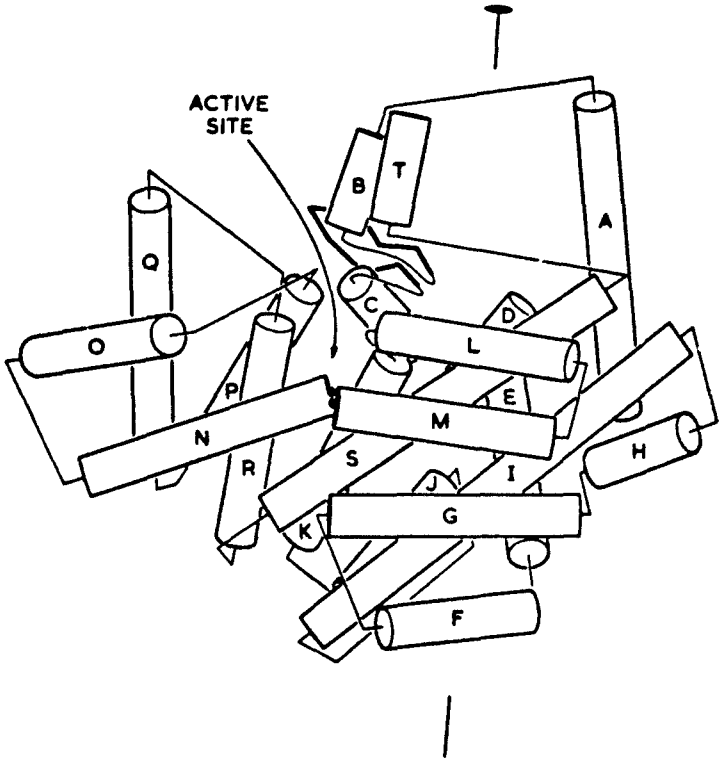


Figure 4. Citrate synthase. The monomer is shown, consisting of two domains with the active site between them. (See ref. [20].)

One crystal structure contains the unliganded form; in the other the protein binds the substrate, citrate, and coenyme A. In the unliganded form the cleft between the two domains is open, in the ligated form the molecule has changed conformation so as to bury almost completely the substrate and cofactor. Some atoms move as much as 10 A. A loop between two helices (O and P) moves by 6 A and rotates by 28 degrees to cover the ligands and form hydrogen bonds to them.

We were curious to look into the conformational change in citrate synthase because its extensive interdomain interface seemed incompatible with the idea of rigid body "hinge-bending" descriptions of relative movements of domains, derived from molecules such as immunoglobulins in which cases they are plausible because the domains are more tenuously connected, by flexible regions of chain. Our analysis of the two structures showed that there is considerable conformational change within the domains, to produce the observed closure of the cleft [20].

Seven helices of the large domain together have a very similar structure in both forms of the molecule. These form a semi-rigid kernel, which one may note contains only about one-third of the helices and is limited to one of the domains. The other thirteen helices reorganize their relative spatial disposition, both with respect to the set of seven and in most cases with respect to one another. At the domain interface, the relative movements of packed helices vary from 0.2 A displacement and 4 degrees of rotation, up to 1.8 A displacement and 11 degrees of rotation.

How then do the large motions required to close the interdomain cleft occur? We found that the set of individual displacements between packed helices is coupled to produce the large shifts as a cumulative effect. Within the limits of the data, the helix movements are consistent with the "helix interface shear" mechanism observed in insulin. The basic point is that during the conformational change there is a limit to the excursion of any pair of packed helices, so that large conformational changes must be built up from the cumulative effects of smaller ones.

We have examined the conformational changes in other proteins see what they add to these conclusions [20-21]. In alcohol dehydrogenase, hexokinase, and haemoglobin, relative helix movements play a role in the mechanism of the conformational change; however, in these cases the interface between the domains is relatively small, compared with citrate synthase, and the movements of the helices are smaller and the effects more localized. It would be interesting to study the analogous conformational changes in beta sheets but adequate data on an appropriate case are so far unavailable.

4. Conclusion

Recent developments in computational molecular biology suggest that it will be possible to build models of proteins from amino acid sequences, to a specifiable degree of accuracy, provided that the structure of another protein fairly closely related to the target molecule is available [10-11]. Other techniques are being developed to analyze unoccupied binding sites to design ligands that might fit into them [22-24]. Both of these are very important but difficult theoretical questions, and we all have a right to be pleased that some progress has been made towards solving them. My purpose today has been to describe certain conformational changes in proteins as seen in crystal structures, which are of course interesting phenomena in themselves, and to suggest that there are cases in which they introduce an extra dimension of complexity into the predictions we want to make.

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Structure and Function of Pneumolysin, the Thiol-Activated Toxin of Streptococcus pneumoniae

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INTRODUCTION

Streptococcus pneumoniae is an important agent of disease in man. The molecular basis of its virulence is unclear, although the capsule plays a key role. The pneumococcus elaborates several proteins, which although no definitive role in vivo has been ascribed to them, do have activities indicative of a role in disease. Many of these proteins interact with components of the host defences known to be important in pneumococcal disease. One such protein is pneumolysin.

Pneumolysin, the thiol-activated toxin of Streptococcus pneumoniae, is one of a family of toxins produced by four different genera of Gram positive bacteria (21). This family of toxins share a variety of physical and biological properties and exert their toxic effects via damage to eukaryotic membranes (3). A striking feature of this family is their pronounced immunological cross-reactivity such that sera raised against one member of this family generally reacts with and often neutralises and precipitates heterologous toxin (21).

These toxins undergo reversible oxidation and reduction (21). This was thought to reflect the formation and breakage of intra-molecular disulphide bridges with concommitant conformational changes in the protein (2). As well as an involvement in mediating such changes, a single sulphydryl was postulated to be essential for activity although its precise role was not defined (7). It has been postulated that the thiol-activated toxins utilise cholesterol as receptor since their cytolytic activity is only manifest on cells which have cholesterol as part of their membranes and because free cholesterol is a potent inhibitor of cytolytic activity (21,3). It has been suggested that the essential sulphydryl group may mediate (or is involved in) the interaction of the toxin and cholesterol (5). Once bound to target cells the toxin is thought to insert into the lipid bilayer and following lateral diffusion form aggregates of toxin molecules (5) which are probably cholesterol-free (2). These aggregates are visible as arc and ring shape structures and it is thought that they represent transmembrane channels through which cytoplasmic molecules can pass leading to cell lysis (4). The relevance of these structures in vivo during infection is unclear since it can be argued that the concentrations of these toxins reached in vivo are insufficient to permit their formation. However, it has long been known that sublytic concentrations of toxin have potent inhibitory effects on cells of the immune system and such effects might have profound consequences for pathogenesis of infection caused by their producing organisms. For example, sub-lethal amounts of

pneumolysin inhibit antibody synthesis by B cells and inhibit the key antimicrobial activities of polymorphonuclear leukocytes (PMNL) such as the respiratory burst (17,18). It is such cytotoxic activities which may be more relevant in vivo.

Complement plays an important role in protection against pneumococcal disease. Pneumolysin activates the classical pathway of complement in an antibody independent fashion (19). When this occurs in the fluid phase it has been proposed that this may consume complement thus diminishing its protective effects (2). Toxin-membrane complexes are potent activators of complement and this may result in assembly of the membrane attack complex on host membranes leading to autolysis (2,4). Each of these events may contribute to the induction of the inflammatory response and damage and contribute to the persistence of pathological signs beyond the time when organisms have been cleared from the body by antibiotic therapy.

To probe the structure and function of pneumolysin and to study its role in the pathogenesis of pneumococcal infection we recently reported the cloning (24) and complete sequence of the pneumolysin gene (24) and its manipulation to yield large amounts of recombinant toxin (15). Subsequently the sequence of three other thiol-activated toxins, Streptolysin O (12), Listeriolysin (14) and Perfringolysin (23) have been deduced. In this article we shall review progress made to date by comparing the available sequences but concentrate in particular our own studies on pneumolysin.

STRUCTURE OF THE THIOL-ACTIVATED TOXINS

Pneumolysin, unlike the other thiol-activated toxins, is not secreted from the producing organism (9) and as expected no signal sequence was found at the N-terminus of pneumolysin in contrast to the other three toxins so far analysed (24). Pneumolysin is 471 amino acids in length and comparison of this sequence with the low molecular weight forms of Streptolysin O, and the secreted version of perfringolysin and listeriolysin reveals that they all share remarkable primary amino acid sequence similarity (14,24,12,23). This accounts for their serological cross-reactivity and reflects conservation of overall structure required for activity.

Each of the toxins so far analysed contains a single cysteine residue towards the C terminus of the molecule. This observation precludes the possibility of intra-molecular disulphide bridge formation as the basis for reversible oxidation and reduction of these toxins. The single cysteine is presumably the essential cysteine residue defined previously. Interestingly, the single cysteine residue lies in an 11 amino acid motif which represents the largest contiguous region of identity between these toxins (Fig.1). When the sequences of the individual toxins are aligned to give maximal similarity, these eleven amino acids are also aligned.

Figure 1 The cysteine motif of the thiol-activated toxins

Pneumolysin ECTGLAWEWWR

The unique cysteine in pneumolysin is residue 428.

THE CYSTEINE MOTIF

We have termed the common 11 amino acid sequence, which contains the single cysteine and three tryptophan residues, the cysteine motif. To explore

the role of this motif in activity we have carried out an extensive analysis (Table 1) using oligonucleotide-mediated, site-directed mutagenesis of the pneumolysin genes to systematically change single residues in this region (20, unpublished data).

Table 1 Mutagenesis of the cysteine motif

Modification	Haemolytic activity	red cell binding	oligomer formation	cholesterol binding
Cys_{428}>ala	100	100	+	100
Cys_{428}>ser	15	ND	+	100
Cys_{428}>gly	1	100	+	100
Trp_{433}>Phe	0.6	ND	+	100
Glu_{434}>Asp	25	ND	+	50
Trp_{435}>Phe	13	ND	ND	ND
Trp_{436}>Phe	100	ND	ND	ND
recombinant	100	100	+	100

Apart from oligomer formation all activities are recorded as the percentage of the activity of wild type, recombinant pneumolysin. Wildtype recombinant pneumolysin and native toxin were indistinguishable in all assays used. ND denotes not determined.
Haemolytic activity was determined by standard methods (ref.24).
Red cell binding was measured by incubating cells with pneumolysin radiolabelled in vitro with ^{35}S and determining number of counts bound.
Oligomer formation was determined quantitatively by separation of toxin oligomers from red cell membranes using sucrose density centrifugation (ref.8) as described previously (ref.24).
Cholesterol binding. Toxin was incubated with radiolabelled cholesterol and toxin-cholesterol complexes separated by sucrose density centrifugation. Binding was measured by counting label associated with toxin.
Complement activation was measured by determination of C3b formation by immuno-electrophoresis as described previously (ref.20).

The Cys_{428}>Ala modified toxin was indistinguishable from native and recombinant, wild-type toxin in all parameters so far examined, including cytolytic and sublethal effects on PMNLs and the ability to activate complement (20). This clearly precludes an essential role for the cysteine residue and hence sulphydryl groups in pneumolysin activity in vitro. It remains possible that the sulphydryl group is important in vivo. In crude cell extracts of E. coli, the Cys_{428}>Ala modified toxin is not susceptible to oxidation and reduction, a situation which contrasts with the wild type recombinant toxin. We have observed that if highly purified pneumolysin loses its activity on storage, it is not possible to resurrect activity by the addition of reducing agents.

The nature of the amino acid at position 428 in pneumolysin is not unimportant since the Cys_{428}>Ser and Cys_{428}>Gly modified toxins had reduced cytolytic activity and this was mirrored by a reduced ability to inhibit the respiratory burst of phorbol ester-stimulated human PMNL (20). This may indicate that the events in cytolysis are fundamentally similar to those which occur when cells are treated with sublytic concentrations of the toxin.

Modification of the tryptophan residues led to an interesting observation (Table 1, unpublished). The Trp_{433}>Phe and Trp_{435}>Phe had reduced cytolytic activity whilst the Trp_{436}>Phe modified toxin was fully active. The Glu_{434}>Asp

modified toxin was also reduced in cytolytic activity. Thus despite their pronounced conservation, specific residues (including the cysteine) are not absolutely required for activity and also no one residue is of overriding importance but the overall structure of this cysteine motif may be important for cytolytic and cytotoxic activity.

Interestingly, all of the modified toxins which we have analysed bind red cell membranes and form oligomers in membranes as efficiently as the wild type toxin (Table 1). Although these assays were not strictly quantitative, we believe a twofold difference in binding and/or oligomer formation would have been detected. The reason why some of these modified toxins exhibit dramatically reduced cytolytic and cytotoxic activity remains unclear. Perhaps the nature of the transmembrane pores formed by these modified toxins are defective. This is presently under investigation.

Preliminary experiments using the Glu_{434}>Asp modified toxin showed that it may have a reduced capacity to bind cholesterol. It is thus tempting to speculate that the 11 amino acid cysteine motif is responsible (at least in part) for interaction with sterols. It is noteworthy that although the Glu_{434}>Asp mutant apparently exhibits reduced ability to bind free cholesterol we could detect no reduction in its ability to bind red cells. Thus we can speculate that the cysteine motif is involved in sterol interactions after initial binding to receptor via a toxin domain different from the cysteine motif (see below). The cysteine motif might mediate recognition of the cholesterol aliphatic side chain as part of the membrane insertion process and this interaction determines the structure of the pore.

The nature of the receptor-binding domain remains unclear. However, treatment of pneumolysin with diethyl pyrocarbonate (DEPC) effectively abolished the cytolytic activity of the toxin and its ability to bind red cells. It is therefore tempting to speculate that such chemical modification affects receptor binding by the toxin. Since DEPC primarily modifies histidine residues and given that each of the sequenced toxins share extensive homology and probably a common mode of action it is of interest that only a single histidine in each of the toxins, which is outside the cysteine motif, is aligned when the toxins sequenced are aligned for maximal sequence similarity (see Fig. 2). This raises the hypothesis that this histidine residue comprises part of the receptor binding domain of the protein, possibly mediating recognition and binding of the hydroxyl group of cholesterol. We have changed this histidine to arginine by site-directed mutagenesis and the modified toxin was devoid of cytolytic activity. We are presently purifying this (modified) toxin in order to carry out red cell and cholesterol binding assays.

Figure 2 The common histidine of the thiol-activated toxins

Pneumolysin	364 - L L D H S G A Y Y A Q - 374
Listeriolysin	420 - L I D H S G G Y V A Q - 430
Perfringolysin	345 - N L D H S G A Y V A Q - 405
Streptolysin O	469 - N L S H Q G A Y V A Q - 479

COMPLEMENT MUTATION

All of the mutants in the cysteine motif we have analysed activate complement as efficiently as the wild-type toxin regardless of their cytolytic activities. This implies that the cysteine motif is not the region of pneumolysin responsible for complement activation.

During the production of monoclonal antibodies to pneumolysin we noticed that the toxin bound efficiently the conjugated secondary antibody used in the ELISA employed for identification of hybridomas. We subsequently showed that the Fc fragment and not the Fab fragments of IgG bound to pneumolysin. This may account for the ability of pneumolysin (and perhaps the other thiol-activated toxins) to activate complement in the absence of anti-toxin antibodies.

We have attempted to localise the Fc binding domain of pneumolysin. A search of protein sequence data bases for sequences similar to pneumolysin reveals little of substantial similarity. The most similar protein was human C-reactive protein. This acute phase protein binds the C-polysaccharide of pneumococci which leads to activation of complement via the classical pathway (11). In addition, several groups have found that passively administered human CRP will protect mice from challenge with virulent pneumococci (8,16,25). The C-terminal third of human CRP has homology with pneumolysin but it is interesting to note that the homologous regions of pneumolysin are not contiguous (Fig. 3).

Figure 3 The CRP-like domains of pneumolysin

```
Domain 1
Human CRP      121  DFSGGNFEGSQSLVGDIGNVNMWDFVLSPDEINTIYLGGPFS  162
                    !* ***!!* * ! *** * !* *!* *!**** !!!  !
                    |  ||    |   |   |  |   |    |  |||  |
Pneumolysin    257  DEVEAAFEALIKGV-KVAPQTEWKQILDNTEVKAVILGGDPS  297

Domain 2
Human CRP      158  GGPFSPN-VLNWRALKYEVQG-EVFTKPQLW - 186
                    *!** **  **! *!*!* !! !! ! !* !
                    |   |    |  |  |  || || | |  |
Pneumolysin    368  SGAYVAQYYITWDELSYDHQGKEVLT-PKAW - 397
```

Fig. 3. Comparison of the amino acid sequences of pneumolysin and CRP. The sequences were aligned using the FASTP program of Lipman and Pearson (13). Numbers refer to amino acid residues in the respective proteins. ¦ and * denote identical or similar residues, respectively.

On the assumption that the common regions in pneumolysin and human CRP are those which mediate complement activation, perhaps via non-immune binding of antibody via Fc, we have carried out site directed mutagenesis of domain 2 in pneumolysin (Table 2). The three replacements made in domain 2 all affect the ability of pneumolysin to activate complement relative to the wildtype recombinant toxin. In the case of Tyr_{384}>Phe this reduced ability to activate complement is mirrored by a reduction in Fc binding. None of the modifications affect the cytolytic activity of the toxin. We propose that the region between residues 368-397 of pneumolysin is an Fc-binding domain, and that non-immune binding of antibody via Fc results in activation of the classical pathway of complement.

Table 2 Mutagenesis of the CRP-like domain 2 of pneumolysin

Modification	Haemolytic Activity (%)	Complement Activation (%)	Fc_γ binding (%)
Trp_{379}>Phe	100	55	ND
Tyr_{384}>Phe	100	14	25
Trp_{397}>Phe	100	80	ND
Recombinant	100	100	100

Haemolytic activity and complement activation were measured as described in Table 1. Fc_γ binding was measured by incubating toxin with purified Fc_γ and measuring Fc_γ bound by ELISA. Results are given as percentage activity of recombinant wildtype toxin. ND denotes not determined.

Whilst we have preliminary data to suggest that the CRP-like domain 2 of pneumolysin is an Fc binding domain we, as yet, have no evidence regarding the properties of domain 1.

This model also predicts that the domains in CRP similar to pneumolysin have the same properties.

IN VIVO ROLE OF PNEUMOLYSIN

A definitive role for pneumolysin in the pathogenesis of pneumococcal disease has not yet been established, although several lines of evidence point to an involvement of the toxin in pathogenesis. Firstly, patients and carriers of pneumococci have anti-pneumolysin antibodies and there is a rise in anti-pneumolysin antibodies during pneumococcal infection (22, unpublished data). Secondly, immunisation of mice with pneumolysin previously inactivated by oxidation, confers some protection to nasal challenge with virulent pneumococci (18). Mice immunised in this way succumb to the infection some 4-5 days after the non-immunised controls. The basis for this protection is not yet clear. Also mice immunised with the Cys_{428}>Gly mutant and challenged intra-nasally with virulent type II pneumococci survived for up to 5-9 days compared with 3-4 days for non-immunised animals (unpublished data). We are currently pursuing this study using other genetically engineered toxoids whose lack of activity permits immunisation with more protein.

The above observations suggest that pneumolysin might be a valuable addition to the existing pneumococcal capsular polysaccharide vaccine perhaps being exploited as a protein carrier for polysaccharide. Pneumolysin is produced by all serotypes of the pneumococcus and alone confers limited protection in a serotype independent manner (8). Conjugated to the existing 23-valent polysaccharide vaccine, the toxin might induce B cell memory to polysaccharide antigen and improve the efficacy of the vaccine in the very young as well as eliciting neutralising toxin antibodies which themselves might be partially protective. Recombinant pneumolysin is the most logical source of the toxin for this purpose since it is available in very large amounts via high level expression in E. coli (15). In addition, genetically engineered toxoids are already available (see above). We have recently constructed a triple mutant with dramatically reduced cytolytic and complement activating activity. Further mutagenesis to abolish biological activity yet retain native conformation and hence immunogenicity is the next phase of these studies. Such modified toxins would form the logical carrier in a pneumolysin-polysaccharide conjugate vaccine.

To explore the basis of the partial protective effect of immunisation with pneumolysin, we performed a time course experiment following intra-nasal inoculation with virulent type III pneumococci and monitoring appearance of bacteria in various organs. Our preliminary results showed that for about 40 hours post challenge pneumococci are found only in the lungs. The challenged animals were healthy and the lungs showed no obvious sign of consolidation. At 44 hrs post infection, bacteria appear in the blood, spleen, liver and brain and following a period of rapid pneumococcal growth the animals die within 6-10 hours. The events which result in translocation of pneumococci from the lung and their explosive growth in other organs are now a topic for detailed study. It will be interesting if immunisation with pneumolysin extends the interval of apparent disease-free colonisation of the lung.

In collaboration with Dr Rob Wilson (Brompton Hospital, London) we have demonstrated that pneumolysin is a potent inhibitor of cilia beating in in vitro organ cultures of ciliated human respiratory tract epithelia (22). Treatment of normal epithelia with pneumolysin resulted in disruption of cilia beat frequency and extrusion of cilia and membrane blebbing from the epithelial cells. This is not a consequence of disruption of cilial structure since in cross-section the cilia have their normal appearance. When pneumolysin alone was instilled into a partially ligated lobe of a rat lung, the lobe had the classical histological appearance of bronchopneumonia, essentially identical to the situation when virulent pneumococci were inoculated into similarly ligated lobes.

Thus pneumolysin may act to compromise the normal, non-specific defences in the lung allowing access of pneumococci to alveoli. In this location pneumolysin may interfere with the antimicrobial activity of resident macrophages and abrogate the protective effects of PMNL infiltration, complement and CRP. In addition, pneumolysin, as well as bacterial cell wall components of the pneumococcus, might contribute directly to bronchopneumonia.

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Structure and Conformational Changes in Cholera Toxin and E. coli LT.

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INTRODUCTION

Cholera toxin (CT) is an enterotoxin secreted by Vibrio cholerae producing its pathological effects by increasing the c-AMP level in intestinal epithelial cells (1,2). It is an oligomeric protein (Mr ∿ 84,000) composed of two structural and functional distinct subunits CT A and CT B (Mr ∿ 29,000 and 55,000 respectively). CT B contains five identical polypeptide chains (Mr = 11,600), most likely arranged in a ring-like pentameric configuration and CT A consists of two non-identical polypeptide chains A_1 or α-chain (Mr = 23,000) and A_2 or γ-chain (Mr = 5,500) linked by a single disulfide bridge (for reviews see refs. 1, 3-5). CT A is synthesized as a single polypeptide chain which is "nicked" between two cysteine residues by an extracellular bacterial protease. During this proteolysis two serine residues are removed at the C terminus of A_1 (6). The subunits are arranged such that CT A occupies the central channel of the CT B pentamer extending well above the plane of the pentameric ring (7,8).
CT action is initiated by rapid binding to the outer cell membrane through interaction between CT B and the monosialoganglioside GM_1 followed by entry of polypeptide A_1 into the cell where it is able to stimulate adenylate cyclase by catalyzing the ADP-ribosylation of the $G_{s\alpha}$ subunit of the stimulatory GTP binding regulatory protein (for reviews, see 3-5).
Chemical, immunochemical and spectroscopic studies have implicated various amino acid residues as critical to the initial highly specific interaction of CT B with the carbohydrate moiety of GM_1 (9-12). More specifically the lone Trp 88 residue (10,12) of each β-polypeptide chain of CT B and one or more lysyl residues (9,10,12) appear to be important for the recognition of the negatively charged receptor. Because the fluorescence properties of CT B are related to the Trp 88 residues more information concerning the microenvironment of these residues and thus the binding sites for GM_1, could be obtained by fluorimetric titration- (13) and quenching experiments (14).
These experiments have further demonstrated that : (i) the intramolecular disulfide bridge of each β-chain is vital for the exact conformation of the binding site; (ii) the Trp 88 residue of each β-chain of CT B appears to be located in a cleft which disappears following dissociation of CT B into its constituent monomers; (iii) receptor binding stabilizes the pentameric structure of CT B.
The latter observations were interpreted in the light of our previous proposal (10) that binding sites for GM_1 are located at the interfaces of ad-

jacent β-chains of the CT B pentamer and that binding requires the participation of amino acid residues from adjoining chains. In order to further test this hypothesis we constructed hybrid CT B preparations from inactive chemically modified parental β-chains.

MATERIALS AND METHODS

CT was obtained from List Biological Laboratories Inc. (Campbell, CA).
CT B was prepared as previously described (15). GM_1 was isolated from bovine brain according to the procedure of Iwanori et al. (16). The oligosaccharide moiety of GM_1 was obtained from an ozonolysis reaction followed by (i) an alkaline fragmentation procedure and (ii) chromatography on Dowex 1-X8 (15).
Formylation of tryptophanyl residues of CT B was performed with HCl-saturated formic acid according to Previero et al. (17). Reversible acylation of lysyl residues with citraconic anhydride was performed in 0.2M borate buffer pH 8.2 at 0°C by the addition of 100-fold molar excess of anhydride over amino groups (18). Irreversible acylation was conducted in 0.2M borate buffer pH 8.2 at 0°C by the addition of 100-fold molar excess of solid succinic anhydride over amino groups (19). Free amino groups were determined by the TNBS assay (20).

RESULTS AND DISCUSSION

Preparation of chemically inactivated derivatives of CT B.

Because of the critical involvement of Trp 88 and lysyl residues in receptor recognition, chemical derivatization of these residues was chosen to prepare inactive derivatives of CT B. Several methods can be applied to modify tryptophanyl residues in CT B. Nitrophenylsulfenylation, dinitrophenylsulfenylation, formylation and oxyindole formation cause a complete loss of binding activity (11,12). Modification of tryptophanyl residues in CT B, depending on the nature of reagent and modification conditions, frequently leads to a loss of pentameric structure. More specifically, modifications destroying the indolic character e.g. by oxyindole formation or modification by nitrophenylsulfenylation, reducing the electron density and causing steric hindrance, indeed result in a loss of pentameric structure. After formylation of Trp 88 the quaternary structure of CT B is preserved as judged by gelfiltration on a Bio-Gel P-60 column and therefore this modification procedure constitutes a suitable method for the preparation of inactive CT B derivatives which can be used for the preparation of hybrid pentamers.
Upon modification of lysyl residues receptor binding is only dramatically impaired when the modification involves a conversion of lysyl residues to anionic derivatives (12). Each β-chain of CT B contains 9 lysyl residues and therefore succinylation or citraconylation of these residues is associated with a major change in charge which might prevent the proper association of monomers after exposure to the denaturating conditions essential for the construction of hybrid CT B from chemically inactivated derivatives. Exposure of fully succinylated CT B to 6M guanidine HCl makes it indeed unable to form the pentamer after removal of the denaturant. Therefore an attempt was made to selectively modify lysyl residues critically involved in receptor recognition. The experimental approach consisted of first blocking through the reversible acylation lysyl residues accessible

in a CT B.GM_1 (oligo-GM_1) complex followed by irreversible modification by succinylation of lysyl residues exposed after removal of the ligand and finally removal of the blocking groups. After each step the number of remaining amino groups was estimated by the TNBS assay (20).
After citraconylation of CT B in the presence of GM_1 (oligo-GM_1) and subsequent removal of the ligand three amino groups are unmodified and the partially citraconylated material is still able to bind GM_1. Further irreversible acylation of these ligand protected amino groups almost completely blocks binding. Decitraconylation of this double modified CT B does not restore binding.
In agreement with the number of GM_1 protected amino groups the TNBS assay demonstrates that three amino groups are modified in site specific succinylated CT B (sss CT B). Exposure of sss CT B to 6M guanidine HCl does not, in contrast with the fully succinylated derivative, affect its ability to form the pentamer.

Formation of hybrid CT B from chemically inactivated derivatives.

To test the hypothesis that binding sites of CT B are not contained within a single polypeptide chain but rather shared between B-chains formylated CT B (f CT B) and sss CT B were mixed under conditions promoting random interchange of B-chains. To this end equal amounts of f CT B and sss CT B were incubated in 0.1M glycine/HCl buffer pH 3.2 containing 6M urea and 0.2M NaCl for 24 h at 4°C. Subsequently the denaturant was removed by stepwise dialysis against Tris-acetate buffers pH 7.5 containing decreasing amounts of urea. Assuming a random reassociation of the two chemically inactivated CT B preparations and further assuming that Trp 88 and the essential amino groups are located on adjacent B-chains 25 percent of the reconstituted binding sites should bind GM_1 (Fig. 1). As shown in Fig. 2 and Table I application of this reconstitution procedure to the inactive derivatives of CT B leads to a marked increase in GM_1 binding as evidenced by the induction of a blue shift in the fluorescence emission spectrum of the hybrid CT B preparation and the ability of hybrid CT B to compete with GM_1 binding of native CT in a solid phase radio binding assay.
When the inactive derivatives are each separately exposed to the acid-urea conditions necessary to resolve B-chains and subsequently subjected to the renaturation procedure no restoration of binding is observed (Table I). f CT B as expected does not show any significant fluorescence but is clearly unable to compete with CT for GM_1 binding (Fig. 2, Table I).
A possibility to be considered is that the construction of hybrids leads to partial reactivation of binding sites through a mechanism of conformational correction. This mechanism, however, implies that the modified amino acids are not essential for binding, which is highly unlikely in view of many experimental data (10-12). Furthermore, as indicated in Table I, neither reactivation of formylated CT B nor sss CT B occurs when they are incorporated into hybrids with native B-chains. The binding activity of hybrid CT B is approximately 25 percent of that of native CT B in agreement with a random association of B-chains and the formation of one or two active binding sites per CT B pentamer. Assuming a binomial distribution 1/16 of the reconstituted pentamers have a composition similar to that of the parental compounds, 10/16 have one binding site and 5/16 have two active binding sites.
A further implication of the successful reconstitution of binding sites is that when all three amino groups located in or near the receptor binding site are equally important for binding they should be located on one side

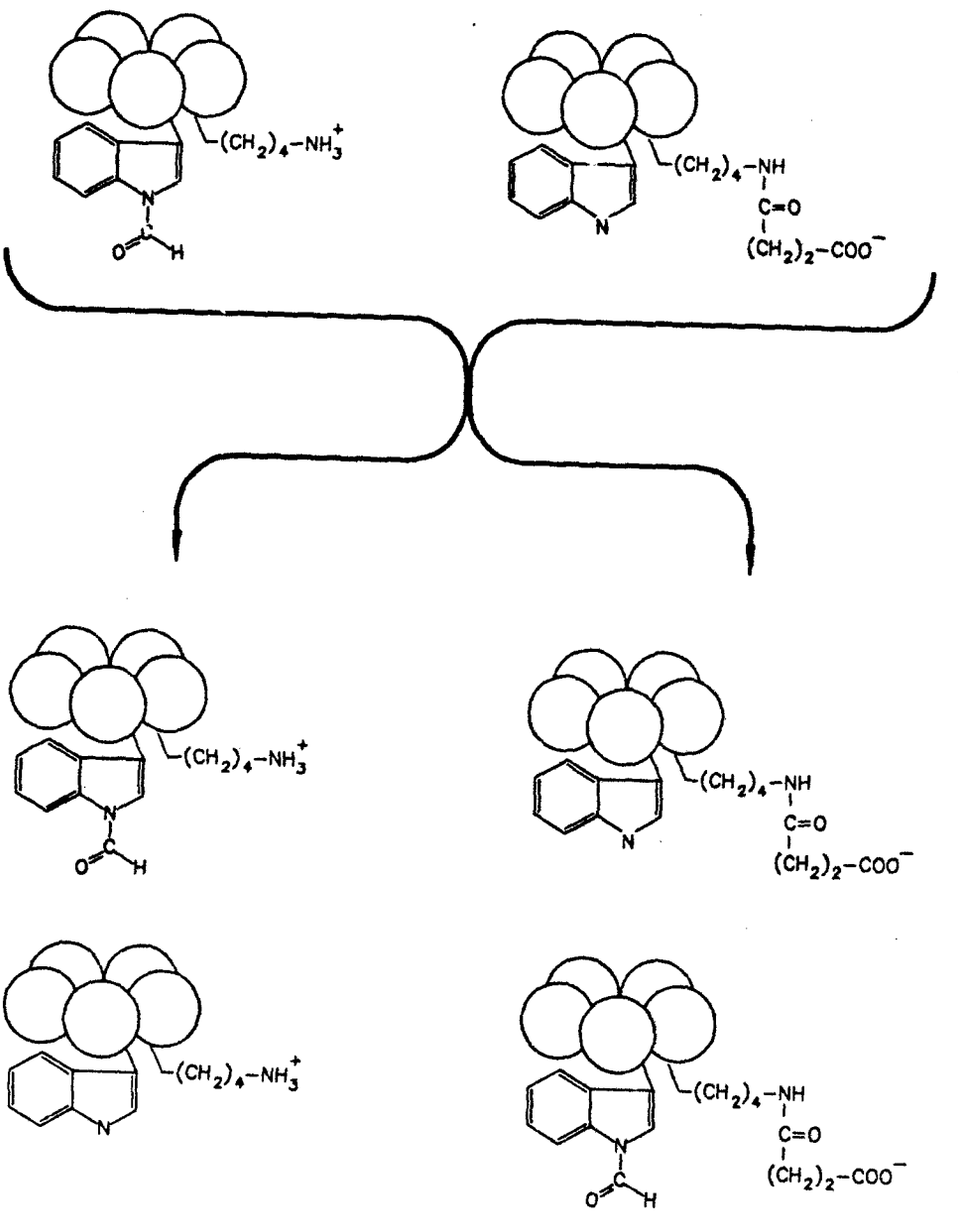


Fig. 1. Schematic representation of the reconstitution experiment used to test the hypothesis that binding sites of CT B are shared by adjacent monomers. Equal amounts of Trp modified CT B and lysine modified CT B are mixed under conditions promoting random interchain association. According to this hypothesis 25 percent of the reconstituted binding sites should be active.

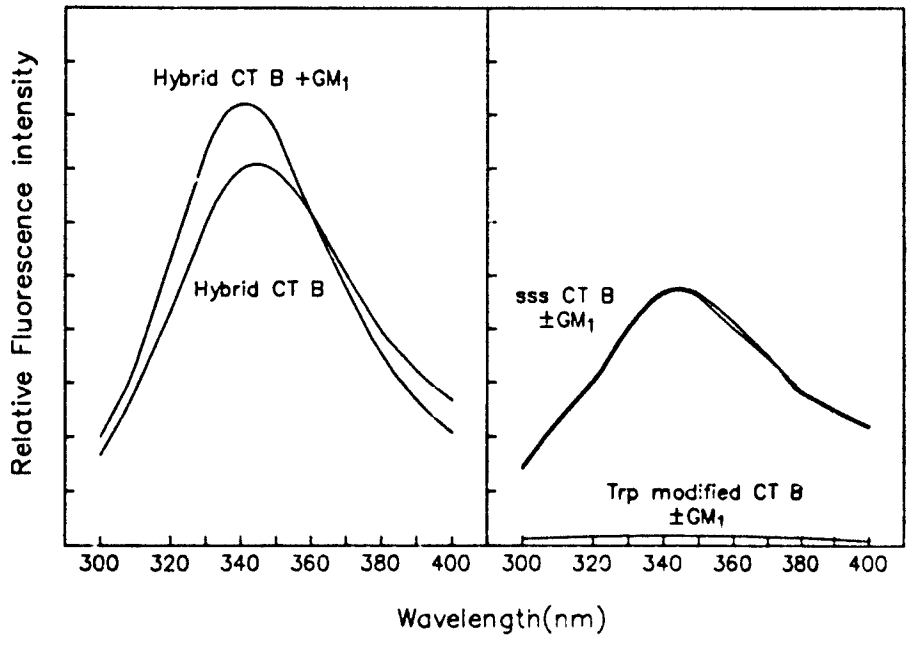


Fig. 2. Effect of GM_1 on the fluorescence emission spectra of sss CT B, Trp modified CT B and hybrid CT B.

Table I. Receptor binding activity of CT B hybrids.

Species	Receptor binding activity (%)
f CT B*	< 0.0001
sss CT B*	0.001
hybrids from f CT B and sss CT B	24
hybrids from f CT B and native CT B	49
hybrids from sss CT B and native CT B	50

Receptor binding activity is defined as the ratio of IC_{50} (native CT B) to IC_{50} (hybrid CT B) multiplied by 100 %, where IC_{50} is the concentration of competitor which produces a 50 % reduction of ^{125}I-CT bound to GM_1 in a solid phase competitive radiobinding assay.

* f CT B and sss CT B were also taken through the denaturation and renaturation cycle used for the preparation of hybrids.

of the interface opposite of the Trp 88 containing side or more likely these amino groups are distributed over both sides of the interface but only those (one or two) located at the opposite side of Trp 88 are vital for GM_1 binding.
End group analysis using dansylchloride reveals that the α-amino group of the N-terminal threonyl residue constitutes one of the modified amino groups in sss CT B. Selective modification of this residue by transamination with glyoxylic, however, does not significantly affect GM_1 binding. Therefore this amino function, probably located in or near the receptor binding site, appears not to be essential for binding.
The ability of oligo-GM_1 to reverse the quenching of the fluorescence of hybrid CT B by potassium iodide was used to determine the number of active binding sites in the hybrid CT B preparation in a more direct way. Upon fluorimetric titration with oligo-GM_1 maximal reversal of iodide quenching of CT B and hybrid CT B occurred respectively at molar ratios of oligo-GM_1 to protein of 5.1 and 1.3 which is again in very good agreement with the view that 25 percent of the reconstituted binding sites is active.
The presence of shared binding sites on CT B provides a structural basis for the observation that complexation of CT B with GM_1 prevents the low pH induced dissociation of CT B (12) and markedly enhances the thermal stability of CT B (21). The stabilizing effect of GM_1 (oligo-GM_1) on the quaternary structure of CT B might offer an explanation for the observation that although a rapid internalization of CT occurs (22), ^{125}I-CT remains persistently (several days) bound to human fibroblasts and is degraded slowly (23). since during this time the toxin is probably recycled back to the cell surface, the stabilizing effect of GM_1 might protect against dissociation and loss of binding at the acidic pH of intracellular compartments such as endosomes and lysosomes. It is therefore not excluded that this persistent binding of CT enables CT A_1 to penetrate into the cell at both the level of the cell surface and the level of the membranes of the vacuolar apparatus, which is in accordance with the partial protective effects of lysosomotropic amines (24,25).
Finally, hybrid CT B pentamers having one or two functional binding sites might be useful to further address the question whether multivalent binding of CT is essential for expression of toxic activity and more specifically the role of this multivalent binding in the translocation of the A_1 polypeptide chain.

Formation of hybrid LT B from chemically inactivated derivatives.

E. coli LT resembles CT not only functionally but structurally and immunologically as well (26). The low pH induced dissociation of LT B into its constituent monomers is also hindered by the addition of GM_1 or oligo-GM_1. This observation prompted us to investigate, using an experimental approach similar to that described for CT, whether receptor binding sites on LT are also located at the interfaces of LT β-chains. Preliminary data show that the lone Trp 88 and one or two lysyl residues of each β-chain of LT B are again essential for receptor binding activity and that construction of hybrids from Trp modified LT B and sss LT B results in a marked enhancement of binding. These results therefore suggest that LT receptor binding sites are also shared between β-chains.

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Molecular Characterization of HlyA and HpmA, the Prototypes for Two Gram Negative Hemolysin Gene Families

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INTRODUCTION

Hemolysins are undergoing increased scrutiny as a class of cytotoxins that have significant pathogenic effects on nucleated cells of different hosts (4, 5,14). The gram positive thiol-activated hemolysins and the Escherichia coli hemolysin (HlyA)-Pasteurella haemolytica leukotoxin (LktA) gene family represent two of the best examples of virulence determinants which arose from unknown primordial sources that are now horizontally disseminated among a broad array of pathogens (10, 15, 16, 21). In this paper we will summarize some of our recent data involving the structure and function of HlyA as well as provide a preliminary account of our discovery of a second gram negative hemolysin gene family. We have found that on the basis of significant amino acid sequence similarity, the calcium independent hemolysin of Proteus mirabilis, HpmA is evolutionarily related to the Serratia marcescens hemolysin (ShlA).

MATERIALS AND METHODS

Escherichia coli DH1 was the common background strain used for the study of hemolysin recombinant plasmids and their products. The construction of the HlyA encoding recombinant pWAM04 was previously described (27). The Proteus mirabilis derived hemolysin, HpmA was produced by pWPM100 (25). The different in vitro methods used to construct mutant strains are standard except for the isolation of a deletion mutation in the hpmA gene of Proteus mirabilis BA6163. The hpmA mutant was generated by substituting a kanamycin resistance gene cassette for ClaI fragments totaling 1689 basepairs within hpmA, using that construction for cloning in a pir-dependent suicide vector system of Peterson and Mekalanos (personal communication)

and finally selecting for the hpmA mutation via allelic replacement in Proteus transconjugants.

Rabbit polyclonal antiserum against HlyA was raised as described earlier (27). The isolation, production and epitope mapping of murine monoclonal antibodies (MAbs) against HlyA will be described elsewhere (Welch et al, manuscript submitted).

The determination of the DNA sequence of hpmB and hpmA will be presented elsewhere (Uphoff and Welch, manuscript submitted). The computer assisted analysis of gene and gene product sequences was performed using the UWGCG software package.

RESULTS AND DISCUSSION

HlyA structure and function. We have used a variety of approaches to characterize the salient features of the E. coli HlyA protein. The computer assisted analysis of the predicted HlyA amino acid sequence results in the identification of a number of structural features such as several large hydrophobic domains and the presence of a tandem array of glycine-rich repeats (10, 26). A review of those physical attributes along with some additional primary structure characteristics are presented in Figure 1 A-H. Since the publication of the E.coli hly gene sequences (9) several new algorithms for predicting secondary and tertiary structure have been developed. One in particular is the algorithm of Emini et al (6) which predicts the probability of surface exposure of a protein region. Such analysis of HlyA provides for some interesting predictions which are shown in Figure 1-C. The potential surface domains in their decreasing order of probability are: aa 507-515 > 553-561 > 672-690 > 69-84 = 426-437 = 457-470 = 607-621 > 161-170 > 115-127 = 327-344. A surprise is that this algorithm results in the prediction that most of the glycine rich repeat area involved in Ca^{2+} binding [aa 721-849] is unlikely to be on the HlyA surface.

Dramatic amino acid substitutions or small deletions in a putative transmembrane domain [#5, aa 303-326, see Figure 1-B] result in a hemolytically inactive forms of HlyA (18). A threonine to proline substitution at HlyA aa# 2 results in increased hemolytic activity (18). That mutation along with the prediction that the HlyA amino terminus can form an amphiphilic helix similar to a typical mitochondrial protein targeting sequence suggests that the amino-terminal region of HlyA may be involved in initiating the insertion of HlyA into host cell membranes (7, 12). Our laboratory has recently constructed a TthIII-1 - TaqI subclone of hlyA [see figure 1-I]. The insert starts at an unique TthIII-1 site corresponding to HlyA aa 66 and then encodes the remainder of HlyA past the HlyA carboxyl terminus through to a TaqI site at the proximal end of hlyB. When this subclone is put in trans to a second plasmid encoding hly C, B and D, the recombinant cells secrete extracellularly a 95 kDa polypeptide which has approximately 10% of the hemolytic activity of wild type HlyA encoded by pWAM04 (Pellett and Welch, in preparation). This result suggests that although the HlyA amino terminal sixty six amino acids may play a role in facilitating HlyA cytolytic activity, that region of HlyA is not required for intact function.

Ludwig et al isolated a HlyA carboxyl terminal 37 amino acid deletion which retained hemolytic activity (18). Felmlee and Welch isolated a HlyA frame shift mutation lacking the 197 carboxyl terminal amino acids of HlyA which also retained hemolytic activity [see figure 1-J] (10). Together with the data discussed above, it is apparent that neither of the HlyA termini are required and that a substantial portion of HlyA can be deleted without loss of complete activity.

HlyA-mediated cytolysis is known to be calcium dependent (3). The discovery of the glycine rich repeats from aa 721-849 led to the early speculation that they may function

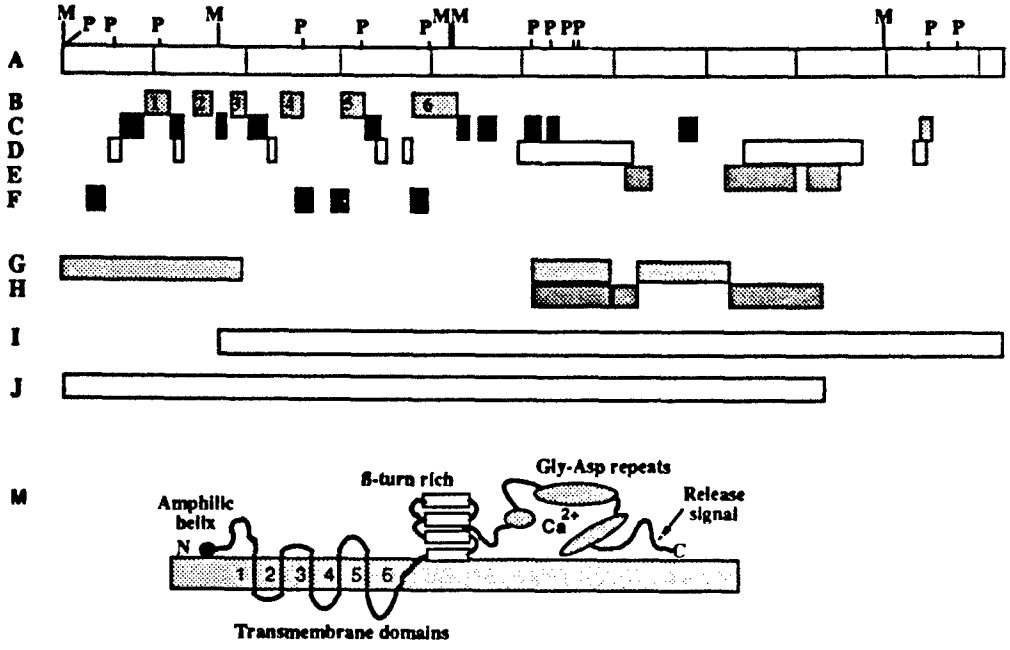


Figure 1. HlyA Structure and Function. Line A shows the length of HlyA [1023 aa] in 100 aa blocks marked by the vertical dashed lines. The marks labelled with M [methionine] or P [proline] show the location of those amino acids within HlyA. Line B shows the location of predicted six transmembrane domains [aa 92-114, 145-160, 180-198, 237-259, 303-326 and 380-410]. Line C shows the location of the eleven most likely surface domains which are described and ranked in the text. Line D shows the areas where ß -turns are likely to occur in HlyA. Line E indicates the location [aa 614-649, 721-801 and 814-849] of three groups of tandem arrays of the LXLLXGNDX repeats. Line F shows the presence of four copies [aa 29-42, 260-273, 295-308 and 381-394] of the ASAAAGAVAAAXG repeat originally recognized in the Bordetella pertussis adenyl cyclase/hemolysin by Glaser et al (11). Lines G and H indicate the general location of epitopes for different anti HlyA MAbs. Line G indicates the three regions where neutralizing MAbs recognize HlyA. Line H indicates the location for the epitopes of seven nonneutralizing MAbs. Line I indicates the encoding region for the hemolytically active 95 kDa HlyA amino-terminal truncate produced by a TthIII1-Taq1 subclone described in the text. Line J shows the encoding region for the hlyA BglII frame shift mutant that produces a surface bound, hemolytically active form of HlyA (10). At the very bottom of the figure (K) is a model for the structure and topology of HlyA inserted in a target membrane. Shown are the six, numbered potential membrane spanning regions; the proline-rich, ß-turn domain [aa 490-580]; the 626-721 domain where the epitope for a completely neutralizing MAb resides; the glycine-rich, ß-turn rich, Ca^{2+} binding domain and lastly, the carboxyl-terminal extracellular release signal (19).

in HlyA binding of Ca^{2+} (26). The repeats are known to be required for detectable hemolytic activity (10). The recent construction of HlyA mutations lacking integral numbers of the repeats resulted in mutants that are dependent on increased concentrations of Ca^{2+} for hemolytic activity (17). Recently one of us has found HlyA which is separated by SDS-polyacrylamide gel electrophoresis can be electrophoretically transferred to nitrocellulose where it is now capable of binding $^{45}Ca^{2+}$ (Boehm et al, manuscript in preparation). Therefore, we believe a direct analysis of the role that the repeats play in Ca^{2+} binding can be studied.

The extracellular secretion of HlyA is a fascinating and complicated event. Advances have been made in the identification of the HlyA intragenic targeting signal(s) for secretion across the E.coli envelope into the media. The export of HlyA clearly does not involve cleavage of an amino terminal leader peptide (8). Mackman et al demonstrated that a signal for release of HlyA from the cell surface resides in the carboxyl-terminal 23 kDa region (19). Deletion of 11 of the 13 repeats in the aa 721-849 region causes a two to three fold reduction in the rate at which HlyA is exported via an apparent nonperiplasmic route of secretion (10).

Two laboratories have reported the isolation of anti HlyA MAbs where in one instance the MAbs neutralized hemolytic activity (24) and in the second, no neutralizing MAbs were found (13). In neither instance were the HlyA epitopes for the MAbs identified. We have recently isolated a panel of 12 MAbs to HlyA (Welch et al, submitted). The MAbs were used in immunoblots containing various HlyA mutants and cyanogen bromide cleavage fragments of HlyA. The immunoblotting reactivity enabled us to map the MAb epitopes to contiguous regions of HlyA ranging in size from 28 to 103 amino acids. In addition, 5 of the 12 MAbs either completely or partially neutralize hemolytic activity. A low resolution map of the location of those five MAb HlyA epitopes is reviewed in Figure 1-G. Three of the MAbs have epitopes within the first 194 amino acids and these MAbs only partially neutralize hemolytic activity. This is consistent with the mutational results described above that indicate the amino terminal region of HlyA may facilitate hemolytic activity. The amino terminal region is likely to aid the formation of a competent HlyA conformation made up of a core functional sequence from approximately aa 100 through to 829. One of the MAbs has as potent a neutralization ability as our anti HlyA polyclonal antiserum. The epitope for this MAb lies between aa 626 and 726 which suggests that a previously unrecognized functional domain exists here. Likely candidates for such additional functional domains are the site of the cation selective pore (1, 20) and the site of HlyC modification (22).

We have devised a model for the structure of HlyA as it is associated with a target cell membrane which is based on the data and predictions described above. That model is shown at the bottom of Figure 1. At this time we do not know the inside-outside orientation of HlyA with the membrane. We predict that there are at least six transmembrane domains. Brandl and Deber hypothesize that proline residues, which are commonly found in the transmembrane region of transport proteins but are only rarely observed in the transmembrane region of nontransport proteins, participate in the cis-trans isomerization of X-pro bond(s). The isomerization results in conformational changes necessary for ion transport or alternatively this leads to the creation of X-pro carbonyl groups within an intramembranous domain which can act as cation ligand sites (2). There are proline residues in three of the putative HlyA transmembrane domains [# 1, 4 and 6, Figure 1-B, K]. Therefore it may be speculated that the #4 and #6 domains comprise the HlyA cation selective channel and that perhaps HlyC is involved in proline modifications at those sites. Downstream from the transmembrane domains are two separate domains which are rich in potential ß - turns. The region from aa 490-580 harbors two of the most likely surface areas predicted from the Emini algorithm [see Figure 1-C]. The epitope for one of the neutralizing MAbs lies within this region. The second ß - turn rich region encompasses the glycine-aspartate repeat region which is

likely to be responsible for HlyA calcium ion binding. It is important to point out that there are three additional copies of the repeats in this area of the HlyA sequence which were previously unrecognized. They occur tandemly beginning at aa 614 and continue through to aa 626.

The core HlyA structure necessary for hemolytic activity based on our mutant analysis would therefore comprise transmembrane domains 3 through 6, the first ß-turn rich domain, the surface domain present within aa 626-721 and the second ß-turn rich domain made up of the glycine-aspartate repeats.

Discovery of a second gram negative hemolysin gene family. An epidemiological survey of different bacterial species producing HlyA-like polypeptides revealed that Proteus vulgaris isolates produce a second, unrelated hemolysin [HpmA] which has calcium-independent activity (25). We find by DNA-DNA hybridization that hpmA-like sequences are present in all of our P. mirabilis isolates [n=65] and in the majority of P. vulgaris strains [n=19] present in our collection (Swihart and Welch, in preparation). This stands in clear contrast to the case for hlyA-like sequences among Proteus species where we find that only rare isolates of P. vulgaris unambiguously possess hlyA and its linked accessory hlyB and hlyD sequences. In terms of the dissemination of hpmA, we do not detect any related sequences among other members of the Enterobacteriaceae using an internal hpmA sequence as a hybridization probe (25). However, as we will describe below any conclusions based on those negative results are premature.

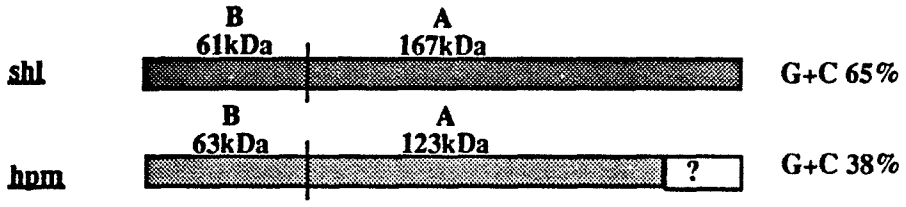
Inorder to examine the relative contribution of HpmA to the hemolytic activity of Proteus mirabilis and to test the role of HpmA in different animal models of Proteus disease, we isolated a P.mirabilis mutant with a 1689 basepair internal deletion of the chromosomal hpmA sequence (Swihart and Welch, in preparation). The mutant produces no detectable extracellular or cell-associated hemolytic activity when late log phase cultures are tested in a quantitative liquid hemolytic assay. Therefore we conclude that even if we are failing to detect hlyA-like sequences in this strain of P. mirabilis because of prohibitive sequence divergence, HpmA alone appears to be responsible for detectable hemolytic activity.

The original HpmA-encoding recombinant plasmid, pWPM100 contained an 5395 base pair XhoI fragment insert (25). The early mini cell analysis of pWPM100-encoded polypeptides indicated that HpmA is 140 kDa in molecular size and apparently is the only nonvector product of pWPM100 (25). We have recently completed analysis of the DNA sequence of the XhoI insert (Uphoff and Welch, manuscript submitted). An open reading frame capable of encoding HpmA is present, however its 3' end stops at one of the XhoI insert sites. At the 5' end of the HpmA sequence there appears to be a typical leader peptide for a secreted protein which suggests that HpmA is secreted extracellularly by a non-HlyA mechanism probably utilizing the common sec gene products. We see within the DNA sequence upstream of hpmA, an open reading for polypeptide with a predicted molecular size of 63 kDa [HpmB]. It seems likely that if HpmB is in fact encoded within this open reading frame it was previously undetected in the mini cell analysis because ^{35}S- methionine was used as the radiolabel and aside from the methionine at the putative start site of this product, there are no other methionines within its amino acid sequence. Compounding the problem is the likelihood that HpmB is also a secreted protein because it possesses a match to the consensus for a leader peptide and its processing site.

We have performed a search of the international data bases containing different DNA and protein sequences for sequence similarity matches for hpmA and hpmB and their products. We have not detected any significant similarities to any entries in those data bases. However, because the hpm size and gene arrangement is reminiscent of the shlA and shlB hemolysin genes of Serratia marcescens (23), a comparison of HpmA and HpmB sequences to the shl products was performed. Despite the fact that the respective

genes have quite different guanine plus cytosine content there is just over 50% identity in their gene and amino acid sequences. A summary of the Hpm and Shl comparisons are shown in figure 2. Therefore our earlier attempts at detecting hpmA-like sequences in Serratia were thwarted because the gene sequence divergence was great enough to make probe hybridization impossible. We are presently examining whether we can detect HpmA-like antigenic determinants among other bacterial species

Comparison of Proteus mirabilis and Serratia marcescens hemolysins



	% Identity
hpm vs shl	54 %
HpmA vs ShlA	50.4 %
HpmB vs ShlB	55.4 %

Poole and collaborators demonstrated that ShlB acts as an activator of ShlA (23). We have made a similar observation where we find that HpmB activates HpmA. This further establishes the pattern initially observed with HlyC and HlyA where hemolysin structural genes are often found closely linked to genes whose products activate the cytolytic toxins by an unknown mechanism(s).

ShlA appears to be in the class of pore forming cytolytic toxins similar to HlyA (1, 20). As of yet we do not have any information regarding the functional consequences of HpmA attack on erythrocytes. The different sequence algorithms applicable to the structure of HpmA do not reveal any striking differences to those projected for ShlA with the exception that HpmA is predicted to have a pI significantly more acidic than ShlA.

In conclusion, we have used a variety of approaches to develop a relatively detailed model for the structure and function of HlyA. HlyA is the prototype determinant for a hemolysin-leukotoxin gene family that is found in a wide variety of gram negative animal and human pathogens. In the future, we will apply similar strategies to the analysis of HpmA which may be found disseminated in genera other than Proteus and Serratia. This seems a likely prospect given the strong Hpm and Shl sequence conservation but wide divergence in their guanine plus cytosine content.

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Molecular Studies of Transport of Hemolysin in E. coli

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ABSTRACT

Hemolysin is transported out of the E. coli cell using a specialized export apparatus consisting of HlyB and HlyD and topogenic signals at the C-terminus of the protein itself. Signal sequences in HlyA were delineated using a combined genetic and immunological approach to construct a nested set of N-terminal truncated mutant proteins. Thus it was possible to functionally reduce a macromolecular protein transport system into one mediating peptide transport. The smallest peptides detected showing transport competence consisted of 60 amino acids. The implication of these observations for an understanding of the mechanism of transport is discussed.

KEYWORDS

Hemolysin - E. coli - Transport - Signalsequences - Peptide transport

INTRODUCTION

Hemolysin of E. coli is among the members of a new and emerging family of bacterial cytotoxins (Rtx-toxins) widely disseminated in gram-negative bacteria. They have in common two characteristic features: the presence of a series of repeated domains rich in glycine and aspartic acid at the C-terminal end of the toxin molecule (2,5) and a unique mode of secretion of that molecule into the extracellular

compartment without the involvement of a classical N-terminal signal sequence (1). The transport of these toxins across the inner and outer membrane of the gram-negative cell is dependent on a specialized export apparatus that is believed to catalyse its translocation through a C-terminal topogenic signal in the toxin molecule itself (3,4,6).
Hemolysin is the only protein genuinely excreted by E. coli. To understand the mechanism whereby hemolysin is secreted, we used a combined genetic and immunological approach to systematically scan the hemolysin molecule for topogenic sequences mediating transport.

RESULTS AND DISCUSSION

Capitalizing on the ability of a bacterial cell expressing HlyB and HlyD to recognize and decode secretion signals in the hemolysin molecule we performed a systematic search for transport-mediating sequences in the C-terminal region of the toxin molecule by deleting unidirectionally the coding region of its gene. Fusions of these mutations to the same restriction fragment harboring transcription and translation initiation signals created a nested set of deletion mutants controllable by identical signals of gene expression that were screened for transport competence using an immunological approach. Colony blots of these bacteria were performed using hemolysin-specific antibodies affinity-purified on one of the smaller peptides. From 3000 clones screened approximately 300 positive clones were obtained. A hundred positive clones were further analysed by SDS polyacrylamide gel electrophoresis and immuno-blotting of TCA precipitated cell-free culture supernatants in order to determine the molecular weight distribution and the amount of protein secreted.

The successful isolation of mutants demonstrated that it was possible to functionally reduce a macromolecular protein transport system into one mediating peptide transport. Hence we found that up to 95% of wild type hemolysin sequences are dispensable with respect to functions involved in transport under conditions of steady state. A subset of clones comprising of the smallest ones secreted most efficiently in the respective molecular weight range were further analysed in detail and their sequence determined. These data revealed (a) no involvement of the repetitive domain of hemolysin protein in its transport, (b) a dependence of transport efficacy on molecular weight with an optimum at about 110 amino acids and (c) a lower limit in size for transport competence at 60 amino acids of length with a smaller region -27 to -37 amino acids from the C-terminal end being absolutely necessary for transport.

We conclude that the C-terminal end of the 1024 amino acids long hemolysin molecule is composed of a distinct domain of at least 60 amino acids. This domain would provide conformational flexibility and hence functions not merely as a topogenic signal in transport but also as a source of energy for extrusion of the remainder of the polypeptide out of the bacterial cell.

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Aspects of the Molecular Architecture of the Channel Formed by E. coli Hemolysin in Planar Lipid Membranes

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INTRODUCTION

A 107 kDa hemolysin causes the virulence of some strains of Escherichia coli (1). This toxin lyses RBC by an osmotic shock due to the formation of hydrophilic pores in the cell wall. The channel can also be studied in model systems such as planar lipid membranes (2,3) and unilamellar vesicles (4). Here we present some information on the molecular architecture of the pore derived from its electrical properties.

MATERIALS AND METHODS

Liophilyzed E.coli hemolysin (donated by S.Bhakdi) was kept frozen, after reconstitution its hemolytic activity was usually 1000-2000 HU/ml. Planar bilayers were prepared apposing two monolayers on a hole in a teflon foil separating two solutions (2,3). Lipids used were phopsphatidylcholine (PC) alone or in a 1:1 mixture with phosphatidylserine (PS). The toxin was added to one side only (called cis), voltage signs referred to this side. The current flowing through the membrane was recorded under voltage clamp conditions.

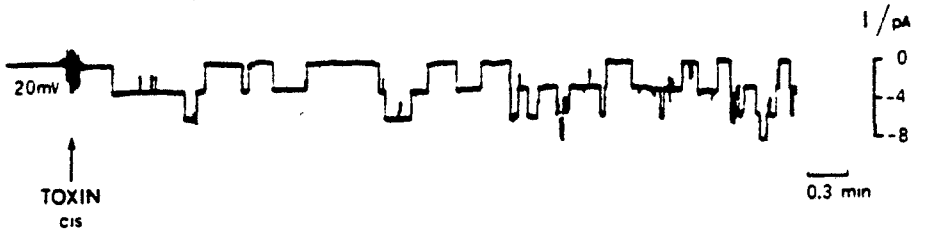


Fig.1. Current steps induced by 2 HU/ml E.coli hemolysin in a PC bilayer bathed by 0.1 M KCl at pH 5.0. Applied voltage was -20 mV. Downward deflections are due to the opening of ionic channels, upward steps indicate closing. Up to three channels, of conductance 100 pS, can be seen fluctuating in this trace.

RESULTS

E.coli hemolysin induces current steps in lipid membranes due to the opening of ionic pores, Fig.1. In neutral membranes the pore is ohmic, cation selective and its conductance is proportional to the salt concentration. We explain these results in terms of a simple model which assumes that the pore is wide and bears a negative fixed charge on its walls (5). We confirmed the presence of a charge by studying the pH dependence of the conductance and the selectivity. Both decrease at low pH because of neutralization of the charge, Table 1. In membranes containing the acidic lipid PS the conductance is non-ohmic in such a way to suggest that the trans (but not the cis) entrance of the pore is affected by the surface potential of the membrane. Applying our model we find that the trans and cis entrances are located respectively about 0.5 nm and more than 5 nm apart from the plane of the membrane. We confirmed the asymmetric disposition of the channel by enzymatic digestion of preformed pores which was effective only from the cis side (5).

Table 1. Effects of pH on the single channel conductance, G, and the ionic selectivity of the E.coli hemolysin pore. P(K+) and P(Cl-) are respectively potassium and chloride permeability

pH	G (pS)	P(K+)/P(Cl-)
4.6 ± 0.2	80 ± 10	1.1 ± 0.1
5.0 ± 0.1	100 ± 10	2.1 ± 0.2
5.5 ± 0.1	160 ± 15	4.7 ± 0.5
7.4 ± 0.1	270 ± 20	14.6 ± 1.9
8.7 ± 0.2	420 ± 30	24.3 ± 4.2

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One Step Affinity Chromatographic Purification of Shiga and Shiga-Like Toxins.

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ABSTRACT

Hydatid cyst fluid from sheep infected with Echinococcus granulosus contains a P1 blood group antigen specific glycoprotein (P1gp) which has a terminal Galα1→4Gal disaccharide moiety. This disaccharide is known to bind Shiga toxin (ShT) and Shiga-like toxin II (SLT II). We have shown that a component of hydatid cyst fluid, likely the P1gp, is able to inhibit the binding and cytotoxicity of ShT to HeLa cells. We have also shown that ShT and SLT II can be quantified by an ELISA using P1gp as the capture molecule. This ELISA is able to detect both toxins in subnanograam amounts. P1gp bound to Sepharose 4B formed an affinity matrix that was capable of purifying both ShT from S. dysenteriae and SLT II from E. coli C600W in one step.

INTRODUCTION

Shiga toxin from S. dysenteriae and Shiga-like toxins I and II from E. coli have the following in common:

- -Biological characteristics (entertoxic, neurotoxic, cytotoxic)
- -Mechanism of action (glycosidase activity at A4324 of 28S rRNA)
- -Binding specificity (Galα1→4Gal)

Hydatid cyst fluid from sheep infected with Echinococcus granulosus contains a glycoprotein(s) that has P1 blood group activity. P1 has a terminal Galα1→4Gal disaccharide and has previously been shown to bind Shiga toxin. We demonstrate that the cyst fluid P1 glycoprotein and Shiga and Shiga-like toxins interact, and investigate the usefulness of this interaction in the quantitation and purification of these toxins.

METHODS

1. Plgp of varying degrees of purity was preincubated with ^{125}I-ShT. The mixture was added to HeLa cell monolayers and both toxin binding and cytotoxicity determined.

2. Crude Plgp from hydatid cyst fluid was bound to ELISA plates, blocked with bovine serum albumin and then exposed to ShT or SLT II. A polyclonal antibody to toxin was followed by a goat anti-IgG alkaline phosphatase conjugate and substrate.

3. An affinity column of purified Plgp bound to Sepharose 4B was prepared and either crude lysate of S. dysenteriae or an ammonium sulfate precipitate of E. coli C600W supernatant was added. This was washed with phosphate buffered saline, 1M NaCl and toxin eluted with 4.5M $MgCl_2$.

RESULTS

1. Plgp inhibited ^{125}-ShT binding to HeLa cells in a dose dependent way and also inhibited ShT related cytotoxicity.

2. Crude Plgp bound to ELISA plates was able to detect as little as 80pg ShT and 130pg SLT II.

3. Plgp bound to Sepharose 4B could be used as a one step affinity matrix to purify:
   -ShT from S. dysenteriae lysate
   -SLT II from E. coli C600W supernatant
Both ShT and SLT II were pure by SDS-PAGE and cytotoxic to HeLa cells.

DISCUSSION

We have been able to use Pl glycoprotein from hydatid cyst fluid to inhibit the binding and cytotoxicity of ShT to HeLa cells. We have also been able to use Plgp in an ELISA that is able to detect both ShT and SLT II. Finally we have purified both ShT and SLT II in one step utilizing Plgp bound to Sepharose 4B as an affinity matrix.

ACKNOWLEDGEMENTS.

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Genetic Analysis of Receptor-Binding Function of Cholera Toxin B Subunit

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INTRODUCTION

Cholera toxin (CT), encoded by the ctx operon of Vibrio cholerae, is an 84 kDa protein composed of one 27 kDa A polypeptide and five 11.5 kDa B polypepcides. Subunit A (fragment A1) has ADP ribosyltransferase activity, and subunit B binds to ganglioside GM1 receptor. Amino-acids in the mature 103 amino-acid CT-B polypeptide that have been implicated in receptor binding include trp-88 (3,6), gly-33 (10), arg-35 (4) and either or both of the cysteines 9 and 86 (6) which form an intramolecular disulfide bond. We have used bisulfite- and oligonucleotide-directed mutagenesis of ctxB gene cloned in Escherichia coli to study the B subunit-receptor interaction.

MATERIALS AND METHODS

Mutagenesis of cloned ctxB was done as described (7,8). Mutants were screened by radial passive immune hemolysis assay (RPIHA) (1). Mutant genes were characterized by DNA sequencing (9) and mutant proteins by sandwich solid phase radioimmunoassay (S-SPRIA)(2) and GM1-SPRIA (5).

RESULTS AND DISCUSSION

Bisulfite induced mutations (C to T) were generated on the coding strand of ctxB. Missense mutations that gave mutants with halo-negative phenotypes on initial screening but positive halos (generally smaller than wild type) when expressed at higher levels in the presence of toxR included pro-2 to leu, ala-10 to val, thr-28 to ile, thr-41 to ile and ala-46 to val. These mutants produced immunoreactive toxin that also bound in a GM1-SPRIA. In contrast, the mutants with mutations pro-93 to leu and pro-93 to ser gave no halos under any conditions and produced only low levels of immuno-reactive toxin by S-SPRIA. This phenotype may reflect a decreased ability of CT-B to pentamerize or an increased sensitivity to proteolysis.

Mutations were generated at cys-9, gly-33, arg-35, cys-86 and trp-88 using synthetic oligonucleotides. Each cys was independently changed to ser and the double mutant also constructed. All cys mutants were halo-negative, and only the ser-9 mutant was immunoreactive in S-SPRIA. Codons 33, 35 and 88 were mutagenized with degenerate oligonucleotides, and individual mutations were sequenced and the phenotypes characterized. All replacements for gly-33 and trp-88 were negative by RPIHA. Most arg-35 replacements were positive, except glu, asp or cys (halos faint, none, or small and turbid respectively).

Mutant proteins were quantitated by S-SPRIA. All mutants with substitutions for gly-33 or arg-35 produced wild-type levels of toxin antigen. However, missense mutants with mutations at position 88 produced substantially less immunoreactive protein than wild-type, ranging from approximately 1000-fold less for ser-88 to approximately 20-fold less for gln-88. No trp-88 substitution produced wild-type levels of protein.

Most of the mutant proteins bound to ganglioside GM1 (in GM1-SPRIA) as well or nearly as well as native CT-B, but substitution of gly-33 by asp-33 or glu-33 almost completely eliminated binding to GM1. Replacement of glu-33 with gln restored binding, indicating that a negatively charged residue at position 33 prevents binding to GM1. All arg-35 missense mutant proteins bound to GM1, implying that residue 35 is not involved in the toxin-receptor interaction. For mutants with mutations at position 88, binding in the GM1-SPRIA was proportional to immunoreactivity in S-SPRIA. Trp-88 is probably essential for structural stability of the CT-B but not for binding to GM1. The cystine bridge formed between cys-9 and cys-86 is also essential for structural stability and antigenicity of CT-B.

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Fragmentation and Purification of Cholera Toxin Active Subunit

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INTRODUCTION

It is well known that cholera toxin is able to permanently activate the adenylate cyclase system, in this respect numerous studies have been carried out (1). In addition cholera toxin elicits a potent immunoadjuvant effect, particularly in the mucosal tissues (2). This phenomenon is likely due to B subunit because the binding to ganglioside GM1 affects the membrane properties and this in turn might trig immunomodulation. However A subunit could also contribute to enhance the immune response because of its ability to activate the adenylate cyclase. Another possibility may be related to the capacity of A1 to insert in the lipid bilayer (3). The aim of the present work is to render feasible the use of the A2 subunit as a natural carrier of other antigens. In fact A2 interacts with B5 oligomer and it represents the anchor domain for the A1. For this reason we carried on experiments designed for preparing fragment of A subunit containg A2.

RESULTS

Fragmentation of A subunit was performed with CNBr at a reagent protein ratio 30:1. This was compared with the CNBr fragmentation of the cholera toxin which was executed at varying molar ratio. namely 10-30-60. SDS-PAGE analysis shows tha among several peptides, one, corresponding to a molecular weight of 16000 (A16) seems the major product. Under A subunit primary structure analysis the A16 is the part of A subunit starting at methionine 94 and comprising the A2 subunit.
In order to purify the fragments we use the HPLC system by employing the Nucleosil C8 reversed-phase columns 10 μm. Two gradient programs were used to obtain the profile shown in Fig. 1. The peak content was analised in SDS-PAGE and it appeared that the 4th peak is mainly formed by A16.
The A16 was assayed for ADP-Ribosylating activity but no

residual activity was found in that peptide.

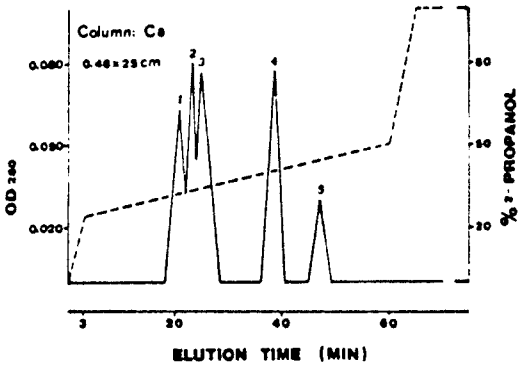


Fig. 1 Fractionation of cholera toxin after digestion of CNBr on HPLC with Nucleosil C8 reversed-phase 10 μm column (25 x 46 mm I.D.). A gradient elution system of 0.1 % (TFA) and 2-propanol, total elution time is 75 min.

CONCLUSIONS:

1) It was set up a method which makes use of HPLC system to seperate and purificate fragments of cholera toxin.
2) The major peptide we obtained is a fragment of A1 subunit starting at the 94th residures (molecular weight 11215) which is jointed to A2 subunit through a disufide bond, all together have 16463 molecular weight and they are named A16.
3) A16 does not elicit any biological activity, this can be due to several reasons. However, we advanced two possible explanations, CNBr may split off the peptide of A1 subunit possesing the active site or the peptide which is crucial for refording of A1 subunit in active form. The second explanation is inferred by the obvervation that the A1 biological activity was lost evenwhen CNBr treatments were mild and did not produce no more than 10% of fragmentation, this seems to suggest that some other secondary reaction occurs, probably, modifications of residues like tryptophan and tyrosin which plan an important role in A1 conformation.

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HPLC Purification of Different Species of Cholera Toxin

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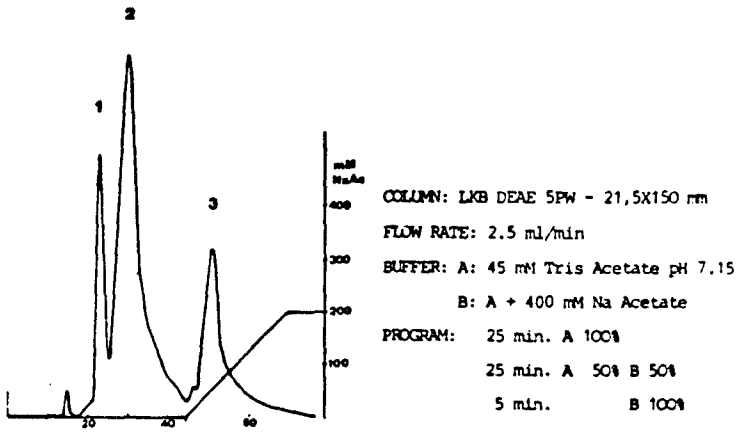
INTRODUCTION

Cholera toxin is a multisubunit protein produced as two peptides A and B which are assembled in the periplasm as AB5. The toxin is exported in the medium and concomitantly A subunit is cleaved by a trypsin like enzyme in two peptides A1 and A2 which are joined by a disulfide bond (1-2). The toxin can be isolated and purified from the bacteria culture medium in both nicked and unnicked forms (1). The percentage of the two forms may vary in dependence of either the presence of EGTA, inhibitor of the vibrio cholerae protease responsible of the specific cleavage or culture conditions (2). An additional heterogeneity of cholera toxin preparation, particularly those which are commercial, is due to different charge content. Firstly described and separated as in (3) their presence seems the cause of poor and unreliable crystal formation (4).
Here we give an account of a method for separating the different cholera toxin species. The method makes use of an anion exchange column in HPLC system.

RESULTS

Fig. 1 shows a typical chromatogram profile of a CT preparation at a purity degree similar to a commercial one. The first two peaks are poor resolved and the second one possesses a shoulder. The protein content was analised in PAGE both in SDS and in native condition with and without reducing agents. The results showed that the first peak is mainly unnicked toxin. The second and the third peaks, when analysed in SDS-PAGE in reducing condition appear to be two identical nicked forms. By contrast native PAGE when reducing conditions were employed evidentiated that the A1 subunit of the third peak was more negative charged than

the A1 of the second peak. In order to establish whether the differences may have a role in a mechanism of action, we carry on experiments with the two different species of cholera toxin. Thus, A1 lipid insertion, circular dichroism and enzymic activity were performed. Only ADP- ribosylation revealed differences in Vmax but no in Km, probably due to greater interaction of A1 subunit with polyarginine,which we used as substrate.



CONCLUSIONS

Different species of cholera toxin were isolated from commercial preparations. The HPLC system we used is also able to separate unnicked and nicked species. Furthermore we characterizes two species of nicked which are different for the negative charge of A1.

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«Checkerboard Immunoblotting» Studies of Cholera Enterotoxin-related Enterotoxins

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INTRODUCTION

The heat-labile enterotoxins (LTs) of E. coli are immunologically, structurally and functionally similar to cholera enterotoxin (CT) (1). In this report, we introduce a simple technique to examine the immunologic cross-reactivities of the B-subunits of CT-related enterotoxins.

MATERIALS AND METHODS

Antigens are immobilized on a solid phase membrane in parallel lanes or channels using a miniblot apparatus. Primary antibodies are applied in channels perpendicular to the antigen and reactions are developed with appropriate secondary antibodies and substrate. The positive reactions are small colored squares that give a "checkerboard" appearance.

Eight different antigens (2) were used in this study: CTB-1 and CTB-2; HLT-B-1 and PLT-B; and four genetically engineered chimeras, pDL-2, pDL-3, pDL-5 and pDL-7 (2), in which individual (or two) amino acids from HLT-B-1 were substituted for the corresponding residue(s) in PLT-B. Antigens, applied in the native state, denatured by 8M urea, or as CNBr-cleavage products, were reacted with monoclonal antibodies (mAbs), with pre- and post-convalescent sera from ten American volunteers with induced cholera (from Dr. M. Levine), and with hyperimmune sera.

RESULTS

As illustrated in the Figure (below), different cholera convalescents responded differently. For example, the convalescent sera of subjects 4, 6, 9 and 10 reacted with all of the native antigens tested. However, subject 6 clearly had a stronger response to CTB-2 than to CTB-1. Interestingly, subjects 2 and 5, apparently exhibited stronger responses to the CT-related toxins than to CT itself. Subject 3, with strong responses to CTB-1, CTB-2 and HLT-B also responded to chimeras with HLT Ala^{46} (i.e., pDL3 and pDL5) better than those with the PLT-Glu^{46} at that position. Individuals responded variously to the urea-denatured antigens (lower half of Fig.) but none responded to CNBr-digested antigens (not shown).

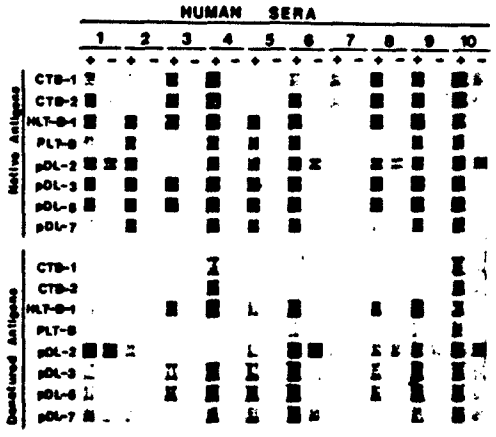


Figure. Reactions of convalescent (+) and acute (-) sera of human cholera patients (L to R) with CT-related antigens (top to bottom).

The reactions with the mAbs in the "checkerboard immunoblot" were practically identical with those reported earlier in ELISA (2). The monoclonal antibodies did not recognize CNBr-digested antigens.

Rabbit, horse and goat polyclonal hyperimmune anti-CTB-1 recognized both CTs but the reaction was somewhat stronger with the homologous antigen. Goat anti-PLT and goat anti-HLT clearly preferred the homologous antigen. The hyperimmune sera, interestingly, recognize some of the CNBr-digested heterologous proteins better than the same proteins in the denatured and native states. Space does not permit discussion of other reactions.

DISCUSSION

It is evident that the "Checkerboard Immunoblot" procedure will be very useful in the further analysis of the shared and unique epitopes in the growing family of the CT-related enterotoxins and should be equally applicable to studies on other antigen/antibody interactions. In the past, emphasis has been placed on the relatedness of members of the CT/LT family to each other. It is clear, however, from the present and previous observations, that the "unrelatedness" of members of the family deserves further scrutiny.

ACKNOWLEDGEMENTS

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Binding of Cholera Toxin to a Monoclonal Antibody: Comparison of Solid-Phase and Solution-Phase Methods

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ABSTRACT

Solution-phase and solid-phase determinations of the binding of a monoclonal antibody against an epitope found on the B subunit of cholera toxin have been made using holotoxin. Although ELISA assays demonstrated a concentration-dependent binding of the MAb to immobilized holotoxin, no significant binding of MAb to holotoxin could be detected by size-exclusion HPLC analyses or ELISA-based competition experiments. Some possible explanations for this discrepancy include recognition of a denatured form of the epitope by the antibody or effective enhancement of a weak binding constant by secondary interactions between the solid phase and the antibody.

INTRODUCTION

Isoelectric heterogeneity of cholera toxin appears to limit the formation of crystals suitable for x-ray diffraction (3). All preparations of the toxin examined by isoelectric focusing were found to be isoelectrically heterogeneous. This condition may be a result of isolation procedure, or the observed heterogeneity might have some functional relevance.

At least two hypotheses can be advanced to suggest functional significance: (A) the toxin may require some post-translational modifications to produce a charge distribution that will more effectively position the oligomer on the membrane receptor, or (B) the progressive degree of heterogeneity may serve to decrease antibody binding to the toxin, thereby reducing the effect of host immune response.

RESULTS AND DISCUSSION

Monoclonal antibody, MAb TE33 (1) was the generous gift of Dr. Chaim Jacob, Stanford University Medical School.

No significant binding of cholera toxin to the MAb TE33 could be demonstrated by the small-zone size exclusion chromatography method (4) used here. The expected high molecular weight peak is not evident and the chromatogram of the experimental mixture coincides with a synthetic sum calculated by adding the peak areas of the two components run as individual controls. The solid-phase ELISA assay, on the contrary, indicates a high degree of binding for the antibody to immobilized antigen. However, holotoxin in solution does not appear to compete effectively with immobilized antigen either in a preincubation competition experiment or a coincubation competition experiment.

It is possible to suggest that MAb TE33 recognizes a denatured epitope on the holotoxin that is conformationally different from its native state and more closely resembles the peptide against which the antibody was generated, whereas polyclonal immunoglobulins may contain antibodies against not one but several conformers adopted by the peptide, one of which may, in fact, resemble the native conformation either in solution or bound to the receptor. In an experiment in which the polyclonal goat anti-cholera toxin antibody was used to coat microtiter plate wells, then holotoxin bound to it, the subsequent binding of TE33 was reduced considerably, compared to that observed when holotoxin was bound directly to the plastic. It is possible that the epitope may lie near the receptor binding site, or that the antigen was not presented in a conformation recognizable to the MAb.

The results suggest that some caution should be exercised in the interpretation of solid-phase assays such as ELISA. Jemmerson (2) has shown that it is more common than usually assumed to obtain antibody to "denatured" antigen by the hybridoma technique when ELISA is used as a screening procedure. ELISA assays, like other solid-phase assays, may also appear to enhance very weak interactions because even a small solvent exclusion process may contribute to the overall energy of binding. The results presented here suggest that solution-phase competition experiments and size-exclusion HPLC analyses (4) may be useful adjuncts to studies of antigen-antibody binding and vaccine analysis.

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Mutations in the S1 Subunit of Pertussis Toxin Affecting the Recognition by Mabs

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INTRODUCTION

A genetically detoxified Pertussis toxin (PT) should serve as a better candidate for a new acellular vaccine against whooping cough. We have constructed mutations in the NH2 terminal region of the S1 subunit. Their effects on the ADPribosyltransferase (ADPRT) activity and on the antigenic properties of the S1 protein using monoclonal antibodies directed against S1 were examined.

MATERIALS AND METHODS

Construction of the mutants

Mutations in position 9 and in positions 11 and 14 were obtained after insertion of mixed oligonucleotides between unique restriction sites (previously created by site directed mutagenesis and changing amino acid 5 from alanine to glycine). All the mutant S1 proteins obtained have lost more than 99,9 % of their ADPRT activity.

Monoclonal antibodies directed against S1

MAbs 3CX4 (strongly neutralizing), 6FX1 (weakly neutralizing) and X2X5 (non neutralizing) were obtained from J.G. Kenimer (4). MAbs 15B6A2, 8B9A1 and 15B6G3 were produced by immunizing BALB/c mice with PT. These last mAbs recognize PT in ELISA and S1 in western blot : they are not protective in the CHO cell assay.

RESULTS

Recognition of the mutated S1 proteins by mAbs were analysed by western blot with purified mAbs against whole cell extracts containing these proteins. The great majority of the mAbs is affected in its recognition of the S1 protein by a least one mutation in the 8-18 amino acid region, with the exception of the non neutralizing mAb X2X5. This result is consistent with the fact that the epitope corresponding to mAb X2X5

has been mapped in the 15-26 aminoacid region (4) and that the NH2 terminal end of the protein is at least part of an immunodominant epitope (1). The single mutated protein (9 Arg→His) retains the recognition by most mAbs (neutralizing or not neutralizing) indicated that this protein should possess one or more protective epitopes. Double mutated proteins (positions 11 and 14) react only with mAb X2X5.

CONCLUSION

The NH2 terminal portion of the S1 protein is critical for its antigenic properties (with mAbs neutralizing or not). However, we have obtained one S1 protein inactivated by mutation in this region and retaining a protective epitope. This mutated S1 gene is a good candidate to return into Bordetella pertussis chromosome.

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Probing the Structure-Function Relationship of the Pertussis Toxin S1 Subunit by Site-Directed Mutagenesis

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INTRODUCTION

Pertussis toxin is the major virulence factor synthesized by Bordetella pertussis, the causative agent of whooping cough[1]. This toxin is the most complex bacterial toxin studied so far. It is composed of five dissimilar subunits designated S1 through S5. Subunits S2 through S5 are responsible for the binding of holotoxin to the target cells, whereas the S1 subunit expresses enzymatic ADP-ribosyltransferase and NAD glycohydrolase activities[2]. In this study we used site-directed mutagenesis and enzymatic analyses to investigate the role of individual residues in the enzymatic activity of the pertussis toxin S1 subunit.

RESULTS AND DISCUSSION

The coding regions for the mature S1 subunit (rS1) and for a carboxy- terminally truncated S1 protein (rS1d) were introduced into M13mp18 and pUC18 respectively, such that the inserted genes were expressed under lacZ transcriptional and translational control. Whereas rS1d was fully soluble, the rS1 protein was found exclusively in the particulate fraction, and could only be solubilized in urea containing buffers. rS1d was purified to homogeneity using two chromatography steps. Both rS1 and rS1d showed NAD glycohydrolase and ADP-ribosyltransferase activities. The ADP-ribosyltransferase activity of rS1d was however approximately 20 fold reduced as compared to that of rS1 or pertussis toxin (Table I). On the other hand, specific NAD glycohydrolase activity of rS1d was very similar to that of pertussis toxin. Furthermore, over 20 monoclonal antibodies, some of which recognize very complex conformational epitopes, react with rS1d as well as with holotoxin. This suggests that the carboxy-terminal portion of the S1 subunit is involved in acceptor protein substrate binding.
Individual amino acid residues of rS1d were altered by site-directed mutagenesis. As shown in table I, deletion of either W-26, or C-41 or E-129 had a dramatic effect on the NAD glycohydrolase activity, whereas alterations of H-83 or E-106 did not affect this activity. Deletion or substitution of E-106 appeared to somewhat diminish the ADP-ribosyltransferase activity, thereby suggesting that the region around E-106 may also be involved in acceptor substrate recognition.
In order to investigate the specific role of W-26, C-41 and E-129 in either NAD binding or catalysis, substitutions of these residues were carried out. Replacement of C-41 by S, G, P or N reduced NAD glycohydrolase activity, without abolishing it, indicating that no side chain is needed at this position. Analysis of the kinetic parameters of mutant C41G as compared to those of rS1d, indicates that the K_m for NAD was increased in the

mutant, whereas the k_{cat} was not significantly changed (Table I). This result shows that C-41 is not a catalytic residue but is at, or close to the NAD binding site. On the other hand replacement of C-41 by E or D reduced the activity to background levels. This indicates a drastic effect of a negative charge at position 41, leading to the suggestion of a charge-charge repulsive interaction and therefore to the speculation that C-41 may be in close proximity to the phosphate poieties of the NAD.
Replacement of W-26 by T did not restore enzymatic activity, indicating that a side chain is essential at position 26. When W-26 was changed to Y, approximately 25 % of residual activity was found. The K_m for NAD was about 25 fold increased, whereas the increase k_{cat} suggests that the mutant has also decreased affinity for one of the products of the reaction. Inhibition kinetics may be useful to further investigate this possibility.
Finally, none of the substitutions made at position 129 have been found to restore measurable activity, and no kinetic studies could be carried out. It is possible that E-129 has a catalytic function similar to E-148 in diphtheria toxin.
The identification of residues involved in catalysis and/or NAD binding is useful for the development of a safer, new generation vaccine against whooping cough, since pertussis toxin is considered the most important antigen in the new acellular vaccines and, due to its enzymatic activities, is also believed to be responsible for the severe side effects of the current whole cell pertussis vaccines.

Table 1 : Activities of truncated and mutant S1 subunit forms

S1 form	ADP-ribosyl-transferase	NAD glyco-hydrolase		K_m (NAD)	k_{cat} (min^{-1})
PTX*	+++	15+	100 %	20 µ	
S1	+++	11.6	77.3 %		
rS1d	++	14.7	98 %	16 µM	.50
rS1d-W26Δ	-	< .05	< .4 %		
rS1d-W26T	-	< .05	< .4 %		
rS1d-W26Y	+	4.8	32 %	500 µM	1.48
rS1d-C41Δ	-	< .05	< .4 %		
rS1d-C41S	+	5.9	39.3 %		
rS1d-C41G	+	6.9	46 %	47 µM	.42
rS1d-C41P	+	4.7	31.3 %		
rS1d-C41N	+	4.1	27.3 %		
rS1d-C41E	-	.1	.7 %		
rS1d-C41D	-	.1	.7 %		
rS1d-H83Δ	+	15	100 %		
rS1d-E106Δ	+	15.6	104 %		
rS1d-E106D	+	17.6	117 %		
rS1d-E129Δ	-	< .05	< .4 %		
rS1d-E129D	-	< .05	< .4 %		
rS1d-E129E	-	< .05	< .4 %		

*PTX = pertussis toxin; +pmol nicotinamide released/min.µg S1

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Site-Specific Antibodies to Pertussis Toxin

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INTRODUCTION

We have examined the immunogenicity of peptides from the amino acid sequence of pertussis toxin (PT) a virulence determinant of Bordetella pertussis.

MATERIALS AND METHODS

ELISA's were performed with murine and rabbit antibodies (Ab) raised to peptide-KLH conjugates. The ability of antibodies to neutralise PT activity in lymphocytosis, CHO cell clumping and glycohydrolysis of NAD^+ (1,) was measured.

RESULTS

All peptides produced an immune response which recognised the homologous peptide on ELISA. The sera to peptides 8(S1 35-52), 9(S1 237-255), 11 (S2 191-209), 13(S2 36-53), 14(S3 153-171) and 15(S4 94-115) also recognised PT on ELISA (Fig.1A). However, purified antipeptide IgG coated onto ELISA plates could recognise KLH or homologous peptide but not native PT (Fig.1B). This explains why none of the peptide antisera could inhibit toxin activity, whereas control McAb L10 (1) neutralised lymphocytosis, CHO cell clumping and enzyme activity.

CONCLUSION

Short peptides do not induce antibodies which recognise native toxin and are therefore unlikely to produce a protective immune response. The results show variation in PT antigenicity which is dependent on assay configuration.

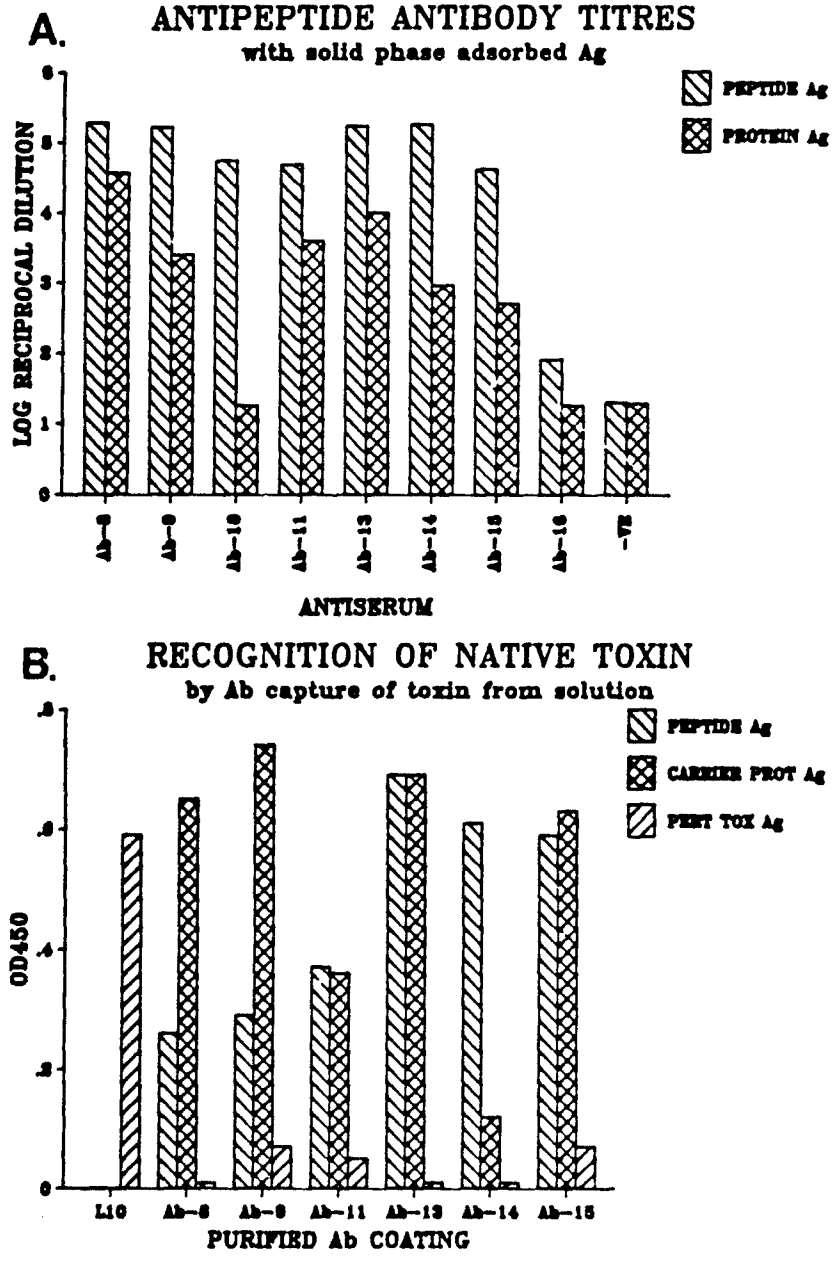


Fig. 1 Antibody Recognition by ELISA

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Identification of an Antigenic Domain in the Pertussis Toxin S2 Subunit Associated with Receptor Binding

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INTRODUCTION

Whooping cough is a human respiratory disease caused by virulent Bordetella pertussis organisms. Concern over adverse reactions following pertussis vaccination has led to reduced acceptance of the current vaccines causing an alarming rise in the incidence of infection. Among the many candidate virulence factors expressed by B.pertussis, the filamentous hemagglutinin and the secreted pertussis toxin (PT) are of prime importance for the pathogenesis of the disease and thus also in the development of new vaccines. The ADP-ribosylation of G_i-proteins by PT interferes with the adenylate cyclase regulatory system of target cells. Immunization with PT and with the isolated B-oligomer (1), but not with isolated subunits induces protective immunity in mice. One way to interfere with the pathologic effects of PT could be the inhibition of toxin interaction with its cellular receptor(s) by preformed toxin-directed antipeptide antibodies. A prerequisite for this approach is the localization of antigenic and "inhibitory" epitopes. Receptor recognition of pertussis toxin (PT) is mediated by its B-oligomer consisting of the five subunits S2, S3, 2xS4, and S5. As the S2 subunit of PT has been implicated with receptor binding, linear antigenic and immunigenic determinants of S2 have been identified (3). Here, we report the inhibition of pertussis toxin binding to model receptors by antipeptide antibodies and the identification of a possible antigenic domain formed by the corresponding segments in the S2 subunit.

MATERIALS AND METHODS

The PT used in this study was either purchased from List Biochemicals (Campbell, USA) or received as agift from the Institut Mérieux (Lyon). Synthetic peptides were prepared as described (3). Antipeptide antibodies were purified by affinity chromatography using the homologous peptide-BSA conjugates coupled to CNBr-Sepharose and elution of bound antibodies with 0.1M glycine followed by immediate neutralization. For solid phase binding assays, ELISA plates were coated with fetuin, blocked with 5% BSA/PBS, and reacted with serial dilutions of PT in 0.01%BSA/PBS. Bound PT was detected with HRP-conjugated purified anti-PT IgG. For inhibition experiments a standard amount ot PT (200 ng) was incubated with serial dilutions of purified antipeptide antibodies and added to fetuin-coated microtiter wells. Bound PT was detected as above. Prior to hemagglutination, goose erythrocytes were sensitized by incubation with chymotrypsin (sGRBC). The additive ELISA was performed as described (2).

RESULTS

Ten peptides corresponding to selected segments of S2 were synthesized. Only antibodies induced by R1-7, R35-50, and R91-106 recognized the S2 subunit in the holotoxin, thus defining the corresponding regions as antigenic epitopes (3).

A) To study the inhibitory effects of antipeptide antibodies PT-binding was assessed in a solid phase fetuin-binding assay after preincubation with serial dilutions of affinity purified antipeptide antibodies. All three antipeptide antibodies showed inhibition of PT binding to fetuin at concentrations larger than 1 µg/ml.

B) Eight ng PT/ml were found to be sufficient for hemagglutination of 10^7 sGRBC. PT was preincubated with serial dilutions of antipeptide antibodies and added to sGRBC in 0.1%BSA/PBS. All three antipeptide antibodies inhibited hemagglutination with minimal inhibitory concentrations ranging from 80 µg/ml to 160 µg/ml.

C) The clustered growth pattern of CHO cells induced by PT, however, could not be inhibited by these antipeptide antibodies, though high concentrations of anti-R91-106 seem to have some effect.

D) In a competition experiment by additive ELISA (2) antipeptide antibodies were applied as sequence specific probes to see wether the linear, sequentially separated antigenic determinants could be juxtaposed in space due to the threedimensional folding of the S2 subunit in the holotoxin. All antipeptide antibodies competed pairwise for binding indicating steric hindrance probably due to close spatial proximity of the respective determinants.

DISCUSSION

Antipeptide antibodies directed at linear antigenic determinants of the S2 subunit inhibited the interaction of PT in two of the model receptor systems used in this study. The induction of the clustered growth pattern in CHO cells could not be inhibited, indicating binding of PT to its cellular receptor(s) and translocation of the S1 subunit through the membrane. It has been shown that the interaction of PT with the N-linked carbohydrate moiety of fetuin or haptoglobin as receptor analogues is dependent on the S2 subunit. To be able to act as hemagglutinin PT must at least be bivalent. Thus, the inhibition of interactions associated with the binding site in S2 abolishes hemagglutination as well as fetuin binding. In the CHO-assay binding cannot be blocked by anti-S2 antibodies alone as only one binding site of the holotoxin is affected. Furthermore, as indicated by the pairwise competition experiments the linear antigenic determinants seem to be in close spatial proximity in the native toxin and thus might constitute distinct segments of a larger (conformational) epitope. This line of evidence is further supported by the lack of crossreactivity between S2 and S3 as demonstrated with antipeptide antibodies (3).

We thus propose that pertussis toxin harbours two distinct, different receptor binding sites in the B-oligomer located in the S2 and S3 subunit. Synthetic peptide antibodies directed at linear antigenic determinants of the S2 subunit can efficiently interfere with interactions linked to the S2 subunit, but for the reasons dicussed above not with binding due to S3.

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The Interaction of Pertussis Toxin with NAD^+

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INTRODUCTION

The Michaelis constant, Km, for the pertussis toxin catalysed NAD^+-glycohydrolase reaction is 30 μM (1). This suggests that the Kd may be of the same order of magnitude. The number of binding sites for NAD^+ has not yet been determined. We investigated the protein ligand interaction using equilibrium dialysis and fluorescence. We calculated Kd values at different temperatures and hence a value for the enthalpy change on binding of NAD^+ to pertussis toxin.

MATERIALS AND METHODS

Equilibrium Dialysis. Equilibrium dialysis was carried out in a Dianorm apparatus as described by Galloway et al. (2) The system was rotated at a constant temperature for three hours, by which time equilibrium was reached. The proportion of ligand bound to the toxin could then be calculated.

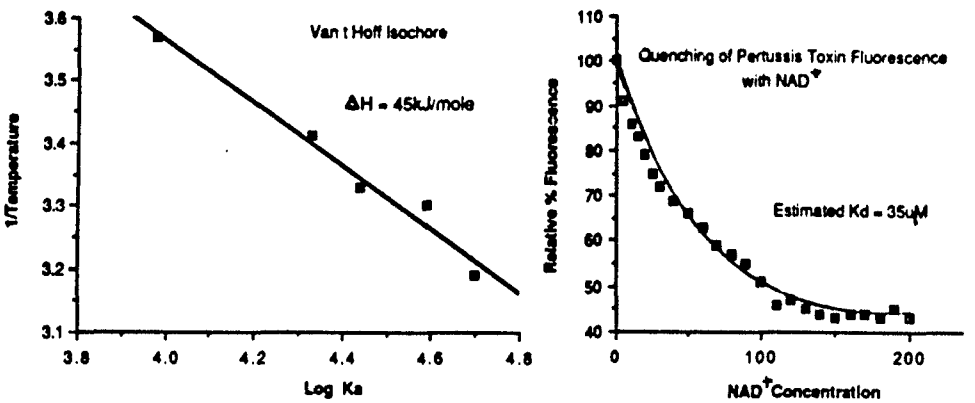
Fluorescence. Quenching of pertussis toxin fluorescence was carried out at emission wavelength 340 nm and excitation 280 nm. The samples were corrected for dilution and inner filter effects, (3, 4 and 5).

RESULTS AND DISCUSSION

1. The results from equilibrium dialysis are shown in Table 1. Kd values and the number of binding sites for ligand, n, are shown.

Temperature	Kd	n
7°C	105 μM	1.00 ± 0.25
20°C	46 μM	1.00 ± 0.15
23°C	37 μM	1.00 ± 0.05
30°C	27 μM	1.00 ± 0.10
40°C	20 μM	1.00 ± 0.10

The van't Hoff Isochore drawn from these results is shown in Fig. 1.



Both of the above methods gave Kd values of the same order of magnitude, and these were in agreement with the previously calculated Km value for the toxin catalysed NAD^+-glycohydrolase reaction. The number of binding sites for NAD^+ was shown to be one, as expected and finally, the enthalpy change calculated from these results gave a value which is typical of that of binding of a ligand to a protein.

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Pasteurella multocida Toxin: its Biological Activities and its Use for Vaccination against Progressive Atrophic Rhinitis in Pigs

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INTRODUCTION

Pigs infected with certain strains of Pasteurella multocida develop progressive atrophic rhinitis (PAR) (6). The lesions of PAR are irreversible atrophy of the nasal turbinate bone, distortion of the snout and reduced growth rates. The atrophy is caused by enhanced osteoclastic bone resorption and impaired osteoblastic synthesis of the osseous core.
Only toxinproducing strains of P.multocida are able to cause PAR. The P.multocida toxin (PMT), which is a protein of 143 kDa (2), induces osteoclastic bone resorption of the nasal turbinates of pigs and rats, and is therefore considered the central aetiological factor in PAR (1,2). This publication describes the physical and biological properties of PMT, and the use of inactivated PMT as a single component PAR-vaccine.

MATERIALS AND METHODS

Anti-PMT monoclonal antibodies (MAbs) were produced as described in (3). Bacterial extract of a toxigenic strain of P.multocida was applicated to an affinity column containing the anti-PMT MAb, P3F51, immobilized on divinyl sulfone agarose (3). PMT was eluted by glycin-HCl, pH 2.8, and the purity and yield determined by, respectively, SDS-PAGE and a sandwich-ELISA based on two MAbs reacting with different epitopes on PMT (3,4).
The minimal doses of PMT resulting in: i) cytopathic effect on embryonic bovine lung cells (7), ii) dermonecrotic effect in guinea pigs and iii) turbinate atrophy in piglets was determined; and the LD_{50} of PMT in mice, rats and pigs was recorded. In vivo neutralisation of the lethal effect of crude extract from toxigenic P.multocida by MAb P3F51 was studied.
Pregnant sows were immunized with inactivated PMT 6 and 3 weeks before expected farrowing (5). The offspring of vaccinated and unvaccinated sows were intranasally infected with Bordetella bronchiseptica and with toxigenic P.multocida. Bacteriological examinations of nasal swabs and determination of serumtiters by ELISA were performed regularly until the pigs were slaughtered at 90 to 100 kg liveweight. At slaughter the snouts were cross-sectioned and turbinate atrophy scored according to Bendixen.

RESULTS

The yield of PMT in the affinity purification was 10.3 mg from 4.3 l of a cell-free bacterial extract, corresponding to 93 % of the PMT applied to the column.

TABLE 1. Characterization of PMT.

Molecular weight (SDS-PAGE):	143 kDa
Isoelectric point (IEF):	4.8
Minimal cytopathic dose on EBL-cells:	30 pg
Minimal dermonecrotic dose in guinea pigs:	50 ng
LD_{50}/bodyweight in mice, rats and pigs:	0.5-1.5 µg/kg
Dose (i.p.) resulting in turbinate atrophy in piglets	2 x 0.5 µg

Passive immunization with an anti-PMT MAb 2 days before challenge of mice with crude extract of toxigenic P.multocida was highly protective.
Pregnant sows vaccinated twice with purified inactivated PMT transferred anti-PMT antibodies to their suckling offspring.

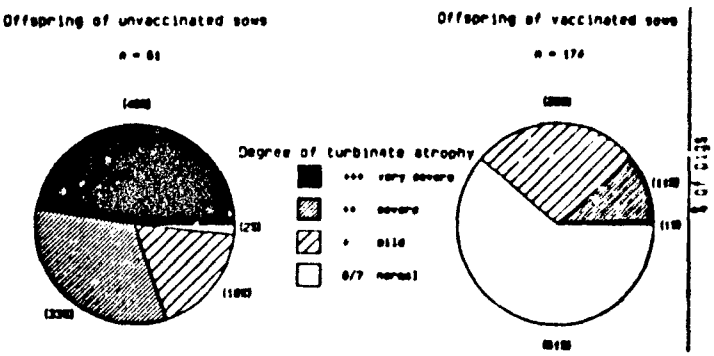


Figure 1. Occurence of turbinate atrophy in pigs at slaughter.

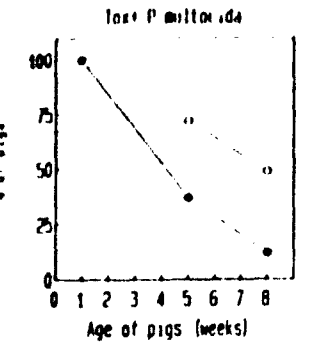


Figure 2. Reisolation of toxigenic P.multocida. Offspring of unvaccinated (o) and vaccinated (●) sows.

DISCUSSION

PMT is a 143 kDa bacterial protein toxin, which can be purified in a single step by affinity chromatography based on immobilised anti-PMT MAb. This method has made is possible to assess the value of protecting pigs against the effects of PAR by vaccination with PMT. We observed that the incidence of turbinate atrophy at slaughter in piglets born by vaccinated sows was significantly reduced when compared to the offspring of unvaccinated sows. Bacterial reisolation rates indicated that this protection might be caused at least in part by a reduction of the colonisation of toxigenic P.multocida.

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Complement Activation by Pneumolysin, Location of Domain and Similarity to Human Acute Phase Protein

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INTRODUCTION

Pneumolysin, the membrane damaging toxin of Streptococcus pneumoniae, has several effects on the components of the immune system which could be of relevance to the pathogenesis of pneumococcal infection. Sub-lytic concentrations of the toxin inhibit antibody synthesis by B-cells (1) and inhibit the key anti-microbial activities of polymorphonuclear leukocytes (PMNL) such as respiratory burst (4). At higher concentrations the toxin activates the classical complement pathway in an antibody independent manner (5). We have attempted to localize the complement activating domain of pneumolysin.

MATERIALS AND METHODS

Computer comparisons were made using the FASTP program of Lipman and Perason (2). Pneumolysin was purified and complement activation was measured as previously described (3). Site-directed mutagenesis was done as previously described (6). Antibody binding was measured by ELISA using goat conjugated second antibody.

RESULTS

Computer searches of protein sequence data bases with the pneumolysin sequence showed the most closely related protein was human C-reactive protein (CRP). Two non-contiguous areas of homology were located in pneumolysin (domains 1 and 2) which were adjacent in the CRP sequence. Domain 1 shows 62% homology (including conserved changes) over a 42 amino acid sequence and domain 2 shows 73% homology over 30 amino acids. CRP was first identified by its ability to bind the C-polysaccharide of pneumococci and activate complement. On the assumption that the common regions in pneumolysin and CRP are those which mediate complement activation, we have carried out site directed mutagenesis of domain 2 in pneumolysin. We changed separately 3 aromatic residues in domain 2 to phenylalanine. All three amino acid changes decreased the ability of the toxin to activate

the complement pathway. The greatest reduction in ability was seen when tyrosine 384 was changed to phenylalanine (86% reduction). Since changing tyrosine to phenylalanine only involves the elimination of a hydroxyl group, it is probable that this OH group is involved in complement activation.
Complement activation by pneumolysin in all cases was abolished by addition of EGTA + Mg ions or by removal of IgG by prior passage of serum over protein A sepharose. Thus complement activation is via the classical pathway and is IgG dependent. However, no specific antitoxin could be detected. We therefore examined the ability of pneumolysin and modified toxin to bind antibody and antibody fragments. We found pneumolysin to bind the Fc portion of antibody. Substitution of tyrosine 384 with phenylalanine reduced the ability of the toxin to bind Fc (8-fold reduction); thus mirroring the effect of this alteration on complement activation.

DISCUSSION

We tentatively propose that the region between rsidues 368-397 (domain 2) of pneumolysin is involved in antibody binding via Fc, and that this non-immune binding of antibody results in the activation of the classical complement pathway. It is possible that the CRP-like domain 1 will prove also to be an antibody binding domain and thus provide a second site for interaction of complement component C1q necessary for classical pathway activation.

ACKNOWLEDGEMENTS

We thank Dennis Burton for providing us with human Fc and Fab and the MRC and Lister Institute for financial support.

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Non Essential Role for Thiol Groups in the in vitro Activity of Pneumolysin, the «Thiol Activated» Toxin of Streptococcus pneumoniae

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INTRODUCTION

Pneumolysin, the thiol activated cytolytic toxin of Streptococcus pneumoniae, is one of a family of immunologically cross reactive toxins elaborated by gram positive bacteria. Thiol activation was postulated to reflect the formation and breakage of intramolecular disulphide bonds. A single free sulphydryl group was thought to be essential for activity. During cytolysis it is assumed that toxin monomers bind to cholesterol in the target cell membrane and insert into the lipid bilayer. They aggregate to form oligomers which are believed to act as transmembrane pores leading to lysis. Cholesterol was proposed as a receptor because these toxins are only active on cells with cholesterol in their membranes and are cytolysis is inhibited by free cholesterol. These toxins also have potent inhibitory effects on various aspects of the immune system. For example, at sublytic concentrations, they inhibit the respiratory burst of polymorphonuclear leukocytes (PMN) and they also activate complement.

MATERIALS AND METHODS

Site directed, oligonucleotide-mediated mutagenesis and purification of wildtype and modified toxins has been described (3).

RESULTS AND DISCUSSION

The thiol-activated toxins for which a predicted sequence is available, pneumolysin (6), streptolysin O (1), listeriolysin O (2) and perfringolysin O (5), have a single cysteine which lies in a conserved 11 amino acid motif which is the single longest region of sequence identity. A modified toxin in which the unique cysteine was replaced by an alanine (cys_{428}>ala) was identical to the wildtype in terms of haemolytic activity, inhibitory effects on PMN, binding to red blood cells (RBC) and oligomer formation. This demonstrates that a sulphydryl group is not necessary for toxin activity in vitro.

A series of modified toxins was produced to investigate the importance in activity of the cysteine and other residues in the cysteine motif (see

Boulnois et al., this volume). In a number of these haemolytic activity was reduced but never abolished. Thus it is the overall structure of this region that is likely to be important rather than specific residues. For cys_{428}>ser and cys_{428}>gly toxins the decrease in haemolytic activity correlated with a similar decrease in activity against PMN at sublytic concentrations. None of the modified toxins studied showed any decrease in ability to bind to RBC or to form oligomers suggesting that the cysteine motif is not involved in receptor binding. It is possible that the cysteine motif is important in the generation of functional pores. One toxin, glu_{434}>asp , showed a decrease in cholesterol binding. Thus the cysteine region may play a role in the toxin-sterol interactions after receptor binding, and this may be involved in pore formation. Treatment of wildtype pneumolysin with diethyl pyrocarbonate (DEPC), which modifies histidine residues, virtually abolished haemolytic activity and the ability to bind to RBC. There is only one histidine residue which aligns in the four toxins in this group which have been sequenced and when this is changed pneumolysin lacks all cytolytic activity. His_{367}>arg modified toxin may define the receptor binding region.

In conclusion we propose the following model for toxin activity. Toxin monomers bind via the histidine to the OH on C3 of cholesterol or to another membrane component. The cysteine motif, via an interaction with cholesterol, may be important in the formation of functional oligomers.

ACKNOWLEDGEMENTS

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A Thiol Group is not Required for the in vitro Activity of the «Thiol-Activated» Toxin Streptolysin O

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INTRODUCTION

Streptolysin O, which is secreted by Streptococcus pyogenes, is one of a family of fifteen biologically related membrane-damaging toxins, which are produced by diverse species of Gram-positive bacteria. These toxins are known as "Thiol-Activated" toxins and their biological properties has led to the assumption that cysteine is essential for their cytolytic activity. Here we report the construction of mutant SLO derivatives that differ only with respect to the unique cysteine and show that the above assumption is not correct.

RESULTS AND DISCUSSION

The TGC (Cys) codon of SLO was altered to GCC (Ala) or TCG (Ser) by site directed mutagenesis. When mutant plasmids were transformed into E. coli, haemolytic colonies were obtained on blood agar. The mutant SLO derivatives, SLO. Ala.530 and SLO.Ser.530, were purified and their properties were compared with those of wild-type SLO (SLO.Cys.530). The results summarized in Table 1 show that there are no significant differences in the specific haemolytic activities of wild-type SLO and the SLO.Ala.530 mutant, while SLO.Ser. 530 has a reduced, but still considerable, activity. Reagents which chemically modify free sulfhydryl groups (iodoacetate and PHMB) cause a marked reduction in the cytolytic activity of wild-type SLO but, predictably, do not significantly change the haemolytic activity of the cysteine-free mutants. Cholesterol is required for SLO interaction with membranes and pretreatment of SLO with cholesterol irreversibly inactivates its cytolytic activity. Current models for cytolysis by SLO suggest that the toxin interacts with membrane cholesterol and oligomerises to form large arc-and ring shaped structures, which result in the leakage of high molecular weight cell components. During these studies we have isolated similar oligomers from membranes lysed with SLO.Cys.530, SLO.Ala.530 and SLO.Ser.530 and shown that cholesterol inhibits the cytolytic activities of the wild-type and mutant toxins to the same extent, suggesting that the mutants cause cytolysis by the same mechanism as the wild-type.

CONCLUSIONS

Although cysteine is not required for the cytolytic activity of SLO, it probably lies close to a functionally important site in the active protein, which is distorted by thiol-modifying agents interacting with the non-essential thiol group. The location of Cys in a 12 amino acid region which is strongly conserved among the thiol-activated toxins which have been sequenced to date (1,2,3,4) supports this conclusion. However, in these studies toxin activity was assayed in vitro and a possible role for Cys in an in vivo activity of SLO cannot at present be ruled out.

Table 1: Specific haemolytic activities of SLO and the mutant toxins.

	Specific Haemolytic Activity (HU/mg)		
	SLO.Cys.530 (wild-type)	SLO.Ala.530	SLO.Ser.530
Toxin alone	850,000	750,000	200,000
Toxin +:			
Cholesterol	3,000	3,000	700
Iodoacetate	0	550,000	200,000
PHMB	25,000	750,000	400,000

A difference of up to two fold in the measured haemolytic activity is within the bounds of experimental error.

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Tetanus and Botulinum A Toxins: Comparison of Sequences and Microinjection of Tetanus Toxin A Subunit-Specific mRNA into Bovine Chromaffin Cells

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INTRODUCTION

It is our final goal to determine the molecular mechanism by which tetanus and botulinum toxins block neurotransmitter release. Our experimental approach involves cloning of the corresponding genes, in vitro synthesis of mRNA encoding specific subdomains and characterization of such mRNA by in vitro translation in a suitable translation system or by microinjection of biologically stable mRNA into secretory cells. We report here the primary sequence of botulinum type A toxin (botox A), as deduced from the nucleotide sequence. We have compared this sequence with that of tetanus toxin (1). We show that microinjection of mRNA encoding the authentic A-subunit of tetanus toxin leads to toxification of the injected bovine chromaffin cells in a time-dependent and cycloheximide-sensitive process.

RESULTS AND DISCUSSION

Fig. 1 shows the complete amino acid sequence of botulinum type A toxin, as deduced from the nucleotide sequence and aligned with the tetanus toxin sequence (1) and partial amino acid sequences of botulinum toxins type B (2), C1 (3), and E (4). It should be noted that our sequence is in complete agreement with partial sequences published previously for botox A (5, 6) Botox A (MW 149,424) contains 1296 amino acids including the N-terminal methionine residue which, however, is apparently removed during translation. The A subunits (L-chains) of botox A and tetanus toxin show 30.8% the B-subdomains 35.9% (46.4% between Arg^{565} and Phe^{787}) and the C-fragments 33.6% homology at the amino acid level. The A-subunits of both toxins contain a particular α-helical domain, each involving 17 amino acids. Both domains contain 3 histidine residues in conserved positions being located at the same face of the α-helix. The histidine modifying reagent diethylpyrocarbonate de-toxifies tetanus toxin and botox A at a 1000 molar excess within two minutes (7). Under such conditions three of the twelve histidine residues of botox A were modified. These data suggest that these histidine residues play a role either in the translocation step of the A-subunit into the cytosol or in the toxification process.

The cysteine residues Cys^{430} and Cys^{454} are likely to participate in the disulfide linkage between the L- and H-chains of botox A, since they can be aligned with Cys residues fullfilling this function in the tetanus toxin sequence.

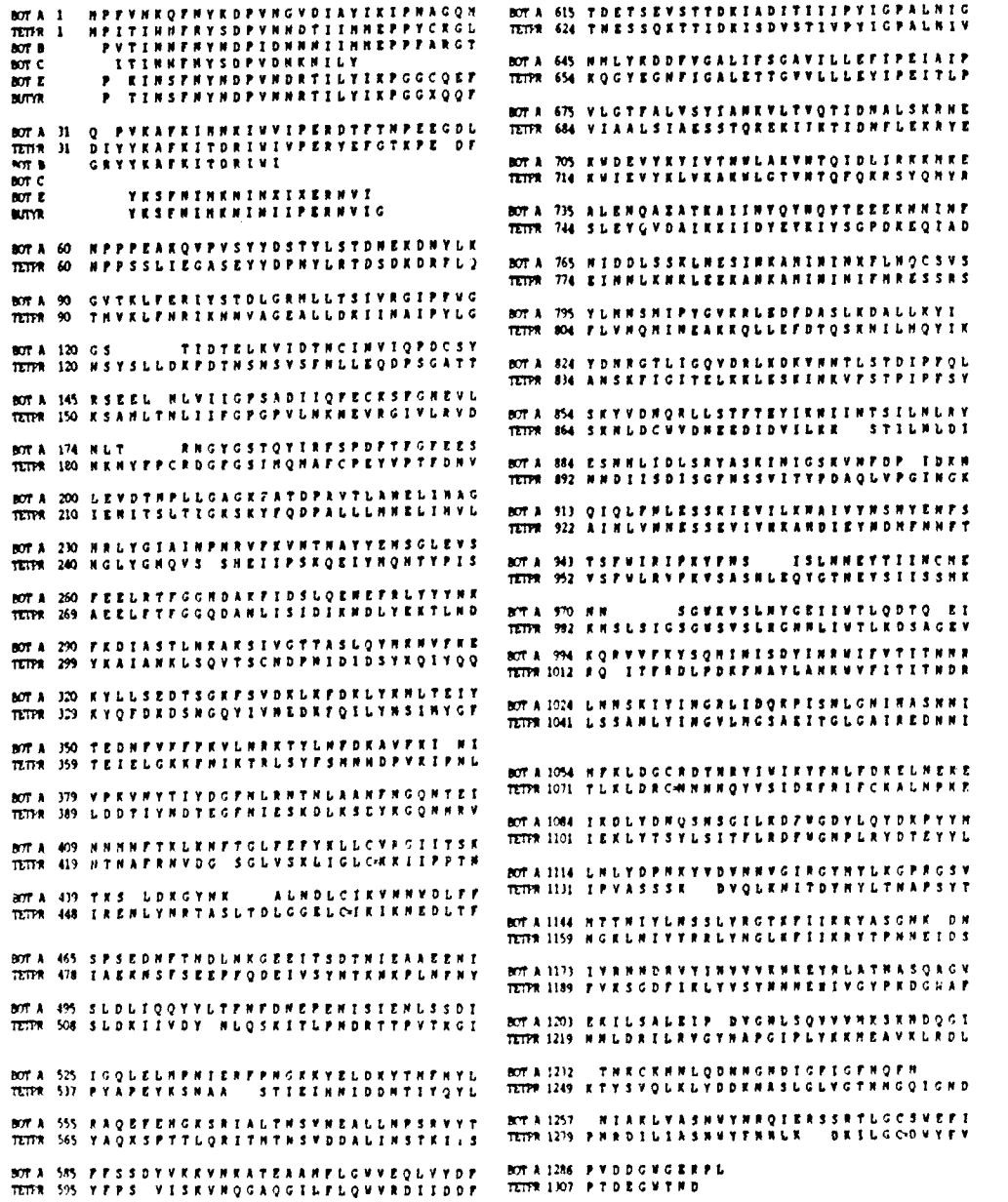


Fig. 1 Comparison of the sequences of Botox A, tetanus toxin (1) and partial amino acid sequences published for botox B (2), C1 (3), E (4), and a neurotoxin from C. butyricum (4).

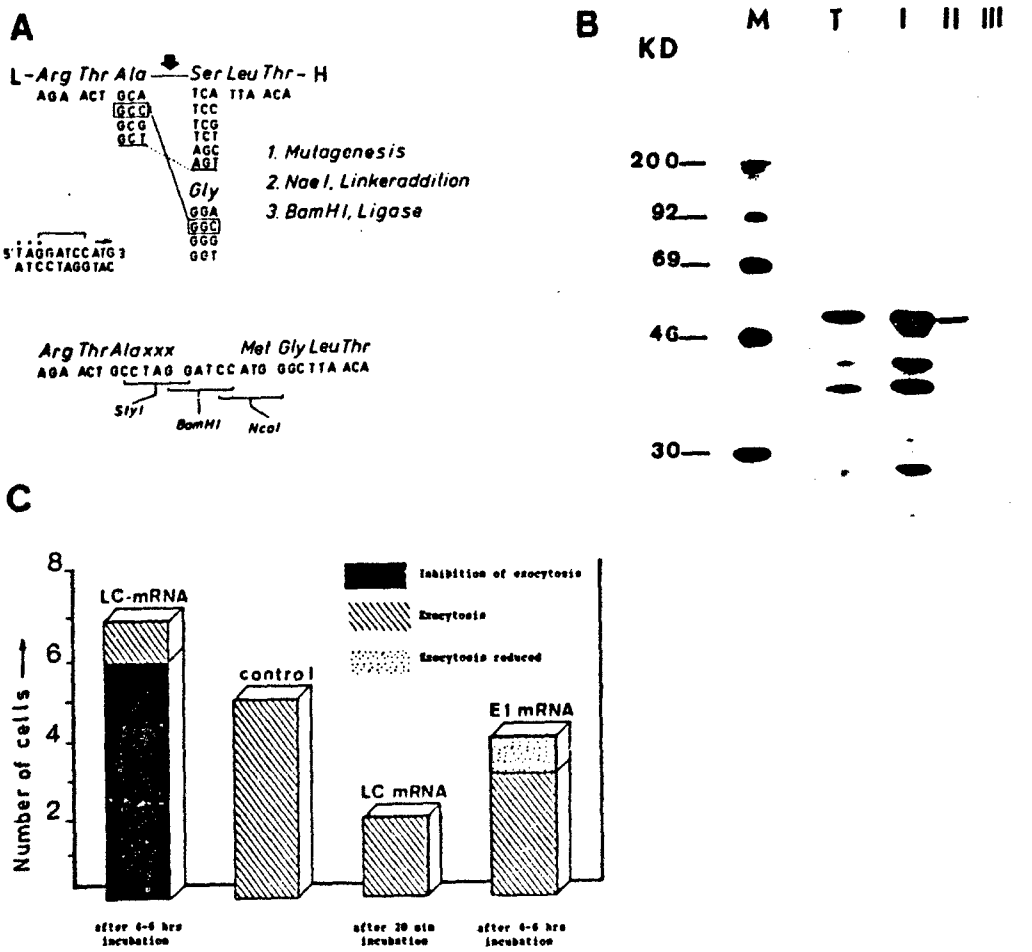


Fig. 2: In vitro transcription/translation of the authentic A-subunit of tetanus toxin from cloned DNA. For details see the text and reference (8). Capped and polyadenylated mRNA encoding fragment A of tetanus toxin or the coronaviral glycoprotein E1 was injected into single bovine chromaffin cells. The cells were incubated for the time indicated and the cell capacitance was measured by the patch clamp technique using the whole cell configuration.

We applied site directed mutagenesis and insertion of a synthetic oligonucleotide linker to introduce a codon for termination of translation into the position corresponding to the proteolytic cleavage site between the A and BC-subunits (Fig. 2A). A DNA fragment encoding the authentic A-subunit of tetanus toxin was generated in this manner and subcloned by means of the newly created BamHI-site into an in vitro transcription vector containing a dA/dT block downstream from a multiple cloning site. 5'-Capped and 3'-polyadenylated mRNA was obtained with SP6-RNA-polymerase and tested by translation in rabbit reticulocyte lysate (Fig. 2B) or microinjection into bovine chromaffin cells (Fig. 2C). Authentic A-subunit was synthesized, as

demonstrated by immunoprecipitation (Fig. 2A). Four to six hours after injection of the biologically stable mRNA into the secretory cells, six out of seven injected cells showed a complete inhibition of exocytosis, as assessed by measurement of the cell capacitance using the patch clamp technique in the WHOLE CELL configuration (Fig. 3C). In contrast, all the control cells (i. e. cells injected with buffer alone or with a control mRNA, or cells measured 20 min after injection) still showed exocytosis. Taken together these results indicated that the A-subunit of tetanus toxin alone is sufficient to block exocytosis. In addition, these data will facilitate future studies involving site-directed mutagenesis to characterize functional subdomains of the A-fragments of tetanus and botulinum type A neurotoxins at the molecular level.

ACKNOWLEDGEMENTS

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Molecular Conformations of Botulinum Neurotoxins

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Botulinum neurotoxins (~150 kDa proteins) are produced by Clostridium botulinum, and are solely responsible for the botulism disease. Seven serologically distinguishable types (A-G) of botulinum neurotoxin have been identified which have similar pharmacological properties (1) with varying degree of toxicity. The neurotoxins exist in single and dichain forms. In the dichain form, the two chains (light chain of ~50 kDa and heavy chain of ~100 kDa) are linked through a disulfide bond. In general, the dichain forms of the neurotoxin are more toxic than the single chain forms (2). Molecular basis of such observation is not clear. In the proposed mode of the neurotoxin action, the neurotoxin binds to the presynaptic membranes of the peripheral nervous system, and the whole or a part of it gets translocated into the cell where it causes the blockage of the acetylcholine release resulting into flaccid paralysis (3). In this process, the two chains seem to act in a coordinated manner that could involve specific structural and functional interactions between them.

In order to understand the structural basis of the common biological activity, secondary structures of types A, B and E neurotoxins were analyzed. Majority of the amino acids of the neurotoxins are in ß-sheet (40-44%) and α-helical (21-28%) conformation, and no obvious correlation was noticeable between the degree of ordered structures and the degree of toxicity. It is interesting to note that the secondary structures of botulinum neurotoxin estimated in this study differ significantly from those of tetanus neurotoxin (35% α-helix and 30% ß-sheets; 4) which is structurally and functionally related to the former. Tertiary structural parameters such as intrinsic fluorescence quantum yield and degree of Tyr exposure indicated considerable differences in the three neurotoxin types studied. For example, Tyr exposure in type E neurotoxin was only 61% whereas it was 84% in type A. Whether or not such a difference has any physiological implications needs further study.

Interactions between the two subunit chains of botulinum neurotoxin may play significant role in its biological functions. An

indication of specific interactions could be derived if the conformations of the two chains are altered upon their separation. Secondary structural analysis of the two chains and the dichain type A neurotoxin indicated virtually no change in any of the estimated secondary structural parameters of the two chains upon their separation. For example, a weighted mean of the α-helical contents of light (22.00%) and heavy (18.75%) chains was 19.83%, virtually same as that observed for the dichain neurotoxin (20.00%). The degree of Tyr exposure was considerably higher in the heavy (81.5%) than in the light (46.4%) chain. However, a weighted mean of the Tyr exposure in light and heavy chains (69.6%) was similar to the Tyr exposure observed for the dichain neurotoxin (70.7%). These observations suggest that the two subunit chains of the neurotoxin exist as two independent domains. But, intrinsic fluorescence studies and the analysis of the circular dichroic signals (CD) of the aromatic amino acid residues indicate a significant alterations in the polypeptide segments containing Trp residues. Trp fluorescence quantum yields of light and heavy chains were 0.072 and 0.174, respectively. The Trp fluorescence quantum yield of the dichain type A neurotoxin (0.197) was ~29% higher than the weighted mean of the quantum yields of the light and heavy chains (0.153). Similarly, the CD signal corresponding to Trp residues (at 292 nm) was ~31% higher in the dichain neurotoxin relative to the weighted mean of the corresponding CD signals of the light and heavy chains. These results indicate significant changes in the polypeptide folding of the two chains upon their separation suggesting specific interactions between them.

The results presented above suggest the following:

1. Botulinum neurotoxins (A, B and E) have similar amount of secondary structures with high ß-sheets and low α-helix.
2. The neurotoxins differ significantly in tertiary structural parameters such as Tyr exposure and Trp fluorescence quantum yield.
3. Secondary structures of the light and heavy chains remain unchanged upon their separation.
4. Tertiary structural folding of the light and heavy chains changes considerably upon their separation.
5. The two subunit chains seem to exist as semi-independent domains in the dichain botulinum neurotoxin.

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Determination of the Complete Nucleotide Sequence for the Neurotoxin of Clostridium botulinum Type A

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INTRODUCTION

Toxigenic strains of Clostridium botulinum produce one or more of 7 immunologically distinct neurotoxins (A-G) which cause paralysis by inhibiting the release of acetylcholine from presynaptic nerve terminals. C. botulinum toxins are thought to be similar in secondary structure and reaction mechanism to C. tetani toxin, and are synthesised as a single polypeptide chain comprising of a light (L-) chain of 55 kDa and a heavy (H-) chain of 95 kDa (for review, see 1). To facilitate our understanding of these toxins we have determined the complete primary amino acid sequence of the type A neurotoxin, by nucleotide sequence analysis of the encoding gene.

CLONING STRATEGY

The amino acid residues which exhibited the least translational degeneracy (6-14) were selected from the determined N-terminal sequence of purified H-chain and used to design a 26-mer oligonucleotide probe. Southern hybridisation studies, using genomic DNA from C. botulinum type A and the radiolabelled probe, enabled the construction of a restriction map of the region encoding the neurotoxin gene.

Toxicity of the molecule is lost if the L- and H-chains are separated, or if the C-terminus of the H-chain is removed (1). The estimated size of the neurotoxin gene is 4 kb. It follows that a 2 kb fragment should prove nontoxigenic when cloned into E. coli. The restriction map showed that amino acids 6 through 14 were encoded by a 5 kb PvuII fragment. Subsequent digestion of purified 5 kb PvuII fragments with TaqI generated a 2 kb fragment which hybridised to the radiolabelled probe. The PvuII/TaqI fragments were isolated, cloned into pMTL23 and a positive clone (pCBA2) was identified by *in situ* colony hybridisation.

Comparison of the nucleotide sequence of the insert with tetanus toxin revealed that 50% of the L-chain and 38% of the H-chain had been

cloned. Specific subfragments of the pCBA2 insert were radiolabelled and used as probes to identify and clone the genomic fragments encoding the 5'- and 3'-end of the toxin gene. The data obtained indicated that a 3.4 kb HpaI/PvuII fragment and a 2.4 kb TaqI/ScaI fragment encoded the 3'- and 5'-end of the gene, respectively. Positive clones containing the 5'-end (pCBA4) and the 3'-end of the gene were identified and the nucleotide sequence of the cloned inserts determined.

The probability of co-cloning of adjacent sequences of the neurotoxin gene was remote because digestion of the genomic DNA with HpaI/PvuII cleaves the 5'-end of the gene resulting in a 0.5 kb HpaI, a 0.9 kb and a 0.25 kb HpaI/PvuII fragment. Similarily cleavage with TaqI/ScaI cleaves the central portion of the gene into a 0.55 kb TaqI/ScaI, 0.55 kb and 0.75 kb ScaI fragment.

FEATURES OF THE TOXIN GENE

The deduced neurotoxin peptide contains 1295 amino acid residues and has a predicted molecular weight of 149580 Da. Discrepancies with previously determined protein sequences were apparent, e.g., Gln at position 2 rather than Pro (2), Glu at position 480 instead of Pro (3) and Thr and Ser at positions 876 and 896 rather than Leu and Lys (4). The L-chain was analysed with amino acid sequence fingerprinting for the presence of a classical ADP ribose binding fold (see 1), but no such sequence was found.

The comparative alignment of botulinum and tetanus toxin showed that they share 33.4% identity. The cysteine residues conserved at position 430 and 454 probably participate in the disulphide bridge linking the L and H-chains. Further analysis by a dot matrix plot demonstrates that the H-chain of the 2 proteins share a greater degree of homology than the L-chains. The most extensive homology occurs at the N-termini of the H-chain where hydrophobic regions are conserved. This domain is implicated in channel forming activities (see 1).

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Partial Amino Acid Sequence of the Clostridium botulinum Neurotoxins B and E

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INTRODUCTION

Clostridium botulinum is able to produce a high molecular weight protein of extreme neurotoxic capacity. This toxin is the causative agent of botulism. Various immunological types (A to G) are known of which mainly types A,B and E are involved in human intoxication. Presence of C.botulinum in a sample is determined by enrichment culturing and subsequent testing of the culturing fluid for botulinum toxin. Uptill now the most reliable method for this test is the mouse bio-assay. An alternative method is the use of DNA hybridization to detect the organism directly. However to develop a suitable DNA probe, information is needed on the nucleotide sequence of the toxin genes. We therefore decided to clone and sequence parts of the genes. As a result of the these data we have been able to deduce the partial amino acid sequence of the toxin type B and E.

MATERIALS AND METHODS

Chromosomal DNA was isolated from C.botulinum (strains B/Okra and E/Beluga) and subsequently digested with various restriction endonucleases. The reaction mixtures were fractionated on an agarose gel, DNA fragments of $\leq$ 1 kb. in size were isolated and ligated into linearized pUC19-vector. By transformation E.coli recombinants were obtained and those containing parts of the C.botulinum toxin genes were identified by hybridization with synthetic oligonucleotide probes, their sequencing being based on reverse translation of concise amino acid sequence data from literature (5). Positive clones were subjected to DNA sequencing and the amino acid sequence encoded is presented here.

RESULTS AND DISCUSSION

In fig. 1 the partial amino acid sequences are given for type B and E toxin, starting at the N-terminal end of the L-chain subunit.
Comparison of these data with direct amino acid sequencing data from literature (1,3,5) shows that:

a) the N-terminal methionine residue is not found in the toxin. This is probably removed by processing.
b) residues 2-17 from the deduced B/Okra sequence are in agreement with the first 16 residues known from direct amino acid sequencing (5). However residue 30 (threonine) differs from the methionine which is found at the corresponding site in neurotoxin from strain B/657 (1). This may cohere with the observation that type B toxins from different strains can display different immunological behaviour (4).
c) residues 2-48 of the deduced E/Beluga sequence match the first 47 known amino acids of the type E toxin (3). This type of toxin, isolated from various C.botulinum strains, has been found to display no immunological variations (personal observation).
d) considerable homology is present between C.botulinum neurotoxins and the tetanus toxin from C.tetani (2).

Elucidation of the complete amino acid sequence of the C.botulinum neurotoxins will enable determination of functional domains and their modes of action.

```
B/Okra:    10          20          30
           |           |           |
1 MPVTINNFNY NDPIDNNNII MMEPPFARGT GRYYKAFK

E/Beluga:  10          20          30          40          50          60
           |           |           |           |           |           |
  1 MPKINSFNYN DPVNDRTILY IKPGGCQEFY KSFNIMKNIW IIPERNVIGT TPQDFHPPTS
 61 LKNGDSSYYD PNYLQSDEEK DRFLKIVTKI FNRINNNLSG GILLEELSKA NPYLGNDNTP
121 DNQFHIGDAS AVEIKFSNGS QDILLPNVII MGAEPDLFET NSSNISLRNN YMPSNHRFGS
181 IAIVTFSPEY SFRFNDNSMN EFIQDPALTL MHELIHSLHG LYGAKGITTK YTITQFQYPL
241 ITNIRGTNIE EF
```

Fig. 1: Partial amino acid sequences.

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The Analysis of the Clostridium Difficile Toxin A Gene Reveals the 3' End to be Composed of a 2499 bp Repetitive Structure

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Clostridium difficile produces two toxins A and B which are major factors of pathogenicity. Because toxin A is a one chain protein of 300 kDa the encoding gene segment should be 8000-9000 bp. We have already cloned a toxin A gene fragment by colony blotting (v. Eichel-Streiber et al., 1989). The aim of the work presented was to isolate clones adjacent to pCd14 and pCd13 and furtheron to sequence parts of the toxin A gene (toxA). These studies should lead to a better understanding of the molecular architecture and the mechanism of action of this potent toxin.
The toxA gene-fragment could be extended from 10 to 20 kb by chromosome walking. The determination of the direction of transcription proves that pCd122 lies upstream of pCd14 and pCd107 lies downstream of pCd13 (Fig.1). This extended DNA-fragment contains the total toxA. To sequence toxA overlapping plasmid clones were created either by

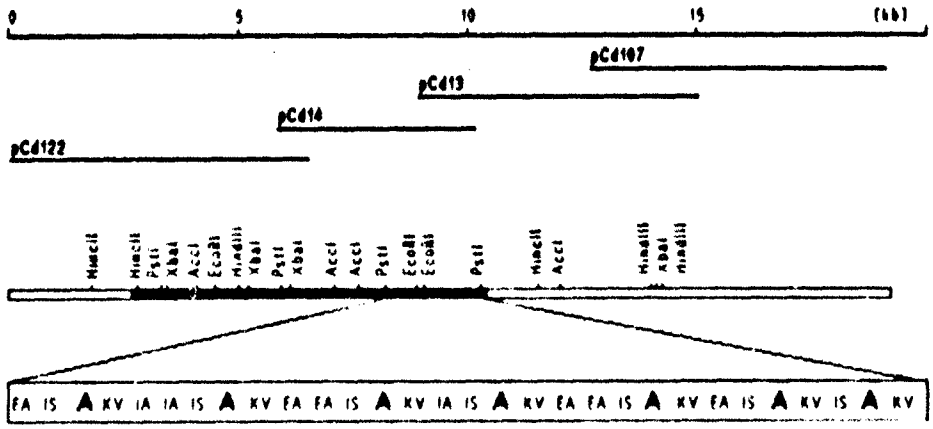


Fig 1: Physical map of Clostridium difficile toxA

subcloning of restriction fragments or according to the method of Henikoff (1987). These clones were used to determine a nucleotide sequence of 8815 bp length by Dideoxy-sequencing of plasmid DNA using T7-DNA-Polymerase. Only one open reading frame was detected which was large enough to cover the toxin A. The molecular weight of the protein deduced from this sequence is 309kDa and the isoelectric point pK_i is 5,35. The ATG-codon of the toxin A gene lies between the two HincII sites at 2,6 kb of the physical map followed by the 8142 bp open reading frame ended by a tandem stop codon. The 3' end of toxin A is located at 10,5 kb of the physical map, 90 bp downstream of the Pst I site. There is a Shine-Delgarno sequence in front of the gene and a rho-independent terminator of transcription at the end. Upstream of the 3' end of toxA a 2499 Bp repetitive segment was identified which consists of 9 different SRON's (shortest repetitive oligonucleotides). The self homology of toxin A at the C-terminus could also be shown by a dot matrix plot, indicating the repetitive protein segment. A group of five CROP's (combined repetitive oligopeptides) was created by combining three individual SRON's. One third of the toxin A protein consists of repeats of the five different CROP's. The box in the physical map marks the protein segment which consists of repetitions of CROP's. Consensus sequences of the five CROP's are presented in Tab.1.

KV	KAVTGWQTIBGKKYYFNPNTA
EA	EAATGWQTIBGKKYYFNTNTA
IA	IAAVGLQTIBNBKYYFBTDTA
IS	IASTGYKTIBGKHFYFBTDGI
ALICE	MDIGVFKGPNGFEYFAPANTDANNIEGQAIVYQNKFLTLNGKKYYFGNRS

Tab 1: Consensus sequences of the five CROP's (combined repetitive oligopeptides)

There are two major hydrophobic blocks within toxin A. The first is positioned in the center of toxin A between amino acids 950 and 1200. The second hydrophobic block is located at the C-terminus close to the repetitive structure. Within the repeats there are no major hydrophilic or hydrophobic areas. Instead there are remarkable drops in hydrophilicity within each of the highly conserved CROP's which we call ALICE (see Tab.1).

Using the "variability index" of the individual CROP's two large and two small clustered domains of conservation and three domains of high variability were detected within the repetitive area. Within the ocean of four relatively homologous CROP's (KV, IA, IS, EA) there lies the fifth highly conserved oligopeptide like an island. We call these islands ALICE ("Antigen Locus by Insistent Computer Evaluation"). ALICE is remarkable because it is highly conserved, always includes a huge drop in hydrophilicity, lacks structured areas, and possibly contains a major antigenic site. This assumption is confirmed by the reactivity of two monoclonal antibodies. The mAbs PCG-4 (Lyerly et al., 1986) and 1337C8 (v.Eichel-Streiber et al.1989) react only with toxin A and precipitate this toxin in the Ouchterlony test. Subcloning experiments show that the mAb 1337C8 binds to an epitope which is part of the repetitive structure.

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Chemical Modification of S. aureus α-toxin by Diethylpyrocarbonate: Effects on its Channel-Forming Properties

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INTRODUCTION

Staphylococcus aureus α-toxin is a cytolytic exotoxin secreted as a single water-soluble 33 kDa polypeptide with pI 8.5 (1). It causes erythrocyte lysis and damage to nucleated cells. Current evidences indicate that α-toxin oligomerizes on the cell surface forming an amphiphilic hexamer that inserts into the lipid bilayer generating a transmembrane channel responsible for ion leakage. A colloid-osmotic shock follows leading to the lysis of the membrane and eventually to the death of the cell (2).
Consistently with this hypothesis we have observed that α-toxin interacts with planar lipid membranes and small unilamellar vesicles by opening ionic channels and that the aggregation of several toxin monomers is required for the formation of one pore (3,4).
These water-filled pores have an average diameter of 11±1 A and are rather anion-selective. The selectivity increases at low pH suggesting that it is due to the overall positive charge of the toxin below pH 8.5.
To investigate the role of the electric charge of this protein on its pore-forming properties we have modified it by treatment with diethylpyrocarbonate (DEPC) which modifies histidyl and lysyl residues removing the positive charge on their amino-group (5).

MATERIALS AND METHODS

Liophilyzed α-toxin from Staphylococcus aureus was kindly gifted by Dr. Hungerer (Behring, Marburg, FRG). Carbethoxylation with DEPC and decarbethoxylation by hydroxylamine were performed essentially as described by Miles (5). The number of modified histidine residues per α-toxin monomer was caculated from the increase in absorbance at 240 nm using an absorption coefficient of 3.2 10^3 M^-1 cm^-1 (5).

RESULTS

The extent of histidyl modification can be followed spectrophotometrically and saturates at about three histidines modified per α-toxin monomer, which is the known histidine content of α-toxin (6). As expected histidine modification is to a good deal reversible by treatment with hydroxylamine.
We have found that modification of even one of the three histidines of this toxin is enough to impair its activity on red blood cells and platelets whereas only two histidines are essential for its activity on model membranes such as lipid vesicles and planar bilayers. Loss of activity is mainly due to defective binding but the oligomerization step is also inhibited. All the activity is restored by hydroxylamine treatment.
We think that the two essential histidines of α-toxin are located one in the lipid binding site and the other in the hexamerization promoting site. Consistently when α-toxin hexamers are assembled onto lipid vesicles two of the histidines become protected from DEPC modification.
Native-page and isoelectric-focusing gel-electrophoresis reveal that DEPC modifies the charge of the toxin in discrete steps, probably by modifying some of its lysyl residues. Consistently these effects are unaffected by hydroxylamine treatment. Modification of a few lysyl residues alone does not reduce the activity of the toxin either on cells or on model systems but decreases the conductance and the selectivity of the pore. Even chemical modification of preformed channels in planar bilayers (where histidines are protected) produces a linearization of their current-voltage characteristic and a reduction of their anionic selectivity. This indicates that positively charged lysins are actually located in the lumen of the pore and thus are important in determining its electrical properties and that they remain accessible from the solution.

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Site-Directed Mutagenesis of eta Gene of Staphylococcus Aureus

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The DNA encoding the exfoliative toxin A gene (eta) of Staphylococcus aureus 50586 was cloned within a 3.5 kb Hind III chromosomal fragment into plasmid pUC 18. The dideoxy chain terminator sequencing method was used to determine the sequence of the eta-containing cloned fragment. The eta gene was expressed from its natural promoter in E. coli JM 83, but the synthesized protein (ETA) was not secreted in culture supernatant. It reacted with polyclonal antibodies and was biologically active. The 3.5 kb Hind III fragment was then cloned into a shuttle plasmid pLP 1202, and S. aureus RN 4220 was transformed by electroporation. The toxin was secreted in the culture supernatant by this transformed strain.

The structure-function relationships of this toxin were investigated by site-directed mutagenesis using several synthetic oligodeoxynucleotides to induce deletions in different regions of the gene. Length of deletions ranged from 4 to 28 aminoacids residues.

Deletions of 15 and 28 aminoacids in the signal sequence prevented the secretion of toxin by S. aureus RN 4220 in culture supernatant, but cell lysates contained an active toxin. Deletions of 10 and 20 aminoacids from the NH_2-terminus of the protein led to the excretion of non active toxin which remained recognized by polyclonal antibodies.

Three homologous regions exist within ETA and ETB protein sequences (1) : the first between the aminoacids + 41 and + 69, the second between + 99 and + 124 and the third between + 198 and + 209. Deletions were carried out in these three sequences. In the first region a deletion of 13 aminoacids (from + 56 to + 69) inactivated the toxin and conserved the immunological reactivity whereas another deletion of 4 aminoacids (from + 41 to + 45) did not suppress the biological activity of the toxin and conserved the immunological reactivity. In the second sequence of homology, a deletion of 11 aminoacids (from + 99 to + 110) inactivated the biological effect and suppressed the immunological

reactivity and another deletion of 9 aminoacids (from + 115 to + 124) suppressed the biological activity but not the immunological reactivity. Finally, a deletion of 15 aminoacids (from + 188 to + 203) in the third zone of homology inactivated the biological and immunological activities of the protein.
Some regions in mature ETA seemed therefore to be important for the biological activity, or have a crucial role for the immunological reactivity but did not interfere with the secretion. Only mutations within the signal sequence alter the export of the molecule. More smaller deletions and point mutations will be done to determine the essential aminoacids for activity or immunogenicity. Further studies are needed to quantify the specific activities of the obtained mutants and to determine the exportation rate of these toxins through the staphylococcal envelopes.

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Putative Functional Domains of Protein B (CAMP-Factor) Predicted from Sequence Comparisons

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INTRODUCTION

CAMP-factor was first described in 1944 by Christie, Atkins and Munch-Petersen as an extracellular product of group B streptococci, which causes hemolysis of sphingomyelinase-treated sheep erythrocytes (1). In addition to its well known co-hemolytic activity a recent study has shown that CAMP-factor binds in a non-immune reaction to the Fc-part of immunoglobulins (4). It hence exhibits similar properties as protein A of Staphylococcus aureus and protein G of group G streptococci. Therefore, CAMP-factor was designated "protein B".

The presence of two different biological activities in protein B such as co-hemolysis and Fc-binding, rises the question whether these functions may be associated with different domains. The knowledge of the complete amino acid sequence of protein B (5) enabled now the application of theoretical approaches (e.g. hydrophobicity analyses, sequence comparisons or secondary structure predictions) for further characterization of these domains. This report describes partial sequence similarities between protein B and apolipoprotein A-IV, tropomyosin 2 and protein A. In addition, the significance of these similarities is discussed with respect to structure/function relationship of protein B.

MATERIAL AND METHODS

The sequence comparisons have been performed as described (6).

RESULTS AND DISCUSSION

Comparison with apolipoprotein A-IV: Comparison of protein B (pos. 1 - 118) and apolipoprotein A-IV (pos. 144 - 256) revealed 32 % similarity between both sequences when 3 breaks were introduced for optimal alignment (alignment score 1.9 SD).

The similarity is restricted to that segment of protein B, which corresponds almost to the 9 kDa CNBr-fragment (6). Interestingly, this CNBr-fragment has been shown to bind to lipid suspensions, whereas the 13.5 kDa CNBr-fragment did not bind (7). In addition, the four potential amphiphilic helices, which may be involved in protein/lipid interaction are located within the same segment of protein B (6).

We think that the similarity between a partial sequence of protein B and a segment of the putative lipid-binding domain of apolipoprotein A-IV reflect a common function of both sequences, namely to interact with lipids.

Comparison with tropomyosin 2: Comparison of protein B (pos. 113 - 166) and tropomyosin 2 (pos. 198 - 253) revealed 51 % similarity between both sequences when 3 breaks were introduced for optimal alignment (alignment score 5.0 SD).

The functional consequences of this obvious similarity are yet not known. With respect to the similarity with tropomyosin protein B resembles staphylococcal protein A and streptococcal M protein, which also exhibit significant similarity with myofibrillar proteins such as tropomyosin, myosin, and actin (3).

Comparison with protein A: Comparison of protein B (pos. 129 - 217) and protein A (pos. 182 - 267) revealed 40 % similarity between both sequences when 2 breaks were introduced for optimal alignment (alignment score 5.5 SD).

The 89-residue string of protein B, exhibiting similarity with a part of the Fc-binding domain of protein A (Fc-binding regions B and C), corresponds almost to the 13.5 kDa CNBr-fragment (5). Interestingly, this CNBr-fragment showed unspecific binding to immunoglobulins, whereas no binding was found for the 9 kDa CNBr-fragment (2).

In conclusion, the experimental data from studies with purified CNBr-fragments (2, 7) and the results from sequence comparisons (5, 6) supports the assumption, that (i) the amino-terminal domain may be involved in lipid-binding whereas (ii) the carboxy-terminal domain may be responsible for Fc-binding.

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Structure/Function Analysis of IL-2-Toxin: Fragment B Sequences Required for Cytotoxicity

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INTRODUCTION

Interleukin-2-toxin (IL-2-toxin) is a 68,142kDa fusion protein in which amino acids 2 through 133 of interleukin-2 are fused to Ala485 of diphtheria toxin (1). This fusion toxin has been shown to combine the receptor binding specificity of IL-2 with the cytotoxic potency of diphtheria toxin. In order to further our understanding of IL-2-toxin and to clarify the role of diphtheria toxin fragment B sequences in the fusion protein, we have used cassette exchange and deletion mutagenesis techniques to assemble a family of IL-2-toxins having lesions within the fragment B domain. Following purification, wild type and mutant proteins were assayed for their ability to inhibit protein synthesis in IL-2 receptor positive Hut102/6TG human T cells. The ultimate goal of these studies is to define the precise location of those domains required for the membrane translocation of fragment A into the eukaryotic cell cytosol.
**NOTE: The nomenclature adopted for "wild type" IL-2-toxin is DAB_{486}-IL2. Amino acid numbering in DAB_{486}-IL2 is out of phase with that of native diphtheria toxin by one amino acid due to the deletion of the diphtheria toxin signal sequence and the addition of a methionine residue at the amino terminus of the fusion protein.

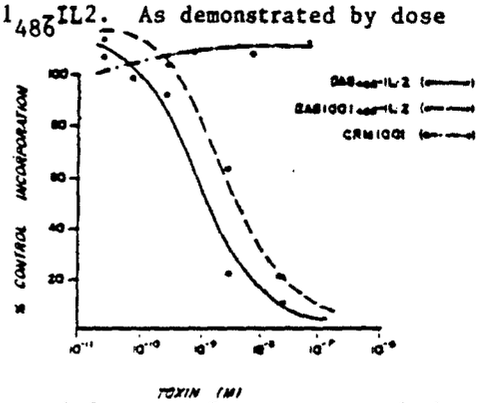
MATERIALS AND METHODS

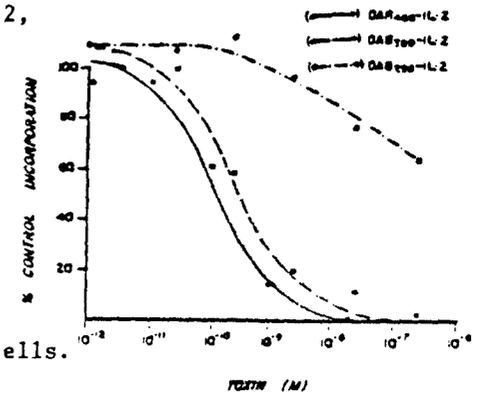
All fusion proteins used in this study were expressed in the cytoplasm of Escherichia coli strain JM101 at 30°C from the inducible promoter,trc(2). Clarified bacterial lysates were applied to an anti-diphtheria toxin immunoaffinity column at 4°C. Immunoreactive material was eluted with 4M guanidine hydrochloride in phosphate buffer, pH7.2, treated with 1% 2-mercaptoethanol, and subjected to size fractionation by HPLC. Cytotoxicity assays were performed as described by Williams et. al. (1). The inhibition of protein synthesis is expressed as the percentage of [14C]-leucine incorporated by toxin treated cells compared with untreated controls. Standard methods were used for all DNA manipulations.

RESULTS AND DISCUSSION

The non-toxic diphtheria toxin mutant, CRM1001, had been found to carry

a single missense mutation, Cys471 to Tyr471, which prohibits the formation of the disulfide bond within fragment B (3). Although CRM1001 is enzymatically active, its lack of cytotoxicity suggests that the Cys461-Cys471 bond is essential for internalization/processing of the toxin. Assuming that the IL-2 component of DAB_{486}-IL2 constitutes a complete and independent binding domain, it was anticipated that the incorporation of the CRM1001 mutation into the gene encoding IL-2-toxin would allow us to evaluate the role of the Cys462-Cys472 disulfide bond in the internalization/processing of the fusion toxin. We have incorporated the Cys to Tyr mutation into the coding sequence for IL-2-toxin by exchanging an AccI-SphI restriction fragment with the analogous fragment from the tox1001 allele. The resulting toxin has been designated $DAB1001_{486}$-IL2. As demonstrated by dose response analysis (Figure 1), the absence of a disulfide bond in $DAB1001_{486}$-IL2 did not effect the potency of the toxin. Given that $DAB1001_{486}$-IL2 retained full cytotoxicity, it was of interest to determine what portion of fragment B was required to facilitate the cytotoxic action of fragment A. Several deletion mutants were constructed by removing restriction fragments within the fragment B encoding portion of the DAB_{486}-IL2 gene. The reading frame between truncated fragment B sequences and the IL-2 encoding domain was restored via the insertion of an oligonucleotide linker. In the first mutant, DAB_{389}-IL2, 97 amino acids were removed between Thr387 and Ala486. The diphtheria toxin component of DAB_{389}-IL2 is analogous to the non-toxic diphtheria toxin mutant, CRM45. The second deletion mutant DAB_{295}-IL2, has an additional 94 amino acids deleted from fragment B. This deletion includes two putative membrane-spanning helices thought to play a role in the membrane translocation of fragment A (4). As can be seen from the dose response curves in Figure 2, treatment of Hut102/6TG cells with full length IL-2-toxin inhibited protein synthesis by 50% (IC50) at a concentration of 4×10^{-10} M. Similarly, the IC50 for DAB_{389}-IL2 was 2×10^{-10} M. In marked contrast, the cytotoxicity of DAB_{295}-IL2 was reduced by 10,000-fold. These results strongly suggest that, in the case of IL-2-toxin, the transmembrane domains between Thr301 and Phe369 are essential for delivery of fragment A to the cytosol of target cells.





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Toxin Binding and Internalization

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Translocation of Diphteria Toxin across the Plasma Membrane

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INTRODUCTION

The normal entry mechanism of diphtheria toxin involves binding to surface receptors, internalization from coated pits and delivery to endosomes (1). Here the low pH induces a conformational change in the B-fragment such that normally hidden hydrophobic domains become exposed and insert themselves into the membrane. If acidification of endosomes is prevented, translocation of the A-fragment to the cytosol does not occur and protein synthesis is not inhibited (2, 3).

Translocation of diphtheria toxin across the limiting membrane of the endosomes is difficult to study in detail, because the conditions on the two sides of the membrane cannot be manipulated in a controlled manner. However, when cells with surface-bound toxin are exposed to acidic medium, translocation across the plasma membrane is induced. This is a system amenable to experimental manipulations (3). To reduce endocytic uptake, the toxin can be bound to the cells at low temperature, and translocation can then be induced by a brief exposure to pH < 5.3 at 37 °C. The experiment mimics at the cell surface a process that normally occurs in the endosomes.

The conditions on the two sides of the plasma membrane can be manipulated in a number of ways. The membrane potential can be altered, and the ionic conditions and the pH can be changed independently in the cytosol and in the external medium. Diphtheria toxin translocation both from endosomes and from the cell surface are not very efficient processes. In fact, the rate of ADP-ribosylation of elongation factor 2 in intoxicated cells can be accounted for by entry of only a few per cent of the total number of surface-bound toxin molecules (4).

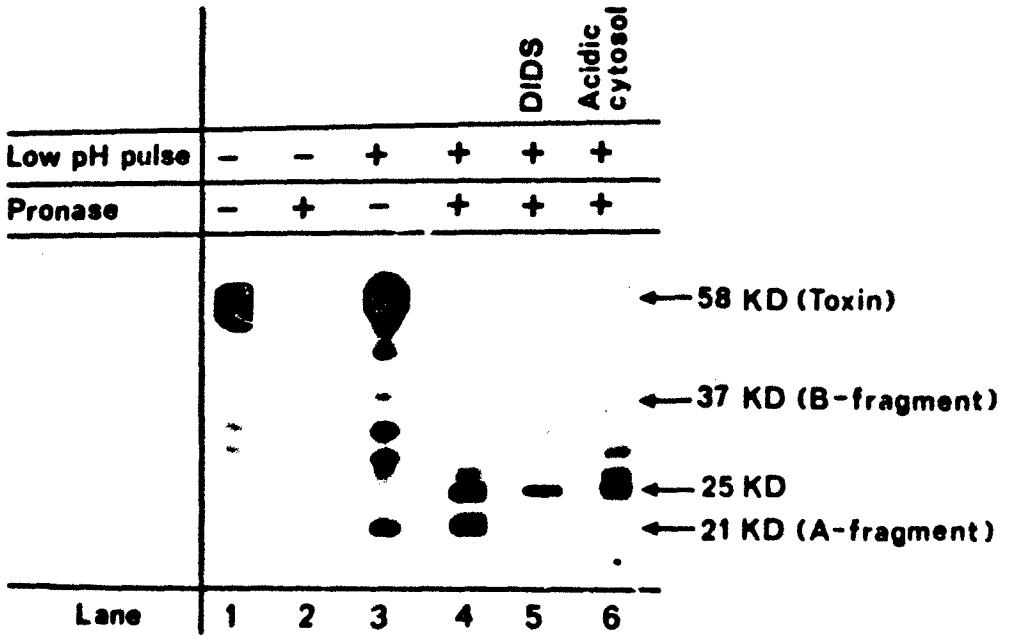


Fig. 1. Translocation of nicked diphtheria toxin across the plasma membrane. Lane 1: ^{125}I-labelled nicked toxin was bound to cells, which were then dissolved under non-reducing conditions and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Lane 2: As lane 1, but the cells were treated with pronase before they were lysed. Lane 3: As lane 1, but the cells were exposed to pH 4.5 for 2 min before lysis. Lane 4: As lane 3, but after being exposed to low pH, the cells were treated with pronase and then lysed. Lane 5: As lane 4, but 0.1 mM DIDS was present during the exposure to low pH. Lane 6: As lane 4, but the cytosol was acidified by treatment with acetate before exposure to low pH. (Data from refs. 4 and 5).

REQUIREMENTS FOR TRANSLOCATION

When toxin entry was induced as described above, the disulfide that links the A- and the B-fragment was reduced in 5-10 % of the toxin molecules (5). The B-fragment was rapidly cleaved to smaller components (Fig. 1, lane 3). The reduction is presumably due to exposure of the inter-fragment disulfide to the reducing conditions in the cytosol. A similar percentage of the toxin molecules were transferred to a position where they were shielded from degradation by externally added pronase (4). It therefore appears that 5-10 % of the surface-bound toxin molecules are translocated to the cytosol. In these molecules, the whole A-fragment was protected against pronase, while only a 25 KD piece of the B-fragment was protected (Fig.1, lane 4).
Permeabilization of the pronase-treated cells with saponin releases cytosolic components such as lactate dehydrogenase. Also the toxin A-fragment was released by the saponin treatment, whereas the 25 KD piece of the B-fragment remained associated with the membrane fraction (4). The A-fragment

therefore appears to be free in the cytosol, whereas the protected part of the B-fragment is presumably inserted into the plasma membrane.

The main requirements for translocation of the toxin appear to be an inward directed proton gradient. In the absence of permeant anions translocation of the A-fragment is inhibited, whereas insertion of the 25 KD polypeptide takes place. Similar results are obtained in the presence of anion transport inhibitors, e.g. 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) (Fig.1, lane 5).

It appears that a transmembrane pH-gradient of at least 1 pH-unit is necessary for entry of the toxin (7). Thus, when the pH-gradient was reduced by acidification of the cytosol, the cells were not intoxicated when exposed to low pH. In accordance with this, the toxin A-fragment was not protected against pronase under the same conditions (4). On the other hand, insertion of the 25 KD piece of the B-fragment into the membrane occurred even in cells with acidified cytosol (Fig.1, lane 6).

Possibly, unfolding of the toxin is induced by the exposure to the low pH. When the translocated toxin is subsequently exposed to the neutral pH in the cytosol, the A-fragment may refold. This refolding could provide energy for the transfer across the membrane.

FORMATION OF CATION SELECTIVE CHANNELS

Diphtheria toxin and toxin fragments containing the hydrophobic regions of the B-fragment are able to form ion-conducting channels in lipid bilayers (8,9). Also when the toxin was inserted into the surface membrane of Vero cells by exposure to acidic medium, ion channels were formed (10). Thus there was a strong increase in the influx of $^{22}Na^+$ and $^{86}Rb^+$ and efflux of K^+ under these conditions.

To study in more detail the formation of channels, a panel of plasmid constructs with N-terminal deletions of the toxin was made. Diphtheria toxin with Glu_{148} changed to Ser_{148} to strongly reduce the toxicity was used (11). Fig. 2 A shows a scheme of the resultant proteins compared to the whole toxin mutant. Plasmid templates were transcribed and translated in vitro in the presence of [^{35}S]methionine (12).

In vitro synthesised whole toxin (A-58) bound specifically to Vero cells. The binding was abolished when the cells were pretreated with excess unlabelled toxin. However, it bound approximately 5-10 fold less well than ^{125}I-labelled natural toxin. This appears to be due to inhibitory factors in the reticulocyte lysate since binding of natural toxin was similarly inhibited by addition of lysate. The nature of the inhibitor is not known, but it could be oligonucleotides formed by the nuclease treatment to deplete the lysate of endogenous mRNA. Those deletion mutants that were tested for

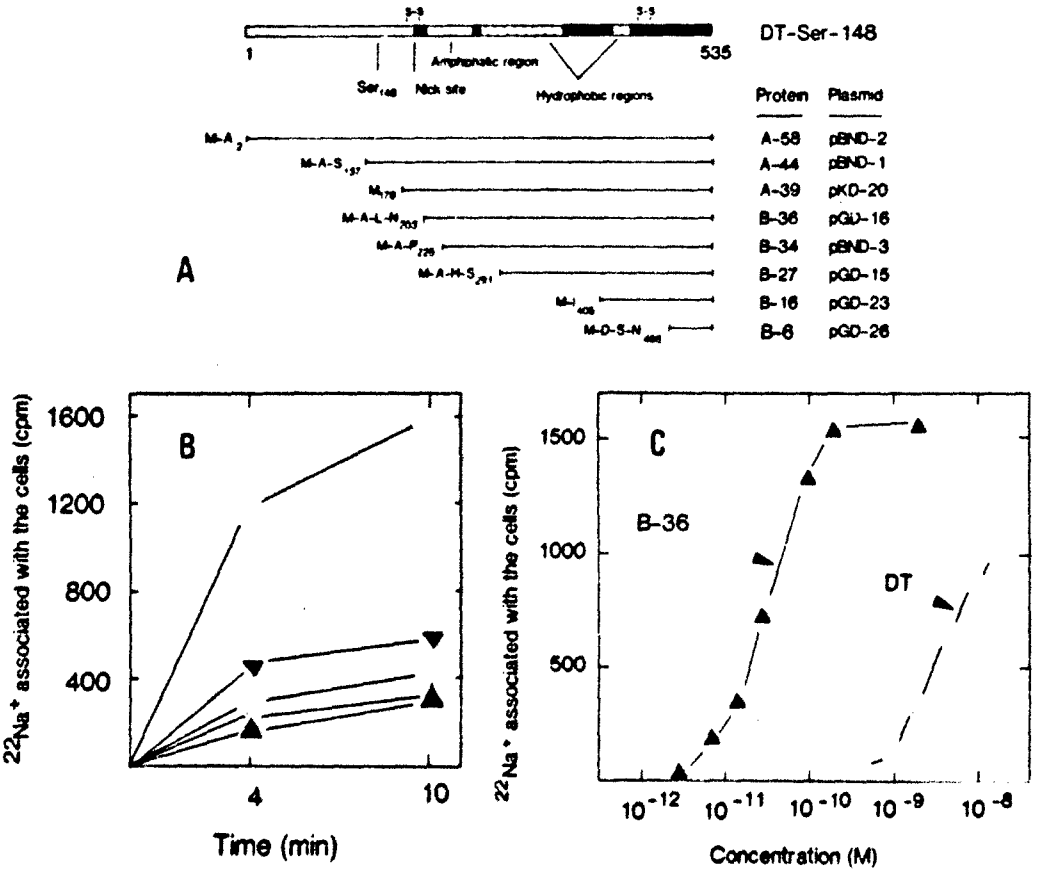


Fig. 2. In vitro synthesised diphtheria toxin mutants. (A) The proteins are shown oriented with respect to a map of whole diphtheria toxin with Glu_{148} changed to Ser (DT-Ser-148). Extra amino acids added to the N-terminal end are shown using the one letter code. (B) Vero cells in 24-well microtiter plates were incubated with and without 100 µl reticulocyte lysate containing 2 nM B-36 for 15 min at 37 °C. The cells were then washed, and Hepes medium, pH 4.8, containing $^{22}Na^+$, was added. The $^{22}Na^+$-influx was measured after the indicated period of time, as described in Materials and methods. (o), B-36; (□), the cells were preincubated with 0.1 µM TPA at 37 °C for 15 min and then incubated with B-36; (△), B-36 in the presence of 1 % horse anti-diphtheria toxin serum; (▲), control, 100 µl reticulocyte lysate that had been incubated without added mRNA; (▼), B-36, 2 mM Cd^{2+} present in the uptake buffer. The symbols represent the mean of two measurements. SEM was in no case more than 9 %. (C) Vero cells in a 24-well microtiter plate were incubated with Hepes medium containing the indicated concentrations of B-36 (▲) or nicked diphtheria toxin (□) for 15 min at 37 °C. The influx of $^{22}Na^+$ during 10 min at pH 4.8 was then measured as described in Materials and methods. (Data from refs. 12 and 13).

trypsin sensitivity (A-44, B-34, B-27) showed almost the same high resistance as diphtheria toxin, indicating that they were folded in a correct manner. On the other hand, cell-bound whole toxin and deletion mutants were usually somewhat degraded, probably by cell associated proteases.

To study whether a truncated toxin molecule devoid of the A-fragment is able to form channels, we studied the mutant B-36, which corresponds to a diphtheria toxin B-fragment where the 9 amino-terminal residues have been replaced by the tripeptide Met-Ala-Leu. The truncated protein binds specifically to toxin receptors and is inserted into the plasma membrane at low pH. B-36 was translated in a rabbit reticulocyte lysate in the absence of radioactive label, then dialyzed and added to Vero cells (13). In a parallel sample, the translation was carried out in the presence of [^{35}S]methionine in addition to the unlabeled methionine, to estimate the amount of translated protein. As shown in Fig. 2B, B-36 increased the influx of $^{22}Na^+$ at low pH, whereas a control lysate (no mRNA added) had no such effect. The $^{22}Na^+$-influx was prevented when the cells were exposed to anti-diphtheria toxin antibodies before acidification. Preincubation of the cells with TPA (phorbol 12-myristate 13-acetate), which strongly reduces the ability of cells to bind diphtheria toxin (14), also prevented the B-36-induced $^{22}Na^+$-influx. These results indicate that B-36 permeabilizes the cells by a receptor-dependent mechanism, as earlier found with whole diphtheria toxin (15).

As with natural diphtheria toxin (10) the formation of channels was strongly reduced in the presence of Cd^{2+} (Fig. 2B). Moreover, channel formation with B-36 depends on the presence of Cl^-, as earlier found with whole diphtheria toxin (10). Also, a transmembrane pH-gradient was required.

The deletion mutants A-44 and A-39 bound specifically and formed channels at low pH in a similar way as B-36. B-34 and B-27 permeabilized membranes in a nonselective way even without exposure to low pH. B-16 and B-6 did not bind to cells in a specific way.

The channels formed with whole diphtheria toxin are closed when a sufficient amount of protons has entered the cytosol (10). Similarly, when $^{22}Na^+$ was added 15 min after exposure of cells with surface-bound B-36 to low pH, very little uptake was observed (data not shown). Altogether, the data indicate that channel formation by the truncated protein B-36 is to a large extent dependent on the same conditions as those required for channel formation by whole diphtheria toxin. The channel formed by natural diphtheria toxin is selective for monovalent cations (10, 16). Although it is not permeable for anions and uncharged solutes such as sucrose, large monovalent cations like choline and glucosamine were found to pass the channel, though at a lower rate than smaller cations, such as $^{22}Na^+$. This was also the case with the channel formed by B-36. B-36 and natural toxin differed in the relative channel-forming efficiency. As shown in Fig. 2 C, about 100 times more diphtheria toxin than B-36 was required to obtain

half-maximal $^{22}Na^+$-influx. The different concentration dependence can partly be explained by the fact that B-36 has about 10 times higher affinity for the receptors than the natural toxin. However, this is not sufficient to account for the ~ 100-fold increase in pore formation, and it can not be the reason why B-36 induces a higher $^{22}Na^+$-influx in Vero cells than a receptor-saturating concentration (20 nM) of natural toxin. It therefore appears that B-36 permeabilizes the plasma membrane more efficiently than whole diphtheria toxin. Consistent with this, B-36 induced a considerably higher $^{22}Na^+$-influx than a saturating concentration of natural diphtheria toxin in A-431 and MDCK cells, which have fewer diphtheria toxin receptors than Vero cells (data not shown).

Native diphtheria toxin forms channels only at pH < 5.5, apparently because a conformational change takes place at this pH (17-19). Since B-36 appears to insert into the plasma membrane at higher pH values than whole toxin, we studied if also the pH dependence of channel formation differed between the two molecules. In fact, at pH 5.6, where B-36, but not whole diphtheria toxin forms channels, the $^{22}Na^+$-influx was strongly inhibited when excess diphtheria toxin was added together with B-36 (data not shown). This indicates that B-36 forms channels only when bound to the specific diphtheria toxin receptors.

The interaction of diphtheria toxin with artificial lipid bilayers has been extensively studied. Although the toxin appears to make channels most efficiently when bound to specific cell surface receptors (15), the whole toxin as well as crm45, a mutant defect in receptor-binding, have the ability to induce ion channels in model membranes at low pH (20, 21). Also a 12 kD cyanogen bromide fragment derived from the middle region of the B-fragment has been shown to induce conductance changes in planar lipid bilayers. It is not known what parts of diphtheria toxin are involved in the channel formation, but a putative amphiphatic helical region at the N-terminus of the B-fragment (22) and a hydrophobic, putative membrane-spanning region in the middle of the B-fragment (11, 22, 23), may play a role. The hydrophobic region is contained within crm45 and partly in the 12 kD channel-forming cyanogen bromide fragment (24). All the membrane-active mutants described in the present paper contain the putative membrane-spanning region, whereas B-16, which did not increase membrane permeability, does not. This supports the idea that the hydrophobic middle region of the B-fragment participates in membrane insertion and channel formation.

FORMATION OF ACTIVE TOXIN IN VITRO

Studies of the entry process has been hampered by the fact that it is considered too dangerous to clone the intact diphtheria toxin gene. Although inactive mutants of the toxin have been cloned, they are of limited value, because the most convenient and sensitive way of measuring translocation is to monitor inhibition of protein synthesis. We have now developed

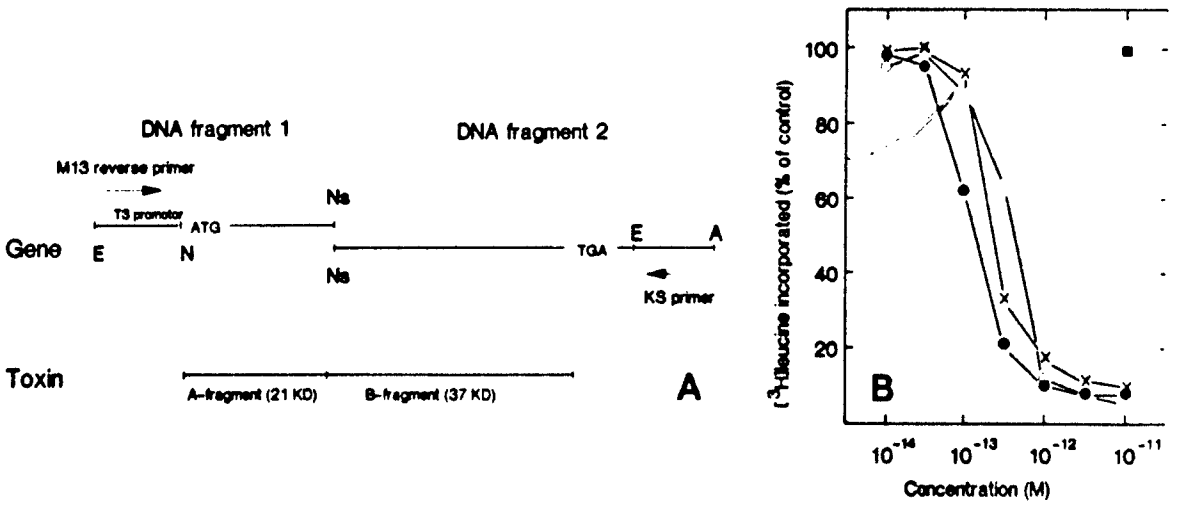


Fig. 3. Production of active diphtheria toxin in a cell-free system. (A) The gene for diphtheria toxin was reconstituted in vitro by ligation of two non-mutated DNA-fragments each coding for a separate part of the toxin. In some cases, the ligated gene was amplified by PCR. The toxin gene was transcribed in vitro with T3 RNA polymerase, and the mRNA formed was translated in a rabbit reticulocyte lysate system. E, EcoRI; N, NcoI; Ns, NsiI; A, ApaI. (B) Increasing amounts of natural diphtheria toxin or toxin formed in vitro were added to Vero cells. After incubation over night, the incorporation of [^{3}H]leucine during 15 min was measured. Serum contains sufficient proteolytic activity to nick the toxin (25). (o), natural toxin; (●), toxin from ligated DNA-fragments; (x), toxin from ligated DNA amplified by PCR; (□), toxin from ligated and PCR amplified DNA + 10 µl horse anti-diphtheria toxin serum; (△), translation product from mRNA transcribed from DNA-fragment 1 alone; (□), 10 µl translation mixture after mock transcription/translation of DNA fragment 2 alone (Data from ref. 26).

a method to produce active toxin without cloning the intact gene.

The gene for the wild type toxin was obtained by ligation of two DNA-fragments coding for complementary parts of the toxin (26). The reconstituted gene was placed behind a T3 RNA polymerase promotor and transcribed and translated in vitro.To increase the amount of toxin gene and to reduce the formation of incomplete toxin, the ligated gene was amplified by PCR. Since amplification is dependent on a primer in each direction, only ligated gene fragments were amplified (Fig. 3 A).

The main translation product coded for by the ligated gene was bound to Vero cells in the same way as ^{125}I-diphtheria toxin and the binding was inhibited by a 100 fold excess of unlabelled toxin. To measure toxicity, dialyzed translation

mixture was added to Vero cells and, after incubation overnight, the ability of the cells to incorporate [^{3}H]leucine was measured. As shown in Fig. 3 B, the translation products from the in vitro spliced gene fragments (both amplified and not amplified) were approximately as toxic as native toxin. The toxic effect was prevented in the presence of anti-diphtheria toxin serum. Translation product from DNA-fragment 1 was not toxic and this was also the case with mock transcription/translation of DNA-fragment 2.

Amplification by PCR with Taq polymerase induces errors at a rate of 1-2 x 10^{-4} mutations per nucleotide polymerised (27). This corresponds to 0.2-0.4 base pair changes in the toxin gene for each replication. In our case, the gene was amplified only ~10 times and this was not sufficient to alter the average toxicity or in other ways change the properties of the toxin. Since it obviates the necessity to isolate and ligate fragments in large amounts, amplification by PCR simplifies considerably the work with in vitro ligated toxin fragments and it gives a more pure translation product. The method can easily be adapted to other toxins, such as tetanus toxin and botulinum toxin that are also considered too dangerous to be cloned in their intact form.

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Cellular Targets of Tetanus Neurotoxin: Further Studies on Binding and Action at Cellular and Molecular Levels

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1. Introduction

Tetanus toxin (TeTox) is a high molecular weight (150 kDa) polypeptide secreted by the Clostridium tetani microorganism and is considered one of the most potent biotoxic agents known to mankind (22). The principal target of TeTox is the central nervous system where it is believed to block inhibitory interneurons (13,30). It also produces a peripheral clinical picture characterized by muscle rigidity and paroxysmal muscle contraction, suggesting a blockade at the neuromuscular junction (13,30).

TeTox interaction with the nervous tissue has been extensively discussed and a number of models have been proposed to explain binding and neurotoxicity (23,28,29,34). Similar to other bacterial toxins (14), TeTox contains at least two functional domains: one which enables specific binding to the neuronal plasma membrane, the result of which the intact molecule is translocated and internalized, and a second domain which causes a disturbance of a normal cellular activity. At this time, little is known as to whether the latter interacts with a specific cell-surface component as a prerequisite for the poisonous action of the toxin.

With the increasing application of cultured nerve cells in tetanus research (10,15,31,33), considerable knowledge has been gained concerning the mechanism of binding/internalization and bioactivity of the toxin. Using a variety of cell culture systems including rat primary neurons (33), somatic neurohybrids (35), and PC12 pheochromocytoma cells (11,25), as well as human erythrocytes artificially supplemented with gangliosides (19), we defined a ganglioside-mediated three-step interaction of [^{125}I]labeled TeTox with these cells (34). The first step involved a reversible low ionic strength and energy-independent binding, followed by a second, salt-insensitive and detergent nonextractable toxin-cell association, operationally defined as sequestration. Finally, in living cells, this process resulted in an energy-dependent internalization defined by the criteria of resistance of the toxin-membrane complex to sialidase.

Despite these observations, it remained unclear whether a sialic acid containing molecule other than ganglioside is the true receptor for TeTox. Recently, using an affinity purified TeTox fraction (18), we have demonstrated that binding to guinea pig synaptic preparations depends on gangliosides but is facilitated by a protease-sensitive and phospholipase-sensitive component (20). Similarly, we have found that TeTox receptors on nerve cells contain a trypsin-sensitive component (36). The data suggested that, in addition to a sialic acid component, binding of TeTox to cerebral neurons was facilitated by a protease-removable and formaldehyde-inactivated component. To extend these studies, we have now examined the effect of acidic pH on the interaction of TeTox with nerve cells.

2. Is Acidic pH Enhancing Binding and Internalization of TeTox in Cultured Nerve Cells?

The role of acidic pH in the endocytic internalization of bioligands such as Semliki Forest virus and diphteria toxin has been documented (27,32). In an analogous fashion, a pH-dependent internalization of other clostridial toxins including TeTox has been proposed (29). Evidence to support this concept has been advanced based on artificial membrane studies using pH gradients which demonstrated toxin-induced channels (6,16). These channels were generated presumably as a result of hydrophobic penetration of the heavy (7), as well as the light toxin chains (24). These ion channels did not necessitate the presence of gangliosides (6,7), in contrast to a ganglioside-dependent formation of channels in planar lipid bilayers at neutral pH (8). The possibility that the ganglioside-mediated TeTox internalization via the three-step sequence (34) may be facilitated by lowering the pH thus deserved a thorough investigation.

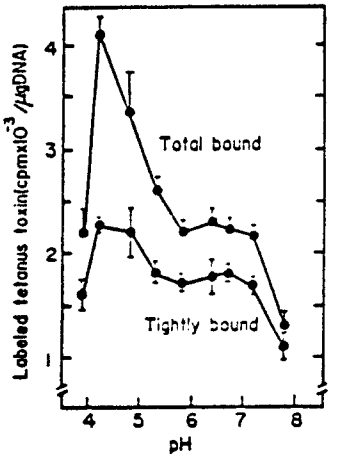


Fig. 1. Effect of pH on [^{125}I]TeTox binding to neurons. Cerebral neurons grown for 1 week or over in culture were incubated for 1 h at 37 °C with [^{125}I]TeTox (40000 cpm/well) added in LIS buffer (25 mM Tris-acetate, 0.25 M sucrose, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1% ovalbumin) at different pH. Incubation was terminated by medium removal followed by 2 washes with the LIS buffer at a suitable pH and once with Pi/saline (PBS) containing 0.5% BSA. Tightly bound toxin radioactivity associated with the cells (internalized) was determined. Total bound toxin represents the sum of the internalized and salt-sensitive toxin.

pH optimum: The effect of pH on [^{125}I]TeTox binding to cerebral cells is shown in Fig. 1. When binding is done at 37 °C using isoosmotic and low ionic strength (LIS) buffer, total toxin bound is elevated between pH 4.2 and 5.4. High ionic strength (Pi/saline) buffer causes a dissociation of a toxin fraction which is bound to cells presumably via weak electrostatic interactions. The remaining (tightly bound) cell-associated toxin cannot be removed by detergents or sialidase and thus is operationally defined as internalized toxin (34). As evident from Fig. 1, this fraction is significantly higher between pH 4.2 and 4.8.

Effect of temperature: Table 1 summarizes the effect of temperature on the cell-associated toxin under low ionic (total) and high ionic (tight) strength conditions. Evidently, at 4 °C, total binding at low pH is about 3-fold greater compared to pH 7.4. This pH-dependent difference is smaller (1.3-fold), however, for the internalized toxin. When binding is performed at 37 °C, a general increase in the total toxin bound to cells is observed. The amount of internalized (salt-resistant) toxin is significantly higher, as anticipated from the raise in temperature. There is, however, no significant pH dependency at this temperature.

Therefore, lowering the extracellular pH has no marked effect on the temperature-mediated internalization of TeTox.

Table 1. Effect of temperature on [^{125}I]TeTox binding

Temperature	pH	Total bound cpmx10^{-3}/well	Fold	Internalized cpmx10^{-3}/well	Fold
4 °C	4.7	15.06	2.92	4.39	1.29
	7.4	5.15	1	3.39	1
37 °C	4.7	24.70	1.39	14.79	1.15
	7.4	17.87	1	12.84	1

Cells were incubated with [^{125}I]TeTox at either 4 °C or 37 °C as described in Fig. 1.

Effect of sialidase: The high affinity interaction of TeTox with polysialogangliosides of the G1b series is now well established (13,29,30). Therefore, utilization of sialidase, which causes an almost complete loss of sialic acid residues from the cell surface, should provide a clue as to whether the external pH affects toxin binding via sialic acid-dependent interactions.

Table 2 summarizes the [^{125}I]TeTox binding capacity of sialidase pretreated cells at various pH. Notably, the remaining binding activity after removal of sialic acid residues is proportionally decreased with a raise in pH. At pH 3.4, the internalization of toxin by desialylated cells is hardly affected (73%). As the pH of the incubation is changed toward a higher value, i.e., pH 4.2 and 4.8, sialidase pretreatment causes a substantial loss of binding (71-74%) compared to control cells. At neutral pH (7.5), the effect of sialidase pretreatment on toxin binding is maximal; only a residual (11%) of the initial binding capacity is retained. This experiment suggests that effective binding and subsequent internalization are highly dependent at physiological pH on the presence and availability of sialic acid residues on the cell surface.

Table 2. Effect of sialidase pretreatment on [^{125}I]TeTox binding

pH	Control	Sialidase treated cpmx10^{-3}/well	Internalization % of control
3.4	7.34	5.37	73
4.2	11.80	3.37	29
4.8	14.23	3.71	26
7.5	12.37	1.35	11

Cells were pretreated with sialidase (2 mU/well) for 2 h at 37 °C and further incubated with [^{125}I]TeTox as detailed in Fig. 1.

Effect of various agents: Cerebral neurons incubated with 2-desoxyglucose, sodium azide or a mixture of both, internalize equally well TeTox at acidic pH although a substantial inhibition is apparent at pH 7.4 (Fig. 2). Thus it would appear that at physiologic pH, toxin internalization requires energy as also evident from the general effect of temperature. Lysosmotrophic agents such as ammonium chloride or chloroquine known to raise the internal lysosomal pH seem to have no effect whatsoever on toxin internalization (Fig. 2). Limited proteolysis of the cell surface or fixation with formaldehyde does not affect toxin internalization at acidic pH but produces a substantial inhibition at neutral pH.

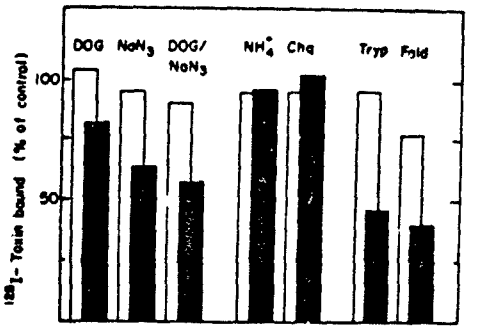


Fig. 2. Effect of various agents on toxin internalization. Cells were preincubated for 15-30 min at 37 °C with either 2-desoxyglucose (DOG) (50 mM), NaN_3 (10 mM), NH_4Cl (10 mM), chloroquine (Chq, 0.1 mM) or 3.5% paraformaldehyde at pH 4.7 (open bars) or 7.4 (closed bars). Labeled toxin was added, cells incubated for 1 h at 37 °C and cell-associated radioactivity determined as detailed in Fig. 1. Trypsinization of the cell surface was performed for 10 min at room temperature using 0.01% trypsin, followed by addition of soybean trypsin inhibitor.

In summary, these experiments illustrate that interaction of TeTox with nerve cells is partially enhanced at low pH but that a sialic acid is still necessary for internalization. Additionally, while at neutral pH toxin internalization appears to depend to a limited extent on energy and to require a protease-sensitive, formaldehyde inactivable component, at acidic pH, this is not the case. The most plausible explanation to account for these observations is that at low pH additional interactions which involve the toxin molecule may take place. Protonation of toxin molecules at acidic pH and the resultant conformational change in the presence of membrane lipids has been proposed as a general mechanism for insertion of bacterial toxins (9). In the case of TeTox, it has been suggested that low pH increases the hydrophobicity of the heavy chain of fragment B, enabling toxin translocation (7). In preliminary results using hydrophobic chromatography, we were able to separate two discrete hydrophylic and hydrophobic populations of TeTox molecules. Elution at acidic pH resulted in a clear increase in the hydrophobic population (data not shown). Nevertheless, the current data suggest that the bulk of toxin is internalized via a sialic-acid receptor containing component.

3. From Binding to Neurotoxicity

Given the conclusion that sialic acid is necessary, a major issue still remains as to whether binding/internalization via the ganglioside or a related sialo-component has any relevance to the mode of action. Undoubtedly, one of the most noticeable characteristics of TeTox is its exceedingly high biotoxicity. As only $8x10^7$ molecules are estimated to cause toxic symptoms and kill a mouse, for example, the signal evoked by so few molecules must be directed to a very specific and highly centralized target or at least activate a transducing system to amplify it. Gangliosides cannot fulfill this task because their ubiquity and relatively lack of specificity (both GD1b and GT1b gangliosides bind avidly the toxin). Neither is there any evidence for an intrinsic enzymatic activity on the toxin subunit, or a secondary activation of a cellular enzyme as a result of toxin binding. In search for an intracellular target which could fit the criteria to amplify the signal and exert an effect on the presynaptic terminals, our attention was drawn on the possibility that protein kinase C (PKC) may serve as a transducer and signal amplifier for TeTox.

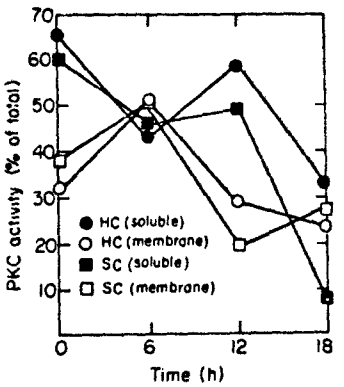


Fig. 3. Time-dependent translocation and loss of PKC activity in rat hippocampus and spinal cord after single injection of TeTox. Soluble and membrane fractions from hippocampus and spinal cord areas of animals subjected to 100 MLD TeTox for designated times, were prepared and assayed, for PKC as described elsewhere (21).

PKC is an enzyme abundant in presynaptic terminals (12) which can be shifted from a cytosolic inactive form to a membrane-bound active form by a variety of stimuli (17). The active form is believed to play an important role in regulation of nerve excitability, most likely through phosphorylation of specific proteins (3,5,17). When applied intraventricularly, TeTox at a concentration of 10 ng/kg body weight (100 MLD), causes behavioral changes in the rat within 4-6 h (2). By this time, the majority of brain structures attain a steady state with respect to [^{125}I]TeTox distribution. Hippocampus, midbrain, hypothalamus, spinal cord and frontal cortex contained each 31, 17.8, 7.7, 4.7 and 5.6% of the total radioactivity, respectively.

Six hours after intraventricular application of 100 MLD of toxin, little or no change in total PKC activity is encountered in either hippocampus or spinal cord structures (Fig. 3). At this time, however, approximately 50% of the enzyme in the hippocampus is associated with the membrane compartment, in comparison to only 33% in control brains. A similar change in distribution of PKC is also evident in the spinal cord, an area distant from the site of injection. The data indicate that TeTox induces in vivo translocation of PKC. At 12 to 18 h after injection, the effect of translocation is less noticeable, since it is accompanied by a substantial decrease in overall PKC activity. In fact, as shown in Fig. 3, by 18 h about 44% and 60% of the total activity is lost in hippocampus and spinal cord, respectively. The latency of PKC down-regulation in hippocampus and spinal cord may be due to a delayed regional-specific internalization of the toxin, although both structures take up labeled toxin as soon as 15 min after injection (unpublished observations).

A marked decrease in PKC levels in the cytosolic fraction of the hypothalamus, an area enriched in biogenic amine terminals, was also noticed. Depletion of the activity is accompanied by a decrease in the PKC, as evident on a Western blot using a polyclonal antibody.

Various biochemical and electrophysiological studies have implicated PKC as a key modulator of neurotransmitter release based on the phenomenon of translocation/activation of the enzyme (3,26,37). Activators currently in use, such as phorbol esters, act at nanomolar concentrations and are not selectively targeted to neuronal membranes. In contrast, TeTox is highly selective for neuronal membranes and is effective at extremely low concentrations (10^{-14} M), thus providing a powerful tool for studying the role of PKC in the regulation of neuronal excitability. TeTox-triggered PKC translocation most likely occurs

at the presynaptic level, based both on enzyme localization (12) and the presumed site of TeTox action (30). Translocation of PKC in the presynaptic membrane is a Ca^{2+} mediated event (4), the magnitude of which may be perturbed by TeTox. The time-dependent down-regulation of PKC may well indicate a process which at the end may impair neurotransmitter release (13,29,30). Recently, using a similar experimental protocol, an increase in serotonin has been shown in rat brain structures after intraventricular administration of TeTox (1). The molecular mechanism and whether one or more neurotransmitters (excitatory and inhibitory) are involved in a PKC-dependent synaptic-vesicle release, remain to be studied.

Summary

pH-dependent binding and subsequent internalization of tetanus toxin in cerebral neuron cells in tissue culture was examined. At acid pH, internalization was partially enhanced and did not depend on the presence of a protease-sensitive receptor component. It did, however, depend on the existence of a sialic acid residue indicating the prime role of gangliosides in binding and subsequent internalization of tetanus toxin.

A toxin-stimulated activation followed by down-regulation of protein kinase C in the adult rat brain after intraventricular administration of the toxin is documented. The data identifies PKC as a possible indirect target which could initiate a pathophysiological cascade characteristic to tetanus neurotoxicity.

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Uptake and Processing of Ricin A Chain by Macrophages

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ABSTRACT

Ricin A chain, a glycoprotein containing high mannose chains, selectively intoxicates macrophages following uptake by the mannose receptor. After internalization A chain is rapidly cleaved by a pepstatin sensitive protease. Incubation of cells with pepstatin leads to intracellular accumulation of toxin and surprisingly, to a decrease in cytotoxicity. Three different MW peptides are generated intracellularly. Characterization of A chain degradation inside endosomal vesicles suggests that more than one protease is involved in A chain cleavage.

KEYWORDS

Ricin A chain, endocytosis, macrophages, mannose receptor, cathepsin D, Fc receptor

INTRODUCTION

Many bacterial and plant protein toxins enter the cell after binding to cell surface receptors. The plant toxin ricin, which is composed by an A and B subunit joined by a disulphide bond,

enter the cell after binding of the B subunit to galactose residues present on the cell surface. Following binding, the intact toxin is rapidly endocytosed and reaches the intracellular compartment from which the A chain translocates to the cytoplasm. Once in the cytoplasm it inactivates the 60S ribosomal subunit by removing a single adenine residue from ribosomal RNA (1). Several studies indicate the Golgi apparatus as the site of translocation of the toxin to the cytoplasm (2,3) but the mechanism of translocation is still greatly unknown. Studies in vivo have shown that ricin is cleared from the circulation preferentially by the liver Kupffer cells (4). In fact, it has been shown that ricin can kill macrophages following uptake via two distinct routes (5), either by the standard pathway, i.e. after binding of the B subunit to galactose residues on the cell surface, or by receptor mediated endocytosis via the macrophage mannose receptor (6). Highly purified A chain is not toxic to most cell types but it kills macrophages, which uniquely express the mannose receptor, following uptake via the mannose receptor. Sugar specific uptake of A chain by macrophages is due to the high mannose chains present on the molecule.
The ability of isolated A chain to selectively intoxicate macrophages allowed us to study, in a model system, some of the events which follow internalization and ultimately lead to translocation of the toxin to the cytoplasm.

RESULTS AND DISCUSSION

Ricin A chain is toxic to macrophages following uptake by the mannose receptor as shown in Fig. 1. Yeast mannan, a ligand for the mannose receptor, decreases cytotoxicity shifting the dose response curve of about 2 orders of magnitude.

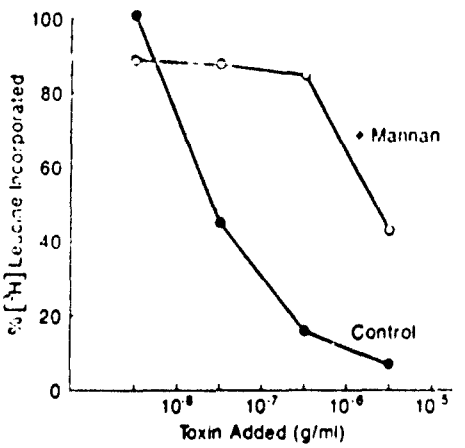


Fig.1 Inhibition of protein synthesis in mouse bone marrow macrophages by ricin A chain either in absence ( ) or in presence ( ) of 1 mg/ml yeast mannan.

Uptake of A chain is saturable and inhibitable by yeast mannan. Following uptake, A chain is rapidly cleaved and degradation products can be detected in the medium as early as 10 min.

Diment et al. (7) have shown that in macrophages endosomes contain cathepsin D, an aspartic protease responsible for degradation of mannosylated-BSA when taken up by the mannose receptor. To test if cathepsin D was also involved in A chain rapid degradation, we preincubated macrophages with pepstatin, a specific inhibitor of cathepsin D, and we then looked at degradation of labelled A chain. The results indicate that in presence of pepstatin degradation of A chain is blocked and undegraded toxin is accumulated intracellularly. Surprisingly, cells preincubated with pepstatin are protected from intoxication by A chain, despite the higher amount of toxin accumulated inside the cell as compared with control cells. Other protease inhibitors have no effect on A chain cytotoxicity. Intact ricin cytotoxicity, on the other hand, was not affected by pepstatin showing that pepstatin does not have a general effect on cytotoxicity. Cleavage of A chain by a pepstatin sensitive protease seems therefore necessary for toxicity, suggesting that peptides generated by partial proteolysis are more toxic and/or more efficient in translocating to the cytoplasm than intact A chain. Three detectable peptides of MW 29, 18 and 14 kDa are generated from A chain in macrophages. Of these peptides only the 29 kDa peptide seems generated by cathepsin D cleavage suggesting that more than one protease is involved in A chain cleavage in vivo.

In order to better study degradation of A chain, we loaded macrophages with radiolabelled A chain and we then isolated endosomal vesicles. The results demonstrate that cleavage of A chain occurs intravesicularly and is only partially ATP dependent. ATP is required to acidify the lumen of endosomes. At acidic intravesicular pH (i.e. in presence of ATP) the protease inhibitors pepstatin and leupeptin both inhibit proteolysis by 40% whereas they do not have any effect in absence of ATP. Incubation of these vesicles with buffers at different pH shows that A chain degradation still occurs at pH 7. These data suggest that both a leupeptin sensitive protease and a protease active at neutral pH are also involved in A chain cleavage. Since leupeptin did not have any effect on A chain cytotoxicity further experiments will be necessary to better characterize the proteolytic activity present at neutral pH.

The route of internalization of A chain is also important. When complexed with anti-A chain monoclonal IgG, A chain is internalized by macrophages via the Fc receptor (8). Following this route, the toxin is very slowly degraded and is not cytotoxic. The antibody by itself does not block A chain enzymatic activity in a cell free system. One possibility to explain these results is that the antibody binds A chain at

cleavage site blocking degradation and therefore cytotoxicity.

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Botulinum Neurotoxin: Present Status of Covalent and Conformational Structures and Structure-Function Relationship

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Certain strains of four different species of clostridia, C. barati, C. botulinum, C. butyricum and C. tetani are now known that produce neurotoxic proteins. Progress in the studies of their structure and structure-function relationship is revealing very interesting similarities within their diversities.

Covalent structure: Each of these neurotoxic proteins is about 150 kD. They are synthesized as single chain proteins and then nicked to the dichain structure composed of a light and heavy chains. The neurotoxin (NT) isolated from C. barati was recently demonstrated as a ~150 kDa molecule composed of a ~50 kDa light (L) and ~100 kDa heavy (H) chain that can be dissociated after reduction (8). Partial amino acid sequences of botulinum NT types A, B, C_1 and E based on Edman degradation are available (4,17). The complete sequence of tetanus NT, based on nucleotide sequence, was reported from two European laboratories (12). A comparison of the available amino acid sequences reveals the following: Each NT is nicked 1/3rd the distance from the N-terminal. In each case the N-terminal amino acid of the mature protein is Pro. In the L chain, -Ile-Trp- pair is found between residues 38 and 43 in each protein. The H chain in each case has a different N-terminal amino acid. In each of the H chain a 1/2 Cys is present within the first 10 residues from the N-terminal. This 1/2 Cys is flanked by Leu or Ile on the amino side and Ile on the carboxyl side. This 1/2 Cys appears to form the disulfide with the L chain. Four residues away from the 1/2 Cys is an Asn. Another four residues away, i.e. 9 residues away from 1/2 Cys is Phe.

As more sequence data become available more of these invariant and unique features may become known that could serve as the "signature sequence" of the clostridial NTs. Our laboratory is awaiting the results of partial sequences of the type F-like NT from C. barati and the type F NT. We are waiting to see if the invariant features noted so

far will hold true. The NT isolated from C. butyricum was partially sequenced and compared with type E; we have found differences in only three residues in the L chain at positions #2, 14 and 27. But so far we have not found any violation of the "signature sequence" (4).

We have progressed using the classical protein chemistry i.e. fragmentation, isolation of the fragments and then Edman degradation; for comparative purposes the following is known at this time from the type A and E NTs. If we assume that the NT has ~1300 amino acids, we have sequenced 27% of the L chain, 14% of the H chain i.e. 18% of the entire type A NT. For type E corresponding numbers are 20.4%, 10.9% and 14.2% (Fig. 1). On type A the numbers 73, 47, 4, 35, 21, and 59 are the number of residues sequenced from the indicated locations. The same is true for type E. These segments show in the tabular form the indicated % homology (positional identity) with tetanus NT.

How correct are these sequences determined by Edman degradation? The 35 residues at the N-terminal of the type A H chain reported by Shone, Hambleton and Melling (15) agree with the 27 residues we have identified (4). Another independent confirmation comes from our nucleotide sequencing of the type A NT gene. In Fig. 2 the N-terminal of the L-chain is examined. Amino acid sequence derived from the DNA matched exactly with the Edman degradation results.

Site of nicking: Which two amino acid residues make the peptide bond that is the nicking site? For this question we have examined type A and E NTs. Fig. 3 shows that in type A NT the H chain, beginning with Ala-Leu-Asn, is connected to the L chain whose C-terminal amino acids are unknown. Similarly, in type E NT, its H chain beginning with Lys-Ser-Ile is connected to amino acid residues of unknown identity. Note that the N-terminal residue of the H chain of these NTs are not identical. Because trypsin nicks single chain type A and single chain type E (to their dichain forms) one would expect Lys or Arg residues to provide the peptide bond as the nicking site. We had proposed several years ago, based on chemical modification studies that an arginyl residue in type E is the nicking site for trypsin (5,6). Studies in another laboratory in this area were interpreted to conclude that trypsin nicks type E NT at a Lysyl peptide because the protease Lys-C (which is highly specific for Lysyl bonds) nicked type E into what appeared as L and H chains (9).

In our laboratory the single chain type E NT was nicked with commercially available Lys-C protease The single chain protein yielded two fragments that resembled L and H chains in SDS-PAGE. We sequenced these fragments. In the H chain we found Gly-Ile-Arg-Lys-Ser-Ile at the N-terminal. Thus the peptide bond in type E that is nicked by trypsin is Arg-Lys (Gimenez, J. and DasGupta, B. R., to be published). Also recently we nicked the type A single chain NT with a protease that we isolated from C. botulinum type A culture (7). The endogenous protease was purified 1000-fold using the synthetic substrate benzoyl arginyl p-nitroanilide--a substrate for trypsin. The single chain type A NT was isolated using a method that was reported by Krysinski and Sugiyama (10). The two chains of the nicked type A were sequenced. The N-terminal residues of the H chain were Gly-Tyr-Asn-Lys-Ala-Leu-Asn (DeKleva, M. and DasGupta, B. R., to be published).

Results from type A NT point out an interesting issue. The H chain of type A isolated from the dichain type A NT begins with Ala-Leu-Asn but not with Gly-Tyr-Asn-Lys. So why is the endogenous protease nicking the single chain protein four residues away from Ala the well-known N-terminal? Two explanations are possible: First, we digested a pure preparation of the single chain protein in vitro with a pure protease preparation. In the bacterial culture the situation is different. The NT remains tightly bound to a hemagglutinin protein. Thus, availability of the susceptible peptide bond in vivo could be different. Second possibility is that, in the bacterial culture, another cleavage occurs between Lys-Ala that removes the tetra peptide Gly-Tyr-Asn-Lys. A word of caution. In the case of type A Gly-Tyr-Asn-Lys need not be considered the C-terminal amino acids of the L-chain, until experimentally demonstrated: One reason for this caution was mentioned above, i.e. these four residues are perhaps temporary N-terminal residues of the H chain that are removed from the mature H chain.

Spontaneous break down of L-chain: We routinely separate the L chain from the H chain using a QAE-Sephadex column and pH 8.4 buffer that contains DTT and 2 M Urea (14). We observed that the L chain of type A after separation from the H chain begins to yield two fragments of size ~27 kDa and ~18 kDa. These were detected in SDS-PAGE. The H chain following its isolation and the parent dichain NT do not yield similar products under comparable conditions. Because the type A NT is isolated from a culture that is highly proteolytic we extended our study of this phenomenon using type E NT which is produced by non-proteolytic culture. The type E L chain after separation from the H chain was also found to yield ~27 kDa and ~18 kDa fragments.

Partial amino acid sequences of these fragments established two points. The ~27 and ~18 kDa fragments come from the N- and C-terminal regions of the L-chain, respectively; the L chains of the type A and E breakdown at precise peptide bonds. These bonds are five residues apart. The 47 residues of the 18 kDa fragment of type A (4) and the 41 residues of the type E align with tetanus NT beginning at its residue #259 and #264, respectively. Out of 47 residues of the type A 18 are identical to tetanus. This gives 38% homology. Out of 41 residues of type E 20 are identical to tetanus; this gives 49% homology. The 13 overlined residue of type E are positionally identical to type A; that gives 32% homology. Homology increases further if single nucleotide base substitution is considered.

Fragmentation of the isolated L chain of botulinum NT is not restricted to type A and E. We have observed the same in type B which is currently under investigation.

We do not have a definitive explanation as to why the fragmentation occurs. Probable causes, i.e. proteolytic or non-proteolytic were considered. Although the presence of traces of a protease in the NT preparation is a possibility which is extremely difficult to disprove; several observations, experiments and arguments argue against a protease catalyzed fragmentation. An explanation alternative to the protease catalyzed fragmentation is that certain aspartyl and asparginyl residues can form succinimide ring that leads to spontaneous degradation of protein. Within the last two years a number of papers have appeared

discussing this issue (see ref. 16). Our experimental results and interpretation have been submitted for publication (DasGupta, B. R. and Foley, J.). Whether in vivo the L chain separates from the H chain is not yet known but the slow in vitro fragmentation we found warrants consideration in the studies where the isolated L chain is to be used.

Structure-Function Relationship: We have utilized three different approaches to study the structure-function relationship of the NT. These are: 1) Chick ciliary ganglion-iris neuromuscular preparation, 2) Bovine chromaffin cells, digitonin permeabilized, 3) Rat pheochromocytoma cell line PC-12, cracked cells.

The bioassay using the chick iris neuromuscular preparation (dissected from 1-10 day old chicks is mounted onto a dish perfused with an oxygenated physiological salts solution, the iris nerve ganglion is sucked into an electrical stimulator and a force transducer is placed inside the ring formed by the iris muscle, the time to inhibit the muscle contraction is noted) show dose-response to the type A and E NT. The mildly trypsinized type E NT is about 20-40 folds more active than the untrypsinized type E. The iris system is sensitive to the NT at concentrations similar to those used in experiments with mouse hemidiaphragm preparations.

Using this system (Lomneth, R., Suszkiew, J. and DasGupta, B. R., to be published) we have repeated our earlier studies made with isolated L and H chains (1): Results were as follows: The isolated chains of type A by themselves did not produce paralysis up to about 180 minutes. The isolated H chain delayed (antagonized) the paralytic effect of the parent dichain. And when the tissue incubated first with H chain was washed to remove free unbound H chain and then further incubated with L chain we observed rapid paralysis. These observations were similar to what we had reported using mouse hemidiaphragm neuromuscular preparation (1).

These results once again indicate that the H chain binds with specific sites on the nerve terminals and competes with the parent dichain NT for these sites. This binding permits the L chain (or some combination of the L and H chain) to bring about paralysis. These experiments do not show if the paralysis requires internalization of the L chain or combination of L and H chains.

Role of L chain in paralysis: Using two independent techniques and different secretory cells (the digitonin permeabilized chromaffin cells and cracked PC-12 cells) we have established that only the L chain segment of the NT causes the biochemical damage inside the cells that blocks neurotransmitter release. In these two different systems we bypassed the "receptor" binding and "passage through lipid membrane" steps.

The chromaffin system: Low concentrations of the detergent digitonin renders the plasma membrane of bovine adrenal chromaffin cells permeable to ions and proteins without altering the integrity of the intracellular storage granules and Ca^{++} dependent catecholamine secretion. In collaboration with Prof. Ronald Holz and Dr. Mary Bittner (University of Michigan, Ann Arbor) we have been examining the effect of the NTs and

their subunit chains on the secretion of catecholamine (2,3). The type A NT and its isolated L chain inhibited Ca^{++} dependent catecholamine secretion in a dose dependent manner, only when semi-permeabilized cells are used. Inhibition was not seen when intact cells were used. Therefore, inhibition required introduction of the NT or its L chain inside the cells. The heavy chain had no effect. The degree of inhibition achieved by type A NT or its isolated L chain was not complete, but they were comparable. The inhibition could not be overcome by increasing Ca^{++} concentration. The inhibition occurs rapidly i.e. within 3 min. and is irreversible. Botulinum type E, the single chain protein was also inhibitory in a dose-dependent manner. Trypsinization nicks the single chain type E protein to the dichain form and increases its mouse lethality. Therefore we wanted to see if the catecholamine secretion inhibition assay will also show similar activation. The trypsinized dichain type E exhibited at least 30 folds higher inhibitory potency than the untrypsinized single chain. We have not yet tested the isolated type E L chain. In contrast to the dichain type A as well as single chain and dichain type E, the single chain type B was not inhibitory. Trypsinization of type B produced the dichain type B, but this was also not inhibitory. Reduction of the dichain type B with DTT produced inhibition. The isolated L chain was inhibitory. Inhibition was complete. Table 1 summarizes these observations (2).

In our second approach PC-12 cell lines are used (Lomneth, R., Martin, T.F.J., and DasGupta, B. R., to be published). The cells are permeabilized by a physical process referred to as cracking. Cracked cells are formed when cells are passed through a ball homogenizer with enough shear force to break open the cell membrane, but gentle enough to leave the secretory apparatus functional and responsive to Ca^{++} stimulation (11). The effect of dichain type B NT and its isolated chains on catecholamine release were examined. As was found with chromaffin cells dichain type B NT without reduction did not inhibit secretion. The results with PC-12 cells once again showed that the Ca^{++} stimulated norepinephrine release is sensitive to the NT only when the PC-12 cells are permeabilized. Most importantly, the L chain alone can cause inhibition as effectively as the parent dichain NT once it is inside the cell. Another important fact is that, once the L chain is internalized, its poisoning effect is not enhanced by the presence of the H chain.

2-Dimensional crystals of the NTs and amphiphilic secondary structure: The first two steps of intoxication are binding of the NT to the membrane surface and then internaliztion through membrane. Both events involve interaction of protein with lipids. A number of laboratories have shown that botulinum NT binds the ganglioside GT1b. The ganglioside binding property has allowed us to prepare two dimensional crystals of type A, B and E NTs. The crystals form at the interface of a solution of NT and a phospholipid monolayer containing ganglioside. This work was accomplished in collaboration with Prof. John Robinson at Vanderbilt University. The method was similar to the procedure used previously to prepare 2-D crystals of tetanus neurotoxin (13).

The crystals of botulinum types A, B and E neurotoxins have been prepared more than once and from more than one independent NT preparation. The optical diffraction patterns of type A and E indicate

that the structures are visibly ordered to about 25-30A°. The type B NT crystals are tubular in form (~1000Å) in diameter and have a visible helical period. These crystals form most abundantly over a pH range 4.5 to 6.0 and the number of crystals decreases rapidly outside this range. The tubular structures also form "most abundantly" at ionic strength below 0.1 at pH 5.6.

To understand how a water soluble protein becomes partially embedded in the membrane lipid and how does it cross the hydrophobic membrane barrier to reach the cytoplasm presence of amphiphilic secondary structures in type A NT was considered.

Dr. Frenec Kezdy and I searched for the amphiphilic structures using the algorithm of K. D. Berndt and F. J. Kezdy. The presence of one amphiphilic α-helix was found in each of the four peptide regions sequenced (see Fig. 1); these are residue #32-44 (-VKAFKIHNKIWVI-), residue #36-47 (-YYNKFKDIASTL-), residue #2-16 (-YNKALNDLCIKVNNW-) and residue #1-16 (-IYLNSSLYRGTKFIIK-). Predictive analysis by the Chou-Fasman method also showed that the amphiphilic segments of the first three regions (residues #32-44, 36-47 and 2-16) also have an α-helix potential, but the fourth segment (residues #1-16) does not. This allows us to predict that at least these three segments are in the helical form in the native NT and they are on the surface of a globular domain; but once they come in contact with a phospholipid bilayer, they are able to insert themselves into the lipid structure. Such an interaction would also be favored by the net positive charge(s) on all these regions (can be seen from their Edmundson projections). The potential structural role of these amphiphilic segments is also supported by the fact that both the amphiphilicity and the helix potential is fully conserved in the corresponding segments (residues 35-46; 296-306; 459-473 and 1163-1177) of the tetanus NT (12).

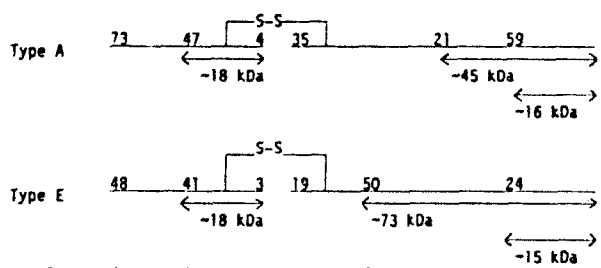
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Fig. 1 Regions of botulinum NT Type A and E sequenced by Edman degradation and their comparison with tetanus



Homology	%	%	%	%	%	%
A:E	8	32	42	-	29	25
A:Tet.	23	38	29	-	-	29
E:Tet.	4	49	42	52	-	33

DasGupta, B. R. & Foley, J. (to be published)
Gimenez, J. & DasGupta, B. R. (to be published)

Fig. 2. Partial nucleotide sequence of botulinum type A neurotoxin gene compared with N-terminal amino acid sequence of the neurotoxin

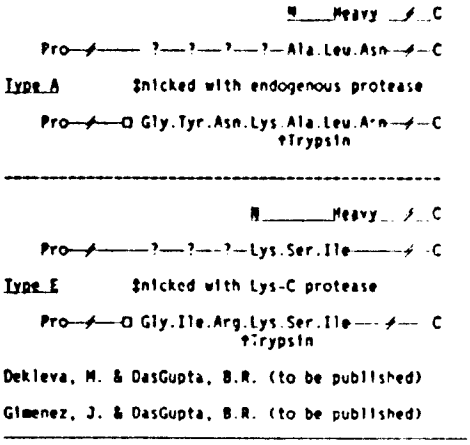
```
DNA                 TAA TTT TAA ATA TTA TAA GAG GTG TTA AAT ATG CCA TTT GTT AAT
Derived A.A.         *  Phe  *  Ile Leu  *  Glu Val Leu Asn Met Pro Phe Val Asn
 sequence
Peptide sequence                                                 P   F   V   N

                    AAA CAA TTT AAT TAT AAA GAT CCT GTA AAT GGT GTT GAT ATT GCT
                    Lys Gln Phe Asn Tyr Lys Asp Pro Val Asn Gly Val Asp Ile Ala
                     K   Q   F   N   Y   K   D   P   V   N   G   V   D   I   A

                    TAT ATA AAA ATT CCA AAT GCA GGA CAA ATG CAA CCA GTA AAA GCT
                    Tyr Ile Lys Ile Pro Asn Ala Gly Gln Met Gln Pro Val Lys Ala
                     Y   I   K   I   P   N   A   G   Q   M   Q   P   V   K   A
```

Betley, M. J., Somers, E. & DasGupta, B. R. (to be published)

Fig. 3 Sites of nicking in type A and E neurotoxin



Dekleva, M. & DasGupta, B.R. (to be published)

Gimenez, J. & DasGupta, B.R. (to be published)

Table 1. Inhibition of Ca^{++} stimulated [^{3}H]norepinephrine release from permeabilized chromaffin cells by clostridial neurotoxins

	Single chain	Dichain	L-chain	Extent of inhibition	Cells treated with	Ref.
Botulinum Type A	ND	Yes	Yes	incomplete	Digitonin	1,2
Type B	No (DTT)	No Yes (DTT)	Yes	complete	"	1,2
Type E	Yes	Yes 30Xmore	ND	complete	"	1,2
Tetanus	ND	Yes	Yes	complete	"	1
Tetanus	No No (DTT)	No Yes (DTT)	Yes	complete	streptolysin O	3

ND = not determined; DTT = reduced with dithiothreitol
ref. 1: Bittner, Holz & DasGupta, Soc. Neurosci. Abstr. 14, 68 (1988)
ref. 2: Bittner, DasGupta & Holz, J. Biol. Chem 264, 00 (1989)
ref. 3: Ahnert-Hilger et al. FEBS Letters 242, 245 (1989)

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Infrared Spectroscopy as a Tool to Determine the Secondary Structure of Proteins and the Orientation of Protein Domains in a Lipid Membrane

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Laboratoire de Chimie Physique des Macromolécules aux Interfaces. Université Libre de Bruxelles, Bd. du Triomphe CP 206/2, B-1050 Brussels, Belgium

INTRODUCTION

The determination of the conformation of biological membrane molecules by experimental means has long been challenged by the difficulties encountered to obtain crystals of proteins suitable for high resolution X-ray crystallography and by the turbidity inherent to membrane fragments which prevents them from being analyze by spectroscopic methods using UV or visible light.

Infrared spectroscopy has been recognized for long as a potentially useful method to gain information on the structure of molecules of biological interest. However, the complexity of the latter molecules, the intrinsic broadeness of the absorbtion bands in the liquid or solid state and the presence of water (H_2O or D_2O) whose bands overlap several interesting regions of the sample spectrum have prevented the technique from a widespread development. The recent availability of FTIR instrumentation along with computer softwares allowing to solve to some extent the problems described above has enable IR spectroscopy to become one of the leading technique in the experimental search of the structure of biological molecules. In some areas, IR spectroscopy has become the only technique available. Let us quote for example the determination on hydrated membranes of the secondary structure of membrane proteins, the determination of the orientation of some protein segments with respect to the plane of the membrane, the evaluation of the structure and orientation of the different phospholipid groups, the determination of the ionization state of various chemical groups of interest, etc... Moreover, results are obtained in a relatively short lag of time if we compare

with other techniques such as NMR of selectively deuterated molecules or X-Ray crystallography.
In this paper, we describe how infrared spectroscopy can be used for the determination of protein secondary structure and of the orientation of protein segments in a lipid bilayer.

MATERIALS AND METHODS

The detailed methodology of I.R. spectroscopy has been described elsewhere (1). Porin, Bacteriorhodopsin and glycophorin were reconstituted according to 2, 3, 4 respectively.

RESULTS

The system (ATR= Attenuated Total Reflection method (5)) we used for recording our spectra is shown figure 1: the sample is deposit on one side of a germanium plate and then slowly evaporated under nitrogen flux. When lipids are present in the suspension, they spontaneously form oriented multilayers on the surface of the plate.

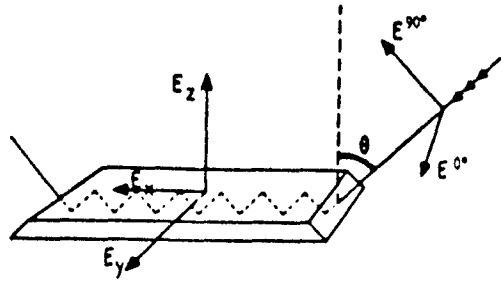


Figure 1 : Internal reflection element (IRE).

A. Determination of protein secondary structure.
It has been known for long that protein IR spectra are sensitive to secondary structure. The amide I band (amide carbonyl stretching) is the most useful band to study since it is the most conformational sensitive and it falls between 1600 cm^{-1} and 1700 cm^{-1}, a region with little lipid absorption (except for phosphatidylserine). The sensitivity of the shape of amide I' (the ' means that a H/D exchange has been realized) on the protein secondary structure appears in figure 2 where the ATR spectrum of 3 soluble proteins are presented.

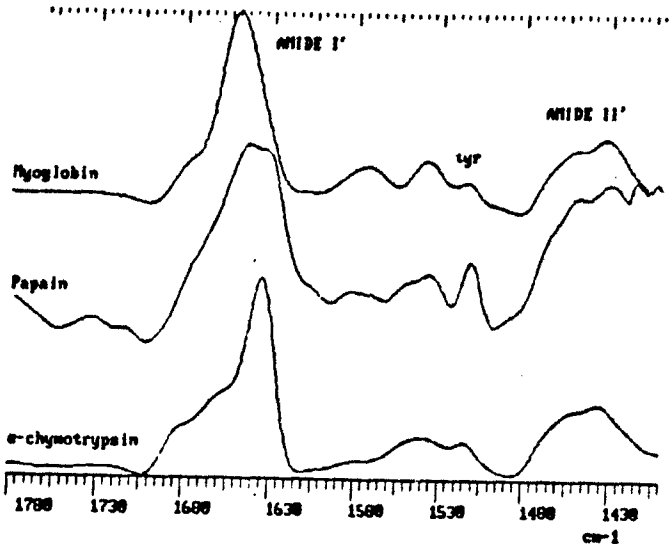


Figure 2 : Infrared spectrum in the amide I region of three soluble proteins prepared for ATR by the solvent evaporation technique. a. myoglobin; b. papain; c. α-chymotrypsin.

The structure of these proteins have been previously determined by X-Ray crystallography. Myoglobin is rich in α helix structure, α-chymotrypsin in β sheets structure and papain contains both α helix and β sheets.
In the case of myoglobin, the frequency of the peptide C=O stretching at 1655 cm^{-1} indicates a largely α helix structure. α-chymotrypsin absorption near 1630 cm^{-1} is characteristic of β sheets and papain which contains both α helix and β sheets shows two peaks, respectively around 1650 cm^{-1} and 1630 cm^{-1}.
Similar data obtained with membrane proteins reconstituted in dimyristoylphosphatidylcholine (DMPC) vesicles are shown in figure 3. (a) is the spectrum of porin , an outer membrane protein of E. Coli, assumed to be associated to the membrane by several β sheets (6). As in the case of α-chymotrypsin, the ATR spectrum of porin shows a proeminent peak near 1630 cm^{-1}. Bacteriorhodopsin, associated to the membrane of Halobacterium holobium by 7 transmembrane helices (7) shows an absorption peak near 1660 cm^{-1} (Fig.3c). For glycophorin, whose structure has been shown by CD to consist of α and β structures (8), two components can be clearly identified in spectrum figure 3b.

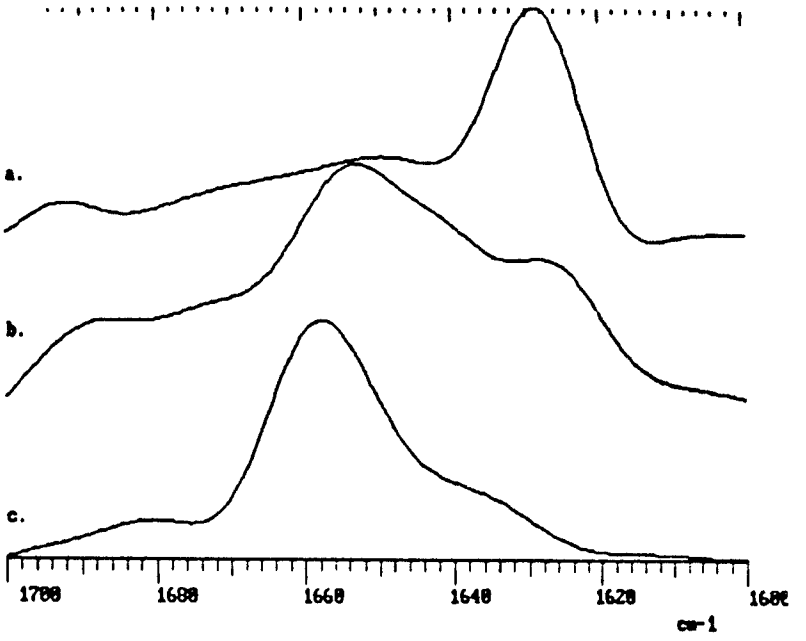
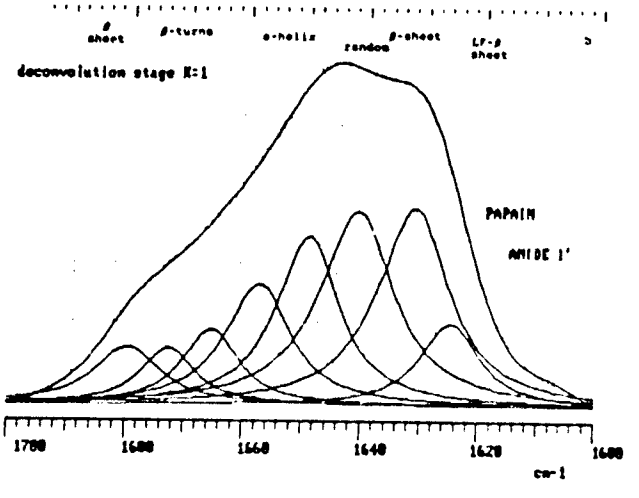


Figure 3 : Infrared spectrum in the amide I region of three membrane proteins reconstituted into liposomes and then prepared for ATR by the solvent evaporation technique. a. porin, b. glycophorin A, c. bacteriorhodopsin.

Assignement of the different frequencies found in amide I' to the different recognized secondary structures were made on empirical basis as well as on theoretical grounds (9). The way the secondary structures were determined for proteins from ATR spectra is illustrated in figure 4 for papain.



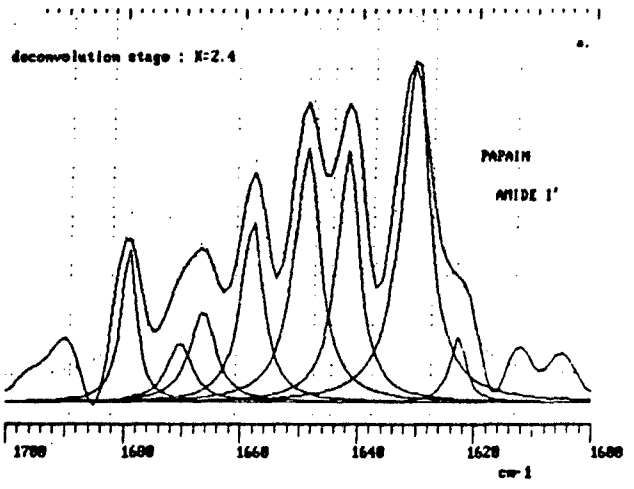


Figure 4 : Fourier self-deconvolution and curve fitting realized on the amide I' band of papain, see text for details of the procedure. K is the resolution enhancement factor. The vertical dotted lines indicate the frequency limits in which each secondary structure is found.

The number and the approximate frequency of the different components are obtained after Fourier self-deconvolution (here K=2.4). A curve fitting using Lorentzian lines is then performed. The results of the curve fitting reported in figure 4a are then used as input parameters for a second curve fitting performed on the original spectrum. This procedure avoids some of the drawbacks of the curve fitting techniques (10) and prevents artifacts possibly introduced by the deconvolution to interfere with the quantitative analysis. The areas of the components assigned to a same structure are added and the percentage of that structure in the protein is taken to be that resulting area divided by the total amide I' area. This method assumes that the extinction coefficient are identical for all the secondary structures. When this procedure was extended to a series of well-characterized proteins, the mean difference between the

values so-obtained and the X-ray values amounted to no more than 2% (S.D. 8%) for all the proteins and all the structures when using a quite automatic procedure which requires no human decision between the recording of the spectra and the obtention of the secondary structure (unpublished results).

B. Orientation of protein secondary structures.

The theory about the determination of orientations by IR-ATR in lipid multibilayers systems has been reviewed by Fringeli and Günthard (5). The amide I vibration is made out of 80%(C=O), 10%(C-N) and 10%(N-H) vibrations. In a alpha-helix, the main transition dipole moment (ν(C=O)) lies close to a parallel to the helix axis while in an anti-parallel beta-sheet the polarisation is opposite i.e. predominantly perpendicular to the fiber axis (11). It is therefore possible to determine the mean orientation of the alpha-helix and beta-sheet structures from the orientation of the peptide bond C=O group. When that information was desired, additional spectra were recorded with parallel (0°) and perpendicular (90°) polarized incident light with respect to a normal to the ATR plate. Polarization was expressed as the dichroic ratio $R_{atr}=A^{90°}/A^{0°}$. The mean angle between the C=O bond and a normal to the ATR plate surface was then calculated from R_{atr} according to Fringeli and Günthard (5). Figure 5 reports the ATR polarized spectra of bacteriorhodopsin incorporated into DMPC multilayers. Polarization appears more clearly on the difference spectrum 90°-0°. The lipid C=O stretching at 1730-1740 cm^{-1} is very weakly polarized, probably because it is composed of several contributions with various orientations. The frequency of the peptidic C=O stretching (amide I) at 1660 cm^{-1} indicates a largely α-helix structure of the protein. The Fourier self-deconvolution/curve fitting analysis (not shown) determines a content of 51% for the α-helix and 14% for the β-sheet structures. The amide I band is strongly polarized at 90°, particularly on its high frequency side, indicating a transmembrane orientation of the helices according to the model proposed for the orientation of bacteriorhodopsin into the membrane. The orientation of the lipid hydrocarbon chains perpendicular to the membrane plane is assessed by 1) the 90° polarization of the CH_2 wagging at 1200 cm^{-1} (dipole $\parallel$ to the all-trans chain axis) and 2) by the 0° polarisation of the CH_2 bending at 1470 cm^{-1} (dipole $\parallel$ to the H-C-H bissector i.e. $\perp$ to the chain axis).

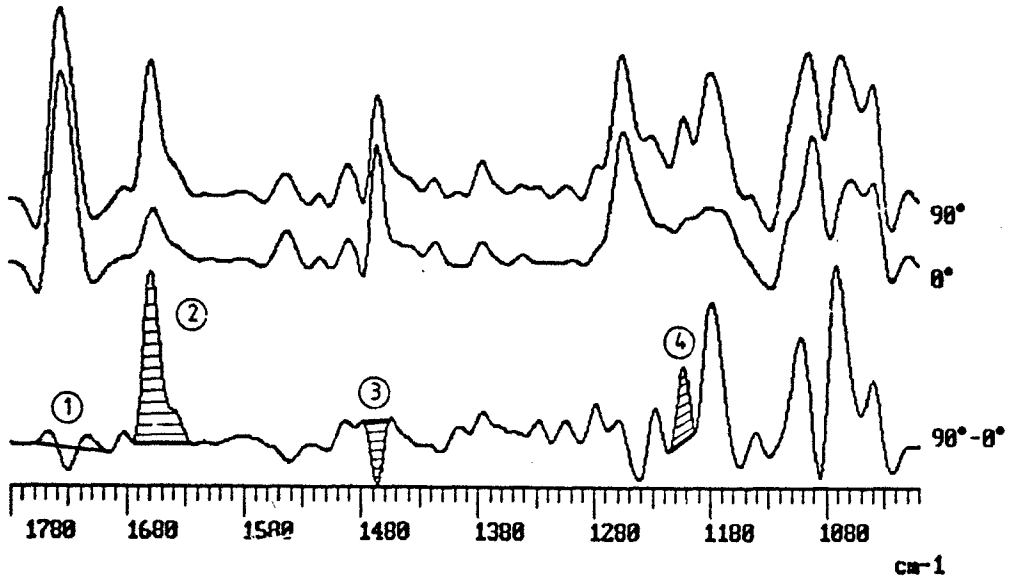


Figure 5 : polarized infrared spectra of bacteriorhodopsin reconstituted in DMPC liposomes. Multilayers were formed by the solvent evaporation technique on a Germanium IRE at 45°. Spectra were recorded by ATR. The difference spectrum 90°-0° is rescaled. A positive deviation indicates that the dipole is oriented preferentially perpendicular to the membrane plane and a negative deviation suggests an orientation parallel to the membrane plane. 1. lipid acyl chain carbonyl stretching, 2. peptidic carbonyl stretching (amide I), 3. lipid hydrocarbon chain CH_2 bending, 4. lipid hydrocarbon chain CH_2 wagging.

DISCUSSION

The data presented in this paper indicate some possible applications of infrared spectroscopy in the determination of conformational and orientational parameters of biological membrane molecules. However, we will briefly discuss here the limitations of the interpretation of the secondary structures of proteins by IR spectroscopy. Indeed, for the quantitative evaluation of each structure, several hypotheses are put forward : the integrated extinction coefficients are supposed to be equal for all structures and all types of proteins; the treatment described allows only for a simple interpretation in which each structure (or family of structures, see below) is characterized by a defined frequency but in fact only 80% of amide I is due to the amide C=O stretching. As a matter of fact, these approximations have not impeded the correct estimation

(within a few percent) of the proportions of each secondary structure for a large number of soluble proteins in several approach essentially identical to ours (9,12). They can therefore be considered as empirically valid with regards to soluble proteins. The variability of the width of the different components of Amide I is likely due to the variability of the different structures found in a complex protein which causes the component maxima to shift within the ranges previously defined and their half-width to be broaden. For instance, the amide I' band of the alpha helix structure which usually falls near 1650-1655 cm^{-1} is located at 1660 cm^{-1} for bacteriorhodopsin. Even for copolypeptides, frequency shifts (13) and considerable band broadening (14) have been associated to small conformational changes of the "alpha helix". Very short alpha helices are also predicted to be shifted (15). Several examples of the variability of the amide I frequency of β sheets type structures can also be found. As a consequence, the components determined for large proteins are likely to represent a family of structures with common features e.g. the "alpha helix" family rather than a single structure.

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Endocytosis and Intracellular Transport of Ricin in Polarized MDCK Epithelial Cells

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INTRODUCTION

MDCK cells grown on permeable filters form monolayers with tight junctions between the cells, and with distinct apical and basolateral membrane domains (1). We have in the present study used the toxic protein ricin to measure the endocytic rate from the basolateral as well as from the apical side, the rate of transcytosis of ricin from the two sides, and the ability of ricin to enter the Golgi apparatus and to intoxicate the cells when added at either side. We have also addressed the question of whether ligands added from the two sides meet in intracellular organelles.

MATERIALS AND METHODS

Preparation of monovalent ricin-horseradish peroxidase conjugates, protein synthesis measurement and electron microscopy were performed as earlier described (2). Ref. 3 describes binding and endocytosis of ^{125}I-labeled ricin.

RESULTS AND DISCUSSION

Ultrastructural studies involving ricin-horseradish peroxidase (Ri-HRP) and biochemical measurements based on ^{125}I-labeled ricin revealed that ricin was bound both to the apical and basolateral surface of the MDCK cells. The toxin was rapidly endocytosed from both sides of the cells, although somewhat more efficiently from the basolateral side. After 1 hour of

incubation with monovalent Ri-HRP, which behaves like native ricin with respect to intracellular sorting (2), the toxin was found in apical vacuoles whether added from the basolateral or from the apical side. Furthermore, when cationized ferritin was added at the apical side and ricin was added at the basolateral side, the two ligands was partially colocalized in the same organelles after 1 hour of incubation. Both biochemical measurements and ultrastructural studies showed that ricin is transferred from the apical side of the cell to the basolateral side and vice versa. Transcytosis of ricin was most efficient from the apical side to the basolateral side. Interestingly, the vesicular profiles which were labeled with transcytosed Ri-HRP were often coated (Fig. 1).

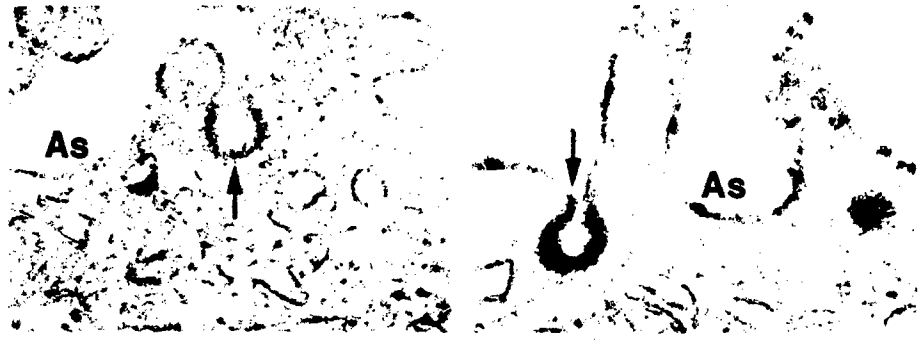


Fig. 1. Left: A coated vesicle (arrow) bringing Ri-HRP from the basolateral to the apical side. Right: Cells incubated with Ri-HRP for 1 hour apically. A coated pit is present (arrow).

Different lines of evidence suggest that ricin has to be transferred to the trans Golgi network in order to intoxicate the cells, and, interestingly, electron microscopical studies showed that ricin entered the Golgi apparatus from both sides. Furthermore, ricin intoxicated the cells with about the same kinetics when added at the basolateral side as when added apically. This result is of interest also in connection with the possible use of immunotoxins.

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Hydrophobic Photolabelling of Staphylococcus aureus α-Toxin in the Membrane-Bound State

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INTRODUCTION

Staphylococcus aureus is known to produce several exotoxins (1). One major component of these exotoxins is the α-toxin, which presumably plays a major role in the pathogenicity of this microorganism. The α-toxin is a soluble protein which interacts with membranes to form transmembrane channels or pores, which leads to cellular damage. The pore formation is said to result from the monomeric -toxin's ability to spontaneously aggregate to a hexamer on interaction with membranes (2). We have recently reported diazofluorene (DAF) as a new photoactivable reagent for labeling membrane hydrophobic core in both artificial and natural membranes (3). These studies indicated that DAF effectively labels the membrane-spanning domains of integral membrane proteins in human erythrocytes (3). In the present study it was observed that while DAF does not label the α-toxin in solution, it effectively labels the α-toxin in the membrane bound state in artificial membranes.

MATERIALS AND METHODS

The α-toxin was kindly provided by Dr. C.A. Pasternak and Dr. S. Bhakdi. Phosphotidylcholine (PC) was purified from egg yolk and vesicles prepared by sonication as described earlier (3). In all experiments 10 mM Tris containing 100 mM NaCl, pH 7 was used. For carrying out the calcein release assay, the PC vesicles were prepared in the same buffer containing 0.8 mM calcein and passed over a G-50 column. The PC vesicles with entrapped calcein were eluted in the void volume. The PC concentration at this stage was 1 mM. A part of this PC (5 nmoles) vesicle preparation was added to 3 ml of Tris buffer and increasing amount of α-toxin (3 - 45 μg) was added. The release of calcein was monitored by following the increase in fluorescence. The 0% lysis value was obtained from the control PC vesicles which did not contain the α-toxin. The 100% lysis was obtained by addition of 5% Triton X-100 to the

control PC vesicles. Diazofluorene tritiated at position C2 was prepared as described earlier (4). An alcholic solution of [3H]-DAF (sp. act. 175 mCi/mmole) was used. On addition to the aqueous suspension it was ensured that the alcohol concentration was below 1% (v/v). [3H]-DAF (0.5 µCi) was added to 1 ml of Tris buffer containing 30 µg of α-toxin and incubated for 20 min at 25°. The solution was then photolysed in a Rayonet minireactor (RMR-500) fitted with four 25 watts 3000 Å lamps, for 3 min. After photolysis the aqueous layer was extracted with diethyl ether and counted. In a similar experiment [3H]-DAF (0.5 µCi) was added to iml of PC (2 mmoles) vesicle preparation and incubated for 20 min. To this suspension 30 µg of α-toxin was added and incubation continued for 10 min. The suspension was then photolysed for 3 min as described above.

RESULTS AND DISCUSSION

In the present work PC vesicles were utilised as the membrane preparation for studying the interaction of α-toxin with membranes. Ikigai and Nakae (4) have recently shown that the α-toxin interacts with vesicles made from PC containing unsaturated fatty acids and forms hexameric channels. The membrane pore formation property of α-toxin was assessed by calcein release assay. Over 90% of calcein was released at a concentartion of 30 µg/ml of α-toxin. When α-toxin was labeled with [3H]-DAF in solution, no radioactivity was found to be associateed with the α-toxin. On the other hand under conditions when α-toxin leads to pore formation in PC vesicles, [3H]-DAF effectively labeled the α-toxin. Photolabeling of PC vesicles and α-toxin together followed by chloroform:methanol (2:1) extraction indicated that both the PC and the α-toxin have been labeled. The aqueous layer obtained after lipid extarction, on Sephadex G-50 column chromatography gave bulk of the radioactivity in the void volume, confirming the labeling of α-toxin in the membrane bound state. It is interesting to note that DAF does label soluble proteins with hydrophobic sites like bovine α-lactalbumin and calmodulin. The labeling of α-toxin only in the membrane bound state indicates that the α-toxin on interaction with membranes, undergoes conformational changes leading to exposure of hydrophobic sites. These newly genearted hydrophobic sites assist in effctive interaction of the α-toxin with itself and other membrane components leading to transmembrane channel formation.

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Effect of pH on Tetanus Toxin Interaction with its Receptor Components

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In previous studies from our laboratory (1), the interaction of tetanus toxin (TeTo) with cerebral neurons in culture was characterized as ganglioside (Glb) mediated three-step interaction. Additionally we have documented the existence of a second non-glycolipid trypsin-sensitive component that facilitates the interaction of TeTo with nerve cells (2).
As TeTo binding capacity is reversibly pH-dependent (3), we have modulated the external pH during binding to further characterize the interaction of TeTo with the different components. Maximal toxin binding at 4°C and 37°C was obtained between pH 4.2 - 4.9. At either acidic or neutral pH TeTo binding is absolutely dependent on the presence of the Glb gangliosides at the cell surface. However the role of the trypsin-sensitive component was more evident at pH 7.4 than at pH 4.7. (As observed by sequential proteolysis and cell fixation which reduced the binding only slightly at pH 4.7). Furthermore, metabolic inhibitors (2-deoxyglucose and sodium azide) reduced toxin-cell association by more than 40% at pH 7.4 while had almost no effect at pH 4.7. Therefore, although TeTo is internalized at both pH (as it became inacressible to MAb's), it seems that different mechanisms account for the processing at acidic and neutral pH. This possibility was studied by testing the effect of lysosomotropic agents (which raise the acidic endosomal pH). In our neuronal cultures such agents (ammonium chloride, chloroquine) were not effective in reducing toxin cell-association at either pH 4.7 or 7.4. Additional analysis of the fragmentation pattern on SDS-PAGE of labeled TeTo bound to cells at 4°C and 37°C; at pH 4.7 and 7.4 revealed similar toxin fragments. These results might imply that the acidic endosomal pH is not required for the processing of TeTo.

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*This work was done as part of the Ph.D. thesis of A. Nathan at the Dept. of Neurobiol., Weizmann Inst. of Science, Rehovot.

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Finally, we have employed hydrophobic chromatography to dissociate between the direct effect of pH on TeTo molecule from its interaction with the receptor components at cell surface. An elution profile was designed to separate between hydrophilic and hydrophobic materials at either pH 4.7 or 7.4. At the neutral pH most of the toxin (ca. 60%) was eluted under hydrophylic conditions while at the acidic pH a clear increase of hydrophobic population was observed. The increase in the hydrophobic properties of TeTo observed at acidic pH is in agreement with previous evidence from other laboratories (for review see (4)). The concept that neurotoxins undergo conformational changes at acidic endosomal pH with exposure of hydrophobic surfaces, has been proposed as a general mechanism for the penetration across the lipid bilayer.

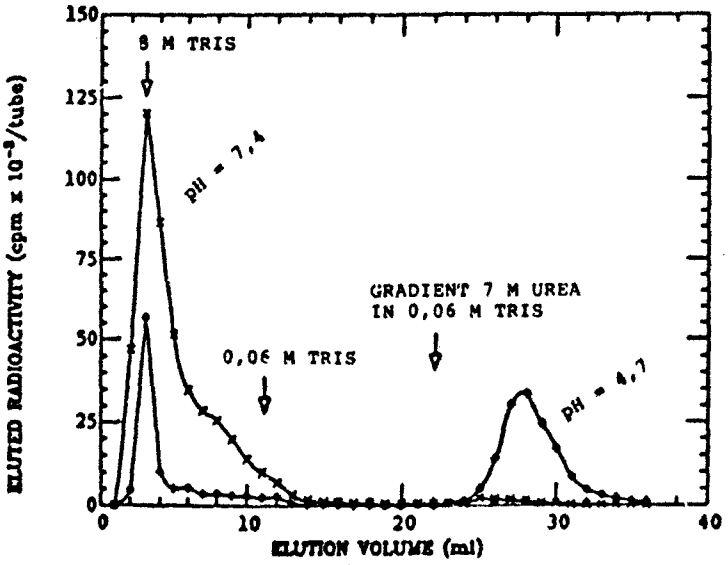


Figure 1 : Hydrophobic chromatography of ^{125}I-TeTo at different pH on phenyl- sepharose (CL-4B) column.

^{125}I-TeTo (1x10^6 cpm) was loaded on a phenyl-sepharose column (2 ml) pre-equilibrated with 2.0 M Tris-Cl (at suitable pH). Radioactivity was eluted from the column starting with 2.0 M Tris, followed by 0.05 M Tris (10 ml each) and then by a linear gradient developed by 7M urea (10 ml) into 0.05 M Tris (10 ml) and finally washed by 7M urea.

Acknowledgment
We thank Dr. P. Lazarovici for his advice on the hydrophobic chromatography technique.

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Kinetics of Interaction of Tetanus Toxin with Lipid Vesicles

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INTRODUCTION

Tetanus toxin (TeTx) is a potent neurotoxin which acts both on the central and on the peripheral nervous system. Its paralyzing effects require several steps: binding to nerve terminals, internalization, retrograde axonal transport, transition to presynaptic neurons (1,2). TeTx forms channels in model membranes (3). The strong pH dependence of the membrane-toxin interaction and the resistence of mutant cells defective in endosomes acidification suggest that TeTx is internalized via endocytic vescicles. The channel forming properties may be related to this step.

MATERIALS AND METHODS

TeTx, B-fragment and toxoid were kindly gifted by prof. B.Bizzini. Small unilamellar vesicles (SUV) were prepared by sonication, loaded with 70 mM calcein, and washed by gel filtration. Phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) were used alone or in 1:1 mixtures.

RESULTS

We studied the interaction of TeTx with SUV of various lipids as a function of pH, toxin concentration, temperature and ionic strength. TeTx increases the permeability of the vesicles, Fig.1A. The time course of permeabilization is described by the sum of two exponential components of which the faster accounts usually for more than 70% of the effect. Both time constants decrease either by lowering the pH or by introducing negative lipids into the vesicles, Fig.1B. We presented a simple model based on reaction rate theory which accounts for these results (4). It assumes that insertion of TeTx into a lipid membrane occurs only when an acidic group of

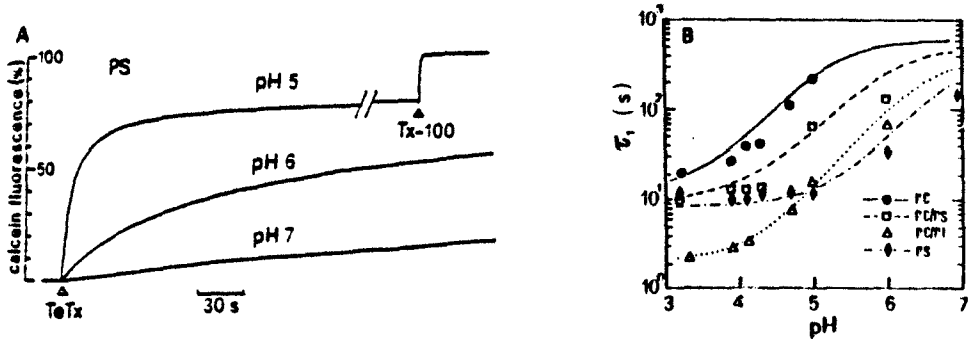


Fig.1. Permeabilization of lipid vesicles by tetanus toxin. A: 278 nM TeTx was added to PS vesicles at different pH. The fluorescence (excitation at 494 nm and emission at 520 nm) increases after toxin addition as the dye is released by the vesicles. 100 % release was obtained by the addition of 0.5 mM Triton X-100. B: pH dependence of the fast time constant measured as in part A. Different symbols are for different lipids as indicated on the figure. Theoretical lines are best fit according to the model described in (4).

the protein with pK 3.4 is neutralized by one proton at the surface of the vesicle. Since the toxin equilibrates with the local pH, the fastening effects of acidic lipids is explained merely by the creation of a negative surface potential which attracts protons locally. This is confirmed by the slowing effects of high Na+ concentration which reduces the surface charge by screening. There are still small differences between the lipids tried which indicate the following order of sensitivity to the action of the toxin: PI > PS > PC. The activation energy for the two time constants is 19.8 and 14.8 Kcal/mole, fast and slow component respectively, i.e. slightly larger than that for pure diffusion through the bilayer. The permeabilization effect is voltage dependent in the sense that vesicles bearing an inner negative potential are depolarized very quickly while those bearing an inner positive voltage are barely depolarized at all. Both H-fragment and toxoid were much less active than whole TeTx.

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Pharmacology of the Chains and Fragments of Tetanus Toxin

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Some properties of the chains and fragments of two-chain toxin (1, 2) have been studied and compared with those of genuine and reconstituted toxin. With respect to general toxicity, reconstituted two-chain toxin differed only marginally from genuine toxin (LD_{50}, mice, s.c. about 2 ng/kg). Toxicity of light (LC) and heavy (HC) chain, and of the fragments β_2 ($F\beta_2$) and C (FC) was minor and probably due to traces of toxin. Fragment B (FB) in the mg/kg range produced the typical (3) run-down syndrome.

Binding on rat brain membranes was assessed in buffers of low and high ionic strength by competition with radiolabelled genuine two-chain toxin. Differences between genuine and reconstituted toxin were absent. On a molar basis, FC did not differ from two-chain toxin in hypotonic buffer but displayed very low affinity in isotonic buffer. HC behaved like FC in hypotonic buffer and was about 100 times less potent than toxin in isotonic buffer. LC failed to compete with radiolabelled toxin or with radiolabelled LC in either buffer.

Inhibition of [^{3}H]-noradrenaline release was assessed on preloaded particulate rat brain (1). Again, genuine and reconstituted toxin were approximately equipotent whereas FB, FC and LC were completely inactive. HC inhibited the release about 100 times less than toxin. HC also decreased, in contrast to toxin, LC, FB and FC, the [^{3}H] noradrenaline content of particulate brain in a manner sensitive to FC.

To abolish the barrier function of the cell membrane, bovine adrenomedullary cells were preloaded with [^{3}H] noradrenaline, permeabilized with streptolysin O, exposed to the

agents to be tested and stimulated with Ca^{2+}. Toxin, in particular if reduced, inhibited the release. HC was inactive whereas LC mimicked the toxin effects (4).
On the buccal ganglion of Aplysia californica toxin, its chains and fragments were applied either extracellularly or intracellularly to cholinergic cells. Before and in the course of poisoning individual cells were stimulated electrically and the evoked potentials were recorded from a pertinent postsynaptic cell. When applied extracellularly, toxin as well as FB, but neither $F\beta_2$, HC or LC inhibited acetylcholine release. Toxin, LC and FB but not HC did so upon intracellular injection (5).
Thus the toxin action on permeabilized and injected cells can be mimicked by its LC. HC or its N-terminal β_2-fragment is apparently necessary for internalization of LC into Aplysia neurones. The complete HC is required, together with LC, to elicit full toxicity, binding in isotonic buffer and inhibition of neurotransmitter release in the murine systems used.

Table: Pharmacology of the chains and fragments - a synopsis

	Toxicity (ng/kg)	Binding [1]	Inhibition of neurotransmitter release		
			in rat brain	bovine cells after permeabilization	Injected Aplysia
Toxin	2	+++	+++	+[2]	+++
LC	≈ 10000	0	0	+++	+++
HC	≈ 10000	+	+	0	0
FC	≈ 10000	(+)	0	n.t.	n.t.
FB	> 10^6	(+)	0	0[2]	+++

[1]) in isotonic buffer [2]) +++ when reduced. n.t. = not tested

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The Chains of Tetanus Toxin and its Fragment B – Preparation and some Biochemical Properties

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INTRODUCTION

Tetanus toxin is synthesized as a single-chain intracellular polypeptide which is converted into two-chain toxins by numerous proteases. Limited proteolysis leads to a 2-20 fold activation (4), depending on the assay system. We now communicate simple, reliable, non-denaturating techniques for separating the chains of toxin and fragment B (5). The polypeptides obtained allow studies on the interaction between the individual chains and their contribution to the pharmacological action of the toxin.

MATERIALS AND METHODS

Single-chain and two-chain toxins (LD_{50} in mice 4-5 and 2 ng/kg, resp.) were isolated from cells or culturefluids of Cl. tetani (4). Fragments B and C were obtained using a modified protocol from a papain digest of single chain toxin (3,5).

Two-chain toxins and fragment B were separated into their constituent chains by isoelectric focussing. A Vesterberg-Svensson column (Pharmacia-LKB) was loaded with cathode solution (0.24M NaOH, 2M urea and 1.5M sucrose). Then a linear sucrose gradient was formed. Its heavy solution (50ml) consisted of 1.9ml ampholyte (equal volumes of Servalyte^R pH 3-6 and pH 6-8), 2M urea, 1.45M sucrose and 50mM dithiothreitol. The light solution (50ml) contained tenfold less sucrose but was otherwise identical. The two-chain toxin or fragment B (10-20mg in 0.5 to 3 ml in buffered saline) were dialyzed against glycine (1%, pH 6) for 3h at 4°C, mixed with the same volume of heavy solution and 200µl ampholyte, and introduced after about 50% of the gradient volume had entered the column. Finally, the sucrose gradient was completed and followed by the anodal solution (20ml 2M urea with 400µl of phosphoric acid). After about 16h of focussing (max. 2000V, 15W at 4°C) the contents of the column was withdrawn in 2ml fractions using a roller pump. To obtain light chain (LC) of increased purity (LD_{50} above 100µg/kg) the polarity of the column may be reversed, so that this chain is eluted first.

RESULTS AND DISCUSSION

Toxin, fragment B and their constituent chains differ by their pI's (toxin 6.0, heavy chain (HC) 7.2, LC 4.8; fragment B 5.6, fragment B2 (the N-terminal moiety of HC) 7.1 and 6.8). In the presence of urea and dithiothreitol chain separation proceeds (see Fig.1) with a yield of about 60-80%, high purity and low residual toxicity. LD_{50} in mice was $1\text{-}3\times10^5$ ng/kg for HC and $3\text{-}4\times10^4$ ng/kg for LC.

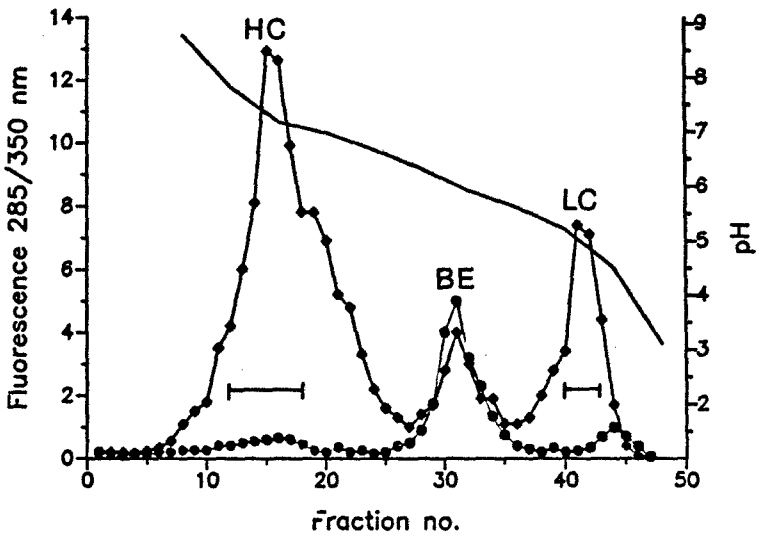


FIG.1: Genuine two-chain toxin (20mg) from bacterial culture filtrate (BE) was separated into HC and LC by isolelectric focussing. A small amount of starting material remained undissociated (◆). The left ordinate gives the fluorescence (285nm/350nm) intensity of the tryptophan (trp) residues. The pH values of the fractions (abscissa) are indicated by the right ordinate (—). Note that HC contains 10 trp residues/molecule but the LC only 1 (1,2). Reconstituted two-chain toxin corresponding to 3mg protein was focussed without dithiothreitol (●).

Reconstitution of the toxin was achieved by mixing equimolar amounts of the chains and dialyzing them under air against isotonic buffers (pH 7-8). Under these conditions the disulfide bridge was re-formed and toxicity was recovered (LD_{50} 3-7ng/kg) almost quantitatively. This indicates that damage to the chains due to the separation procedure is minor. By the same technique Fragment B was reconstituted from light chain and B2 fragment of HC, whereas no association occurred between B2 and purified fragment C. This indicates that LC and B2 are counterparts in the tertiary structure of the toxin molecule.

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Modification of Tetanus Toxin by Transglutaminase: Effect of Gangliosides.

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The nucleotide sequence of the gene encoding for tetanus toxin has been recently published (1) . We have computer searched the deduced primary structure with the aim of finding regions of homology with domains already known to be associated with specific functions in other proteins.

As an example of this approach we report the existence of transglutamination sites in the toxin molecule, as suggested by sequence analysis and confirmed by experimental tests.

Experimental methods

Computer analysis was carried out with the 4.1 release of GENEPRO Software Package, and by using the PIR Sequences Data Bank running on an IBM AT computer. Homology search was enhanced by the implementation of a BASIC routine able to search homologous domains also by referring to known chemical-physical aminoacidic similarities (3). For the biochemical approach, tetanus toxin, purified from a toxigenic non-sporulating strain of Clostridium Tetani, was incubated with Guinea pig liver transglutaminase. The reaction was carried out in the presence of ^{14}C spermidine (S.A. = 110mCi/mmole), 3mM $CaCl_2$, 100 mM Tris HCl pH 8.0 and 2mM DTT. Incorporation was measured after incubation at 37 °C followed by 10% TCA precipitation and liquid scintillation count.

Results

A computer assisted examination of the toxin primary structure indicated the presence of two sequences, the first one in the light chain and the second one in the heavy chain that showed good structural homology with specific domains in proteins previously known to possess transglutaminase substrate features, such as β-Casein and β-Bp Crystallin (2)(3)(fig. 1).

MSP	L G L S Q S K V L G	
CASEIN	S V L S L S Q S K V L P	167
TETANUS	G F G S I M Q M A F C P	195
UTEROGLOBIN	M K D A G M Q M K K V L	40
TETANUS	L L E F D T Q S K N I L	823
BP CRYSTALLIN	Q K G A Q T E M S A	7
RSBP	T K E K Y S Q S E E V V	9

Fig.1 Alignment of putative transglutamination sites of tetanus toxin with known transglutaminase substrates (4). The numbers on the right side indicate the position of the reactive glutamine.

Tetanus toxin was thus tested as a substrate of transglutaminase (fig. 2) by evaluating the incorporation of ^{14}C labeled spermidine in the presence of Guinea pig liver transglutaminase. The kinetic features of the reaction were studied by varying the concentrations of spermidine and tetanus toxin. The apparent Km for spermidine was about 5 μM, in good accord with literature data. The Vmax was 0.1 nmol spermidine/min/mg enzyme (fig. 2A), at a toxin concentration of 1 μM. (Reaction volume was 80 μl). SDS PAGE analysis of tetanus toxin after transglutamination showed formation of high molecular weight aggregates suggesting the possibility of cross linking, probably as a result of transglutamination, between light and/or heavy chains of tetanus toxin. Interestingly , preincubation of tetanus toxin in the presence of micellar gangliosides, previously suggested to be membrane receptors for tetanus toxin (5), showed a higher than 80% inhibition of the incorporation of spermidine, whereas dimethylcasein, a very powerful substrate for transglutamination, when preincubated in the same way, did not show a significant decrease of incorporation (fig. 3).

Discussion

Transglutamination is a common intracellular reaction that has been implicated in the process of endocytosis and vesicular membrane traffic (6). The present results suggest that tetanus toxin might be modified by cellular transglutaminases during the process of intoxication, since it is known that the toxin must be internalized and probably modified by the target cells to become active. Thus, there is a possibility that the activation step might involve transglutamination. With regard to this hypothesis, it is interesting to note that one of the sites identified by us is adjacent to the putative NAD binding region suggested by Montecucco (7). We plan to test whether transglutamination might lead to expression of an ADP ribosylating activity by the toxin. Gangliosides are known to be part of the receptor for tetanus toxin and to induce changes in its secondary structure (5). Their inhibitory effect on toxin transglutamination indicates a marked dependence of this reaction on the conformation of the toxin substrate, and is in line with the hypothesis of a functional role for the transglutamination sites .

Acknowledgements

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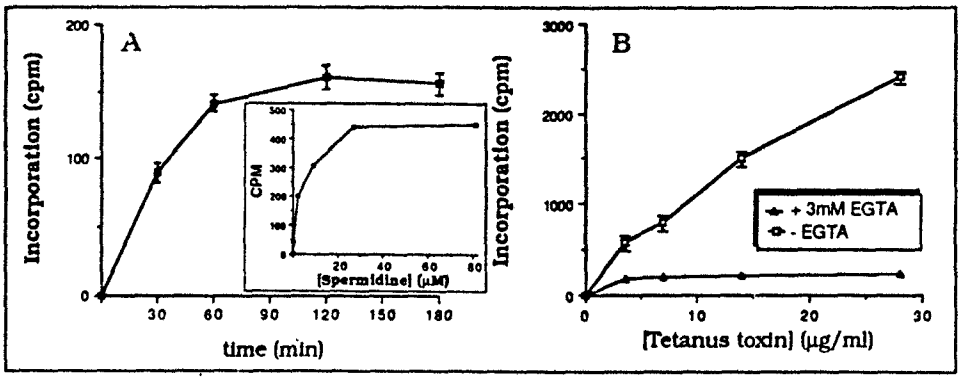


Fig. 2 Incorporation of spermidine in tetanus toxin. A. Time course of the reaction. The toxin concentration was 1 μM . Inset: dependence on spermidine concentration after a 2 hours incubation. B. Dependence on toxin concentration after a 2 hours incubation. Values are means ± SD (n=5)

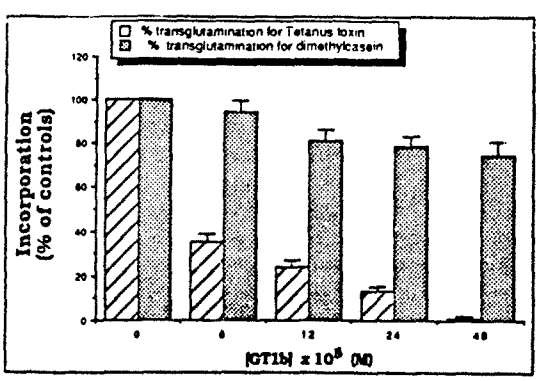


Fig. 3 - Inhibitory effect of micellar gangliosides (CMC=10^{-5} M) on spermidine incorporation in tetanus toxin and dimethylcasein after a 2 hours incubation. Experimental conditions were as in fig. 2. Bars are means ±SD (n= 7).

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Membrane Interaction of Botulinum Neurotoxins

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ABSTRACT

The interaction of botulinum neurotoxin (BoNT) serotypes A, B and E with membranes of different lipid compositions was examined by photolabelling with two photoreactive phospholipid analogues that monitor the polar region and the hydrophobic core of lipid bilayer respectively.

At neutral pH the neurotoxins interact both with the polar head groups and with fatty acid chains of phospholipids. At acidic pHs the botulinum neurotoxins appear to undergo a structural change characterized by a more extensive interaction with lipids. Both the heavy and light chain subunits of the neurotoxins are involved in the process. The low pH acquired hydrophobicity of BoNT falls in a pH range overlapping that found in endosomes.

KEYWORDS

Botulinum neurotoxins, protein-lipid interactions.

INTRODUCTION

Botulinum neurotoxins (BoNT) block acetylcholine release at the neuromuscular junction thereby causing a flaccid paralisys. There are seven BoNT serotypes with similar macrostructure and mechanism of action, but with different immunological, biochemical and neuropathological properties. The three NT serotype most frequently associated with human botulism are: A (BoNT-A), B (BoNT-B) and E (BoNT-E).

BoNTs are synthesized as a single chain (150 kDa) and are nicked by proteolytic cleavage that generates a heavy chain (H, 100 kDa) and a light chain (L, 50 kDa); the fragments remain held together by interchain disulphide bridge(s) and not-covalent forces.

The cellular mechanism of action is unknown, but experimental work and a supposed similarity with diphtheria toxin suggest that the cell intoxication by BONT proceeds in three main steps: (a) binding, (b) internalization, (c) block of neurotransmitter release. Binding appears to be followed by a receptor-mediated endocytosis that brings the NT into closed vescicles. The effect of lysosomotropic substances and the ability of BoNT to form ion-conducting channels at low pH promoted the idea of a low-pH-driven escape of the NT from an endosomal compartment, similar to that found with diphtheria toxin. This process can be very conveniently monitored by membrane photolabelling with two photoactive phospholipid analogues.

RESULTS AND DISCUSSION

At neutral pH BoNTs interact with asolectin liposomes at superficial and deeper level and both the heavy and light chains are involved in the lipid interaction. At pH 4.5 both chains of the BoNTs are much more extensively labelled by both the surface and deeper probes. The presence of polysialoganglliosides in the asolectin liposomes causes an increase in the amount of radioactivity bound to heavy chain.

The pH-dependence of labelling with PC I and PC II dispersed in asolectin vesicles shows a large increase of radioactivity incorporated into H and L chains at low pH. This increase in the fragments interaction with phospholipids at the surface and deeper level suggest that the BoNTs at acidic pHs undergo a structural change, with the exposure to phospholipids of previously hidden hydrophobic surface. Within this general feature, the BoNTs tested here differ noticeably: BoNT-B shows a larger hydrophobic interaction than BoNT-A and BoNT-E especially at the level of glycerol moiety.

The PC I/PC II ratio of labelling, measured as the $^{3}H/^{14}C$ ratio of toxin-bound radioactivity after illumination with respect to the starting ratio present in liposomes, changes with pH. The most likely explanation is that NTs expose aminoacid residues of different reactivity at the two levels monitored by PC I and PC II when pH is lowered. These findings also indicate that the increased labelling of BoNT at acidic pHs is related to a conformational change of the NT, characterized by a more extensive hydrophobic interaction with phospholipids, rather than in a larger number of toxin molecules interacting with lipids.

Membrane photolabelling was also used here to characterize the interaction with membranes of the isolated carboxymethylated chains of BoNT-A. Both the H and L chains show a higher level of photolabelling with respect to the labelling of the same chains in the native toxin both at pH 7.5 and pH 4.0. The reconstitution of H and L subunits cause a decrease of the labelling and a reappearance of the pH dependence of the interaction with the membrane. These findings suggest that both fragments in the isolated form expose some hydrophobic patches hidden in the native and reconstituted toxins and probably involved in protein-protein interactions.

The distribution of radioactivity along the BoNT-A chains as determined after pepsin cleavage and separation of B and C fragment, shows a high level of labelling of fragment C compared with the total amount of radioactivity associated with whole molecule in the same conditions. Moreover C fragment presents a clear pH dependence of the interaction with lipids and a change in the PC I/ PC II ratio of labelling at low pH. These results suggest that fragment C at low pH undergoes a structural change with exposure to phospholipids of hydrophobic surface. The comparison of the radioactivity bound to fragment C and whole toxin at acidic pH indicates that the carboxy-terminus of heavy chain may be involved in the insertion of the toxin into the membrane.

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Cytotoxicity of Clostridium Novyi Type A Alpha-Toxin. Pharmacological and Morphological Studies

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INTRODUCTION

Clostridium novyi (Cl.novyi) is classified into four types designated A, B, C and D, according to the respective soluble antigens. Types A and B only synthesize the lethal α-toxin (1.). The actions of apparently homogeneous α-toxin from C.novyi type A (5) were studied in order to develop an in vitro system which closely mimicks the in vivo effects, and to search for the mode of poisoning (2).

RESULTS AND DISCUSSION

Time to death (i.v. injection, mice) was inversely related to dose with a detection limit of about 200 ng/kg at 100 hours. Injections of 2.5 ng and more into the rat paw led to a slowly (maximum after about 30 hours) developing, dose-dependent edema which was useful as a quantitative in vivo assay based on volumetry (6). Vascular leakage was due to gap formation between endothelial cells as demonstrated by the Evans blue test (4) and by vascular labelling with carbon ink (3).
Similarly endothelial cells cultured from pig pulmonary artery lost their "cobble-stone" arrangement, retracted and finally became round after a dose-dependent lag period of some hours after poisoning. The morphological changes were accompanied by depression of uptake or incorporation of [^{3}H]uridine. A quantitative in vitro assay was established on the inhibition of [^{3}H]uridine incorporation. Like in animals, the action of α-toxin started with a few ng/ml and proceeded slowly for at least one day. It became resistant to antitoxin within two hours exposure. The toxin action is not limited to endothelial cells since chicken embryonic cells, L-929 (a mouse fibroblast line), T-84 cells (an epithelial cell line), FaO-cells (a hepatoma cell line) and a rat phaeochromocytoma line (PC-12) behaved similarly, but with differing sensitivities.

α-Toxin was found to differ from other bacterial toxins investigated whose mode of action is already known. Unlike botulinum C_2 toxin it was no ADP-ribosyltransferase. Unlike clostridial neurotoxins it did not inhibit noradrenaline release. Unlike diphtheria toxin it failed to inhibit protein synthesis in a cell free system. Early membrane damage was apparently not involved. Search for broad spectrum proteolytic activity was negative. Thus α-toxin differs basically from bacterial cytolysins.
One of the earliest sequelae of poisoning observed in endothelial cells was the disintegration of the F-actin fibers in the cell periphery, called dense peripheral band (DPB). Depending on dose and time the remnants of the DPB were shifted centripetally (2 µg/ml α-toxin: 40 minutes; 20 ng/ml: 5 hours) and disappeared after 2 hours exposure to 2 µg/ml. Within 12 hours incubation with 2 µg/ml, F-actin condensed around the nucleus and concentrated in perinuclear caps. Even at this stage cell rounding was not yet complete, as processes containing very little F-actin were still visible. The unknown target of the toxin action appears to be linked to the cytoskeleton. Final proof for internalisation is lacking so far, and specific binding in isotonic buffer is still to be shown (5).

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Alpha-Toxin of Clostridium Novyi Type A: Purification, Iodination and Binding Properties

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INTRODUCTION

Clostridium novyi is classified into four types (A-D) based on the synthesis of different soluble antigens (4). C. novyi type A causes malignant edema and death by action of alpha toxin (3). Endothelial and chicken embryonic cells lose their arrangement when poisoned in vitro. The morphological changes are accompanied by depression of uptake and incorporation of [^{3}H]uridine (1).

METHODS

C. novyi type A (strain ATCC 19402) was cultured anaerobically 3 days at 37°C in 0.4-liter portions of TYG medium (3% trypticase, 2% yeast extract, 0.4% glucose, pH 7.8, supplemented with 0.5ml/100ml 20% cysteiniumchloride, pH 7.4, after sterilisation). Cultures were pooled, clarified by centrifugation, and the toxin was precipitated with $(NH_4)_2\ SO_4$ (40% saturation, buffered with Na_2HPO_4 to pH 7.2) for 3h at 4°C. The precipitate was redissolved, dialyzed against 10mM Na-phosphate, pH 7.8 and applied to a DEAE cellulose column. Bound alpha-toxin was eluted with a NaCl gradient (0-140mM, 400ml) at about 80mM. Minor impurities were removed by gelfiltration on Sephacryl S 300 column (LD_{50} = 200ng/kg). Alpha-toxin was iodinated with Bolton Hunter reagent. Binding properties were evaluated as described (5) in hypotonic (50mM TRIS acetate, pH 6.0) and isotonic buffer (50mM TRIS HCL, pH 7.4, 150mM NaCl) with membranes (2.5µg/ml for hypotonic, 150µg/ml for isotonic buffer) isolated from cultured chicken embryonic cells.

RESULTS AND DISCUSSION

Recovery from the ammonium sulfate precipitate after DEAE ion-exchange chromatography and gelfiltration was between 10 and 20% with respect to alpha-toxin. A purification step on CM-Sephadex (2) could be omitted.

Purification step	Total protein (mg)	Recovery (% of protein)	Lethal activity (LD_{50}/mg)	Purification (fold)
Ammonium sulfate precipitate	105	100	3.9×10^4	1
DEAE (DE 52)	27	25	1.1×10^5	2.8
S 300	6	5.7	1.6×10^5	4.1

Under non-reducing conditions SDS-PAGE revealed a faster main band (MW 200 kD) followed by a multiple number of weaker ones in the molecular range of more than 200 kD. Reduced alpha-toxin formed a single band, indicating homogeneity and an apparent MW of 200 kD, as well as the absence of disulfide-linked chains. Toxin was stable at least for 10 months at -70°C. Iodination with the Bolton Hunter technique yields specific radioactivity of 6.6-8.8 µCi/µg. 20-50% of biological activity was lost, whereas nearly 100% was lost when the IodoGen technique was applied, probally due to oxidation. Binding experiments in hypotonic buffer gave evidence for a single binding site with a K_D of 12-20nM and a B_{MAX} of 1.2-1.5 pmoles/mg as estimated from competition experiments by LIGAND analysis. Binding experiments in isotonic buffer were negative, even when membrane concentration was increased up to 150 µg/ml. Therefore it is questionable, if the results in hypotonic buffer reflect the physiological binding properties in vivo or in cell culture.

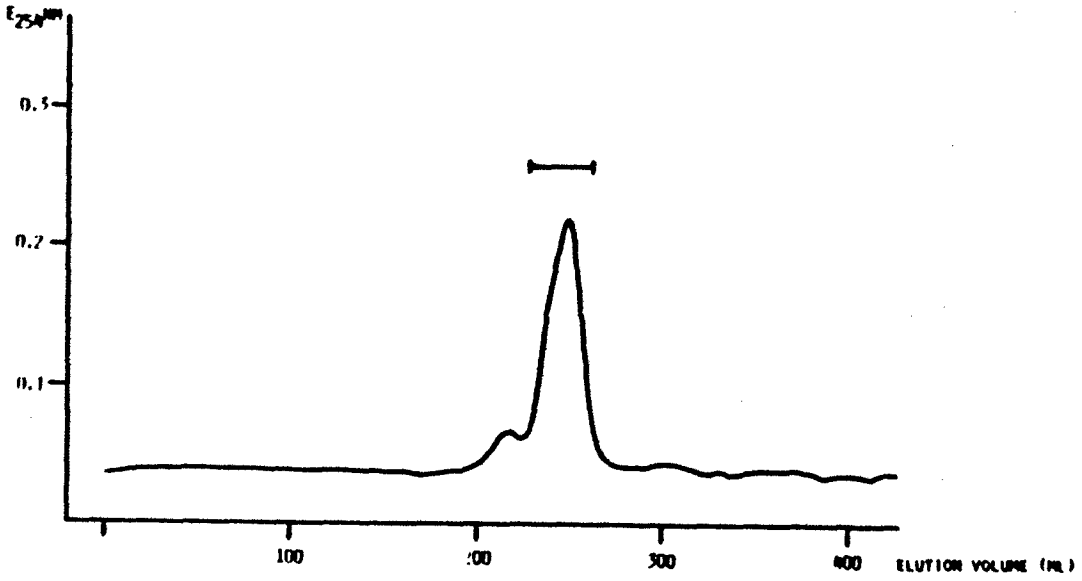


Figure. Gelfiltration of alpha-toxin (6 mg) on a S 300 column (2.5x90cm) after DEAE ion exchange chromatography and concentration. The buffer was 50 mM Na-phosphate, 150 mM NaCl, pH 7.4, flow was 14 ml/h. The major peak represents alpha-toxin. The fractions indicated by the bar were pooled and stored frozen at -70°C.

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Immunoprecipitation of a Diphtheria Toxin Binding Protein from Vero Cells

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ABSTRACT

A monoclonal antibody (1A2) that inhibits binding of radiolabeled diphtheria toxin (DT) to Vero cells and protects Vero cells from the inhibition of protein synthesis mediated by DT was used to immunoprecipitate a DT binding protein from biosynthetically labeled Vero cells. SDS-PAGE of the immunoprecipitates under reducing conditions followed by autoradiography revealed a band corresponding to 23 kDa. Preincubation with DT prior to immunoprecipitation caused a decrease in the precipitation of the 23-kDa band. The decrease ("down regulation") in radiolabeled DT binding seen after incubation of Vero cells with CRM 197 was not associated with a decrease in the labeling of the 23-kDa protein.

INTRODUCTION

DT binding to receptors on the plasma membrane of target cells is thought to be the first step in expression of the biological activity of this inhibitor of protein synthesis (2, 4). A polypeptide that specifically binds DT has been identified as a 150-kDa membrane component in guinea pig lymph node cells (5). Subsequent studies with primate cell lines demonstrated a DT binding protein with a molecular weight of 10,000-20,000 (1). However, further progress in characterization and initiation of purification efforts has been limited by the lack of availability of a highly specific tool for separating the DT receptor from other components of cellular membranes. We have produced and characterized monoclonal antibodies against Vero cells that protect the cells from the toxin (6) and used one of these monoclonal antibodies to develop immunoaffinity techniques for identifying the DT receptor.

MATERIALS AND METHODS

A detailed description of the preparation of radiolabeled DT and the toxin receptor binding assay has appeared previously (3). Production, purification, and characterization of

monoclonal antibodies against Vero cells that protect from DT have been recently described (6). Vero cell monolayers were labeled with [^{35}S]methionine and solubilized in NP-40. Cell lysates were incubated with monoclonal antibody 1A2 and immunoprecipitation was then effected by addition of goat anti-mouse IgG conjugated to Protein A-Sepharose CL-4B. In some experiments incubation with monoclonal antibody 1A2 was preceded by incubation of cell lysates with an excess of unlabeled DT. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography. During down regulation of DT receptors, Vero cells were incubated with CRM 197 for 22 h at 37°C, then labeled with [^{35}S]methionine in the presence of CRM 197. After biosynthetic labeling, cells were solubilized and further processed as described above. CRM 197 was the generous gift of Rino Rappuoli, Sclavo Research Center, Siena, Italy.

RESULTS AND DISCUSSION

A DT binding protein has been isolated by solubilizing membranes from biosynthetically labeled Vero cells and immunoprecipitating the DT binding protein with a monoclonal antibody. This antibody inhibits binding of radiolabeled DT to Vero cells and protects Vero cells from the inhibition of protein synthesis induced by the toxin (6). Analysis of the immunoprecipitates on SDS-PAGE followed by autoradiography revealed the presence of a band that migrated as a 23-kDa protein. Preincubation of labeled solubilized membrane preparations with the toxin prior to immunoprecipitation caused a decrease in the precipitation of the 23-kDa band. The decrease ("down regulation") in radiolabeled DT binding seen after incubation of Vero cells with CRM 197 was not associated with a decrease in the labeling of the 23-kDa band. These and other results (6) suggest that the 23-kDa protein is the DT receptor (or a component thereof), and that our monoclonal antibody offers a powerful tool to study its structure, biosynthesis, and processing.

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Structure of DT and Fragments Studied by Polarized Infrared Spectroscopy

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Until now, the study of the secondary structure of diphtheria toxin (DT) in the presence of phospholipid vesicles as a function of the pH has been prevented by the optical turbidity of the solution. This problem has been overcome by the use of infrared attenuated total reflection spectroscopy, to study the structure of DT and its fragments A, B, CB1 and CB4 as a function of the pH in the absence and in the presence of phospholipids vesicles.

Incubation of DT with asolectin or DPPC-DPPA liposomes at pH 7.3 results in the binding of DT onto the liposomes and a 10% increase in its alpha-helix content. At pH 4, in the presence of liposomes, the secondary structure of DT is characterized by the appearance of a beta-sheet structure with strengthened hydrogen bonds which did not exist before pH lowering. The exact nature of this new type of beta-sheet structure which is called here low frequency beta-sheet (LF beta sheet) because of its stretching (C=O) frequency below the frequency expected for the "classical" beta-sheet structure, has not been elucidated.
DT fragment B displays little conformational change upon pH lowering in the presence of liposomes. However, the conformation of its cyanogen bromide fragments CB1 and CB4 is significantly modified when studied in the same conditions. The alpha-helix content of CB1 increases by 10% and polarisation measurements indicate that the average tilt between the alfa-helices of CB1 at pH 4 and a normal to the bilayer plane is 0°, confirming the hypothesis that several CB1 segments are transmembrane helices. On the other hand, the alpha-helix content of CB4 decreases dramatically while the LF beta-sheet content increases. Dichroism measurements

demonstrate that this sheet lies close to a parallel to the bilayer surface.
The fragment A of DT, which is thought to be transported into the cell cytoplasm with the help of fragment B, experiences a large conformational change upon pH lowering. Similarly to CB4, the LF beta-sheet content increases and the alfa-helix content decreases. In the low pH conformation, DT fragment A binds to the liposome membrane even in the absence of DT fragment B. The conformational modification of DT fragment A is fully reversed when pH is brought back to 7.3.

The abbreviations used are :
DPPC : DL-dipalmitoylphosphatidylcholine, DPPA : DL-dipalmitoylphosphatidic acid, DT : diphtheria toxin, DTA : fragment A of diphtheria toxin, DTB : fragment B of diphtheria toxin, CB1, CB4 : cyanogen bromide subfragments 1, 4 respectively of DTB.

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Requirements for Diphtheria Toxin A-Fragment Translocation into the Cytosol

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ABSTRACT

The 25 kD B-fragment derived polypeptide that is inserted in the plasma membrane when cell-bound diphtheria toxin is exposed to low pH, was mapped. It was found to consist of the residues from aminoacid ~300 to the C-terminal end. In addition to insertion of the 25 kD polypeptide, translocation of diphtheria toxin A-fragment was found to be dependent on a specific A-fragment C-terminal sequence, ending at Arg_{190}.

INTRODUCTION

When receptor-bound diphtheria toxin is exposed to low pH, uptake of the toxin occurs directly from the cell surface (1). By using ^{125}I-labelled nicked diphtheria toxin and a protease protection assay, both intact A-fragment and a part of the B-fragment became protected against externally applied proteases. The A-fragment has been shown to be released into the cytosol under these conditions (2). Several requirements for translocation has been found; insertion of the B-fragment into the membrane (2), a proton gradient of at least 1 pH unit (3), reduction of the interfragment disulfide bond (4) and anion transport (5). We have now mapped the part of the B-fragment that inserts into the membrane and characterized one of the factors that limit the amount of A-fragment that is translocated at low pH.

RESULTS AND DISCUSSION

The B-fragment derived 25 kD polypeptide protected against proteases after exposure of receptor-bound radioactively labelled nicked diphtheria toxin or in vitro made truncated

B-fragment to low pH, was isolated and mapped by cleavage with sequence specific reagents. Both o-iodosobenzoic acid, which cleaves at Trp-residues, and hydroxylamine which cleaves between Asn and Gly, yielded a peptide pattern indicating that the 25 kD polypeptide spans the region from residue ~300 to the C-terminal end. This region contains CB 1, a CNBr B-fragment derived peptide, that has been shown by Cabiaux et al. to interact with lipids at low pH (6).

Diphtheria toxin requires nicking between A- and B-fragment to be toxic. The toxin molecule contains three sensitive trypsin sites in that region, Arg_{190}, Arg_{192} and Arg_{193}. When A-fragment translocation was induced at low pH, only 5-10 % of the bound toxin molecules resulted in translocation. We therefore decided to investigate the role of nicking site on A-fragment translocation. A-fragments from total diphtheria toxin and A-fragments translocated to the cytosol at low pH were isolated and isoelectrically focused. We found that A-fragments from total toxin focused as four bands, whereas A-fragments translocated to the cytosol focused as one band with lowest pI, compared to the four bands from total toxin. This indicate that only A-fragments with one Arg in the C-terminal end was translocated. This was confirmed by sequencing data. Increasing the amount of A-fragments cleaved next to Arg_{190} by treatment of diphtheria toxin with carboxypeptidase B before binding, also increased the amount of A-fragment translocated to the cytosol.

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Structure-Activity Relationships of Diphtheria Toxin Fragment B: Interaction with Receptors and Protein Kinase Activity

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Diphtheria toxin (DT ; Mr 58390) is a bacterial protein toxin that consists of two polypeptide chains linked by a disulfide bridge : fragment A (A ; Mr 21150 ; 193 amino acids), the N-terminal enzymatic part, and fragment B (B ; Mr 37240 ; 342 amino acids), the C-terminal part.

B binds the whole toxin to specific eukaryotic cell surface receptors as well as to the charged head groups of the membrane phospholipids. It, then, mediates, via acid endosomes, the transmembrane transport of A into the cytosol where A catalytically ADP-ribosylates elongation factor 2 and, thereby, inhibits protein synthesis of the cell (6).

We have recently determined the complete primary structure of B and identified, in its N-terminal and central regions, several amphiphilic surface and transverse lipid-associating domains that have been made responsible for the strong destabilization B induces at low pH in lipid bilayers (1,2,4).

Concerning the interaction of B with the cell surface receptors (which have not yet been clearly identified), at the present time, only the C-terminal region of B is known to be involved since a mutant toxin (CRM45) that lacks this region is unable to bind to the toxin-specific receptors on sensitive cell membranes.

In this work,we show that the N-terminal moiety of B is also involved in the interaction of B with its receptors since a mixture of the CNBr peptides located in this region of B was able to compete with diphtheria toxin for these receptors. As B is also able to compete toxin toxicity, the mixture was completely freed from contaminating B by affinity chromatography on anti-DT antibodies-Sepharose 4B. The IC_{50} of DT in the presence of the competitive CNBr peptide mixture (3×10^{-6} M) was 8×10^{-12}M, compared to

$7x10^{-11}$ M in the presence of B (10^{-7}M) (IC_{50} of DT=$1x10^{-12}$M). A mixture of the CNBr peptide of bovine serum albumin, used as a control, was totally non-competitive, whatever the concentration.

On the other hand, we have identified, in the C-terminal region of A and in the Mr 8000 C-terminal region of B, three short amino acid sequences (A : ^{144}SER-SER-SER-VAL-GLU-^{148}; B : ^{494}SER-SER-SER-GLU-^{497} and ^{505}SER-SER-ASP-SER-^{508}) of the kinds that are specifically phosphorylated by protein kinases like casein kinase II, enzyme located in the endosomal membranes. Phosphorylation is likely to play a role in the interaction of DT with its receptors since compounds that are known to induce phosphorylation of proteins eliminate the binding properties of the cells (5). In this context, it has been demonstrated that DT is a protein kinase with autophosphorylating activity (3). We have now shown that, in autophosphorylation of DT, both A and B are phosphorylated, as well as DT. However, only B is able to bind ATP : when nicked and reduced DT is affinity-chromatographed on ATP-sepharose in 20 mM Tris-HCl pH 7.4, 2 mM dithiothreitol, B is retained while A is eluted ; B is eluted with 20 mM Tris-HCl pH 7.4, 0.5 M NaCl.

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Receptor Binding and Membrane Translocation of in vitro Translated Diphtheria Toxin Mutants

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INTRODUCTION

Diphtheria toxin (DT) is secreted from Corynebacterium diphtheriae as a polypeptide chain of 58 kD, which is nicked by proteases into two disulfide-linked fragments, termed A and B. At low pH, the A-fragment is translocated into the cytosol, where it inhibits protein synthesis. The mechanism by which the B-fragment inserts into membranes to facilitate the translocation of the A-fragment is subject to investigation.

MATERIALS AND METHODS

Deletion mutants were constructed by modifying the gene for the nontoxic full-length mutant DT-Ser-148 (1), and then transcribed and translated in vitro. The in vitro translated proteins were compared to wild-type DT with respect to their binding to receptors on Vero cells, their protease accessibility after receptor binding and subsequent exposure to low pH, and their ability to permeabilize cells to a number of radiolabeled solutes.

RESULTS

The results are summarized in Table 1. Deleting all or most of the A-fragment yielded molecules with higher receptor affinity than native DT. Mutants containing small parts of the A-fragment (A-44, A-39) as well as mutants lacking the very N-terminal region of the B-fragment (B-36, B-34) became protected against pronase after binding and subsequent

exposure to low pH. A-44, A-39 and B-36 were much more effective than DT in selectively permeabilizing Vero cells to cations at low pH. Like DT, these molecules did not permeabilize L cells, which lack receptors. In contrast, the shorter B-fragment-derived mutants B-34 and B-27 even permeabilized L cells. Cells treated with these proteins were permeable to anions and uncharged solutes as well as to cations.

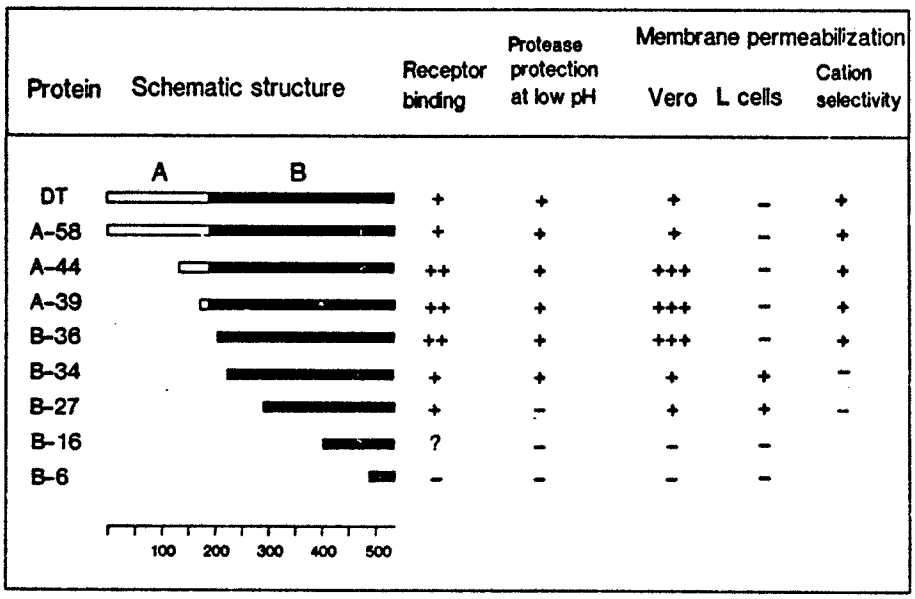


Table 1. Structure and properties of DT and in vitro translated mutants.

DISCUSSION

The present results show that an N-terminally truncated B-fragment alone is able to bind to receptors and insert into the plasma membrane. Clearly, the formation of ion channels can occur even in the absence of A-fragment translocation. The increased affinity of A-44, A-39 and B-36 as compared with DT could be due to increased interactions between the N-terminal region of the B-fragment and membrane lipids. The superior membrane-permeabilizing effect of these molecules could only partly be accounted for by their increased affinity. It appears that the presence of the intact A-fragment actually results in less effective channels. B-34 and B-27 probably permeabilize cells by a different mechanism than DT, since the permeabilization by these molecules was nonselective and receptor-independent.

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Interaction of Protein B (CAMP-Factor) with Artificial Membranes as Studied by Hydrophobic Photolabeling

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INTRODUCTION

Protein B (CAMP-factor) of group B streptococci is active in synergistic hemolysis (4, 9) and unspecific binding of immunoglobulins (7). Binding of protein B to ceramide liposomes (1) as well as to lipid suspensions and liposomes of varying lipid composition (9) has been demonstrated. A "membrane-binding domain" of protein B is discussed in respect to the sequence data (8).
To investigate the interaction of protein B with membranes we have used a liposome model (5) containing either radioactive photoactivatable phospholipids (2) or a radioactive lipophilic photolabel (3).

MATERIAL AND METHODS

Unilamellar ATP-containing liposomes consisting of cholesterol : sphingomyelin = 43 : 57 MOL% were prepared as previously described (5). [^{3}H]-PC I (1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-sn-glycero-3-[^{3}H]phosphocholine; SA = 2.6 Ci/mmol) and [^{14}C]-PC II (1-myristoyl-2-[12-amino(4-N-3-nitro-1-azidophenyl)]dodecanoyl-sn-glycero-3-[^{14}C]phosphocholine; SA = 174 Ci/mol) have been introduced in the liposomes at a molar ratio of 0.16 % and 0.6 %, respectively (2). Both lipid derivatives were a kind gift of Prof. C. Montecucco.
The liposomes were incubated with sphingomyelinase (E.C. 3.1.4.12.) prior to the addition of protein B. Illumination and identification of the cross-linked protein by SDS-PAGE were performed according to Bisson & Montecucco, 1981 (2). Hydrophobic photolabeling with TID (3-(Trifluoromethyl)-3-(m-[^{125}I]iodophenyl)diazirine; SA = 10 Ci/mol) was done as described (3).

RESULTS AND DISCUSSION

To identify hydrophobic interaction of protein B with target liposomes we used TID a lipophilic photolabel. Sphingomyelinase treated liposomes (target liposomes) were incubated with TID followed by the addition of protein B. After illumination of the sample and gel filtration on a Sepharose 4 B column a radioactive liposome peak was obtained containing 3 % of the initial TID. Analysis of the sliced SDS-PAA gel demonstrated the association of radioactivity with protein B suggesting an interaction of the protein B with artificial membranes. However, this statement is limited

because TID is known to partition into hydrophobic pockets of proteins which are not in contact with the bilayer (6).
To investigate the depth of penetration of protein B into the lipid bilayer we prepared liposomes (5) traced with radioactive lipid derivatives. The photoreactive group was introduced at a defined position of the fatty acyl chain for [^{3}H]-PC I at the polar headgroup and for [^{14}C]-PC II at the methyl terminus of one of the fatty acid (2).
The pattern of radioactivity upon SDS-PAGE is depicted in Fig. 1.

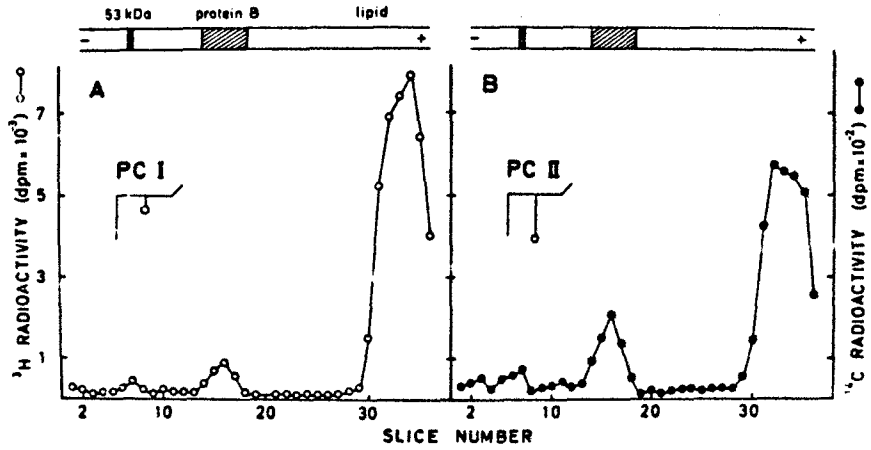


Fig. 1: Labeling patterns of liposome-bound protein B with [^{3}H]-PC I (A) and [^{14}C]-PC II (B).

The Coomassie blue-stained gel (top) was cut into 2.5 mm thick slices before solubilizing and counting for radioactivity (bottom). Inset: schematic graph of the PC-structures.
Both radioactivity profiles show one peak corresponding to the protein B position on the gel. The result indicates that protein B is able to interact with the polar head group as well as with the hydrophobic membrane phase. The extent of cross-linking measured by SDS-PAGE for PC I and PC II was 0.5 % and 2 %, respectively. There was no cross-linking obtained in the control experiments with either sphingomyelinase in the absence of protein B or with protein B alone.

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Cellular Distribution of Protein B (CAMP-Factor) in Group B Streptococci during the Logarithmic Growth Phase

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INTRODUCTION

Protein B (CAMP-Factor) of group B streptococci (Streptococcus agalactiae) is an extracellular polypeptide which acts as a cocytolysin on red blood cells (3) and which binds in a non-immune reaction immunoglobulins of different mammalian species (5). It has been shown that protein B was released in large amounts from bacterial cells into the medium especially during the logarithmic and post-log growth phase (6).

MATERIAL AND METHODS

Group B streptococci, GBS (NCTC 8181, type IIb) were grown aerobically and in the presence of CO_2 (0.1 l/h) in Trypticase peptone-yeast extract broth supplemented with 2 % (w/v) maltose (6). Cell fractionation was achieved with either mechanical cell disruption or protoplast formation with mutanolysin (1). Cocytolytic activity of protein B was measured in the culture supernatant and in the cell fractions as reported earlier (4).

RESULTS AND DISCUSSION

Table 1 shows the relative distribution of protein B activity in individual fractions during the log growth phase of GBS. The release into the medium started in the early log phase. Protein B was thereafter mainly produced during the mid- to late log phase of growth with a maximal intracellular accumulation during the late log phase. With respect to the different cell compartments the distribution of protein B at this stage of growth was as follows: cytoplasm, 76 %, cytoplasmic membrane, 9 %, and cell wall, 15 %. A CO_2-gas atmosphere enhanced the production of protein B by increasing the total amount of proteins synthesized during growth (6). During the post-log growth phase the intracellular CAMP-factor activity decreased of about 55 %, whereas, concomitantly, the amount of protein B in the culture supernatant increased. This observation may possibly be explained by an increase in defective cell wall structure of streptococci due to autolytic phenomena in the post-log phase (2). Up to 95 % of the synthesized protein B were released into the culture supernatant during growth (6).

Table 1: Distribution of protein B within the cell of group B streptococci (type IIb) during logarithmic growth phase

A. AEROBIC GROWTH

	growth phases											
	early log phase			mid-log phase			late log phase			post-log phase		
cell fractions	CS	CP	CE	CS	CP	CE	CS	CP	CE	CS	CP	CE
HA* (HU)	1.7×10^5	4.5×10^2	1.6×10^2	2.5×10^5	4.3×10^2	0.5×10^2	2.8×10^5	2.2×10^4	7.2×10^3	3.8×10^5	1.1×10^4	2.0×10^3
SA (HU/mg pr.)	8.6×10^2	0.2×10^2	0.1×10^2	1.0×10^3	0.1×10^2	0.5	1.0×10^3	1.2×10^2	0.6×10^2	1.4×10^3	0.6×10^2	0.2×10^2
total HA (%)	99.6	0.3	0.1	99.8	0.18	0.02	90.1	7.4	2.5	98.7	2.8	0.5

B. MICROAEROPHILIC GROWTH (CO_2:0.1 l/h)

	growth phases											
	early log phase			mid-log phase			late log phase			post-log phase		
cell fractions	CS	CP	CE	CS	CP	CE	CS	CP	CE	CS	CP	CE
HA* (HU)	1.8×10^5	4.5×10^2	1.5×10^2	4.1×10^5	3.0×10^2	0.4×10^2	4.5×10^5	2.4×10^4	8.5×10^3	5.0×10^5	1.1×10^4	2.3×10^3
SA (HU/mg pr.)	8.2×10^2	0.2×10^2	0.1×10^2	1.8×10^3	0.1×10^2	0.3	1.8×10^3	1.2×10^2	0.4×10^2	1.8×10^3	0.7×10^2	0.2×10^2
total HA (%)	99.6*	0.25	0.08	99.92	0.07	0.01	93.6	5.08	1.35	97.4	2.15	0.45

* Total co-hemolytic activity/800 ml of broth (mean values of 2 cultures in Trypticase peptone-yeast extract medium containing 2 % maltose).
HA: co-hemolytic activity; HU: co-hemolytic unit; SA: specific activity; CS: culture supernatant; CP: cytoplasm; CE: cell envelope

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Importance of the Disulfide-Bridge within Domain II of Pseudomonas Aeruginosa Exotoxin A

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INTRODUCTION

Pseudomonas Exotoxin A (ETA) kills eukaryotic cells by catalyzing the ADP-ribosylation of elongation factor-2 within the cytosol(3). ETA consists of three distinct domains as determined by X-ray crystallography(1). Data indicating that domain II is responsible for membrane insertion and translocation have been presented. To probe the function of the disulfide bridge within domain II we applied directed mutagenesis to the cloned gene. We hypothesized that the disulfide provides a conformational constraint on the molecule and that its removal might influence the conformational change at low pH. An intracellular compartment with acidic pH is required for intoxication of cells (2).

MATERIALS AND METHODS

Cysteines 265 and 287 were changed to Serine by oligonucleotide directed mutagenesis in the M13 system. The mutant toxin was purified by immunoaffinity-, anion exchange-, and gel filtration chromatography.

RESULTS AND DISCUSSION

Mutant toxin was less stable than wild-type toxin and was readily degraded by proteolytic enzymes. The susceptibility to proteolysis reflects the importance of the disulfide-bridge for correct folding of the protein. Mutant toxin had the same in vitro ADP-ribosyl transferase activity as wild-type toxin. Since the enzyme activity is located to domain III, it was not surprising that a mutation in domain II did not affect this function.

The toxicity of mutant toxin on mouse L-cells was reduced ca. 80-fold compared to wild-type toxin. The addition of protease inhibitors slightly decreased the ID_{50} (toxin concentration reducing protein synthesis 50%) in the presence of serum. This was not observed with wild-type toxin.

ETA has been shown to bind Triton X-114 at pH values below 5.0, implying that cryptic hydrophobic regions of ETA become exposed at low pH. The titration curve for detergent-binding of mutant and wild-type ETA as a function of pH was sharp, and the mutant toxin entered the detergent phase at a higher pH than wild-type toxin.

Entry of ETA into the cytoplasm of eukaryotic cells occurs by an acid-dependent mechanism. NH_4Cl at a final concentration of 15mM, protected L-cells more than 100-fold against intoxication by wild-type ETA, but only 10-fold against intoxication by the mutant ETA.

The decreased cytotoxicity of the mutant toxin can possibly be explained by decreased stability, since degradation of proteins can take place within endosomes. The observation that NH_4Cl has less effect on cytotoxicity of the mutant ETA than the wild-type ETA could be explained by the possibility that an increase in intravesicular pH due to the presence of NH_4Cl stabilizes mutant ETA that would otherwise be susceptible to degradation or to unfolding causing premature, abortive membrane insertion. The Triton X-114 experiments indicate that less pH-reduction is required for the protein to undergo a conformational change and thereby expose cryptic hydrophobic regions.

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Insights into Membrane Insertion Based on the Structure of the Colicin A Pore-forming Domain

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INTRODUCTION

Colicins are bactericidal proteins which kill sensitive *E.coli* cells. Their mode of action comprises three steps: 1) binding to a specific receptor located on the outer membrane; 2) translocation across the membrane(s); 3) interaction with their target in the cell. Most of them are large proteins (60-70 Kda) and the domains associated with the steps are organized in three distinct regions of the polypeptide chain. Colicins of the E1 group (A,E1,Ia,Ib, B,K,N) form voltage-dependent pores which lead to depolarization of the cytoplasmic membrane[1,2]. The C-terminal domain of pore forming colicins, responsible for channel formation, can be easily purified by limited proteolytic digestion[3].

RESULTS

The structure of the colicin A thermolysin peptide has been solved to 2.5 Å resolution[4]. It consists of 10 alpha-helices packed in a three layer structure. Two helices are completely buried within the structure and form a hydrophobic loop. This peptide was found to interact strongly with negatively-charged lipids to form a well defined disk-like complex with those lipids. Circular dichroism and fluorescence spectroscopy studies carried out with the soluble and the membrane-bound form of the peptide suggest that the membrane-bound state is almost entirely alpha-helical with the three tryptophans in an hydrophobic environment. The tryptophans are more mobile in the membrane-bound state than in the soluble state. Studies on the accessibility of the protein to trypsin or carboxypeptidase digestion and to histidine chemical modifications show that a large portion is accessible at the membrane surface but the protein's C-terminus is buried within the membrane. Based

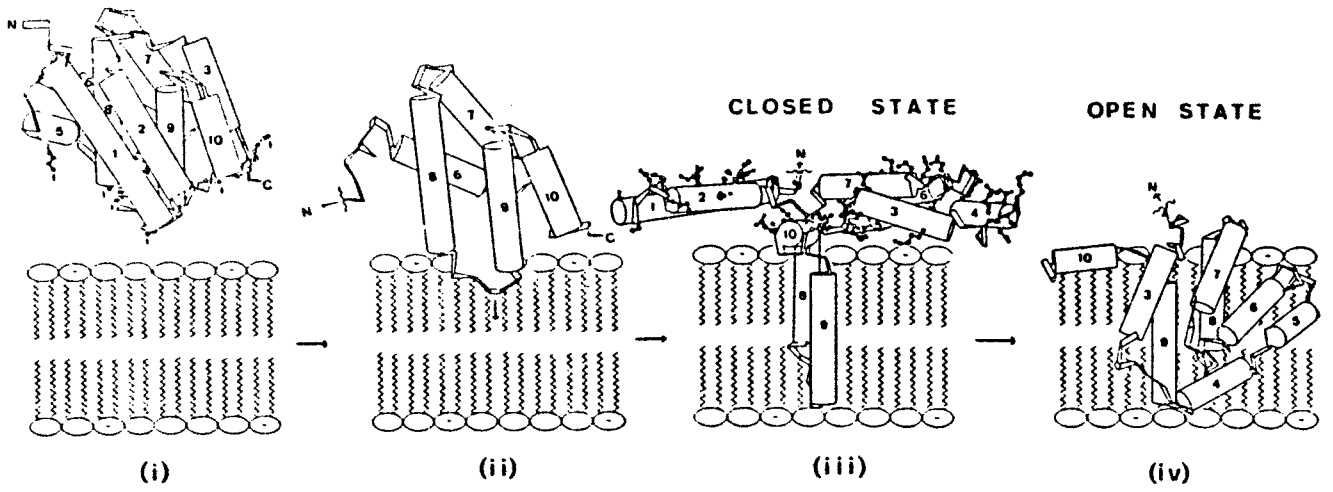


Figure 1 : A model for protein insertion into membranes suggested by the structure of the pore-forming domain of colicin A. i) Initial interaction with the membrane occurs through electrostatic interaction. This first step orientates the hydrophobic helical hairpin (helices 8 and 9) perpendicular to the membrane surface.
ii) The hairpin penetrates into the membrane and initiates opening of the structure.
iii) "Open umbrella" structure of the membrane-bound state of the peptide. This state corresponds to the closed state of the ion channel (adapted from ref. 4).

digestion and to histidine chemical modifications show that a large portion is accessible at the membrane surface but the protein's C-terminus is buried within the membrane. Based on these studies and the three-dimensional structure of the pore-forming domain of colicin A, we present a model for membrane insertion of the protein into lipid bilayers (Figure 1). The features of this model may be applicable in other biological systems involving protein insertion or translocation across membranes.

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Interactions of a Cytolytic Toxin from Bacillus thuringiensis var. israelensis with Liposomes and Membranes

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INTRODUCTION

The 27 kDa δ-endotoxin is one of four mosquitocidal proteins synthesised by Bacillus thuringiensis (Bt) var. israelensis. This protein is toxic to mosquito larvae and is cytolytic to a wide range of insect and mammalian cells in vitro (1,4). Its broad activity spectrum has been attributed to the fact that this toxin binds certain ubiquitous phospholipids; phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin, provided they possess unsaturated fatty acids (5). We have proposed that this protein, along with other Bt δ-endotoxins, acts by colloid-osmotic lysis (2) following insertion into the plasma membrane of susceptible cells to generate a pore of 0.6-1 nm radius. In this study, we have used liposomes as a simple model membrane system to extend our knowledge of the mechanism of toxin binding, insertion and pore formation.

MATERIALS AND METHODS

The 27 kDa toxin was purified from recombinant Bacillus subtilis (7). Methods for liposome preparation (5) and cell assays (4) were described previously.

RESULTS AND DISCUSSION

Binding of the 27 kDa toxin to liposomes depended on both the pH of the incubation and the charge on the liposome: maximal binding to negatively charged liposomes was at pH 7, to neutral liposomes at pH 7 or 8, while positively charged liposomes were still able to bind some toxin at pH 10.5. The decrease in binding at high pH may be due to an increase in the negative charge on the toxin, and can be partially overcome by a positive charge on the liposome. The haemolytic ability of the toxin also displayed pH dependence.

Toxicity was similar at pH 7, 8 and 9.5, with the maximum effect at pH 8, while at pH 10.5 the first lytic effect was seen only after a lag of 30 min at 100 µg/ml toxin, compared to less than 5 min at pH 8. After 2 h lysis was seen at a toxin concentration of 1.5 µg/ml at pH 8 and 6.25 µg/ml at pH 10.5 for human or rabbit red blood cells. Assay of cytolysis of the Aedes aegypti Aa(s) cell line in vitro could not be carried out at pH 10.5 since the cells did not survive at this pH. Assays at pH 7 and 9.5 showed no difference in the cytolytic effect of the 27 kDa toxin, 50% cell lysis occuring at 2.25 µg/ml after 30 min at either pH.

In order to investigate the insertion of the 27 kDa toxin into the lipid bilayer, we studied the protease resistance of the toxin after its interaction with liposomes. Treatment with proteinase K of the toxin bound to liposomes generated two protease resistant fragments of 8.5 and 9.5 kDa. Comparison of the N-terminal amino acid sequences of the proteinase K fragments with the sequence of the native protein (6) revealed that cleavage occurred at the N-terminal sides of amino acids 45 and 154 of the 248 residue protein. The predicted M_r of peptides containing amino acids 45-153 and 154-248 are 11674 and 10633 Da respectively. It appears that only a few amino acids were removed from the C-termini of these fragments by proteolysis.

In order to investigate the formation of pores in liposomes by the 27 kDa toxin, we employed an assay for release of entrapped glucose. The maximal rate of glucose release from liposomes was directly proportional to toxin concentration, but even at the highest doses of toxin used there was a time lag before release of the marker. The lag time doubled with a 2-fold decrease in toxin concentration, a result also shown for the activity of the same toxin on Malpighian tubules (3). These results are consistent with a model in which more than one toxin molecule is required to make a functional pore, but further work is required to confirm this theory.

In summary, binding of the 27 kDa toxin to a lipid membrane is dependent on pH and charge. Only a small proportion of the molecule remains exposed in the aqueous phase after binding, and the toxin generates pores in the membrane, permeable to glucose.

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Mechanisms of Cell Intoxication

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Physiological Role of Endogenous Mono (ADP-ribosyl) ation of G Proteins

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INTRODUCTION

Mono(ADP-ribosyl)ation of proteins is catalyzed by mono(ADP-ribosyl)transferases, which are present in both prokaryotic and eukaryotic cells (1). Prokaryotic mono(ADP-ribosyl)transferases, bacterial toxins, are known to interrupt signal transduction in eukaryotic cells through mono(ADP-ribosyl)ation of G proteins that serve as transducers in a variety of transmembrane signaling systems (2,3). Among these toxins, at least three types of amino acid-specific ADP-ribosyltransferase are known at present (Table I). Diphtheria toxin and Pseudomonas aerginosa exotoxin A utilize a modified histidine, termed diphthamide, residue in elongation factor 2 (EF-2) as the acceptor amino acid (4). This modification inactivates the polypeptide-translocase activity of EF-2, thereby inhibiting protein synthesis, ultimately causing cell death (5). Cholera toxin and Escherichia coli heat-labile enterotoxin modify an arginine residue of a stimulatory G protein (Gs) of the adenylate cyclase system, leading to an increase in cellular cAMP (1). Pertussis toxin catalyzes the ADP-ribosylation of a cysteine residue of an inhibitory G protein (Gi) of the adenylate cyclase system and of the other G protein (Go) that is abundant in brain (2,6).

Enzymes similar to these bacterial toxins have been identified and purified from several eukaryotic cells (7-11). Like bacterial toxins, the eukaryotic mono(ADP-ribosyl)transferases so far purified are classified into three types according to the amino acids that accommodate mono(ADP-ribosyl)ation: diphthamide- (7), arginine (8,9), and cysteine (10,11) specific mono(ADP-ribosyl)transferases. These transferases can be separated by column chromatography and are designated as ADP-ribosyltransferase D, A, and C, respectively (10,11). However, the physiological roles of the eukaryotic mono(ADP-ribosyl)transferases are unknown. Recently, mono(ADP-ribosyl)ation of Gi by ADP-ribosyltransferase C has been shown to attenuate the inhibition of adenylate cyclase activity by epinephrine (12). This finding suggests that endogenous mono(ADP-ribosyl)ation of G proteins may be important in the regulation of signal transduction in eukaryotic cells. Here we summarize recent results obtained in our laboratory on the physiological role of endogenous mono(ADP-ribosyl)ation of G proteins catalyzed by ADP-ribosyltransferase C.

Table I. Mono(ADP-ribosyl)ation Reaction in Prokaryotes and Eukaryotes

Class	Target		ADP-ribosyltransferase	
	Amino acid	Protein	Prokaryotes	Eukaryotes
I	Diphthamide	EF-2	Diphtheria toxin *Pseudomonas aeruginosa* exotoxin A	ADP-ribosyltransferase D
II	Arginine	Gs transducin 21 - 26 KDa membrane proteins P21*ras*	Cholera toxin *Escherichia coli* heat-labile enterotoxin	ADP-ribosyltransferase A
III	Cysteine	Gi transducin Go	Pertussis toxin	ADP-ribosyltransferase C

MATERIALS AND METHODS

ADP-ribosyltransferase C activity was determined by measuring the radioactivity of the reaction product, ADP-ribosylcysteine methyl ester, as described in (10,11). The standard reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mg/ml bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM cysteine methyl ester, 0.15 mM [^{3}H]-NAD (6.7 μCi/μmol), and a suitable amount of the enzyme source. The reaction was carried out at 30°C.

ADP-ribosyltransferase C was purified from human erythrocytes as described previously (11). This enzyme, present in the cytosol, was purified by sequential chromatographic steps on phenyl-Sepharose, phosphocellulose, Sepharose CL-6B, blue-Sepharose and cysteine-agarose.

Adenylate cyclase activity in human platelet membranes was assayed in buffer solution consisting of 50 mM sodium phosphate (pH 7.4), 0.3 mM PMSF 1 mM ATP, 0.1 mM GTP, 2 mM $MgCl_2$, 0.2 mM 3-isobutyl-1-methylxanthine, 1 mg/ml membranes and other reagents as indicated in the text. Incubation was carried out at 30°C. The rate of cAMP production was measured by radioimmunoassay with an Amersham cAMP[^{125}I]kit (12).

The membranes were incubated with purified ADP-ribosyltransferase C in buffer solution consisting of 50 mM sodium phosphate (pH 7.4), 0.3 mM PMSF 1 mM ATP, 0.1 mM GTP, 2 mM $MgCl_2$, 10 μM [^{32}P]NAD and 1 mg/ml membranes. ADP-ribosylation was carried out at 30°C.

RESULTS

Properties of ADP-ribosyltransferase C. ADP-ribosyltransferase C, present in the cytosol of human erythrocytes, was purified by column chromatography (11). The specific activity of the final preparation was 8.3 μmol/min/mg protein, showing 35,000-fold increase over that of the cytosol. The purified ADP-ribosyltransferase C gave one main protein band on SDS-

polyacrylamide gels with an estimated Mr = 28,500. On gel permeation chromatography, the transferase C appeared as a single peak of Mr = 27,000, suggesting that the ADP-ribosyltransferase C is a monomeric polypeptide. Similar enzymatic activities have been detected in extracts of human lymphocytes and platelets, HeLa S3 cells, and Ehrlich ascites tumor cells. ADP-ribosyltransferase C is, therefore, probably ubiquitous in eukaryotic cells (10, 11).

ADP-ribosyltransferase C catalyzes the reaction between two substrates, NAD and cysteine methyl ester (Cys), to yield two products, ADP-ribosyl-Cys and nicotinamide (Nm). Kinetic analysis of the bireaction system revealed that the apparent Km value for one substrate increased as the concentration of another fixed substrate increased (Fig. 1a). The results indicate that the binding of one substrate to the free enzyme decreased the affinity for the second substrate for factor α. These kinetic studies suggest that the mechanism of ADP-ribosylation of cysteine methyl ester by ADP-ribosyltransferase C may be consistent with a sequential rapid equilibrium random mechanism rather than a sequential ordered one (Fig. 1b). The Km values for NAD and cysteine methyl ester were determined to be 65 and 4,400 μM, respectively.

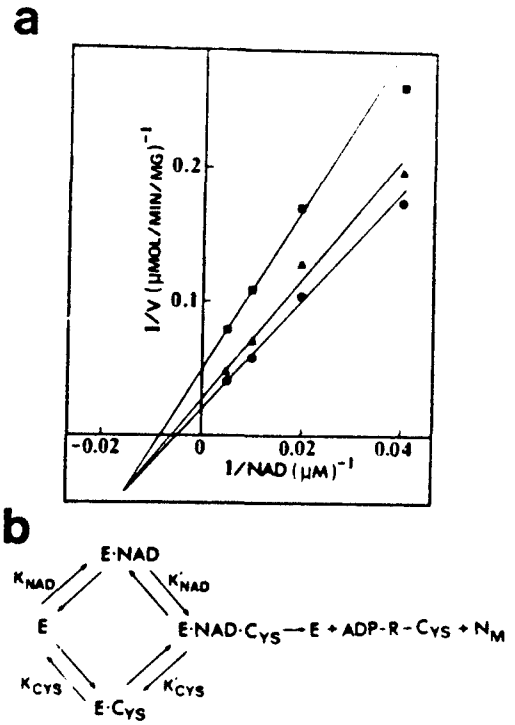


Fig. 1. Kinetic mechanism of ADP-ribosyltransferase C. Plots of 1/v versus 1/[NAD] at fixed concentrations of cysteine methyl ester (20 (-■-), 40 (-▲-), and 80 (-●-) mM) (a). Mechanism of ADP-ribosylation of cysteine methyl ester by ADP-ribosyltransferase C (b).

Targets of ADP-ribosyltransferase C. By using human erythrocyte inside-out membrane vesicles, ADP-ribosyltransferase C was found to ADP-ribosylate a Mr = 41,000 protein, which had a mobility identical to that of protein (Giα) ADP-ribosylated by pertussis toxin (11). The ADP-ribosylated protein was clearly separated from the substrate (Mr = 45,000 protein, Giα) for cholera toxin. The ADP-ribosylation of 41,000 protein by ADP-ribosyltransferase C was inhibited by pre-ADP-ribosylation of membranes with pertussis toxin. The protein ADP-ribosylated by ADP-ribosyltransferase C is, therefore, believed to be the α subunit of Gi.

The restriction of modification by ADP-ribosyltransferase C to cysteine residues in Giα was confirmed by the following observation. The linkages of ADP-ribose - amino acid residues in proteins have been classified according to their sensitivities to hydroxylamine; that is, on neutral hydroxylamine treatment, the ADP-ribose - cysteine linkage formed by pertussis toxin is considerably stable, whereas the linkage of ADP-ribose - arginine formed by cholera toxin is unstable. As shown in Fig. 2, the ADP-ribose - Giα linkage formed by ADP-ribosyltransferase C was as stable to hydroxylamine as that formed by pertussis toxin. In contrast, about half of that of ADP-ribose - Gsα formed by cholera toxin was degraded by hydroxylamine treatment for 50 min. These data are consistent with the ADP-ribosylation by ADP-ribosyltransferase C of a cysteine residue in Giα.

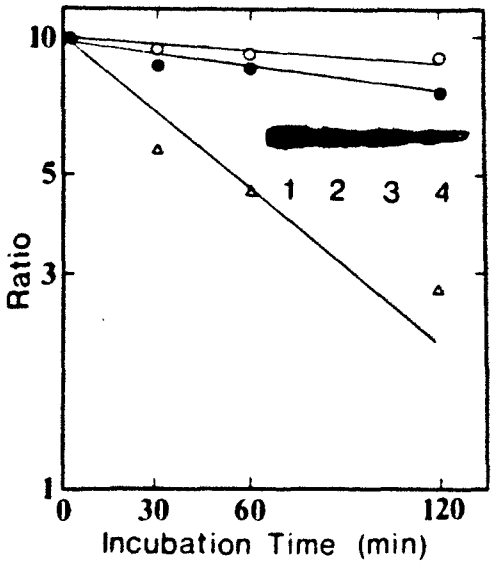


Fig. 2. Hydroxylamine sensitivity of ADP-ribose - Giα linkage. Membranes [^{32}P]ADP-ribosylated with ADP-ribosyltransferase C (-●-), pertussis toxin (-○-), or cholera toxin (-△-) were incubated in 0.5 M hydroxylamine (pH 7.4) and 0.5 M NaCl for the indicated times. The initial value was defined as 10 for each sample. Inset, autoradiographs of ADP-ribosyl - Giα treated with hydroxylamine for 0, 30, 60 and 120 min (lane 1, 2, 3, and 4, respectively).

Function of ADP-ribosyltransferase C. We developed a system to investigate the effect of mono(ADP-ribosyl)ation of Gi by ADP-ribosyltrasferase C on adenylate cyclase activity (12). Adenylate cyclase activity in human platelet membranes was stimulated by forskolin, which is a direct activator of the catalytic subunit. Using this system, the effect of epinephrine, an α2-adrenergic receptor agonist, on forskolin-stimulated adenylate cyclase was examined. Epinephrine inhibited adenylate cyclase activity with half-maximal inhibition at about 0.5 μM. Interestingly, the epinephrine-induced inhibition of adenylate cyclase activity was much less in membranes that had been preincubated with ADP-ribosyltransferase C than in control membranes (Fig. 3a). Preincubation with the transferase C had little effect on the stimulatory effect of forskolin. In the absence of NAD, ADP-ribosyltransferase C showed no attenuating effect. These results indicate that ADP-ribosyltransferase C attenuates receptor-mediated inhibition of adenylate cyclase and does not affect adenylate cyclase directly.

To determine the target protein for ADP-ribosyltransferase C, we incubated human platelet membranes with the enzyme in the presence of [^{32}P]-NAD and analyzed the membrane proteins by SDS-polyacrylamide gel electrophoresis. As observed in human erythrocyte inside-out membranes, in human platelet membranes, ADP-ribosyltransferase C predominantly ADP-ribosylated Giα (Fig. 3b). The dose-dependent ADP-ribosylation of Giα by ADP-ribosyltransferase C was correlated with the attenuating effect of the enzyme on the inhibitory action of epinephrine on adenylate cyclase activity. This finding supports the idea that the attenuation of the inhibitory effect of epinephrine on the adenylate cyclase by pretreatment with ADP-ribosyltransferase C is due to mono(ADP-ribosyl)ation of Giα.

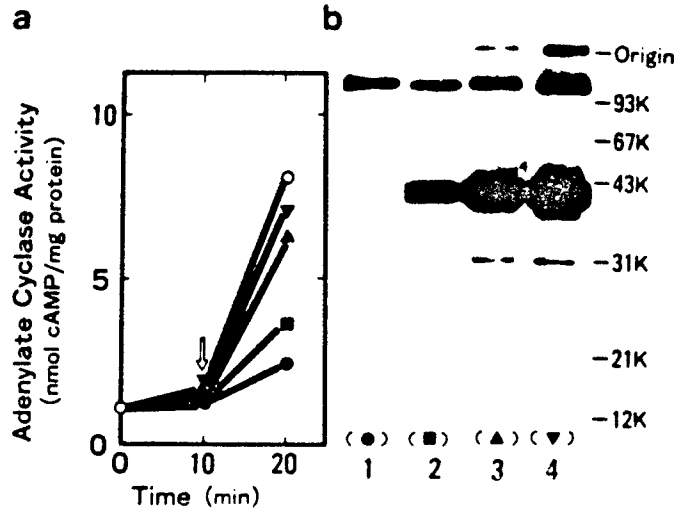


Fig. 3. Attenuation of epinephrine-induced inhibition of adenylate cyclase by ADP-ribosyltransferase C. Human platelet membranes were preincubated with ADP-ribosyltransferase C (0 (○, ●), 0.1 (■), 0.3 (▲), and 1.0 (▼) μg/ml) for 10 min at 30°C and then exposed to 10^{-5} M forskolin in the presence (●, ■, ▲, ▼) or absence (○) of 30 μM epinephrine for 10 min at 30 °C. The rate of cAMP production was measured by radioimmunoassay with a cAMP[^{125}I]kit from Amersham (a). The membranes were subjected to 10% acrylamide/0.1% SDS slab gel electrophoresis and gels were autoradiographed (b).

DISCUSSION

It is very intriguing that eukaryotic cells contain at least three species of ADP-ribosyltransferase (D, A, and C), enzymes that can recognize a specific amino acid residue in G proteins, as do bacterial toxins. Among these eukaryotic ADP-ribosyltransferases, ADP-ribosyltransferase C can ADP-ribosylate the α subunit of Gi. Furthermore, this enzyme attenuated the inhibitory effect of epinephrine on the adenylate cyclase system in human platelet membranes. The attenuation was due to mono(ADP-ribosyl)-ation of Giα. Thus, this modification may decrease the inhibition of adenylate cyclase by Gi by uncoupling Gi from the inhibitory receptor. These studies provide experimental support for the hypothesis that mono-(ADP-ribosyl)ation of Gi catalyzed by ADP-ribosyltransferase C endogenously modulates the adenylate cyclase system.

Recent studies have revealed that Gi and Go, which are the substrates of pertussis toxin, act as transducers between receptors and effectors in adenylate cyclase, phospholipase C and A2 and ionic channel systems. These G proteins are ADP-ribosylated by ADP-ribosyltransferase C. ADP-ribosyltransferase C may, therefore, play an important role in the control mechanism of not only the adenylate cyclase system, but also these other signal transduction systems via mono(ADP-ribosyl)ation of Gi or Go.

We also detected and purified ADP-ribosyltransferase A from human platelets and Ehlrich ascites tumor cells. The target proteins of this enzyme were membrane proteins of Mr = 21,000 - 26,000 and the p21*ras* oncogene product. These results indicate that multiple species of ADP-ribosyltransferase with diverse structures and functions exist in eukaryotic cells. On the basis of these observations, we propose that ADP-ribosyltransferase C and A may have different functions in the regulation of transmembrane signaling systems through mono(ADP-ribosyl)ation of respective G proteins.

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Clostridial Actin-ADP-Ribosylating Toxins

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INTRODUCTION

Several microbial toxins transfer ADP-ribose from NAD to specific protein substrates, thereby affecting the functions of their target proteins. Diphtheria toxin and Pseudomonas exotoxin A ADP-ribosylate elongation factor 2, a modification which inhibits protein synthesis (6, 10). Cholera toxin and pertussis toxin ADP-ribosylate heterotrimeric G-proteins involved in transmembrane signal transduction (19, 28). GTP-binding proteins are also the substrates of botulinum ADP-ribosyltransferase C3 (3, 5). This exoenzyme is structurally and functionally distinct from botulinum neurotoxins (23) and ADP-ribosylates the ras-homologous rho protein (5). Recently, it has been shown that various clostridial bacteria produce ADP-ribosylating toxins, which common target is actin (1, 21, 24, 27). Best studied example of this novel class of actin-ADP-ribosylating toxins is botulinum C2 toxin.

BOTULINUM C2 TOXIN

Botulinum C2 toxin is produced by various strains of Clostridium botulinum types C and D. In contrast to neurotoxins C1 and D, botulinum C2 toxin is apparently not phage-encoded, since bacteria which were cured from their phages, stopped neurotoxin but not botulinum C2 toxin production (7).

Several toxins such as pertussis-, cholera- and diphtheria toxins are constructed according the A-B-model consisting of two types of toxin subunits or chains (9, 28). One subunit binds to a cell surface receptor, whereas the other cross the

cell membrane and is responsible for the biological effect of the toxin. Similarly, botulinum C2 toxin is comprised of two components (II and I), which are involved in binding and biological activity, respectively (12). However, while most toxins which can be described by the A-B-model, consist of connected chains or subunits, botulinum C2 toxin is binary in structure and consists of two separated proteins.

Component II of botulinum C2 toxin is responsible for the binding of the toxin to the eukaryotic cell surface (12, 17). The molecular weight of the binding component is about 100,000 on SDS-PAGE. Component II is activated by trypsin treatment to gain full binding activity (18). So far the nature of the cell surface receptor for component II is not known. It has been reported that the activated proteolytic fragment (74,000 Da) of component II forms oligomers with a molecular weight of 365,000 having hemagglutinine and hemolytic activities, while the monomer possesses only hemagglutinating activity (18).

Botulinum C2 toxin component I, but not component II, owns ADP-ribosyltransferase activity and modifies actin (25). Various findings indicate that modification of actin is due to mono-ADP-ribosylation: Firstly, the phosphodiesterase treatment of ^{32}P-ADP-ribosylated actin releases labelled 5'AMP and secondly, no increase in the molecular weight of labelled proteins occurs during ADP-ribosylation. The K_M of the ADP-ribosylation reaction for NAD is 1-5 µM (2). As found for other bacterial ADP-ribosyltransferases, botulinum C2 toxin possesses glycohydrolase activity and splits NAD into ADP-ribose and nicotinamide.

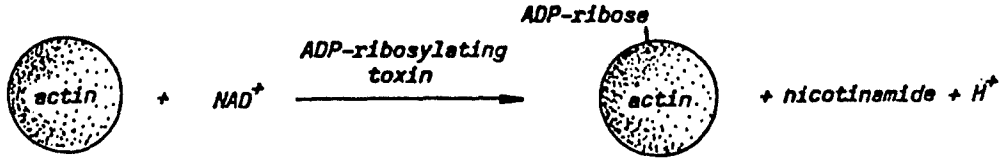


Figure 1. General scheme of ADP-ribosylation of actin

OTHER ACTIN-ADP-RIBOSYLATING TOXINS

ADP-ribosylation of actin is not unique for botulinum C2 toxin. Clostridium perfringens iota toxin (24, 26), C. spiroforme toxin (27) and an ADP-ribosyltransferase from C. difficile (21), which is distinct from C. difficile toxins A and B, possess ADP-ribosylating activity and modify actin. These "iota-like" toxins are also binary in structure and are immunologically closely related representing a sub-family of actin-ADP-ribosylating toxins (27). Botulinum C2 toxin is not recognized by antibodies raised against these toxins. More-

over, the binding components of the "iota-like" toxins can substitute each other in their transport ability. In contrast, botulinum C2 toxin does not facilitate the transport of the other toxins into the cells (27).

ADP-RIBOSYLATION OF ACTIN BY BOTULINUM C2 TOXIN

ADP-ribosylation by botulinum C2 toxin is highly specific for actin (1). Tubulin, transducin or other G-proteins are not modified by botulinum C2 toxin. Moreover the modification of actin by the toxin exhibits an interesting actin isoform specificity: Whereas both isoforms (β and ν) of non-muscle actin are good substrates of C2 toxin (30), skeletal muscle actin is only marginally modified by the toxin (1). Substrate of botulinum C2 toxin and of the other toxins is monomeric G-actin, not polymerized F-actin. In line with this finding is the observation that phalloidin, which induces polymerization and stabilizes F-actin inhibits actin ADP-ribosylation (2). About 0.9 mol ADP-ribose per mol protein can be incorporated into freshly prepared actin. Recently, it has been shown that botulinum C2 toxin ADP-ribosylates nonmuscle actin in arginine-177 (30). Surprisingly in skeletal muscle actin, which is a poor toxin substrate, amino acid 177 is also arginine. It has been speculated that the preceeding amino acid, methionine, in skeletal muscle, which is replaced by leucine in non-muscle actin, blocks ADP-ribosylation. However, recent finding that C. perfringens iota toxin modifies arginine 177 in skeletal muscle actin, too, indicate that methionine-176 does not principially prevent ADP-ribosylation (29).

ADP-RIBOSYLATED ACTIN BEHAVES LIKE A CAPPING PROTEIN

Several findings indicate that ADP-ribosylation of actin blocks its ability to polymerize. Treatment of liver non-muscle actin with botulinum C2 toxin reduced the viscosity of the actin solution after induction of polymerization measured by the falling ball device (1). Electron microscopy studies supported the view that toxin-catalyzed ADP-ribosylation drastically decreased actin polymerization and formation of filaments (2). Moreover, recent investigations indicate that ADP-ribosylated actin largely affects polymerization of un-modified actin. Polymerization of actin onto actin filaments was monitored by an increase in the fluorescence of polymerized NBD-actin. These studies demonstrated that ADP-ribosylated actin inhibited nucleated polymerization of unmodified actin in a manner typical for capping proteins (31, 32). In order to characterize whether ADP-ribosylated actin acts on the barbed and/or the pointed end of actin filaments, the influence on polymerization was studied with gelsolin capped actin filaments (31). Gelsolin binds to the barbed end

of actin filaments thereby blocking polymerization at this end, whereas the pointed end is still capable of filament elongation (24). Under these conditions ADP-ribosylated actin did not influence nucleated polymerization of unmodified actin at the pointed filament end (31). These findings can be interpreted to indicate that actin acts like a barbed end capping protein.

ADP-RIBOSYLATION INHIBITS ACTIN ATPase AND IS REVERSABLE

Cholera- and pertussis toxins ADP-ribosylate GTP-binding proteins participating in signal transduction (19). These G-proteins are known to be regulated by associated GTPase activities (19). The toxin-induced ADP-ribosylation causes drastical changes in the rates of GTP hydrolysis associated with the regulatory functions of these proteins. Actin is not a GTP but an ATP-binding protein having ATPase activity (20). ADP-ribosylation of actin by botulinum C2 toxin or iota toxin inhibits ATP hydrolysis by actin (8). Several findings indicate that inhibition of ATP-hydrolysis is not simply due to blockade of actin polymerization: First, inhibition of actin ATPase activity is observed even at low concentrations of Mg^{2+} (50 µM) and at actin concentrations below its critical concentration. Second, ADP-ribosylation inhibited ATP hydrolysis by actin even in the DNAse I-actin complex and, finally, also the cytochalasin-stimulated actin ATPase was impaired by toxin treatment.

ADP-ribosylation of actin by clostridial toxins is a reversable reaction. In the presence of high concentration of nicotinamide (30 mM) and low pH (6.5) botulinum C2 toxin and iota toxin cleaved the ADP-ribose-actin bound and formed NAD. Reversal of actin ADP-ribosylation was accompanied by a functional reconstitution of actin, e.g. a decrease in the amount of ADP-ribose attached to actin correlated with an increase in ATPase activity. ADP-ribosylation of actin and toxin-catalyzed reversed reaction exhibited identical substrate specificity. While ADP-ribosylation of non-muscle actin was reversed by iota toxin and C2 toxin, modification of skeletal muscle actin was cleaved only by iota toxin but not by C2 toxin (K. A. and I. J., unpublished observations).

PHARMACOLOGICAL ACTIONS OF BOTULINUM C2 TOXIN

According the A-B model of botulinum C2 toxin, the pharmacological effects of the toxin depend on the presence of both components. Injection of botulinum C2 toxin into the intestinal loop of mice causes fluid accumulation after a delay of 1-2 h (13). Morphological changes following toxin application indicate induction of an acute inflammation (15). Botulinum C2 toxin dramatically increases the vascular

permeability (14). The toxin causes rounding up of cells in culture (16, 22). Variably, 24 to 48 h after rounding up of cells lysis occurs.

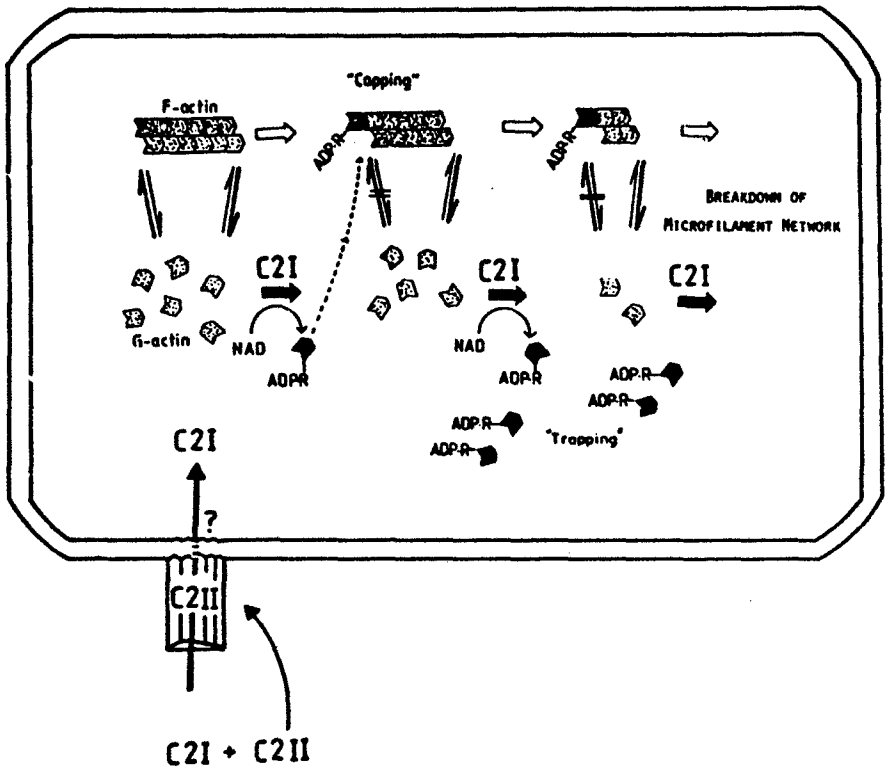


Figure 2. Model of the action of botulinum C2 toxin on the microfilament network of cells. Component II of botulinum C2 toxin (C2 II) binds to the cell membrane and facilitates the transport of component C2 I into the cell. ADP-ribosylation of actin converts actin into a capping protein, which binds to the fast growing ends of actin filaments and inhibits further polymerization. Depolymerization at the pointed end increases the G-actin concentration. The released actin monomers are ADP-ribosylated by the toxin. Since ADP-ribosylated actin is not able to polymerize, it accumulates which finally causes destruction of the microfilament network.

Cholera toxin can ADP-ribosylate cytoskeletal proteins in vitro but not in intact cells (11). Therefore, it was of particular importance for the pathophysiological relevance of actin ADP-ribosylation to proof its modification in intact cells. Treatment of intact cells with botulinum C2 toxin or with other actin modifying toxins reduced or blocked subsequent ^{32}P-ADP-ribosylation of actin in cell lysates (1, 22). Thereby it has been shown that in chicken cells rounding up correlates with toxin-induced ADP-ribosylation of actin in a time and concentration dependent manner. A more direct approach was reported by Reuner et al. (22): Intact ^{32}P-loaded chicken cells were treated with botulinum C2 toxin resulting in the specific labelling of a 43 kDa protein, which was identified as actin by its ability to bind to a DNAse column and by subsequent protease mapping. These studies showed that G- but not F-actin is the main toxin substrate in intact cells, because the botulinum C2 toxin-induced label was exclusively found in the Triton-soluble G-actin fraction but not in the Triton-insoluble F-actin fraction.

Rounding up of cells by botulinum C2 toxin is associated with destruction of the microfilament network of intact cells (22). This was demonstrated by staining microfilaments of fibroblasts with fluorescein-labelled phalloidin. By using DNAse I inhibition test, it was shown that the G-actin content of toxin-treated fibroblasts increased concomitantly with ADP-ribosylation of actin (4). Thus, all these findings were interpreted to indicate that botulinum C2 toxin treatment of intact cells causes depolymerization of actin filaments.

The following model for the cytotoxic effects of actin ADP-ribosylating toxins has been proposed (4). It is generally accepted that in nonmuscle cells monomeric and polymeric actin is in a dynamic equilibrium, which is mainly regulated by actin binding proteins (20). The actin-modifying toxins apparently interfere with this equilibrium by ADP-ribosylation of G-actin. The ADP-ribosylated actin caps the barbed end of actin filaments thereby blocking further polymerization at this end of filaments and increases the critical concentration of actin. In contrast, depolymerization at the pointed end is not impaired and still proceeds. The cellular pool of G-actin, which serves as substrate for toxins, increases. As ADP-ribosylated actin is not able to polymerize the toxin traps actin in its monomeric form, which is not available for formation of microfilaments. All these events finally cause destruction of the microfilament network (Figure 2).

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The Use of Patch Clamp Technique for the Study of the Mode of Action of Bacterial Toxins

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INTRODUCTION

Bacterial toxins can be classified according to their different sites and modes of action (Fig. 1). The pore forming toxins constitute one class (3). Prototypes are the α-toxin produced by most strains of Staphylococcus aureus, and streptolysin-O product by β-hemolytic group A streptococci. They are able to form pores or ion channels. Depending on diameter the pores allow the exchange of small ions and molecules and the efflux of large intracellular proteins leading eventually to cell death.

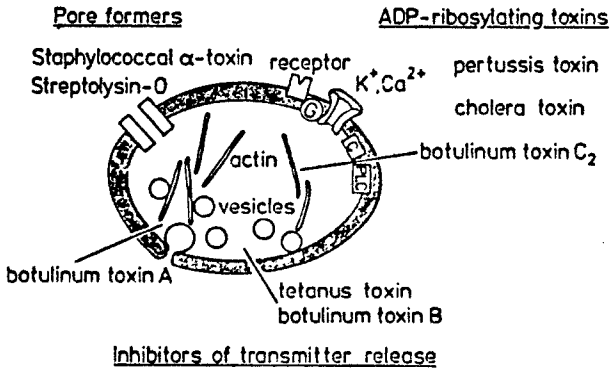


Fig.1: Schematic classification of bacterial toxins according to their different sites and modes of action. G = nucleotide binding protein, C = catalytic subunit of adenylate cyclase, PLC = phospholipase C.

The ADP-ribosylating toxins constitute another class. The most known and used toxins are pertussis toxin and cholera toxin since they affect nucleotide binding proteins (G-proteins) which have a key role in signal transduction. The G-proteins couple receptors on the cell surface with membrane-bound effectors such as subtypes of ion channels, the phospholipase C, or the catalytic subunit of adenylate cyclase. The ADP-ribosylation of actin by botulinum C2 toxin largely affects the ability of the microfilament protein to polymerize (2) thereby also modulating the evoked transmitter release (8). To reach their sites of action in the cytosol it is suggested that the ADP-ribosylating toxins or toxin fragments form open cation-selective channels or pores to facilitate the translocation across the membrane.

A third class forms the clostridial neurotoxins such as tetanus toxin and the botulinum toxins type A to G. Although their molecular mechanism of action is still unknown, several evidences support the view that botulinum B toxin/tetanus toxin and botulinum A toxin act at different sites in the chain of events that results in transmitter release (4,5). The pore former streptolysin-O has been used as a membrane-permeabilizing agent for the introduction of macromolecules such as tetanus toxin into bovine adrenal medullary chromaffin cells to study tetanus toxin effect on exocytosis (1).

RESULTS AND DISCUSSION

STUDY OF PORE FORMATION. Several methods are in use to characterize pore formation and pore diameter of bacterial toxins (for review see (3)). The first successful method to study the action of single toxin molecules was their incorporation into planar phospholipid bilayer membranes, and the electrophysiological recording of opening and closing of formed pores/ion channels. Electrophysiological recording of pore formation of bacteria toxins on biological membranes has so far not been reported. The patch clamp technique developed by Neher and Sakmann (9) is an ideal tool for such purposes. Figure 2 shows schematically the procedures which has been described in detail (6, 12).

The cell-attached recording configuration (Fig. 2) has the advantage that the "milieu interieur" of the cell is untouched. This may be important if second messengers or other cell components are involved in the gating of ion channels or receptors. Further if the amount of available toxin is limited then the pipette which has a volume of about 15 µl, can be filled with toxin and the effect on a small membrane patch can be studied.

In the whole-cell recording configuration there is a low-resistance access to the cell interior. This allows to measure the resting potential of the cell and under voltage-

clamp conditions the membrane currents flowing in response to membrane potential changes. Further there is a rapid diffusional exchange between the cytoplasm and the pipette solution. The advantage of this configuration is that the cell cytoplasm is under experimental control. Toxins can be applied intracellularly to study their action from the cytoplasmic side. The disadvantage is that the physiological state of the cell is changed. Important signal mediators (second messengers, regulatory proteins) may be lost from the cytoplasm.

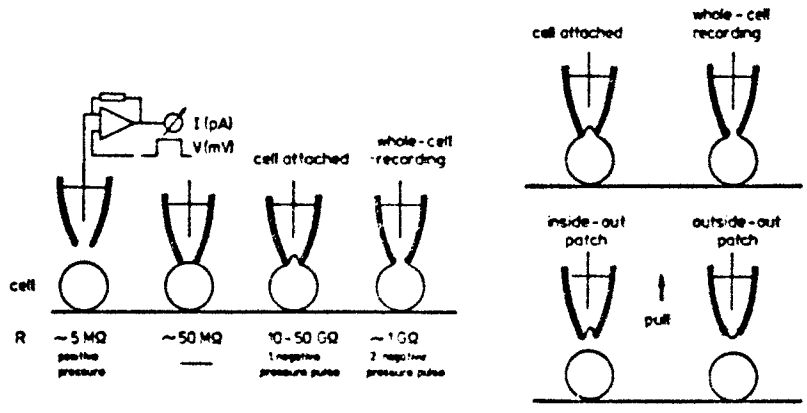


Fig.2: Schematic drawing of the procedures that lead to different recording configurations of the patch clamp technique such as cell-attached and whole-cell recordings as well as inside-out and outside-out membrane patches. The pipette resistance R is measured by the current flow I (pA) through the glass pipette in response to a small voltage pulse V (mV). Note the huge change of the resistance due to the forming of a giga-seal between the tip of pipette and the cell membrane. Withdrawal of the pipette from a cell when a cell-attached or whole-cell recording configuration was established leads to inside-out and outside-out membrane patches, respectively. In the inside-out patch the cytoplasmic side and in the outside-out patch the external side of cell membrane are exposed to the bath solution. For excellent details see (6, 12).

The whole-cell configuration is the starting point for the investigation of pore forming toxins. Because of the large surface area the probability of pore formation is high. It will provide us with initial information about the potency and efficacy of a toxin. After toxin application to the extracellular medium pore formation will decrease the membrane resistance, i.e. under voltage-clamp condition the membrane current increases. We studied the effects of a cytotoxin from Pseudomonas aeruginosa (7) which is liberated during autolysis and appears to be produced by all strains (7). Whole-cell recordings in cultured bovine adrenal medullary chromaffin cells showed that bath application of the toxin (1 µg/ml) changes the membrane currents (Fig. 3). The increase in outward current is larger at positive voltages than at negative ones suggesting that the open probability of the newly formed toxin pores is dependent on membrane potential.

In the outside-out patch configuration the external side of the cell membrane is directed to the bath medium, while the cytoplasmic side faces the pipette solution. This configuration allows to study single ion channels/pores in a very small membrane patch similar to the method of planar lipid bilayers. Like in the whole-cell configuration the membrane potential can easily be changed and the extra- and intracellular solutions are under experimental control.

Pore formation of P. aeruginosa cytotoxin molecules in an outside-out membrane patch of a bovine chromaffin cell is shown in Fig. 4. The holding potential of the membrane patch was here -40 mV. After variable latency the current flow through newly formed pores can be recorded. After change to the usual bath solution the further process of pore formation stops while the already incorporated toxin molecules remain in the membrane. Such recordings performed at negative and positive membrane potentials allow to determine single channel properties such as the unitary conductance of the pore, the appearance of subconductance states, the ion selectivity and the open probability of the pores. The pore formed by P. aeruginosa cytotoxin is a non-selective ion channel with a slope conductance of 120 pS.

Increasing the number of pores in the membrane patch by longer exposure to the cytotoxin, the voltage-dependent gating of the pores can be demonstrated (Fig. 5). A depolarizing voltage step to +20 mV from a holding potential of -60 mV increases tremendeously the open probability of the pores. Stepping back from +20 mV to -60 mV a large instantaneous current flows through the open pores which then mostly close within 10 s.

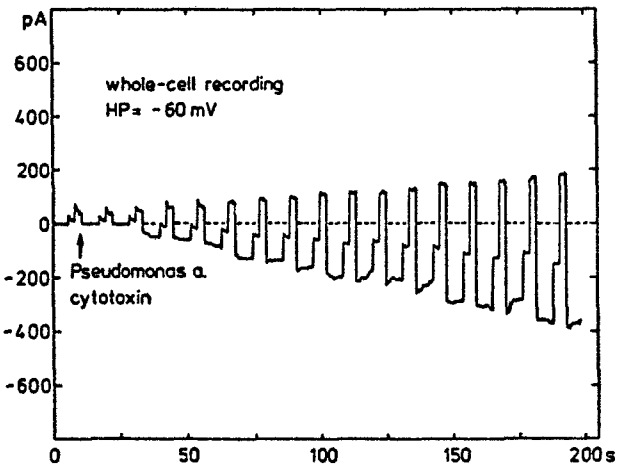


Fig.3: Whole-cell membrane currents in cultured bovine chromaffin cells before and after application of Pseudomonas aeruginosa cytotoxin (1µg/ml). Each of the family of currents was generated by membrane depolarization from the holding potential of -60 mV to -20 mV and +20 mV. The bath solution had the composition (mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.3. The pipette filling solution contained in mM: 140 K-gluconate, 20 NaCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5 EGTA, 10 HEPES, pH 7.3.

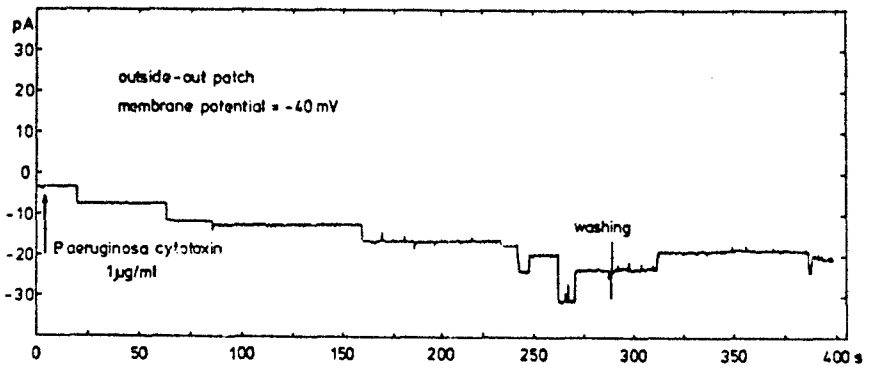


Fig.4: Elementary currents through pores formed by P. aeruginosa cytotoxin (1 µg/ml) in outside-out patch from bovine adrenal chromaffin cell. Change of the bath solution to toxin-free solution stops the further formation of pores.

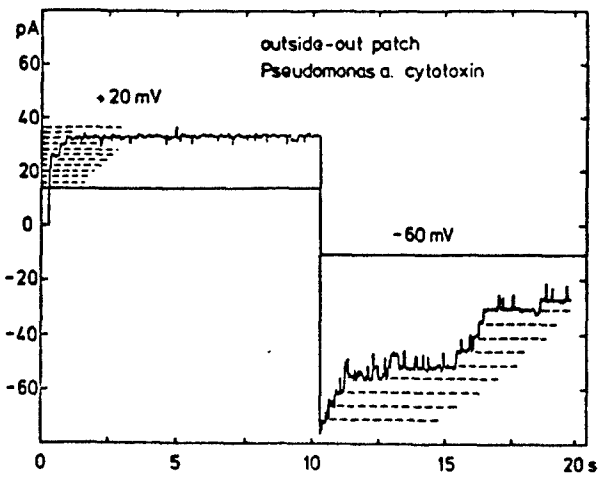


Fig.5: Voltage-dependent activation and inactivation of pores formed by P. aeruginosa cytotoxin (1 µg/ml) in an outside-out patch of bovine chromaffin cells. The holding potential was -60 mV. Then a depolarizing pulse to +20 mV was applied for 10 s, followed by repolarization to -60 mV.

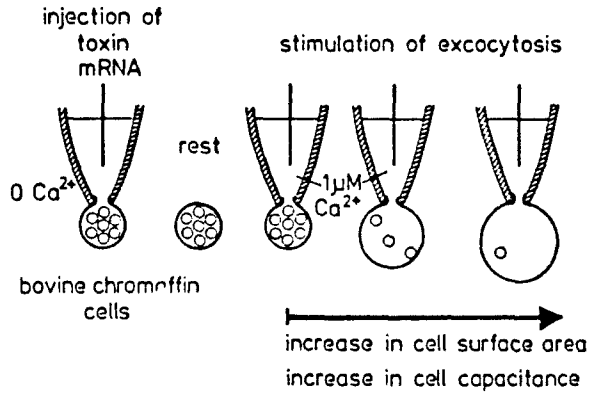


Fig.6: Schematic illustration of the whole-cell recording as a tool to study the effect of clostridial neurotoxins and their fragments on exocytosis in bovine adrenal chromaffin cells. The exocytosis is monitored by the increase of cell capacitance as the cell surface area increases during incorporation of granule membrane into the plasmalemma.

STUDY OF EXOCYTOSIS. The patch clamp technique has also successfully been used for the study of exocytosis in mast cells and bovine adrenal chromaffin cells (10). In the whole-cell recording mode the release of vesicles/granules can be stimulated by raising the Ca^{2+}-concentration of the pipette solution to 1 µM. Neher and Marty (10) showed that then the fusion of the large granules with the cell membrane increases the cell surface area thereby increasing the capacitance of the cell (Fig. 6). So the measurement of cell capacitance is a measure of the secretion of granules. The capacitance of a cell can be estimated from membrane currents in response of test voltage pulses.

The clostridial neurotoxins tetanus toxin and botulinum A toxin are known to inhibit transmitter release from peripheral as well as central nerve terminals so far investigated suggesting that the neurotoxins act on basic processes of exocytosis. The chromaffin cells of adrenal glands, however, often used as a model for secretion studies, are insensitive to externallly applied tetanus toxin and botulinum A toxin as the cells lack the capacity to bind the toxins. We used the whole-cell recording to apply the toxins internally and to measure the inhibition of exocytosis by means of capacitance measurement (11). This is schematically illustrated in Fig. 6. The cells were preloaded with tetanus toxin or botulinum A toxin using patch-pipettes filled with the toxins, but containing no Ca^{2+} to avoid the stimulation of release. After loading of a cell the pipette was lifted up and the cell was incubated for 1 h at 37°C because the inhibitory effect is strongly temperature dependent. Then in a 2. step the secretion is stimulated using a patch-pipette filled now with a solution containing 1 µM free Ca^{2+}. Tetanus toxin and botulinum A toxin effectively inhibited the secretory response. The conclusion from these experiments is that the clostridial neurotoxins interfer with so far unknown steps of exocytosis and that these crucial steps are quite generally associated with vesicular/granular release. Fragments of tetanus toxin have also been tested (11). While fragment C was unable to alter the secretion, fragment B effectively suppressed the exocytosis. Recently it has been shown that the paralytic action of tetanus toxin is associated with the light chain of the toxin (1).

The whole-cell recording also allows to inject mRNA to express smaller parts of the light chain to find the minimal peptide chain which is still effective.

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Mechanism of Action of Clostridium Difficile Toxins A and B

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INTRODUCTION

Clostridium difficile is the cause of nearly all cases of pseudomembraneous colitis as well as being involved in many cases of antibiotic-associated diarrhoea and some cases of sporadic diarrhoea (2). The evidence is that most of the gastrointestinal tissue damage is mediated by toxin A, which is also the cause of the diarrhoea. The toxins have a variety of biological activities in addition to the above including cytotoxicity and haemagglutination. This has led to a number of approaches to the understanding of the mechanism of action of these toxins. This brief overview will first present data on the physicochemical properties of these two toxins, then assess their activities in animal models. This will be compared to findings in in vitro models eg Ussing chambers and explant organ culture. Finally the mechanism of action of the toxins in cell culture will be reviewed.

PHYSICOCHEMICAL PROPERTIES

The physicochemical and biological activities of toxins A and B have recently been expertly reviewed elsewhere (15,18). Both toxins are extremely large, native toxin A having an Mr in the range of 400,000 to 600,000, and native toxin B an Mr in the range of 360,000-500,000. Under denaturing conditions both toxins have an Mr in excess of 250,000. It was recently reported that toxin B consists of sub-units which have an estimated Mr of c. 50,000 (23,24), though Meador and Tweten (19) demonstrated that a non-cytotoxic protein with physicochemical properties identical to those described by Pothoulakis (23) readily contaminates toxin B preparations. However, the controversy has been

re-activated by a recent publication providing evidence for the existence of two forms of toxin B (30); form 1 having the characteristics of the toxin B described by the majority of workers, and form 2 being of much lower molecular weight though distinct from the 50 kDa protein described above. Fortunately, much less controversy is associated with the physicochemical properties of toxin A. However, Rihn and colleagues (25) have reported an Mr of 52,000 and a subunit composition of A_1 (Mr = 41,500) and A_2 (Mr = 16,000).
Toxin A is less susceptible to pH extremes and proteases than toxin B (1,26,27) though both are inactivated by oxidizing agents but not reducing agents (17). One of the minor inconsistencies, ie the differing results on susceptibility of toxin A to trypsin, has been resolved by the demonstration that the reported inactivation of toxin A by trypsin is due to chymotrypsin contamination (14,31). The toxins are both acidic molecules with isoelectric point values of 5.2-5.7 for toxin A and 4.1-4.5 for toxin B (17), and are both rich in aspartate, glutamate and glycine with low amounts of histidine and sulphur containing amino acids (1,16,17).

EFFECTS IN VIVO

Administration of toxigenic strains of C. difficile to a variety of antibiotic pre-treated animals, in particular hamsters, results in diarrhoea and gross intestinal tissue damage. Similar findings occur following administration of crude culture filtrates. Work with purified toxins has helped to clarify their respective roles in the disease process. The general concensus from the studies is that toxin A in the small bowel of rabbits causes haemorrhage, villous architecture damage, a polymorphonuclear leucocyte infiltration, increases in permeability, total protein (especially albumin), osmolality, sodium, chloride and potassium, with bloody fluid accumulation and a decrease in pH (12,20,22,29). In constrast to these findings Lönnroth and Lange (13) found that toxin A caused a clear fluid accumulation in the mouse small bowel loop, which became haemorrhogic only if it was adminsistered with toxin B.
The effects of toxin A in the large bowel differ markedly to those noted in the small bowel. Lima and colleagues (12) found that crude culture filtrate resulted in a delayed accumulation of fluid with increases in pH, bicarbonate and potassium but that pure toxin A did not unless at a concentration of 50μg/ml. These findings are in broad agreement with those of Mitchell et al (20) who also demonstrated the appearance of large intercellular spaces between the epithelial cells, removal of colonocytes but an intact basement membrane.
Toxin B alone on the other hand appears to have no measurable effect in either the small or large bowel.
The use of animal gut tissue in vitro has also yielded interesting information. Guandalini et al (8) removed gut tissue from C. difficile infected rabbits and used the material which had been exposed to C. difficile products in vivo for in vitro nutrient influx ion transport and cyclic nucleotide studies. They found increased tissue permeability in the ileum and a decrease in chloride absorption. There was net chloride secretion for caecal tissue. No increase in cAMP or cGMP was detected in either ileal or caecal mucosa, in keeping with findings in

the mouse (13). This appears to contradict the findings of Vesely *et al* (32) who noted that partially purified toxin(s) stimulated guanylate cyclase in the hamster intestine, though due to the 24h delay between exposure to toxin and measurements the findings are considered to represent secondary compensatory effects rather than primary events (4).

Two groups have used Ussing chambers to study the effects of C. difficile toxins on rabbit ileum (10,21). Hughes and colleagues (10) found that crude broth filtrates of C. difficile, prevented absorption of sodium and increased secretion of chloride ions. Mitchell and colleagues (21) found that pure toxin A had similar effects to crude culture filtrates, but concluded that the apparent net secretion of sodium and chloride were due to toxin induced changes in tissue permeability and not a toxin mediated stimulation of an active secretory pathway.

One of the most interesting in vitro studies is that of Triadafilopoulos et al (29) who compared their in vivo findings in rabbit ileal loops to those found in vitro with ileal explants in short-term organ culture. In contrast to the in vivo findings they found that toxin A had no effect on epithelial cell permeability, protein synthesis, release of alkaline phosphatase or morphology. The proposed explanation for these observations is that the changes observed in vivo are a consequence of toxin A triggered infiltration of the ileum with neutrophils.

EFFECTS IN VITRO

Although some work has been done in vitro with organ culture and ileal tissue in Ussing chambers it was more appropriate to deal with those in the above section. This section will restrict itself to findings in tissue culture, which represent the vast majority of in vitro work with these toxins. Both toxins A and B cause rounding in a large number of cell types, giving the appearance of a cytotonic effect. As the changes are due to effects on the cell cytoskeleton the effect was called actinomorphic (3). The cells do eventually die so the toxins are also cytotoxic. An acceptable generalised term for the action of the toxins is cytopathic. For the toxins to have a cytopathic effect they must interact with the cell (bind), become internalised or induce a transmembrane signal, and if internalised interact with an intracellular target with or without intracellular modification/ activation. Some information on these processes is available for both toxins (see 4,6,9,28). Both toxins bind to cells in a temperature independent manner within 30s and have a latency period of about 45min. The identity of the specific cell surface receptor, if any, is unknown, though it is thought that it is unlikely to be a lipid. In addition, the inhibition of cytopathic effects with concanavalin A are now thought to be due to interference with endocytosis and not binding. The observation that polyphosphorylated compounds delay the appearance of the cytopathogenic effect induced by toxin B and caused the toxin to remain accessible to neutralisation with antitoxin implies that toxin B contains a phosphate binding site which may be involved in the interaction of the toxin with the cell surface other than its initial binding step (5). It is interesting to speculate that this may be

similar to the P-site on diphtheria toxin.

There is very good indirect evidence that the receptor in the gut for toxin A is Galα1-3Galβ1-4 N-acetylglucosamine (11), but that this is not the receptor on cells in tissue culture.

Both toxins A and B have to be endocytosed and require a low endosomal pH for internalisation. Neither toxin can be transferred directly to the cytosol from the endosomes. However on reaching the endosomes the two toxins appear to proceed along different pathways. Although both toxins seem to require enzymatic processing besides a low pH the necessary enzymes may be located in different cellular compartments, these probably being lysosomes for toxin B and maybe components of the Golgi complex for toxin A. Interestingly, unlike toxin B but similar to diphtheria toxin, exposure of toxin A to low extracellular pH facilitates transfer of active toxin across the plasma membrane and overcomes inhibitors of intracellular processing. However, the reasons why plasma membrane-bound toxin A can be transferred in active form directly across the membrane to the cytosol following exposure to low pH, though seems to be unable to be transferred to the cytosol from endosomes (which are acidic) across the endosomal membrane has not yet been adequately explained.

Although the microfilaments are the primary target of the toxins the specific intracellular target that mediates these changes remains unknown. As there is evidence for a role for calcium from the work in mouse ligated loops (13), rabbit ileal tissue in Ussing chambers (10) and vero cells in tissue culture (7) Donta (4) has speculated that the calcium dependent regulatory protein, villin, which is one of the major proteins associated with intestinal microvillus core filaments, is a likely specific target.

CONCLUDING COMMENTS

There are a number of problems, which are insufficiently highlighted in the literature, associated with trying to interpret the information available. Firstly, most of the work on determining the mechanism of action of the toxins in vivo has been conducted in animal small bowel, whereas in man the disease is almost invariably restricted to the colon. In addition, none of the animal models show classical pseudomembranes/ plaques which are features of pseudomembranous colitis in humans. Secondly, findings in tissue culture which are then extrapulated to in vivo findings have been derived almost exclusively from work with toxin B, the potent cytotoxin, whereas most in vivo animal work has been with toxin A. A more systematic approach to the study of these toxins to determine mechanism of action is required.

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Diphtheria Toxin Receptor and a Nonproteinous Diphtheria Toxin-binding Molecule in Cell Membrane

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INTRODUCTION

Diphtheria toxin (DT) is a cytotoxic protein which inhibits cellular protein synthesis in eukayotes by catalyzing the ADP-ribosylation of EF-2, which results in its inactivation. The first step of intoxication by DT is binding of the toxin to a susceptible cell. A specific receptor for DT is believed to be involved in this step (4,19). Cells from a number of mammals including humans and monkeys are sensitive to DT, but mouse and rat cells are insensitive (14). Several lines of evidence show that the difference in sensitivity to DT between species is primarily determined by the presence or absence of a cell surface receptor (7,11,15,22). However, this receptor has not been isolated. A strategy to isolate the DT receptor has been to explore its binding activity for DT in isolated membrane or using solubilized membrane fractions. However, the specific binding of labeled DT to isolated membrane has not been observed as yet.

In this paper we will show two DT-binding molecules that are present in isolated Vero cell membranes. One of these substances is referred as inhibitor, because it inhibited the cytotoxic effects of DT and it is found on both DT-sensitive cells and DT-insensitive cells. This inhibitor bound to DT but could not bind to CRM197, the product of a missense mutation in the DT gene. The other substance, which is the DT receptor, was found to bind to both DT and CRM197 with high affinity and is present only on DT-sensitive cells. Using the CRM197 the receptor was partially purified and a 14.5kD protein was identified to be the receptor, or at least a component of it.

MATERIALS AND METHODS

Preparation of Cell Membrane: Vero cells and other cultured cells were grown on plastic dishes, collected with rubber policemen, and stored at -80C until use. Ehrlich ascites tumor cells were grown in and harvested from mouse abdomen. The membrane fractions were obtained by an alkali-extraction method essentially as described by Thom (18) with slight modifications (13).

Inhibition of the Cytotoxicity of DT: Cytotoxicity of DT was measured by assaying the rate of protein synthesis in cultured cells as described previously (10). The inhibitory activity was calculated as described previously (13). The inhibitory activity of samples containing octylglucoside was measured after removing the detergent by dialysis.

Effect of the Inhibitor on the Binding of DT or CRMs to Vero cells: Indirect assay of binding of DT or CRMs was carried out using ^{125}I-labeled anti-DT monoclonal antibody as described previously (12).

Binding of ^{125}I-CRM197 to Isolated Membranes: Nicked CRM197 was labeled with Na^{125}I using Enzymobeads (Bio-Rad) as described (12). Membranes were washed twice and resuspended with Buffer D (130mM NaCl, 50mM MES (2-(N-morpholino)-ethanesulfonic acid), 10mM KCl, 0.5mM $CaCl_2$, 1mM NaN_3, 1mg/ml BSA, pH6.1) by repeated aspiration through a 26-gauge needle. The binding reaction was carried out in a 200μl reaction volume (0.5-1 mg of protein) with 2-20 ng of ^{125}I-CRM197 at 24C for 4h unless otherwise stated, with gentle shaking. Then the mixture was rapidly filtered on Millipore GVWP filters, and each filter was washed with 10 ml of cold Buffer D. The amount of radioactivity retained on each filter was counted in a γ-counter. Non-specific binding was assessed in the presence of a 1000-fold excess of unlabeled CRM197.

Binding of ^{125}I-CRM197 to Solubilized Membrane: Membrane fractions solubilized with octylglucoside were mixed with phosphatidylcholine, then the mixture was precipitated with acetone as described previously (16). The precipitates were collected by centrifugation and resuspended with Buffer D. The suspended materials were incubated with ^{125}I-CRM197 in the presence or absence of excess unlabeled CRM197. After 4hr incubation at 24C, the mixture was filtered as described above. The amount of radioactivity retained on each filter was measured.

Radioiodination and Immunoprecipitation of DT Receptor: A fraction of DT receptor eluted from CM-Sepharose was radioiodinated by Bolton-Hunter reagent. The labeled sample was incubated at 4C for 14h with DT or one of the CRM proteins at the concentration indicated, followed by the addition of horse anti-DT antibody conjugated with CNBr-activated Sepharose beads. The mixture was incubated at 24C for 5h with gentle shaking. The Sepharose beads were pelleted, washed as described previously (13). The precipitated materials were analyzed by SDS-PAGE (8) under reducing condition and by autoradiography.

Western Blot Analysis of DT Receptor: The DT receptor fraction from CM-Sepharose was incubated with SDS gel sample

buffer at 37C for 3h, two sets of protein samples were run on 15% SDS-PAGE and then transfered to Durapore filters (type GVHP, Millipore Co.). Following treatment with the blocking solution, one of the filters was treated with ^{125}I-CRM197 and the other with ^{125}I-CRM197 plus 1,000 times excess unlabeled CRM197. The filters were washed, dried and autoradiographed.

RESULTS

An Inhibitory substance to DT activity is contained in cell membrane:
We found that a membrane fraction isolated from Vero cells inhibited the cytotoxicity of DT. Addition of DT at 40 ng/ml to FL cell cultures reduced protein synthesis to about 10% of the value in control cultures. When the membrane fraction was added with DT to FL cell cultures, the rate of cellular protein synthesis was increased in a dose-dependent manner, indicating that the cytotoxicity of DT was blocked (Fig. 1). The membrane fraction per se had no effect on cellular protein synthesis. We next tested whether membrane fractions isolated from other cell lines with different sensitivities to DT show inhibition of the cytotoxicity. HeLa cells are about 100 times and mouse L cells about 10^5 times less sensitive than Vero cells (11). Ehrlich ascites tumor cells are more resistant than L cells (5). Membrane fractions from all these cell lines showed similar inhibitory activity, indicating the inhibitor was present both in DT-sensitive and in DT-insensitive cell lines.

DT Inhibitor Binds to DT but not to CRM197:
We examined whether the inhibitor could bind to DT. The inhibitor was solubilized from Vero cell membrane(13), and the solubilized inhibitor was incubated with Sepharose beads

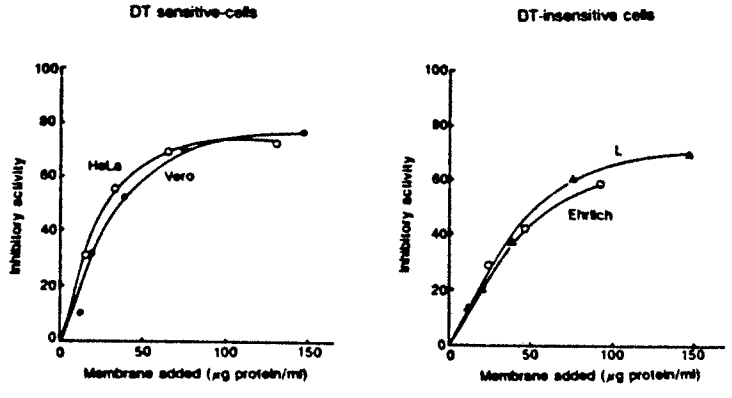


Fig. 1 Inhibition of DT-cytotoxicity by membrane fractions of DT-sensitive and DT-insensitive cells. FL cells were incubated for 2h at 37 C with DT (40ng/ml) and various amounts of membrane fractions from cultured cells Then the cells were labeled with [^{3}H]-leucine for 1h at 37 C, and the rate of protein synthesis was measured.

conjugated with DT. The gels were washed and the inhibitor bound to gels was eluted with 4M-KSCN. The inhibitory activity was bound to DT-beads. Next we used DT fragment A and CRMs to determine the region of DT responsible for the binding of the inhibitor. CRM45 is a premature termination protein of DT containing enzymatically active fragment A and about half of the B fragment (19). The inhibitor was not bound to beads conjugated with fragment A or with CRM45, indicating that at least the C-terminal 15K-region of DT is required for binding of the inhibitor. CRM197 is a product of a missence mutation in the DT gene (20) and differs from wild type toxin only in one amino acid residue in fragment A (2). CRM197 is known to bind to DT-sensitive cells with an affinity similar to or higher than that of native toxin (12). Interestingly, the inhibitor was not adsorbed with CRM197-beads. Therefore, not only fragment B but also fragment A is required for the binding of the inhibitor.

Effect of the Inhibitor on the Binding of DT and CRMs to Vero Cells:

The effects of the inhibitor on the binding of DT and CRM proteins to Vero cells were examined by an indirect binding assay using a ^{125}I-labelled monoclonal antibody against DT (12). This antibody, referred to as #2, binds to DT but does not inhibit the binding of DT to cells (3). As shown in Fig. 2, the inhibitor blocked the binding of DT to Vero cells but not the binding of CRM197. Moreover, the assay showed that the inhibitor blocked the binding of CRM176 and CRM228 to the cells.

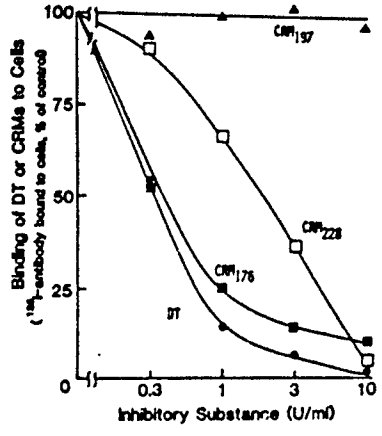


Fig. 2 Effect of DT inhibitor on the binding of DT or CRMs to Vero cells measured by an indirect binding assay using ^{125}I-anti-DT antibody. ^{125}I-Antibody was incubated with DT or CRMs. The mixture and various amounts of DT inhibitor were added to Vero cells, and the cells were incubated for 90 min at 37 C. Then specific cell-associated radioactivity was measured.

^{125}I-CRM197 Binds to Vero Cell Membrane but not to L Cell Membrane:

The inhibitor was present even in the membrane fractions of toxin-insensitive cells. Moreover, although CRM197 binds to Vero cells with an affinity similar to, or greater than that of DT (12), the inhibitor did not bind to CRM197 and did not inhibit the binding of CRM197 to Vero cells. These facts suggest that the inhibitor is not the receptor responsible for the difference in sensitivity to DT between species, and

that another DT-binding substance would be present in the membrane of DT-sensitive cells. As CRM197 did not bind the inhibitor, we used CRM197 to look for a DT receptor in Vero cell membrane preparations.

Vero cell membrane was incubated with ^{125}I-CRM197 and then the radioactivity associated with the membrane was measured. As shown in Fig. 3, saturable specific binding of ^{125}I-CRM197 was observed. Analysis by Scatchard plots indicates a single class of binding sites with a Ka value of 2.1×10^9 liter/mole. This value is consistent with the value determined by us in intact Vero cells (12). We also examined the binding of ^{125}I-CRM197 to mouse L cell membrane. Over the range of CRM197 concentration used for Vero cells, no specific binding was observed. Thus, the sites specific for CRM197 binding are either not present on DT-insensitive L cells, or exist in very low density to be detected in our binding assay. These results clearly show that the molecule which binds CRM197 differs from the inhibitor, because the inhibitor exists on L cell membrane in densities similar to those on Vero cell membrane.

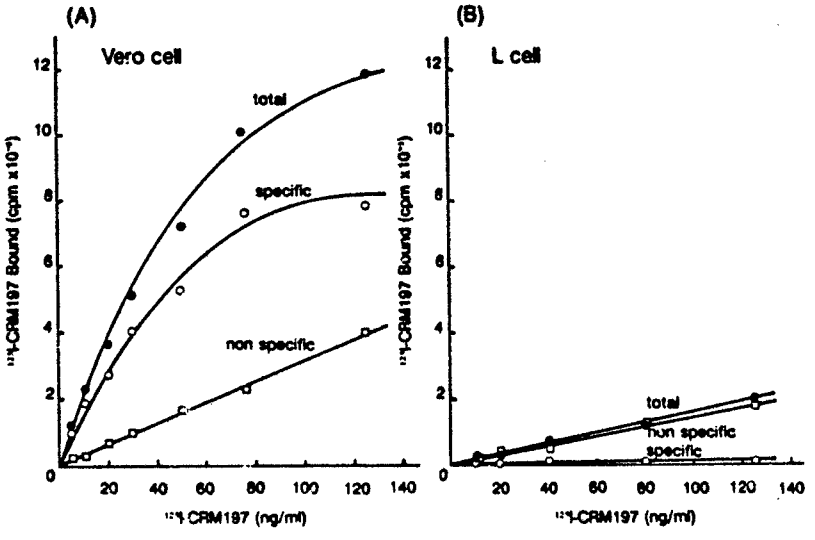


Fig. 3 Binding of various concentrations of ^{125}I-CRM197 to Vero cell membrane (A), or L cell membrane (B).

To examine whether the observed binding of ^{125}I-CRM197 to Vero cell membrane is truely specific for DT, we performed a competition assay using unlabeled DT or CRMs. As shown in Fig. 4, the binding of ^{125}I-CRM197 was most strongly inhibited by nicked CRM197. Native DT was about 3 times less potent and CRM228 was about 200 times less potent than nicked CRM197. CRM45 did not inhibit the binding of labeled CRM197. These results strongly suggest that the binding sites for CRM197 observed in membrane fractions were DT receptors.

Partial Purification of DT Receptor:
The receptor was partially purified by column chromatography. We used an acetone-precipitation method to assay the binding of solubilized membrane fractions. Vero cell membrane was treated with 60 mM octylglucoside, then centrifuged to remove insoluble materials. The solubilized cell membrane material was applied on a CM-Sepharose column. CRM197-binding activity was retained on the column and found to be eluted with 0.4-0.6M NaCl. Then the eluted materials were applied on a CRM197-conjugated affinity column. Most of the proteins appeared in the flow-through fractions, but the receptor was retained and eluted with acidic buffer. Although by these purification steps the receptor was purified from crude solubilized membrane fraction by at least 2,000-folds, several protein bands were still observed when the purified materials were radioiodinated and analyzed by SDS-PAGE.

Identification of DT Receptor:
To identify the receptor, the CM-Sepharose fraction containing DT receptor was radioiodinated and immunoprecipitated using either DT or other CRM proteins and immobilized anti-DT antibody. The immunoprecipitated materials were analyzed by SDS-PAGE. In the samples precipitated with DT or CRM197 and anti-DT antibody, three major bands with 14.5kD, 47kD and 62kD and several minor bands are seen in a reducing condition. When neither DT nor CRM197 was added, or DT and a non-specific antibody was added, such major three bands were not seen.

Although the results of immunoprecipitation study suggest that the three major proteins seen on SDS-PAGE are related to DT receptor, there is the possibility that the receptor coprecipitated with contaminating cellular materials. To identify which molecule on SDS-PAGE has the property to bind to DT, we carried out a Western blot

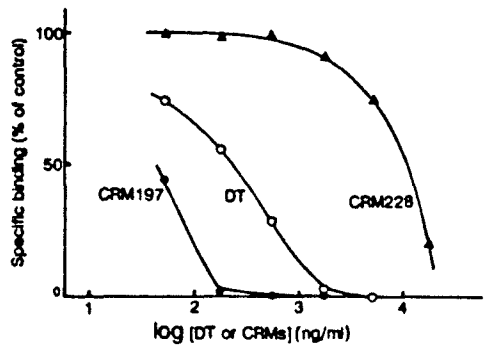


Fig. 4 Competition for binding of ^{125}I-CRM197 to Vero cell membranes by unlabeled DT or unlabeled CRMs. Vero cell membranes was incubated at 24 C for 4h with ^{125}I-CRM197 at 30 ng/ml and various concentrations of one of the unlabeled protein. The specific radioactivity associated with Vero cell membranes was measured. The data are expressed as a percentage of the specific binding.

analysis of the receptor fraction eluted from CM-Sepharose using ^{125}I-CRM197 as probe. Only a single band with 14.5kD was observed. This band was identical to the 14.5kD band seen in immunoprecipitation study. When the probe contained an excess amount of unlabeled CRM197, no significant bands were observed. Thus we concluded that this 14.5kD band is the DT receptor or at least a component of it. This finding also suggested that 14.5kD DT receptor is associated with the 47kD and the 62kD proteins, and these proteins were co-precipitated by the addition of DT or CRM197 and the antibody.

DISCUSSION

We have shown that an inhibitor present in a membrane fraction of cultured cells blocks the cytotoxicity of DT by preventing its binding to target cells. We suggest that the inhibitor is not a molecule with a specific role in the binding DT to the surface of DT-sensitive cell. Does the inhibitor exist on the cell surface? The plasma membrane-rich fraction used in this paper contained inner membranes. Although there are several lines of evidence to support the existence of the inhibitor on the cell surface (6,9,17,21), we have not obtained any direct evidence on location of the inhibitor. It has also been is unclear whether the inhibitor plays any role in DT intoxication.

The second DT-binding molecule present in the membrane fraction has characteristics of the surface receptor for DT. This molecule actually defines the sensitivity of cells to DT in different species. In fact, (I) this substance is present only in DT-sensitive cells, (II) it binds to CRM197, and its binding affinity is similar to that obtained with intact Vero cells. Furthermore, the results of the competition experiments in which binding of ^{125}I-CRM197 to Vero cell membrane was measured in the presence of DT and various CRMs are consistent with CRM197 binding experiments with intact Vero cells. Therefore, we conclude that this binding activity is due to the DT receptor present in DT-sensitive cells. Specific binding of DT to the receptor has not been reported using isolated membrane or a solubilized membrane fraction. The presence of the inhibitor might have interfered in the binding of DT to its receptor and made the interpretation of such experiments hard (1,6). The immunoprecipitation study and Western biot analysis revealed that a protein of 14.5kD in SDS-PAGE is the DT receptor or a component of it. Is this 14.5kD protein a functional DT receptor? The possibility that 14.5kD protein was a processed form of the precursor cannot be ruled out. By the immunoprecipitation study, two proteins of 47kD and 62kD precipitated prominently with 14.5kD protein. These proteins may also be related to DT receptor, but much more extensive studies will be required to elucidate the relationship of these components to the DT receptor.

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Enzyme, Toxin, Liposome-Disrupting and Hemolytic Activities of Adenylate Cyclase Toxin from Bordetella pertussis

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INTRODUCTION

Bordetella pertussis produces a calmodulin-activated adenylate cyclase (AC) which is able to enter eukaryotic target cells where it catalyzes the production of intracellular cyclic adenosine monophosphate (cAMP) (1,2). This protein toxin is a virulence factor for B. pertussis, due at least in part to the inhibition of phagocytic leukocyte function caused by the accumulated cAMP (1-7). While a variety of different sized molecules from B. pertussis possessing AC enzyme activity (ability to catalyze cAMP production in a cell-free assay) have been described (2,8-12), much less is known of the holotoxin molecule which is capable of eliciting cAMP accumulation in intact target cells. Data from several laboratories suggest that the active form of AC toxin has a molecular mass of slightly greater than 200-kDa (9,13,14). Furthermore, the gene which appears to encode the AC has been cloned and sequenced and the derived amino acid sequence yields a calculated mass of 177-kDa (15).

The AC holotoxin has been the object of study in this laboratory and it has recently been isolated in homogeneous form (16). The properties and activities of this 216-kDa protein are the subject of this presentation.

IDENTIFICATION AND CHARACTERIZATION OF THE AC HOLOTOXIN

Monoclonal antibodies which immunoprecipitate AC enzymatic activity from extracts of B. pertussis recognize a band of 216-kDa on Western blot (Figure 1). Using purification by urea extraction, hydrophobic chromatography, preparative sucrose gradient centrifugation and affinity chromatography with either calmodulin-Sepharose or monoclonal

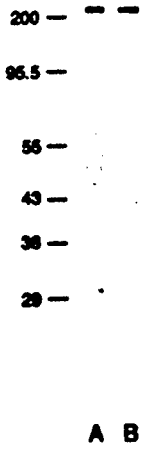


Figure 1. Monoclonal antibodies that immunoprecipitate AC enzymatic activity identify a 216-kDa band on Western blot. Sucrose gradient-purified, B. pertussis AC toxin was run on a 12.5% SDS-PAGE. The proteins were transferred to a PVDF membrane and incubated with two AC (+) antibodies A) 9D4 and B) 1H6, as well as an irrelevant antibody C) MHS-5.

antibody-Sepharose as the final step, the 216-kDa protein can be isolated to > 85% purity (16). As shown in Figure 2, this preparation of the 216-kDa protein has its AC enzymatic activity increased ~1000-fold by calmodulin in a dose-dependent manner. The ability to elicit cAMP accumulation in J774 cells, however, is essentially unaffected by calmodulin (Figure 2).

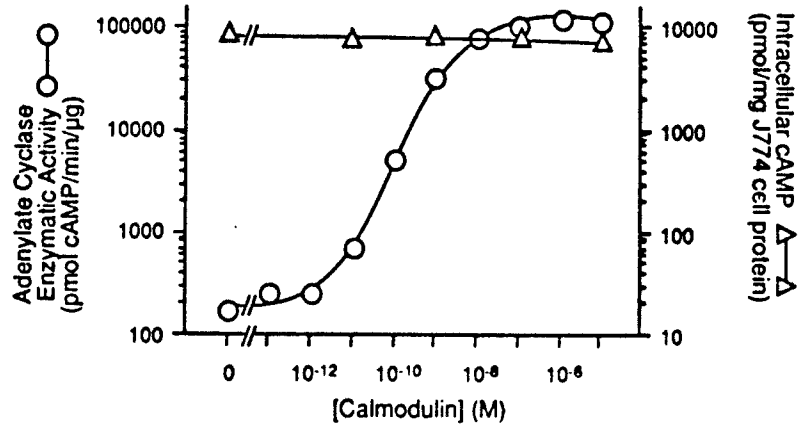


Figure 2. Effect of calmodulin on AC enzymatic and toxin activities. Sucrose gradient-purified, B. pertussis AC toxin was incubated with the indicated concentration of calmodulin for 10 min. at 25°C and then assayed for enzymatic activity (O - O) or for toxin activity (Δ - Δ) in J774 cells (1 hr incubation at 37°C).

This is in contrast to the data of Shattuck and Storm who showed calmodulin inhibition of the intoxication of human erythrocytes and N1E-115 cells by AC toxin (17). Gentile et al., however, have found that calmodulin inhibition of AC toxin action is limited to only a few cell types, thus potentially explaining the apparent discrepancy (18). Finally,

electroelution of the 216-kDa band from SDS-polyacrylamide gel yields a protein which has an enzyme activity of 589 μmol cAMP/min/mg protein and a toxin activity of 21 μmol cAMP/mg target cell protein/mg toxin (16). These data, obtained with the use of the purified 216-kDa protein, establish that it is the AC holotoxin.

LYSIS OF LIPOSOMES BY B. PERTUSSIS AC TOXIN

In a study of the entry mechanisms used by AC toxins from Bordetella pertussis and Bacillus anthracis, Gordon et al. (19) demonstrated that anthrax AC toxin, but not pertussis AC toxin, enters target cells using receptor-mediated endocytosis. Based on these data, it was hypothesized that pertussis AC toxin inserts itself directly into the lipid bilayer. Using a model system of liposomes containing a trapped marker, as shown in Figure 3, Gordon et al. found that pertussis AC toxin releases up to 40% of the trapped marker in a time and dose-dependent manner (20). Liposome lysis by AC toxin

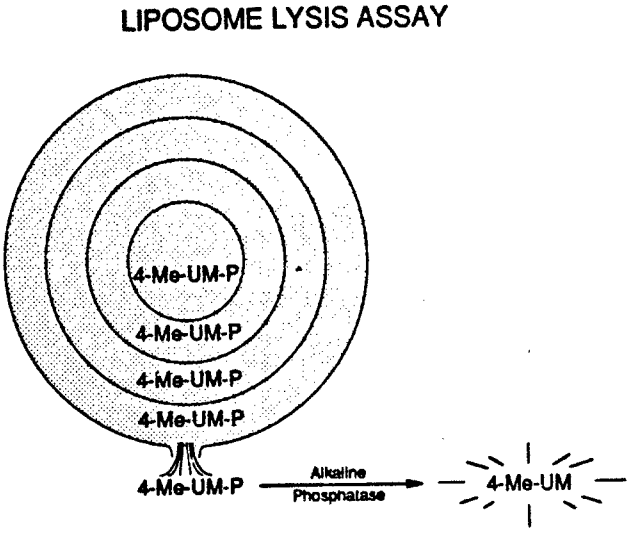


Figure 3. Schematic representation of liposome lysis assay (20). Multilamellar liposomes consisting of DMPC: cholesterol:glycolipid (molar ratio 1.0:0.75:0.03) contained trapped 4-methyl umbelliferone phosphate. Each assay tube contained AC toxin or control and alkaline phosphatase (12 U/ml). The reaction was initiated with the addition of liposomes and damage to the liposomes was quantitated by spectrofluorometric determination of marker release.

is calcium-dependent, as is the intoxication of target cells (1,9,20). This system provides a model for study of the interaction of pertussis AC toxin with target cells which may reflect the mechanism by which the catalytic AC enters.

HEMOLYTIC ACTIVITY OF AC TOXIN

Colonies of B. pertussis are surrounded by zones of hemolysis on Bordet-Gengou blood plates. The molecule responsible for that hemolysin, however, has not previously been identified. Mutants of B. pertussis which are deficient in AC are non-hemolytic, suggesting a genetic linkage between the AC enzyme/toxin and hemolysin (6). The first data indicating a possible functional association between AC toxin and hemolysin is the demonstration that the toxin can disrupt multilamellar liposomes (20).

Analysis of the sequence of the AC gene from B. pertussis by Glaser et al. reveals an internal homology with repeating elements found in α-hemolysin from E. coli (15). This sequence homology is supported by the recent observation by Ehrmann et al. (manuscript in preparation) that monoclonal antibody against pertussis AC toxin reacts with E. coli α-hemolysin on Western blot. Analysis of DNA sequences from regions surrounding the apparent structural gene for AC in B. pertussis demonstrates additional homologies (21). There are open reading frames which are homologous with the ancillary genes involved in secretion of E. coli hemolysin and Pasteurella haemolytica leukotoxin (21).

An investigation of the relationship between hemolysis and AC toxin activity indicates that these functions may reside on the same molecule, as illustrated by the following data. Urea extracts from wild type B. pertussis, but not mutants lacking AC toxin, are hemolytic for sheep erythrocytes. As shown in Figure 4, purification of the 216-kDa holotoxin yields increasingly potent hemolytic activity. This hemolytic activity is

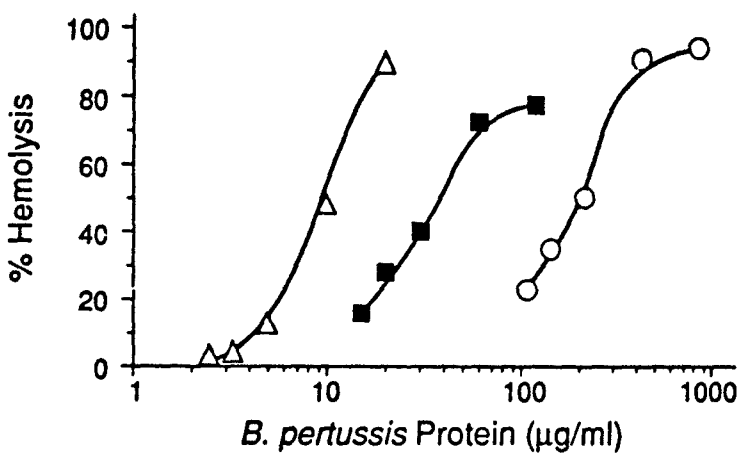


Figure 4. Hemolytic activities of fractions from AC toxin purification. Sheep erythrocytes (2 x 10^8) were incubated with urea extract of B. pertussis (O-O), phenyl-Sepharose purified AC toxin (■ - ■), or sucrose gradient purified AC toxin (Δ - Δ) for 5 hrs at 37°C. Hemolysis was quantitated by hemoglobin release as % of total hemoglobin released by NH_4OH using spectrophotometric reading at 540 nm.

calcium-dependent, as are cell intoxication and liposome disruption. This calcium dependence is not unexpected since one of the regions of E. coli with homology to AC toxin (nonameric repeats) is felt to be the calcium-binding domain (22). Finally, the electroeluted 216-kDa protein is hemolytic, indicating that this activity is the result of the interaction between the AC holotoxin and sensitive erythrocytes.

CONCLUSIONS

The 216-kDa AC toxin produced by B. pertussis is novel in that it is a calmodulin-activated AC which is able to catalyze intracellular cAMP accumulation, disrupt liposomes, and lyse erythrocytes. All of the actions of this molecule which involve membrane interactions require calcium, probably reflecting at least in part a function of the calcium-binding repeats which are homologous to E. coli hemolysin.

One postulated role of the AC toxin in clinical pertussis is to paralyze phagocytes in order to prevent removal of B. pertussis organisms (1-4,23). Since pertussis is a localized infection of the respiratory tract, it is unlikely that the AC toxin encounters erythrocytes frequently and

thus the hemolytic activity is not likely to be an essential feature. On the other hand, the hemolytic and liposome-disrupting activities probably reflect the mechanism by which the toxin interacts with membranes, with the domain homologous to other bacterial hemolysins responsible for creating a pore or channel through which the catalytic fragment is transferred to the cell interior.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the contributions of Barbara Taylor, Susan Roscoe, Virginia Estabrook, Donna Taylor, Martha MacLeod and George Vandenhoff. This work was supported by grants from the National Institutes of Health (AI 18000 and DK 38942).

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Pore Formation by Escherichia coli Hemolysin in Lipid Bilayer Membranes

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INTRODUCTION

The membrane-active cytolysins (hemolysins) are extracellular toxic proteins which are produced by a large number of Gram-positive and Gram-negative bacteria (1). Cytolysin-secreting bacteria are frequently pathogenic and the direct involvement of these extracellular proteins in pathogenesis has been demonstrated. Pore-formation in the target cell membrane is a well established mechanism for some of the toxins. We present lipid bilayer data that clearly indicate that hemolysin of E. coli forms transient pores in membranes formed of asolectin whereas membranes of pure lipids are relatively inactive targets for HlyA. We describe an analysis of the unitary (single-channel) conductance formed by HlyA in asolectin membranes. The channels with an average single-channel conductance of about 500 pS in 0.15 M KCl have a mean lifetime of about 2 s at 20 mV.

MATERIALS AND METHODS

Hemolysin was isolated and purified essentially as described from E. coli 5K (containing the plasmid pANN202-812 (2) or supernatents of cell cultures were directly used. Black lipid bilayer membranes were formed as described previously (3). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by small circular holes. Membranes were formed across the holes by painting on a 1% solution of different lipids in n-decane. The aqueous salt solutions were used unbuffered and had a pH around 6. The membrane current was measured with a pair of calomel electrodes switched in series with a voltage source and a current amplifier.

RESULTS AND DISCUSSION

Lipid bilayer experiments were performed in the presence of small concentrations of hemolysin (around 200 ng/ml) of E. coli. The toxin had a rather low activity in membranes formed of pure lipids, such as phosphatidyl-choline or phosphatidylserine. In membranes from asolectin, a crude lipid mixture from soy bean, hemolysin was able to increase the conductance by many orders of magnitude in a steep concentration-dependent fashion which suggested that several hemolysin molecules could be involved in the formation of the conductive unit (4). The much higher toxin activity in asolectin membranes is consistent with the assumption that this lipid may contain a receptor needed for membrane activity of the toxin.

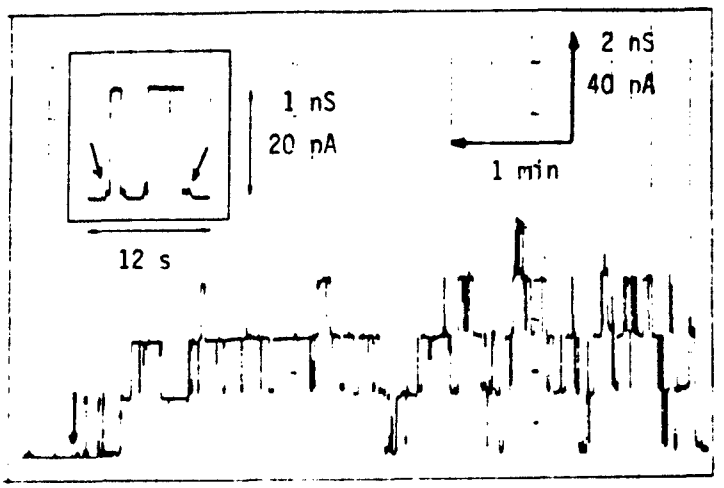


Fig. 1. Single-channel recording of hemolysin channels in the presence of 0.5 M KCl. The membrane was formed from asolectin/n-decane. The applied voltage was 20 mV; T = 25° C. The insert shows two channels on a faster time scale. The arrows indicate the small conductance fluctuations.

Single-channel records in the presence of small toxin concentrations showed that the membrane activity of hemolysin is due to the formation of ion-permeable channels with a single channel conductance of about 1 nS in 0.5 M KCl (Fig. 1). The hemolysin channel seemed to be formed by a toxin oligomer because smaller channels with a single-channel conductance of about 100 pS were also observed besides the large conductive unit (see Fig.1). The lifetime of the hemolysin channel was limited which is consistent with an association-dissociation reaction of the pore-forming oligomer. The mean lifetime of the channel was about 2 s at small transmembrane voltages. Larger voltages decreased the lifetime of the open channel and additional substates were observed. The conductance of the hemolysin channels was only moderately dependent on the salt concentration in the aqueous phase. This result indicated that charged groups inside the channel were involved in ion transport. Zero-current membrane potential experiments showed that the hemolysin channel is cation-selective. The mobility sequence of different cations in the channel was similar to their mobility sequence in the aqueous phase. This result suggested that the hemolysin channel is wide and that the field strength inside the channel is not very high. From the single-channel conductance a lower limit for the effective channel diameter of about 1.0 nm could be estimated by assuming that the channel is a cylinder filled with an salt solution of the same conductivity as the bulk aqueous conductance. This method yielded satisfactory results in the case of bacterial porins (5).

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Unique Secretion and Pore Formation of the Serratia marcescens Hemolysin

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Two proteins, ShlA and ShlB, are encoded by the S. marcescens hemolysin determinant. Both polypeptides are synthesized with signal sequences. ShlA, the hemolysin proper, is only active if expressed in the presence of ShlB. ShlB was localized in the outer membrane. ShlA produced in the presence of ShlB was found in the culture supernatant and on the cell surface as shown by immunoelectron microscopy and trypsin degradation. ShlA* (ShlA expressed in the absence of shlB) formed in the absence of ShlB accumulated in the periplasmic space. Therefore, we propose a secretion pathway where ShlA is translocated through the cytoplasmic membrane in a signal sequence dependent manner. ShlB facilitates the specific transport through the outer membrane. The latter step is accompanied by a change in hemolytic activity and in the conformation of ShlA. Deletion analyses revealed that only the N-terminal 260 amino acids, less than 1/5 of ShlA, are necessary for secretion. These results, obtained with the cloned S. marcescens genes in E. coli, were supported by studies with the original S. marcescens strain.

The ShlA-protein was shown to generate transmembrane channels in erythrocytes. To study these pores in more detail, bilayer experiments were performed with purified hemolysin as well as hemolysin from freshly prepared spent medium.
Single chanel experiments found that the ShlA-protein formed pores with an average size of 0.9 nS. Control experiments with inactive ShlA* and supernatants of uninduced cells showed no conductance under these conditions.
Macroscopic conductance measurements indicated an non-linear increase of conductance as a function of time after adding hemolysin to membranes. This suggested pore formation by hemolysin oligomers. Pores formed by monomers would yield a linear dependence.

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Tetanus Toxin Channels in Planar Lipid Bilayers

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Tetanus toxin (TeTx) and its B-fragment form voltage dependent ionic channels in planar lipid bilayers (1,3). In contrast, the light chain and the C-fragment do not form channels. It follows then, that the channel forming domain must be located in the amino terminus of TeTx heavy chain. Channel open-closed transitions occurr in bursts of activity; within a burst the channel current undergoes rapid transitions between closed and open states.

The rate of TeTx incorporation into bilayers is affected by the pH of ionic solution. Ten fold higher concentrations of TeTx are necessary to detect single channel signals if the ionic solution pH is increased from pH 4 to pH 7 in phosphatidylserine (PS) membranes. Moreover, TeTx channel conductance (in PS membranes) is three times higher at neutral pH than at pH 4, in otherwise identical conditions. In contrast, the voltage dependence of the channel is independent of pH.

Channel conductance and voltage dependence are modulated by the lipid composition of the membrane (2). Conductance augments almost linearly with increases in the content of charged phospholipid in diphytanoylphosphatidylcholine (dphPC) bilayers. This effect can be accounted for by the enrichment of counterion concentration at the membrane-water interface consequent the increase in the membrane surface potential.

The phospholipid composition of the membrane affects also the gating properties of the channel and its kinetics. In PS membranes, TeTx channels flicker among the closed state and several open states at positive applied transmembrane voltage. Following a switch of polarity, the channel closes

in few milliseconds and does not reopen until the transmembrane potential is switched to positive values. Such property is clearly demonstrated in I-V curves generated by a continuously cycled potential. The probability of the channel being open is very high at positive voltages ($P \geq 0$), whereas it is almost zero during the negative branch of the cycle. Similar properties were measured in egg PC. In contrast, an opposite probability of channel opening was observed when TeTx was incorporated in dphPC membranes. This modulatory role of lipid membrane composition on TeTx channel gating is remarkable and has not been hitherto reported for other ionic channels. Presumably, phospholipid hydrocarbon chains influence channel gating.

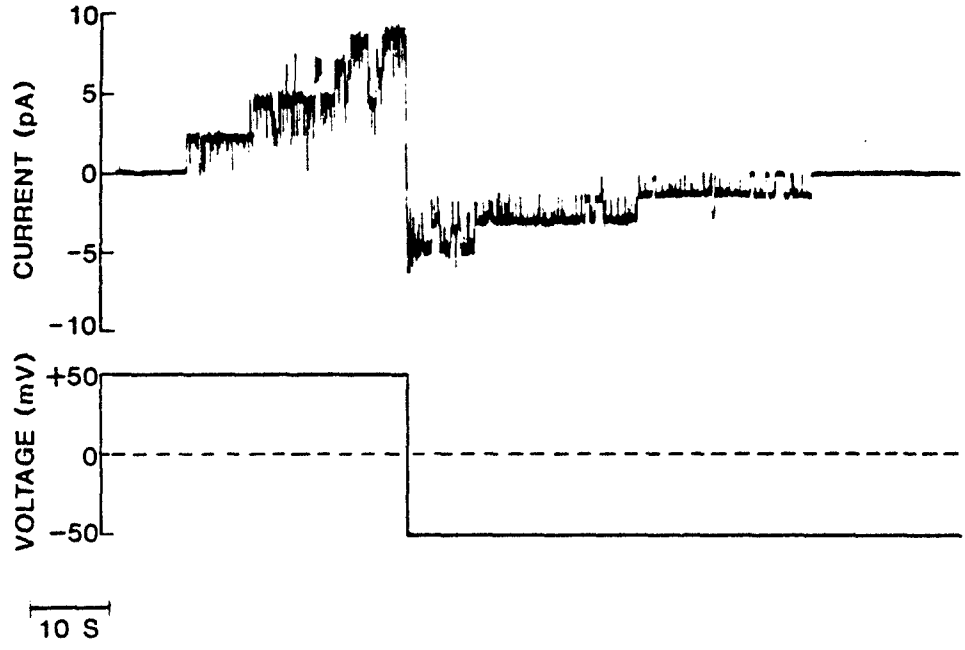


FIG.1., Single tetanus toxin channel current records in asolectin membrane. Positive transmembrane potential activate the channel, whereas when the voltage is switched to negative values the channel closes. The voltage of the tetanus toxin free compartment was defined as the reference voltage. Ionic solution was 0.5 M KCl, 10 mM Hepes, pH 7

TeTx channels undergo rapid transitions into and out from several intermediate states between the fully open state and the closed state, that, due to their fast kinetics, are rarely identified. Accordingly, TeTx channel states were characterized by best fitting current histogram

distributions with the sum of three gaussians. One represents the closed state, the others identify the full open state ($\gamma \approx 90$ pS in PS membrane) and an intermediate lower conductive level ($\gamma \approx 60$ pS in PS membranes). The ability of tetanus toxin to form channels in lipid bilayers suggests possible mechanisms for tetanus intoxication based on persistent depolarization at postsynaptic membrane level after the protein undergoes internalization and retrograde axonal transport.

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Inhibition of Noradrenalin Release from PC12 Cells by Botulinum Type A Neurotoxin

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Botulinum type A neurotoxin, a 145000 dalton disubunit protein, acts presynaptically at the neuromuscular junction blocking calcium-mediated release of the transmitter acetylcholine. Blockade of transmitter release is accomplished in at least three stages: binding to acceptor molecules on the presynaptic nerve surface, energy dependent internalisation and finally one or more steps in which the transmitter release mechanism is disabled. There is now considerable evidence that botulinum toxin A acts to block the release of a broad range of transmitter substances and the neurotoxin may indeed act on a process fundamental to all calcium-mediated secretory processes. Recent studies on the mode of action of botulinum type A neurotoxin using the buccal ganglion of Aplysia as a model suggest that active site regions on both the light subunit and the carboxy-terminal half of the heavy chain (H_1 fragment) are involved in the intracellular blockade of calcium-mediated transmitter release (1).

The rat adrenal pheochromocytoma cell line PC12 releases both acetylcholine and noradrenalin in a calcium-dependent manner thus making it an excellent model for the study of the mode of action of botulinum type A neurotoxin. In the presence of high potassium concentrations (60mM), PC12 cells loaded with [^{3}H] noradrenalin, release the transmitter in a calcium dependent manner.

Incubation of intact PC12 cells with botulinum type A neurotoxin for 16 hours at 37°C markedly reduced the calcium dependent release of noradrenalin. Inhibition of transmitter release was evident at toxin concentrations of 30nM. At toxin concentrations of 1μM, between 72 - 83% of the calcium dependent release was inhibited. In the presence of an excess of botulinum type A specific antibody the inhibitory effect of the neurotoxin was abolished. The toxin had very little affect on the calcium independent transmitter release and did not appear to affect significantly the uptake of noradrenalin by the PC12 cells.

The presence of acceptors for botulinum type A neurotoxin on PC12 cells was investigated using ^{125}I-labelled toxin. The binding of labelled toxin to PC12 cells grown on collagen was measured using concentrations of [^{125}I]-neurotoxin of 1nM and 10nM in the presence and absence of a 100-molar excess of cold toxin. No specific saturatable binding was observed to PC12 cells at either toxin concentration suggesting either the

complete absence of acceptors or the presence of very few acceptor molecules per cell. The presence of small quantities of a high affinity acceptor seem unlikely in view of the relatively high toxin concentrations required to induce intoxication. Similar results were obtained with PC12 cells treated with nerve growth factor for up to 28 days.

Supportive of the lack of acceptors is the long time course required for intoxication. Blockade of transmitter release was not observed for 4 - 6 hrs in the presence of 1μM neurotoxin and did not reach a maximum for 10-16 hrs. Collectively the data suggest the absence of high affinity acceptors for botulinum toxin on PC12 cell even after prolonged treatment with nerve growth factor.

Various fragments of botulinum type A neurotoxin were tested for their ability to block transmitter release from PC12 cells. Neither the heavy subunit nor the H_2L fragment, which contains the light subunit linked to the amino-terminal half of the heavy chain (2), were effective in blocking noradrenalin release from PC12 cells. A combination of both these fragments was similarly ineffective. The inability of the H_2L fragment to block transmitter release was not due to damage to the light chain component by trypsin (used in the production of the fragment (2)) since light chain purified from this fragment could be successfully reconstituted with heavy chain to give the active toxin.

Using ^{125}I-labelled H_2L fragment no difference in the amount of cell associated fragment compared to the intact neurotoxin was observed after 8 hours incubation with PC12 cells suggesting little difference in the extent of the internalisation between the two proteins.

CONCLUSIONS

Botulinum type A neurotoxin effectively blocks the calcium-dependent release of noradrenalin from intact PC12 cells. Approximately 1 μM neurotoxin was required to achieve an 80% reduction in transmitter release.

No high affinity acceptors on the PC12 cells were apparent even after prolonged treatment with nerve growth factor. This apparent lack of acceptors is reflected in the relatively high concentration of neurotoxin required to produce blockade of transmitter release.

Neither the H_2L fragment nor heavy chain had any effect on the release of noradrenalin from PC12 cells suggesting that both the light chain and H_1 portion of the heavy chain play a role in the intracellular action of the toxin.

Previous studies suggest that the H_1 fragment of type A neurotoxin is involved in acceptor binding. In view of the apparent lack of external acceptors on PC12 cells a secondary role must be predicted for this fragment. One possibility is that H1 fragment directs the neurotoxin into the relevent internal cellular compartment. A direct action of the H_1 fragment on the calcium-dependent release mechanism is also a possibility.

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Inhibition by Tetanus Toxin of Dopamine Release Induced by the Presynaptic Neurotoxin, Alpha-Latrotoxin, in NGF-Differentiated PC-12 Cells

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INTRODUCTION

α-latrotoxin (αLTx) from black widow spider venom is a presynaptic neurotoxin which stimulates neurotransmitter release via both a Ca2+ dependent and a Ca2+ independent mechanism (1). Tetanus toxin (TT), the neurotoxin from Clostridium tetani has the opposite effect as it inhibits neurotransmitter release. We have studied the effect of TT on αLTx induced dopamine release in an NGF-differentiated cell line highly sensitive to TT (2).

METHODS

Dopamine release measurements were made in Richelson's medium on adherent PC-12 cells which had been differentiated for 8-11 days with NGF. Tetanus toxin was added directly to the growth medium and was removed before cells were labelled for 2 hours with 3H dopamine.

RESULTS

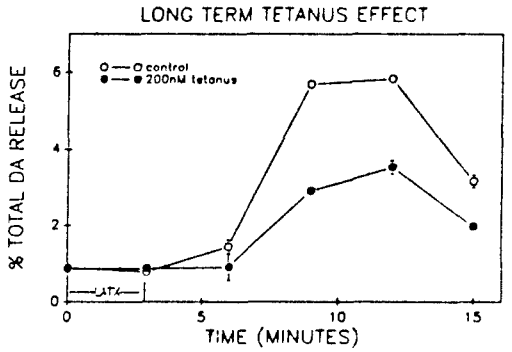


Figure 1.

Preincubation of NGF differentiated PC-12 cells with 200 nM tetanus toxin for 4 hours caused significant inhibition of αLTx (0.1 nM) induced dopamine release (Fig.1). Tetanus toxin appears to decrease the total amount of dopamine released without affecting the overall kinetics of release.

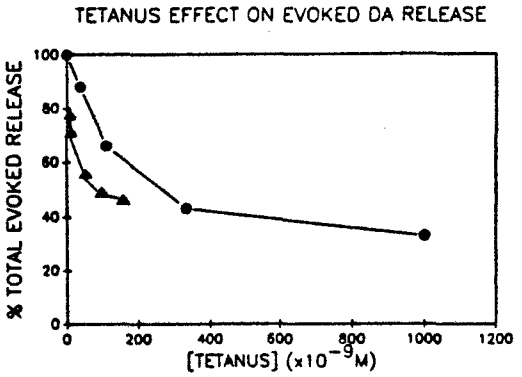


Figure 2.

The inhibition by TT is already clearly visible at 10 nM TT (30% inhibition) after an overnight incubation, and is both time and dose dependent (Fig 2). However there is a considerable fraction of αLTx-stimulated release which remains apparently insensitive to TT's inhibitory effect (approx. 40% at 0.2 nM αLTx).

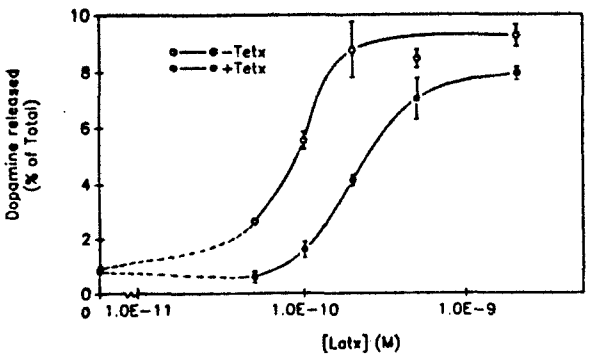


Figure 3.

An overnight incubation with 150 nM TT causes a clear shift in the αLTx dose-response curve (Fig 3). In addition, at concentrations of αLTx above 1 nM the fraction of release inhibited by TT becomes fairly small and explains why in these conditions αLTx would indeed appear to override tetanus toxin inhibition, as has been described previously(3,4).

Conclusion: αLTx-stimulated dopamine release has at least two components, one which is TT sensitive and the other which is TT insensitive.By determining which pathway (Ca2+ dependent or independent) is inhibited we can learn more about the site of action of TT and the multiple intracellular pathways leading to neurosecretion.

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Inhibition of Hormone Release from Isolated Nerve Endings of the Posterior Pituitary by Tetanus Toxin

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INTRODUCTION

Tetanus toxin is a potent neurotoxin produced by *Clostridium tetani*. Tetanus toxin intoxicates cells by inhibiting the release of neurotransmitter. The molecular basis of intoxication by tetanus toxin is poorly understood. The neural lobe of the pituitary secretes two neuropeptide hormones, oxytocin, and vasopressin. Recently, Nordmann and coworkers demonstrated that isolated nerve endings (neurosecretosomes) prepared from the neural lobe of male rat pituitaries retain the ability to secrete vasopressin and oxytocin in a calcium-dependent fashion in response to various stimuli. We have examined the effect of tetanus toxin on hormone release from neurosecretosomes (NSS). The data presented here demonstrate that in NSS, tetanus toxin is a potent inhibitor of hormone release.

MATERIALS AND METHODS

Neurointermediate lobes were dissected from pituitaries of male rats, and rinsed with modified Ringer's solution. Neurosecretosomes were prepared according to the method of Cazalis et al., and incubated 18-24 hours in Ringer's solution in the presence or absence of tetanus toxin. This preparation consists mostly of purified nerve endings (neurosecretosomes). The ability of the NSS to release vasopressin (VP) or oxytocin (OT) in response to various stimuli was measured in a perfusion experiment. Briefly, the NSS were loaded onto a 0.45 µm pore, 13 mm diameter Millex-HV filter, and

perfused with Na-free (Na replaced with N-methyl-D-glucamine) Ringer's solution at a flow rate of 0.1 ml/min. After a 40 minute wash period, the perfusate was collected in 10 minute time intervals. The NSS were stimulated with Ringer's solution containing 30 mM K^+ for 10 minutes. The perfusate was assayed for OT or VP by radioimmunoassay. The amount of hormone released during the stimulation is expressed as the percent of total hormone present in the NSS at the beginning of the experiment.

RESULTS AND DISCUSSION

When NSS were incubated with tetanus toxin overnight, depolarization-evoked release of oxytocin and vasopressin was inhibited. The effect of tetanus toxin was dose-dependent. Approximately 10% inhibition was seen with 0.01 µg/ml, and maximal inhibition was reached at ~1 µg/ml. Tetanus toxin which was pre-incubated with a neutralizing monoclonal antibody or heated to 100 °C had no effect on hormone release.

The inhibition of hormone release by tetanus toxin may be related to a inhibition of the rise of intracellular calcium in response to depolarizing stimuli. The rise in intracellular calcium is essential for exocytosis. To test this, two ionophores which are known to increase intracellular Ca^{++} were examined for their effect on NSS that had been treated with 5 µg/ml tetanus toxin for 18 hours. A23187 and ionomycin (10 µM each) were more effective secretagogues in control NSS than 45 mM K^+, but were not able to stimulate secretion in NSS that had been treated with 2 µg/ml tetanus toxin. Tetanus toxin may be preventing the ionophore induced rise in Ca^{++}, or the increase in Ca^{++} due to the ionophores may not be sufficient to overcome the block caused by tetanus toxin. Further experiments are being conducted to address this question.

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The Interaction of Tetanus Toxin with Intact Chromaffin Cells

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INTRODUCTION

Tetanus toxin is an inhibitor of the release of catecholamines evoked by nicotine from bovine adrenal chromaffin cells (which develop from the neuronal crest) (1,2). Although other authors (e.g. 2,3) have reported that the toxin is inactive with intact cells, and has to be introduced into them by microinjection or by permeabilizing the cells, we find that this is not necessary.

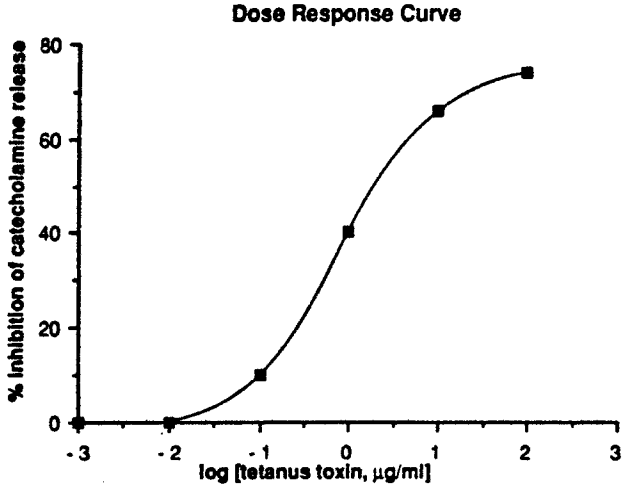
MATERIALS AND METHODS

Toxin was incubated overnight with primary cultures of bovine adrenal chromaffin cells (grown for between 4 and 7 days in Dulbecco's modified Eagle's medium); the cells were then washed once with Locke's solution (154 mM NaCl, 5.6 mM KCl, 5.6 mM glucose, 5 mM HEPES, 2.2 mM $CaCl_2$, 12 mM $MgSO_4$), pH 7.6, stimulated with 10 μM nicotine for 10 min at room temperature, and lysed with 1% Triton. Released endogenous catecholamines were measured fluorimetrically (4).

RESULTS AND DISCUSSION

The figure shows the inhibition by un-nicked tetanus toxin of the release of catecholamines from intact adrenal cells; 50% of maximum inhibition was obtained at about 1 μg/ml (7 nM). Results were essentially the same with intact or "nicked" (two chains) toxin, perhaps because of proteolytic activity in the toxin preparation or in the cell culture. The maximum inhibition ever obtained was about 70% (with 70 nM toxin), possibly because some secretory granules are already too close to the cell membrane for their exocytosis to be affected by toxin. The time course of this inhibition is relatively

slow: very little effect was seen after incubation with toxin for 10 h or less. Preincubation with ganglioside GT1 did not consistently increase the inhibition: indeed, ganglioside alone at <10 μg/ml significantly inhibited nicotine-evoked exocytosis.



We (and others, e.g. 2) have not been able to show any high-affinity binding of [^{125}I]-labelled toxin to these cells. However the toxin concentrations that are active are comparable to those found effective in intact PC12 cells (5) and considerably lower than is required with permeabilized adrenal cells (our published results and 3,6).

These results do not agree with several other authors who find the toxin to be ineffective with intact cells. We have found inhibition in many experiments over two years, but not with every cell preparation that we try. It may be that some as yet undiscovered property of the cells can affect the response (cf PC12 cells which are responsive to toxin only when grown for some time in the presence of NGF, 6). A possibly important difference in our methods is that we measure directly the release of endogenous catacholamines, where others measure the release of [^{3}H]-labelled catecholamine with which the cells have been prelabelled.

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Botulinum ADP-Ribosyltransferase C3 Modifies the GTP-Binding Protein rho

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In addition to botulinum C2 toxin which ADP-ribosylates actin, certain strains of Clostridium botulinum type C and D produce the ADP-ribosyltransferase C3 (3). C3 has a molecular weight of about 25 kDa and is functionally and structurally distinct from botulinum neurotoxins (1-4, 6). C3 ADP-ribosylates 21-24 kDa GTP-binding proteins in all tissues studied so far. Here we report on the purification of the protein substrate of C3 and on the biological activity of C3 transferase.

The substrate of C3 was purified from the porcine brain cytosol by acetone precipitation, CM-Sephadex, octyl-Sepharose and TSK Phenyl-5PW HPLC chromatography to apparent homogeneity. Purification was about 4000-fold with a recovery of about 2.3 %. About 0.6 mol ADP-ribose/mol purified protein was incorporated by C3. The purified C3 substrate binds GTP and possesses GTPase activity. Amino acid sequence determined from two tryptic peptides of the ADP-ribosylated substrate covered corresponding sequences of the human rho protein. Furthermore, rho A protein expressed in E. coli was ADP-ribosylated by C3. Antibodies raised against the purified C3 substrate from porcine brain cytosol recognized rho protein expressed in E. coli but no other GTP-binding proteins. All these findings indicate that the rho protein is a substrate of C3.

ADP-ribosylation by C3 neither influenced guanine nucleotide binding nor GTP hydrolysis by purified C3 substrate or E. coli expressed rho. Recently, it has been shown that rho associated GTPase activity is severalfold increased by a specific rho

GTPase-stimulating protein (rho-GAP) (5). Therefore, the influence of ADP-ribosylation was studied on rho GTPase in the presence of GAP from human spleen. However, GAP stimulated GTP hydrolysis of control and ADP-ribosylated rho to the same extent.

In order to get more insight into the pharmacological actions of C3, the ADP-ribosyltransferase was microinjected into Xenopus oocytes. Xenopus oocytes are arrested in the prophase of meiosis and can be triggered to undergo meiosis by treatment with progesterone. Injection of C3 induced the migration and the breakdown of the germinal vesical of Xenopus oocytes. Progesteron-induced maturation was largely accelerated by C3. These effects were apparently caused by ADP-ribosylation since coinjection of [^{32}P]NAD labelled an about 22 kDa protein.

Microinjection of C3 into Swiss 3T3 cells resulted in dramatic changes in the morphology of cells. C3 induced rounding up of cells in a dose dependent manner. This effect was not due to a possible contamination with the cytotoxic botulinum C2 toxin. Antibodies directed against C2 toxin blocked the cytotoxic effects of microinjected C2. In contrast, the C3-induced morphological changes were not affected.

The data available indicate that C3 modifies the GTP-binding ras-related protein, rho, and suggest that rho is involved in the regulation of the cytoskeleton.

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The Reversal of Actin ADP-Ribosylation

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Various clostridial toxins possess ADP-ribosyltransferase activity. Among these toxins are Clostridium perfringens iota and Clostridium botulinum C2 toxin. Both toxins belong to a new class of actin-ADP-ribosylating toxins (1,3). Iota toxin and C2 toxin are binary in structure and consist of two non-linked protein components. Component-I possesses ADP-ribosyltransferase activity, component-II is responsible for binding of the toxin to the eukaryotic cell surface. In order to elicit the toxic effects on intact cells both components have to act together. The substrate of iota and C2 toxin is monomeric G-actin, not polymerized F-actin. The toxins differ in their substrate specifity: Whereas C2 toxin ADP-ribosylates only the non-muscle isoforms of actin, iota toxin modifies skeletal and non-muscle actin. Interestingly, both toxins ADP-ribosylate actin in Arg-177 (4).

The ADP-ribosylation of actin causes functional effects: The modified actin loses its ability to polymerize. Furthermore, the ADP-ribosylated actin inhibits the fast polymerization at the barbed end of actin filaments like a capping protein (5). Recently, it has been shown that the ADP-ribosylation of skeletal and non-muscle actin inhibits the actin ATPase (2). Inhibition of ATP hydrolysis correlates with the toxin-induced ADP-ribosylation of actin. The reduction of ATPase activity is not simply due to the inhibition of actin polymerization: First, impairment of ATP hydrolysis was observed at concentrations below the critical actin concentration. Second, ADP-ribosylation blocked ATP hydrolysis of DNAse-I-bound actin. This complex is incapable of polymerization. Third, ATPase activity stimulated by cytochalasins was inhibited by ATPase. ADP-ribosylation did not affect the binding of cytochalasins to actin. All these findings strongly indicate that ADP-ribosylation inhibits the ATPase of monomeric actin. Under certain conditions cholera toxin and pertussis toxin catalyze the reverse reaction of ADP-ribosylation. Therefore,

we studied the reversal of actin ADP-ribosylation by clostridial toxins. In the presence of a high concentration of nicotinamide and at low pH the clostridial toxins caused the reversal of ADP-ribosylation and formation of NAD. The reverse reaction was more effective after removing of NAD by NADase treatment or by gel filtration. Under optimal conditions (pH 6.5; 30 mM nicotinamide) incorporated ADP-ribose was cleaved by about 70% . The reversal of actin ADP-ribosylation was specific, since the cleavage of the ADP-ribose-actin bound was inhibited in the presence of toxin antibodies. Exclusively NAD but no other nucleotides were formed. The reverse reaction was accompanied by functional reconstitution of actin ATPase activity. The decrease of ATP-ribose attached to actin is correlated with an increase in ATP hydrolysis.
The actin isoform specifity of the C2 toxin induced by ADP-ribosylation was also valid for its reverse reaction: ADP-ribosylation of skeletal muscle actin by iota toxin was not reversed by C2 toxin. In contrast, non-muscle actin ADP-ribosylated by iota toxin or C2 toxin was cleaved by either toxins.

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Characterization of Actin ADP-Ribosylating Toxins in Clostridia

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Clostridia produce numerous protein toxins. Up to now, the molecular basis of their mechanism of action are only known for few of them. ADP ribosylation seems a common molecular mechanism for many Clostridia toxins. The substrates of Clostridia ADP ribosylating toxins are either G actin which is an ATP binding protein, or a p21 GTP binding protein (rho) which is the substrate of botulinum C3 ADP ribosyl transferase (2).

The first ADP ribosylating toxin described was botulinum C2 toxin. This toxin is produced by certain strains of C. botulinum C and D, it is not a neurotoxin but instead increases intestinal secretion, vascular permeability and causes hypotensive effects. It was shown that non-muscle G-actin is the substrate for ADP ribosylation by botulinum C2 toxin (1). ADP ribosylated actin is unable to polymerize, and the dynamic equilibrium between G and F actin in the cell is disturbed. Like other ADP ribosylating toxins, botulinum C2 toxin is a binary toxin consisting of two components C2-I and C2-II with molecular weights of 45 and 80 kDa respectively, which are neither linked by covalent nor disulfide bonds.

Recently, it has been demonstrated that the iota toxin secreted by C. perfringens E has a structure and activity similar to C. botulinum C2 toxin (5,7). We have now shown that several C. spiroforme strains produce also a toxin closely related to C. perfringens E iota toxin. Iota and C. spiroforme toxins consist of a light chain {ia and sa (MW 44 kDa)} and a heavy chain {ib (MW 67 kDa) and sb (MW 76 kDa)} respectively. The light chain ADP ribosylates non muscle G-actin and the heavy chain which is active after partial proteolysis, allows the light chain to penetrate and intoxicate the target cells (3,6). Moreover, we have found that one C. difficile strain (CD 196) exhibits an ADP ribosyl transferase activity (CDT) analogous to the light chains of iota and C. spiroforme toxin (4). But no heavy component has been found in this particular strain.

Using 12% PAGE with 0.1% SDS and Western blotting, Clostridia ADP ribosylating toxins might be classified in two groups : the first one consists of botulinum C2 toxin while the second encompasses iota, C. spiroforme toxin and CDT (and called C2-like toxins).

Antibodies against botulinum C2-I only recognize botulinum C2-I, antibodies against ia or sa however indistinguishly recognize ia, sa and CDT but not botulinum C2-I. Accordingly immunological cross reactions between ib and sb are observed, but antibodies against botulinum C2-II only react with botulinum C2-II. Moreover, we observed a cross complementation between the light and heavy chains for biological activities (cytotoxicity on Vero cells, mouse lethality) within the members of C2-like toxins ; ia, sa and CDT can interact with ib or sb to produce biological activities, but botulinum C2-I only interact with C2-II.

We have studied the cell entry of C. spiroforme toxin in Hep 2 cells. Ammonium chloride (10 to 30 mM) and low temperature (15°C) do not block C. spiroforme toxin activity but potassium depletion which blocks coated pit formation prevents the cytotoxicity induced by C. spiroforme toxin. From these data, we suggest that the C. spiroforme binary toxin might enter the cytosol by the coated pit coated vesicles pathway. Since this toxin does not require an acidic compartment as shown by the lack of activity of ammonium chloride, it might gain entry into the cytosol before or during transfer to early endosomes. The absence of temperature block, suggests that routing of the toxin to a more central cell compartment (i.e.: golgi, ER, lysosomes) is not required for toxin entry. Therefore C. spiroforme toxin may be transferred into the cytosol in the vicinity of the cell periphery where G actin is mostly localized (especially in intestinal brush border cells) and therefore could be in close proximity of its target molecule.

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Mechanisms Underlying the Cytoskeletal Changes Induced by Clostridium difficile Toxins

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INTRODUCTION

Clostridium difficile produces at least two different protein toxins, named toxins A and B, implicated in the pathogenesis of the pseudomembranous colitis (1). Toxin A elicits severe epithelial damages when injected into rabbit ileal loops, while toxin B appears to be devoid of enterotoxic activity (1). Both toxins induce, in different degrees, cytophatogenic effects in tissue cultured cells (2). Results obtained by a comparative study on the effects of these toxins on different cultured cell lines are here reported.

MATERIALS AND METHODS

Toxins were purified as previously described (3). Different epithelial cell lines (HeLa, HEp2 and CG5), were treated with different doses of toxin A and toxin B. In consideration of the results obtained only doses of 4 ug/ml of toxin A and 0.15 ug/ml of toxin B are here reported. For fluorescence microscopy and scanning electron microscopy (SEM) samples were processed as previously described (2). Briefly, cells were fixed with paraformaldehyde and permeated with Triton X-100 for fluorescence studies on cytoskeleton and fixed with glutaraldehyde, critical-point-dried with CO_2 and gold coated by sputtering for cell surface studies by SEM.

RESULTS

Exposure of cell monolayers to 0.15 ug/ml toxin B induced cell retraction, cell rounding and the formation of bulb-like structures on the cell surface. The bleb matrix appeared to be filled with ribosomes and devoid of other cell organelles; F-actin was completely absent as well as intermediate filament types keratin and vimentin; the actin-bin-

ding proteins, alpha-actinin and filamin, and tubulin were revealed by immunocytochemical staining. Human cell lines exhibited a different sensitivity undergoing multiple surface protrusions in different extent (TABLE I). Toxin A treatment (4 ug/ml) of cultured epithelial cells induced a characteristic series of morphological changes mainly represented by cell rounding and subsequent nuclear displacement. Cytochalasin B and phalloidin were ineffective in protecting the cells against toxin while demecolcin completely inhibited the nuclear polarization (Table II). The microfilaments and the intermediate filament type vimentin were localized in the Golgi area near the nucleus whereas tubulin was diffused in the cytoplasm and still organized in a thin meshwork around the nucleus.

TABLE I

	(a)	(b)
HeLa	100%	5%
HEp2	100%	30%
CG5	100%	35%

TABLE II

	(a)	(b)
Cytochalasin B	100%	100%
Phalloidin	100%	100%
Demecolcin	100%	5%

TABLE I. Percentage of roundish (a) and blebbing cells (b) in different cell lines after toxin B treatment (0.15 ug/ml).
TABLE II. Percentage of cells with roundish shape (a) or nuclear displacement (b) after toxin A treatment (4 ug/ml).

DISCUSSION

Both toxins caused cell retraction and rounding in all cell types considered and seemed to exert their effect on cell morphology via a rearrangement of some cytoskeletal components. However, differences have been observed among the various cell lines after exposure to toxin B. This could also depend on different adhesion pattern and on intercellular relationships as well as on differences in cytoskeletal organization. Furthermore, the toxin B-induced blebbing and the toxin A-induced nuclear polarization seem to depend on different cytoskeletal elements (2). In particular, toxin B probably exerts its cytotoxic action by an impairment of the relationships between actin and actin-binding proteins while the displacement of the nucleus caused by toxin A appears to require the microtubular apparatus integrity and function. Hence, a different mechanism could be considered for C. difficile toxins playing a role in the cytopathogenesis of the disease.

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Histidine-21 is at the NAD Binding Site of Diphtheria Toxin

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SUMMARY

Fragment A of diphtheria toxin (DT-A) was treated with the histidine reagent diethylpyrocarbonate (DEPC) (Dominici et al., 1985). This treatment caused specific modification of His-21, the single histidine residue present in DT-A, without modification of other residues. Parallelv to DEPC modification, DT-A loses its NAD-glycohydrolase and ADP-ribosyltransferase activities. NAD^+ and adenine are equally effective in protecting DT-A from DEPC modification, while adenine is uneffective. The pH-dependence of DEPC modification and of NAD^+ binding indicate that the pKa of histidine 21 of DT-A is around 6.3.

Key words: diphtheria toxin; NAD^+ binding; histidine; pH dependence

INTRODUCTION

Diphtheria toxin (DT), the clinical agent of diphtheria, is produced by pathogenic strains of *Corynebacterium diphtheriae* as a single chain of 58 kDa (Pappenheimer, 1977). Proteases cleave DT at a single site yielding two chains: A (21,164 Da) and B (37,194 Da). Fragment A is an enzyme that ADP-ribosylases specifically EF-2 with a consequent block of cellular protein synthesis (Pappenheimeir, 1977). DT-A also possesses a NAD^+-glycohydrolase activity and binds NAD^+ at a single site with high affinity (Kandel et al., 1974). There is evidence that Lys-39 and Glu-148 interact directly with NAD (Carrol & Collier, 1984; Zhao & London, 1988).

Here we present evidence that His-21 is at the NAD binding site of DT and that its pKa value is around 6.3.

RESULTS AND DISCUSSION

Treatment of DT-A with 80 µMolar DEPC at 25°C leads to the modification of around 75 % of the histidine of DT-A within 10 min, as determined by following the increase of absorbance at 243 nm, which is diagnostic of the formation of N-carbethoxyhistidine. DEPC is rapidly hydrolysed in water and a complete modification of DT-A is attained after a further addition of 80 µMolar reagent. When 1 histidine residue per DT-A is modified, no other residue appear to have been modified by the DEPC treatment.

Parallelv to histidine modification, DT-A loses both its NAD-glycohydrolase and ADP-ribosyltransferase activities. Since His-21 is the sole histidine present in DT-A, these results indicate that His-21 is important for the activity of DT-A.

To try to localize this residue with respect to the functional sites of DT, we performed a series of protection experiments, where DT-A was treated with DEPC in the presence of NAD^+ and of its various moieties known to be competitive inhibitors of DT-A activity and NAD^+ binding. We found that, while adenosine was as effective as NAD^+ in protecting DT-A from histidine modification, adenine was without effect.

This result, together with an inspection of the available 3D structure of the closely related exotoxin A secreted by *P. aeruginosa* exotoxin A, indicate that the ribose group of adenosine and of NAD^+ is involved in an interaction with the imidazole ring of histidine-21.

By performing DEPC modification at different pHs and by plotting the corrected rates of modification at the various pHs versus pH, it was found that His-21 has a pKa around 6.3. The same result was found by applying the analysis of Dixon (1953) at the binding of NAD^+. This analysis also showed that protonation of His-21 strongly affects NAD^+ binding whose Kd increases upon protonation from 14 μM to 125 μM and that, conversely, NAD^+ binding shifts the pKa from 6.3 to 5.4.

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The Mechanism of Action of the Osteolytic Toxin of Pasteurella multocida

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INTRODUCTION

Atrophic rhinitis of growing pigs is a disease where the nasal turbinate bones can be completely destroyed, the snout twisted or shortened and animal weight gains reduced (2). Toxigenic isolates of P.multocida isolated from cases were identified by the toxicity of crude extracts for mice, cultured embryonic bovine lung (EBL) cells and pigs where sublethal injections reproduced the disease. The toxin was purified to characterise its mode of action (1).

MATERIALS AND METHODS

Confluent monolayers of EBL cells (3) in dishes 2.5cm dia. were pre-labelled with ^{14}C thymidine and 150 ng of purified toxin (1) was added. At time 0 and 2, 4 and 6 hours after the addition of toxin ^{3}H labelled leucine, thymidine or uridine was added for 30 minutes: cycloheximide, mitomycin C and actinomycin D were used as positive controls of inhibition of metabolism. Trichloracetic acid insoluble ^{3}H labelled protein or nucleic acid was measured and normalised for ^{14}C content. Intracellular ATP was measured by luminometry using firefly luciferase (Sigma). cAMP was measured in cells treated with toxin for 3 hours (Amersham). Toxin treated cells which had begun to separate were examined for ultrastructural changes by transmission electron microscopy. The minimum toxic period of exposure was determined using washoff experiments. Cells treated with toxin for 24 hours were stained with trypan blue or neutral red.

RESULTS

Two hours after addition of toxin, cells had started to separate and continued to separate until 6 hours after addition of toxin. Differences were not detected between controls and toxin treated cells for uptake of ^{3}H uridine or thymidine and differences were not detected in ATP content. Two hours after exposure to toxin, uptake of ^{3}H leucine was reduced in toxin treated cells which continued to decline until 6 hours after addition of toxin when it was 50% of the control cells. The cycloheximide control, however, ceased to incorporate leucine immediately but changes in the cells were not detected until 2 hours after addition of the drug. Differences in cAMP content or ultrastructure were not detected between controls and toxin treated cells. Cells stained with neutral red but not trypan blue 24 hours after treatment with toxin. Toxin reacted with cells within 5 minutes and could not be washed off.

DISCUSSION

The toxin did not cause changes in nucleic acid turnover or intracellular concentrations of ATP or cAMP. The reduction in protein synthesis was probably a secondary effect because cells treated with cycloheximide did not change shape until 2 hours after protein synthesis had ceased. Changes in ultrastructure were not seen at the time cells had started to change shape and cells which had changed shape many hours previously did not have a damaged cell membrane detected by staining.

These results indicate that in EBL cells the toxin has a mechanism of action unlike that of many other bacterial toxins in other cell systems because it did not involve shutdown of protein turnover, cAMP metabolism or membrane damage.

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Bacillus cereus Enterotoxin: On the Mechanism of Action

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INTRODUCTION

Bacillus cereus diarrhoeal type food-poisoning is caused by an enterotoxic component(s) that is secreted during vegetative growth, mainly at late logarithmic phase. It has for some time been speculated that the toxin involved is a multi protein component. Thompson et al (4) reported three different antigens with molecular weights of 43,000, 39,500 and 38,000 that all could be involved in the biological activity during B. cereus food-poisoning. Molecular weights ranging from 38,000 to 57,000 have been reported for the enterotoxin(s) (2). The symptoms of the B. cereus food-poisoning are almost indistinguishable from the Clostridium perfringens food-poisoning, and it would therefore be of interest to see if differences in the mode of action could be detected.

In this study we report on the number of antigens involved, their relation, and on some differences between B. cereus and C. perfringens enterotoxin mode of action.

MATERIALS AND METHODS

A strain of B. cereus isolated from an "Oriental stew", involved in food-poisoning in Norway during the autumn 1988, was used throughout this investigation. This strain produces enterotoxin in very high amounts. As reference strain B. cereus F 4433-73 from Central Public Health Laboratories, London, UK, was used. Antiserum produced against enterotoxin (purified by isoelectrofocusing) from the same strain, was a gift from J. Kramer, Central Public Health Laboratories, London, UK.

SDS polyacrylamide gel electrophoresis and Western immunoblots were carried out as described before (5). Crossed immunoelectrophoresis was done according to Hagen et al (1), using a Pharmacia Phast Gel System . All experiments were carried out using a 70% ammonium sulfate precipitate of the crude cell-extract after the B. cereus strain was grown on BHI with additional 10 g/l of glucose, at 30° C for 6 hours. The toxin extract was dissolved in 20 mM phosphate buffer pH 7.2 after dialysis (20 x concentrated). The anti-enterotoxin neutralized all biological activities in this crude extract, on the Vero cells toxicity test. Toxicity was determined under various conditions using a Vero cell assay according to Sandvik and Olsnes (3).

RESULTS AND DISCUSSION

Three distinct different antigens were found when the crude cell-extract was used in crossed immunoelectrophoresis, against the anti-enterotoxin (results not shown). No cross-reaction could be detected. This is partly in agreement with Thompson et al (4), who also found three different proteins, but reported that antibodies raised against either of them could neutralize the other two.

We believe that their results must be due to impurities from the other two proteins during immunization. The molecular weights of the three proteins in the crude extract, as judged from the immunoblots were about 40,000, 50,000 and 58,000 respectively. It is therefore possible that Thomson et al (4), have worked with processed proteins after proteolysis. This may also be the reason for the lack of activity on two of the proteins when tested alone, although it is possible that all three proteins are subunits in one enterotoxin complex.

Figure 1 shows the influence of trypsin and chymotrypsin digestion (1 mg/ml, pH 7.2 at 37° C) on the biological activity of the enterotoxin(s). After 60 minutes of proteolysis, the toxic proteins are still visible on the immunoblots (data not shown), and the biological activity is also present, although reduced to about 6-10% of the initial activity. This is in contrast to earlier conclusions that preformed enterotoxin could not result in food-poisoning as the protein was rapidly degraded by proteolytic enzymes. This may well be the case with purified enterotoxin, but obviously not when tested in a more "natural" environment.

Figure 1

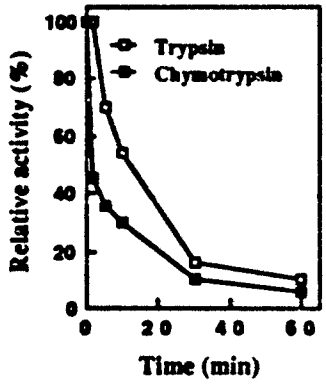
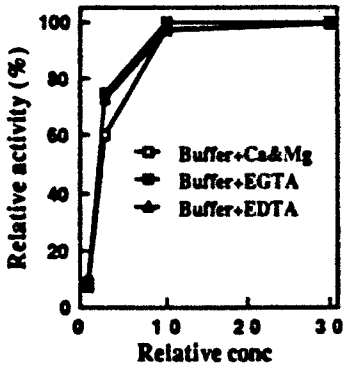


Figure 2



Since it is known that the C. perfringens enterotoxin depends on calcium to interfere with the cell-membranes, we wanted to test if this was true for the B. cereus enterotoxin(s). As shown on Figure 2 (The activity was tested in 20 mM Hepes buffer containing 0.14 M NaCl, calcium and magnesium concentration was 0.1 mM when present), it is clear that neither calcium nor magnesium is essential for toxin activity. Not only was the protein synthesis inhibited just as well without cations, but the Vero cells were also killed at about the same rate with and without the cations, as judged from uptake of tryphan blue, by the Vero cells (data not shown).

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Activation of Guanylate Cyclase in T84 Human Colonic Cell Line by Heat-Stable Enterotoxins

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INTRODUCTION

The heat stable toxins (ST) are a family of small peptides produced by pathogenic organisms such as E.coli, Non-agglutinating (NAG) Vibrios and Yersinia enterocolitica. Their elaboration in the human intestine is a major cause of diarrhoea . T84 cells were chosen to study the activation of particulate guanylate cyclase by a few bacterial toxins to understand their mechanism of action. Activation of guanylate cyclase by ST in T84 cells has been reported previously (3).

MATERIALS AND METHODS

T84 cells were cultured in a 1:1 mixture of F12:DMEM containing 5% foetal calf serum. ST_h was purified from a strain overproducing ST_h (1). ST_p and NAG ST were prepared synthetically and ST from Yersinia purified by a modification of the procedure used for ST . Toxins were added in serum free media, and cGMP produced was monitored by radioimmunoassay.

RESULTS AND DISCUSSION

Low amounts of ST_h activated guanylate cyclase rapidly and specifically in T84 cells (Fig 1,2) whereas cholera and pertussis toxins did not show similar activation (data not shown).

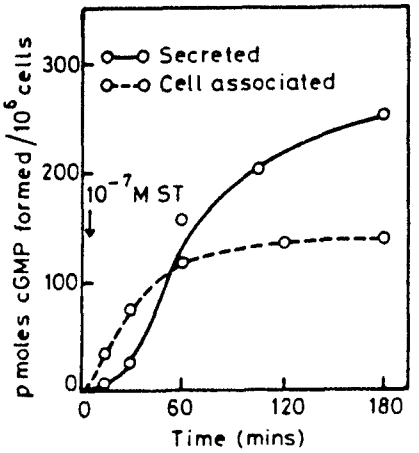


Fig.1. Time course of response of ST_h

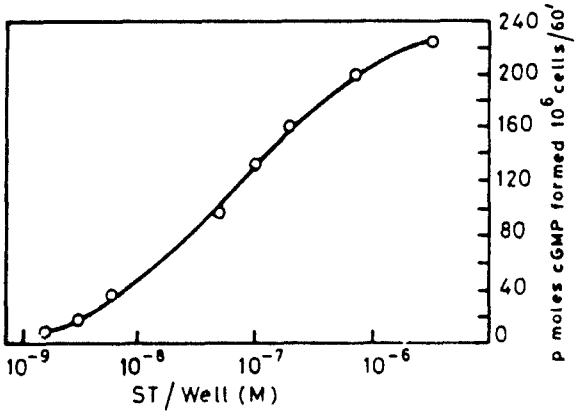


Fig.2.Dose response to ST_h

Incubation with an antiserum to ST_h did not significantly decrease cGMP production, but a C-terminal specific monoclonal antibody which neutralises ST_h activity in the suckling mouse (2) inhibited cGMP production (Table 1). Sulfhydryl or disulfide containing compounds inhibited cGMP production (Table 2).

Table 1

Effect of antibodies on activity of ST_h

	Intestine: cGMP produced/ body wt	10 cells/h(pmol)
Polyclonal antibody	0.11	85
Monoclonal antibody	0.077	25
Control serum	0.11	100

a)Ratio of 0.083 (one mouse unit)is considered positive in the bioassay.
b)Basal levels of cGMP are 10-15 pmol.

Table 2.

Effect of Disulfide and Thiol compounds on ST_h mediated guanylate cyclase activation

Compound	pmol cGMP produced 10 cells/h	Inhibition %
Nil	102.1	
Cystamine	30.1	71
Cystine	80.8	21
L-Cysteine	47.1	54

Values represent the mean of two assays

ST_p, NAG ST and Yersinia ST activated guanylate cyclase, but to various extents suggesting different affinities and interactions of the toxins with the receptor/cyclase (Fig.3). These results suggest that cGMP production may be the common mode of action of all ST's and activation of guanylate cyclase in T84 cells is a sensitive assay which could serve as an alternative to the suckling mouse bioassay for detection of toxigenic peptides of the ST family.

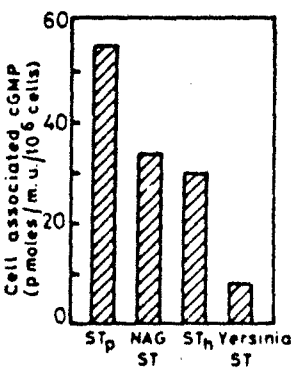


Fig.3.Activation of guanylate cyclase by ST peptides.

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In vivo Effect of Staphylococcal Enterotoxin A on Natural Killer Cells

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Keywords: Staphylococcal enterotoxin A (SEA), Natural Killer Cells (NK), Interleukin-2 (IL-2).

Staphylococcal enterotoxins, apart from being highly potent toxins causing food poisoning in humans, are also very efficient immunomodulators. This family of structurally related proteins induce in-vitro a whole series of lymphokines (1), suppressor cells (2), cytotoxic cells (4), and natural killer cells (3).

In this report, we present studies describing the enhanced cytotoxic activity of natural killer cells (NK) isolated from peripheral blood lymphocytes (PBL) of baboons, following experimental SEA toxemia. The cytotoxic activity of NK cells, isolated 24h after SEA challenge (0.5 µg SEA per kg monkey i.v.), increased significantly (Fig. 1). Peak NK activity was observed 4 days after SEA administration, followed by a decline in activity, approaching normal values, one to three weeks after the onset of toxemia. The enhancement of NK activity in PBL, isolated from SEA challenged monkeys, was essentially the same as that found in PBL stimulated by this toxin in-vitro (compared data in Fig. 1 to Ref. 3).

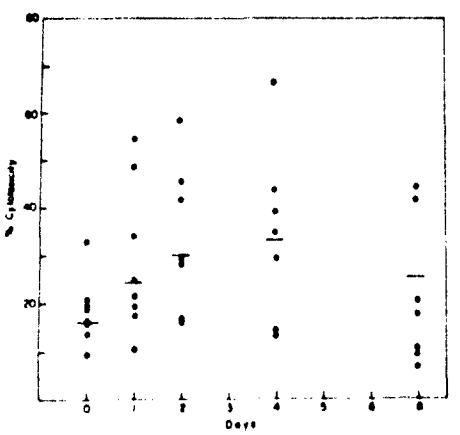


Fig. 1. The NK activity of cells from monkeys challenged with SEA. PPL (10^6 cells/ml), isolated from control and SEA challenged monkeys were tested for NK activity against K-562 target cells, at the indicated times, following SEA exposure. Effector target ratio was 20:1. Each dot represents NK activity of an individual monkey's PBL at an indicated time. Lines represent mean NK activity of all the monkeys at a given time.

The main difference between the in-vivo and in-vitro SEA induced NK activity was in the kinetics of induction when the in-vitro peak activities were reached a few hours after SEA exposure and the in-vivo NK peak activities were reached only after 2-4 days.
The induction of IL-2 synthesis in lymphocytes of SEA exposed monkeys, as a prerequesit of NK cell activation, was studied as well. Serum from monkeys withdrawn before and up to 24 hours after SEA administration, consistently contained minute amounts of IL-2 (0.1-0.5u IL-2 per ml. serum). In two monkeys, levels of 5 u IL-2 per ml serum were detected at 7 h after challenge. 2-7 days after SEA exposure, these small amounts of IL-2 were completely depleted from the serum, followed by a raise in IL-2 to initial levels, 2-3 weeks thereafter. No measurable amounts of IL-2 were secreted in-vitro by either control PBL or PBL isolated from SEA challenged monkeys (tested at time intervals 7 h to 96 h past SEA administration). However, PBL from monkeys 2-7 days after SEA challenge, aquired the capacity to absorb IL-2 from incubation medium, indicating SEA dependent induction of IL-2 receptors on part of the lymphocytes' population (Fig. 2).

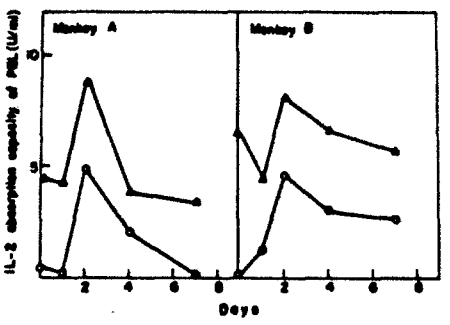


Fig. 2. The capacity of IL-2 absorbtion of PBL isolated from two monkeys at various times after SEA challenge. PBL isolated from SEA challenged monkeys were incubated ($2x10^6$ cells/ml) in triplicates with 5(O) or 10(A) units IL-2 per ml. Following a 24 h incubation, the cells were removed from the incubated suspensions and IL-2 was determined in the media.

The fact that no IL-2 secretion from lymphocytes was observed after SEA challenge may be the consequence of its consumption by the SEA activated cells. It is not clear from these studies whether during SEA toxemia, the enhanced NK activity is directly stimulated by SEA, or it is mediated by lymphokines (IL-2) induced by this toxin.

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Mode of Action of Bacillus thuringiensis Delta-Endotoxins

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INTRODUCTION.

Bacillus thuringiensis is commonly used as a microbial insecticide. Upon sporulation different strains of Bacillus thuringiensis produce crystalline inclusions consisting of insecticidal crystal proteins or delta-endotoxins. The protoxins are solubilized in the insect midgut at alkaline pH and proteolytically activated to active toxins of a smaller molecular weight. The toxins bind to receptor sites in the brush border membrane of midgut epithelial cells and disrupt the integrity of this membrane. The nature of the receptor site is unknown and the physiological effect of the toxin is incompletely understood.

METHODS.

Brush border membrane vesicles were prepared the Mg^{2+}-precipitation and differential centrifugation method. Recombinant delta-endotoxin was obtained from a transformed E. coli according the method described in (1). The uptake assay was performed by measuring the radioactive amino acid content of the brush border membrane vesicles. Binding of the toxin was measured by a filter assay.

RESULTS and DISCUSSION.

Whereas several older papers suggest that delta-endotoxins interact with the active transport of potassium recent data support an effect at the level of passive transport. More specifically the toxin is presumed to cause a selective increase in the potassium permeability of the brush border

membrane. Evidence was obtained from K^+ driven amino acid uptake.
We have used the cotransport system to study the effect of Bt2-toxin on Manduca sexta brush border membranes. K^+ permeability is increased by Bt2-toxin in concentrations of 0.1 ug/ml and more. However our experiments indicate no ion-specificity of the toxin. Na^+ permeability also appaers to be increased, since Bt2-toxin affects the Na^+ driven cotransport. Preliminary results even show a permeability change for lager molecules. This is in good agrement with the findings in (2), where a colloid osmotic lysis is indicated to contribute to cell lysis.
In order to understand the physiology of the toxin, characterisation of the receptor is an essential element. Recently evidence was given that the specificity of the delta-endotoxins is not only situated at the level of solubilization and activation of the delta-endotoxins but also requires the presence of a receptor molecule. We found a reduction in the binding capacity of brush border membrane for the delta-endotoxin by treatment with proteases and by mixed glycosidases. KNOWLES et al. (3) suggested a lectin like binding for the Bacillus thuringiensis delta-endotoxin. In our model system we could inhibit the binding of the delta-endotoxin to brush border membranes by certain lectins but not by simple sugars. These results indicate that the receptor for the delta-endotoxin is a glycoprotein and that the sugar moiety of the receptor may be important in the specific insecticidal action of the delta-endotoxin. Still further research of the detailed structure of the receptor is nessecary and will be undertaken in our lab.

ACKNOWLEDGEMENTS

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Regulation of Toxin Expression

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DNA Supercoiling: a Role in the Regulation of Gene Expression and Bacterial Virulence

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Introduction

It has been known for many years that transcription can be highly sensitive to the level of supercoiling of the DNA template (reviewed in 32). Chromosomal supercoiling was thought to be maintained at a constant level by a homeostatic control process. However, recently it has become apparent that DNA in bacterial cells can vary in response to growth conditions. These changes in DNA topology appear to be specifically responsible for regulating the expression of a number of genes. There is now compelling evidence that environmentally-induced changes in DNA supercoiling play an underlying role in the regulation of gene expression in response to a variety of environmental stresses including osmolarity, nutritional status, anaerobiosis and temperature. Superimposed upon this global control network are the more specific regulatory events with which we are all familiar. In this article we review briefly the evidence that environmentally-induced DNA supercoiling changes play a central role in the regulation of gene expression and outline a number of important and unanswered questions. One possible function for this topological control network is to achieve the gross physiological adaptation required when cells pass from a free-living to a host-associated state. We propose here that changes in DNA supercoiling play a crucial role in the regulation of bacterial virulence. Evidence which leads us to this model is presented and discussed.

DNA supercoiling and gene expression

Closed circular DNA isolated from bacterial cells is negatively supercoiled. Although isolated DNA is protein-free, and therefore in a rather different state from DNA in the cell, all available evidence suggests that DNA *in vivo* is also under net torsional stress (reviewed by Lilley, 24). In *E. coli*, *S. typhimurium*, and probably all other eubacteria, the level of DNA supercoiling is determined, to a first approximation, by the opposing activities of two enzymes, DNA gyrase and DNA topoisomerase I. Non-enzymatic factors can also affect the level of supercoiling *in vivo*. For example, DNA binding proteins such as the histone-like HU proteins can constrain superhelicity (3,12) and local unwinding of the DNA helix, such as occurs during transcription, also affects the supercoiling of DNA located immediately upstream and downstream of the unwinding event (39).

Changes in the free energy of negative supercoiling can potentially influence a number of cellular processes including DNA replication, recombination, transposition and transcription (reviewed by Drlica, 11). It is well established that artificial perturbation of DNA supercoiling, for example with inhibitors of DNA gyrase, or by introducing *gyr* or *topA* mutations, can affect transcription from many promoters. Many promoters are highly sensitive to the level of DNA supercoiling in *in vitro* systems. Given the economy of function of bacterial cells it would be suprising if the potential for gene regulation in response to changes in DNA supercoiling were not put to advantage. However, only recently has evidence in support of this idea been forthcoming.

DNA supercoiling levels vary in response to environmental stimuli.

Several studies have now demonstrated a difference in the linking number of plasmid DNA purified from bacterial cells grown under different regimes. For example, high extracellular osmolarity leads to an increase in mean negative superhelix density (9,19). Anaerobic growth (8,40), growth phase (8), temperature (14) and growth transitions between carbon-rich and carbon-poor media (1,31) also appear to affect the supercoiling of plasmid DNA isolated from the cell. These studies have all been based on plasmid DNA, principally because of the difficulties inherent in studying the supercoiling of chromosomal DNA. However, direct measurement has shown that chromosomal supercoiling also changes during anaerobic growth and during growth under osmotic stress (K. Drlica; unpublished results). Indirect evidence, based on studies of gene expression are also entirely consistent with changes in chromosomal supercoiling under different growth regimes (19,20, 31). Although these conclusions must be considered within the limits of the techniques involved (discussed in detail elsewhere; 20) there now seems to be little doubt that, rather than being homeostatically determined, the supercoiling of cellular DNA is in a highly dynamic state.

Changes in DNA supercoiling regulate gene expression *in vivo*.

The first evidence directly supporting the idea that changes in DNA supercoiling play a role in the regulation of gene expression during normal growth came from studies on the *gyr* and *topA* genes themselves (28,37). Each gene responds to changes in DNA supercoiling in a manner appropriate to maintain a homeostatic balance of the two enzymes and, hence, a relatively constant level of supercoiling. However, this is a 'closed' system and does not imply a role for supercoiling in the differential regulation of gene expression during adaptation to environmental changes.

More recently, compelling evidence has accrued that alterations in DNA supercoiling brought about by environmental stresses can be directly responsible for the specific control of gene expression (8,19). This is best illustrated for the *proU* gene which encodes a transport system for the uptake of the osmoprotectant glycine betaine (5). The *proU* locus is not expressed during growth at low osmolarity but is induced over one hundred fold if the osmolarity of the growth medium is increased. Despite extensive efforts in a number of laboratories, no classical regulatory protein has been identified which might mediate *proU* regulation. Instead, *trans*-acting mutations selected for altered regulation of *proU* have been mapped to the *gyr* and *topA* loci and to an additional locus, *osmZ*, which also influences DNA supercoiling (see below). There is excellent correlation between the level of supercoiling and *proU* expression, whatever means is used to perturb DNA supercoiling (19). Detailed analysis of the *proU* promoter *in vivo* and *in vitro* is also consistent with a primary role for DNA supercoiling in its regulation (unpublished results). Changes in DNA supercoiling in response to osmotic shock appear to be sufficient to explain the osmotic regulation of *proU* expression. There is less comprehensive, but accumulating evidence that changes

in DNA supercoiling also play a role in the regulation of other osmotically controlled genes including the *bet* betaine biosynthetic genes and the *ompC* and *ompF* genes encoding the major outer membrane porins (15).

Anaerobically-induced changes in DNA supercoiling also appear to be play a role in regulating expression of several anaerobically regulated genes. This is perhaps best established for the *tonB* gene, which is required for iron uptake (8) and is probably also the case for a subset of other anaerobically regulated genes (31).

There is, therefore, compelling evidence that environmental stresses can affect DNA supercoiling. There is equally compelling evidence that these changes in supercoiling can affect gene expression in an appropriate manner. It therefore seems highly likely that such environmentally-induced supercoiling changes play a role in the regulation of expression of these genes although a direct link between these two observations remains to be established. Final proof of the model requires reconstitution of some of these regulatory systems *in vitro*.

Some unanswered questions.

The establishment of a role for environmentally-induced changes in DNA supercoiling in the regulation of gene expression does, of course, raise a number of questions which must be addressed. The answers to many of these questions can, as yet, be based only on informed speculation.

(i) Is DNA supercoiling homogeneous or is there local heterogeneity within a single DNA molecule? All measurements of supercoiling to date provide a global estimate of the state of affairs; it is not yet possible to measure directly the levels at different positions on a single molecule. There is good evidence that the chromosome is divided into topologically separate domains (35) although it is not known whether the level of supercoiling differs between domains. There is also considerable indirect evidence that local heterogeneity of supercoiling exists. For example, it is now well established that the unwinding of the helix during transcription affects supercoiling immediately upstream and downstream of the RNA polymerase molecule (39). The nucleoid of bacteria contains many histone-like DNA binding proteins such as HU or IHF (reviewed by 12,13). Many of these proteins are loosely bound to the DNA and upon binding can affect local DNA topology. However, little is actually known about how these local changes might be constrained or disseminated throughout the entire molecule. One possiblilty is anchoring through membrane attachment sites (25). In this regard there is an urgent need for a more complete understanding of chromosome structure and function.

(ii) How do environmental stresses alter DNA supercoiling? Again we do not yet have an answer to this question. It is conceivable that the activities of DNA gyrase or topoisomerase I are affected. However, there is no evidence for this and there is now some evidence to the contrary (9; unpublished results). An attractive possibility is that the interaction of one or more of the histone-like proteins with DNA might be affected. Such a model, which is currently being tested, suggests a mechanism whereby different environmental factors could differentially affect supercoiling, and allow the possibility of local variations in topology in response to different stimuli.

(iii) What is *osmZ*? Mutations in *osmZ* were identified by their effects on *proU* expression, appearing to be altered in the cells ability to sense osmotic changes (19). *osmZ* mutations are highly pleiotropic, altering the expression of a wide variety of chromosomal genes as well as affecting site specific recombination events. Many of these *osmZ*-sensitive functions were also known to be supercoiling sensitive and it *osmZ* mutations alter the level of plasmid and chronmosomal supercoiling. It appears this is the primary defect in *osmZ* mutants and that the other phenotypes are secondary consequences of the altered supercoiling. It is apparent that *osmZ* is a complex locus, with different mutations exhibiting different phenotypes. The locus cannot be cloned

stably in multicopy. As yet the nature of any gene product(s) encoded by the locus is unknown although it does not appear to encode a topoisomerase activity. Most probably, *osmZ* encodes a general DNA binding protein(s) (histone-like?) which, upon interaction with DNA alters its topology.

(iv) If gross changes in chromosomal supercoiling occur, for example, in response to osmotic upshock, why are not all genes osmotically regulated? There are several possible reasons for this. First, many promoters are relatively insensitive to changes in DNA supercoiling, at least over the range which is observed *in vivo*, and would therefore be unaffected by any changes that occurred during gowth. Secondly, many promoters do in fact respond to osmolarity even though they would not normally be classified as osmotically regulated genes. Rather their "constitutive" level of expression shows some variation as the osmolarity of the medium is changed. Indeed, osmotically induced supercoiling changes now provide a means whereby such changes can be explained. Thirdly, it is entirely possible that, as discussed above, although osmotic upshock results in a global change in supercoiling there may be local heterogeneity to the response and thus, differential effects on different promoters.

(v) Given that a number of different stimuli such as anaerobiosis or osmolarity affect DNA supercoiling, why are genes only regulated by one or other of these signals? Again, the answer to this question is in multiple parts. Firstly, we do not know how the different stimuli alter supercoiling. If they achieve their effects by different means then it is entirely possible that, even though at a global level they appear to have similar effects, local differences in supercoiling may be induced by different stimuli. Secondly, it turns out that many supercoiling regulated genes are in fact sensitive to more than one environmental parameter; specificity is less than might generally be supposed (31). Thus, many osmotically regulated genes respond to anaerobicity or other stresses, and vice versa. There is increasing, although often ill-defined evidence for an overlap in the responses to all types of stresse such as pH, anaerobicity, starvation osmolarity and temperature (e.g. 18,22,31,34,36). Indeed, we normally tend to consider many regulatory processes in too simplistic a fashion. The cell rarely (except in the laboratory) experiences changes in a single parameter but is stressed in many ways and must respond accordingly by a general change in its physiological state. We have proposed (31) that there is a class of stress-regulated promoters which play a role in such adjustments for which environmentally-induced changes in DNA topology play a major role in regulation. This provides an underlying control system upon which the specific control processes with which we are more familiar may additionally be superimposed.

A potential role for DNA supercoiling in bacterial pathogenesis

Upon first consideration, regulation of gene expression by changes in chromosome structure might appear rather crude. However, a global control mechanism upon which specific regulatory processes may be superimposed does provide for maximum flexibility. It is also perhaps worth considering that regulatory processes are often considered in the wrong way. We tend to think of genes responding to a single stimulus. However, when outside the laboratory does such an event occur? Normally, the cell must adapt to environments in which many factors fluctuate. We have suggested that a subset of genes in the cell responds to many different stresses via a common mechansim: changes in DNA supercoiling. When might a cell be exposed to all these stresses and when might a cell require a major adjustment of its metabolism? One obvious circumstance is during the transition from free-living to host-associated growth. Since this transition is important in the infective process, we propose that the environmental control of DNA supercoiling plays a crucial role in bacterial pathogenesis.

What evidence is there for this conjecture? First, many genes which play an important role in infection are supercoiling-sensitive. For example, the major outer

membrane porins of *E. coli* , which are regulated by many environmental parameters and are supercoiling sensitive (15) play an important role in *Salmonella* virulence (10). The supercoiling-regulated *tonB* gene (8) plays a central role in iron uptake; iron is the nutrient most frequently limiting during infection. Fimbrial genes, which are essential for initial bacteria-host interactions, are also sensitive to DNA topology (7,19). Another illustration is the functions affected by *osmZ* mutations which alter chromosomal supercoiling (19) many if not all of which play a role in infection and colonization. For example, *osmZ* mutants show altered regulation of fimbiae and surface polysaccharides. Even the *proU* locus may be important for infectivity as glycine betaine plays a role in *vir* expression during Agrobacterial infections (38).

Secondly, many stress-regulated proteins play a key role in virulence. For example, heat-shock proteins are major antigens in mycobacterial infections and their expression affects the pathogenicity of Histoplasma spp. (6,27,41). Although designated 'heat-shock' proteins, there is increasing evidence that their synthesis is affected by various stresses. An overlap between heat shock protein expression, the stringent response and osmolarity is certainly apparent (4,17,18) and, as DNA topology appears to play an important role in the stringent (e.g. 23,33) and osmotic responses (19) a role in heat shock expression would not be unexpected.

Thirdly, many virulence factors which play important roles in infectivity are regulated by a multiplicity of environmental signals. The best characterized examples are cholera toxin, the pilus colonization factor TcpA and the major outer membrane proteins OmpT and OmpU of *Vibrio cholerae* which are coordinately regulated and whose expressions are dependent upon the *toxR* gene product (29,30). Many different environmental signals affect expression of these virulence factors including osmolarity, temperature, oxygen tension and pH. Genes required for virulence in *Bordatella pertussis* (16), *Shigella, Yersinia* and other pathogenic bacteria are also coordinately regulated in response to a variety of different environmental signals. It is not immediately obvious how a single environmental sensor (ToxRS in *V. cholerae*) can respond to these diverse signals and achieve an appropriate response. An alternative and simpler explanation is that environmentally-induced changes in DNA supercoiling also play a role in the regulation of the virulence genes. It is striking that the factors which affect expression of the *V. cholerae* virulence genes are the same as those which alter DNA supercoiling in *E. coli* and *S. typhimurium* . In addition, the ToxR and ToxS proteins required for expression of the *V. cholerae* virulence genes are similar to the pairs of phosphorylated proteins such as OmpR-EnvZ and NtrB-NtrC which regulate expression of many bacterial genes (reviewed in 2). Like the *V. cholerae* virulence genes, the *E. coli* OmpC and OmpF porins which are OmpR-EnvZ-dependent are sensitive to many environmental parametrs including pH, temperature and osmolarity. It is clear that supercoiling plays a central role in porin regulation (15); regulation can occur in the absence of OmpR and the requirement for OmpR can even be overcome by altering growth parameters and supercoiling. Thus, there appears to be two levels of control; a general effect mediated by environmentally-induced supercoiling changes and the more specific requirement for a regulatory protein. Indeed, the reason that the OmpR-EnvZ ToxR-ToxS class of regulatory proteins are rather different from other known regulatory proteins may be because of a required adaptation to supercoiling-sensitive promoters.

Finally, we have recently obtained more direct evidence of a role of DNA supercoiling in pathogenicity. In *Shigella flexnerii* , the *virR* gene regulates expression of many virulence factors in response to temperature (26). At 37°C, the virulent, invasive phenotype is expressed while at 30°C the same strains are non-virulent.*virR* mutants express virulence factors at both 30°C and 37°C. The chromosomal *virR* locus has been mapped to between *trp* and *galU* in *Shigella flexneri* (26) and complementation analysis suggests that an equivalent gene exists in *E. coli* (21). As the *osmZ* gene is located between *trp* and *galU*, we examined the possibility that *virR* was in fact the *S. flexneri* equivalent of *osmZ*. This appears to be the case (Manuscript

in preparation). First, *virR* mutants show altered supercoiling of the native *Shigella* plasmids (Figure 1).

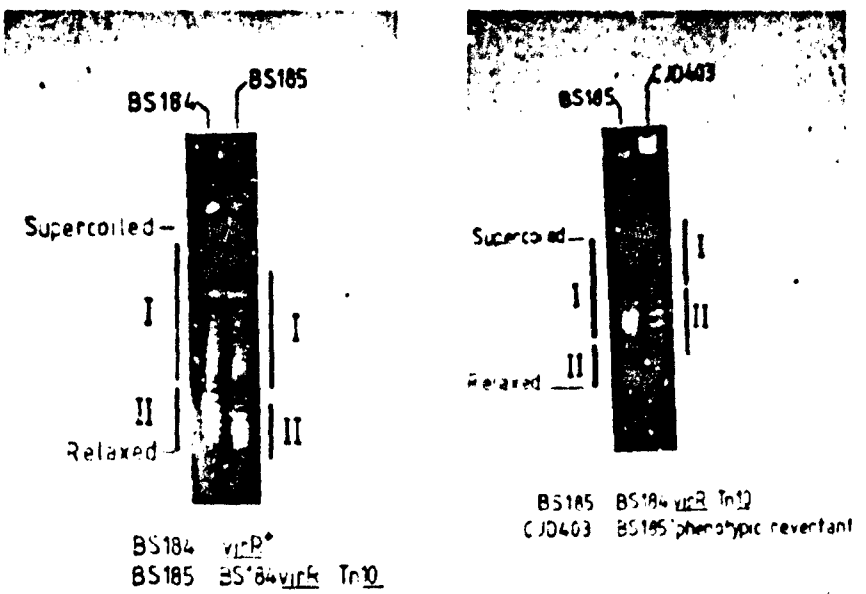


Figure 1.
Chloroquine-agarose gel showing differences in topoisomer distribution of native *S. flexneri* plasmids in a *virR* strain (BS184) and a *virR* ::Tn*10* derivative (BS185). Panel B shows a similar gel for BS185 and a phenotypic revertant of the *virR* mutation(CJD 403) in which supercoiling is simultaneously restored.

Plasmid DNA from *virR* mutants is relaxed compared with the wild-type. While this appears to be opposite to *osmZ* mutants, certain *S. typhimurium osmZ* mutants also relax DNA rather than increase negative supercoiling. Supercoiling in *S. flexneri* is also affected by growth temperature. Secondly, when introduced into *E. coli*, a *virR* ::Tn*10* causes an increase in negative supercoiling of plasmid pACYC184 DNA, similar to that seen for *osmZ* mutants. Thirdly, the *virR*-dependent promoters are sensitive to gyrase inhibitors. Fourthly, the *virR* mutation in *E. coli* activates the *proU* gene at low osmolarity, the basic phenotype of *osmZ* mutants. Thus, *osmZ* and *virR* appear to be one and the same locus. As the primary defect in *osmZ* mutants appears to be a change in chromosomal and plasmid supercoiling, it seems highly likely that the *virR* locus functions in a similar way to regulate expression of the virulence genes. Finally, a rather different line of experimental evidence which implicates DNA supercoiling in virulence comes from studies on virulent strains of *S. typhimurium*. When an *osmZ* lesion is introduced into virulent *S. typhimurium* strains they become attenuated in mouse assays.

Conclusions

It is now apparent that the level of DNA supercoiling in the cell varies in response to environmental stimuli including osmolarity, anaerobiosis, temperature, growth phase and probably other factors such as pH and medium composition. While little is known about how external factors alter DNA supercoiling, a role for such changes in the regulation of gene expression is rapidly being established. A whole new facet of cellular control is emerging. Genes and environmental parameters can no longer be

considered in isolation. Environmentally induced supercoiling changes appear to provide an underlying regulatory network upon which more specific regulatory events are superimposed. This network enables the cell to adjust coordinately and appropriately its physiology in response to multiple environmental changes. One such occasion may be during the invasive process; we suggest that the ability of the cell to adjust DNA supercoiling plays a crucial role in bacterial virulence.

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Coordinate Regulation in Bordetella pertussis

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ABSTRACT

Bordetella pertussis strains haboring transposon TnphoA insertions in vir regulated genes have been analysed for loss of virulence factors and for sequences involved in regulation by the vir locus. Fusions have been identified in previously uncharacterised vir activated and repressed genes.

KEYWORDS

B. pertussis, modulation, regulation.

INTRODUCTION

Pathogenic microorganisms coordinately regulate the production of virulence factors according to the environment in which they find themselves. The regulatory systems of V. cholerae and A. tumefaciens are well characterised examples. B. pertussis, the causative agent of whooping cough, coordinately regulates the expression of pertussis toxin, filamentous haemagglutinin, haemolysin and agglutinogens. These virulence factors are regulated in response to environmental signals in a phenomenon known as phenotypic modulation (3). In the laboratory, B. pertussis no longer expresses virulence determinants in the presence of low temperature (25°C), high $MgSO_4$(20mM) or high nicotinic acid (5mM). This is controlled by two genes vir and mod (2). It has recently been shown that in addition to genes activated by vir (vags) others are repressed (vrgs) (2). The function of these vrgs has not been determined but it is tempting to speculate that they may be involved in adaptation to an environment other than the disease state.

RESULTS

TnphoA insertions in B. pertussis strain 18323 were screened for alkaline

phosphatase activity in media containing high and low concentrations of modulators. As has been previously reported (2) 14 vir regulated fusions were identified, 9 of these were activated by vir (vags) and 5 were repressed (vrgs). The positively regulated TnphoA fusions were characterised for loss of known virulence determinants: pertussis toxin, fha, adenyl cyclase and dermonecrotic toxin. In addition fusions were screened for loss of outer membrane proteins. Results are shown in Table 1.

TABLE 1

STRAIN	FUSION	PHENOTYPE	COLONIZATON
18323	-	wt	+
SK39	vag-39	ptx-	+/-
SK75	vag-75	fha-	+
SK91	vag-91	fha-	-
SK16	vag-16	fha-	+
SK97	vag-97	hly-	ND
SK8	vag-8	66kd major OMP -	-
SK34	vag-34	65kd minor OMP-	+
		40kd minor OMP-	
SK49	vag-49	?	+
SK25	vag-25	?	+
SK6	vrg-6	?	-
SK18	vrg-18	?	+
SK25	vrg-25	?	+

+ = cfu in the lungs of mice 2 weeks after challenge equal to 1 log or greater than the challenge dose
(approx. 5.10^5)
- = cfu in the lungs 3 logs or less than the challenge dose
+/- = cfu in the lungs equal to the challenge dose

SK39 was defective in a fetuin ELISA and was also negative in the CHO cell assay. Using a pertussis toxin-derived probe we have determined that the TnphoA is inserted into the S1 subunit of the toxin. Three strains (SK16, SK75, and SK91) were negative for FHA on the basis of goose blood agglutination, and this was confirmed by SDS gel electrophoresis. However none of these fusions appear to be in the structural gene for FHA (C. Locht, unpublished infomation). However, mouse lung colonization data has shown that one of these fusion strains (SK91) is defective. While FHA has been shown to be required for in vitro adherence of B. pertussis to cultured cells (4), FHA-negative strains have not previously been shown to be defective in colonization in an animal model. However, this defective phenotype may be unusual in that two other FHA- mutants (SK16 and SK75) did not show colonization defects. Interestingly, serotyping using the standard sera against type 2 and type 3/6 agglutinogens show SK91 to be negative for both agglutinogens while SK16 and SK75 react with sera to both agglutinogens. Perhaps 3/6 corresponds to a fimbria which can substitute for FHA.

The role of haemolysin in the pathogenesis of B. pertussis infection is

unclear, although its production is associated with virulence(6). One fusion, SK97, was nonhaemolytic, although it had adenyl cyclase enzymatic acivity. This is in contrast to the nonhaemolytic mutants isolated by Weiss et al.(6) which were all deficient in adenyl cyclase enzymatic activity.

Of great interest to us was the possibility of isolating fusions in previously unidentified virulence genes. We screened for these firstly by looking for loss of outer membrane proteins (OMPs) on SDS-PAGE. This identified one fusion SK8 defective in production of a major OMP of approximately 66kd. Colonization studies indicate that this strain has greatly reduced colonization ability when compared to 18323. Another fusion, SK34, had a normal SDS-PAGE profile but a Western probed with anti-phase I sera showed that three highly immunogenic bands were missing when compared to the wild type. Neither SK8 nor SK34 are defective in the 69kd protein which is thought to be an important antigen of B. pertussis vaccine preparations (1)

Two fusion strains, SK49 and SK25, showed no defects in the above assays.

CLONING OF vir ACTIVATED phoA FUSIONS

We were interested in regulation of vir activated and repressed genes in addition to any novel virulence factors. We chose to study two vags, vag34 and vag49. SK34 was chosen because, although it appeared to be a minor membrane component, it was highly immunogenic. SK49 has not been assigned a phenotype but alkaline phosphatase activity was strongly regulated under modulating and nonmodulating conditons. The fusion junctions of these two mutants were cloned in E.coli using the vector pUC18. The resultant clones pTF100 (from SK34) and pTF101 (from SK49) both produced alkaline phosphatase activity in the E. coli, but this activity was not modulated in the presence of the cloned vir locus (Table 2). The higher activity seen with pTF100 is probably due to read through from the lacZ promoter; the insertion in pTF101 is in the opposite orientaton and readthrough from the lacZ promoter unlikely. This is in contrast to results of Stibitz et al. (5) who have shown modulation of fha expression by the vir locus in E. coli.

TABLE 2

ALKALINE PHOSPHATASE ACTIVITY IN E.COLI

STRAIN	UNITS	
	NA	$MgSO_4$
CC118 pTF100	1514	-
CC118 pTF100 pGB304	1833	1957
CC118 pTF100 PLAFC1	1139	744
CC118 pTF101	49	-
CC118 pTF101 pGB304	47	54
CC118 pTF101 pLAFC1	42	40

NA - no modulators, $MgSO_4$ - 20mM

Similar experiments were performed in a B. bronchoseptica IT-2 background. The fusions were subcloned from pTF100 and pTF101 onto pLAFR2 and the resultant plasmids termed pTF001 and pTF003 respectively. As can be seen from Table 2, the alkaline phosphatase activity was modulated in a B. bronchoseptica background.

TABLE 3

ALKALINE PHOSPHATASE ACTIVITY IN B. bronchoseptica

STRAIN	UNITS		
	NA	$MgSO_4$	Nic
IT-2	9	ND	ND
IT-2 pTF001	3083	17	468
IT-2 pTF003	3156	27	770

NA - no modulators added, $MgSO_4$ - 20mM, Nic - nicotinic acid 5mM

These results demonstrate that the region of the B. pertussis chromosome isolated from these fusions contains all the infomation necessary for interaction with vir gene products to regulate their expression under modulating and nonmodulating conditions.

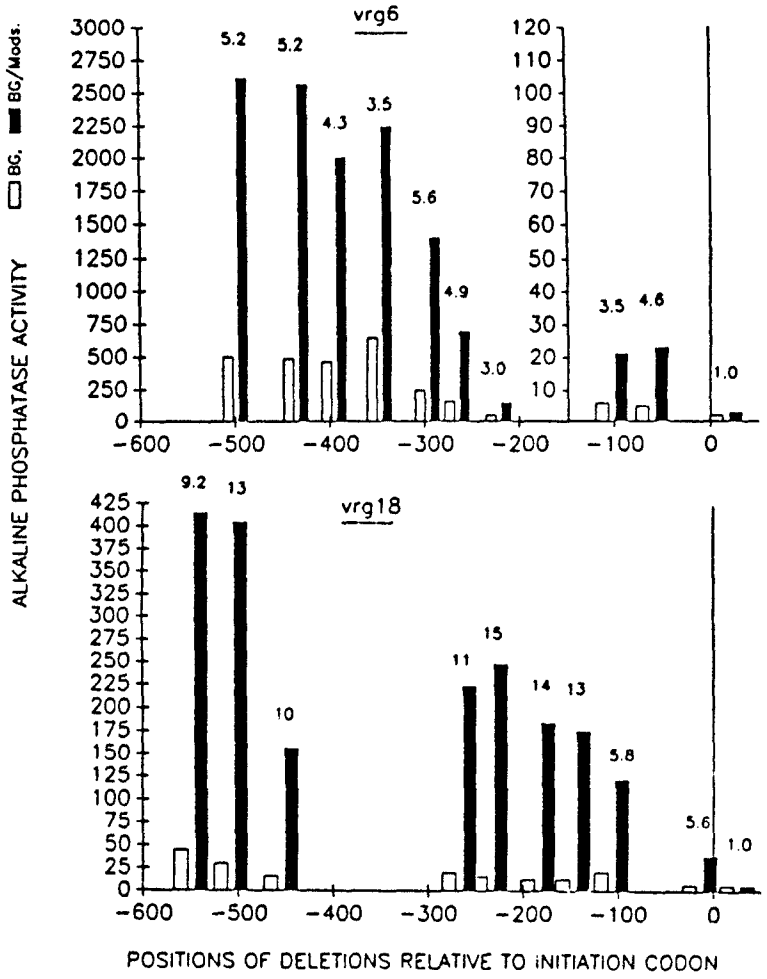
CLONING OF vir REPRESSED GENES

Strains SK6 and SK18 harbor negatively regulated TnphoA fusions, designated vrg6 and vrg18. Both of these fusions have been cloned into the vector pLAFR2 and mobilized into B. pertussis. In the Tohama I derivative 348 (hly1::Tn5) there was strong induction of alkaline phosphatase activity in the presence of modulators. In strain 347 (Tohama I vir1::Tn5) the levels were high and unregulated.

The fusion junction and upstream DNA from both clones were sequenced, and nested deletions created using Exonuclease III. Measurement of alkaline phosphatase activity from these deletion clones in B. pertussis 348 revealed that regulation was maintained even when all but 14bp upstream of the initiation codon were removed (Fig.1) This suggests that regulation of these genes occurs at sites very near or within the coding region. A nearly perfect 20bp dyad repeat is found within the coding sequence of vrg6 and is superimposed by an 8bp direct repeat. Insertion of a SmaI linker in this region abolishes regulation. A similar sequence showing 75% identity to the dyad/direct repeat is found in an identical location in vrg18. Sequences with lesser identity are found upstream of the coding regions of two vir activated genes, ptx and fha, implying that the binding sites for regulatory proteins may be conserved among both categories of genes and differential regulation may be determined by location relative to the promoter.

The function of vir repressed genes is not known. However they appear to play a role in the pathogenesis of B. pertussis as strain SK6 was found to be defective in colonization of mouse lungs (Table 1).

FIGURE 1



Strains grown on Bordet-Gengou plates in the absence or presence of 20mM $MgSO_4$ and 5mM nicotinic acid
Numbers above bars indicate induction ratios

DISCUSSION

Many aspects of Bordetella pertussis pathogenesis remain unclear despite progress in the genetic characterization of several virulence factors and the vir regulatory locus. We have used TnphoA insertional mutagenesis to identify and to analyze coordinately regulated genes. Using this approach, we have isolated strains harboring fusions in both vir activated and vir repressed genes. These two groups appear to include previously unidentified virulence factors. Phenotypic characterization of these fusion strains in animal and tissue culture models, as well as molecular characterization of vir regulated genes should increase our understanding of how B. pertussis causes disease.

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Regulation Studies and Structure-Function Analyses on Shiga Toxin and the Shiga-Like Toxins of Escherichia coli

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I. INTRODUCTION

The Shiga-like toxins (SLTs) of Escherichia coli are cytotoxins related to Shiga toxin of Shigella dysenteriae 1 (14). All members of the Shiga toxin family are bipartite molecules comprised of an enzymatically active A subunit, which can inhibit protein synthesis, and multiple copies of a B or binding subunit. Shiga-like toxin type I (SLT-I) and Shiga-like toxin type II (SLT-II) are produced by enterohemorrhagic E. coli (EHEC) strains associated with diarrhea, hemorrhagic colitis, and the hemolytic uremic syndrome in humans (13), whereas Shiga-like toxin type II variant (SLT-IIv) is produced by E. coli strains responsible for edema disease of swine (11). The Shiga-like toxins are similar in structure and biological activities but vary with respect to immunological relatedness and cytotoxic specificity. Hence, antiserum to purified Shiga toxin neutralizes SLT-I (14) but does not neutralize the immunologically cross-reactive toxins SLT-II or SLT-IIv (11,19). In addition, Shiga toxin, SLT-I, and SLT-II are equally cytotoxic for HeLa and Vero cells, whereas SLT-IIv is markedly more cytotoxic for Vero cells (11).

The functional receptor for the B subunits of Shiga toxin, SLT-I, and SLT-II is globotriaosyl ceramide (Gb_3) (9,10,21). However, Gb_3 does not appear to be the functional receptor for the SLT-IIv B subunit which may explain why the variant toxin differs from the other members of the Shiga toxin family with respect to cytotoxic specificity (J.E. Samuel, D.L. Weinstein, V. Ginsburg, and H. Krivan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B73, p.70). The purpose of this report is to summarize our current understanding of the molecular genetics of the Shiga-like toxin family and to provide insight into the relationship between toxin structure and function.

II. MOLECULAR GENETICS OF THE SHIGA TOXIN FAMILY

A. Genetic Relationship Among Shiga Toxin Family Members

The nucleotide sequences have been determined for all the members of the Shiga toxin family identified to date (1,3,7,17,22). The sizes of the A subunit and B subunit polypeptides of the family members as calculated from the deduced amino acid sequences are approximately 32,200 daltons and 7,700 daltons, respectively. The genes coding for the A subunit and B subunit of these toxins are arranged in tandem as an operon with a gap of 12 to 15 nucleotides between the open reading frames. A putative ribosomal binding site exists immediately upstream of the A subunit coding region with a second putative Shine-Dalgarno site located in the gap between the A subunit and B subunit genes. The nucleotide sequences of Shiga toxin and SLT-I are essentially identical (17). These sequences differ by only three nucleotides which results in a single conservative amino acid change within the A subunit. The structural genes for Shiga toxin/SLT-I share 55% nucleotide sequence homology with SLT-II (7) and 57% nucleotide sequence homology with SLT-IIv (22).

B. Characterization of Promoters for the SLT Operons

The promoter for the stx/slt-I operon was previously mapped by primer extension analysis (3) and its location confirmed by deletion analysis (2). We have used primer extension and S1 nuclease protection analyses to determine that the transcription start site for the SLT-II operon is an adenine residue 118 nucleotides upstream of the slt-IIA initiation codon (L.M. Sung, M.P. Jackson, D.L. Weinstein, and A.D. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D77, p.84). In contrast to the stx/slt-I promoter, the nucleotide sequence of the putative slt-II promoter is not highly homologous to a consensus sequence established from a compilation of defined E. coli promoters. Thus, the slt-I promoter might be a more effective transcription start signal than the putative slt-II promoter. Indeed, the levels of SLT-I observed in cell lysates of SLT-producing EHEC strains are 100 to 1000-fold higher than for SLT-II (19). To examine the possibility that this difference in toxin levels reflects a difference in transcriptional efficiency between the stx/slt-I promoter and the putative slt-II promoter, the promoters will be exchanged to create hybrid operons. These hybrid operons will consist of the stx/slt-I promoter fused with the slt-II structural genes and the putative slt-II promoter fused with the stx/slt-I structural genes. To facilitate this promoter exchange, compatible restriction endonuclease sites bracketing the Shiga toxin promoter and the putative SLT-II promoter have been created by cligonucleotide-mediated site-specific mutagenesis.

C. Iron Regulation of the Shiga-like Toxins

The role of iron regulation in the virulence of SLT-producing E. coli is of considerable interest to our laboratory. Shiga toxin/SLT-I production has been shown by several groups to be repressed by high levels of iron (2,3,23). Iron regulation of the SLT-I operon is mediated by the binding of the fur protein and its iron co-repressor to a site within the slt-I promoter which prevents transcription

of the toxin genes (2). SLT-II and SLT-IIv production are not influenced by the concentration of iron in the media (22). Recent studies in our laboratory using E. coli fur mutants have also shown that, as expected, the slt-II operon is not subject to regulation by the fur repressor (Table I). As discussed above, we are currently replacing the putative slt-II promoter with the stx/slt-I promoter which should convert slt-II to an iron regulated operon under fur repressor control. Using this construct we will examine the relevance of iron-regulation in the pathogenesis to SLT-producing E. coli.

TABLE I

Comparison of the stx/slt-I promoter and the putative slt-II promoter.

Toxin promoter	Transcription start site	Homology to E. coli promoter consensus	Toxin synthesis inhibited by iron	Acted on by fur repressor
stx/slt-I	-106	HIGH	YES	YES
slt-II	-118	LOW	NO	NO

D. Transcriptional Regulation of SLT-II

Previous studies by DeGrandis et al. (3) on the slt-I operon suggested that slt-IA and slt-IB are transcribed as a single bicistronic mRNA from the promoter identified upstream of slt-IA. However, this study and previous findings (12,23) still left open the possibility that a second promoter existed within the downstream sequences of the slt-IA open reading frame. An independent transcriptional start site for slt-IB could lead to increased expression of the B subunit gene and help explain the single A subunit to multiple B subunit protein stoichiometry observed in the holotoxin (4,14).

Recently, we used Northern blot analysis to investigate the transcriptional regulation of the slt-II operon. When total cellular RNA isolated from the SLT-II clone, E. coli HB101(pNN76), was probed with a DNA fragment comprising slt-IIA and slt-IIB, a single band corresponding in size to a bicistronic mRNA was observed (L.M. Sung and M.P. Jackson, Abstr. Annu. Meet. Soc. Microbiol. 1989, H89, p.184). A DNA probe containing only slt-IIB hybridized to the identical transcript. Furthermore, primer extension analysis did not identify a transcription start site within the downstream sequences of slt-IIA. Therefore, the possibility of the independent transcription of slt-IIB seems unlikely.

III. RELATIONSHIP BETWEEN TOXIN STRUCTURE AND FUNCTION OF THE SHIGA TOXIN FAMILY

A. The A Subunits of the Shiga Toxin Family

The A subunit of Shiga toxin/SLT-I, SLT-II, SLT-IIv and the plant lectin ricin have

identical enzymatic activities (5,15). These cytotoxins are rRNA N-glycosidases that cleave a specific adenine residue from the 28S rRNA of the eucaryotic ribosome (5). By comparing the A subunit sequences of SLT-I and SLT-II with a putative active site cleft in the A subunit of ricin, Hovde, et al. (6) provided evidence that glutamate 167 in the SLT-I A subunit (the A subunits of Shiga toxin and SLT-I are identical at this position) is an active site. A mutation induced at the corresponding glutamate in the SLT-II A subunit supports the hypothesis that this residue is the active site for all members of the Shiga toxin family (M.P. Jackson and S.B. Calderwood, manuscript in preparation).

B. The B Subunits of the Shiga Toxin Family

Experiments using hybrid toxins comprised of the A and B subunits of Shiga toxin, SLT-I, SLT-II, and SLT-IIv demonstrated that the B moiety dictates cytotoxic specificity and extracellular localization in E. coli (D.L. Weinstein, M.P. Jackson, L.P. Perera, R.K. Holmes, and A.D. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, D207, p.117, and manuscript submitted). In an attempt to identify domains of the Shiga toxin and SLT-IIv B polypeptides involved in receptor binding and toxin localization in E. coli, oligonucleotide-directed site-specific mutagenesis was used to change specific amino acid residues.

1. Induction of Missense Mutations in the Shiga Toxin B Subunit

A hydrophilic sequence between residues 10 and 20 of the Shiga toxin B subunit has been proposed to be an antigenic region of the polypeptide (16). This region is highly conserved in the B subunits of Shiga toxin, SLT-I, SLT-II, and SLT-IIv (7,17,22). Amino acid substitutions designed to reduce the hydrophilicity of this conserved region were induced at aspartate residues 16, 17, and 18 of the Shiga toxin B subunit (Table II). Only the double mutation aspartate 16 and 17 to histidines (D16H,D17H in Table II) had a significant effect on activity. This double mutation resulted in the total loss of cytotoxic activity on HeLa cells and Vero cells. However, the mutated polypeptide was fully immunoreactive in the colony blot ELISA using three monoclonal antibodies that were specific for the native Shiga toxin B subunit (data not shown). Because these monoclonal antibodies recognize conformation-dependent epitopes (18), this result indicates that the conformation of the B polypeptide was not drastically changed. Furthermore, immuno- precipitation using monoclonal antibodies against the individual subunits demonstrated association between the A subunit and the mutated B subunit. Therefore, the loss of activity was not due to failure of the holotoxin to assembly.

2. Induction of Missense Mutations in the SLT-IIv B Subunit

Although Shiga toxin and SLT-IIv differ with respect to cytotoxic specificity, there are relatively few significant amino acid differences between the B subunits of these two toxins when their nucleotide sequences are aligned. To identify a receptor binding site, we changed several residues in the B subunit of Shiga toxin to the corresponding residue at the same position in the SLT-IIv B subunit. Conversely, residues in the SLT-IIv B subunit were altered to resemble the Shiga toxin amino acid sequence (one example, N17D in Table III). While none of the

amino acid changes altered cytotoxic specificity, the glutamine 64 to glutamate mutation at the carboxyl-terminus dramatically affected the localization of SLT-IIv in E. coli; the level of extracellular cytotoxin was reduced 1000-fold (Table III). The SLT-IIv B subunit is a basic molecule with an isoelectric point (pI) of 10.2 (22). Replacement of glutamine 64 with a glutamate alters the charge of this molecule at neutral pH and therefore may have affected the localization of SLT-IIv by disrupting ionic interactions with the E. coli cell envelope.

TABLE II
Missense mutations in the Shiga toxin B subunit.

Residue[a]	Cytotoxicity[b]
pEW3	7
D16H,D17H	0
D16N,D17N	7
D16N	7
D17N	7
D18N	7

TABLE III
Missense mutations in the SLT-IIv B subunit.

Residue[a]	Cytotoxicity[b] CA[c]	EC (%)[d]
pDLW5.3	4	5 (91)
N17D	3	4 (91)
Q64E	4	2 (1)
N17D,Q64E	3	0 (0)

[a] Nomenclature of Knowles (8). One letter amino acid abbreviations: asparagine, N; aspartate, D; glutamate, E; glutamine, Q; and histidine, H.
[b] log 50 % cytotoxic doses per milliliter
[c] cell-associated toxin
[d] extracellular toxin and (percent of total)
Plasmid EW3 expresses wild-type Shiga toxin. Plasmid DLW5.3 expresses wild-type SLT-IIv.

3. Nonsense Mutations in the Shiga Toxin B Subunit

Previous studies using fluorescence spectroscopy demonstrated that tryptophan 34 is at or very near the Gb_3 receptor binding domain (20). Expression of the amber mutation induced at position 34 in the E. coli tRNA suppressor mutant LE392 resulted in the replacement of tryptophan 34 with a glutamine or a tyrosine. This mutation (W34 in Table IV) did not prevent binding to the receptor analog, which indicates that this residue is not directly involved in receptor recognition. A single intrachain disulfide bond links two identically located cysteine residues found in the B subunits of Shiga toxin, SLT-I, SLT-II, and SLT-IIv (7,16,17,22). This disulfide bond is eliminated when the amber mutation induced in the Shiga toxin B subunit at cysteine 57 is expressed in the nonsuppressor host MC4100. This mutation resulted in the total loss of cytotoxicity, receptor analog binding (Table IV), and immunoreactivity (data not shown). In addition, expression of the amber mutation induced at position 65 (E65) in E. coli MC4100 resulted in the truncation of the polypeptide from 69 to 64 amino acids and rendered the Shiga toxin B subunit inactive in all assays. Thus, the elimination of the single intrachain

disulfide bond, as well as truncation of the polypeptide by five amino acids, may have altered the conformation of the B subunit and prevented holotoxin assembly. When the E65 amber mutation is expressed in the E. coli suppressor host HB101, glutamate 65 is replaced with a glutamine, the residue found at the corresponding position in the SLT-IIv B subunit. However as shown in Table IV, cytotoxicity and receptor analog binding could not be restored. The corresponding mutation in SLT-IIvB (Q64E in Table III) affected extracellular localization of the holotoxin in E. coli without causing a significant effect on the levels of cell-associated cytotoxicity. These results indicate that the carboxyl-terminus of the SLT-IIvB is important for release of holotoxin from the periplasm, while the carboxyl-terminus of the Shiga toxin B subunit is a domain important for the structural integrity of the holotoxin.

TABLE IV

Amber termination mutations in the Shiga toxin B subunit.

E. coli strain[a]	Residue[b]	Cytotoxicity[c]	Receptor-analog binding(%)
HB101	pEW3	7	100
LE392	W34	6	52
MC4100	C57	0	0
MC4100	E65	0	0
HB101	E65	0	0

[a] MC4100, nonsuppressor; HB101, supE mutant; and LE392, supE supF mutant.
[b] One letter amino acid abbreviations: cysteine, C; glutamate, E; and tryptophan, W.
[c] log 50 % cytotoxic doses per milliliter
Plasmid EW3 expresses wild-type Shiga toxin.

IV. CONCLUSIONS

The structural genes for Shiga toxin/SLT-I and SLT-II are highly related in nucleotide sequence and are similar in operon arrangement. Recent studies in our laboratory on characterization of the slt-II operon indicate that transcription of slt-IIA and slt-IIB occurs as a bicistronic message, a finding consistent with published observations on the transcription of the stx/slt-I operon. However, the efficiency of the putative slt-II promoter as a transcriptional start signal appears to be less than that of the slt-I promoter based on a comparison to an E. coli promoter consensus sequence. Furthermore, the putative slt-II promoter is not iron- regulated, in contrast to the fur protein-repressible slt-I promoter. These differences in genetic regulation between stx/slt-I and slt-II are being explored in our laboratory through construction of promoter/structural gene hybrids.

Site-directed mutagenesis was used to alter the amino acid sequence of the A subunit of SLT-II and the B subunits of Shiga toxin and SLT-IIv. The results of

the A subunit gene mutagenesis support the hypothesis that glutamate 167 may be the active site for all members of the Shiga toxin family. While a single amino acid could not be identified as the B subunit receptor binding moiety, the hydrophilic region near the amino terminus of the Shiga toxin B subunit was determined to be important for receptor binding. In addition, we demonstrated that the carboxyl terminus is important for the structural integrity of Shiga toxin and the extracellular localization of SLT-IIv in E. coli. Multiple residues distantly separated in the primary structure of the Shiga toxin and SLT-IIv B subunits may be required for receptor recognition.

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Antigenic Modulation and Phase Variation of Bordetella Species

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THE GENUS BORDETELLA

The genus Bordetella is composed of four pathogenic species. B. avium is a pathogen for birds, B. bronchiseptica for several mammalian species, B. parapertussis can occasionally cause disease in man, and B. pertussis, the etiological agent of whooping cough, is a pathogen exclusively for man (1,5,13). While all species produce many virulence factors which are involved in adhesion to epithelial cells, in local damage, in evasion of host defense mechanisms and in systemic intoxication (13), pertussis toxin (PT) is produced only by B. pertussis. PT is an ADP-ribosylating enzyme believed to be responsible for most of the systemic manifestations of whooping cough. Interestingly, also B. parapertussis and B. bronchiseptica encode the PT operon, but they do not express it (2).

COORDINATE REGULATION OF THE VIRULENCE FACTORS

Most of the virulence factors such as PT, filamentous haemagglutinin (FHA), adenylate cyclase/haemolysin, fimbriae, outer membrane proteins and others are regulated coordinately. A spontaneous loss of the production of these factors has been observed after passage of Bordetella species in the laboratory, which is rarely reversible. This phenomenon has been called phase variation and leads to avirulent (phase III) variants which differ strongly in their immunological properties from the virulent (phase I) bacteria. A similar avirulent phenotype has been described by Lacey after growth of the bacteria in presence of

nicotinic acid, salts such as $MgSO_4$, or after growth at low temperature (8). This phenomenon (antigenic modulation) is reversible and reflects a change in gene expression as a response to changes in the environment. Following transposon mutagenesis of B. pertussis, Weiss and Falkow identified a locus (vir), which is necessary for the expression of the virulence factors and they proposed a model where the vir locus encodes a transactivating factor(s) which interacts with the promoter regions of the virulence genes and activates their transcription (17).
This chapter is focused on the identification of the DNA sequences required in cis and in trans for virulence gene expression in B. pertussis.

THE PERTUSSIS TOXIN PROMOTER

The pertussis toxin promoter has been identified after the cloning and sequencing of the PT operon (11). It shows -10 and -35 regions which are very similar to the canonical Escherichia coli promoter sequences, but it has a distance of 21 bp between the two regions instead of the typical 17 bp (12). A transcript directed from the PT promoter could be found only in phase I bacteria, but not in phase III bacteria, in vir insertion mutants, nor in bacteria grown under modulative conditions (5 mM nicotinic acid, 30 mM $MgSO_4$, or at low temperature). This showed that both mechanisms, the phase variation and the modulation, act at the transcriptional level (7).

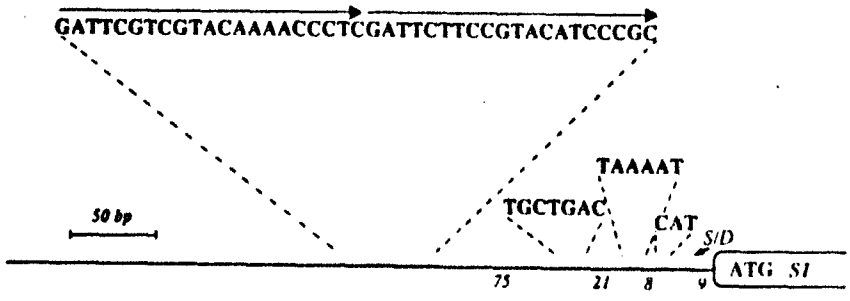


Fig.1: Schematic presentation of the PT promoter and its regulatory sequences. S/D marks the ribosomal binding site. Transcription starts with an adenosine residue within the sequence CAT. The -10 and -35 sequences are shown. The directly repeated sequence at position -117 to -158 is important for the vir-mediated transactivation of the promoter.

A deletion analysis of the pertussis toxin promoter revealed that at least 170 bp upstream from the transcriptional start site are necessary for its vir-dependent activity (4). Fig. 1 shows that a directly repeated sequence is located at the position where the deletions impair the promoter activity (4).

In several other positively regulated promoters direct repeats have been shown to be binding sites for transactivating factors. It is therefore possible that the direct repeat in the PT promoter represents a binding site for a vir encoded activator protein. DNA binding studies are under way to clarify the function of this repeated sequence in the PT promoter.

The analysis of the activity of the PT promoter and its various deletion derivatives in phase I and phase III variants of the species B. pertussis, B. parapertussis and B. bronchiseptica grown under various modulative and non-modulative conditions revealed that the modulation occurs through the same cis-acting sequences which are required for the transactivation by the vir system (7). Furthermore, phase III variants did not show a modulation of the PT promoter activity. This demonstrated that the modulation also acts through the vir system (7).

Interestingly, the species B. parapertussis and B. bronchiseptica containing the silent PT operons were able to express the PT promoter of B. pertussis. This showed that they are endowed with a functional vir system (4). Their inability to express their own PT operons is due to a strong accumulation of point mutations in the 170 bp region upstream of the transcription initiation site, which is exactly the region important for transactivation by the vir system (4).

THE NUCLEOTIDE SEQUENCE OF THE BORDETELLA VIR/BVG LOCUS

The B. pertussis vir locus has originally been cloned by complementation of a Tn5 insertion mutant in vir (15). It is closely linked to the structural gene encoding FHA (15).

The nucleotide sequence of the vir loci of the Bordetella species revealed that the vir system is composed of three genes called bvgA, bvgB and bvgC (bvg = Bordetella vir gene) (3). Fig. 2 shows the genetic organization of the bvg operon with the direction of transcription from left to right. No typical transcriptional signals have been identified so far. The BvgA protein has a size of 23 Kd and is very likely a cytoplasmic protein. The bvgB gene encodes a 30 Kd protein which is probably located in the periplasm because of the presence of a leader sequence and its general hydrophilic character. The third protein BvgC has a size of 102 Kd and

is probably a transmembrane protein with a periplasmic and a cytoplasmic domain. Non polar linker insertions in each of the three open reading frames destroyed the vir activity which demonstrated that all three genes are essential for vir function (3). All characterized mutants or spontaneous phase variants so far had mutations in the region of the three open reading frames. Interestingly, the only description of a reversible phase variation, observed with the Tohama strain, involved a reversible frameshift mutation in the bvgC gene (16). However, most of the spontaneous phase III derivatives of B. bronchiseptica analysed so far had non reversible deletions in various parts of the vir/bvg region (10).

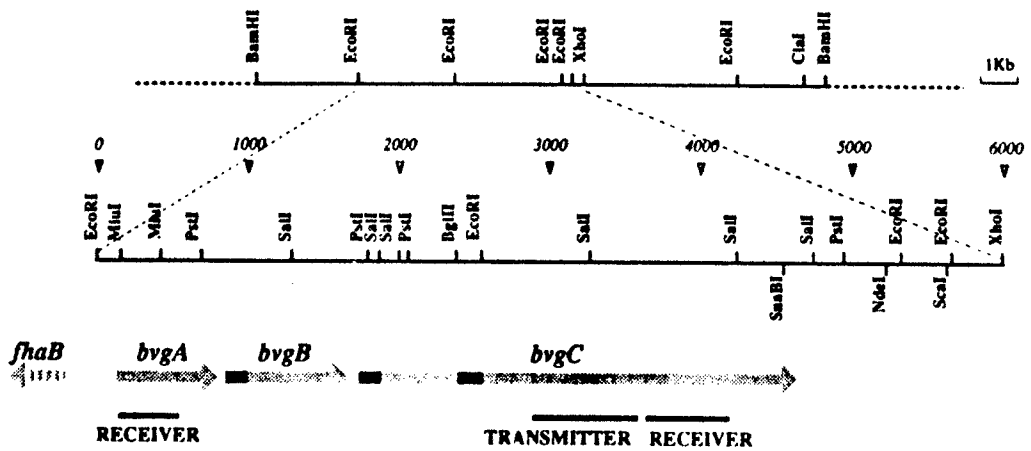


Fig.2: Restriction map and structure of the vir/bvg operon of B. pertussis. The three open reading frames coding for BvgA, BvgB and BvgC are shown. The homologies to the proteins of the two-component systems are indicated (receiver/transmitter). Hydrophobic parts of the proteins are shown as black bars.

THE BORDETELLA BVG SYSTEM BELONGS TO A FAMILY OF BACTERIAL SIGNAL TRANSDUCING PROTEINS

The search for homologies with other proteins revealed that the Bordetella bvg system belongs to a family of prokaryotic proteins which are involved in signal transduction and gene regulation (3). These systems respond to changes in the

enviroment by regulation of gene expression (6,9,14). They are typically composed of two proteins: a sensory protein in the cytoplasmic membrane and a cytoplasmic DNA binding regulatory protein (Fig. 3). The sensor proteins generally have two domains, a periplasmic sensor domain and a cytoplasmic domain which is conserved in all the sensor proteins. This conserved domain is called "transmitter" domain and has a kinase activity which phosphorylates the activator protein. The activator has typically a C-terminal domain which binds to specific DNA sequences and a strongly conserved N-terminal domain called "receiver" which is phosphorylated by the transmitter. In the phosphorylated state the regulator protein is believed to activate transcription from its target promoters (Fig. 3).

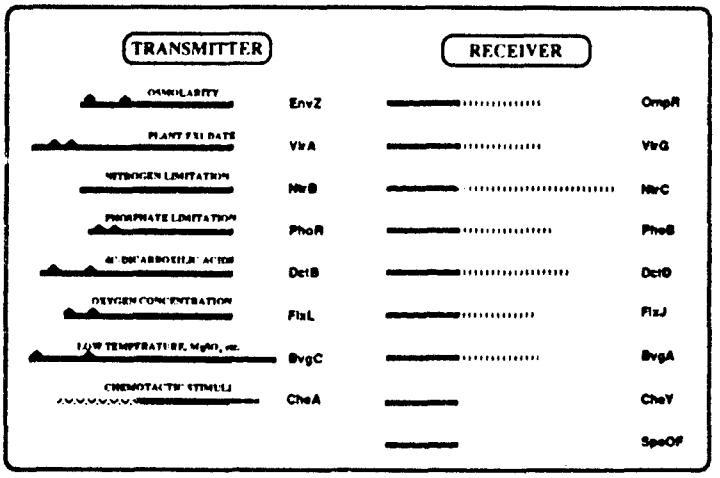


Fig.3: Schematic presentation of some proteins belonging to the two-component systems. On the left sensor proteins such as EnvZ are shown, which have the homologous transmitter domain in their C-terminal parts. On the right the corresponding regulators such as OmpR with the conserved N-terminal receiver domain are shown.

The BvgA protein of Bordetella shows strong homologies with the "receiver" domain of the regulator proteins and it is therefore probably a DNA binding activator protein, which interacts with various promoters of the Bordetella virulence factors. The BvgC protein shows the typical overall structure of a sensor protein with a periplasmic domain and a cytoplasmic part with a strong homology to the transmitter domain. However the BvgC protein is unique in the fact that it contains also a perfect homology to the receiver domain

fused directly to the C-terminus of the transmitter domain (Fig.3 and Fig. 4). A second peculiarity of the vir/bvg system is the presence of a third essential component, the periplasmic BvgB protein. BvgB shows a strong aminoacid homology with the periplasmic portion of the BvgC protein, which suggests an interaction of the two proteins in sensing the environment (Fig. 4). A model for the action of the Bordetella vir/bvg system deduced from the analysis of the sequencing data is proposed (Fig. 4):
The periplasmic domain of the sensor protein BvgC, together with the BvgB protein, senses the environment and transduces signals to its cytoplasmic transmitter domain, perhaps via conformational changes or by changing the equilibriuum between a monomeric and an oligomeric form. The transmitter domain transmits the signal by phosphorylation of the receiver module of the regulator protein BvgA, which stimulates transcription in the activated form. The role of the additional receiver domain of the sensor protein BvgC is not clear, but it could be that BvgC is also a DNA binding protein or that the activity of the BvgC sensory protein is modulated at this receiver module by other unidentified factors.

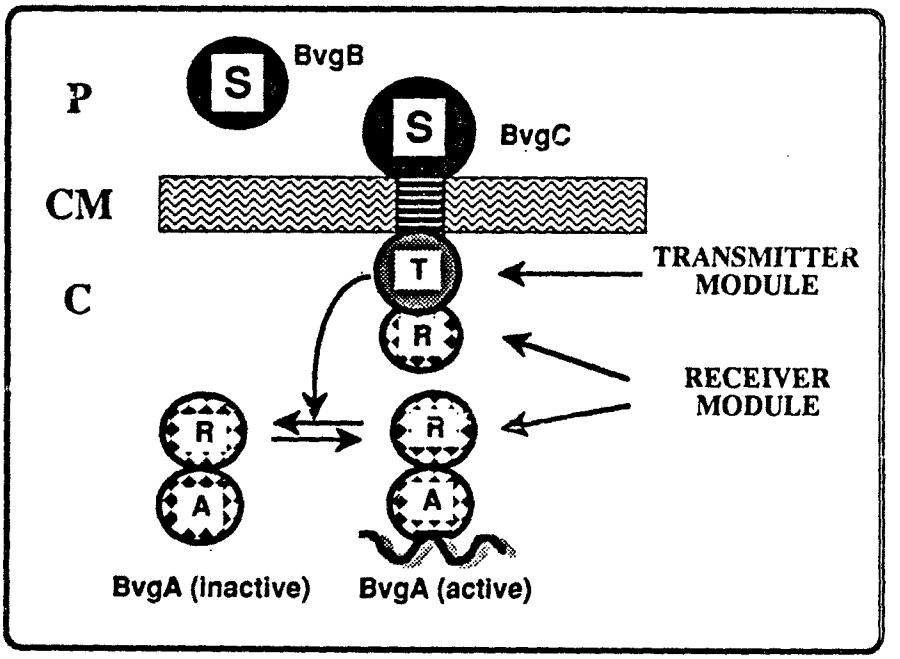


Fig. 4: Model of the Bordetella vir/bvg system. Abbreviations: C, cytoplasm; CM, cytoplasmic membrane; P, periplasm; R, receiver module; S, sensor domain; T, transmitter module; A, DNA binding domain

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Molecular Studies on the Aerolysin Gene of Aeromonas Sobria

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ABSTRACT

The presence of the aerolysin gene in 242 Aeromonas strains comprising all known species, was probed using five non-overlapping probes derived from a 6kb DNA fragment haboring the aerolysin gene and adjacent regions from an Aeromonas sobria strain AB3. Sequence homologous to the aerolysin gene were present in all species of Aeromonas tested regardless of the source of isolation. Only three strains were found to hybridize to a DNA probe haboring the aerolysin promotor and its regulatory sequences; however, DNA probes flanking this region showed homology with almost all strains tested. To facilitate studies on the regulation of expression of the aerolysin gene in A. sobria AB3 we constructed isogenic strains of the parental strain was replaced by an aerolysin-alkaline phosphatase mutant allele by marker -exchange mutagenesis. Production of aerolysin was sensitive to levels of iron in the medium, varying salt concentrations and repressed by glucose in the growth medium. Additionally, aerolysin production was found to be growth phase regulated. Furthermore using purified aerolysin and planar lipid membranes we showed that aerolysin is capable of pore formation in artificial lipid bilayers. Single-channel conductance revealed a pore size of at least 2nm diameter. The pore had a lifetime of several minutes and zero-current membrane potential experiments revealed that the aerolysin channel is anion selective.

Aerolysin- DNA hybridization studies- Regulation of expression- Function

Aerolysin, a potentially important virulence factor of Aeromonas spp. is a single polypeptide with a molecular weight of 54000, and is secreted as a proenzyme. Upon proteolytic processing of the protoxin at its carboxy-terminal end (5) it becomes an active toxin. The biological properties of this toxin include lethality of mice, enterotoxicity in rabbit ileal segments (1), release of inflammatory mediators from granulocytes and mast cells (13) at sublethal concentrations and cytotoxcity to a large variety of cell lines in vitro. All these events may be attributed to the insertion of the aerolysin molecule into the membrane of the target cell. Using marker-exchange mutagenesis it was demonstrated that aerolysin is an important virulence factor required for both the establishment and subsequent maintenance of Aeromonas-associated systemic infections (4).

We recently reported the cloning of the aerolysin gene from a clinical isolate of A. sobria and its entire nucleotide sequence. In these studies, it was shown that two regions upstream (aerC) and downstream (aerB) affect aerolysin production (3,7). The aerC region was shown to contain two divergent non-overlapping promotors and elements required for the regulation of the aerolysin production. The nucleotide sequence of aerolysin from a A. hydrophila strain has also been reported (6).

While there is a good deal of information regarding the biochemistry and properties of aerolysin, much less is known about the genetics of aerolysin regulatory and structural genes. Individual strains vary widely in their ability to produce the toxin in vitro and little is known of the environmental conditions and factors that influence the amount of toxin produced. Aerolysin production could be regulated by repression as has been proposed for iron-mediated regulation of diphtheria toxin (8) and Shiga-like toxin (2), or a positive regulatory mechanism such as been recently described for cholera toxin (11). It has also been suggested that large differences in the absolute amounts of aerolysin produced in vitro by different strains could be a result of duplication of the gene in some strains. This mechanism has recently been shown to occur in some strains of Vibrio cholerae encoding enterotoxin (10). With the availability of cloned aerolysin and information of its nucleotide sequence it is now possible to address specific questions regarding the presence of this gene in Aeromonas spp. isolated from a wide geographical distribution.

In order to select DNA probes for the detection of aerolysin-homologous sequences we first compared the nucleotide sequences of the cloned A. sobria and A.

hydrophila aerolysin genes (Fig.1). The region encoding the aerolysin structural gene shows an overall homology of 77% at both nucleotide and amino acid level. This analysis also revealed a higher level of homology at the C-terminal end of the molecule compared to its N-terminus (81% vs 73%) between the two sequences. The region 3' to the aerolysin gene showed an even higher homology at 83%. In contrast, sequences upstream of the structural gene showed an abrupt change in the level of homology between both genes. The corresponding aerC regions showed only 46% homology and further upstream it was only a little higher at 63%. This suggested that aerolysin may represent a relatively homologous family of cytolytic toxins in Aeromonas.

Because of the great differences in regional homologies along the length of the aerolysin gene between the two published sequences, we used several representative fragments as DNA probes. These are depicted in Fig.2. A total of 242 strains comprising of both clinical and environmental isolates of all known species from different geographical regions were tested using colony blot hybridization. A summary of the results obtained with all DNA probes is shown in Fig.2. They show that sequences homologous to the aerolysin gene and its flanking regions are present in all Aeromonas spp. regardless of their source of isolation. While probes denoted 4 and 5 hybridized readily to whole cell DNA from all isolates under stringent conditions, hybridization signals with probes 1 and 3 were detected only when the blots were washed at a lower stringency. All three strains reacting with probe 2 did so at conditions of high stringency. The gene was present in all three species A. hydrophila, A. sobria and A. caviae. This result was unexpected for A. caviae strains, since this species are considered to be non-pathogenic for humans. Nevertheless, of the 43 strains that failed to hybridize to the aerolysin probe, 35 were of the species A. caviae, 7 were A. sobria strains, and one strain belonging to the species A. hydrophila. The identification of the three mesophilic, motile Aeromonas species is complex. Each of the three species contain different hybridization groups that are biochemically indistinguishable from each other (12). Also, three additional species, A. media, A. veronii and A. schubertii have also recently been proposed. Hence it is possible that the non-hybridizing strains are true A. caviae strains and those strains haboring aerolysin-homologous sequences belong to species other than A. caviae and may have to be reclassified.

Environmental isolates of all three species also habored sequences related to the aerolysin gene suggesting that human infections with Aeromonas may be environmentally acquired. The DNA hybridization data obtained with probe 2 show that there is considerable heterogeneity in sequences encoding aerolysin, since many strains were only detected

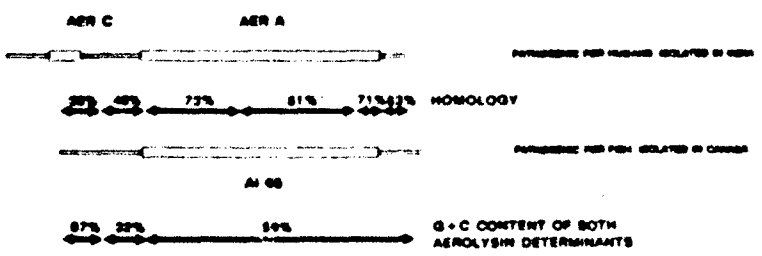


FIGURE 1. Homology levels between two sequenced aerolysin genes at the nucleotide level.

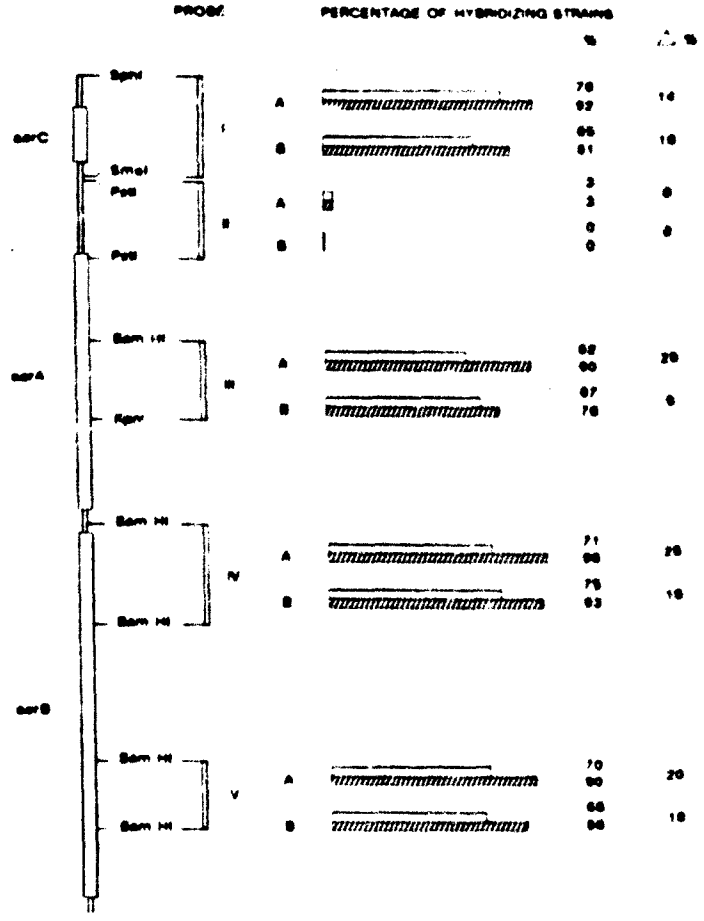


FIGURE 2. Cumulative results of DNA hybridization data obtained with 242 Aeromonas strains from diverse geographical locations using probes derived from the aerolysin region of A. sobria AB3.

A STRAINS ISOLATED IN AUSTRALIA (125) AND USA (15)
B STRAINS ISOLATED IN THE NETHERLANDS (102)
ENVIRONMENTAL ISOLATES
CLINICAL ISOLATES

when hybridizing under conditions of lower stringency than that used with the other probes used. While, homology to the aerC probe was rare, it was observed under conditions of high stringency. Taken together, these results suggest that the aerolysin gene(aerA) and its regulatory region (aerC) are under different evolutionary pressures and seem to vary independently of each other. One implication of the results is that the aerolysin gene may be differently regulated in individual strains of any one species.

We next examined the regulation of the aerolysin gene in A. sobria strain AB3. Aerolysin is exported as an inactive protoxin that must be proteolytically cleaved to give active toxin. Assaying the hemolytic activity especially at early phases of bacterial growth does not accurately reflect expression of the gene because extracellular proteases that cleave the protoxin are only produced at later stages of growth. In order to facilitate our studies on the regulation of gene expression we constructed a mutant strain, designated AB3-34, where the resident aerolysin gene was replaced by an aerolysin-alkaline phosphatase mutant allele. The mutated aerolysin allele consisted of 46 amino acids from the N-terminal end of aerolysin fused to a signal-peptide truncated alkaline phosphatase gene of Escherichia coli (9). This fusion is secreted as efficiently as aerolysin (B. Huhle and T.C., manuscript in preparation). Since C-terminal processing of the fusion protein is not required for the expression of alkaline phosphatase activity in the mutated strain AB3-34, this activity provided a sensitive assay for monitoring expression of the aerolysin gene.

When the strain AB3-34 was grown in LB medium with increasing amounts of the metal chelator 2',2'dipyridyl 3 fold higher alkaline phosphatase activity was obtained as compared to those cultures grown in LB alone. To further investigate the role of iron in the regulation of aerolysin expression we studied its effects on cultures growing in a defined low-iron media. When strains were grown in MOPS minimal media containing glucose, only low levels of alkaline phosphatase activity was detected. Addition of increasing amounts of iron to the media showed little effect on this already low level of alkaline phosphatase activity. However, when strains were grown in minimal media supplemented with glycerol, alkaline phosphatase activities were 10 fold higher than those obtained in glucose supplemented media. This occurred despite a three fold longer generation time for cells grown in the poore media. The alkaline phosphatase activity in glycerol growing cells was extremely sensitive to added iron. It decreased with increasing iron concentrations, and was almost entirely abolished when 100uM iron was present in the media. These experiments revealed that aerolysin expression in strain AB3 is subject to glucose inhibition.

Aeromonas spp. are natural inhabitants of fresh water

environments and it was therefore of interest to study the effects on the expression of the fusion protein in AB3-34 under conditions that may exist in such environments. No differences in expression was observed when strains were grown in complex media with different starting pH values. When AB3-34 was grown in complex media containing different salt concentrations , expression of the aerolysin-alkaline phosphatase fusion was found to increase with increasing salt concentrations of the growth media. The activity was optimal at about 170mM NaCl, and was more than three fold higher compared to strains grown in media containing no NaCl. The effect was specific with respect to the salinity of the media and was not observed when other osmoactive substances such as non-metabolizable sugars were used. There was no effect on the expression of the gene fusion when bacteria were grown at different growth temperatures between 20 and 40^{0}C.

The results presented here show that the aerolysin gene is subject to regulation by certain nutritional and physical conditions that may prevail in the diverse ecological niches where Aeromonas bacteria are found. The optimal salinity for aerolysin production in A. sobria AB3 is also the optimal concetration at which it grows. Significantly, this optimum is close to the concentration of serum which is about 150mM. This result coupled with the observation that iron-deprivation induces aerolysin production suggests that it is in this host compartment where aerolysin is most effectively expressed.

Many of the biological properties of aerolysin can be attributed to the insertion of the aerolysin molecule into the membrane of the target cell. To study the nature of the transmembrane pores generated, we purified aerolysin and analyzed the pores formed in planar lipid bilayers. When purified aerolysin (10-100ng/ml) was applied to voltage clamped lipid bilayersconsisting of a 1% solution of phytoanoylphosphatidylcholine in n-decane, current steps of homogenous size were observed after a few minutes indicating the formation of ion channels in the membrane. The time course of increase of the increase was gradu. suggesting thaat pores of homogenous sizes were formed. The conductivity measurements led to an estimate of effective pore diameter of at least 2nm. The channel formed was stable and zero-current membrane-potential experiments revealed that the aerolysin channel is anion selective. Since aerolysin is capable of insertion into bilayers of pure phosphatidylcholine, it would appear that surface receptors on the eucaryotic cell are not essential for its action. Aerolysin can generate stable transmembrane pores that could allow flux of ions and small molecules across a bilayer membrane. As a result of the lesions, cytolysis of cells that are devoid of membrane turnover and repair such as erythrocytes will occur. Nucleated cells are in general capable of repair of a limited number of lesions, but

transiently formed ion fluxes may nevertheless trigger secondary responses, such as the release of inflammatory mediators from target cells, which are of pathophysiological relevance in the infected host.

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Concomitant Control of Expression of Staphylococcus aureus Beta-Toxin, Enterotoxin A and Fibrinolysin Mediated by Serotype F Lysogenic Converting Bacteriophages

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SUMMARY

The mechanism of triple-lysogenic conversion of Staphylococcus aureus β-toxin, enterotoxin A and fibrinolysin by a newly found group of serotype F bacteriophages was determined, and compared with the mechanism of β-toxin and fibrinolysin double-conversion caused by the serotype F phage, φ13. In the case of the former, the determinants encoding enterotoxin A (*ent A*) and fibrinolysin (*sak*) were linked in the phage DNA near the attachment site (*att P*). The *sak* determinant of φ13 was also located near *att P*. For both types of phage β-toxin conversion was due to the orientation-specific integration of phage DNA into the β-toxin genetic determinant.

KEYWORDS: Staphylococcus aureus. Lysogenic conversion. Enterotoxin A. Beta-toxin. Fibrinolysin. Serotype F bacteriophage.

INTRODUCTION

Only a minority of human S.aureus isolates produce β-toxin, whereas the majority produce fibrinolysin (7,12). The β-toxin-negative (Hlb$^-$), fibrinolysin-positive (Sak$^+$) phenotype in the majority of cases is directly associated with the carriage of lysogenic converting phages (13). The most frequently encountered of these phages are serotype F double-converting phages, which mediate the concomitant loss of expression of β-toxin (negative-conversion) and acquisition of the capacity to express fibrinolysin (positive-conversion) (7,13). Some serotype B phages mediate positive-conversion for fibrinolysin expression only

(7), whereas other serotype A phages mediate negative-conversion for β-toxin expression only (3). Converting phages which mediate positive-conversion for enterotoxin A expression have also been described (1,2). However, these reports did not describe which serotype these phages belonged to and whether they affected expression of other S.aureus characteristics.

Positive-conversion for fibrinolysin and enterotoxin A expression, respectively, has been shown to be due to the location of the respective genetic determinants in the DNA of converting phages, which are expressed by lysogenic S.aureus derivatives (1,7). This has been established only for a single phage in respect of fibrinolysin . Previous studies from my laboratory demonstrated that negative-conversion of β-toxin expression mediated by ϕ13, the serotype F, double-converting (β-toxin and fibrinolysin) phage, and by ϕ42E, the serotype A, single-converting (β-toxin) phage , was caused by insertional inactivation of the chromosomally located β-toxin determinant. Phage genomic DNA was insertionally integrated during lysogen formation (3). Casman (2) reported the isolation of a carried phage from S.aureus strain PS42-D, which mediated positive-conversion for enterotoxin A expression. However, Kondo & Fujise (7) reported that PS42-D carried a serotype F double-converting (β-toxin and fibrinolysin) phage. The possibility that these carried phages of strain PS42-D are the same or similar, and thus, are in fact triple-converting was recently investigated in this laboratory (4). These studies demonstrated that strain PS42-D indeed harboured a serotype F carried phage mediating the coincident triple-lysogenic conversion of β-toxin (negative-conversion), fibrinolysin (positive-conversion) and enterotoxin A (positive-conversion). Studies also revealed that serotype F triple-converting phages, mediating conversion of the same three properties, were prevalent in methicillin-resistant S.aureus (MRSA) isolates recovered from cases of nosocomial infection in Irish hospitals between 1971-1988 (4,5).

The present study was undertaken to determine and compare the molecular mechanisms of double- and triple-conversion mediated by serotype F bacteriophages.

MATERIALS AND METHODS

The bacterial strains and phages used are shown in Table 1.

DNA techniques: Purification of chromosomal, plasmid and bacteriophage DNA and transformation of protoplasts of S.aureus strain RN4220 was performed as described by Coleman *et al.* (3). Molecular cloning, restriction mapping, transformation of Escherichia coli, agarose gel electrophoresis and hybridization analysis were carried out as described by Maniatis *et al.* (11). Enterotoxin A, β-toxin and fibrinolysin assays: These were performed for S.aureus isolates and E.coli derivatives harbouring recombinant plasmids as described (3,4). Clinical and carriage S.aureus isolates: 212 clinical S.aureus isolates, including 102 MRSA isolates, which were recovered from cases of infection in Irish hospitals between 1971-1988 and 108 isolates from the anterior nares of separate,

healthy individuals were screened for production of β-toxin, enterotoxin A and fibrinolysin.

Western immunoblotting: Polypeptides were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12.5% (w/v) gels and were electrophoretically transferred onto nitrocellulose filters, which were processed as described (3,4). Production of enterotoxin A by E.coli strain DS410 harbouring recombinant plasmids was detected by colony immunoblotting as described (4).

Cloning vectors: Plasmids pBR322 and pAT153 (11) were used in molecular cloning experiments. The shuttle plasmid pDC020 (3) which can replicate in E.coli and S.aureus was used as a vector to transfer cloned phage DNA fragments into S.aureus.

RESULTS AND DISCUSSION

The vast majority of the hospital and nasal carriage strains produced fibrinolysin (Sak^+) and were β-toxin deficient (Hlb^-), in agreement with previous reports (7,12). The Hlb^-, Sak^+ phenotype exhibited by 10 representative carriage and 10 non-methicillin-resistant hospital isolates was found to be due to the carriage of serotype F double-converting phages. Recent studies from this laboratory demonstrated that the Hlb^-, Sak^+, enterotoxin A-positive ($Ent\ A^+$) phenotype exhibited by representative methicillin-resistant isolates (MRSA) was due to the carriage of serotype F triple-converting phages (4).

Strain/phage	Phenotype/genotype	Comments	Source/reference
80CR3	$Hlb^+Ent\ A^-\ Sak^-$	Restriction impaired strain	(4)
13CR3-L	$Hlb^-\ Ent\ A^-\ Sak^+$	80CR3 lysogenized with φ13	This study
42CR3-L	$Hlb^-\ Ent\ A^+\ Sak^+$	80CR3 lysogenized with φ42	(4)
A1CR3-L	$Hlb^-\ Ent\ A^+\ Sak^+$	80CR3 lysogenized with φA1	(4)
A3CR3-L	$Hlb^-\ Ent\ A^+\ Sak^+$	80CR3 lysogenized with φA3	(4)
φ13	*sak*$^+$	Serotype F double- converting phage	(3,4)
φ42	*sak*$^+$, *ent A*$^+$	Serotype F triple-converting phage	(4)
φA1	*sak*$^+$, *ent A*$^+$	Serotype F triple-converting phage	(4)
φA3	*sak*$^+$, *ent A*$^+$	Serotype F triple-converting phage	(4)

Table 1. S.aureus strains and phages.

These studies confirmed that serotype F converting phages were prevalent among human S.aureus isolates and that the capacity of these organisms to express or not to express β-toxin, fibrinolysin and enterotoxin A, respectively, was directly associated with the carriage of lysogenic prophages.

Molecular analysis of positive-conversion for fibrinolysin and enterotoxin A expression: A detailed description of these experiments has been described recently (4). In separate experiments, *Hin*dIII-generated fragments of genomic DNA of phages ϕ13, ϕ42, ϕA1 and ϕA3 (Table 1) were cloned in Escherichia coli strain DS410 (Table 1). Recombinants expressing fibrinolysin activity were detected on fibrin agar plates. Analysis of the cloned DNA harboured by representative Sak+ recombinants revealed that each contained a single *Hin*dIII fragment which corresponded to a *Hin*dIII-generated fragment of the respective parental phage genomic DNAs.

E.coli DS410 recombinants harbouring cloned *Hin*dIII fragments of phages ϕ42, ϕA1 and ϕA3 were screened for enterotoxin A production by colony immunoblotting with specific anti-enterotoxin A serum. Putative Ent A+ recombinants were tested for enterotoxin A production by Western immunoblotting of polypeptides from 50-fold concentrated cell lysates, following SDS-PAGE gel electrophoresis. In each case, a single polypeptide of 27 kDa was detected, which corresponded in molecular mass to purified staphylococcal enterotoxin A. Analysis of the cloned DNA of representative Ent A+ recombinants revealed that each harboured a single *Hin*dIII fragment of 2.5 kb which corresponded in size to a single *Hin*dIII fragment of the respective native phage DNAs.

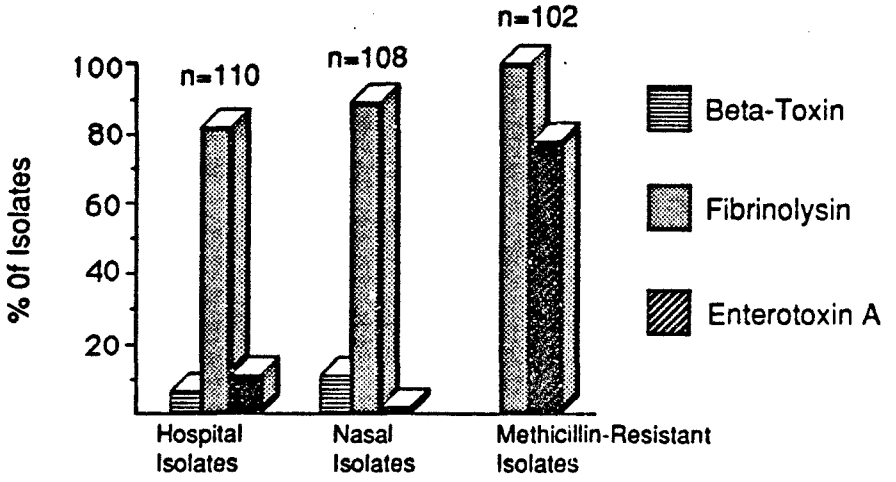


Figure 1. Production of β-toxin, fibrinolysin and enterotoxin A by S. aureus isolates.

No detectable homology was observed when *Hin*dIII-cleaved ϕ13 DNA (double-converting) was probed with the 2.5 kb *Hin*dIII fragment containing the cloned enterotoxin A determinant (*ent A*) from ϕ42, indicating that ϕ13 did not encode an *ent A* determinant. Expression of the cloned fibrinolysin determinants (*sak*) of ϕ13, ϕ42, ϕA1 and ϕA3 and the cloned *ent A* determinants of phages ϕ42, ϕA1 and ϕA3 was readily detected in S.aureus following transformation of protoplasts of S.aureus strain RN4220 (4) with derivatives of shuttle vector pDC020 containing the cloned *sak* - and *ent A* - containing *Hin*dIII fragments, respectively.

These findings indicated that the mechanism of positive-conversion for fibrinolysin and enterotoxin A expression, respectively, mediated by the double- and triple-converting phages was due to the presence of the *sak* and *ent A* (in the case of ϕ42, ϕA1 and ϕA3) determinants in the genomic DNA of the respective phages.

Mechanism of negative-conversion of β-toxin mediated by triple-converting phages: This was determined by comparing the structure of the β-toxin determinant (*hlb*) of S.aureus strain 80CR3 with lysogenic derivatives. A detailed description of these studies has been reported recently (4). The *hlb* determinant of 80CR3 is located on a 2.2 kb *Hin*dIII fragment and is homologous with, and has an identical structure to the *hlb* determinant cloned in plasmid pDC007 (Fig. 2), which was used as the source of a *hlb* probe (3). A single fragment of 2.2 kb hybridized in *Hin*dIII-cleaved 80CR3 genomic DNA but this was replaced by two junction fragments in the DNA of the lysogens 42CR3-L, A1CR3-L and A3CR3-L (Table 1; Fig. 3, panel A). Similar results were obtained with *Hin*dIII-cleaved DNA of lysogen 13CR3-L (Table 1; Fig. 3, panel A). The smaller junction fragment was identical in size (1.7 kb) for all of the lysogens whereas the larger junctions varied in size for three of the four lysogens (Fig. 3, panel A). These experiments were repeated with three independently generated lysogens for each phage and identical results were obtained in each case.To confirm these findings, the attachment site-(*att P*) containing *Hin*dIII fragments of ϕ13 ϕ42, ϕA1 and ϕA3 were cloned and used to probe *Hin*dIII-cleaved DNA of the corresponding 80CR3 lysogens. In each case, two junction fragments identical in size to those detected with the *hlb* probe were detected for each lysogen (Fig. 3, panels B & C).

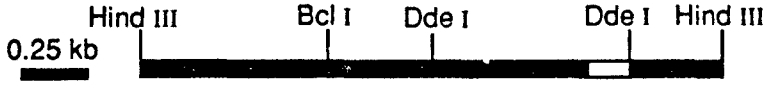


Figure 2. Restriction map of the 2.2 kb *Hin*dIII fragment of plasmid pDC007 (3) which encodes a cloned S.aureus β-toxin determinant (*hlb*), and which was used as a *hlb* probe.The unshaded portion of the figure refers to a 0.15 kb sequence to which the insertion site of phages ϕ42, ϕA1, ϕA3 and ϕ13 was localized in the corresponding genomic DNA of S.aureus strain 80CR3.

These results demonstrated that the mechanism of β-toxin conversion mediated by the triple-converting phages and ϕ13 was due to insertional inactivation of the *hlb* determinant following lysogenization.

Recent studies have shown that the genomes of phages ϕ42, ϕA1, ϕA3 and ϕ13 consist of linear molecules with cohesive termini which can anneal to form circular molecules (4). Because the sizes of the small junction fragments detected in the DNA of the lysogens were identical and consistent, it is likely that these phages integrated at the same or closely linked sites in the bacterial chromosome in one orientation only. Similar findings have been described for the S.aureus phages L54a and ϕ11 (9,10).

Organization of *sak*, *ent A* & *att P* sequences in phage genomic DNA: A restriction map of the triple-converting phage ϕ42 is shown in Fig. 4. The position of the *ent A* -, *sak* - and *att P* -containing regions were established by cloning and subcloning experiments and by hybridization analysis as described in detail elsewhere (4). The *ent A* and *sak* determinants were found to be closely linked in the phage genomic DNA adjacent to the phage attachment site, *att P*. Similar findings were obtained for the triple-converting

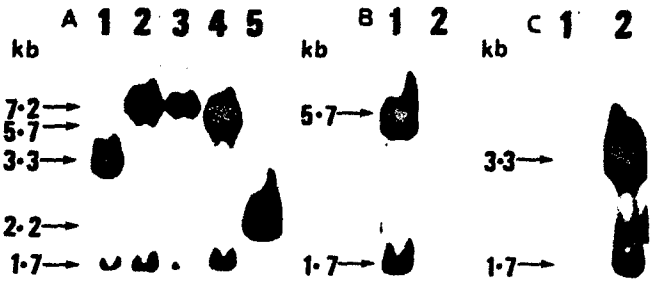


Figure 3. Hybridization analysis of restriction-enzyme *Hin*dIII-digested chromosomal DNA of S.aureus strain 80CR3 and lysogenized derivatives. The source of DNA in each track with phages harboured by individual strains shown in parenthesis is as follows: **panel A** track 1, 13CR3-L (ϕ13); track 2, A1CR3-L (ϕA1); track 3, A3CR3-L (ϕA3); track 4, 42CR3-L (ϕ42); track 5, 80CR3 (phage-free); **panel B** track 1, 42CR3-L (ϕ42); track 2, 80CR3 (phage-free); **panel C** track 1, 80CR3 (phage-free) ; track 2,13CR3-L (ϕ13) . DNA in the panels was hybridized with the following probes: **panel A**, 2.2 kb *hlb-Hin*dIII fragment of plasmid pDC007 (Fig. 2); **panel B**, 5.0 kb *att P-Hin*d III fragment of ϕ42 (4); **panel C**, 2.9 kb *att P-Hin*dIII fragment of ϕ13 (4).

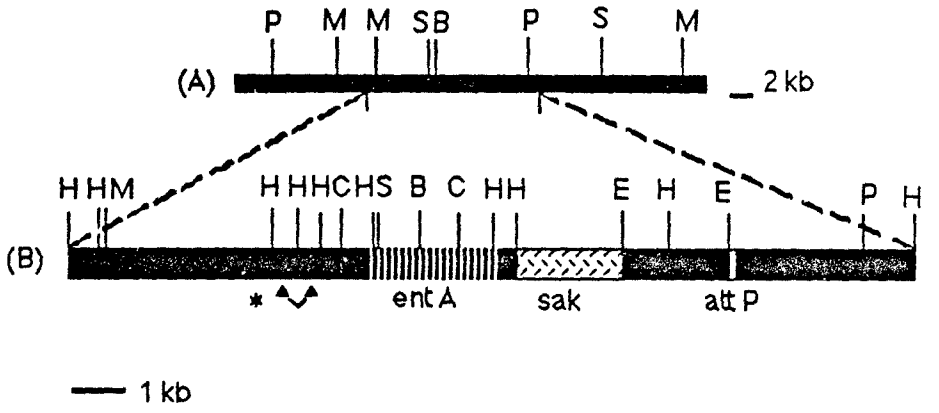


Figure 4. **(A)** Restriction map of linear ϕ42 genome, which is represented as a rectangular box. **(B)** Restriction map of a 17.4 kb DNA region from within the ϕ42 genome, which is represented by the large rectangular box.. The DNA sequences to which the *ent A* (vertical shading) and *sak* (cross-hatched shading) determinants were localized are shown. The small unshaded area represents a 0.15 kb DNA sequence to which *att P* was localised. Abbreviations: P, *Pst* I; M, *Mlu* I; S, *Sal* I; B, *Bgl* I; E, *Eco*RI; H, *Hin*dIII; C, *Cla* I.* The juxtaposition of the two small *Hin*dIII fragments was not determined.

phages ϕA1 and ϕA3 (Fig 5). In the case of the double-converting phage ϕ13, the *sak* determinant was also encoded in close proximity to *att P* (Figure 5).

These findings suggested that both the triple- and double-converting phages may have acquired their *ent A* and *sak* determinants, respectively, by imprecise excision events between chromosomally located *sak* and *ent A* determinants and closely integrated prophages. Similar mechanisms have been suggested to explain the findings that corynephage β and Streptococcus pyogenes phage T12 encode the genes for diphtheria toxin and erythrogenic toxin near to their respective *att P* sites (6,8).

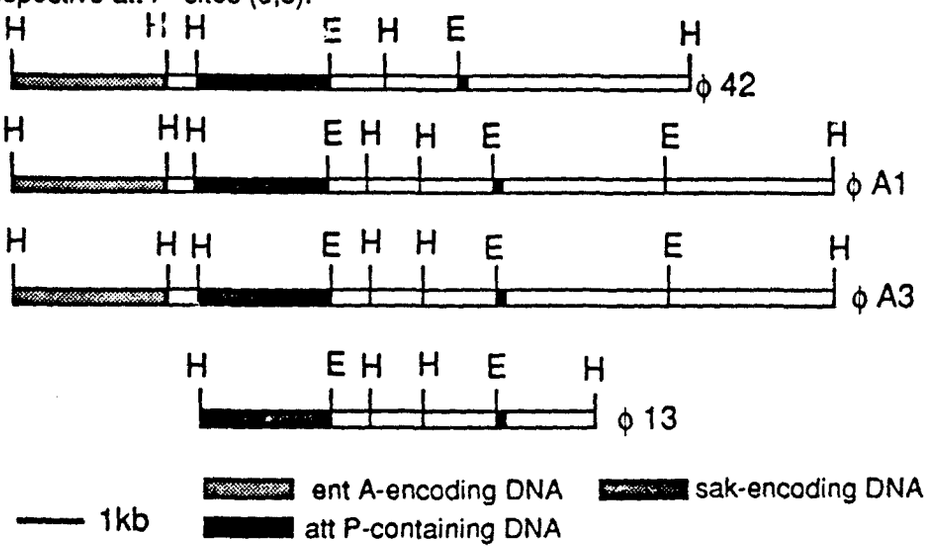


Figure 5. *Eco*RI and *Hin*dIII restriction maps of the *ent A* -, *sak* -, and *att P* -encoding DNA sequences of the triple-converting phages ϕ42, ϕA1 and ϕA3 and the *sak* - and *att P* -encoding DNA sequences of the double-converting phage, ϕ13. The 2.5 kb *Hin*dIII fragments to which the *ent A* determinants were localized and the 2.2 kb *Eco*RI-*Hin*dIII fragments to which the *sak* determinants were localized are shown. For each phage *att P* was localized to a 0.15 kb sequence of DNA.

The phenomenon of triple-conversion and the associated concomitant control of expression of three S.aureus virulence determinants raises the possibility of the introduction of new virulence factor combinations into clinically significant S.aureus isolates by phage conversion. This may contribute significantly to the generation of more virulent derivatives. In this light, the prevalence of these phages in methicillin-resistant S.aureus (MRSA) isolates may be particularly significant, due to their importance as nosocomial pathogens, world-wide. Indeed, recent studies on septicaemic isolates from this laboratory revealed that enterotoxin-producing MRSA were significantly associated with patient mortality (5).

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A Genetic Approach to Demonstrate the Role of Listeriolysin O in the Virulence of Listeria monocytogenes

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ABSTRACT

The gene coding for listeriolysin O, hlyA, was shown to be the target of a transposon which inhibited the hemolytic phenotype and the virulence of Listeria monocytogenes. Transcriptional mapping and DNA sequence analysis of the region containing hlyA revealed that hlyA is a monocistronic unit and that the transposon inserted in the structural gene unlikely exerted a polar effect on adjacent genes. In addition, when a transposon-induced, non hemolytic and avirulent mutant of Listeria monocytogenes was transformed with a plasmid carrying the wild type gene, it recovered both the wild type hemolytic phenotype and the virulence. These data demonstrate that listeriolysin O is essential for the virulence of Listeria monocytogenes.

INTRODUCTION.

Listeria monocytogenes is a facultative intracellular bacterium responsible for severe infections in human and animals (16, 17). Its virulence is generally attributed to its capacity to survive and replicate inside macrophages (11). Although the macrophage is the main cell involved in the in vivo multiplication of Listeria, a number of other cell types such as fibroblasts and epithelial cells have been successfully infected in vitro (3, 6, 10, 14) and it was demonstrated in the case of the human enterocyte like

Caco2 cells that after phagocytosis, bacteria leave the phagosome and multiply within the cytosolic compartment of these host cells. Therefore, identification of the factors which allow bacteria to invade cells, to escape from the phagosome compartment or to multiply intracellularly is therefore essential to understand the virulence of the organism.

The observation that all strains of L. monocytogenes isolated from natural infections produce a zone of hemolysis on blood agar medium and are virulent in the mouse model, whereas non hemolytic strains isolated after mutiple subcultures (7) or from the environment are avirulent, first suggested that a hemolysin might be a relevant factor (15). The hemolytic factor, listeriolysin O has been purified (5) and shown to belong to the family of thiol activated toxins. Interestingly, among these cytolysins, listeriolysin O is the only one to be produced by an intracellular bacterium and to be active at acid pH. Recently, genetic and physiopathological studies using avirulent mutants obtained by transposon mutagenesis (4, 8, 14) gave strong support to the hypothesis that secretion of a hemolytic exotoxin is a crucial event promoting intracellular growth of L. monocytogenes in host tissues (3, 10, 14). This conclusion was based on the finding that non hemolytic mutants were avirulent in the mouse, whereas virulence was restored in hemolytic revertant strains obtained by spontaneous loss of transposons. Moreover, non hemolytic mutants invade different cell lines, as efficiently as the wild type (3, 10, 14) but in most cases, do not survive or replicate intracellularly. Since only hemolytic bacteria escape from the phagosome compartment into the cytosol of Caco2 cells, it was proposed that listeriolysin O mediates virulence by damaging the phagosome membrane (3).

Localization of transposon insertions within hlyA,the gene coding for listeriolysin o in several non-hemolytic, avirulent mutants (12, 1, 8) strongly suggested that this region of the chromosome is crucial for virulence. However , these data did not rule out the possibility that disruption of hlyA exerted a polar effect on genes adjacent to hlyA that could be involved in the expression of other virulence factors required for entry in the host and intracellular survival and multiplication. We report here two types of approaches which show that listeriolysin O is essential for virulence.

RESULTS

Transcriptional mapping, RNA sizing and DNA sequence analysis reveal that hlyA is a monocistronic unit.

We previously reported the DNA sequence of hlyA (13). The gene had first been identified after cloning the region proximal to Tn1545 from a non-hemolytic mutant (12, 1). This transposon had inserted in an open reading frame (ORF) whose deduced sequence was highly homologous to streptolysin O (SLO) and pneumolysin (PLY) (9). This cloned fragment was then used to localize on a cosmid (21) the listeriolysin O gene, then called hlyA (12). The deduced sequence of the protein confirmed that listeriolysin O belongs to the class of thiol activated cytolysins (18). The sequence of a fourth SH-activated hemolysin, perfringolysin (PLO) has recently been reported (20). It is also very similar to SLO, PLY and LLO and contains the conserved undecapeptide E-C-T-G-L-A-W-E-W-W-R (Figure1).

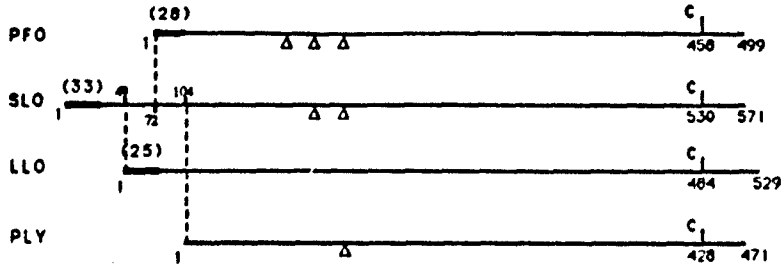


Fig. 1. Schematic representation of the four known thiol-activated hemolysins.

As spontaneous deletions had occurred on the original cosmid, a 3454bp BamHI fragment containing hlyA was recloned in pUC18, giving rise to pLis3. Using as a probe an oligonucleotide internal to the hlyA gene, a 1886bp EcoRI fragment containing part of hlyA and its upstream region was then cloned, giving rise to plasmid pLis8. Inserts of pLis3 and pLis8 span 4223bp of the hlyA region. The DNA sequence of this region was determined and revealed the presence of two open reading frames adjacent to hlyA, one ORF D is located 331bp downstream from hlyA and the other ORF U is located 224bp upstream from and in opposite direction to hlyA. Promoter mapping performed with RNAs extracted from cells growing exponentially in rich medium, showed that the three ORFs are transcribed independently. hlyA appears transcribed from two promoters separated by ten basepairs and located in the intergenic hlyA-ORF U region. ORF U is transcribed in the opposite direction from an adjacent promoter. Finally, ORF D is transcribed from a promoter located downstream from hlyA (Figure2).

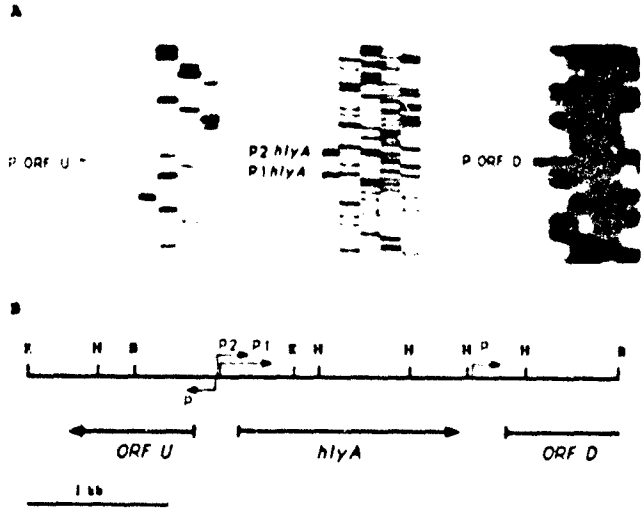


Fig. 2. Transcriptional map of the hlyA region.

Northern blot analysis indicated that hlyA transcripts were of a size expected if they started at the identified promoter region and if they ended at the terminator like structure identified 50 basepair downstream from hlyA.These data strongly suggest that hlyA is a monocistronic unit and that the transposon insertions in hlyA unlikely exert a polar effect on adjacent genes expression.

Direct evidence for the role of listeriolysin O by gene complementation.

In order to evaluate the role of listeriolysin in virulence, we also performed a gene complementation experiment. For this purpose, from an easily transformable virulent strain of L. monocytogenes, a new non hemolytic mutant was constructed by means of transposon Tn917. This strain, as other non hemolytic mutants previously isolated (4, 8, 14), was avirulent in the mouse. The single copy of Tn917 was inserted after codon 503. This strain LO28 (hlyA::Tn917) as well as its parental strain, LO28, was then transformed with a shuttle plasmid, pLis4 containing only the listeriolysin O gene or with the vector, i. e. pMK4 (19). Results are summarised in Table I.

TABLE I

Hemolysin Production and Virulence in the Mouse.

STRAIN	HEMOLYTIC TITER	LD_{50}
LO28	900 HU/ml	$10^{5.2}$
LO28 (pMK4)	1000 HU/ml	$10^{6.5}$
LO28 (pLis4)	900 HU/ml	$10^{6.8}$
LO28 (*hlyA* ::Tn*917*)	< 10 HU/ml	$10^{9.5}$
LO28 (*hlyA* ::Tn *917* , pMK4)	< 10 HU/ml	$10^{9.5}$
LO28 (*hlyA* ::Tn *917* , pLis4)	1200 HU/ml	$10^{6.8}$

Strain LO28 (hlyA::Tn917, pLis4) displayed a stable hemolytic phenotype identical to the wild type: its maximal hemolytic titer in the culture supernatant did not significantly differ from that of strains LO28, LO28 (pMK4) or LO28 (pLis4) taken as controls. The result obtained in the last case as well as in the case of the mutant complemented with pLis4 was somewhat intriguing since presence of a multicopy plasmid carrying the hemolysin gene does not increase the hemolytic titer. It seems, at least in the case of strain LO28, that a maximum level of hemolytic activity is reached regardless of the number of copies of the hlyA gene, implying that hemolysin production must be precisely regulated. It is indeed already known that there is an inverse correlation between iron concentration and hemolysin production (2). Other factors are probably also involved.

Assays for virulence were performed by i.v. infection of Swiss mice.The hypothesis that the presence of a plasmid in strain LO28 might affect virulence, was first investigated by analyzing the virulence of strain LO28 harboring pMK4 or pLis4. Indeed, introduction of either one of the two plasmids in LO28 significantly reduced the level of virulence: the LD_{50} values dropped by more thant 1.0 log unit, in both LO28 (pMK4) and LO28 (pLis4), with a parallel reduction of their replicating capacity in the spleen and liver of infected mice. It appears that presence of a plasmid impairs bacterial multiplication. It is likely that under the stress conditions encountered in hos. issues, bacterial survival and replication involve activation and repression of several genes, a mechanism which is apparently affected by the presence of a pl. id. As in the case of other mutants already described (4, 8, 14), the non hemolytic mutant LO28 (hlyA::Tn917) and its transformant derivative LO28 (hlyA::Tn917, pMK4) were avirulent in the mouse. Even at high infecting doses (108 per mouse), bacteria were rapidly eliminated from the spleens and the livers within 48 h. In contrast, the capacity to grow in these host tissues was restored in the complemented strain LO28 (hlyA::Tn917, pLis4). Virulence estimated by LD50 values was expressed at the same level as that of the control strains LO28 (pMK4) and LO28 (pLis4). At high infecting doses strain LO28 (hlyA::Tn917, pLis4) induced visible abscesses in liver and spleen and ultimately killed mice. On the basis of the antibiotics resistance pattern and analysis of the DNA, it was verified that the plasmids were stably maintained in vitro and in vivo.

In conclusion, our results show that expression of the hemolysin gene was necessary and sufficient to restore virulence in a non hemolytic mutant. One can conclude that the transposon in the Tn917 insertion non hemolytic mutant did not exert any polar effect on adjacent genes and that hlyA was the only gene affected by the insertion. It is also probably the case for other non hemolytic mutants previously isolated (4, 8, 14).

CONCLUSIONS

The implication of this genetic study is that the hemolysin gene product is absolutely required for the virulence of L. monocytogenes. This supports the hypothesis that production of a cytolysin inside the phagolysosomal compartment, would allow bacteria to escape this hostile intracellular environment to freely multiply in the cytosol (3). Listeriolysin O now appears as the first key factor for intracellular growth identified at the molecular level, in L. monocytogenes. Detection of sequences homologous to the hlyA region in virulent and avirulent species of the genus Listeria and presence of unusual structural features in the promoter regions of hlyA, ORF U and ORF D (our unpublished results) are an indication that the in vivo expression of hlyA in Listeria monocytogenes must be precisely regulated.

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AGR Regulated Alpha Toxin Promoter of Staphylococcus aureus

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INTRODUCTION

In S. aureus, a system termed the Accessory Gene Regulator (AGR), acts in trans to coordinately control transcription of a variety of unlinked virulence genes (1,2). Although the mechanism is not understood, it is known that at the end of exponential phase in batch cultures AGR represses expression of certain secreted proteins (e.g. Protein A) and acts as a positive regulator to turn on expression of others (e.g. alpha-toxin). Here we summarise preliminary studies aimed at identifying the target for AGR regulation on an alpha-toxin gene (hla), cloned in the E. coli - S. aureus shuttle plasmid pDU1212 (Fig. 1).

RESULTS AND DISCUSSION

Expression of the cloned hla gene in pDU1212 has previously been shown to be under AGR control in S. aureus (3,4). This plasmid includes ca. 1.8 kb of S. aureus DNA 5' to the hla gene (Fig. 1). The sequence of the hla gene and 330 bp of the adjacent region, extending to the 5' Cla I site in Fig. 1, have been described previously (5). The sequence of the remaining 1.4 kb region between the Hind III and Cla I sites was determined in this study. To locate the sequences required for hla expression, pDU1212 was linearised by cleavage at a unique Lsp I site and a nested set of Bal 31 deletion mutants were constructed, introduced into S. aureus and tested for their abilities to express the alpha-haemolysin (Fig. 1). These mutants showed that most of the 5' sequences between the Hind III and Cla I sites could be deleted without reducing hla expression. The endpoint of deletion 34, which was located precisely by sequencing, defined a 89 bp region immediately proximal to the Cla I site that is required for hla expression in S. aureus (Fig. 1). Deletions extending to or beyond the Cla I site are Hla$^-$. This is consistent with Northern Blots which showed that probes 3' to the Cla I site readily hybridize to Hla mRNA, whereas probes from the 5' side of the Cla I site did not. These experiments suggested that the transcriptional start for the Hla message is located very close to the Cla I site, approx. 330 bp 5' to the hla gene.

To locate the start of the hla transcript more precisely, reverse transcriptase and a primer corresponding to sequences ca. 50 bp downstream of the Cla I site were used in primer extension mapping with RNA isolated from late exponential phase cultures of Agr$^+$ S. aureus expressing the hla gene. The extended primers were sized by comparison with a sequencing reaction employing the same primer. The highest band obtained corresponds to a G, located 3 bp 5' to the Cla I site (Fig. 1) and is consistent with preliminary S_1 mapping experiments. This places the AGR regulated alpha-toxin promoter within 86 bp of the endpoint of deletion 34. Perhaps surprisingly for a regulated promoter, the putative transcriptional start aligns with reasonable consensus -10 and -35 sequences, but there is no evidence that these are involved in AGR controlled expression. A detailed analysis of this region is in progress, in order to identify the sequences required for AGR control.

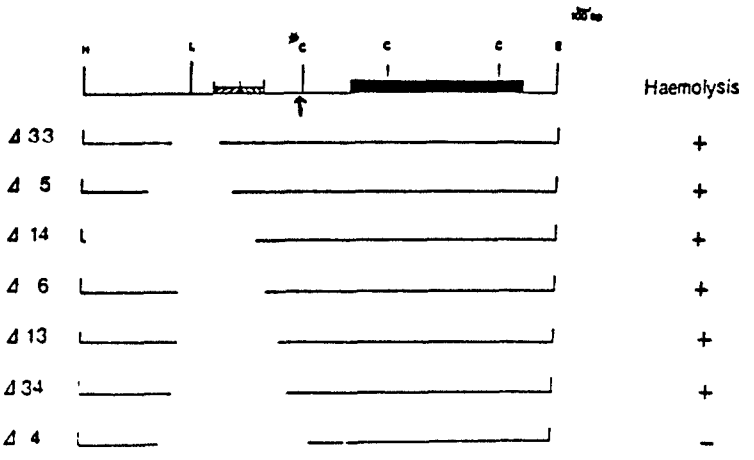


Fig. 1 : Preliminary mapping of Alpha-toxin promoter.

The upper line represents the cloned S. aureus sequences in pDU1212 and the hla gene is depicted by the black box. Selected Bal 31 deletion mutants are depicted by the lower lines. Cleavage sites for restriction endonucleases are: H Hind III; L Lsp I; C Cla I and E EcoR I. The 5' Cla I site referred to in the text is marked with a *. The arrow indicates the putative transcriptional start for the Hly message.

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Expression of Plasmid-Borne Pneumolysin Gene in Wild-Type and Pneumolysin-Negative Pneumococci

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INTRODUCTION

Pneumolysin, a "thiol-activated" cytolytic protein, is produced by virtually all strains of Streptococcus pneumoniae. The experiments described below were intended to yield hyper-producers of this toxin and to investigate possible mechanisms of regulation of pneumolysin production.

METHODS

Growth of Bacteria

Pneumococci were grown in peptone-yeast extract-glucose media supplemented, when necessary, with 10 ug/ml erythromycin. Crude extracts were prepared by sonication.

Isolation of Pneumolysin-negative Strains

Nitrosoguanidine mutagenesis was used to produce pneumolysin-negative mutants, which were scored on Brucella-horse blood agar plates containing 0.015 U/ml of penicillin and incubated anaerobically (1).

Transformation of Pneumococci

Cells were rendered competent by the method of Yother et al. (2).

Cloning of Pneumolysin Gene

From a library of pneumococcal chromosomal DNA in EMBL 3 . bacteriophage, phage carrying the pneumolysin gene was detected by overlaying the plaques with a layer of sheep blood agar. The gene was sub-cloned in several steps, using the low-copy number vector pLG339 at an intermediate step and pUC 18 in the final steps (pMK2).

Construction of Recombinant Shuttle Vector
Shuttle plasmid pVA838 was digested with PvuII and blunt-end ligated to the 2 kb fragment bearing the pneumolysin gene, which had been isolated from the recombinant plasmid pMK2 after digestion with PvuII.

Plasmid Isolation
Plasmids were isolated from pneumococci with a modification of the Birnboim-Doly method which involved lysing the cells with 0.1 % deoxycholate.

RESULTS

Construction of Deletions
In vitro deletions were obtained by digestion of the recombinant plasmid bearing the pneumolysin gene with AccI.
The ends were filled using Klenow fragment, blunt-end ligated, and used to transform E. coli JM83. The deleted DNA was introduced into pneumococcal strain R36A (Hly^+) and WU2R (Hly^+) by transformation, followed by selection of Hly^- colonies.

Introduction of Plasmid-borne Pneumolysin Gene into Pneumococci
By plasmid transformation, the shuttle vector bearing the pneumolysin gene was introduced into wild-type pneumococci and pneumolysin-negative mutants. Transformants were selected with erythromycin and tested for hemolytic activity and plasmid content. The transformants were found to contain a plasmid of the expected size (11.2 kb). In the case of Hly^+ strains, presence of the plasmid did not increase the level of hemolysin production. In the case of Hly^- recipients, hemolysin production was restored, but only to the base level. Recombinant plasmids were reisolated from the pneumococcal transformants and transformed back into E coli to confirm that they functioned normally.

CONCLUSIONS

The level of pneumolysin was not increased over the wild-type base level upon introduction of recombinant shuttle plasmid bearing the pneumolysin gene into wild-type and pneumolysin-negative strains. These results may reflect the existence of a negative control determinant on the chromosome, which encodes a factor capable of operating in trans.

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Molecular Cloning of the Listeria monocytogenes DNA Fragment Presenting Strong Hybridization with V. cholerae Toxin Genes

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INTRODUCTION

Listeria monocytogenes is a gram positive microorganism responsible for a variety of diseases of medical and veterinarian importance. After several recent foodborne outbreaks, Listeria monocytogenes has been recognized as a possible cause of diarrheal illness. A recent study has demosntrated DNA:DNA homology between the genes encoding for cholera toxin and a fragment of the Listeria monocytogenes chromosomal DNA (3).

The advanced studies concerning the structure, mode of action, biosynthesis, release, and pathogenic power of Listeria cholera toxin (LCT) will be improved by the isolation and characterization of the Listeria genes containing the DNA sequences homologous with the ctxAB genes of V cholerae.

MATERIALS AND METHODS

The bacterial strains E. coli TG1 and Listeria monocytogenes L028 were previously described (1). The plasmid pJM17 was obtained from Dr. J. Mekalanos (2). Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. Restriction digest and ligation procedures were performed according to the manufacturers recomemmendations. T7 sequencing kit was obtained from Pharmacia. DNA techniques and Southern blot hybridization were as previously described (1).

RESULTS

Southern blot analysis of *Listeria monocytogenes* L028.-

Listeria monocytogenes L028 is a strain that has been previously used in the studies on the genetics of *Listeria* hemolysin. Plasmid pJM17, composed of CT-coding sequences (ctxAB) was used as a probe on dot blots of purified DNA from *L. monocytogenes* L028 strain. Before electrophoresis, chromosomal DNA was restricted by EcoRI and BamHI. A single chromosomal DNA fragment hybridized with the pJM17 probe, which suggest that the *L. monocytogenes* L028 strain contains a DNA sequence that may encode a CT-related toxin.

Cloning of the CT-related genes.-

Strain *L. monocytogenes* L028 DNA, digested with EcoRI was mixed with a preparation of pUC18 that has been previously digested with EcoRI and treated with alkaline phosphatase. The mixture was treated with T4 ligase, introduced in *E. coli* TG1 by transformation and plated on LB plates containing ampicillin (100 µg/ml). Ampicillin-resistant colonies were tested in colony hybridization for the presence of the gene encoding CT-related cholera-toxin by using as a probe a pJM17 fragment (5.1 Kb PstI/EcoRI).

The recombinant plasmid DNA present in the cells of a colony displaying a positive hybridization signal was isolated. Southern blot analysis confirmed that this plasmid, pJC-1 contained a 0.8 Kb EcoRI fragment that hybridized with the 5.1 Kb PstI/EcoRI probe. This probe contain both toxin A and B genes (3).

SEQUENCE HOMOLOGIES BETWEEN LISTERIA TOXIN AND CT-A

The sequence of approximately 70% of the cloned fragment has been determined. We found a G+C% content around 46% in contrast with the previously known G+C % of 36% characteristic of the *L. monocytogenes* chromosome (4).

We have compared the primary structure of ctxA with the parcial sequence of LCT. A 40% homology with the N-terminal part of ctxA was found. The percentage of amino acid homology between both sequences at the NH terminus region is about 39%.

DISCUSSION

The results show the possibility of cloning the genetic determinant of the LCT, a possible factor of the pathogenicity of Listeria, in the strain E. coli TG1. The interest of our work lies in the fact that it offers the possibility of reintroduction of the LCT genes in a non-LCT Listeria recipient strain, which could be investigated for cell of animal models.

We will try to further localize the position of the LCT-A gene or a fragment of this gene on the cloned Listeria monocytogenes DNA complete sequencing analysis. With the datas we have now there is a chance of cloning only a small piece of the gene. Due to this we are now trying to clone a bigger fragment of L. monocytogenes DNA.

The discovery of this homology between the ctxA region of V. cholerae and a fragment of the Listeria monocytogenes chromosome may suggest that: a) this region may contribute to the pathogenicity of Listeria; b) an exchange of toxin genes has occured between gram negative and gram positive organisms.

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The Osteolytic Toxin Gene from Pasteurella multocida is Expressed and Transcriptionally Regulated in Escherichia coli

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INTRODUCTION

Toxin producing Pasteurella multocida is the causative agent of progressive atrophic rhinitis in pigs (1). Diseased animals are characterized by shortened or deformed snouts. This feature is due to an increased nasal bone resorption brought into effect by the P. multocida toxin (PMT) (1). The toxin triggers proliferation of preosteoclasts to osteoclasts (2). We here report the isolation of the toxin encoding gene as well as its expression and regulation in E. coli.

MATERIALS AND METHODS

Strains, media, growth conditions, and general procedures were as previously described (3). Primer extension was performed using the first step in Amersham's (UK) cDNA synthesis kit, and Northern blotting was done according to Williams and Mason (4).

RESULTS

Two toxin producing recombinant E. coli clones were isolated from a chromosomal DNA library of a toxigenic type D strain of P. multocida.
Extracts of the recombinant clones were examined for protein profiles in Western blotting and for biological activity in a cytopathicity test. A protein of 143 kilo dalton specifically recognized by anti-PMT MAbs could be detected in both extracts which furthermore exhibited cytopathic activities

identical to those of P. multocida extracts. Affinity purified recombinant PMT (rPMT) and native PMT behaved identically in a series of quantitative biological tests for cytopathicity, dermonecrosis and lethality as well as in a quantitative ELISA based on combinations of 10 MAbs recognizing different epitopes on PMT (3).
The conservation of the structure of the cloned DNA region was confimed by Southern blotting using P. multocida DNA as a control. An 18 kb DNA probe containing the toxin encoding toxA gene hybridized to DNA from toxin producing isolates of P. multocida but not to DNA from non-producing isolates. DNA from P. multocida was prepared by a chromosomal DNA preparation technique, and by a plasmid DNA preparation technique. No enrichment of the toxA gene could be detected in the last sample, which suggests a chromosomal location of the gene.
Primer extension of toxA RNA from E. coli and P. multocida showed that the native toxin promoter is functional in E. coli. Transcription from this promoter in E. coli was repressed by a P. multocida factor effective in trans, as shown by Northern blotting. The effect of this factor, encoded close to toxA, could be abolished by a frame shift mutation within its putative structural gene.

DISCUSSION

We have isolated the toxin encoding gene from P. multocida in E. coli and named it toxA. The recombinant toxin produced is identical to native PMT and is expressed due to P. multocida regulatory sequences. We have isolated a putative repressor gene close to toxA, and presented data tentatively suggesting that these genes could be located on a mobile genetic element integrated in the Pasteurella chromosome.

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Genetic Organization of the Yersinia yop Regulon

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INTRODUCTION

Growth of yersiniae is restricted at 37°C in the absence of calcium ions. This phenomenon correlates with the massive release of a set of 11 proteins called Yops. Several of the Yops have been shown to be essential virulence determinants. Growth restriction and Yops production are governed by a 70kb plasmid called pYVe. Five yop genes were mapped so far on pYVe227, the natural plasmid from a typical serotype 9 Y. enterocolitica strain. The yop genes constitute a thermoactivated regulon controlled by gene virF. The transcription activator VirF is a 30kDa protein related to the regulators of the arabinose and rhamnose operons (1). The production of the Yops involves other vir genes, also localized on pYV. So far, we identified virA, virB and virC. The loss of any of these genes abolishes the Ca^{2+} dependency phenomenon and the production of the Yops. Exact role of the Yops has not yet been defined. The exact role of Ca^{2+} ions on the release of Yops also remains largely unknown.

RESULTS

1.MAPPING OF YOP GENES USING Tn2507. In order to map more yop genes, we constructed Tn2507, transposon carrying a chloramphenicol acetyl-transferase gene (cat) to be transcribed from external promoters (2). Analysis of insertion mutants allowed to map the genes yop20, yop18 and to unravel the organization of an operon encoding Yop14, Yop11 and Yop37 (figure 1). Any insertion in this operon of pYVe227 makes the Y. enterocolitica host strain unable to grow at 37°C, even in the presence of Ca^{2+}. In the absence of Ca^{2+}, however, these mutants release all the Yops (apart from those encoded by genes downstream from the insertion). The distal mutations in this operon make the host strain not only unable to grow at 37°C but even sensitive to Ca^{2+} at this temperature. These particular phenotypes remain unaccounted for but they suggest that (i) a specific factor could be involved in the restriction of growth and (ii) that this operon is somehow involved in the Ca^{2+} response. Hence, we called it the car operon (for Ca^{2+} regulation).

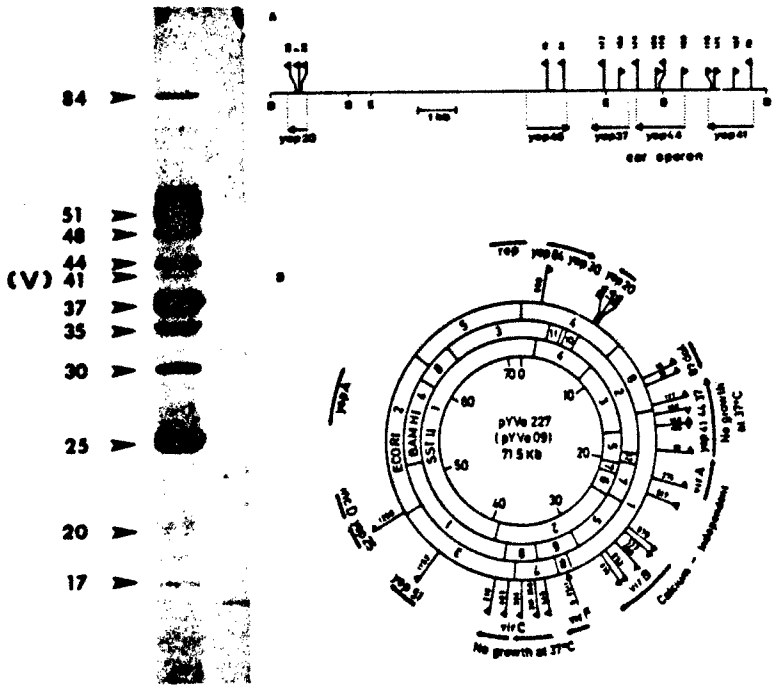


Figure 1 : Yop proteins and map of pYVe227. Left : SDS-PAGE of Yops produced by pYVe + and - variants of Y.enterocolitica W227. Right : map of pYVe227. All the flags identify insertion mutants obtained with Tn813. Mini-mu dlac or Tn2507. Black triangles indicate that the operon fusion led to expression of the probe gene (lac or cat). White triangles indicate lack of transcription. The shaded triangles indicate poor transcription. The triangles give also the orientation of the inserted probe genes while the arrows give the orientation of pYV genes. YopA encodes P1. rep : replication genes; incD : stabilization region

2. PROTEIN VirF BINDS TO Yop PROMOTERS. Gene virF was cloned downstream a T7 promoter and overproduced. Gel mobility shift experiments showed that VirF binds to DNA fragments containing the promoter of yop51. DNaseI footprinting showed that this binding occurs between nucleotides -64 to -34.

3. GENE virF IS NOT REQUIRED FOR TRANSCRIPTION OF THE OTHER vir GENES. We cloned the central part of virF on a suicide vector derived from pJM703 (2) and the recombinant plasmid served to mutate virF by homologous recombination. We mutated virF on pYVe plasmids carrying either a virA-lacZ fusion or a virB-lacZ fusion and we measured the ß-galactosidase activity at 28°C and 37°C. This activity was found to be of the same order in the virF+ and virF- isogenic strains, indicating that transcription of virA and virB is not regulated by virF. Since transcription of these genes is thermoregulated, one must postulate the existence of another regulator thermoregulator of pYV genes.

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Expression of Recombinant Pseudomonas Exotoxin Using High Density Growth of E. coli

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INTRODUCTION

Non-active forms of Pseudomonas exotoxin (PE) could be useful for vaccine development or as a toxoid. Collier and colleagues(1) have shown that deleting the glutamic acid at position 553 of PE eliminates the toxin's ADP-ribosylating activity. Plasmid pVC45D, which encodes PE lacking glutamic acid at position 553, was used to transform E.coli BL21(\DE3). An OmpA signal sequence was included prior to the coding sequence for the toxin so that the expressed protein is directed to the periplasm.

To obtain large amounts of non-active PE (PE553D) using this recombinant strain, a production process was developed based on a strategy to grow the bacteria to high density and induce the expression of the cloned gene during the logarithmic growth phase. Since the induction favors the expression of the cloned gene (the toxin production can be as much as 20% of the final protein), the process was expected to yield large amounts of toxin and simplify the purification and recovery procedures.

MATERIALS AND METHODS

Fermentation: E. coli cultures were grown to high density at 37°C at pH 6.95 in modified LB medium containing in g/l: tryptone (Difco) 10, yeast extract (Difco) 5, NaCl 5, K_2HPO_4 2, $MgSO_4$ 0.45, ampicilin 0.05, glucose 40 and 2 ml trace elements solution. Fermentations were performed according to Fass et. al.(2)

E. coli BL21(\DE3) transformed by the plasmid pVC45D were innoculated into the fermentor. Induction with IPTG (300 mg/l) was used to initiate production of the toxin when the culture reached a density of 20 g dry weight/l (30 OD 600).

Expression: A schematic diagram for the expression of PE553D protein in E.coli BL21(\DE3) is shown in fig.1. When IPTG is added to the culture medium, the lac promoter is derepressed, the T7 RNA polymerase is transcribed and acts on the T7 promoter to transcribe PE553D. The OmpA signal sequence directs the produced toxin to the cell periplasm.

Purification: Cells were centrifuged, suspended in 20% sucrose, centrifuged again and resuspended in chilled water. The supernatant containing the periplasm fraction was centrifuged, membran filtered, dialyzed against 20 mM Tris pH 7.6 and applied to silica-based anion exchange (quaternary methylamine, Waters) column. The PE553D material was eluted with discontinuous NaCl gradient with an isocratic step at 170 mM NaCl. PE553D eluted near the end of this step.

RESULTS

The fermentation system could support the growth of the recombinant E.coli strain at a constant growth rate of 0.35 hr^{-1} until the culture reached a density of 110 OD600 (50 g dry weight/l). However, the induction step with IPTG, performed at a cell density of 30 OD, caused a substantial decrease in growth rate after 30 minutes. The culture was harvested 90 minutes after the induction. At that time, the cell concentration achieved 40 OD and the growth had ceased (fig. 2). The toxin that was produced could be isolated from the periplasm in high yields. The 1.8 l periplasmic fraction contained 3.6 g of total protein. From that, 300 mg of material that was substantially pure was obtained.

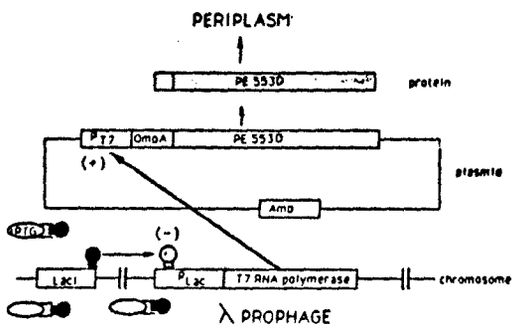


Figure 1. Expression system of the PE553D protein in E. coli.

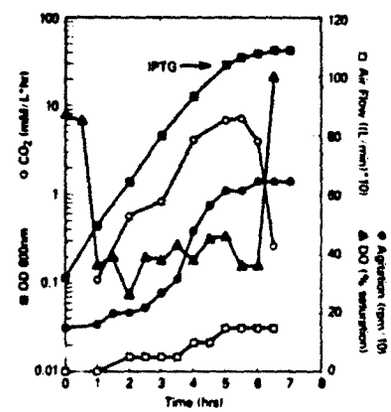


Figure. 2. High density growth of E.coli BL21(\DE3) in modified LB medium before and after induction with IPTG.

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Genetic Studies on the vir locus of Bordetella pertussis

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INTRODUCTION

The *vir* locus of *Bordetella pertussis* regulates the expression of multiple virulence-associated traits, including filamentous hemagglutinin (FHA), pertussis toxin, extracytoplasmic adenylate cyclase/hemolysin, a 69 Kd outer membrane protein (also known as pertactin or agglutinogen 3), and dermonecrotic toxin.

The DNA sequence of the *vir* locus predicts the presence of three genes (1). To test this hypothesis genetically, a complementation analysis of the *vir* locus was undertaken.

MATERIALS AND METHODS

Nonpolar linker insertion mutations in each of the putative *vir* genes were constructed by insertion of 12 bp synthetic oligonucleotides, or by insertion of a kanamycin resistance cassette at *Sal*I sites, and subsequent excision using *Pst*I sites, to leave a 12 bp insertion. These mutations were crossed onto the *B. pertussis* chromosome using a derivative of pRTP1 (2). Each mutation gave a Vir^- phenotype when present in the chromosome. Each mutation was then crossed onto a *vir* containing plasmid which can be maintained extrachromosomally in *B. pertussis*. The mutant alleles were tested in all pairwise combinations for their ability to complement each other to restore a Vir^+ phenotype.

RESULTS AND DISCUSSION

The data which were obtained did not neatly define three complementation groups. Instead, they showed that some of the mutations, when present on the plasmid, had a negative effect on *vir* activity which was *trans*-dominant over wild-type. This was true for the single mutation constructed in *bvgB*, which is postulated to code for a periplasmic protein by virtue of the presence of a consensus signal peptide at its amino terminus. The *bvgC* gene is predicted to code for a large, transmembrane protein which interacts with the *bvgB* product in the periplasm and relays extracytoplasmic signals to the *bvgA* product in the cytoplasm. Three of four mutations inserted in the predicted periplasmic domain of *bvgC* also had a dominant Vir$^-$ phenotype. These data provide evidence for intermolecular interactions of the *vir* encoded proteins. In addition, two mutations, one in the predicted periplasmic domain of *bvgC* and one in its predicted cytoplasmic domain were able to complement each other to restore *vir* activity. This intramolecular complementation is again indicative of interactions between the *vir* encoded proteins. A model is put forward to explain these experimental results.

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Regulation of the Expression of FHA in B. pertussis

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Filamentous Hemagglutinin (FHA) is one of the virulence factors produced by B.pertussis when infecting a host organism.
There is evidence that it is responsible for the adherence of bacteria to ciliated cells in the human respiratory tract.
This adhesin, whose mechanism of action is still unknown, is encoded by an open reading frame 12 Kb long, and the putative 360 Kda precursor seems to be processed post-translationally into a few mature polypeptide chains which are probably involved in the expression/secretion of the functional fragment of 220 kda.
The expression of FHA and nearly all the other virulence factors of Bordetella is under the control of a central regulatory locus, Bvg, which activates/disactivates every virulence related gene depending upon the environmental conditions (growth temperature and presence of MgSO4 or nicotinic acid) (phenotypic modulation) and/or the integrity of the locus (phase variation). Bvg is a complex, positive, trans-acting system, whose coding region is adjacent to FHA gene.

We demonstrate that a 3 kb fragment at the 5' end of the FHA locus contains the region directly controlled by Bvg for the regulation of the entire gene.
Furthermore, that the FHA gene has a promoter completely different from any other known Bordetella or Escherichia promoter sequences.

We have recently constructed a Bvg-dependent regulatory system in E.coli using a transcriptional genomic fusion between a FHA 5′ fragment (Eco-Bam 2.8 Kb long) and lacZYA. Beta-galactosidase activity is evident in the presence of Bvg in trans on multicopy plasmids, and is reduced several hundred fold in the absence of Bvg. Even phenotypic modulation by Temperature, MgSO4 and nicotinic acid is observed in E.coli, as in B.pertussis.
This suggests that the expression of FHA is completely under the control of Bvg, and that the 5′ end of the FHA gene, presumably upstream of the coding region, contains the sequence elements responsible for this trans-regulation.

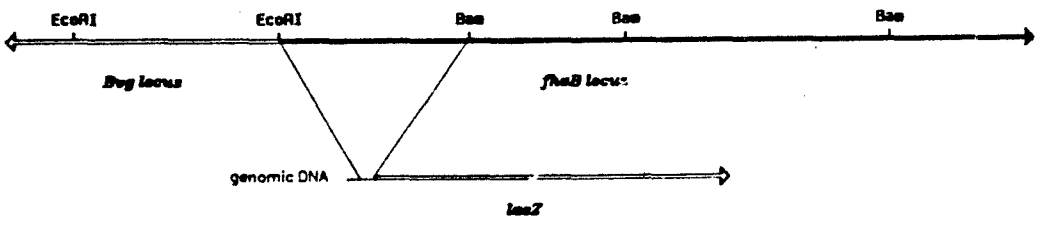


Fig. 1. fragment of FHA cloned into E.coli

By using the S1 nuclease digestion, we have recently mapped the in vivo transcription initiation site of the FHA gene. Both in E.coli containing Bvg + FHA and B.pertussis, the transcription starts at position 177 from a well-localized EcoRI site.
DNA sequence upstream of the transcription startpoint does not share homology with any other known promoter region in B.pertussis.
Details of the promoter structure are under investigation.

```
EcoRI   -240              -220              -200              -180
/
GAATTCCTGCGCTGGCACCCGCGGCGGGCCGGGGAGCGGGTTGTCGGCGCACGCCTATACGTGCCGGACAGGGTTTGATGGTTTGACTAA

        -150              -130              -110              -90
GAAATTTCCTACAAGTCTTGTATAAATATCCATTGATGGACGGGATCATTACTGACTGACGAAGTGCTGAGGTTTATCCAGACTATGGCA
                                                                                  transcription
                                                                                  startpoint
        -60               -40               -20               0
CTGGATTTCAAAACCTAAAACGAGCAGGCCGATAACGGATTCTGCCGATTACTTCACTTCGCTGGTCGGAATATGAACACGAACCT....
                                                                translational
                                                                initiation site
```

Furthermore, since the recombinant plasmid contains the 5'end of the FHA gene and the entire Bvg locus, it has been possible to evaluate the expression of FHA in the absence/presence of phenotypic modulators (Temperature, MgSO4 and nicotinic acid). Both in E.coli and in B.pertussis, RNA was normally synthesized in the absence, but not in presence, of MgSO4.

	B.pertussis	E.coli	
no MgSO4	+	+	RNA synthesis
MgSO4	-	-	

Thus, we can conclude that a) the promoter of FHA is localized 75 nucleotides upstream of the translational start codon ATG, and b) the regulation of FHA gene expression occurs at the level of transcription.

The results of the FHA promoter are in contrast with those of the PTx operon in E.Coli, where, in presence of Bvg in trans on multicopy plasmids, there is no transcription of the operon (in the clone, the non-coding region upstream of the transcriptional start site was 500 bp long). (1)
From other data (2), there is evidence that a region located 170 bp upstream of the transcriptional start site is required for trans-activation.

However, although both PTx and FHA are under the control of Bvg, it is clear that these two genes have different mechanism of regulation.

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A Two Component Regulatory System (phoP phoQ) Controls Salmonella typhimurium Virulence

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A number of mutations in S. typhimurium are known to affect virulence. Recognizing that many bacterial virulence factors are coordinately regulated we wished to identify a regulatory mutation that reduced virulence. We found that strains with mutations in the positive regulatory locus phoP are markedly attenuated for virulence in Balb/c mice and are reduced in survival in cultured bone marrow derived macrophages. This virulence defect is greater than 10,000 fold when organisms are administered by intraperitoneal or peroral routes despite normal growth in rich and minimal media. Independently similar observations have been reported by Fields et. al., who additionally showed that phoP mutants are sensitive to exposure to the rabbit defensin NP-1 (1).

The phoP is one of two loci, (phoP and phoN), found by Ames and colleagues (2) to be necessary for the production of a periplasmic acid phosphatase. Ames postulated that phoP was a regulatory locus and phoN was the structural gene. We have confirmed these predictions by DNA cloning and sequencing of these loci. The DNA in the phoP locus contains at least two genes that we term phoP and phoQ located in an operon. These two genes encode polypeptides of 224 and 487 amino acids respectively. The predicted PhoP amino acid sequence has marked similarity at both the carboxyl and amino termini to other bacterial transcriptional activators such as OmpR, PhoB, and VirG which are known to be members of two-component regulators that respond to environmental signals. The carboxyl-terminal region of PhoP is also similar to the amino-terminal domain of ToxR that has been implicated in transcriptional activation and DNA-binding activity of this Vibrio cholerae virulence regulator. Analogously PhoQ showed similarity in its carboxyl-terminal portion to proteins such as EnvZ and VirA which are the second regulator components. These proteins are envelope proteins which function as protein kinases that detect environmental signals and then phosphorylate themselves and the amino terminal domains of their paired transcriptional activators. Consistent with

this, the sequence of PhoQ predicts two hydrophobic transmembrane segments, and we have isolated several TnphoA fusions to the intervening periplasmic domain which have alkaline phosphatase activity, suggesting that this domain has a transmembrane topology similar to other sensor-kinases.

The contribution of the acid phosphatase gene (phoN) to virulence was tested by construction of several phoN mutants. These strains have no virulence defect, indicating that the avirulence of phoP mutants is likely do to the positive regulation of other genes essential to macrophage survival and virulence. Consistent with this, we have isolated a number of strains with gene fusions to E. coli beta-galactosidase and alkaline phosphatase that require an intact phoP locus for expression. These mutations are unlinked on the Salmonella chromosome indicating that this system is a regulon. Two of these strains with mutations in genes termed pagA and pagB (phoP-activated genes) do not have a virulence defect. Another strain with a TnphoA insertion in a gene termed pagC has attenuated virulence in BALB\c mice, as well as reduced survival in cultured macrophages. Based on the properties of TnphoA we can predict that this strain has a mutation in an envelope protein.

The structures of the predicted PhoP and PhoQ gene products coupled with the virulence defects of phoP, phoQ, and pagC mutants suggest a model for the function of this regulon. PhoQ likely functions as a membrane associated protein kinase that phosphorylates PhoP in response to environmental signals in the intracellular environment. Phosphorylated PhoP would then activate pag gene promoters for synthesis of proteins necessary for survival in the hostile environment of the macrophage phagolysosome. The predicted periplasmic domain of PhoQ would contain receptor function for signals such as defensins, cations, and low pH. Interestingly this region contains a remarkable stretch of negatively charged amino acids that might function to recognize these signals. We have noted the derepression of pag fusions by 2-3 fold in media of low pH and Ames and colleagues noted an increase in PhoN expression in starvation media. Hence low pH and starvation are conditions that might exist within the macrophage phagolysosome.

Lastly we have investigated phoP mutants as live vaccines in mice and find that they afford some protection to wild type challenge. These strains when administered orally efficiently colonize the gut and confer substantial protective immunity. Further analysis of this virulence regulon should increase our knowledge of the molecular basis of Salmonella pathogenesis and could lead to the development of safe attenuated Salmonella strains suitable for the delivery of heterologous antigens to the immune system.

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The Organization of The Operons Coding for Shiga and Shiga-Like Toxins

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INTRODUCTION

Shigella dysenteriae strains produce a cytotoxin, SHT, which is similar in its biochemical properties to the shiga-like toxin (SLT) produced by other enteropathogenic bacteria (1).

MATERIALS AND METHODS

All methods mentioned have been described in ref. 2-3.

RESULTS AND DISCUSSION

Nucleotide (nt) sequences were determined for the toxin operon (sht) present in the chromosome of S.dysenteriae and for shiga-like toxin operon (slt) found in the phage H30 genome. The coding sequences of sht and slt genes differ in four nt's with one nt change responsible for the amino acid replacement. SHT-specific mRNAs were detected by blot-hybridisation with a sequence specific DNA probes; one of them was more abundant and coded only the B subunit of SHT while the other (bi-cistronic mRNA) encoded both subunits. The 5' ends of sht mRNAs, whose transcription seems to be determined by two different promoters, were mapped by the primer extension method with the synthetic oligodeoxynucleotides served as specific primers (Fig.1). The start points of two bi-cistronic mRNA are located before the AUG codon of the A subunit coding sequence. The start point of monocistronic mRNA is localized at 97 nt upstream from the AUG codon of the B subunit coding sequence. The existence of the promoter for monocistronic mRNA was proved by insertion the 411 bp Alu-fragment, witch contains the putative promoter (P_B) and the

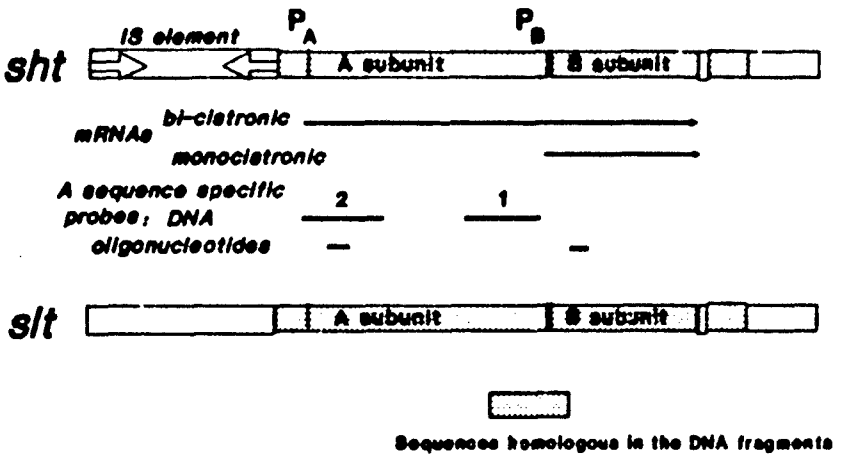


Fig.1. Schematic sructure of DNA fragments containing the *sht* operon, the *slt* operon and their flanking sequences

sequence for seven N-terminal amino acids of the B subunit, into plasmid YEp357 upstream from lacZ gene deprived of the promoter. Such plasmids, when transformed into E. coli JM103 and grow in presence of X-gal,yielded blue colonies. Control plasmids (without insertion) yielded white colonies.

It is noteworthy that the same DNA fragment of S.dysenteriae accommodates an IS element near the sht operon; this element is nearly identical to Shigella sonnei IS600 and is related to E. coli IS3. As it was estimated by dot-blot hybridisation, the sht operon is present in genomes of the different clinical isolates of S.dysenteriae in the number from one to four copies. Southern blot hybridization has showed that despite of the differences in copy number, the sht sequences seems to be organized as a structurally identical units the minimal size of wich 20 KB. The IS element may be a structural component of the transposon responsible for sht operon amplification in the chromosome of Shigella.

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Toxins as Virulence Factor

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Bordetella pertussis Adenylate Cyclase

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KEY-WORDS: calmodulin activation-binding domain; precursor; haemolysin; immunological cross-reactivity; secretion.

ABSTRACT

Bordetella pertussis adenylate cyclase is synthesized as a bifunctional precursor of 1706 residues endowed with both cAMP synthesizing and haemolytic activities. The calmodulin-dependent catalytic activity is located within the first 399 residues: the segment situated between residues 1-235/237 harbors the ATP-binding site, the segment situated between residues 236/238 - 399 is the main calmodulin-binding domain. B. pertussis adenylate cyclase displays immunological cross-reactivity with two other calmodulin-activated adenylate cyclases, one from B. anthracis and one from rat brain. The secretion of the adenylate cyclase-haemolysin bifunctional protein requires the products of three genes located downstream of the adenylate cyclase gene.

INTRODUCTION

Calmodulin-dependent adenylate cyclase toxin, secreted by Bordetella pertussis, is one of the factors implicated in the virulence of this bacterium (22). The "invasiveness" of adenylate cyclase, i.e. its ability to enter eukaryotic cells, has been ascribed to different molecular forms of this enzyme (9, 10, 15). Uncovering of the mechanism of invasiveness and of the toxic effects of the enzyme in the pathophysiology of whooping cough requires a full understanding of the structure-function relationship of adenylate cyclase.

We have cloned B. pertussis adenylate cyclase (*cya*) gene, determined its nucleotide-sequence, and studied the secretion of the protein (5, 6). Our results established that adenylate cyclase is synthesized as a bifunctional precursor endowed with both adenylate cyclase and haemolytic activities. We have also shown that the secretion of the bifunctional protein requires the products of three genes located downstream from the adenylate cyclase gene.

CALMODULIN-ACTIVATED ADENYLATE CYCLASE: STRUCTURE-FUNCTION RELATIONSHIP

Biochemical characterization of B. pertussis adenylate cyclase was hampered by the fact that enzyme obtained from both culture supernatants and bacterial extracts was markedly unhomogenous (10, 12, 19). Thus, the "low" molecular weight form of enzyme varied in size between 43 and 60 kDa, whereas the "high" molecular weight form has been described as ranging between 120 and 700 kDa. We prepared a homogenous, low molecular weight form of B. pertussis adenylate cyclase (43 kDa) by mild trypsin digestion of a mixture of three structurally related peptides (50, 45 and 43 kDa) (13). The amino acid composition of the 43 kDa protein was closely related to that of the peptide corresponding to the NH_2-terminal 399 amino acids (Mr = 42,939) of the adenylate cyclase precursor. The structural organization of the 43 kDa form of adenylate cyclase, which exhibits full calmodulin-activated catalytic activity, (k_{cat} around 2,000 s^{-1} at 30°C and pH 8) was further dissected using a variety of approaches: (i) cleavage of enzyme with proteases or chemical reagents (N-chlorosuccinimide and cyanogen bromide) and separation of the products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; (ii) labeling of adenylate cyclase with photoactivable derivatives of ATP and calmodulin; (iii) synthesis of peptides containing between 16 and 24 residues capable of interacting either with ATP or with calmodulin; (iv) site-directed mutagenesis. Trypsin digestion of the 43 kDa adenylate cyclase allowed isolation of two complementary fragments of 25 kDa (T25) and 18 kDa (T18). T25 represents the N-terminal domain of the 43 kDa protein and harbors the ATP-binding site of adenylate cyclase, whereas T18 represents the main calmodulin-binding site (Fig. 1). ^{125}I-azido-calmodulin binds preferentially to the C-terminal fragment of adenylate cyclase (T18) upon photolysis (11).

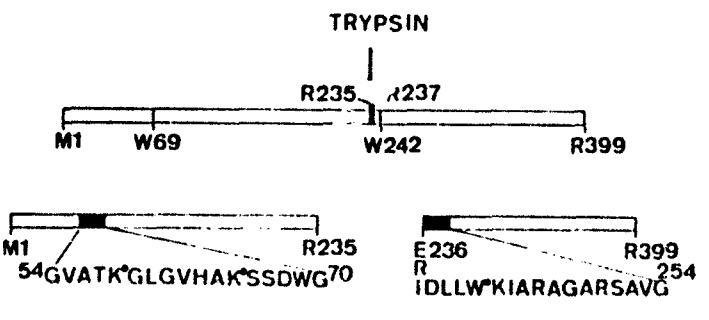


FIG.1: Structural organization of the low molecular weight (43 kDa) form of adenylate cyclase. The black bars correspond to sequences involved in the interaction with ATP and calmodulin. Asterisks indicate the residues submitted to site-directed mutagenesis.

In order to obtain more information about the putative calmodulin-binding site of B. pertussis adenylate cyclase, a peptide of 20 residues corresponding to the segment situated between Arg 235 and Gly 254 ($P_{235-254}$) in the primary structure of the enzyme was synthesized. This peptide has some features characteristic to calmodulin-binding sequences such as presence of basic and hydrophobic residues forming amphiphilic α-helices (Fig. 2A). The fluorescence emission spectrum of $P_{235-254}$, which has a maximum at 350 nm, was shifted to 325 nm in the presence of equimolar concentrations of calmodulin, whereas total fluorescence intensity was increased by a factor of 1.8. EGTA promoted dissociation of the $P_{235-254}$/calmodulin complex (Fig. 2B).

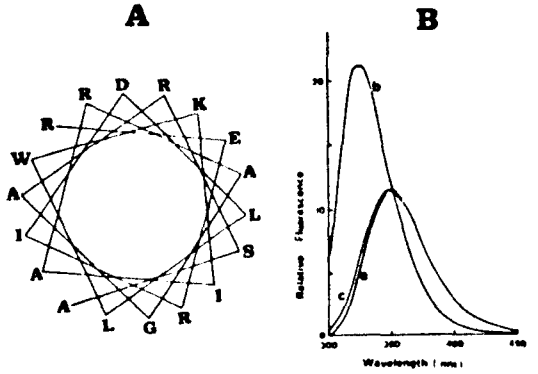


FIG. 2:
(A), A helix wheel representation of the putative calmodulin-binding sequence of B. pertussis adenylate cyclase.
(B), Ca^{2+}-dependent binding of synthetic $P_{235-254}$ peptide to calmodulin as shown by fluorescence analysis.
(a), synthetic peptide alone;
(b), synthetic peptide + Ca^{2+}-calmodulin;
(c), same as (b) after addition of excess EGTA.

To further strengthen the location of the calmodulin-binding site of B. pertussis adenylate cyclase within the segment situated between residues 235 and 254, Trp242 was chosen as the first target for analysis by site-directed mutagenesis. For this purpose, a truncated recombinant B. pertussis adenylate cyclase was expressed in E. coli (4). Replacement of Trp242 in wild-type adenylate cyclase with Val, Arg, Gly and Asp (W242V, W242R, W242G and W242D mutants) had little effect on maximal catalytic activity. However, the Kd of adenylate cyclase for calmodulin was increased by a factor which varied between 2.5 (W242V mutant) and 1750 (W242D mutant), as compared to wild-type protein. By comparing the sequence situated between residues 54 and 70 of B. pertussis adenylate cyclase (Fig. 1) with the well-known motif GXXXGKT(S) in ATP-binding proteins (17) one might surmise that the polyphosphate-binding site in the bacterial enzyme is located near Lys 65. Lys 65, as well as Lys 58, were replaced by site-directed mutagenesis with Gln. Both mutants (K65Q and K58Q) showed drastically reduced catalytic activity (0.05 % and 0.02 %, respectively, as compared to the wild-type enzyme) with unimpaired calmodulin-binding properties.

SECRETION OF BIFUNCTIONAL ADENYLATE CYCLASE-HAEMOLYSIN PROTEIN

We have recently cloned the adenylate cyclase gene (*cya*) of B. pertussis, determined its nucleotide sequence, and expressed it in Escherichia coli (5). The protein is synthesized as a large precursor of 1706 amino acid residues. In vitro deletion experiments and determination of the amino acid composition of the pure enzyme have shown that the 399 amino acid N-terminal end of the precursor corresponds to the calmodulin-activated catalytic domain of the protein. Comparison of carboxy-terminal amino acid residues of adenylate cyclase precursor with the E. coli alpha-haemolysin and with the Pasteurella haemolytica leucotoxin revealed striking similarities (6). The degree of similarity was 25 % between B. pertussis adenylate cyclase and E. coli alpha-haemolysin, 22 % between B. pertussis adenylate cyclase and P. haemolytica leucotoxin, and 37 % between E. coli haemolysin and P. haemolytica leucotoxin. Based on this sequence homology, it seemed reasonable to suppose that the adenylate cyclase precursor is a bifunctional protein carrying the adenylate cyclase activity at its N-terminal end and the haemolytic activity at the C-terminal part. Two lines of evidence corroborated this hypothesis: (i) an in-phase deletion of a 471 bp nucleotide fragment between amino acid residues 623 and 779 of the precursor abolished haemolytic activity without interfering with adenylate cyclase activity (6 and see Table 1, and Fig. 3); (ii) the secreted 200 kDa precursor exhibited both adenylate cyclase and haemolytic activities (Bellalou et al., this volume).

Adenylate cyclase does not contain a signal sequence at the N-terminal end, and its secretion requires products of additional genes. The molecular organization of adenylate cyclase secretory genes has been established by complementation and the nucleotide sequence of secretion genes determined (6).

TABLE 1
Adenylate cyclase and haemolytic activities of transconjugants[a]

Strain	Plasmid	Adenylate cyclase activity[b]		Haemolysis[c]
		Bacterial extracts	Supernatants	
Tohama 81-32	-	110	3	+
BP348	-	< 0.01	< 0.01	-
BP348	pDIA5214	190	< 0.01	-
BP348	pDIA5211	275	43	++
BP348	pDIA5210	103	< 0.01	-
BP348	pDIA5212	110	8	-

[a]Bacterial mating was performed as described in (5).
[b]Measured in the presence of 1 µM calmodulin and expressed in nmol of cAMP/min/mg protein in bacterial extracts and nmol of cAMP/min/ml in supernatants.
[c] -:no haemolysis, +: haemolysis after 48 h, ++: haemolysis after 24 h.

Table 1 shows complementation studies using different B. pertussis DNA fragments. Restoration of the haemolytic phenotype of strain BP348 (21) was achieved with plasmid pDIA5211 (see Fig. 3), where the inserted fragment comprises the *cya* gene and a 5.5 kb fragment of downstream sequences. Three open reading frames were identified, designed *cyaB*, *cyaD* and *cyaE* which code for polypeptides of 712, 440 and 474 amino acid residues, respectively. The gene products of *cyaB* and *cyaD* are highly similar to the *hlyB* and *hlyD* genes of the E. coli *hly* operon, required for the transport of alpha-haemolysin across the cell envelope (7, 14, 20, 23). Overall similarity of *cyaB* and *hlyB* gene products is greater than 50 % and the *cyaD* and *hlyD* gene products share a 32 % similarity. Unlike for E. coli alpha-haemolysin, the secretion of adenylate cyclase-haemolysin (*cyaA* gene product) requires the product of an additional gene, *cyaE*. Structural similarities between the B. pertussis *cya* operon and the E. coli *hly* operon (Fig. 4) strongly suggests that the secretion of B. pertussis adenylate cyclase occurs by a mechanism similar to that of E. coli haemolysin.

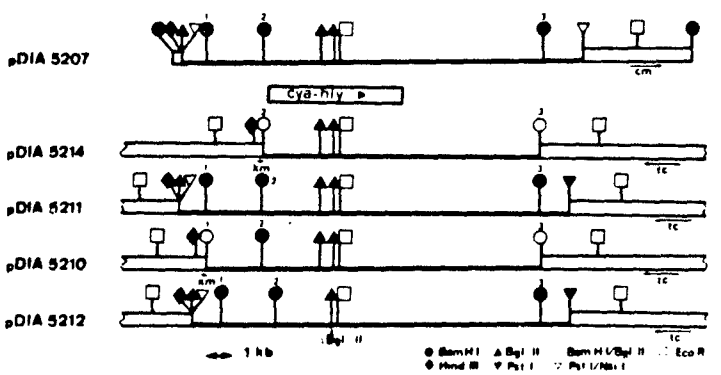


FIG. 3: Restriction map of plasmids encoding the *cya* locus of B. pertussis. Plasmid pDIA5207 was constructed by insertion of the NsiI DNA fragment encompassing the *cya* region at the PstI site from plasmid pGA46 (2). All other plasmids are derivatives from pLA2917 (1). The inserted DNA fragment in plasmid pDIA5214 was obtained by BamHI digestion of plasmid pDIA8 (5). The DNA fragment inserted in plasmid pDIA5211 was obtained after partial digestion by PstI and digestion by HindIII of plasmid pDIA5207. The DNA fragment inserted in plasmid pDIA5210 was obtained after partial digestion by BamHI of plasmid pDIA5207. Plasmid pDIA5212 was obtained by partial digestion by BglII and ligation. In plasmid pDIA5214 the expression of the *cya* gene is under the control of the Km promoter, whereas in the other plasmids the expression of the *cya* gene is under the control of its own promoter. BamHI restriction sites are ordered following *cya* transcription orientation.

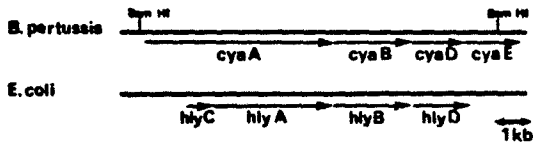


FIG. 4: Comparison of B. pertussis *cya* operon and E. coli *hly* operon. The homologous genes *cyaA/hlyA*, *cyaB/hlyB* and *cyaD/hlyD* are organized in the same manner.

RELATEDNESS WITH OTHER CALMODULIN-SENSITIVE ADENYLATE CYCLASES

We have recently shown that B. pertussis adenylate cyclase displays immunological cross-reactivity with two other calmodulin-stimulated adenylate cyclases, namely with the rat brain catalytic subunit (18) and with Bacillus anthracis edema factor (16). The nucleotide sequence determination of the two bacterial adenylate cyclases has revealed that, although the overall sequences are very different, they contain a well-conserved stretch of 24 amino acids (3; Mock et al., this volume). Making use of antibodies raised against a synthetic peptide corresponding to part of the conserved sequence, we showed that these antibodies recognized both bacterial enzymes as well as the eukaryotic catalytic subunit (Fig. 5). Several lines of evidence indicated that this peptide is part of the catalytic domain of the bacterial enzymes (8). These data suggest that the two bacterial, calmodulin-activated adenylate cyclases and the rat-brain catalytic subunit have a common origin.

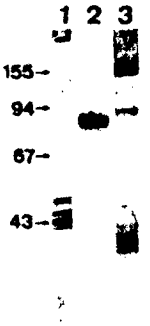


FIG. 5: Immunochemical detection of bacterial and eukaryotic adenylate cyclases by anti-peptide immune serum. Lane 1, B. pertussis enzyme; lane 2, B. anthracis enzyme; lane 3, rat-brain synaptosomal preparation. Proteins were purified as described in (8), separated by SDS-PAGE, transferred to nitrocellulose and reacted with anti-peptide immune serum. The corresponding pre-immune serum gave no reaction.

CONCLUDING REMARKS

Studies of structure-function relationship of the B. pertussis adenylate cyclase allowed localizating the sites involved in the interaction of the enzyme with substrate and with calmodulin. Mutants obtained by site-directed mutagenesis which had decreased affinity for substrate or calmodulin, offer a useful tool for studying the mechanisms of invasiveness and the toxic effects of adenylate cyclase.

The finding that the *cya* gene product is a bifunctional protein endowed with both adenylate cyclase and haemolytic activities raises important questions as to the molecular structure of toxic forms of the protein. If both activities are necessary for invasiveness, it is tempting to suppose that the haemolytic activity is involved in pore formation in host cells, thus permitting the entry of the catalytically active adenylate cyclase. Further analysis of the bifunctional protein should contribute to a better understanding of the toxic effects of adenylate cyclase.

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Thiol-dependent exotoxin as a promoting factor for intracellular growth of Listeria monocytogenes

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The first major contribution in our knowlegde of pathophysiology of listeriosis comes from the pioneer work of GB Mackaness in 1962 who demonstrated that bacteria were capable of surviving and even of multiplying within resident macrophages (1). From this finding originate several major concepts in the field of infectious diseases and immunology : (1) certain microorganisms can take advantage of macrophages as replicating sites, thus escaping to immune system (concept of intracellular pathogen) ; (2) the host can react through T-cell dependent macrophage activation , allowing these scavenger cells to ultimately destroy intracellular bacteria (concept of activated macrophage). Henceforth, *L. monocytogenes* has been extensively used by immunologists as a model to study the induction of T cell-mediated immunity against intracellular parasites. Recent dramatic outbreaks of human listeriosis occuring in several developed countries such as USA, Canada, France and Switzerland, were an inducement to obtain new information on the molecular mechanisms prevailing in the virulence of *L. monocytogenes* . The present report will briefly review the main break-throughs in this field.

Listeria monocytogenes is virulent in immunocompetent host

Often considered as an opportunistic pathogen because many human cases concern newborns infected in utero and immunocompromised patients, clinical reports undoubtedly demonstrate that infection can proceed in healthy people. For example, in a serie of 824 cases (2), it has been found that more than 50 % of infections reported in children and adults were observed in apparently healthy persons. Likewise, outbreaks occur in apparently healthy animals (cattle...), involving most animals of herds. These considerations strongly suggest that *L. monocytogenes* is a virulent organism capable of infecting immunocompetent organisms.

Listeria monocytogenes is a ubiquitous and invasive pathogen

Few bacteria display a so wide array of natural hosts than *L. monocytogenes* . This pathogen is responsible for severe infections for, at least, 37 species of mammals, including humans, 17 species of birds, fish, shell-fish and insects (3). Obviously, this might be related to the fact that *L. monocytogenes* is widely spread in nature as saprophyte in the soil, thus increasing the probability of contamination. However, this ubiquitous pathogenicity is meaningful in terms of virulence, indicating that bacteria can invade many different hosts, regarless of body temperature, reactivity of immune system and many other differences which usually interfere with the expression of virulence. This implicates that the receptors allowing cell-bacterium interaction are probably highly conserved through many susceptible animal species. Likewise, the process of intracellular multiplication can take place in many different hosts, suggesting the existence of a permissive mechanism in most living species. Many clinical features illustrate the existence of an invasive process during the course of *Listeria* infection. The intestine is the usual site of entry of this microorganism, but conjonctival, nasal and respiratory routes have been occasionally mentioned as natural or artificial means of exposure. Also

indicative of invasiveness is the propensity of *L. monocytogenes* to infect placenta and the central nervous system.The high frequency of meningoencephalitis in listeriosis is one of the most remarkable features of this disease observed in about 70 % of human cases, meaning that bacteria can cross endothelial cells to multiply in extravascular sites surrounding brain capillaries.The same process must occur in the placenta and the skin, when cutaneous metastasis are found in severe forms observed in newborns (*granulomatosis infantiseptica*).

Evidence that *Listeria monocytogenes* produces a toxin promoting intracellular multiplication

The observation that virtually all strains isolated from natural infections produce a zone of hemolysis on blood agar, in contrast to the lack of virulence of nonhemolytic species (4-6) was indicative for a relationship between production of hemolysin and virulence. However, despite many attempts in the past, the hemolysin was not purified at homogeneity and no convincing evidence was brought up to believe for a major role of hemolysin in the virulence of *L.monocytogenes* . Even, the existence of hemolytic strains (now identified as *L. seeligeri*) that revealed totally avirulent added to the confusion, insuch as the role of hemolysin in virulence remained controversial until recently .

The molecular mechanisms promoting intracellular multiplication of *L. monocytogenes* has been recently investigated by a genetic approach (7). Our strategy was to obtain well-characterized avirulent mutants, differing from the parental strain by a very limited number of genes (ideally one gene), then to clone and to sequence the gene(s) responsible for virulence, and in the same time, to characterize the product of this gene through purification procedures. This was achieved by transposon mutagenesis on a virulent strain of *L. monocytogenes* (7). The 26-kb conjugative transposon Tn *1545* was transferred from a donor strain designated BM4140 to a recipient virulent strain EGD SmR of *L. monocytogenes* . In a first step, we screened 2500 transconjugants for the production of hemolysin, thus isolating one nonhemolytic mutant. This nonhemolytic mutant was totally avirulent and failed to multiply in host tissues when the kinetics of survival was followed in organs. Its LD_{50} was estimated at $10^{9.6}$ per mouse. It was remarkable that virulence was restored in the hemolysin-producing revertant strain obtained by spontaneous loss of transposon Tn *1545* . These results were then confirmed, using the same or another transposon to induce mutagenesis : all nonhemolytic mutant obtained were avirulent (8, 9).

We then established that this avirulent, nonhemolytic mutant resulted from the insertion of a single copy of Tn *1545* , as shown by Southern-blotting analysis using an intragenic probe specific for the kanamycin resistance gene of Tn *1545* . To eliminate the possible insertion inside a regulatory gene controlling a multigenic operon implicated in virulence, it was important to make sure that the insertion of Tn *1545* took place inside the structural gene of hemolysin. This was achieved by two approaches : (1) by immunoblotting using a rabbit antiserum against highly purified hemolysin (see later), the nonhemolytic mutant was found to secrete an inactive, truncated, 52 kD hemolysin (10) ; (2) by cloning the left end of Tn *1545* encoding for kanamycin resistance and the flanking region of the chromosomal DNA in the plasmid pBR322, it was found that the transposon Tn *1545* had inserted in an opened reading frame sharing striking homologies with two close-related exotoxins, streptolysin O and pneumolysin (10).

Meanwhile, the hemolysin of *L. monocytogenes* has been purified by C. Geoffroy et al. (11). This was achieved by using thiol-disulfide exchange affinity chromatography, taking advantage of the putative presence of free-SH on the protein to be purified. The

hemolysin was characterized as a 58 kD protein, termed listeriolysin O, sharing the classical properties of sulfhydryl-activated exotoxin, including inhibition by cholesterol and antigenic cross-reactivity with streptolysin O and other sulfhydryl-activated toxins. It is interesting to mention that cholesterol molecules are constitutive of lipid bilayer in all eukaryotic cells, but they are not found in procaryotic cells, except in mycoplasmas. This protein was highly toxin in the mouse (LD 50 : 0.8 µg per mouse) and displayed a remarkable feature : its cytolytic activity towards erythrocytes from various animal species was maximum at low pH (~ 5.5). It is tempting to relate this finding to the fact that *L. monocytogenes* is the only intracellular facultative microorganism among the bacterial pathogens secreting SH-activated toxins.

The listeriolysion O gene has been recently cloned and sequenced (12, 13). The deduced protein sequence reveals the presence of a 25 amino-acid signal sequence, associated to the secreted form of the toxin (504 aminoacids), which corresponds to a molecular weight of 58 kD (13). The protein sequence is highly homologous to those of streptolysin O and pneumolysin (14, 15). A peptide of 11 amino-acids conserved in the three proteins contains the unique cysteine of these exotoxins. By DNA probing (13), as well by immunoblotting analysis with a rabbit anti-listeriolysin O (16), it was found that all strains of *L. monocytogenes* produced listeriolysin O. The final step to demonstrate the role of listeriolysin O in the virulence of *L. monocytogenes* was to eliminate the existence of a polar mutation involving the genes nearby the transposon insertion. For this purpose, the listeriolysin O gene with its promoter was cloned in a shuttle vector and reintroduced into a nonhemolytic, avirulent strain of *L. monocytogenes* obtained by transposon mutagenesis by insertion of transposon Tn *917* inside the structural gene of listeriolysin O. This genetic complementation test results in restoration of virulence, demonstrating that the listeriolysin O is a major factor promoting intracellular growth of *L. monocytogenes* (17).

The use of an in vitro model of infection allowed us to confirm the mechanism of intracellular growth at the cellular level. By infecting in vitro the human enterocyte-like cell line Caco-2 with *L. monocytogenes* , it was found that bacteria from the nonhemolytic mutant penetrate the cells as well as the hemolytic revertant bacteria, but this nonhemolytic mutant revealed unable to replicate in these nonprofessional phagocytes (18, 19). This means that listeriolysin O does not interfere in the step of invasion, but only during the phase of intracellular multiplication. Quantitative electron microscopy brought direct evidence that hemolytic bacteria are released free within the cytoplasma through vacuolar membrane disruption, as opposed to nonhemolytic bacteria which remained confined inside phagolysosomes (18, 19).

The use of nonhemolytic mutants revealed a powerful tool to demonstrate that intracellular growth of *L. monocytogenes* is essential to induce the emergence of protective T cells in the mouse (20). Whereas minute doses of hemolytic bacteria capable of growing in host tissues easily induced the expression of T cell-mediated immunity, it was found that nonhemolytic bacteria unable to multiply in host tissues totally failed to initiate the expression of T cell-mediated immunity in vivo. This failure was even observed when mice were repeatedly infected by high doses of nonhemolytic bacteria, allowing to maintain a significant amount of viable bacteria for several days in host tissues. Therefore, the presence of viable bacteria at a significant level in the host is not sufficient per se to induce detectable T cell clonal expansion in the in vivo setting, implying that the process of bacterial growth inside macrophages is required to initiate in vivo the expression of T cell-mediated immunity. In addition, it was found that infection with viable, replicative bacteria induced in vivo the emergence of T cells specifically reacting against highly purified listeriolysin O as demonstrated by eliciting delayed-type hypersensitivity to listerolysin O in *Listeria* -immune mice (21). The expression of T cell-mediated immunity to listeriolysin O was generated by very low amounts of

replicative bacteria, indicating that the exotoxin released in host tissues during the process of intracellular growth is highly immunogenic. The binding of listeriolysin O to the membrane cholesterol might be a critical event potentiating the in vivo expression of delayed sensitivity against this exotoxin. Indeed, the insertion of listeriolysin O into the cell membrane induced resistance to enzymatic proteolysis and membrane-bound listeriolysin O was significantly more effective in inducing delayed inflammatory reaction in *Listeria* -immune mice (21).

Model of intracellular multiplication of *Listeria monocytogenes*

From the above considerations, we have proposed a model of intracellular growth of *L. monocytogenes* (22, 23), confirming an hypothesis proposed long ago (24-26). Bacteria internalized by professional or non-professional phagocytes are confined inside phagosomes. Indeed, the replication site cannot be the phagolysosome since *L. monocytogenes* does not replicate under pH 5.6 (3). Very quickly, the phagolysosomal fusion occurs, inducing an acidification of the phagolysosome at pH 5.5, allowing the toxin molecules to bind to cholesterol at low pH. This leads to disruption of intracellular membranes and toxin export to the cytoplasm, inducing rapid bacterial replication. Therefore, the basic mechanism allowing intracellular multiplication is to escape to the microbicidal mechanisms of macrophages (oxydative burst, enzymes), and to find inside the cytoplasma a favorable environment, including pH, nutriments and iron.

INVASIVENESS OF *LISTERIA MONOCYTOGENES*

Although of crucial importance, the interaction of *L. monocytogenes* with professional phagocytic cells represents only one phase of the entire infectious process. The strategy developed by *L. monocytogenes* in vivo includes at least two additional steps, i.e., the penetration into the host and the invasion of target tissues such as central nervous system and placenta. The primary event occuring during the infectious process is the penetration into the host through the intestine. In a recent work using human enterocyte Caco-2 cells infected with various strains of *L. monocytogenes* (18, 19), we demonstrated that bacteria easily invade enterocytes, but this process required an active process of phagocytosis by the cells (directed phagocytosis), which is inhibited by cytochalasin D, a poison of cytoskeleton. Interestingly, the directed phagocytosis only concerns the pathogenic species (*L. mono- cytogenes*, *L. ivanovii*) and not the nonpathogenic species (*L. seeligeri* , *L. innocua* , *L. welshimeri*), suggesting that this process requires a specific interaction between invasive bacteria and enterocytes. These results give an explanation to the lack of virulence of certain *Listeria* species. The invasive process is probably a general phenomenon concerning many tissues in the host taking place to the penetration sites, and during bacterial dissemination inside the host tissues. Indeed, bacteria are visible inside hepatocytes surrounding infectious foci (26, 27) and are capable to gain access to target organs, including placenta, nervous central system (meningoencephalitis) or skin.

CONCLUSION : SCENARIO OF THE INFECTIOUS D.SEASE

Finally, it is possible to propose a plausible scenario of the infectic us disease induced in the host by *L. monocytogenes* (28) *L. monocytogenes* as saprophyte of the environment, is orally ingested with contaminated food. In most cases, the infecting dose is probably low and bacterial multiplicaiion is easily controlled in immunocompetent hosts without clinical expression, as opposed to immunocompre:nised hosts. Moreover, this low-level contamination might contribute to create a significant level of acquired resistance in many hosts. High infectious doses are presumably needed to induce clinical infection in normal hosts, as demonstrated by oral infection in experimental animal. The key-mechanism to understand the pathophysiology of listeriosis is related to the constant process of invasion-intracellular multiplication. Indeed, bacteria temporarily escape host defences at the primary replication site of the intestine, then escape again inside macrophages, hepatocytes, presumably endothelial cells proceeding through multiple amplification steps at the various replication sites. This results in formation of multiple granulomatous foci where the immune mechanisms ultimately take place to control the disease. The severity of listeriosis is mainly due to the rapid extension of infectious lesions to the central nervous system and to the placenta.

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Evaluation of Eukaryotic Cell Attachment and Internalization by Bordetellae

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KEYWORDS

Bordetella pertussis, Adherence, Invasiveness

INTRODUCTION

The ability of B.pertussis to adhere to cell surfaces has been proposed as an important factor associated with the virulence of this organism.
Recently Ewanowich et al.(4) reported that B.parapertussis commonly regarded as non invasive, is able to invade HeLa cell monolayers. In this study the ability of various strains of B.pertussis to invade eukaryotic cells was investigated. The invasion of the respiratory tract mucosa could be in fact an important step in the pathogenesis of Bordetellae.

MATERIALS AND METHODS

Bacterial strain and growth condition: B.pertussis strains 338(vir+), 359(vir-), 348(Adc-,Hly-), 349(Hly-),536 FHAΔ101, W28(vir+), W28 ΔTox PT were grown on Bordet Gengou agar containing 15% defibrinated sheep blood. The organisms used in the "invasion assay" were grown in Stainer Scholte modified liquid media containing nalidixic acid (20μg/ml) and harvested in log fase.
HeLa cell culture: The HeLa human epithelium-like cell line

was mantained as monolayer in MEM containing 10% foetal bovine serum (FCS), L-glutamine (2mM), Hepes (25mM) and nalidixic acid (20μg/ml). Cells were trypsinized for detach them from the plastic flasks washed twice and adjusted to the right concentration for the "invasion assay".
Monocyte separation: Heparinized venous blood diluted 1:3 with phosphate buffered saline (PBS) was placed on Ficoll-Hypaque and then centrifuged at 400 x g for 30 min at room temperature. Peripheral blood mononuclear cells (PBMC) were collected at the interface, washed and suspended in RPMI-1640 supplemented with 10% FCS. To obtain cells enriched in monocytes, a one-step discontinuos gradient of Percoll was employed as previously described (3).
Invasion assay: HeLa cells or monocytes ($2x10^6$) were incubated at 35°C with B.pertussis organisms ($\approx 1x10^8$)for different periods of time. The cells were then washed twice to remove unbound or losely bound bacteria and then incubated for 3 additional hours at 37°C with medium containing gentamicin (75 μg/ml) to inactivate the extracellular organisms. Viable bacteria were recovered following cell lysis with saponin (0.1%) and enumerated by plating appropriated diluitions on Bordet Gengou agar.

RESULTS AND DISCUSSION

The recovery of B.pertussis strain 338 (vir+) and 359 (vir-) from HeLa cell lysates after incubation for different times is shown in fig.1.

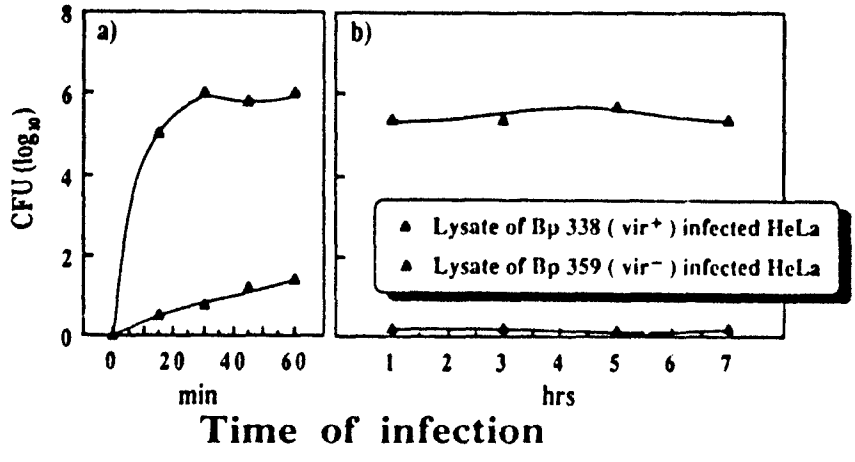


Fig.1. Kinetics of B.pertussis uptake by HeLa cells.

The number of organisms per lysate increased with time, reaching a plateau at 30 min. The viable bacteria of the

virulent strain 338 in the lysate was always much greater than that of the non virulent strain 359.
The ability of B.pertussis to invade eukaryotic cells was further investigated by employing several transformed cell lines as well as fresh monocytes. The results shown in fig.2 once again indicate that the recovery of B.pertussis strain 338 following 1 hr of incubation was markedly higher than that observed with the strain 359.

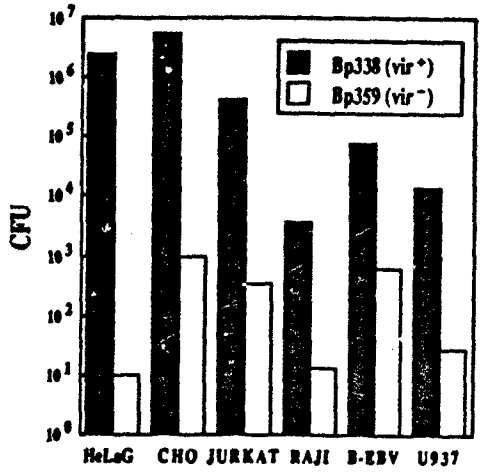


Fig.2. B.pertussis invasion of transformed eukariotic cell lines.

Microscopic examination of HeLa cell viability after coincubation with B.pertussis showed a very small number of dead cells. The results regarding the intracellular growth of B.pertussis, assessed by quantitative bacteriology are shown in fig.3.

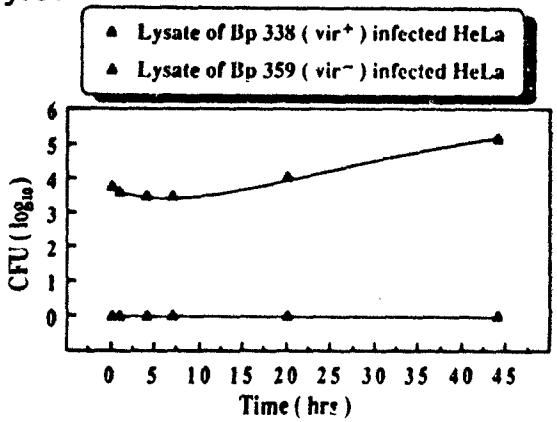


Fig.3. Time-course of B.pertussis surviving in infected HeLa cells.

Although the number of intracellular virulent bacteria progressively increased, the growth appeared limited. In

fact cells did not contain large numbers of intracellular organisms even 44 hours postinfection. With strain 359 the number of bacteria recovered was very low or undetectable throughout the experiment.
The results of representative experiment performed to determine the invasiveness of several B.pertussis mutants are shown in fig.4.

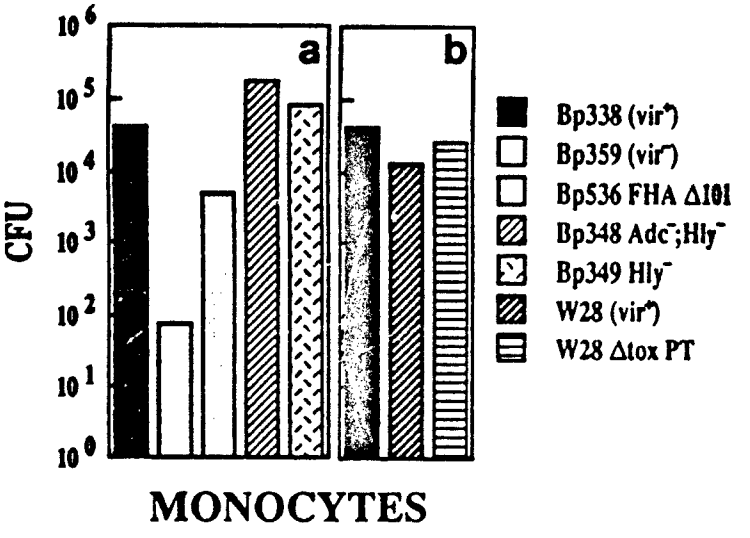


Fig.4 Comparison of the invasive capability of B.pertussis mutants for monocytes.

These mutants were tested for their ability to penetrate into freshly isolated monocytes. The recovery from cell lysates of viable organisms of a B.pertussis strain lacking the toxin gene (W28 ΔTOX) was similar to that of the wild type. The recovery of the adenylate cyclase and hemolysin deficient mutants was similar. The results obtained would suggest that pertussis toxin, which is responsible for most of adverse systemic reactions associated with whooping cough, is not mainly involved in the processes of adherence and invasiveness.
The most difficult problem in assessing cell culture penetration is the determination of the intracellular bacteria compared to those residing between cells or attached to them. The disruption of cells after infection and plating of the resulting lysates is a method to enumerate internalized bacteria. To this regard, most investigators have used antibiotic counterselection to remove or reduce adherent and non internalized bacterial population. Using this procedure they evaluated the number of viable intracellular bacteria. However, our results indicate that the antibiotic treatment is not completely effective (data not shown). In fact, we found that infected HeLa cells exposed to gentamicin for 3 hrs at 37°C and then maintained for additional 2 hrs at 4°C,

released in their supernatant a significant number of viable bacteria. A reduction of the metabolism of those bacteria attached to eukaryotic cell membrane may explain their decreased susceptibility to the antibiotic treatment.
In order to discriminate between attachment and internalization we have used as different approach a flow cytometric fluorescence technique together with trypan blue to quench the extracellular fluorescence of fluorescein isothiocyanate (FITC) labeled B.pertussis. Trypan blue in fact is able to quench the fluorescence of FITC-labeled particles(2). Since the dye is not taken up by viable cells the fluorescence of internalized particles remains unaltered whereas fluorescence of surface bound particles is quenched. This method was in fact succesfully employed by Bjerknes (1) to study the phagocytosis of Candida albicans. Our preliminary results would indicate that this method might be feasible also with B.pertussis. However, technical problems still prevent us from a definitive conclusion. In fact a prolonged incubation (24 hrs) with fluorescein causes bacterial death which may impair a correct evaluation, whereas a short term incubation with fluorescein (1-3 hrs) results in good viability but in a low degree of bacterial labeling which needs signal amplification to be monitored.
In the light of the results obtained, we could speculate that the method of cell lysates is not completely satisfactory to discriminate between adhesion and invasiveness of B.pertussis. On the other hand, our preliminary results obtained with flow cytometric fluorescence technique could shed light on a better understanding of the mechanism underlying the pathogenicity of B.pertussis.

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Secretion of Bordetella pertussis Adenylate Cyclase Haemolysin Bifunctional Protein

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INTRODUCTION

Bordetella pertussis, the ethiological agent of whooping cough, synthesizes a calmodulin-sensitive adenylate cyclase, suspected to play a major role in the virulence of this bacterium (4). Recent genetic and biochemical studies established that the protein is synthesized as a large precursor; the catalytic activity resides in the 400 N-terminal amino acids (3). The C-terminal part of the protein showed striking similarities with the Escherichia coli alpha-haemolysin, and it has been suggested that the precursor is a bifunctional protein carrying both adenylate cyclase and haemolytic activities (1). However, adenylate cyclase isolated from culture supernatants was shown to be a 43-50 kDa protein corresponding to the N-terminal part of the *cyaA* gene product (2). The absence of haemolytic activity of these supernatants was consistent with the hypothesis that the precursor form of the protein was processed and the haemolytic activity, linked to the C-terminal part of the protein, has been lost. The aim of this study was to search for a secreted, high molecular weight form, of adenylate cyclase and to investigate its potential haemolytic activity.

IDENTIFICATION OF A 200 kDa ADENYLATE CYCLASE (AC 200) IN B. PERTUSSIS CULTURE SUPERNATANTS

B. pertussis, grown in Stainer and Scholte (SS) medium supplemented with bovine serum albumin secretes a 200 kDa polypeptide endowed with adenylate cyclase (AC) activity; this high molecular weight AC is indistinguishable from the intracellular AC, as evidenced by Western

blot analysis. Addition of calmodulin to the SS medium resulted in an increase of the extracellular AC. The secreted 200 kDa form of AC could be converted to the 45 kDa form by limited proteolytic digestion. These results show that (i) AC is secreted as a 200 kDa protein, and (ii) the 45 kDa form, generally found in culture supernatants, is the result of proteolytic cleavage of the large form.

IN VITRO HAEMOLYTIC ACTIVITY OF B. PERTUSSIS CULTURE SUPERNATANTS

Crude supernatants of B. pertussis cultures have been tested for haemolytic activity, using sheep erythrocytes. Only culture supernatants containing the 200 kDa form of AC exhibited haemolytic activity which was Ca^{++}, dose- and time-dependent. The haemolytic activity, but not AC activity, was abolished after limited proteolysis by trypsin. Western blot analysis revealed that at the same time the 200 kDa AC was converted into 43-50 kDa protein species, indicating that the haemolytic activity was correlated with the integrity of the precursor. This is consistent with the previous hypothesis, based on sequence data, according to which the C-terminal part of the precursor protein carries the haemolytic activity.

In summary, these data demonstrate that the *cyaA* gene product of B. pertussis, is secreted as a 200 kDa protein, and show that proteolytic processing of the intracellular AC is not a prerequisite for secretion, as was previously reported. The in vitro haemolytic activity, found exclusively in supernatants containing the 200 kDa AC form suggests that this protein may be endowed with both AC and haemolytic activities. Further biochemical characterization of the purified 200 kDa form of AC is in progress.

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Bordetella Adenylate Cyclase: Cytopathic and Immunoprotective Properties

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INTRODUCTION

Studies mainly conducted on B.pertussis, the causative agent of whooping cough, in humans, have led to identify several products as virulence factor. Among those, the filamentous hemagglutinin (FHA) and the pertussis toxin (PTx) are considered as major determinants of pathogenesis and as efficient immunogens (5). However, Weiss et al (6), using Tn5 induced mutants of B.pertussis, provided the first evidence that an other factor, the adenylate cyclase (AC) plays an important role in the virulence of this bacterium.
In the present study, we show by using B.pertussis and B.parapertusssis (another human pathogen expressing the same virulence factors as B.pertussis except the PTx [1]) infections of mice, that AC can be the major effector in the pulmonary cytopathic syndrome. Furthermore, active immunizations with purified AC protect mice against B.pertussis challenge.

RESULTS AND DISCUSSION

Is Bordetella adenylate cyclase a cytotoxin?
Pathogenic derivatives of B.pertussis 18323 Phase I (ATCC 9797) and B.parapertussis (CIP 63.2) were freshly isolated on Bordet Gengou medium supplemented with 15% final defibrinated horse blood (BGB) from lung homogenates of mice, infected for 48 hours, by intranasal injection (2) with either one of these strains. Parental as well as lung-passaged derivative of B.parapertussis induced, after intranasal injection of mice, an acute edematous hemorrhagic alveolitis (AEHA) similar to the AEHA we observed with B.pertussis (2). Thus B.parapertussis, although not expressing PTx was able to evoke pulmonary lesions analogous to those observed after B.pertussis challenge. This result suggests that PTx may only play a minor role in the acute pulmonary syndrome observed after Bordetella infection.

As shown in Table I, lung-passaged derivatives are more pathogenic than the parental strains (10 times differences in the LD50 for B.pertussis and 2 times for B.parapertussis). The increase in pathogenicity is correlated with a 5 to 9 fold increase in the amount of AC. This result suggested that AC could be the main cytotoxin responsible for the AEHA. However, since we could not check if B.pertussis and B.parapertussis pathogenic derivatives produced more of the other virulence factors such as heat-labile toxin or tracheal cytotoxin, we decided to test the efficiency of active immunizations of mice with purified AC (3) against B.pertussis lethal challenge.

Table I: AC, PTx, FHA activities and pathogenicity of B.pertussis and B.parapertussis strains

	AC[1] U/ml	PTx[2]	FHA[3]	LD50[4] No of bacteria/mouse
BP18323	20	64	4	$1.10 \times 10^8 \pm 0.3$
BP18323S	180	250	4	$0.09 \times 10^8 \pm 0.01$
BPP63.2	5	0	64	$2.35 \times 10^8 \pm 0.32$
BPP63.2S	25	0	64	$1.20 \times 10^8 \pm 0.09$

[1] AC activities were measured in culture supernatants (1 unit corresponds to 1nmol of cAMP formed per min at 30°C at pH8) [3]
[2] Reciprocal dilution titer of culture supernatant producing 50% clustering in the CHO cell clustering test [4]
[3] Reciprocal dilution titer of end point dilution of bacterial suspension showing disruption of erythrocyte settling
[4] Three weeks old, female Swiss mice were infected by intranasal injection of different bacterial suspensions in a volume of 50µl. Bacterial counts from the inocula were performed by sampling 10-fold serial dilutions on BGB.

Is AC a protective antigen of B.pertussis?

AC was purified from B.pertussis culture supernatant as described previously (3). The efficacy of active immunization with pure AC was evaluated by comparaison to whole cell vaccine in mice injected subcutaneously and subsequently challenged by intranasal infection with B.pertussis pathogenic derivative. As shown in Table II, a 80% protection, against lethal respiratory infection, was obtained after immunization of the mice with purified AC, a protection rate which was equivalent to that obtained with the classical whole cell vaccine.

TABLE II Active immunizations with purified AC or whole cell vaccine against lethal respiratory infection

GROUP	ANTIGEN		No OF MICE		% SURVIVAL
	AC (µg/injection)	VACCINE (No bact./injection)	INOCULATED	SURVIVORS	
A	2	-	46	37	80
B	-	5×10^8	30	27	90
Control	-	-	50	7	14

Three weeks-old Balb/c mice were immunized three times, at one week interval, with purified AC or bacterial suspension heated, 20 minutes, at 56°C, complexed with aluminum hydroxide. Two weeks after the last injection, lethal challenge was performed by intranasal injection of 10^8 bacteria in a volume of 50µl.

The present results suggest that AC is the primary cytotoxin responsible for the acute pulmonary lesions after respiratory infection with B.pertussis or the related specie B.parapertussis in mice and is a protective antigen of B.pertussis.
Studies are now undertaken to compare the protective efficacy of AC and the two other major virulence factors of B.pertussis, PTx and FHA and to study whether immunization with purified AC results in reduction in bacterial colonization or only in inhibition of AC cytopathic effects.

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Properties of Bordetella pertussis Adenylate Cyclase expressed in E.coli

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INTRODUCTION

B. pertussis produces an adenylate cyclase (AC) toxin which is able to enter mammalian cells where it is activated by calmodulin. Previous work (1) achieved efficient expression of cloned B. pertussis AC in E. coli to a specific activity comparable to that in native B. pertussis. Here we report invasive and immunogenic properties of AC purified from E. coli.

MATERIALS AND METHODS

Plasmids pRMB3 and pRMB9 encode the entire AC gene and 2.6 kb from the 5' end respectively (1). The expression of AC in E. coli from these plasmids depended on the lac promoter contained in the vector. All experimental procedures have been previously described (1,2).

RESULTS AND DISCUSSION

pRMB3 and pRMB9 were transformed into Lon$^-$ E. coli H1469 to minimise proteclytic degradation of AC. SDS-PAGE followed by renaturation of calmodulin-dependent AC activity revealed major peaks of activity at 94 and 200 kDa for AC purified from pRMB9 and pRMB3 respectively (Fig. 1).

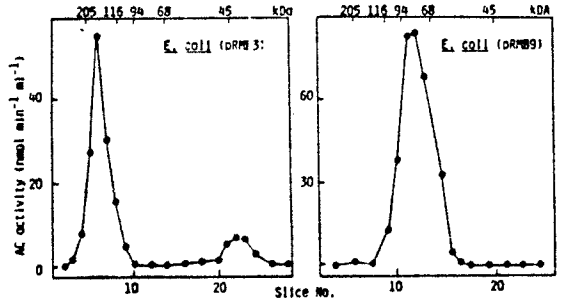


Fig. 1 Purified AC from E. coli pRMB9 (1 µg) or pRMB3 (2 µg) was subjected to SDS-PAGE together with molecular weight standards. The gel was cut into 0.4 cm slices and protein eluted and assayed for AC activity in the presence of calmodulin.

The 200 kDa protein purified from E. coli (pRMB3) migrated on SDS-PAGE to an identical position to the high molecular weight AC purified from B. pertussis, but was unable to raise the intracellular cAMP concentration of S49 lymphoma cells (a value of 10.6 μmol cAMP 10^7 cells mg^{-1} protein was obtained for the penetrative capacity of AC purified from B. pertussis 165). Expression of the entire AC gene in E. coli was therefore not sufficient to produce the invasive form of the enzyme.

Preliminary evidence (Table 1) established partial protection in 3 week old mice vaccinated with AC (200 kDa) purified from E. coli (pRMB3) and challenged intranasally at 5 weeks. No protection was afforded by the 94 kDa protein purified from E. coli (pRMB9), which lacked the C-terminal portion of AC.

Table 1 **Mouse protection with B. pertussis AC purified from E. coli.**

Vaccine preparation[a]	Lungs as % body wt. ± S.D.)	Mean wt. change (g) days 0-14 post-challenge
E. coli (lon)pRMB3 10μg	2.1 (1.25)	+1.7
E. coli (lon)pRMB9 10μg	3.7 (0.9)	-0.1
E. coli (lon)pRMB9 25μg	3.7 (1.3)	-0.3
Whole cell B.pertussis vaccine	1.2 (0.6)	+2.5
Controls : not challenged	0.7 (0.1)	+2.6

[a] Three week old female CD-1 mice, randomised in groups of 10, were vaccinated with 10 or 25 μg per mouse of AC preparation or 5 ou ml per mouse for the whole cell vaccine. Two weeks after vaccination the mice were challenged intranasally with $\geq 10^6$ c.f.u. B. pertussis 18323. Mice were assessed for infection two weeks after challenge.

ACKNOWLEDGEMENTS

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Structural Homology between Two Calmodulin Sensitive Adenylate Cyclase Toxins: Bacillus anthracis Edema Factor and Bordetella pertussis Adenylate Cyclase

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The adenylate cyclase gene of Bacillus anthracis, encoding the edema factor (EF), a component of anthrax toxin, has been cloned and expressed in Escherichia coli (1). Clones were selected by their capacity to complement the cyclase deficiency (cya$^-$) of an E. coli strain expressing the eukaryotic protein calmodulin, an essential activator of B. anthracis adenylate cyclase. The protein expressed in E. coli was shown to exhibit adenylate cyclase activity only in the presence of calmodulin.

The primary structure of the calmodulin-sensitive adenylate cyclase from Bacillus anthracis has been determined from the corresponding nucleotide sequence and compared to that of the homologous toxin secreted by Bordetella pertussis (2, 3). The cya gene of B. anthracis encodes an 800 amino acid (aa) protein beginning with an N-terminal signal peptide. The central part of B. anthracis adenylate cyclase includes a region of striking homology with the N-terminal part of the B. pertussis enzyme. In this region a particularly well conserved 24-aa peptide and two other less similar peptides have been identified. The first 17 amino acids of the conserved 24 aa stretch (situated between residues 342-358 in B. anthracis enzyme and between residues 54-70 in B. pertussis adenylate cyclase) (Fig. 1) contained a sequence that resembled the generally accepted binding site for ATP (G----GKS). It has been demonstrated in the case of B. pertussis adenylate cyclase that, indeed, this region is part of the active centre of the enzyme (4). From comparison of the amino acid sequences and analysis of truncated proteins we can therefore confidently infer that the cognate site is also involved in the catalytic site of B. anthracis adenylate cyclase.

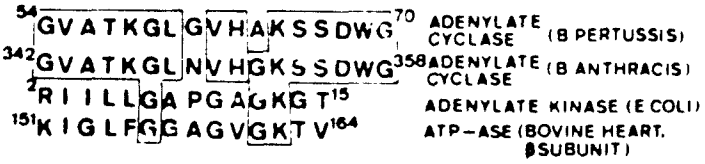


Fig. 1. Alignment of the putative polyphosphate-binding site of B. anthracis and B. pertussis adenylate cyclases with sequences belonging to well characterized ATP-binding enzymes.
Amino acid residues are represented by the standard one-letter code.

Antibodies have been raised against two synthetic heptadecapeptides corresponding to the B. anthracis and B. pertussis sequences represented in Fig. 1. The anti-peptide antibodies specifically reacted in Western blots with the rat brain adenylate cyclase as well as with the two bacterial enzymes (5). In addition anti-rat brain adenylate cyclase serum contained antibodies which were retained by the immobilized peptides. The corresponding affinity-purified antibodies yielded the same recognition pattern of the eukaryotic enzyme as did the unfractionated serum. These results indicate that the eukaryotic adenylate cyclase contains an epitope closely related to that specified by the conserved bacterial sequence and suggest that the three enzymes might have a common origin.

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Basis of Macrophage Sensitivity and Resistance to Anthrax Lethal Toxin

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INTRODUCTION

The two anthrax protein exotoxins share a common B or binding protein. Lethal toxin consists of the B protein, protective antigen (PA83, 83 kDa), together with a second protein, lethal factor (LF, 83 kDa). The same B protein, PA83, together with a third protein, edema factor (EF, 89 kDa) comprises the edema toxin. EF has been identified as an adenylate cyclase (2) while the presumed enzymatic nature of LF remains unknown. Macrophages are uniquely sensitive to the acute cytolytic effects of lethal toxin while non-macrophage cell lines are resistant (1; Singh et al., in press). We now show that some macrophages are resistant to lethal toxin and report our studies to determine the basis of this resistance.

MATERIALS AND METHODS

Peritoneal exudate macrophages cultured from C3H or A/J mice were analyzed for cytotoxicity by release of cellular lactic dehydrogenase or inhibition of protein synthesis. Purified PA and LF were radioiodinated by standard procedures. Binding studies were analyzed by the method of Scatchard. The cAMP response to edema toxin (PA + EF) was determined by radioimmunoassay. The procedure of osmotic lysis of pinosomes (4) was used to introduce LF directly into the cytosol.

RESULTS AND DISCUSSION

Peritoneal macrophages from sensitive C3H mice were lysed by LF at a concentration of 0.001 ug/ml in the presence of excess PA, while macrophages from A/J mice were resistant to

LF at 10 ug/ml. To determine the basis for this resistance we analyzed the various stages in the intoxication process. PA binds to a single class of high-affinity, cell-surface receptors. The PA receptor on both sensitive C3H and resistant A/J cells has the same affinity (K_d = 0.5-0.6 nM) and number of binding sites per cell (25,000-33,000). After PA binding, proteolytic activation by a cell-surface protease converts PA83 to a 63-kDa species, generating a site on PA to which LF or EF bind (3). Proteolytic activation of PA83 was normal in the resistant A/J macrophages and LF receptors generated were identical in sensitive and resistant cells. Thus, resistance was not due to a defect in toxin binding.

The resistant A/J macrophages were not cross-resistant to other toxins, such as Pseudomonas exotoxin A, modeccin, and a transferrin-CRM 107 conjugate. However, they were relatively resistant to edema toxin (PA + EF), suggesting a defect common to both LF and EF. To determine whether LF resistance could be due to a defect in the internalization or translocation of toxin, we introduced LF, in the absence of PA, directly into the cytosol, using the method of osmotic lysis of pinosomes. LF was cytotoxic for the sensitive C3H macrophage, showing that LF itself, possesses toxic activity. The A/J macrophage, however, remained resistant. These results suggest that resistance is not due to a defect in internalization alone. A/J macrophages may be defective at a stage, common to both LF and EF, involving further processing or activation in the cytosol or within vesicles (which may also occur in pinosomes). It is also possible that resistant cells lack the putative LF target and are defective at more than one stage of the intoxication process.

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Changes in Erythrogenic Toxin Patterns in Group A Streptococci: Results from Scarlet Fever Patients in Correlation to the Antibody Response

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ABSTRACT

Group A streptoccoccal strains were isolated from the throat of 46 children suffering from scarlet fever. For detection of erythrogenic toxins (ET) the culture filtrates were concentrated 100 times by ethanol precipitation. ET type A (ETA) was identified by ELISA (detection limit 0.5 ng/ml). The ETA gene was identified by a specific DNA probe.
ET type B (ETB) and ET type C (ETC) were detected by immuno precipitation (detection limit 50 ng/ml). ETA alone or in combination with B and/or C was found in 51.9% of the strains, ETB (alone or in combination with A and/or C) in 75.9%, ETC (in combination with A and/or B) in 28.9%. During the time of observation (21 days after the onset of the disease), one third of the children developed antibodies to SLO and DNase B; 45% developed antibodies to erythrogenic toxins.

INTRODUCTION

At the end of the last century, scarlet fever mortality was very high. The steady fall in mortality began long before an effective therapeutic treatment was introduced. Nowadays, scarlet fever is characterized by a mild course, but the disease is still common (5). The erythrogenic toxins (ET) enact an important part in the pathogenesis of scarlet fever. There are at least 3 types of erythrogenic toxins, type A (26 kD), type B (30 kD) and type C (25 kD) (1, 2).

MATERIALS AND METHODS

Patients: 46 children (3 to 11 years), suffering from scarlet fever. Sera were taken on day 3, 10 and 21 of hospitalisation. Streptococcal strains were isolated from throat swabs. The streptococci belonged to 20 different types of M and T proteins. The ETs determined in 100-fold concentrated culture filtrates (CF) after ethanol precipitation. The detection of the ETA gene was done by hybridization with a specific DNA probe. ETA was detected by ELISA, ETB and ETC by immunoprecipitation with monospecific antisera. Antibodies to streptolysin O, deoxyribonuclease B and ETs were titrated.

RESULTS AND DISCUSSSION

The erythrogenic toxin types, detected in the CF of scarlet fever strains are summarized in Table 1.

Table 1 Percentage of ET positive streptococcal strains

n	A	B	C	AB	AC	ABC	B	Negative	Mean amount of ETA
52	17.3	28.8	no	19.2	no	15.4	13.5	5.8	0.88 ng/ml

The amount of ETA produced by the strains was relatively low: 0.88 ng/ml. Streptococcal strains isolated in GDR during scarlet fever epidemics in 1972 and 1982 produced in average 67.9 and 7.9 ng/ml, respectively. In the time of observation (1961-1968), the amont of ETA produced declined and also the percentage of strains producing ETA. The percentage of streptococcal strains producing ETB increased significantly: 74.2% of strains isolated during the outbreak 1988/89 in GDR found to be positive. Similar trends have been reported (3).
The antibody response of children is shown in Table 2.

Table 2 Antibody reactions in scarlet fever patients

	n	Positive	Percentage
AntiDNase B	83	29	35
ASO	83	26	31
Anti ET (ELISA)	83	37	35

The pathogenic mechanism of erythrogenic toxins in inducing scarlatiniform rash remains still unclear, but other signs of the disease, e.g. fever, can be explained by the production of IL-1 and TNF. The interaction of erythrogenic toxins with the immune cells of the host with subsequent abnormal regulation of mediator production may lead to the scarlet fever symptoms. Antibodies to erythrogenic toxins

were found to be protective in an experimental model in rabbits (4).

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Partial Characterization of Highly Toxic Group A Streptococcal Strains

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ABSTRACT

Streptococcus pyogenes type 1 strains were isolated from 17 patients suffering from a toxic shock-like syndrome, 9 of them died. All strains contained the gene for erythrogenic toxin A (ETA), but the amount of ETA elaborated in vitro, is low (mean value 1.8 ng/ml). Sixteen strains produced ETB and 15 ETC. The LD50 of the strains was not different from "nontoxic" strains.

INTRODUCTION

The history of scarlet fever remains, that a single disease can appear in various syndromes. Scarlet fever could occur as a mild disease with sore throat or it could be associated with sepsis and shock, formerly named as septic or toxic scarlet fever. Cases of septic scarlet fever used to carry a grave prognosis, but such cases were extremely seldom in the antibiotic era.
Cases of staphylococcal toxic shock syndrome and recently of streptococcal toxic shock syndrome reawakened the interest in the relationship between the toxins of S. aureus and S. pyogenes. Similarities in biological activities of TSST-1 and erythrogenic toxins, e.g. mitogenicity, pyrogenicity, induction of interferon γ, IL-1 and TNFα(1,3,5) led to the conclusion, that staphylococcal and streptococcal toxic diseases are related.

MATERIAL AND METHODS

Patients: Seventeen patients (2 from France, 1 from GDR and 14 from Sweden) attended the hospital with a toxic shock-like syndrome.
S. pyogenes was isolated from blood cultures, except for one patient (no culture).
For determination of erythrogenic toxins, streptococcal strains were cultivated in Todd-Hewitt broth, the supernatant was precipitated with ethanol at -20°C and dissolved in citrate buffer, pH 4.2 (concentration factor 75). ETA was identified by ELISA, ETB and C by immunoprecipitation and immunoblotting. ETA gene detection was performed with a specific DNA probe.

RESULTS AND DISCUSSION

All streptococcal strains were identified as M type 1. Strains of this type seem to possess a highly invasive power for a suceptible host and caused the most deaths by S. pyogenes in the last 9 years (2).

Table 1

Characteristics of streptococcal strains isolated from toxic shock-like syndrome

n	M type 1	ETA gene	Detection of erythrogenic toxin in culture filtrate concentrate			
			ETA	ETB	ETC	Average amount of ETA
17	17	17	14	16	15	1.8 ng/ml

The LD50 of the highly human virulent streptococcal strains was comparable with that of other streptococcal isolates. LD50 after i.p. challenge was approximately 5 x 10^6. TNF was induced with the supernatant of both strains from France, but not with the culture filtrate of a type 3 strain (S84). The clinical symptoms: fever, shock, renal failure were accompanied by septicaemia (except one case). Bacteriaemia does not belong to the diagnostic criteria of the classical staphylococcal toxic shock syndrome, but the symptoms of the reported streptococcal toxic shock syndrome are more likely explainable by a toxic than by an solely invasive reaction.
Even under a prompt antibiotic therapy, 9 out of 17 patients died. It should be noted, that an additional therapy with a powerful antitoxic serum may keep the patients alive.

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Biological Properties of a Cytotoxin Produced by Bacillus cereus in Milk at Low Temperatures

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Introduction

Bacillus cereus is an aetiological agent of two forms of food poisoning and severe non-gastrointestinal infections (7). Various extracellular factors such as necrotizing/diarrhoeal enterotoxin, and emetic toxin produced by B.cereus were attributed in the pathogenesis (5, 7). Furthermore, the pathologic role, if any, of cereolysin, protease(s) and phospholipase-C complex is still not known (5). Sporeforming and psychotrophic properties enable B.cereus to survive pasteurization, as well as to grow in milk at low temperatures. The toxin producing ability of B.cereus in milk under different dairy processing and storage conditions is not known. We have recently reported the ability of B.cereus dairy isolates to produce cytotoxins at 8°C (1). We have further investigated the biological properties of such cytotoxins produced at low temperatures.

Materials and Methods

B.cereus strains were grown in brain heart infusion broth containing 0.1% glucose (BHIG), pH 8.0, with aeration and harvested according to the optimal conditions described by Glatz and Goepfert for enterotoxin production (3). Strains were grown in skim milk at 30°C and 8°C (after acclimatization) and tested for cytotoxin production (1). In vitro cytotoxicity experiments on HeLa S3, Vero and human embryonic lung (HEL) cell monolayers, and vascular permeability reaction tests in rabbit skin were performed (1, 2).

Table 1. Cytotoxin production by Bacillus cereus in milk at 8°C

Cell monolayer	Cytotoxicity		
	Positive No. %	Weak No. %	Negative No. %
HeLa	31 (12.7)	39 (38.2)	32 (31.4)
Vero	63 (61.8)	14 (13.7)	25 (24.5)
HEL	75 (73.5)	19 (18.6)	8 (7.8)

A total of 102 strains positive for extracellular toxins at 30°C in milk were acclimatized to 8°C before testing for cytotoxin production. All strains were inoculated as 1 per cent inoculum in milk and incubated with constant shaking (200 rpm) for 72 h.

Results

A total of 136 strains of B.cereus isolated from milk and cream were evaluated for toxin production based on HeLa S3, Vero and HEL cell cytotoxicity in vitro. HEL cell monolayers were more susceptible to B.cereus toxins compared to HeLa and Vero cell lines. The percentage of isolates exhibiting HEL cytotoxicity was similar (43.0 and 48.4%) in BHIG growth conditions at 7 and 24 h harvest time. In milk at 30°C growth temperature, only 21.8% strains showed a HEL cytotoxicity at 7 h, however a larger number of strains demonstrated cytotoxin production (73.2%) at 24 h harvest time. Further, a 102 toxin positive isolates were acclimatized to grow at 8°C in milk. Ninety four (92.2%) of the strains produced HEL cytotoxicity of varying degree (Table 1) with no strict correlation to bacterial cell numbers and also elicited vascular permeability reaction (VPR) in rabbit skin. Laboratory reference strains 4433/73 (enterotoxin positive) and 4810/72 (emetic toxin positive) failed to multiply or demonstrate cytotoxicity at low temperatures.

Histopathological effects of B.cereus cytotoxins in rabbit skin during vascular permeability reaction were studied. Bacterial free culture supernatants were tested for VPR. Crude enterotoxin (from strain 4433/73 grown in BHIG) elicited a distinct VP reaction in the rabbit skin with pronounced oedema in the deep subcutaneous tissue with fresh thrombi in dilated venules. Total necrosis of the panniculus carnosus (muscle layer) was distinct with a scarce infiltration of polymorphonuclear (PMN) cells at the necrotic zone. Milk induced cytotoxin (dairy isolate BC-131), produced at low temperature, showed rather pronounced oedema with distinct infiltration of PMN cells in deep subcutis. Slight degenerative changes in the superficial part of panniculus carnosus with certain degree of PMNs infiltration was evident. Control reaction with skim milk showed no significant histological changes, except, moderate infiltration of PMNs was observed. The tissue trauma elicited by milk induced cytotoxin is shown in Figure 1. Our preliminary data also suggests that this milk induced cytotoxin of B.cereus is distinct from emetic toxin and hemolysin.

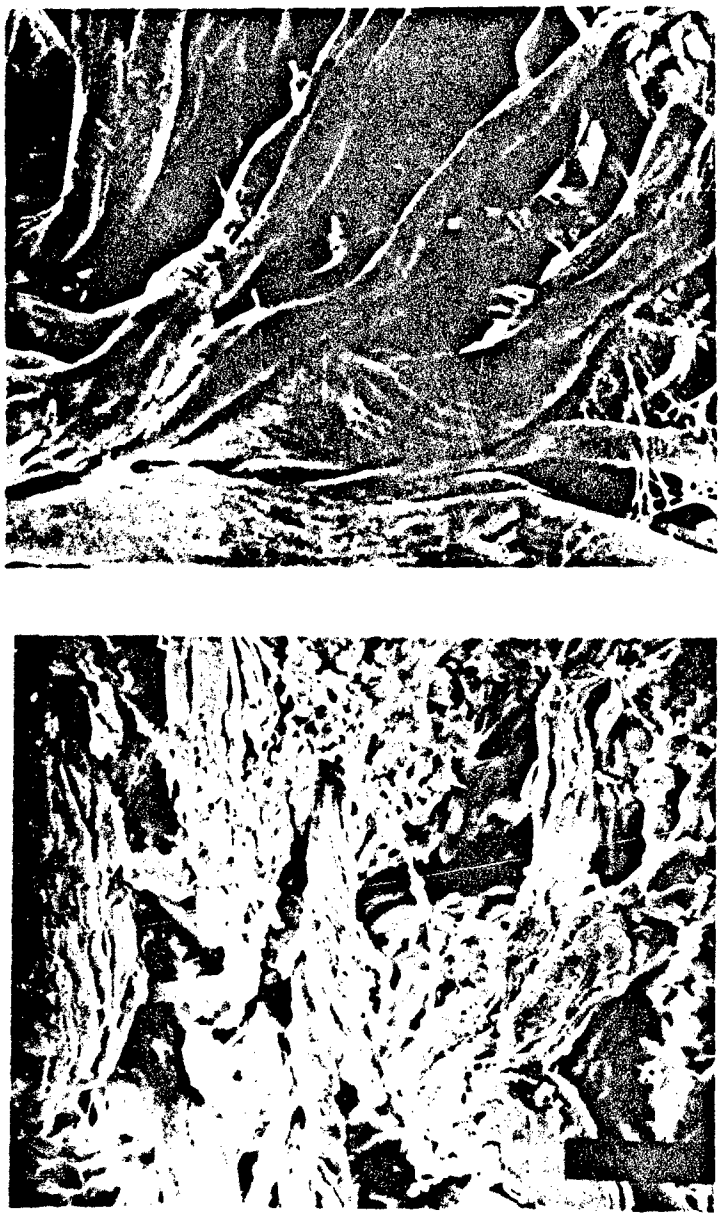


Figure 1. Scanning electron microscopy of rabbit skin showing connective and vascular tissue; Intact in negative control (Top); degenerative changes caused by milk induced B. cereus cytotoxin produced at 8°C (Bottom).

Discussion

Though B.cereus occur in milk at a high frequency, the reports on milk borne toxicosis are few (6). We have demonstrated the ability of B.cereus to produce toxins at storage temperatures. Unlike diarrhoeagenic, necrotic and emetic toxins the milk induced cytotoxins did not show severe pathobiological effects. The ability of these toxins to destroy the subcutaneous tissue matrix is however, interesting. Such condition may possibly allow specific interaction of the gut microbial flora with connective tissue matrix components such as collagens and amplify the adhesion and colonization. B.cereus has also been implicated as a pathogen of bovine intramammary infections, giving rise to increased somatic cell counts (mainly PMNs) in mastitic milk (4). Contact of bacterial pathogen with milk in the bovine udder has been argued as an important event in the pathogenesis of bovine mastitis. Our preliminary data suggests that milk induced toxins were highly chemotactic to PMNs compared to other toxic factors described in B.cereus.

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Molecular Cloning of Listeriolysin O Gene Fragments from Listeria ivanovii

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INTRODUCTION

Listeria ivanovii (formerly L.monocytogenes serotype 5) is a gram positive bacterium pathogenic for animals . Most probably an extracellular cytolytic protein called listeriolysin O is involved in pathogenicity of L.ivanovii as shown for the listeriolysin O (LLO) of L.monocytogenes (2,3). It has been shown recently that both listeriolysins O differ at least in their aminoterminal amino acid sequence, but cross react immunologically using polyclonal anti-listeriolysin O serum (1). To compare the two cytolysins on a molecular genetic level, we are attempting to isolate the LLO gene from L.ivanovii by molecular cloning in E.coli.

MATERIALS AND METHODS

A library of chromosomal DNA of L. ivanovii has been established in E. coli DH5alpha. Chromosomal DNA was partially digested using Sau3A and resulting DNA fragments were ligated into the compatible BamHI site of the plasmid pTZ19R following standard procedures.

An internal 651bp HindIII fragment of the listeriolysin O gene isolated from L. monocytogenes Sv1/2a (pLM47) has been used as a DNA probe to detect recombinant E. coli clones bearing the homologous part of the LLO gene of L. ivanovii (2,3). Screening has been accomplished by DNA colony blot analysis. Under the conditions used only one HindIII fragment of L.ivanovii chromosomal DNA (1.7kbp) has been shown to hybridize with this DNA probe.

RESULTS AND DISCUSSION

Of 6,500 recombinant E. coli clones investigated two have been identified which both react with the DNA probe and also produce proteins which can be detected by Western-Blot analysis using polyclonal antiserum raised against denatured L.ivanovii LLO.

The inserted DNA in the clone bearing the plasmid pAHA10 is approximately 2.6kbp in length, the one in pAHA11 has a molecular weight of approximately 1.4kbp. The corresponding proteins detected by Western blot-analysis have molecular weights of 47kDa and 44kDa , respectively.

DNA/DNA-hybridization experiments using HindIII/EcoRI fragments of the DNA inserted in pAHA10 and pAHA11 revealed that both plasmid inserts are homologous to a 1.7kbp EcoRI fragment of L.ivanovii chromosomal DNA as well as to a 2.4kbp HindIII fragment of L.seeligeri chromosomal DNA. Fragments of the same molecular weight hybridize when the HindIII DNA probe of pLM47 is being used. In pAHA10, a clear DNA sequence homology to the 410bp HindIII fragment of pLM47 is also present. No homology of the inserted Listeria DNA to L. innocua chromosomal DNA (does not produce a LLO) could be detected under the conditions used. Furthermore, it could be shown by restriction analysis and DNA/DNA hybridization studies that both recombinant plasmids (pAHA10 and pAHA11) have an overlapping DNA sequence in common.

The data presented above indicate that most probably fragments of the listeriolysin O gene from L.ivanovii have been cloned in E.coli DH5alpha.

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Characterization of Hemolysin-Mutants of Listeria ivanovii

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INTRODUCTION

Listeria ivanovii (formerly classified as Listeria monocytogenes Sv5b) is clearly distinct from L. monocytogenes based on biochemical and serological characteristics. Interestingly this species is pathogenic for some animals but only mildly pathogenic (if at all) for man, though it exhibits a pronounced hemolysis on blood agar plates. This hemolytic phenotype is at least partially caused by the synthesis and secretion of a SH-activated cytolysin, which has been characterized and has several properties in common with listeriolysin O from L. monocytogenes (1). There is good evidence that listeriolysin O is an important virulence factor of pathogenic Listeriae (2). Apparently L. ivanovii produces a second hemolytic activity which might be related to the CAMP-phenomenon observed with L. ivanovii and Rhodococcus equi (3). In addition to listeriolysin O in culture supernatants of L. ivanovii a protein of Mr 24KD is found in large amounts, the role of which still has to be determined.

MATERIALS AND METHODS

L. ivanovii (ATCC 19119, SLCC 2379) and R. equi (NCTC 1621) were from the Institute of Hygiene and Microbiology, L. monocytogenes BM4140 (with Tn1545) was donated by P. Courvalin (Paris). Bacteria were grown in brain heart infusion broth (BHI, Gibco). Filter matings for transposon mutagenesis were done as previously described (4). SDS-PAGE of TCA-precipitated proteins and immunoblotting was performed by standard procedures, as well as Southren hybridizations. In enzymatic tests for sphingomyelinase and phospholipase the substrate analogues TNPAL-sphingomyelin and p-nitrophenyl-phosphorylcholine, respectively, have been used (5,6).

RESULTS AND DISCUSSION

L. ivanovii was successfully mutagenized by filter mating with L. monocytogenes BM 4140 carrying the conjugative transposon Tn1545 on its chromosome; screening of the recipient bacteria for an altered phenotype yielded five mutants among 3000 transconjugants. These mutants have been analyzed for their hemolytic and phospholipolytic phenotype and their behaviour in the CAMP-test with R. equi. SDS-PAGE of supernatant and total cell proteins, immunoblotting with antisera against listeriolysin O and the 24 KD protein and enzymatic tests for extracellular sphingomyelinase (Smase) and phospholipase C (Plase) were performed. Some of the results of this analysis are summarized in the following table:

Strain	WT	20/24	8/6	44/2
Listeriolysin	+	-	-	+
24 KD protein	+	+	-	-
Smase	+	+	+	-
Plase	+	+	-	+
CAMP	+	+	+	-

This analysis showed that i) listeriolysin O production is indeed only partially responsible for the hemolytic phenotype of L. ivanovii, ii) the transposon mutants are not transport-deficient and iii) most presumably Smase is responsible for the CAMP-effect. Furthermore a region 5'-upstream of the listeriolysin O gene from L. ivanovii has been cloned (data not shown) and will be further analyzed.

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Purification and Characterization of Phospholipases C from Listeria monocytogenes and Listeria ivanovii

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INTRODUCTION

Strains of Listeria monocytogenes are known to produce an extracellular lipolytic activity producing a zone of opalescence in yolk-egg agar or lecithovitellin (6). Clearly, this enzyme is distinct from listeriolysin O (2). Several reports suggest that the extracellular lipolytic activity released in culture supernatant might be due to a phospholipase C (4). However, the enzyme has not yet been purified and characterized. This was achieved in the present study. The lipolytic enzymes secreted by L. monocytogenes and L. ivanovii were purified and characterized as Ca^{++}-independent phospholipase C (PLC).

MATERIALS AND METHODS

Bacterial Strains

We used strain EGD-SmR of L. monocytogenes previously described (1) and L. ivanovii CIP 7842T.

Purification Procedure

L. monocytogenes was grown on charcoal-treated broth and L. ivanovii on proteose peptone N°3, yeast extract, as previously described (3). Culture supernatants concentrated on Pelikon system with a membrane cut-off 10 kD and equilibrated in Tris buffer, 0.075 M pH 7.5, were applied to DEAE 52 (Whatman) column in the same buffer, after which the lipolytic fractions were applied on Biogel P100 (Biorad), equilibated in PBS pH 6.8. SDS-PAGE of proteins and Western-blot analysis were made as described in (3).

Other Methods

Phospholipase C activity was titrated turbidimetrically. The hemolytic assay was made in microtiter plates. TLC was made according to Marrinetti (5). Silica gel was stained by iodine vapour.

RESULTS

1) Purification of Lipolytic Enzymes from Culture Supernatant of L. monocytogenes and L. ivanovii

The purification was achieved in a three-step procedure. Concentrated supernatants in Tris buffer were applied to DEAE-52 column. The unretained fractions containing 90% of the lipolytic activity were pooled, concentrated and passed through a Biogel P100 column. The lipolytic activity peak was again applied to a second Biogel P100 column, thus yielding a pure 28-29 kD protein in the case of L. monocytogenes. With regard to L. ivanovii we obtained a highly enriched 26 kD lipolytic protein, contaminated with a 43 kD protein, in spite of ultrafiltation through Amicon-PM 30.

2) Characterization of the Enzymes

The two enzymes were characterized as PLC by TLC using pure lecithin as substrate. These enzymes expressed a weak sphingomyelinase activity. Using a specific rabbit antiserum raised against PLC from L. monocytogenes, we could not detect any cross-reaction with PLC from L. ivanovii, C. perfringens and B. cereus, as shown by Western blot analysis. These enzymes were Ca^{++}-independent and displayed a weak cytolytic activity against red blood cells containing high amounts of lecithin (guinea pig, rabbit, horse, human). Sheep red blood cells were resistant to these phospholipases. However this hemolytic activity is slowly expressed in vitro, requiring an 18 h incubation at 37°C.

CONCLUSION

These results show that the phospholipases C from L. monocytogenes and L. ivanovii are most likely heterogenous, in contrast to the thiol activated toxins produced by the hemolytic species of Listeria (3).

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Purification of Leucocidin from Staphylococcus Aureus V_8 and Detection of other Leucocidin-Producing Strains

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The Panton-Valentine leucocidin of Staphylococcus aureus (S. aureus) is an exotoxin which has a cytopathogenic effect on monocytes, macrophages and polymorphonuclear (PMN) cells only. Its biological activity is known to require two components inactive separately, but capable of acting synergistically (3,4). The leucocidin mechanism of action is not elucidated so far, and the role of this toxin as a virulence factor has yet to be determined. The aim of this work was to purify the two components of leucocidin from V_8 strain and screen for other strains producing V_8-like leucocidin.

The V_8 strain was cultivated in liquid CCY medium (4). The proteins were obtained by 70 % ammonium sulphate precipitation and cation exchangé chromatography (FPLC, Pharmacia). The yields of purified F and S components were 60 % and 6 mg of each protein were obtained from 1 litre of culture supernate. The specific activity of the mixed components was about forty times that of the supernatant. The F component (M_r 38 kd, pI 9.2) and the S component (M_r 32 kd, pI 8.8) were more than 95 % pure as estimated by SDS-polyacrylamide gel electrophoresis. Each protein had a very little activity by itself, due to up to 3 % contamination with the heterologous component.

Morphology changes of human PMN cells and macrophages were observed by the slide microscopic adhesion method using phase-contrast microscopy (1). The cells adhering to a glass slide were incubated with leucocidin for 30 min at 37°C. The two components had a synergistic effect on PMN cells and monocytes at concentrations as low as 15 ng/ml each. On the opposite, no haemolysis of rabbit, man and sheep erythrocytes occured with either component ; moreover no synergistic effect was observed, even at concentrations such as 140 µg/ml.

Rabbit antisera were raised against each component, then purified by affinity chromatography. They neutralized the two purified components activity as well as the biological activity of the V_8 crude culture supernate. Each component

gave a single immunoprecipitation line with the homologous antibody and none with the heterologous. The V_8 strain supernatant also gave a single line with each antibody. A screening of 33 S. aureus strains from hospitalized patients by immunoprecipitation did not show significant positive reactions. Nevertheless, some of these strains, as P_{83}, had a cytotoxic effect that was not neutralized by leucocidin antibodies ; this P_{83} strain, referred to as leucocidin producer (2), seems therefore to excrete a different leucocidin or membrane-damaging toxin. Moreover, strains with strong haemolytic activity also had strong biological effect on the leukocytes.

These preliminary observations indicate that leucocidin from V_8 strain is not frequently encountered. The leucocidin-like activity often observed for other strains may be due to other known or unknown membrane-damaging toxins.

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Interaction of Toxins with the Immune System

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Degradation and Presentation of Tetanus Toxin to Specific Human T Cell Clones

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INTRODUCTION

Protein antigens are primarily recognized by antigen-specific T cells as proteolytic fragments of 10-15 amino acids in conjunction with products of the major histocompatibility complex (MHC). These products have been called class I or class II antigens. In general, helper T cells are generated by soluble antigens which undergo proteolytic degradation in an endosomal compartment, as yet unidentified, where they are thought to interact with class II antigens. Data obtained in the mouse system using a number of protein antigens indicated that for each mouse haplotype, a restricted set of peptides, were immunogenic and that this set varied according to the mouse haplotype used (1). At the time we began our analysis of human T cell epitopes in humans, little was known as to whether a similar phenomenon existed in this system.

RESULTS

The antigen of choice for our studies was tetanus toxin (t.t.) since a large number of individuals are immunized against this protein, thus facilitating the isolation of specific T cell clones. In addition, t.t. specific B cells can be obtained in humans which are extremely useful in studying antigen internalization and processing. The specific B and T cell clones used in these studies were originally obtained by Lanzavecchia (6). Nine different T cell clones were investigated and grossly mapped by using B and C t.t. fragments and recombinant proteins (Fig. 1).

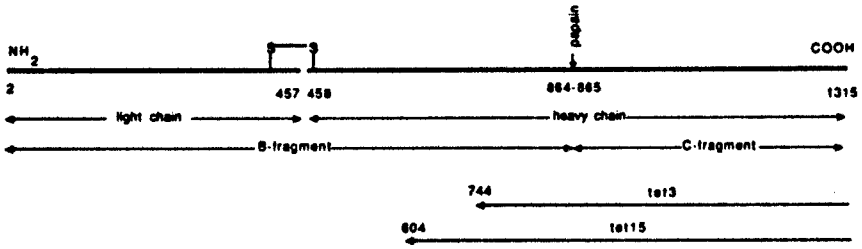


Fig.1. Schematic representation of tetanus toxin. The regions corresponding to the H and L chains. B-fr and C-fr as well as recombinant fragments tet3 and tet15 are shown.

It is interesting to note that four T cell clones were not specific for t.t. but for t.toxoid or bacterial proteins which are part of the t. toxoid preparation. The first epitope specific for the clone KT-4 was mapped by chymotrypsin digestion of t.t. fragment C (Fig. 1) and subsequent HPLC separation of the complex peptide mixture. Each fraction was tested in a T cell proliferation assay.

The resulting positive peaks were rechromatographed, tested and their sequence determined by amino acid and sequence analysis. The epitope thus indentified was formally proven to be correct by synthesis of the corresponding peptide 1273-1284. In addition, peptide analogs were also made and the fine specificity of clone KT-4 was determined (Fig. 2).

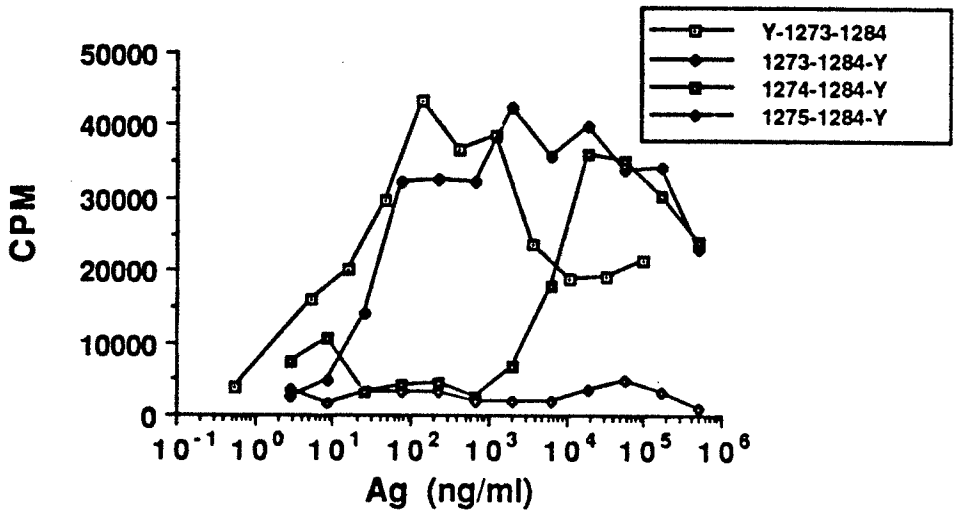


Fig.2. Fine specificity of clone KT-4. Peptides as indicated were incubated at different concentration with $2x10^4$ T and B cells per well.

As shown, deletion of the residue 1273 reduced the potency of the epitope by 100 fold. Peptides of shorter length were not stimulatory. Truncations of the C-terminal side were not performed.

Two additional epitopes were mapped by a totally different method (2,3). We made use of highly purified and specific endoproteases to determine which amino acid residues were present in the various epitopes. If treatment of a protein with a specific protease abolishes the proliferative response, the residue(s) cleaved by that particular protease must be contained in the epitope in question. On the contrary, if the treatment does not affect the proliferative dose response, we assume that that particular amino acid residue is not present. Reduced and carboxymethylated t.t. were first treated with CNBr. No loss of activity was observed after this treatment indicating that all epitopes were contained within CNBr fragments.The CNBr preparation was then treated with different proteases (i.e. trypsin, Lys C, V8, endo asparaginase) and an enzymatic map for five epitopes was thus obtained (Table I).

Table I: Amino acid residues present or absent in the various epitopes

	Amino acid residues	
Clone T	present	absent
KT-2	Lys	Asp, Glu
KT-4	Asp	Lys, Glu
KT-30	Lys	Asp, Glu
KT-42	Lys, Asp	Glu
KT-40	Lys, Asp, Glu	-

The epitope specific for clone KT-2 which was previously mapped within fragment 744-864 was determined by synthesizing only two peptides (830-843 and 849-864) which corresponded to the characteristics shown in table II, namely the presence of lysine and absence of glutamic and aspartic acids (2). The epitope specific for clone KT-30 was similarly mapped (3) together with the use of nitrocellulose strips containing fragments derived from CNBr digestion of the t.t. C-fragment which were separated by SDS-PAGE electrophoresis and blotted on nitrocellulose according to the method of Lamb and Young (5). In this case, synthetic peptide 947-967 was proven to specifically stimulate clone KT-30. Table II shows the various epitopes thus mapped together with the restriction element utilized.

Table II: Summary of t.t. fragments recognized by the various T cell clones and the restriction elements utilized

T cell Clone	Shortest defined t.t. fragment containing T cell epitope	Restriction Element
KT-2	830 - 843	DR5
KT-4	1273 - 1284	DRw52a
KT-30	947 - 967	DR5
KT-40	2 - 602	DR5
KT-42	604 - 742	DRw52a

This type of analysis can be of course extended to other proteins. The quantity needed is about 0.1-1.0 mg and the preparation does not have to be pure.

Epitope specific to clone KT-42 could not be found yet in spite of the fact that synthetic peptides corresponding to the data shown in Table I were synthesized. Our explanation is that asparagine or glutamine residues contained in the fragment have been modified to the resulting acid, thus rendering inoperative our approach.

A similar situation was found for epitope 1273-1284 (2). In this case, the conversion of asparagine to aspartic acid was localized on residue 1272 which is outside the epitope and of little consequence for T cell proliferation. In these cases, the only method available to localize the epitopes is that followed for the caracterization of antigenic determinant 1273-1284.

At this point, the frequency by which these epitopes are recognized by peripheral blood lymphocytes (PBL) in the general population was determined. To our surprise, the two epitopes 830-843 and 947-967 were recognized by PBL of all individual tested. This situation is clearly different from that established in the mouse in which one epitope is usually restricted to one or few restriction elements (1).

The third epitope 1273-1284 was seen only by individuals carrying the DRW52a and 52c restriction elements (4), a situation which is comparable to that seen in the mouse system. The two universal epitopes could in principle be used as T cell carriers for general immunization.

Another interesting observation made in the course of these studies is the different capacity of various antigen presenting cells to present intact t.t. to T cell clones (4).

Table III: Differential presentation of t.t. by two LCL B cell lines

	3H-thymidine, incorporation cpm x 10-3		
	KT - 2	KT - 30	-
t.t.	36.8 ± 4.4	82.5 ± 8.8	2.7 ± 0.7
CNBr RCM-t.t.	46.0 ± 8.3	71.5 ± 2.9	2.7 ± 0.3
t.t.	62.4 ± 5.7	1.1 ± 0.1	1.0 ± 0.1
CNBr RCM-t.t.	72.1 ± 3.6	91.1 ± 7.7	0.9 ± 0.1

B cells were pulsed with t.t. at 40 µg/ml or with CNBr-RCM t.t. at 75 µg/ml for 6h at 37°. After washing, B cells ($2x10^4$ /well) were added to $2x10^4$ cloned T cells.
^{3}H-thymidine was added after 48h and cells were harvested after 72h.

The basic phenomenon is described in Table III. Two Epstein-Barr virus transformed B cell lines which had been pulsed with intact t.t. present the antigen equally well to clone KT-2 but not to clone KT-30. This failure could be bypassed if synthetic peptides or CNBr treated t.t. were used. We hypothesize that different cell lines have a different set of enzymes available for antigen processing and that this difference may represent the first level of antigen selection. We have now extended these results to PBL, indicating that the defect is not linked to the transformation event that took place in these cell lines. This observation may have far reaching implications either at the level of auto-immune diseases or in the design of synthetic subunit vaccines.

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Degenerate binding of immunogenic peptides to HLA-DR proteins on B Cell Surfaces

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Introduction

The identification and sequencing of the antigen receptor of T cells (1-3) coupled with the demonstration that MHC proteins specifically bind immunogenic peptides (4-6), and the solution of the crystal structure of HLA A2 (7,8) collectively have led to a working model of how T cells recognize protein antigens (9). This unique recognition mechanism apparently has evolved to allow receptors on two separate cells to contact a common peptide ligand. As valuable as these experimental results have been for increasing our understanding of T cell recognition, they also have raised several different, but equally perplexing questions. The principal issue our laboratory has concentrated on is how can a single, conserved MHC antigen combining site specifically recognize a very large number of diverse peptides? Although the MHC molcules are members of one of the most polymorphic families of proteins yet defined, the amino acids composing the combining site of any one allele are constant. This is in stark contrast to the other proteins known to bind antigen, antibodies and the antigen receptors of T cells, which both create diverse binding sites by genetic rearrangement and, in the case of antibodies, additional somatic mutations (9-11).

In this report, the ability to detect binding of biotinylated peptides to MHC class II proteins on cell surfaces was used to examine the degree of degeneracy between the ligand and different MHC alleles. This rapid, simple, and quantitative assay allowed a large number of HLA DR alleles to be screened for their ability to bind the peptide. The ability of both a surprisingly large number of DR alleles to bind the peptide and any one MHC protein to bind a large variety of peptides is discussed in the context of MHC restriction of T cell recognition.

Materials and Methods

Peptides

The peptide corresponding to residues 307-319 of influenza hemagglutinin (PKYVKQNTLKLAT), which previously was shown to be recognized by DR1 restricted T cells (12), was the parent sequence for the analogues used in this study. Long chain biotin (13) was placed unambiguously at each position by substituting lysine at the desired position, replacing lysines 308, 311, and 316 with arginine, and acetylating the α-amino group. The peptides were synthesized using standard solid-phase methods as previously described (12) on an Applied Biosystems 430 A synthesizer, and biotinylated with excess sulphosuccinimidyl 6-(biotinamido) hexanoate (Pierce). The biotinylated peptides were

purified by reversed phase HPLC and analyzed by amino acid analysis and fast atom bombardment mass spectrometry. Biotinylation was further confirmed by positive reaction with dimethylaminocinnamaldehyde (14).

Binding assay

The assay has been described in detail elsewhere (15). Briefly, two EBV-transformed B cell lines homozygous for HLA-DR1 Dw1, MAJA and METTE, and the class II deficient EBV-B cell line , RJ 2.2.5 (16), were incubated at 3 x 10^5 cells per well with each biotinylated peptide (50μM) in 96-well plates (200 μl) at 37 °C for four hours, followed by FITC-streptavidin (4.22 μg/ml; Calbiochem). Cell surface DR expression was quantified by staining with fluoresceinated L243 anti-DR monoclonal antibody (17)(Becton-Dickinson; 30 min., 4 °C). After each incubation, cells were washed with PBS containing 0.1% bovine serum albumin. Stained cells were analysed by flow cytometry using a FACScan analyser (Becton-Dickinson). Only viable cells, identified on the analyser by their ability to exclude propidium iodide, were included in the analysis. In inhibition studies, competing peptides or unlabelled L243, were included in the assay. To determine whether differential proteolysis was a factor, a mixture of protease inhibitors (TPCK, 10 μg/ml; PMSF, 50 μg/ml; leupeptin, 1 μg/ml; aprotinin, 1 μg/ml; soybean trypsin inhibitor, 10 μg/ml) were coincubated with cells and the biotinylated peptide.

Results and Discussion

To determine whether immunogenic peptides could be shown to bind MHC class II molecules on the surface of intact cells, a T cell determinant from influenza hemagglutinin (HA; residues 307-319) (12), which is recognized by a HLA-DR1 restricted T cell clone, was assayed for its ability to bind Epstein-Barr virus transformed human B lymphocytes (B-LCL) homozygous for HLA-DR1. These cells were chosen because they were homozygous, well characterized cell lines that express unusually high levels of HLA class II proteins. Because of concerns of high background fluorescence and a low specific signal, the peptide was not directly fluoresceinated. Instead, it was conjugated to a biotinyl group to take advantage of the amplification that can be obtained by using multiply fluoresceinated streptavidin.

When the DR1-homozygous B-LCL, MAJA, was incubated with the peptide, stained with fluoresceinated streptavidin, and analysed for green fluorescence by flow cytometry, the cell surface fluorescence was approximately five times higher than in the absence of peptide (Figure 1a). The signal was two orders of magnitude less intense than that obtained by indirect immunofluorescence with a monoclonal antibody specific for a determinant present on many human class II molecules.

All detectable fluorescence with this peptide was shown to be specific by using RJ 2.2.5 cells (16). These cells, like the MAJA line, are human B-lymphoblastoid cells transformed by Epstein-Barr virus, except that they do not express class II proteins because of a mutation in a regulatory protein required for class II expression (18). Consequently, they provide an excellent control for the specificity of the fluorescent signal, because they should display a similar cell surface except for the absence of MHC class II proteins and any allelic variation of surface proteins between the cell lines. When RJ 2.2.5 cells were used in the assay, the fluorescence in the presence of peptide was indistinguishable from background (Figure 1b). This demonstrated that the large majority of the fluorescent signal on the cells expressing DR1 was specific and strongly suggested that the peptide bound to class II MHC proteins or other proteins whose expression was coregulated.

L cells transfected with genes encoding α and β chains of human class II molecules were used to demonstrate that the peptide binds to HLA-DR. A distinct fluorescent signal was observed when the HA peptide was incubated with cells transfected with DR1, but absent on normal L cells (Figure 1c). In contrast, the fluorescence on L cells expressing DQw1 was indistinguishable from that of untransfected cells (data not shown). However, because the level

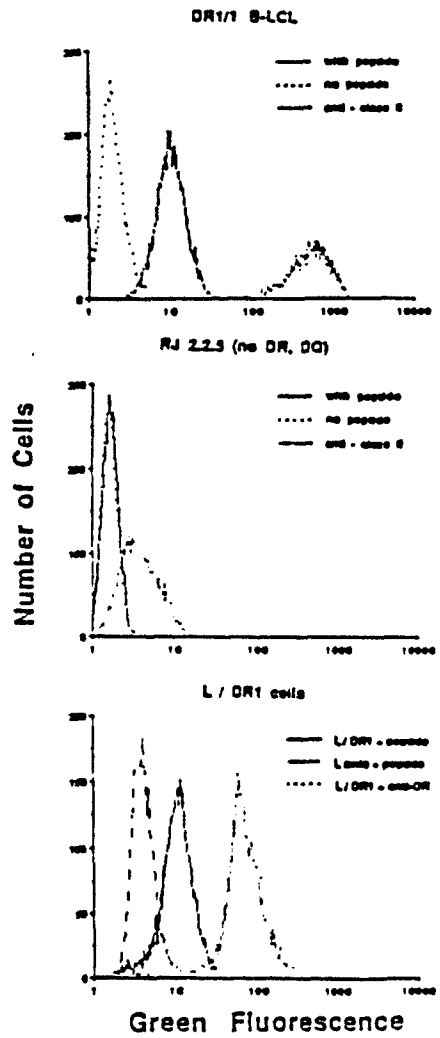


Figure 1. Binding of a biotinylated analogue of HA 307-319 to cell surfaces. *A*, Binding of the HA307-319 analog to The DR1-homozygous B cell line, MAJA (———). Surface expression of class II MHC proteins was shown by indirect immunofluorescence using the anti HLA-D region antibody, TAL 31.1(·····). Background fluorescence in the absence of biotinylated peptide was determined by incubation only with streptavidin (· · · · ·). *B*, Failure of the HA analogue (———) to bind to the class II-deficient B-cell line, RJ 2.2.5, above background (· · · · ·). Residual staining with TAL 31.1 antibody (·····) may be due to the presence of small amounts of HLA-DP. *C*, Binding of the biotinylated HA peptide to L cells transfected with HLA-DR1. Transfectants (clone 5-3.1) (———) or untransfected L cells (- - -) were incubated with 100 μM peptide overnight. The level of staining for DR (.....) on the transfected L cells (indirect immunofluorescence using the anti-DR monoclonal antibody, L243) was about ten times lower than on B-lymphoblastoid cells.

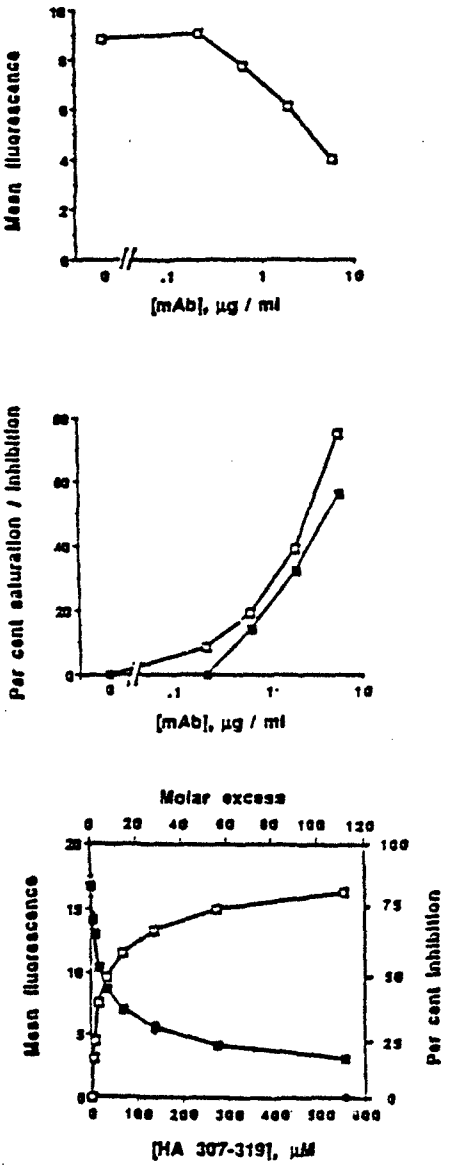


Figure 2. Inhibition of peptide binding by an anti-DR monoclonal antibody and the natural determinant, HA307-319. *A*, peptide binding to MAJA cells in the presence of varying amounts of protein A-sepharose purified L243 anti-DR antibody. *B*, Comparison of the reduction in fluorescent signal by L243 (■) and the fraction of DR molecules bound by antibody (□) determined by indirect immunofluorescence using varying amounts of L243 as in (*A*), followed by fluoresceinated rabbit anti-mouse Ig. *C*, Inhibition of peptide binding (5 µM) to MAJA cells by varying concentrations of HA 307-319 (shown as molarity and molar excess on bottom and top axes, respectively). Both the fluorescence (■; scale on left) and the fractional reduction in fluorescent signal (□; scale on right) are shown. RJ 2.2.5 cells incubated with biotinylated peptide and competitor gave no signal (●).

of DQ expression on these transfectants was low, the possibility that the peptide can weakly interact with DQ cannot be ruled out.

Further evidence that the peptide directly interacted with HLA-DR on the surface of B-LCL was that the fluorescent signal could be modulated by coincubating the B-LCL with peptide and an anti-DR monoclonal antibody (17). Increasing amounts of antibody progressively reduced the fluorescent signal (Figure 2a). However, complete inhibition of the signal by incubation with this antibody could not be obtained due to the difficulty of getting sufficiently high antibody concentrations that blocked the binding without crosslinking the cells and removing them from the gate of the FACS analyzer. Using Fab fragments of the antibody did not solve the problem. Nevertheless, the reduction of the fluorescent signal closely paralleled the saturation of cell surface DR1 by the antibody (Figure 2b), indicating that the majority of the fluorescent signal was due the formation of a peptide-MHC class II complex and not nonspecific binding on the cell surface.

The natural determinant also was shown to compete with the biotinylated peptide, demonstrating that both occupy the same site on DR1. Half inhibition could be obtained at relatively low molar excess of the competitor over the biotinylated peptide (sevenfold) (Figure 2c). As the concentration of the competitor was increased, the fluorescence decreased, reaching 80 % inhibition at a molar ratio of 110. A higher excess of competitor could not be obtained because of the limited solubility of the natural determinant and the need for relatively large amounts of biotinylated peptide in order to observe a distinct fluorescent signal.

Binding of the biotinylated peptide was detectable only after 30 minutes, and continued to increase even after a 16-hour incubation (data not shown). Because binding to the class II-deficient cells was not detectable, even at the longest incubation time, we can assume the increase in fluorescence with time, reflects the kinetics of association of the peptide with HLA-DR. The low rate of cell surface binding is consistent with the previously reported kinetics of association between peptides and purified class II molecules in detergent (5). When cells were incubated with peptide, stained with straptavidin, washed and further incubated at 37 °C, a gradual, slight reduction in cell surface fluorescence was observed over several hours (data not shown). The kinetic data demonstrated that binding does not approach a true equilibrium over the time of the assay and that dissociation is negligible on the time scale of the staining procedure.

The incubation temperature was a critical factor in the generation of the fluorescent signal. Peptide binding to DR1 was halved at 25 °C and reduced by two thirds at 4 °C relative to 37 °C . However, a variety of inhibitors of antigen processing had no effect (sodium azide, colchicine, cytochalasin B, chloroquine, and ammonium chloride), suggesting that internalization of the peptide was not required for binding.

The ease of the assay and the availability of the homozygous B-LCL allowed us to examine the ability of the biotinylated HA peptide to bind to twenty-two cell lines expressing different DR types (Figure 3). Remarkably, even though the fluorescent signal (Figure 3a) significantly varied between cell lines, detectable fluorescence distinct from background was present in each case with the exception of the class II-deficient cell line. The variation in fluorescence could not be explained by differences in DR expression between cell lines (shown in Figure 3b). When peptide binding was corrected for these variations by dividing the fluorescence obtained with peptide by that obtained with the anti-DR monoclonal antibody (Figure 3c), significant differences between cell lines remained, which allowed classification of the cells into groups having haplotypes with high (DR14a Dw16, DR13a Dw18, DR13b Dw19, DR16 Dw21) low (DR17 Dw3, DR9, DR4 Dw15), or intermediate (all others) capacity for associating with the HA peptide.

The broad range of binding exhibited by this peptide is surprising particularly in the context of MHC restricted recognition. However, two important features of this assay should be

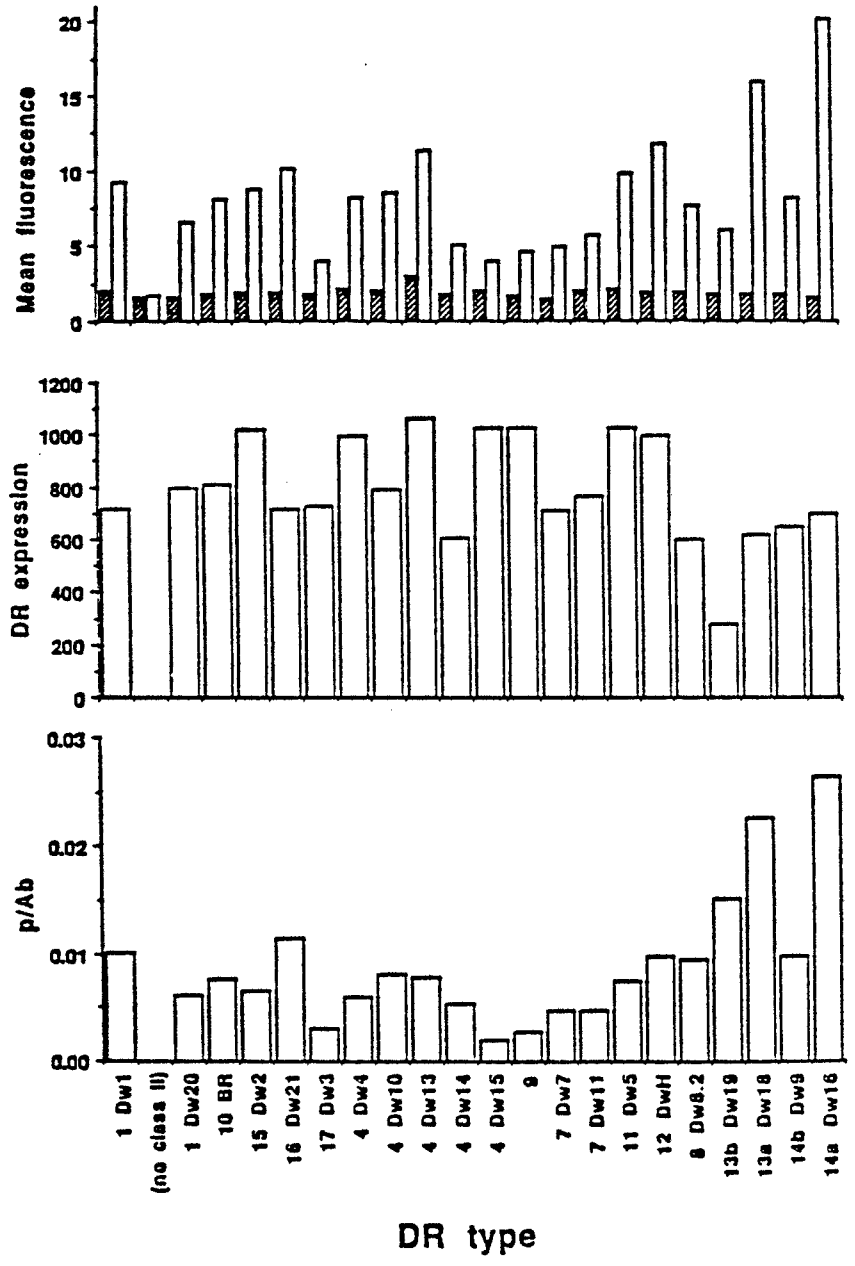


Figure 3. Binding of the HA peptide to B cell lines homozygous for different DR alleles. *A*, Mean fluorescence on cell lines homozygous for the DR types indicated, when incubated with peptide (□) and without peptide (▨). *B*, Mean fluorescence on the same cells stained with directly fluoresceinated L243 anti-DR monoclonal antibody. *C*, Relative peptide binding (from panel *A*) divided by relative antibody binding (from panel *B*).

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The broad range of binding exhibited by this peptide is surprising particularly in the context of MHC restricted recognition. However, two important features of this assay should be

emphasized, which might contibute to the large degeneracy of binding (i.) the assay requires a large amount of peptide (50μM) relative to that necessary for stimulation of T cells and (ii.) the assay is performed in the absence of any competitive peptides. At physiologically relevant concentrations of peptide the binding to the different cell lines might be quite different. In addition, when incubated with a variety of peptides, as in the case of a natural influenza infection, this particular peptide might not occupy the majority of sites on the class II molecules. However, the lowest levels of cell surface fluorescence measured in this assay are relevant to T-cell responsiveness: DR4 Dw15 expressing cells, which bound the biotinylated HA analogue most weakly, presented the natural HA determinant to a HA-specific T lymphocyte clone equally well over a range of concentrations as the autologous restriction element, DR1 Dw1, which binds the biotinylated peptide at intermediate levels (J. Rothbard et al., in press). In addition, the broad range of binding reported here is unique to the HA peptide. Four other T cell determinants also bind the twenty-two B-LCL, but the high binding alleles are not identical for each peptide (manuscript in preparation). If generally true, these results indicate that a major factor in MHC restriction of T cell recognition must arise from MHC-T cell receptor interactions and not simply different capacities to bind peptide.

Acknowledgements

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Delineation of T Cell Epitopes of Pertussis Toxin for the Design of Synthetic Vaccines against Whooping Cough

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Whooping cough, a respiratory infection caused by Bordetella pertussis can be life threatening when acquired within the first year of age. The "cellular" vaccine composed of inactivated B.pertussis cells, although efficacious in protection, has to be substituted because of its side effects. Several "acellular" vaccines, containing purified B.pertussis antigens have been proposed. Pertussis toxin (PT) is the main component of such vaccines, beeing a protective antigen in both animal models and humans, as demonstrated by a recent field trial held in Sweden(1,2). PT is a complex molecule composed of five different subunits, S1, S2, S3, S4, S5 in ratio 1:1:1:2:1. Subunit S1 is an ADP-ribosyltransferase which has as target GTP-binding proteins on eucaryotic cells from different tissues. The other subunits are involved in cell binding. It is very likely that the next generation of anti-whooping cough vaccines will contain chemically or genetically detoxified PT purified from culture supernatants. As an alternative approach, a third generation vaccine may be conceived, based on recombinant or synthetic non-toxic peptides of PT that mimic the entire molecule for the immune system. The immune response to a protein antigen is generated by the cooperation of mainly two cell types: B and T lymphocytes. B lymphocytes generally recognize conformational epitopes on a protein and produce specific antibodies following "help" signals by T lymphocytes specific for the same antigen. On the other hand, T lymphocytes recognize short peptides that are generated by the degradation of the antigen and that are exposed on the surface of the antigen presenting cell, in association with polymorphic ClassII molecules encoded by the Major Histocompatibility Complex (MHC). The identification of B and T cell epitopes allows the formulation of synthetic constructs that could be used for vaccination. The aim of our study was the identification of T cell epitopes of PT recognized by human T lymphocytes(3).

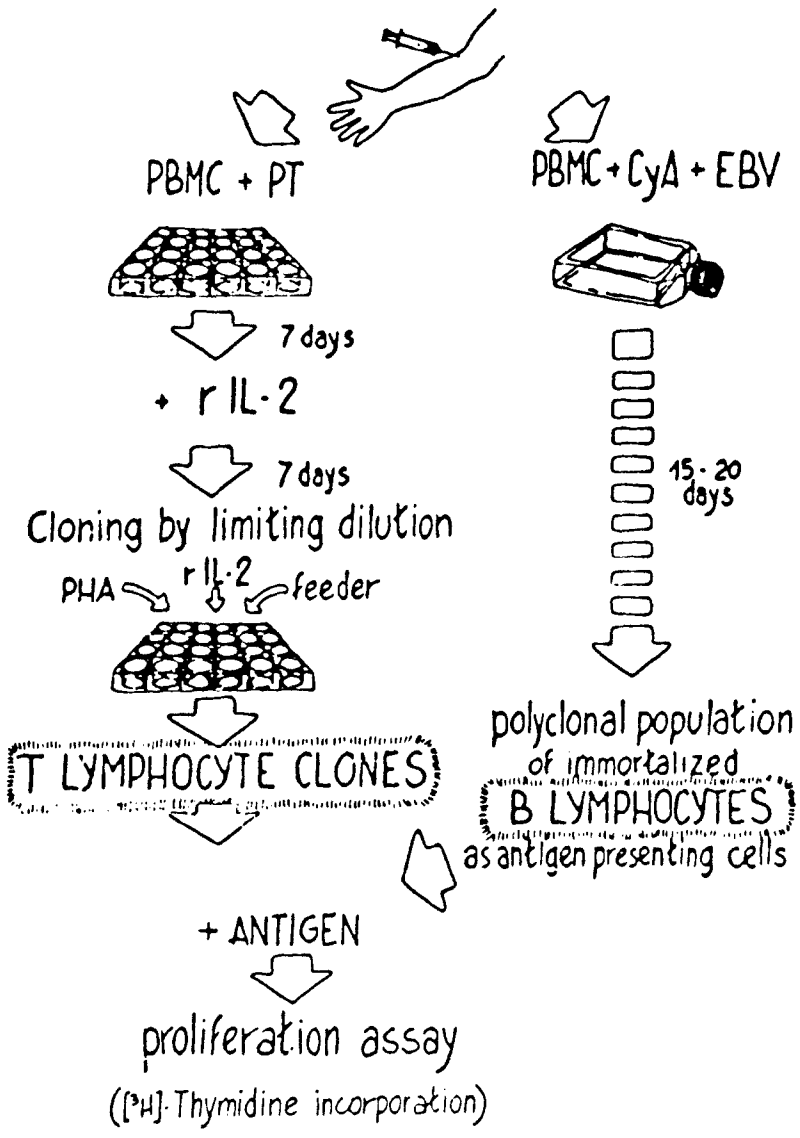


Fig.1. Schematic rapresentation of the method used to obtain human T lymphocyte clones specific for PT and to test their fine specificity (see text).

RESULTS AND DISCUSSION

1) Generation of anti-PT T cell clones.

Clones of T lymphocytes specific for PT were obtained from the peripheral blood of a donor immune to pertussis. The method is shown in Fig.1. Peripheral blood mononuclear cells (PBMC) were cultured with purified and heat-inactivated PT. Proliferating T lymphocytes were expanded in the presence of interleukin-2 (IL2) and phytohaemagglutinin (PHA) and than cloned by limiting dilution. At the same time, B lymphocytes of the same donor were immortalized in vitro with Epstein Barr Virus in the presence of Cyclosporin A, in order to produce a continuos source of antigen presenting cells. Several T cell clones were obtained, each virtually originating from a different memory T lymphocyte. The clones were tested for recognition of PT and its derivatives in proliferation assays.

2) Fine specificity of anti-PT T cell clones

Twelve T cell clones specific for PT were found (Table 1.) Their fine specificity was then investigated using the five recombinant subunits of PT obtained as recombinant proteins in E.coli(4). Surprisingly, ten of the twelve clones (83%) were specific for subunit S1 (Table 1.) ; the other two clones recognized S4 and S2 plus S3 (data not shown). These results suggested immunodominance of S1 over the other subunits of PT and confermed in man similar results obtained in animal models, where S1 has been shown to be also a protective subunit of PT. In order to map the T cell epitopes of this subunit we generated more T cell clones against S1 itself. A total of eighteen T cell clones, all of the helper phenotype $CD3^+$ $CD4^+$ $CD8^-$, were studied.

3) Mapping T cell epitopes on S1 subunit

To this purpose both recombinant and synthetic peptides of S1 were prepared. Nine E.coli strains were ingegnerized to produce fragments of S1 representing NH2- and COOH-terminal truncations of the protein (Fig.2). These were used to localize the regions of S1 containing T cell epitopes. On the basis of recognition of some fragments (indicated with ⊕ in the figure) and non-recognition of others (indicated with ⊖), it was possible to deduce three sequences of S1 containing residues 1-42, 181-211 and 212-235, which are recognized by respectively eleven, five and two clones.

Table 1. Proliferation of human T cell clones to purified PT and recombinant S1

Clone	Antigen		
	None	PT	S1
T106	2.9 ± 0.3[a]	5.1 ± 0.6	2.2 ± 0.2
T207	2.3 ± 0.4	7.7 ± 1.3	1.9 ± 0.1
T209	2.4 ± 0.5	5.8 ± 0.5	18.7 ± 1.7
T215	2.3 ± 0.2	55.8 ± 2.4	59.4 ± 1.6
T216	2.3 ± 0.3	5.8 ± 0.8	14.6 ± 0.2
T217	2.0 ± 0.7	4.7 ± 0.8	16.1 ± 2.3
T218	2.1 ± 0.2	11.3 ± 0.6	20.5 ± 2.6
T219	2.6 ± 0.2	9.9 ± 1.7	10.4 ± 0.4
T220	1.2 ± 0.1	14.2 ± 1.7	18.9 ± 0.6
T226	2.4 ± 0.2	57.3 ± 2.4	56.0 ± 2.2
T227	2.8 ± 0.2	12.0 ± 2.2	12.7 ± 2.1
T229	2.5 ± 0.1	32.6 ± 1.6	31.7 ± 1.3

[a]Mean cpm ($x10^{-3}$) ± SD of triplicate cultures
Boxed values are statistically significant

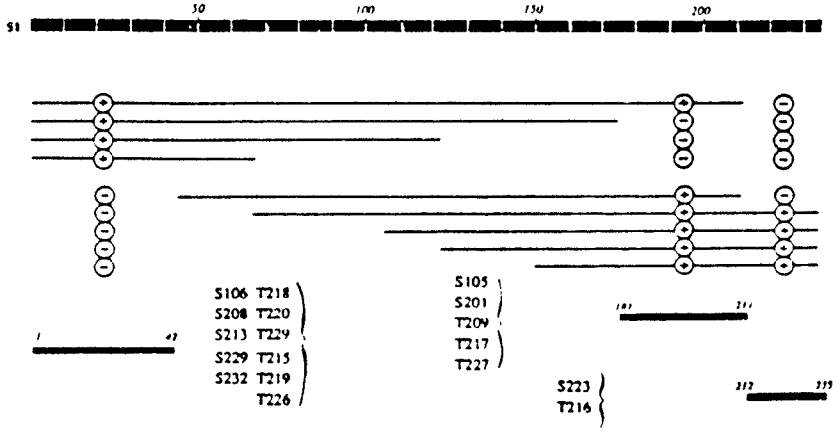


Fig.2: Recombinant deletion fragments of subunit S1 and sequences recognized by T-cell clones. Fragments designed with ⊕ are recognized by the clones listed in the lower part of the figure.

To identify the minimal sequences stimulating T cell proliferation within these regions, synthetic peptides were then used. Among several tested (results not shown), a NH2-terminal peptide of 13 residues, 27-39 was recognized by three clones mapping in region 1-42 (Table 2.). In the region 181-211, peptide 180-194 stimulated four out of five clones. This peptide was synthesized following the previous observation that the longer peptide 171-194 was first identified as stimulatory for the four clones mentioned. The peptides 27-39 and 180-194 were studied for their MHC restriction and resulted to be recognized in the context of class II DR molecules, in particular of DR1 (not shown).

Table 2. Synthetic peptides of S1 recognized by T cell clones

A) Peptide 27-39: GNNDNVLDHLTGR

Clone	Medium	27-39 (10 µg/ml)
S232	0.8 ± 0.2[a]	9.2 ± 1.1
T215	1.8 ± 0.4	45.9 ± 10.4
T226	0.9 ± 0.2	44.3 ± 4.2

B) Peptide 180-194: SRRSVASIVGTLVRM

Clone	Medium	180-194 (10 µg/ml)
S105	2.1 ± 0.1	18.0 ± 1.1
T209	0.5 ± 0.1	30.8 ± 0.8
T217	1.9 ± 0.1	12.3 ± 0.1

[a]Mean cpm ($x10^{-3}$) ± SD of triplicate cultures

CONCLUSIONS

The identification of three regions of S1 containing T cell epitopes and the definition of two short synthetic peptides as stimulatory sequences for T lymphocytes are useful for the development of a synthetic vaccine against whooping cough. Furthermore, the polymorphism of class II molecules imposes that peptides to be included in a vaccine have the capacity to bind to molecules of different specificity, in order to be stimulatory for most of the individuals in a population. We have preliminary evidence that the two peptides that we have identified have this capacity.
Finally, we would like to remark that the localization of T cell epitopes on subunit S1 provides useful information for the production of second generation vaccines containing chemically or genetically detoxified PT. Such modified molecules should keep unaltered their T cell epitopes as well as their tridimensional structure in order to stimulate an immune response capable of recognizing native PT. The PT-specific T cell clones that we isolated from a donor immunized by the disease represent a useful tool to test in vitro the antigenicity of such modified PT molecules.

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Immunocytotropic Bacterial Proteins Toxins

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INTRODUCTION

The study of the interaction of bacterial protein toxins with the immune system has been rather less developed in the past decade as compared to the great expansion of other areas of bacterial toxinology particularly those relevant to molecular genetics, structural biochemistry and cell biology. The main reasons are very likely the high complexity of the immune system network and inappropriate experimental approaches for probing toxin interactions with the various cellular and molecular elements of that system. Fortunately this situation has been rapidly changing over the past five years and interesting information has been obtained concerning the effects of a number of toxins on certain functions and properties of immune system cells and molecular effectors.

DEFINITION OF IMMUNOCYTOTROPIC TOXINS

We propose to coin the denomination "immunocytotropic protein toxins" for those toxins acting directly as molecular signals on immunocytes and their precursors leading to the expression of biological activities and functions on target cells and/or induction of the expression of molecular structure(s) at their surface, independent of their behavior as antigens processed and recognized for immune response to their epitopes.
Among the immunocytotropic toxins are those toxins known to behave as polyclonal mitogens for T lymphocytes (Table 1) leading to their proliferation with concomitant release of various cytokines from monocytes (monokines) and/or lymphocytes (lymphokines).

Table 1. Immunocytotropic bacterial protein toxins acting as T-lymphocyte mitogens

STAPHYLOCOCCAL ENTEROTOXINS A, B, C, D, E.
STAPHYLOCOCCAL TOXIC-SHOCK SYNDROME TOXIN-1
STAPHYLOCOCCAL PYROGENIC (ERYTHROGENIC) EXOTOXINS A, B
STREPTOCOCCAL ERYTHROGENIC (PYROGENIC) TOXINS A, B, C
OTHER STREPTOCOCCAL MITOGENIC PROTEINS
PERTUSSIS TOXIN
CHOLERA TOXIN
PSEUDOMONAS AERUGINOSA EXOTOXIN A

STAPHYLOCOCCAL AND STREPTOCOCCAL IMMUNOCYTOTROPIC TOXINS

Staphylococcus aureus and Streptococcus pyogenes produce an array of immunocytotropic toxins of primary clinical and biological importance. This group is that constituted by staphylococcal enterotoxins (SE), staphylococcal toxic-shock syndrome toxin-1 (TSST-1) and streptococcal erythrogenic toxins (ET) also known as pyrogenic exotoxins (see ref. 1,3,15,21,27,29 for recent reviews). All these toxins are single-chain polypeptides with molecular weights ranging from ca. 22 to 28 kDa. Staphylococcal enterotoxins are classified into five serological groups: A, B, C, D and E (referred to as SEA, SEB, SEC, SED, and SEE) respectively. SEC is further subdivided into three groups, SEC1, SEC2 and SEC3 based on differences in minor epitopes (15,27). Streptococcal erythrogenic toxins comprise three serological types; A, B and C (1,21,29). Enterotoxins A to E, TSST-1 and ETA, ETB and ETC share a number of common biological properties including pyrogenicity and T mitogenicity (Table 2).

Table 2. Biological properties shared by streptococcal erythrogenic toxins, staphylococcal enterotoxins and toxic-shock syndrome toxin-1

1. PYROGENICITY
2. POLYCLONAL LYMPHOCYTE MITOGENICITY (MONOCYTE COOPERATION)
3. INDUCTION OF CYTOKINES (MONOKINES, LYMPHOKINES)
4. ENHANCEMENT OF HOST SUSCEPTIBILITY TO LETHAL SHOCK BY ENDOTOXIN
5. SUPPRESSION OF IgM SYNTHESIS AGAINST SRBC IN MICE
6. SUPPRESSION OF ALL Ig CLASSES IN HUMANS
7. ENHANCEMENT OF DELAYED TYPE HYPERSENSITIVITY
8. INDUCTION OF TOLERANCE IN CERTAIN RABBITS

References: 1,3,4,6,27,29.

The three groups of table 2 toxins exhibit also common clinical and pathological features (table 3).

Table 3. Clinical and pathological manifestations of staphylococcal enterototoxins and TSST-1 and of streptococcal erythrogenic (pyrogenic) (exo)toxins

ENTEROTOXINS (A to E)	EMESIS, DIARRHEA IN HUMANS (FOOD POISONING) AND EXPERIMENTAL ANIMALS, SHOCK-LIKE SYNDROME IN ANIMALS (PYROGENICITY, HYPOTENSION, ERYTHRODERMA)
TSST-1:	MAJOR FACTOR IN TSS AND TSS-LIKE DISEASES, FEVER, HYPOTENSION, SCARLATINIFORM SKIN RASH, DESQUAMATION, EMESIS AND DIARRHEA, MULTIPLE-ORGAN-SYSTEM-DYSFUNCTION.
ERYTHROGENIC TOXINS (A, B, C)	SCARLET FEVER, TOXIC-SHOCK SYNDROME, HEART DAMAGE, SKIN RASH, DESQUAMATION, MULTIPLE-ORGAN-SYSTEM DYSFUNCTION.

References: 1,3,15,21,27,29.

Structural homology in amino acid and nucleotide sequences have been shown between the enterotoxins and erythrogenic toxins but not for TSST-1 (table 4).

Table 4. Structural relationship between staphylococcal enterotoxins TSST-1 and streptocccal erythrogenic toxins

- AMINO ACID (AA) and NUCLEOTIDE SEQUENCES KNOWN FOR: SEA, SEB, SEC1, SEE ; TSST-1; ETA, ETC.
- COMPUTER - AIDED ANALYSIS OF MATURE TOXIN AA SEQUENCES AS WELL AS NUCLEOTIDE SEQUENCES INDICATE SIGNIFICANT HOMOLOGY AND HIGHLY CONSERVED REGIONS BETWEEN EACH OF THESE TOXINS EXCEPT FOR TSST-1. HOWEVER CONSIDERABLE SEQUENCE DIVERGENCE HAS OCCURED WITHIN THESE FAMILIES OF IMMUNOCYTOTROPIC TOXINS.
- THE HOMOLOGY IS REFLECTED BY IMMUNOLOGIC CROSS-REACTIVITY.
- A COMMON EVOLUTIONARY ANCESTOR IS SUGGESTED.

References: 3,26,30.

In the next paragraphs we describe some experimental aspects of the interaction of ETA and TSST-1 with immune cells investigated in our laboratory. We shall summarize in the discussion some of the recent data concerning the mitogenic properties of these toxins and of SE in the context of their behavior as superantigens.

MITOGENIC EFFECTS OF ERYTHROGENIC TOXIN A

We have previously shown that this toxin is a potent polyclonal mitogenic activator of T cells in mice, rabbits and humans (6,8). It also acts as an immunomodulator (immunosuppression/adjuvant properties depending on our experimental conditions) (8) and triggers the release of interferon γ upon T cell stimulation (7). It stimulates B cell polyclonal secretion of immunoglobulins in mice, rabbits and human (8). The study of toxin mitogenicity on $CD4^+$ and $CD8^+$ T lymphocyte subpopulations separated from human peripheral blood lymphocytes clearly showed that ETA almost exclusively induced the proliferation of the helper ($CD4^+$) T cell subset. In contrast the cytotoxic/suppressor (CD8) T cell subset did not significantly proliferate (2). Accessory non T cells (a mixture of B cells and monocytes) were necessary to induce the proliferation of CD4 T lymphocytes. The eventual role of interleukin-2 (IL-2)-activation pathway in the reactivity to ET was investigated. IL-2 was measured in the supernatants of cell cultures stimulated with the toxin. We also determined the number of IL-2 receptor ($Tac^+/CD25^+$) expresing cells. Cells expressing the receptor were noted as early as day 2, and their number peaked on day 5. IL-2 activity in culture supernatants was low peaking on day 2 suggesting that this lymphokine was progressively adsorbed on activated T cells at the same rate as that of its production (Korinek, Brisson, Geoffroy, Alouf and Gluckman, in preparation). The apparent specificity of ETA for $CD4^+$ T cells led us to investigate toxin effect on the production of AIDS virus HIV-1 peripheral blood incubated with mononuclear and $CD4^+$ cells and stimulated thereafter with either ETA or phytohemagglutinin (PHA). HIV-1 production assessed by the assay of reverse transcriptase in cell supernatant appeared earlier after stimulation with ETA and was 6 to 10-fold greater than after stimulation by PHA (2). This finding may have important clinical significance for HIV-infected subjects who acquire S. pyogenes infections with in vivo production of ETA. The toxin may enhance HIV-1 replication and contribute to the development of immunodeficiency.

INTERACTION OF TSST-1 WITH LYMPHOCYTES, MONOCYTES AND THEIR PRECURSORS

This toxin is known to stimulate both CD4 and CD8 T cells (3,4,26). The pyrogenic effects and shock induction led us to investigate whether tumor necrosis factor α (TNFα, also known as cachectin) might be released by TSST-1 challenged monocytes similarly to the case of endotoxins for which TNFα was shown to be an important mediator of its toxicity and pyrogenicity. Indeed, TSST-1 induced at very low doses (0.1-1000 ng) the production of high level of TNFα from human monocytes (9,19) and rabbit monocytes (9) in a dose-dependent manner. This finding was confirmed by other authors for TSST-1, ETA, SEB and SEC1 (10,18,25). Enriched preparations of lymphocytes challenged with TSST-1 were shown to produce interferon γ, and TNFβ (19). The production of IL-2 was also demonstrated (19,22). When the lymphocytes were stimulated with phorbolmyristate acetate, TSST-1 effect was strongly potentiated and the levels of the above-mentioned lymphokines was greatly increased (19). Massive amounts of interleukin-1 have been also shown to be produced by incubation of TSST-1 with monocytes (16,17,18,24,26).

Recently the effects of TSST-1 on murine hematopoiesis were investigated (16) particularly as regards the proliferation and differentiation of murine granulocyte-macrophage progenitor cells (CFU-culture) and the eventual role of endotoxin. TSST-1 had no direct effect on the proliferation on CFU-culture and was unable to influence the CSF-induced proliferation and differentiation of these progenitors. In contrast, the toxin was a potent inducer in spleen cell cultures of a factor with an ability to induce both colony formation by bone marrow cells and proliferation of an IL-3-dependent cell line. Nanogram amounts of TSST-1 were able to induce the release of CSF activity in spleen cell cultures from both normal and LPS-hyporesponsive mice. Cells from C3H/HeJ mice were as responsive as cells from C3H/He Pas mice. Furthermore, in spleen cell cultures from normal mice, TSST-1 and LPS did not act synergistically to induce CSF activity. Nanogram amounts of TSST-1 were also able to induce CSF activity in vivo but failed to induce IL3-activity in the serum and organ-conditioned media from TSST-1-treated mice.

TOXIN RECEPTORS ON MONOCYTES AND LYMPHOCYTES

A great advance has been made in the past two years concerning the receptors involved in T cell proliferation in response to stimulation with staphylococcal enterotoxins and TSST-1. The data obtained (5,11,12,13,14,20,28,31) may be summarized as follows:

1. Enterotoxins A to E and TSST-1 are extremely potent polyclonal mitogens for human and murine T lymphocytes. However they are not indiscriminate mitogens stimulating all T cells. On the contrary each toxin tested reacts only with T cells (CD4 and CD8) expressing particular $V\beta$ domains in their α/β receptor (V_α, J_α, $V\beta$, $D\beta$, $J\beta$) almost regardless of the other components of the TCR. Each toxin is specific for a selective $V\beta$ sequence (SEB: V.3, V.8; SEA: V.3, V.11 in murine system) thus mimicking a strong alloreactive response.

2. SE and TSST-1 are unable to bind directly and stimulate T cells. They require prior binding to accessory cells (monocytes, B cells, RAJI cells,...) that express class II MHC molecules (HLA-DR, IA-IE) to interact with and activate the appropriate $V\beta$ specific T cells.
Accessory cell binding is not restricted by the haplotype of the responder and is independent of class II allotype or isotype. Any allogeneic or xenogeneic class II molecules can reconstitute T cell response to SE (no restriction by polymorphic determinants of HLA molecules).

3. The stimulation of T cells by SE and TSST-1 is presently considered as a process inolving physical association of the toxins with a non polymorphic part of accessory cells, HLA class II molecules. SE do not require processing to stimulate T cells. The toxin is presented by HLA-DR or IA-IE antigens to the appropriate $V\beta$ elements of the TCR through a selective cross-linked TCR/CD3-CMH class II associated structure.

4. The specificity of these toxins for $V\beta$s puts them in the recently

described class of the so-called "superantigens" and may account for the differential sensitivity of different individuals to the toxic effects of these proteins.
The enterotoxins share the "superantigenic" properties with the enigmatic self antigens in mice: Mls-1^a and Mls-2^a. This shared property is unlikely to be coincidental.

5. Neonatal mice given enterotoxin B eliminate all mature and some immature T cells bearing the particular Vβs, demonstrate that tolerance to exogeneously administered antigen could be caused by clonal deletion of reactive T cells.

6. These recent results provide new unexpected insights into the mechanisms of action of certain highly pathogenic bacterial toxins. The ability of SEA to bind class II molecules and activate large numbers of T cells with overlapping specificities raises the possibility that exposure to such bacterial products in vivo may underlie diseases, including several autoimmune disorders in which T cells that share common receptor determinants are activated. The hypothesis that MHC class I and II molecules are receptors for bacterial products has long been postulated, for example, in the pathogenesis of ankylosing spondylitis.

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IgA Protease-associated α-Proteins of Pathogenic Neisseria: Nuclear Transport and Speculations on Their Role in Pathogenesis

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ABSTRACT

The iga genes of pathogenic Neisseria code for large precursor proteins that give rise to two extracellular products, the mature IgA protease and the α-protein. Our mutational analysis of the iga gene from N. gonorrhoeae MS11 revealed that the α-protein is not essential for either IgA protease secretion or for enzymatic activity. Instead, the primary structure of α-proteins shows two striking features: (i) amino acid sequences reminiscent of nuclear transport signals from eucaryotic cells, and (ii) apparent homology with the nucleic acid binding moieties of proteins. The ability of the α-protein to function as a nuclear transport signal has been demonstrated using chimeric proteins produced in eucaryotic cells, where the hybrids localized exclusively in the nuclei. This finding suggests a messenger-like function for the α-proteins in the interaction of pathogenic Neisseria with eucaryotic cells.

INTRODUCTION

IgA proteases are extracellularly secreted by a variety of medically important gram positive and gram negative bacteria (9, 20, 21). These enzymes specifically cleave IgA1 into Fab and Fc fragments and hence probably interfere with the action of one of the predominant immunoglobulins in mucosal secretions (10, 18, 27). The specificity of IgA proteases for human immunoglobulin and their association with pathogens infecting the human mucosal tissues suggests that these enzymes play a role in bacterial pathogenesis (11), although direct evidence for their contribution to virulence is missing.

The IgA proteases of Neisseria gonorrhoeae and the iga genes have been studied extensively (4, 12). Characterization of the mature extracellular enzymes and analyses of cloned iga genes from various strains reveals that the proteases are initially produced as large precursor proteins with molecular weights of approximately 170 kDa (5, 22). In these precursors four domains can be distinguished on the basis of functional and structural properties: (i) an amino terminal signal peptide which is assumed to direct transport through the inner bacterial membrane, (ii) the protease domain of 100 to 110 kDa, (iii) the α-domain, which is strongly hydrophilic and predicted to be α-helical, and (iv) the carboxy terminal ß-domain (previously called helper) harbouring the functions essential for the transport of the protease and the α-domain across the outer membrane (17, 22). In addition to the processing of the amino terminal signal peptide by a signal peptidase, autoproteolysis of the precursor plays a key role in the secretion and maturation of IgA protease. Three proline-rich regions in the precursor, homologous to the cleavage site in the hinge region of IgA1, are used as autoproteolytic sites (22, 23). Cleavage at

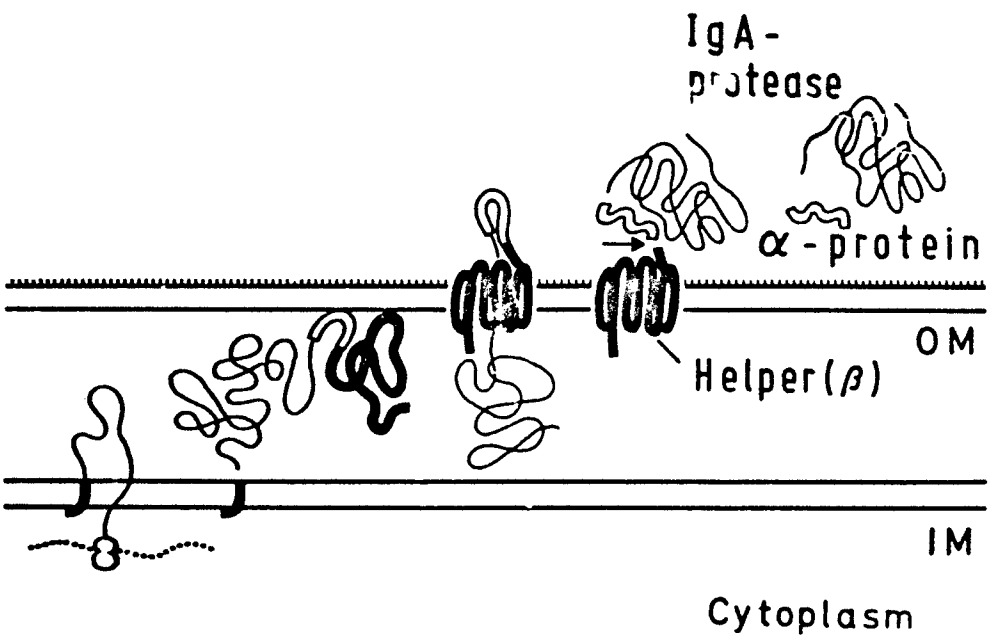


Figure 1. Model for the extracellular secretion of IgA protease and α-proteins. Arrow indicates autoproteolytic site 'c'. (For further details see Text and Ref. 22)

these sites leads to the separation of an extracellular intermediate, P121, consisting of IgA protease- and α-domains, from the outer membrane-associated ß-domain. The extracellular P121 finally developes into the two stable polypeptides, the mature IgA protease and the α-protein.

Two groups of iga genes (H_1 and H_2) can be distinguished in N. gonorrhoeae on the basis of the structure and size of their α-proteins (5). H_1 iga genes specify an α-domain of 15 kDa and a ß-domain of 45 kDa, while H_2 genes code for an α-domain of 26 kDa and a ß-domain of 33 kDa. Further variations have been observed in N.meningitidis (unpublished data). This structural polymorphism suggests that conservation of the α-protein is not required for the extracellular transport or the activity of IgA protease. In fact, our analyses rule out active participation of the α-proteins in protein secretion. Our detection of functional nuclear transport signals instead suggests a role for α-proteins in the interaction of the pathogen with eucaryotic cells.

MATERIALS AND METHODS

Construction and analysis of deletion derivatives.

Deletions were introduced at the unique BstXI site in the α-protein encoding region of the iga gene of N. gonorrhoeae MS11. Bal31 exonuclease was used essentially as recommended in the laboratory manual of Maniatis et al., (16). Recombinant E.coli clones containing the plasmid-encoded iga deletion derivatives were tested for IgA protease activity (4). The amounts of secreted IgA protease in culture supernatants of positive clones were analysed by SDS polyacrylamide gel electrophoresis (14). The deletions in the iga genes of positive subclones were characterized by restriction mapping of the plasmids and double stranded DNA sequencing (28).

Construction of hybrid genes.
The α-protein encoding DNA fragment was amplified from the iga gene of N.gonorrhoeae R16 (pIP2, ref. 5) using the polymerase chain reaction (PCR) technique (25). Taq polymerase and two synthetic oligonucleotide primers were used in a thermal cycler (Perkin Elmer) according to the manufacturers protocols. The amplified DNA fragment was digested with appropriate restriction enzymes and inserted into the iel gene on a plasmid digested with the same endonucleases. After transformation into E.coli, subclones were identified by physical mapping of the plasmid DNA. The correct insertion of the amplified DNA fragment was confirmed by double stranded DNA sequencing (28).

Expression and localization of chimeric proteins.
Control plasmids and the plasmids containing the hybrid iga genes were prepared and transfected into a mouse fibroblast cell line (Ltk^-) using standard methods. Cells were fixed with a mixture of paraformaldehyde and glutaraldehyde (1%/0.5%), permeabilized with 0.5% Triton X-100, and the fusion proteins were localized by indirect immunofluorescence. For detection of pp89 a monoclonal antibody was used in combination with FITC-conjugated polyclonal anti-mouse IgG serum. In addition a polyclonal rabbit serum (α-fp80, ref. 5) was used in combination with FITC-conjugated Protein A to detect the α-portion in the chimeric proteins.

RESULTS

Gonococcal iga genes have the unique ability to direct selective extracellular secretion of active IgA protease in other gram negative bacteria, including E. coli (2, 17). The E. coli system provides a suitable environment for the analysis of α-protein participation in the secretion process and in the enzymatic activity of IgA protease. To study this we made Bal31 exonuclease deletions from the BstXI site, which is located in the region of the iga gene encoding the α-domain (Fig. 2). Deletion mutants giving rise to active IgA protease

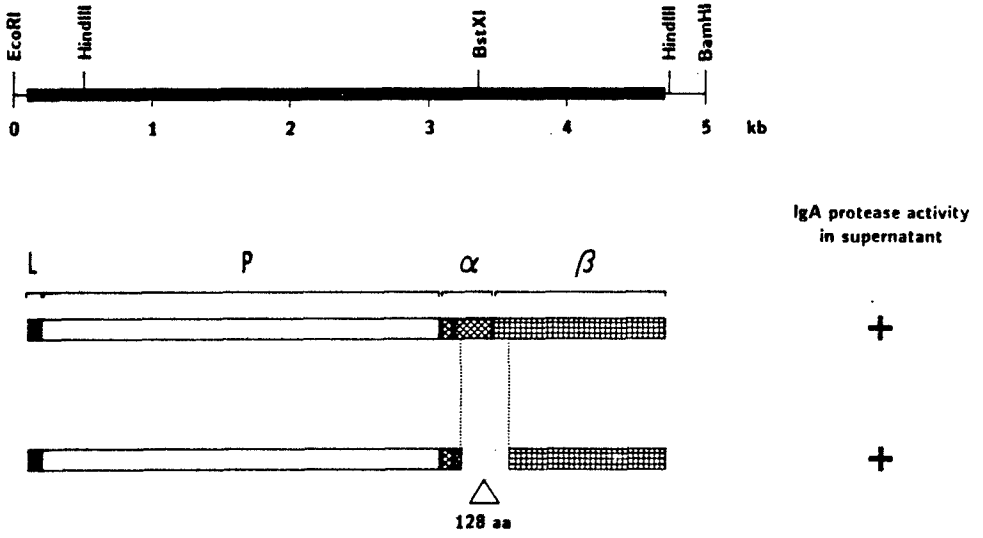


Figure 2. The top line shows the physical map of the cloned iga gene fragment from N.gonorrhoeae MS11. The solid line indicates the open reading frame encoding the IgA protease precursor. The linear structure of the precursor consisting of the leader peptide (L), the protease domain (P), the α-domain (α) and the ß-domain (ß) are indicated below. Location and extent of the largest IgA protease positive deletion in the precursor are shown in the lower part of the figure.

in culture supernatants were identified by SDS polyacrylamide gel electrophoresis and in an enzyme activity assay (4).

An example of a deletion removing the majority of the α-domain and an amino terminal part of the ß-domain is presented in Figure 2. This deletion, of 128 amino acids, does not cause loss of active extracellular IgA protease, even though the autoproteolytic cleavage site 'c' has been lost. While most deletions in the α-domain lead to an increase in protease secretion, deletions extending further into the ß-domain result in decreasing amounts of extracellular protease (data not shown). This clearly demonstrates that the α-domain has no essential role in the secretion and/or maturation of the IgA protease.

Nearby the carboxy terminus of the MS11 α-protein a remarkable sequence consisting of six positively charged amino acids preceeded by a proline residue is located (Fig. 3a). Similar clusters of positively charged amino acids in combination with a proline residue are found in the nuclear location signals responsible for the transport of proteins into the nucleus of eucaryotic cells. A well characterized signal is contained in the Simian Virus 40 T antigen (Fig. 3a) (6, 7). While the MS11 α-protein, encoded by an iga-H_1 gene, carries only one such sequence, we found four similar sequences in the H_2 α-proteins produced by N. gonorrhoeae strains R16 and NG74 (Fig. 3a), even though the overall sequence composition of H_1 and H_2 α-proteins differs extensively (5). Gonococcal iga genes share

a

N.gonorrhoeae MS11	NLS	:	**Pro Lys Arg Arg Arg Arg Arg Ala**
N.gonorrhoeae R16/NG74	$NLS_{1,3}$	:	**Pro Gln Arg Arg Lys Arg Arg Ala**
	NLS_2	:	**Pro Lys Arg Arg Gly His Arg Ser**
	NLS_4	:	**Pro Lys Arg Arg Gly Arg Arg Ser**
N.meningitidis B1939	NLS	:	**Pro Lys Arg Arg Gly Arg Arg Ser**
SV40 T	NLS	:	**Pro Pro Lys Lys Lys Arg Lys Val**

b

MS11 $DNAB_3$		-Lys-Arg-Lys-Ala-Ala-Glu-Ile-Ala-Arg-Gln-Gln-----Glu-Glu-
R16 $DNAB_4$		-Lys-Arg-Lys-Ala-Ala-Glu-Ser-Ala-Lys-Arg-Lys-Ala-Glu-Glu-Glu-
IF-2	E.coli	-Lys-Arg-Lys-Ala-Glu-Glu-Glu-Ala-Arg-Arg-Lys-Leu-Glu-Glu-Glu-
Histon 1	P.angulosus	-Lys-Arg-Lys-Ala-Ala-----Leu-Ala-Lys-Lys-Lys-Ala-
ftz	D.melanogaster	-Lys-Arg-Lys-Ala-Glu-Asp-Asp-Ala-Ala-
HMG 17		-Lys-Arg-Lys-Ala-Glu-Gly-Asp-Ala-Lys-
cI	P22	-Gln-Arg-Lys-Val-Ala-Asp-Ala-Leu-Gly-
Tet R	pSC101	-Thr-Arg-Arg-Leu-Ala-Glu-Arg-Leu-Gly-

Figure 3: Sequence characteristics of α-proteins. (a) Comparison of putative nuclear location signals (NLS) of Neisseria proteins and SV40 T antigen. Amino acid sequences of proteins were deduced from N. gonorrhoeae MS11/R16/NG74 (5, 22) and N. meningitidis B1939 (R.Halter, unpublished) DNA sequences. The nuclear transport signal (NLS) of the SV40 T antigen has been described in several publications (6, 7). (b) Comparison of putative DNA binding domains. Amino acids printed in bold indicate identical residues, while underlined positions mark similar amino acids. The amino acid sequences are derived from: IF-2, E. coli initiation factor-2 (24); sea urchin histon H1 (1); ftz, Drosophila fushi tarazu (15); HMG 17, high mobility group protein 17 (3) and the procaryotic repressor proteins cI and TetR (19).

homology with the iga genes of N. meningitidis (13). Therefore we performed a partial DNA sequence analysis of the iga gene from N.meningitidis B1939 and found a similar transport signal at the carboxy terminus of the predicted α-protein (Fig. 3a).

The α-proteins are strongly basic, with a pI between 10 and 12, and their sequence predicts an extraordinary long α-helix of approximately 80 amino acids. This helical structure consists of short amino acid sequences that are repetitively arranged and show homologies with the putative binding motifs of nucleic acid binding proteins. Figure 3b provides examples of such putative DNA binding motifs in different gonococcal α-protein variants compared with the binding domains of various procaryotic and eucaryotic proteins. Homologies are particularly evident for one of the α-helices in the so-called 'helix turn helix motif', that is common to many procaryotic and eucaryotic DNA binding proteins such as cI, TetR, ftz (15, 19). Homology also exists with the translation initiation factor IF-2 of E. coli, and the DNA binding proteins HMG17 and histon H1. For histon H1 the sequence shown is found in a large region which is predicted to take up an α-helical conformation and to be responsible for the interaction with DNA (1).

To assess the question of whether the observed sequence homology to nuclear location signals is sufficient for the nuclear transport of α-proteins, we constructed hybrid genes. An approach commonly used to identify potential nuclear location signals (NLS) is to construct hybrid genes containing the putative NLS sequence fused to a test gene that encodes a cytoplasmically located protein. For this purpose we constructed fusions substituting the R16 α-protein for different portions of the mouse cytomegalo virus IE pp89 protein, which is encoded by the viral ieI gene (8). Expression of the wildtype ieI gene (contained on plasmid p57/3) leads to a nuclear location of the pp89 protein in eucaryotic cells, presumably due to a NLS sequence coded by the 3'-part of the ieI gene (8); in contrast, mutant construct p60/1, containing a gene devoid of putative NLS coding sequences, gives rise to a protein exhibiting cytoplasmic location (H. Volkmer et al., manuscript in preparation). The mutant pp89 protein, against which monoclonal antibodies have been raised, was therefore used as an immunological reporter for the location of chimeric α-proteins (Fig. 4).

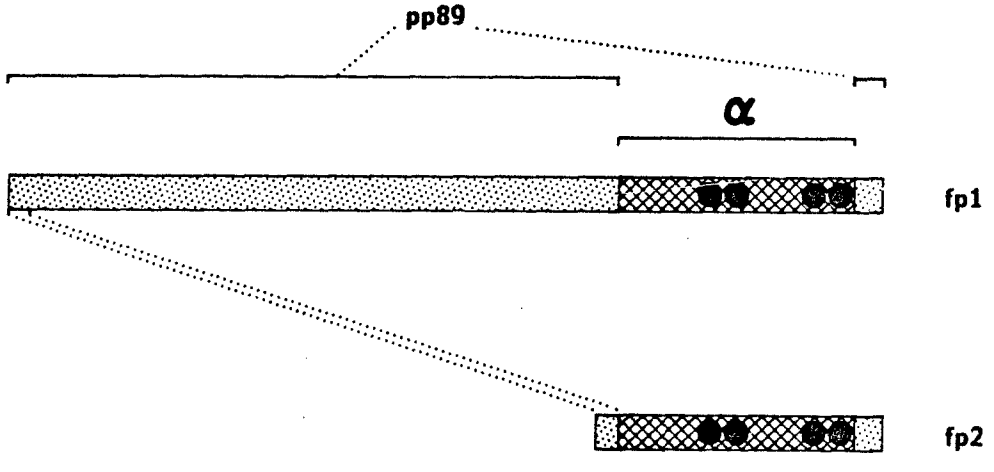


Fig. 4. Structure of chimeric pp89/α-proteins. The fusion proteins fp1 and fp2 are encoded by plasmids pUL1 and pUL2, respectively. The dotted sections indicate the pp89 portions while the hatched sections represent the H_2 type α-protein of N. gonorrhoeae R16. The black dots indicate the positions of the putative nuclear transport signals in the α-protein. The approximate molecular weights of fp1 and fp2 are 90 and 30 kDa, respectively.

To achieve this, the gene fragment encoding the α-protein was enzymatically amplified from the N. gonorrhoeae R16 iga gene using the PCR technique (25). The oligonucleotide primers included flanking restriction sites and thus facilitating subcloning. The resulting plasmid, pUL1 contains a heterologous gene in which 7% (41 codons) of the iel gene was substituted by 205 α-protein codons (Fig. 4). In pUL2, 93% (555 codons) of the viral gene was replaced by the gonococcal iga_{α} gene fragment. In both constructs, the hybrid genes are flanked by the original 5' and 3' sequences of the viral gene and hence contain all necessary expression and processing signals.

The plasmids pUL1, pUL2 and the control plasmids p57/3 and p60/1 were transfected into mouse Ltk^- cells in order to determine the subcellular localization of the chimeric and control proteins by indirect immunofluorescence microscopy. Figure 5 shows representative micrographs of the transfected cells. For detection of the proteins, polyclonal rabbit antiserum raised against the R16 α-protein, and a monoclonal antibody directed against the pp89 protein were used. Cells transfected with plasmids pUL1 and pUL2, which direct the expression of chimeric proteins, showed a characteristic staining of the nuclei. The large fusion protein fp1 (pUL1) caused an even distribution of fluorescence throughout the nucleoplasm, including nucleolar structures (Fig. 5a). In contrast, the small hybrid protein fp2, expressed from pUL2, led in many cases to small areas of bright staining, which possibly correspond to nucleolar regions (Fig 5b). The monoclonal antibody raised against pp89 demonstrated the nuclear localization of the wildtype pp89 protein (Fig. 5c) and fp1 (data not shown), which also contains the pp89 antigenic epitope. In contrast, the mutant pp89 protein expressed from p60/1 was localized, using the monoclonal antibody, to the cytoplasm of transfected cells. Cells transfected with p57/3, p60/1 or pUL2 did not stain with either anti-α-protein serum (p57/3 and p60/1) or the anti-pp89 monoclonal antibody (pUL2). These results clearly demonstrate that the α-protein of N.gonorrhoeae R16 can substitute for the natural NLS of the viral pp89 protein, and consequently direct the chimeric proteins to the cell nucleus.

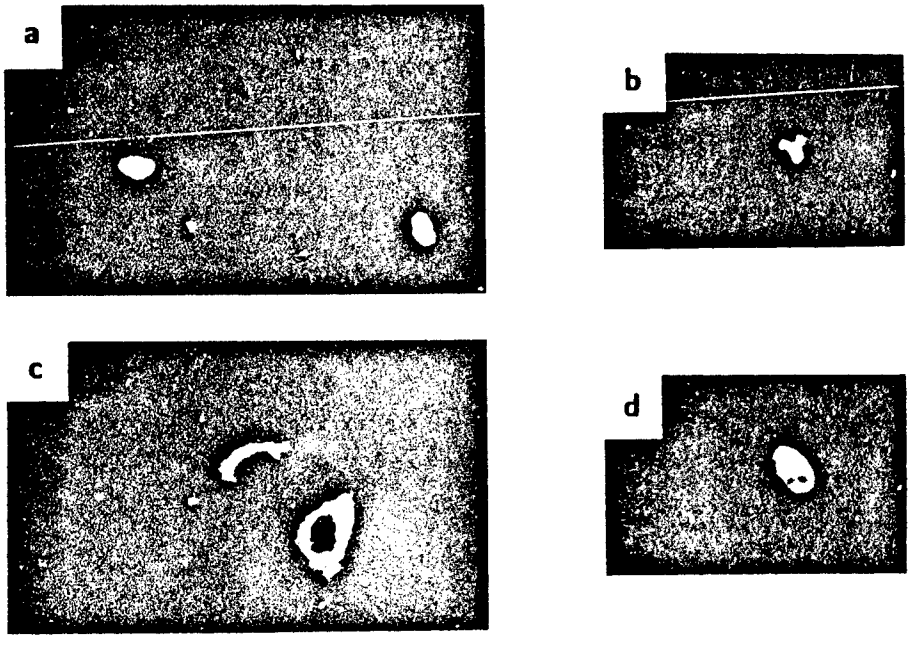


Fig. 5. Subcellular location of chimeric proteins. Mouse fibroblasts (Ltk^-) were transfected with four different plasmids. The location of plasmid-encoded proteins was analysed by indirect immunofluorescence microscopy. Panels (a to d) show cells transfected with pUL1 (fp1), pUL2 (fp2), p60/1 (pp89-NLS^-) and p57/3 (wt pp89), respectively.

DISCUSSION

We investigated the influence of α-domain deletions in the IgA protease precursor on the extracellular secretion of the protease from gram negative bacteria, and on its enzymatic activity. For this analysis we used an E. coli system which offers advantages in the genetic manipulation and in the detection of secreted proteins. Since the IgA protease is selectively secreted from recombinant E. coli cells, and the same transport intermediates and end products appear in the supernatant, as compared with Neisseria, it is a powerful system for such analyses (22). We found no negative effect of α-protein deletions on the secretion or on the enzymatic activity of IgA protease. In contrast, we observed increased IgA protease secretion from some of the α-mutants. This supports the hypothesis, that the α-protein has no helper function in secretion, as was initialy supposed (22), but instead is a factor co-secreted with IgA protease, which has no role in the cleavage of IgA1. Therefore another selective advantage ought to exist that maintains the association of α-protein with the IgA protease. Since pathogenic Neisseria exist exclusively in close relation with the human host, this advantage could be related with an aspect of virulence other than IgA1 cleavage.

The structural characteristics which are commonly found in α-proteins of pathogenic Neisseria match well with this hypothesis. Although there are extensive variations found in the size, and in the overall amino acid sequence of neisserial α-proteins (5), the putative functional domains consisting of the nuclear location signals and DNA binding domains seem to be highly conserved. The results derived from the expression and localization of α-protein hybrids in eucaryotic cells, clearly show that there is a nuclear location signal in the α-protein of N. gonorrhoeae R16. However, it remains to be shown if the short amino acid sequence which is conserved among α-proteins, and shows sequence homology to characterized nuclear location signals (NLS), is sufficient for the transport effect observed with the chimeric proteins. This question is better addressed using the α-protein of N. gonorrhoeae MS11, since this protein contains only one potential NLS, in contrast to the four possible signals in the R16 polypeptide. In addition to the putative nuclear location signal, the basic feature of the α-proteins, their predicted α-helical conformation and the repetitions of amino acid sequences homologous to nucleic acid interacting proteins, suggest a function for α-proteins in the nuclei of host cells.

During the initial steps of cellular invasion bacteria are surrounded by a phagosomal membrane. Pathogens, however, have evolved strategies either to survive inside the vacuoles or to destroy the membrane and therefor get access to the cytoplasm. For Neisseria it is still not clear whether they are always enclosed by an intact vacuole membrane (26, 29). If the bacteria are enclosed, α-proteins would have to overcome a third membrane after leaving the bacterial cell to reach the cytoplasm and the nucleus of the host cell. Alternatively α-proteins might be taken up from the extracellular environment by non-invaded host cells. The recent development of cell culture models for bacterial invasion for a number of different pathogens should provide a better insight into the function of α-proteins.

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We would like to thank Drs. U.H. Koszinowski and H. Volkmer for the generous gift of plasmids p57/3 and p60/1, and monoclonal antibody directed against pp89, S. Lawson for excellent technical assistance in the cell culture experiments, C. Müller for photography, and Dr. B.D. Robertson for critical suggestions on the manuscript.

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T Cell Stimulation by Staphylococcal Enterotoxin A in Man[1]

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A variety of bacteria produces toxins with polyclonal activating potential for T lymphocytes of several species. Important examples of these mitogenic toxins are the staphylococcal enterotoxins (SE) produced by certain strains of Staphylococcus aureus, which cause food poisoning in humans. Several characteristics of these toxins have been reported and have been summarized at this conference by Dr. Joseph E. Alouf. Therefore we here confine ourselves to a few aspects of the induced T cell activation and to various features of TNF production induced by SEA, which has not previously been reported.

An extraordinary feature is the extremely high T cell activating potency of the toxins, being active over a wide range of concentrations from nanogram/ml to fractions of a picogram/ml (1). Despite this high efficiency no direct binding of SE to T cells can be detected and no activation occurs in the absence of accessory cells (1, 2). Besides monocytes normal B cells and various B cell lines (e.g. RAJI) are efficient accessory cells (1). These cells all bind the SE efficiently and they all express MHC class II molecules. Binding of SE to the surface of the cells without processing appears to be sufficient for the activation of added T cells since formalin-fixed RAJI cells are as efficient as

1) This work was supported by grants from the Swedish Medical Research Council, the John and Augusta Persson's Foundation and the Medical Faculty, University of Lund.

viable cells (1). The two monocytic cell lines U937 and THP-1, which lack HLA-DR expression but function as efficient accessory cells supporting PHA-induced T cell activation, cannot support SE-induced activation and fail to bind SE to their surface (1). Similarly, whereas SEA binds well to RAJI cells it fails to bind to the RJ2.2.5 cell line, which is a class II-negative mutant of RAJI (3).

On the basis of the mentioned circumstancial evidence we have investigated the molecular basis for binding of SEA to accessory cells and have identified HLA-DR as the molecule mainly responsible for binding of SEA to accessory cells (4). One distinct SEA-binding molecular band of M_r 60-65 kDa was demonstrated by electrophoresis of detergent extracts of the B cell lymphoma line RAJI. A newly established mAb G8 selected to block the binding of SEA to RAJI cells was shown to bind to the HLA-DR molecule. Similarly, another anti-HLA-DR mAb 9-49 was demonstrated to block the binding of SEA to RAJI cells. Both these mAb also blocked SEA-induced, but not PHA-induced, T cell activation. Immunoprecipitation with specific anti-HLA-DR and anti-HLA-DQ mAb indicated that SEA binds to HLA-DR but not to HLA-DQ. To confirm this further it was shown that binding of SEA to RAJI cells followed by cross-linking and detergent solubilization of the cell membranes, PAGE, and Western blotting resulted in two SEA-containing bands (90 and 105 kDa). The M_r of 90 kDa corresponds well to the predicted size of HLA-DR + SEA. Both the molecular bands contained HLA-DR as demonstrated by binding of an anti-HLA-DR mAb. Further evidence implicating HLA class II molecules in the binding of SEA has been reported (3) showing that mouse fibroblasts transfected with DR, DP or DQ bound SEA and also supproted T cell activation, indicating that besides DR also DP and DQ may be involved in binding of the SE to accessory cells. It is of interest that both CD4 and CD8 T cells and even $\gamma\delta$ TCR-bearing $CD4^-CD8^-$ cells are activated by SE (2), despite the fact that only MHC class II molecules can bind the SE.

The nature of the interaction between SE and the T cells that results in activation of these cells is only partially clarified. A remarkable extensive clonal heterogeneity in the T cell response to SE has been demonstrated (2). Certain T cell clones respond to one SE but not another, whereas other clones respond to the latter SE but not to the former. In both mice and man, T cell responsiveness to a certain SE has been shown

to correlate with the expression of particular V_β sequences (5, 6). This indicates that T cell activation is triggered by interaction of SE with variable parts of the TCR. Triggering of a full T cell activation is entirely dependent on SE being bound to MHC class II molecules. However, it has been reported that SE may interact directly with T cells in the absence of class II molecules resulting in a partial response detectable only as a rise in the cytosolic calcium concentration (2, 7). The role of the class II molecules of accessory cells in the full T cell activation is still to be clarified.

It has previously been established that the toxic shock syndrome toxin 1 (TSST-1) induces human peripheral blood mononuclear cells to produce tumor necrosis factor (TNF) (8) and it has been suggested that TNF is involved in the generation of shock. We have investigated the potency of SEA to induce TNF-α and TNF-β production by human monocytes and T lymphocytes. It has been established that SEA at concentrations of less than 1 picogram/ml induces significant TNF production by human mononuclear leukocytes. Similarly to interferon-gamma (IFN-γ) (9), TNF production requires endogenous or exogenous IL-2, but not IL-4, although T cell proliferation is promoted by both IL-2 and IL-4. Kinetic studies showed that maximal TNF activity in cell supernatants is reached after 72 hours of culture. Intracytoplasmatic staining of TNF-α and TNF-β by selective mAb (10) revealed an early production of TNF-α after 6 hours both in monocytes and in T cells, whereas a late production of TNF-β after 24-48 hours was recorded almost exclusively in the T cells. Corresponding kinetics of released TNF activity was demonstrated in experiments making use of selective inhibition by monospecific mAb to distinguish the two types of TNF. The main lytic component produced by human mononuclear cells was shown to be TNF-β. Equal amounts of TNF-β were produced by CD4 and CD8 T cells whereas a fourfold higher frequency of TNF-β producing cells was recorded among CD4 cells using the intracytoplasmatic staining technique. Within the CD4 cell population the $CD45R^-$ cell subset was an efficient producer of both TNF-β and IFN-γ, whereas the $CD45R^+$ subset produced only TNF-β in more than marginal amounts. Production of TNF-α by monocytes was dependent on the presence of T lymphocytes.

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Binding of Pertussis Toxin to Human T Lymphocytes

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INTRODUCTION

Pertussis toxin (PT), one of several toxins produced by Bordetella pertussis, is composed of an enzymatically active A (S1) subunit and a B oligomer made up of five subunits (S2S4 and S3S4 dimers connected by S5), which is responsible for binding of the toxin to the eucaryotic cell surface (2). Both dimers have been implicated in the binding process (3), and direct binding of both dimers to glycoproteins has been demonstrated (4). Among many other biological activities, PT has been shown to be a T cell mitogen. The toxin's mitogenicity appears to be independent of its enzymatic activity since the isolated B oligomer can mimick the effect of the holotoxin (1,3). A positive correlation between the presence of the CD3 molecule on the cell surface and susceptibility to the mitogenic action of the toxin was also reported (1). In this study we have investigated the binding of PT to human T lymphocytes using Fluorescence Activated Cell Sorter (FACS) techniques.

MATERIALS AND METHODS

Peripheral blood lymphocytes from healthy volunteers were isolated on a Ficoll-Hypaque gradient and were incubated with PT (0.5 µg/10^6 cells) in RPMI medium for 30 min. Cells were washed with RPMI and subsequently incubated with rabbit anti-PT serum and FITC labeled mouse anti-rabbit antibodies to detect binding of PT to the cell surface, in combination with phycoerythrin (PE) labeled anti-CD3, PE anti-CD4 or PE anti-CD8 monoclonal antibodies (MAB's) (Becton Dickinson) as cell markers. Binding of labeled antibodies was evaluated with a FACSCAN flow cytometer (Becton Dickinson). In inhibition experiments, PT was preincubated with unlabeled anti-S2 (18C9H3) or anti-S3 (2131G7) MAB's for 30 min in RPMI before being added to the cells.

RESULTS AND DISCUSSION

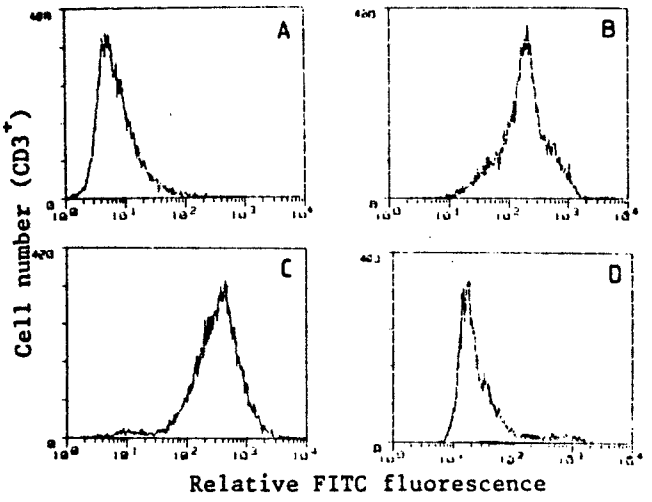


Figure 1.FACS analysis of the binding of PT to $CD3^+$ cells. Human lymphocytes were incubated without PT (A), PT only (B), PT preincubated with MAB 18C9H3 (anti-S2) (C), or PT preincubated with MAB 2131G7 (anti-S3) (D) as described in Materials and Methods.

Dual parameter analysis of the $CD3^+$ population revealed strong binding of PT to human T cells (figure 1A and 1B, histograms shown). Binding could also be detected when using anti-CD4 or anti-CD8 as cell markers (data not shown). The binding to $CD3^+$ cells could be inhibited by preincubating the toxin with an anti-S3 MAB (figure 1D). No inhibition was seen after preincubation with an anti-S2 MAB (figure 1C). These data suggest a role for the S3 subunit in the binding of PT to lymphocytes.
Identification of the lymphocyte receptor and the exact location of the receptor binding site on the PT molecule are being further investigated in our laboratory.

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Application of Toxins

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Development of IL-2-Toxin (DAB486-IL-2) for Human Clinical Trials in Interleukin-2 Receptor Positive Malignancies

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INTRODUCTION

In the case of both diphtheria toxin and Pseudomonas exotoxin A, the genetic substitution of their respective native receptor binding domains with sequences encoding polypeptide hormones has resulted in the construction of a series of "new" toxin molecules (1-6). In each case that has been described these genetically engineered toxins have been shown to be selectively targeted towards only those eukaryotic cells which bear appropriate hormone receptors. These fusion proteins have been found to be potent cytotoxins for target cells, but lack generalized systemic toxicity in vivo.

Williams et al. (2) and Bacha et al. (7) have recently described the genetic construction and selective toxicity of a diphtheria toxin-related interleukin-2 fusion protein, IL-2-toxin. In these studies, IL-2-toxin was shown to be selectively cytotoxic for IL-2 receptor bearing T-lymphocytes. The IL-2-toxin mediated inhibition of protein synthesis in target cells was shown to (i) require the presence of the IL-2 receptor, (ii) upon receptor mediated endocytosis the fusion protein required passage through an acidic vesicle, and (iii) once delivered to the T-cell cytosol, elongation factor 2 was shown to be the target of ADP-ribosylation by the fragment A associated ADP-ribosyltransferase of the fusion protein. Moreover, animal studies have demonstrated the ability of IL-2-toxin to effectively target IL-2 receptor positive cells in vivo. Reduction in tumor load in a murine model of IL-2 receptor positive lymphoma has been observed (Bacha, unpublished observations), and IL-2 toxin has been used effectively in a series of immunosuppression disease models (8-10). Furthermore, Phase I human clinical trials for the

treatment of IL-2 receptor positive leukemias and lymphomas with IL-2 have been initiated.

RESULTS AND DISCUSSION

Molecular biology, expression, and purification of DAB486-IL-2: Williams et al. (2) have previously reported the genetic construction and purification of IL-2-toxin. This construct employed the diphtheria tox promoter and tox signal sequence; the resulting fusion protein was exported to the periplasmic space of recombinant Escherichia coli K-12. IL-2-toxin was secreted in mature form with a molecular weight of 68,086. Moreover, IL-2-toxin was found to retain immunologic determinants of both its diphtheria toxin and IL-2 components. In addition to the full length M_r 68,000 dalton fusion protein, two major degradation products of M_r 49,000, and 47,000 were detected.

As described by Bishai et al. (11) the expression of diphtheria toxin-related fusion proteins in recombinant E. coli K-12 results in marked proteolytic degradation of the full length fusion protein in the periplasmic compartment. Bishai et al. (12) compared the expression of a series of chimeric tox gene constructs in E. coli using the native tox promoter, the λ-phage P_r promoter under the control of the temperature cI857 allele, and the trc promoter which is inducible with isopropyl-ß-D-thiogalactopyranoside (IPTG). Since incorporation of the λ P_r and trc promoters resulted in over expression lethality, these constructs were modified by the deletion of the tox signal sequence which resulted in accumulation of the hybrid toxin in the cytoplasm of recombinant E. coli. These studies clearly demonstrated that maximal yields of diphtheria toxin-related fusion proteins were obtained from the trc promoter.

A partial N-terminal amino acid sequence of IL-2-toxin purified from either the periplasmic space or cytoplasm is shown in Table 1. It is interesting to note that diphtheria toxin-related proteins which are

strain	site of accumulation	partial N-terminal amino acid sequence
E. coli (pABI508)	periplasmic space	Gly-Ala-Asp-Asp-Val-Val-Asp..
E. coli(pABI6508)	cytoplasm	Met-Gly-Ala-Asp-Asp-Val-Val-Asp..

secreted into the periplasmic compartment of E. coli are processed by the signal peptidase at the Ala_{-1} Gly_1 junction used by the Corynebacterium diphtheriae signal peptidase. In contrast, the N-terminal amino acid analysis of diphtheria toxin-related proteins expressed from the trc promoter that are devoid of the signal sequence accumulate in the cytoplasm and contain an N-terminal methionine residue (Table 1).

IL-2 receptor binding requirements for productive entry of DAB486-IL-2: Both Williams et al. (2) and Bacha et al. (7) have shown that IL-2-toxin is selectively active against lymphocytes that bear the IL-2 receptor. It is well known that the IL-2 receptor is composed of at least two

subunits: the 55,000 dalton Tac antigen (p55) which binds IL-2 with low affinity (13), and a 75,000 dalton glycoprotein which binds IL-2 with intermediate affinity (14). The heterodimeric structure, p55/p75, has been shown to bind IL-2 with high affinity (15). Following binding to the high affinity receptor, IL-2 has been shown to be rapidly internalized by receptor mediated endocytosis (16). Waters et al. (17) have recently analyzed IL-2-toxin sensitivity of cell lines which bear either high, intermediate, or low affinity forms of the IL-2 receptor.

The dose response analysis of DAB486-IL-2 for cell lines which bear either the high, intermediate, or low affinity form of the IL-2 receptor is shown in Figure 1. In the case of cell lines which bear either the low affinity p55 or intermediate affinity p75 subunit, the IC_{50} for DAB486-IL-2 is greater than 5×10^{-8} M. In marked contrast, the DAB486-IL-2 IC_{50} for T-cell lines which bear the high affinity form of the IL-2 receptor is $5 - 10 \times 10^{-11}$ M. It is known that the p55 subunit of the IL-2 receptor does not mediate internalization of bound ligand (18), while IL-2 bound to the p75 subunit has been reported to be internalized (19). As a result of the latter observation the failure of DAB486-IL-2 to intoxicate p75 only bearing T-cells was not anticipated.

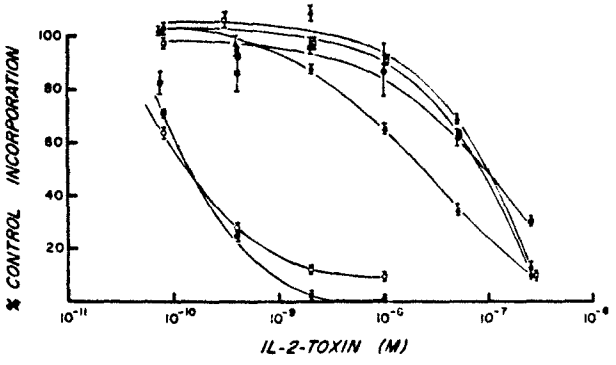


Figure 1: DAB486-IL-2 mediated inhibition of protein synthesis in cell lines which carry the high affinity (C91/P1 [●], Hut102/6TG [○], intermediate affinity (YT2C2 [▲], SKW6.4 [□], MLA-144 [■]), and low affinity (MT-1 [△]) receptor for interleukin 2.

In a series of preliminary experiments, the ability of DAB486-IL-2 to displace [^{125}I]-labeled IL-2 from the high, intermediate, and low affinity receptor has been examined (Schimke, Waters, and Murphy, unpublished). These studies suggest that the binding of DAB486-IL-2 to all forms of the IL-2 receptor are impaired when compared to native IL-2. However, binding to the p75 subunit appears to be most dramatically altered. These results are not surprising since Collins et al. (20) have shown that the N-terminal region of IL-2, particularly Asp20, is essential for binding to the p75 subunit of the IL-2 receptor. Since Asp20 of native IL-2 corresponds to Asp505 of the 619 amino acid DAB486-IL-2, it is likely that this region of the fusion toxin is constrained and may result in stearic hindrance of receptor binding.

Selective toxicity of DAB486-IL-2 for leukemic cells from adult T-cell leukemia patients: Since DAB486-IL-2 is a highly selective cytotoxic agent for retrovirus HTLV-I infected T-cell lines <u>in vitro</u> which

constitutively express the high affinity form of the IL-2 receptor, the effect of this hybrid toxin on leukemic T-cells freshly withdrawn from patients with adult T-cell leukemia (ATL) has been examined. This leukemia is directly associated with infection by the retrovirus HTLV-I. The clinical presentation of ATL has been classified into four types: acute, lymphoma, chronic, and smoldering. Kiyokawa et al. (21) has shown that DAB486-IL-2 is a potent cytotoxic agent for leukemic cells freshly purified from patients with acute and lymphoma type ATL. As shown in Figure 2, the IC_{50} of DAB486-IL-2 for T-cells purified from lymph node aspirates of patients with either acute or lymphoma type disease is similar to that obtained with high affinity IL-2 receptor bearing continuous T-cell lines. It is of interest to note that the IC_{50} of DAB486-IL-2 was approximately 5 - 10 x 10^{-10}M, although T-cells purifed from peripheral blood were not as metabolically active as lymph node T-cells. Since it has been previously shown that the apparent sensitivity

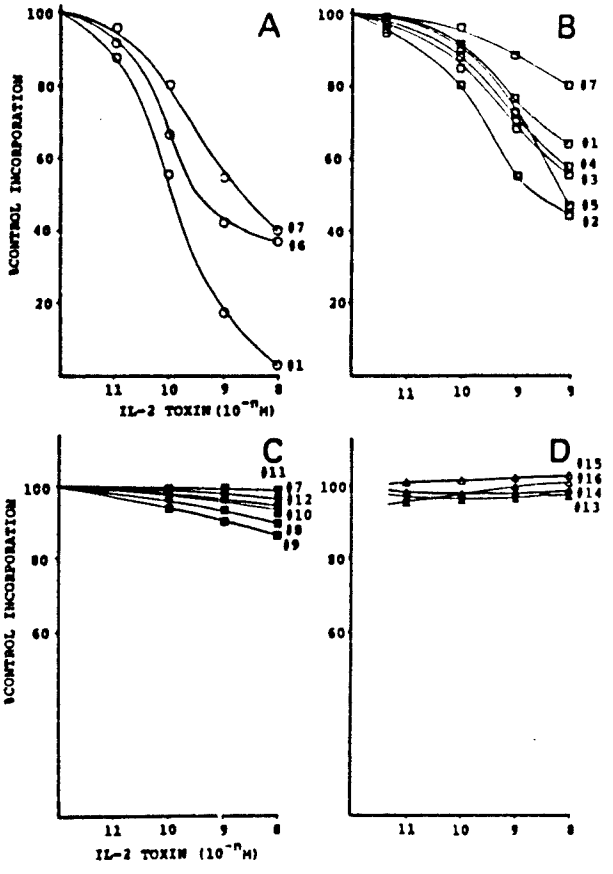


Figure 2: DAB486-IL-2 mediated inhibition of protein synthesis in lymphocytes withdrawn from patients with adult T-cell leukemia. Panel A: lymph node T-cells from acute and lymphomas type ATL; Panel B: peripheral blood T-cells from patients with acute ATL; Panel C: peripheral blood T-cells from patients with chronic and smoldering ATL; Panel D: peripheral blood T-cells from control patients.

of cells to diphtheria toxin is directly related to the endogenous rate of protein synthesis, the decreased potency of DAB486-IL-2 for peripheral blood T-cells vis-a-vis lymph node T-cells is likely related to differences in basal rates of protein synthesis.

Acute toxicity of DAB486-IL-2: As described above, DAB486-IL-2 has been shown to be a potent cytotoxic agent for T-lymphocytes that bear the high affinity form of the IL-2 receptor. Bacha and coworkers (unpublished observations) have examined the acute toxicity of diphtheria toxin, CRM45, and DAB486-IL-2 in several animal species. As shown in Table 2, the LD_{50} of diphtheria toxin in the guinea pig is between 100 - 150 ng/kg; whereas, the LD_{50} of DAB486-IL-2 is greater than 3 mg/kg. The LD_{50} of DAB486-IL-2 in the monkey is also greater than 3 mg/kg. Thus, in diphtheria toxin sensitive species, DAB486-IL-2 is at least 20,000-fold less toxic than native toxin, while in the case of diphtheria toxin resistant species (i.e., mouse and rat), the LD_{50} of DAB486-IL-2 is between 40 - 50-fold less toxic than native diphtheria toxin.

species	LD_{50} diphtheria toxin	CRM45	DAB486-IL-2
guinea pig	150 ng/kg	350 µg/kg	3 mg/kg
monkey	150 ng/kg	n.d.	3 mg/kg
mouse	100 µg/kg	350 µg/kg	5 mg/kg
rat	100 µg/kg	350 µg/kg	4 mg/kg

n.d., not determined

Bacha and coworkers (unpublished observations) have also measured the biological half-life of DAB486-IL-2 in mice, rats, and monkeys following administration of a single intravenous injection. In all three species the $t_{1/2}\alpha$ phase is 5 to 10 minutes, and the $t_{1/2}\beta$ phase is approximately 20 minutes. Using internally labeled [^{35}S]-DAB486-IL-2 to determine the *in vivo* distribution of chimeric toxin following i.v. administration, Bacha and coworkers (unpublished) have found rapid uptake and clearance by the liver.

Phase I human clinical trial of DAB486-IL-2 for IL-2 receptor positive leukemias / lymphomas: The rationale for the use of DAB486-IL-2 as a therapeutic for the treatment of IL-2 receptor expressing luekemias and lymphomas is as follows: (i) HTLV-I infected transformed cells carry large numbers of high affinity IL-2 receptors on their surface and these cells have been shown to be specifically targeted and killed by DAB486-IL-2 in ng/ml concentrations, (ii) significant prolongation of life expectancy has been observed in animals that carry tumors which express the high affinity IL-2 receptor following treatment with DAB486-IL-2, and (iii) IL-2 receptor positive leukemic cells from patients with adult T-cell leukemia are specifically targeted and killed by ng/ml concentrations of DAB486-IL-2. Moreover, DAB486-IL-2 is extremely well tolerated in a variety of animal species at concentrations which are efficacious.

Administration of 0.05 mg/kg DAB486-IL-2 results in a serum concentration of approximately 50 ng/ml, or 10^{-9} M, at 5 minutes. At these concent-

rations there is no measureable toxicity to any organ system; whereas, exposure of high affinity IL-2 receptor bearing lymphocytes to 10^{-9} M DAB486-IL-2 for 5 minutes results in greater than 50% inhibition of protein synthesis (Waters, unpublished).

Based upon this data, as well as additional observations, the Food and Drug Administration has approved an Investigational New Drug application submitted by Seragen Inc. for a Phase I study of DAB486-IL-2 in patients who present with refractory IL-2 receptor positive leukemias / lymphomas. This study involves a dose escalation of DAB486-IL-2 to cohorts of three patients each as shown in Figure 3. At each dosage level patients will be treated with a single dose, observed for 24 hours, given 3 daily doses, observed for one week, treated with 7 daily doses, and then observed for two weeks. Upon completion of each cohort, DAB486-IL-2 will be

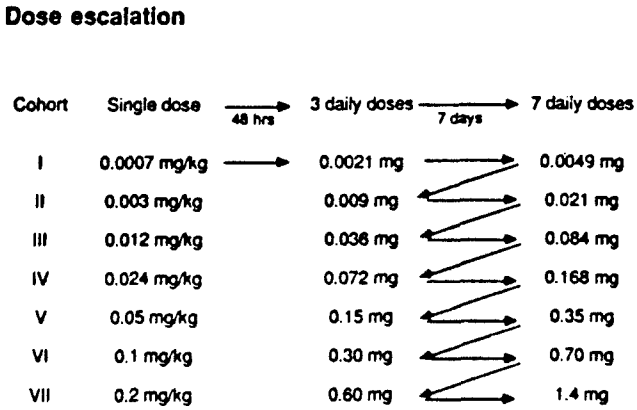


Figure 3: Phase I clinical protocol for determination of safety and tolerability of DAB486-IL-2.

administered at the next higher dosage level until patients present with a grade II level toxic reaction, or a dosage level of 0.20 mg/kg is reached. At present the three patients in the first cohort have been treated with DAB486IL-2 and no adverse reactions have been observed.

SUMMARY AND CONCLUSIONS

Genetic substitution of the native diphtheria toxin receptor binding domain with human interleukin-2 has resulted in a unique fusion protein, DAB486-IL-2. This fusion protein combines the targetting specificity of IL-2 with the potent cytotoxic properties of diphtheria toxin. DAB486-IL-2 has been shown to specifically intoxicate cells which bear the high affinity form of the IL-2 receptor and spare cells which are devoid of the receptor, or express only one chain of the high affinity complex. Furthermore, freshly harvested leukemic T-cells from patients with adult T-cell leukemia are specifically targeted and killed by DAB486-IL-2. In addition, the tumor burden in a murine model of IL-2 receptor expressing malignancy is significantly reduced after treatment with DAB486-IL-2. Food and Drug Administration approval has been obtained for a Phase I clinical trial of DAB486-IL-2 in patients with refractory IL-2 receptor expressing malignancies. DAB486-IL-2 represents the prototype of a new

class of genetically engineered drugs and is the first drug of its class to be approved for clinical evaluation. DAB486-IL-2 and other genetically engineered chimeric toxins are unique biological response modifiers and offer the promise of drugs which possess extraordinary potency and specificity with minimal non-specific adverse reactions.

ACKNOWLEDGEMENTS

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The Use of Pseudomonas aeruginosa Toxin A as a Carrier for Polysaccharide and Peptide Antigens

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INTRODUCTION

Recent efforts directed at developing vaccines against a number of infectious agents have focused on the use of well-defined and highly purified antigens. In many instances, synthetic peptides, recombinant proteins, oligosaccharides, or small-molecular-weight polysaccharides are non- or weakly immunogenic due to their size or their composition (being of a limited number of amino acid or monosaccharide repeats) (2, 5, 18). Large-molecular-weight bacterial polysaccharides, in general, induce a poor antibody response in children less than 24 months of age due to their T-independent nature (10).

The covalent coupling of such antigens as described above to protein carriers has been shown to be an effective means to dramatically increase their immunogenicity (5, 12, 13, 18). A Haemophilus influenzae type b polysaccharide-diphtheria toxoid conjugate has recently been licensed for use in 18-month-old children based upon its superior immunogenicity as compared to native capsular polysaccharide (16). The same vaccine has also been shown to provide a high degree of protection in infants (8).

A number of proteins has been used to produce conjugate vaccines including tetanus toxoid (Ttxd; 9, 12, 13), cholera toxin (CT; 12, 13), diphtheria toxoids (Dtxd; 1, 8), choleragenoid (CG; 1, 8), horseshoe crab hemocyanin (13), and Pseudomonas aeruginosa toxin A (TA; 4, 5). Of these, Ttxd and Dtxd would appear to be the most attractive for large-scale use due to their documented safety. However, the use

of Dtxd and, especially, Ttxd in adults may lead to hyperimmunization with attendant consequences. Two bacterial polysaccharide-Ttxd conjugates have been found to evoke a somewhat surprising high rate of adverse reactions in humans (14). In addition, there is evidence to suggest that elevated levels of preexisting tetanus antibodies will suppress the immune response to the hapten moiety of a Ttxd conjugate (7, 15).

As an alternative to Dtxd and Ttxd, we have explored the feasibility of using other bacterial toxins or toxoids, specifically CT, CG, and TA, as carrier proteins for a variety of polysaccharide and peptide antigens. In our experience, we have found that conjugates synthesized with TA are capable of inducing the highest anti-hapten responses in animals and in humans. Results from several human studies employing TA conjugates are summarized in this report.

MATERIALS AND METHODS

Bacterial strains. All strains of P. aeruginosa employed have been previously described (5). Escherichia coli strain 205 (O18: K-nontypeable) and strain "BORT" (O18:K1) were supplied by A.S. Cross, Walter Reed Army Institute of Research, Washington, D.C.

Purification of carrier proteins. TA was purified as described elsewhere (12).

Haptens. O-polysaccharide (O-PS) was isolated from P. aeruginosa and E. coli lipopolysaccharide (LPS) as described by Cryz et al. (5). R3 is a synthetic peptide corresponding to $NANP^3$ [(asparagine-alanine-asparagine-proline)^3] and is the conserved immunodominant repeat region expressed by the circumsporozoite protein (CSP) of Plasmodium falciparum (18). R32 is a purified recombinant protein with the one letter code, $MDP[(NANP)_{15}\text{-}NVDP]^2LR$ where M = methionine; D = aspartic acid; V = valine; L = leucine; and R = argenine.

Conjugate synthesis. Coupling of R3 or R32 to various carrier proteins using adipic acid dihydrazide (ADH) as a spacer molecule and carbodiimide as a linking agent was performed as described by Que et al. (12). P. aeruginosa O-PS-TA conjugates were synthesized as previously described (5). E. coli O-PS-TA conjugates were prepared in an identical manner with the exception that the O-PS was exposed to $NaIO_4$ for only 2-5 minutes.

Characterization of conjugates. Analysis of the various conjugates as to chemical composition, physicochemical properties, and biological activities was as described elsewhere (5, 12).

ELISA. Anti-NANP, LPS, and carrier protein antibody levels were determined by ELISA as previously described (5, 12).

Immunofluorescent antibody assay (IFA). The ability of anti-NANP antibody induced by vaccination to bind to the surface of P. falciparum sporozoites was determined according to Young et al. (17).

Biological activities of conjugates. TA conjugates were tested for enzymatic activity using wheat germ extract as a source of elongation factor 2 (5).

Clinical studies. Conjugates for human studies were evaluated for pyrogenicity in rabbits, for lack of toxicity in mice and guinea pigs, and for sterility according to the methods described in the U.S. Code of Federal Regulations under articles 610.11 and 610.12. Informed consent was obtained from healthy adult volunteers who received the vaccine (0.5 ml) intramuscularly in the deltoid region. Reactions to vaccination were recorded for 5 days post-immunization.

RESULTS

The characteristics of various TA-containing conjugate vaccines evaluated for safety and immunogenicity in humans is shown in Table 1. Depending upon the hapten, the ratio of hapten to carrier ranged from approximately 1:2 (P. aeruginosa O-PS-TA) to 1:1 (E. coli O-PS-TA). These findings illustrate the utility of the TA-ADH intermediate in the formation of conjugates comprising various types of haptens (polysaccharides, peptides, and recombinant proteins). The molecular weights of the conjugates ranged from 98,000 (R3-TA) to $>10^6$ (P. aeruginosa O-PS-TA). All conjugates were nontoxic for animals and nonpyrogenic in rabbits. In addition, all conjugates lacked detectable ADPR-transferase activity expressed by TA and were stable to toxic reversion after storage at 37°C for 4 weeks.

To date, 119 subjects have received an octavalent P. aeruginosa O-PS-TA vaccine composed of Fisher immunotypes 1, 2, 3, 4, 5, and 7, and Habs serotypes 3 and 4 O-PS (25 µg/serotype). About half of the volunteers reported a mild, transient local reaction, while approximately 10% noted a systemic reaction (most often headache and/or malaise). No pronounced episodes of fever or chills were reported and no reactions were debilitating.

A single dose of vaccine resulted in a significant ($p < 0.05$) rise in IgG antibody to all 8 LPS serotypes and to toxin A. The magnitude of the overall immune response was greatly influenced by preexisting antibody titers. For example, the greatest increase in geometric mean (GMT) antibody levels was seen for immunotype 5 (18-fold), to which volunteers had the

lowest baseline titer (2 μg/ml). In contrast, there was only a 3-fold rise in GMT to IT-3 LPS where the baseline titer was the highest for all antigens (GMT = 40.2 μg/ml). Subjects who possessed <10 μg anti-LPS IgG/ml responded with a more vigorous antibody response than those with >10 μg/ml (Table 2). Immunization gave rise to an increase in opsonic antibody titer to all serotypes. In addition, the passive transfer of pooled post-immunization sera to mice increased the LD_{50} value following challenge from 10-fold (IT-2) to 2900-fold as compared to a pre-immune serum pool. No increase was seen for Habs 4. However, the Habs 4 challenge strain was weakly virulent with an LD_{50} of 3.3×10^6 for control mice. This value was raised to 9×10^6 when pre- or post-immune serum was transferred.

A Phase I trial is currently underway to evaluate the safety and immunogenicity of an E. coli O18 O-PS-TA conjugate in humans. To date, the vaccine has been administered to 7 healthy adults with no reactions reported. The IgG antibody response has been analyzed from 4 of these volunteers and is shown in Table 3. All 4 subjects showed a $\geq$ 4-fold rise in titer following vaccination. A greater than 25-fold mean titer rise was achieved after a single dose of vaccine was administered.

The immune response to R3-TA and R32-TA P. falciparum conjugate vaccines is shown in Table 4. Although both vaccines induced a significant rise in anti-NANP antibody titers, the R32-TA conjugate evoked higher levels than the R3-TA conjugate. While only 1 of 5 subjects who received the R3-TA conjugate had a positive IFA reaction, the serum from most (7 of 10) vaccinees who were immunized with the R32-TA conjugate were positive in this assay system.

Table 1. Characteristics of TA-containing conjugate vaccines

Conjugate	Composition[1] (% dry weight)		Molecular weight	Toxicity[4]	Pyrogenicity[5] (μg/kg)
	Hapten	Carrier			
P. aeruginosa O-PS-TA	37.2	62.8	>10^{6}[2]	Nontoxic	>18
E. coli O-PS-TA	50	50	>600,000[2]	Nontoxic	>10
R3-TA	40	60	98,000[3]	Nontoxic	>5
R32-TA	45	55	119,000[3]	Nontoxic	>5

[1] For O-PS-TA conjugates determined by quantitative analysis of protein and O-PS. For R3-TA and R32-TA conjugates calculated by molar ratio values from amino acid analysis.

[2] Determined by high-pressure liquid chromatography.

[3] Determined by SDS-PAGE.

[4] Determined by intraperitoneal injection of mice.

[5] Expressed as highest quantity of antigen injected intravenously into rabbits which did not evoke a pyrogenic response.

Table 2. Effect of baseline IgG levels on the immune response to O-PS-TA vaccine

Serotype	Vaccinees	GMT µg IgG/ml serum (range)		% ≥ 4-fold rise
		pre-immune	post-immune	
Habs 4	All	12 (0.8-22)	43 (2.8-244)	46
	<10 µg IgG/ml	4 (0.8-9)	42 (2.8-220)	82
IT-3	All	40 (1.2-220)	134 (25-400)	35
	<10 µg IgG/ml	3.8 (1.3-7.9)	83 (33-112)	100
IT-4	All	19 (0.9-147)	60 (1.5-284)	49
	<10 µg IgG/ml	3.4 (0.9-9.7)	38 (1.5-170)	85

Table 3. Immunoglobulin G (IgG) response following immunization with E. coli 018 O-PS-TA conjugate[1]

Mean IgG anti-LPS ELISA titer (range)[2]		No. ≥ 4-fold rise in titer
pre-immune	post-immune	
92 (27-139)	2502 (1723-3302)	4/4

[1] Volunteers each received 100 µg of conjugate (equivalent to 50 µg of O-PS) intramuscularly. Serum samples were obtained on days 0 and 14.

[2] ELISA titers are expressed as the reciprocal of the highest dilution of serum which yielded an $A_{405} \geq 0.4$.

Table 4. IgG antibody response following immunization with R3-TA and R32-TA conjugates[1]

Vaccine	GMT µg IgG/ml serum to NANP repeat		% > 4-fold rise in antibody titer
	pre-immune	post-immune[2]	
R3-TA	0.12	0.69	4/5
R32-TA	0.05	2.4	10/10

[1] Volunteers each received 100 µg of conjugate intramuscularly in the deltoid region on days 0 and 56.

[2] Values given are for day 70 serum samples.

DISCUSSION

TA is synthesized as a single polypeptide with a molecular weight of approximately 60,000. TA is highly toxic for a variety of animal species and cultured cell lines due to its ability to inhibit eucaryotic protein synthesis by inactivating elongation factor 2 (3). Elevated titers of anti-TA and anti-LPS antibody have been found to correlate with an increased survival rate in cases of P. aeruginosa bacteremia (11). Therefore, we initially sought to utilize toxin A as a carrier for P. aeruginosa O-PS antigens in an effort to stimulate 2 "classes" of protective antibodies (5). The incorporation of ADH into the TA molecule was found to result in an irreversible detoxification. A monovalent IT-5 O-PS-TA conjugate vaccine was subsequently shown to be safe and immunogenic in humans (4).

Based upon these promising results, we were interested in evaluating the potential of TA as a carrier for other nonimmunogenic or weakly immungenic candidate vaccine antigens. TA possesses several traits which make it particularly suitable for use as a carrier, especially in adult populations, including: i) lack of detectable biologic activity after coupling to ADH; ii) safety upon parenteral administration; and iii) low levels of baseline antibody titers in the general population which would circumvent the problems of inadvertent hyperimmunization (a concern with the use of Ttxd or Dtxd) and epitopic suppression (6, 7, 15).

In the present report, we have found TA to be an excellent carrier for O-PS from P. aeruginosa and E. coli, and for peptides or recombinant protein expressing the protective NANP epitope present on the P. falciparum sporozoite surface. All 4 conjugate vaccines were well tolerated upon parenteral administration to humans. None of the approximately 200 subjects immunized experienced a reaction which interfered with their daily activities. There was no evidence of toxin

A-mediated effects on any organ system as determined by blood or serum analyses.

All 4 TA conjugates induced a significant anti-hapten antibody response in humans. Anti-P. aeruginosa LPS antibodies engendered by immunization were highly protective in preventing fatal experimental burn wound sepsis. An E. coli 018 O-PS-TA conjugate produced in a similar manner induced high levels of anti-LPS antibody in 4 volunteers. The functional attributes of these antibodies are presently being determined.

In an effort to stimulate an immune response to the NANP epitope expressed, R3 or R32 was coupled to toxin A. The R32-TA vacine appears to hold much promise based upon its ability to stimulate good levels of anti-NANP antibody which recognizes the CSP on the surface of P. falciparum sporozoites. Preliminary results from ongoing trials indicate that approximately 10-fold higher antibody titers can be engendered in humans when the dose is increased to 400 µg, which is well tolerated.

In conclusion, TA appears to serve as an excellent carrier for a variety of potential vaccine candidate antigens. The outstanding acceptability of TA conjugates to date may make it suitable for use as a generic vaccine carrier protein.

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Genetic and Biochemical Studies of the Mechanism of Action of Bacillus thuringiensis Entomocidal δ-Endotoxins

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INTRODUCTION

Bacillus thuringiensis (BT) strains synthesise a cytoplasmic protein crystal during the sporulation phase of their development (1,3). This crystal (∂-endotoxin) consists of one or more polypeptides of varying M_r (4) that are toxic to insect larvae inluding lepidoptera, diptera and coleoptera. The native crystal ∂-endotoxin is a protoxin which requires solubilization and activation by proteolytic processing in the larval midgut (11, 20). For many of the toxins proteolytic activation involves removal of the C-terminal half of the protoxin (18,29,30). The activation can be duplicated in vitro using alkaline buffers together with a combination of proteases (20,22,31). When the protoxin is activated in vivo in the larval midgut, it causes disruption of the gut epithelial cells which leads to larval mortality . The first observed effect is destruction of the brush border of columnar cells followed by swelling and lysis of goblet cells . This leads in turn to a rapid equilibration of ions and pH levels between gut contents and blood, cessation of feeding and death.

BIOLOGICAL ROLE OF THE ∂-ENDOTOXIN

A clue to the biological role of these toxins may come from the fact that they are synthesised only during sporulation of the bacterium. Despite the fact that sporulation is the response of the organism to starvation and is characterised by extensive protein turnover, these crystal ∂-endotoxins frequently comprise one third of the weight of the sporulating cell, i.e. the same weight as the spore itself. Clearly therefore their synthesis during this time suggests they are important to spore survival or germination. When the spore and crystal are released together at the completion of sporulation by lysis of the parent cell, the crystal is of no value to the spore in an environment of neutral pH , since these endotoxins characteristically require alkaline conditions for solubilisation and activation . However the midgut region of a variety of insect larvae is highly alkaline (7) and if the spore is ingested together with a ∂-endotoxin crystal by these larvae , the toxin can first be solubilised and then activated by gut proteases. The specialisation of these toxins to bind to receptors on midgut epithelium cells (see below)

and trigger cytolysis ensures that the high initial gut pH is rapidly reduced. Feeding ceases and the spore is thus retained in the alimentary tract. The additional equilibration of gut and blood contents creates a rich nutrient environment that is 'sensed' by the dormant spore which is stimulated to germinate. The ∂-endotoxin may therefore enable B. thuringiensis spores to initiate a new vegetative colony in an environment inimical to the germination of other bacterial spores and as such confers a valuable selective advantage.

TABLE 1*

Bacillus thuringiensis insecticidal toxins

Designation	Subspecies	Target+	Molecular Mass (kDa)
cryIA(a)	kurstaki; aizawai; sotto; entomocidus.	L	133.2
cryIA(b)	kurstaki; aizawai; berliner.	L	131.0
cryIA(c)	kurstaki.	L	133.3
cryIB	thuringiensis; entomocidus.	L	138.0
cryIC	aizawai; entomocidus.	L	134.8
cryID	aizawai.	L	132.5
cryIIA	kurstaki	L/D	70.9
cryIIB	kurstaki	L	70.8
cryIIIA	san diego; tenebrionis.	C	73.1
cryIVA	israelensis	D	134.4
cryIVB	israelensis	D	127.8
cryIVC	israelensis	D	77.8
cryIVD	israelensis	D	72.4
cytA	israelensis; morrisoni.	D	27.4
cytB	darmstadtiensis	D	~23

* Modified from Reference 19

+ L = Lepidoptera; L/D = Lepidoptera & Diptera; D = Diptera; C = Coleoptera.

MOLECULAR MECHANISM OF ∂-ENDOTOXIN ACTION

The wide differences in molecular weights of the BT ∂-endotoxins, and their different insect targets (Table 1) raises a question as to whether they all share the same toxic mechanism. Recent biochemical studies with toxins of different specificity pointed to just such a common mechanism and as a result we have proposed a general model for the action of all ∂-endotoxins (10,13,23). According to this model (Fig 1), the toxin first binds to a specific membrane receptor (R) on the target cell. Putative receptors have been identified both in vivo on the midgut epithelium of susceptible insects (17) and on the plasma membrane of susceptible insect cell lines (22). In a second step the toxin either individually or in the form of oligomers, partitions into the membrane to create a pore/leakage channel in the membrane which ultimately leads to colloid osmotic lysis.
Recent data in support of this model are described below under 4 headings:

1. EVIDENCE FOR SPECIFIC ∂-ENDOTOXIN RECEPTORS

Studies of the binding of kurstaki, aizawai and sotto toxins to susceptible insect cell lines

(13,22, W.Ahmad unpublished) have shown that the activated forms of these toxins bind to one, or in some cases two, distinct plasma membrane proteins. In one case (22), the single binding component was identified as a 146kDa N-acetylgalactosamine -containing protein.

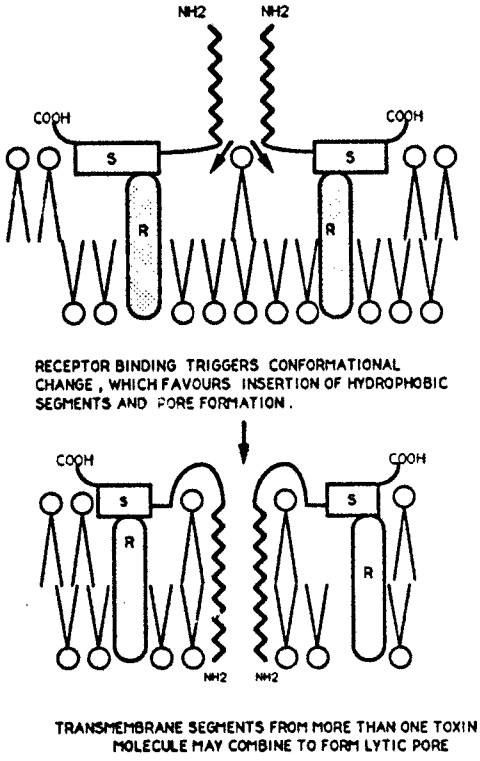


Figure 1 Hypothetical general model for ∂-endotoxin membrane interaction.

Support for the receptor function of this protein came from protection experiments in which N-acetylgalactosamine and N-acetylgalactosamine-specific lectins were found to protect the cells from cytolysis (21). Interestingly, N-acetylgalactosamine was also found to protect a mosquito cell line against the cytolytic effect of a Bacillus sphaericus toxin (6). Similar experiments with aizawai (13) and entomocidus (Knowles, B.H. unpublished) toxins have shown that D-glucose is an important component of the receptor for these toxins. Because of the undefined nature of these insect cell lines, results from such in vitro studies must be supplemented by experiments with gut epithelium preparations. Recently Hoffman et al (17) showed the existence of high affinity saturable binding sites in a vesicle preparation from the larval midgut brush border and have found a correlation between this specific binding and toxin specificity. Despite the acknowledged limitations of the insect cell lines, in two instances we have been able to reproduce in vitro the dual activity spectrum (Lepidoptera and Diptera toxicity) observed in vivo (12, Nicholls, C.N. et al. , J. Bacteriol. in press).

Early work with the cytA (Table 1) toxin showed that it was rapidly lytic to all eukaryotic cells tested (32). This broad activity in vitro was attributed to its high hydrophobicity and the discovery that it binds to certain ubiquitous membrane phospholipids provided they are substituted with unsaturated fatty acids (32,33). These data however failed to explain the fact that in vivo the cloned cytA toxin is highly specific for dipteran larvae (33). Recent studies in which this cloned toxin was modified by site-directed mutagenesis (34, G. Armstrong; unpublished) suggest that like the other toxins in Table 1, an insect specific receptor may be necessary for this toxin to be active in vivo. When Glutamate 204 in this toxin was replaced

by alanine , the resulting protein was still toxic to dipteran cells in vitro, but no longer bound phospholipids and had lost toxicity to lepidopteran cells. We interpret these results to suggest that the wild type cytA protein can insert into membranes by two mechanisms: (a) simple partition into the bilayer by virtue of an appropriate configuration of highly hydrophobic segments. (This would be the basis for the broad in vitro activity of the wild type toxin); (b) a two-step membrane insertion in which step (a) is preceded by toxin binding to an insect specific receptor. (This is the mechanism we believe occurs in vivo). The results with the Glu204 mutant suggest that this amino acid replacement interferes with step (a) so that unless tha tissue specific receptor is present to assist in targetting the toxin to the bilayer, it is unable to insert and trigger cytolysis. An explanation for the failure of the wild type toxin to kill lepidoptera in vivo by mechanism (a) may reside in the observation that spontaneous insertion of this toxin into lipid bilayers (i.e. mechanism (a)) is dependent both on bulk pH and net charge on the lipid headgroups (24, Knowles, B.H. & Carroll, J., unpublished). In the insect gut, conditions for spontaneous insertion may be unfavourable (local pH, surface charge etc.) and the close juxtaposition of toxin and lipid molecules brought about by receptor binding may thus be the only route by which the toxin can insert into the lipid bilayer in vivo.

2. EVIDENCE FOR ∂-ENDOTOXIN INSERTION INTO MEMBRANES

Reference was made in 1 above to our early studies (31,32) that showed the 27 kDa cytA protein (Table 1) binds to lipid vesicles (liposomes). An immunologically distinct 28 kDa toxin from a darmstadtiensis strain with very similar cytolytic properties has been described (9) and designated cytB in accord with the nomenclature proposed by Hofte and Whiteley (19). To investigate the topology of this lipid binding we treated liposomes that had bound the cytA toxin with proteinase K. Two large peptides (8.5 kDa and 9kDa) were protected from digestion , with N-termini corresponding to residues 45 and 154 of the 248 residue toxin. This result suggests that the bulk of this toxin is inserted into the lipid phase consistent with its high hydrophobicity. Conceivably only a segment at the N-terminus (residues 1-44) and a predicted extramembranous loop region containing residues 153-172 , may be exposed in the aqueous phase after insertion. For the other ∂-endotoxins the topological details of the membrane association are not yet available, but data from other experiments indicate that they also can interact intimately with phospholipid bilayers.

Conditions that influence liposome aggregation or lipid bilayer structure result in changes in the turbidity of liposome suspensions and this approach has been used previously for investigating the mode of action of some bacterial protein toxins including diphtheria toxin and its mutants (27). We recently employed this strategy to study wild type and mutant Bt toxins (16, Haider,M.Z. & Ellar, D.J., J. Mol. Biol. in press). Addition of wild type toxins to liposome suspensions lead to an immediate increase in the turbidity of the suspension. The nature of the turbidity changes further suggested that the BT toxins were partitioning into bilayers to form stable pores which would be expected to allow leakage of liposome contents to the exterior. This leakage was confirmed in subsequent experiments (see 3 below).

3. CHARACTERISTICS OF THE ∂-ENDOTOXIN PORE IN THE MEMBRANE

In our general model for BT ∂-endotoxins (10,13,23) we propose that these toxins kill cells by colloid osmotic lysis as do other bacterial toxins such as Staphyloccoccus aureus a-toxin and Pseudomonas aeruginosa cytotoxic protein. Toxin binding to membrane receptors leads to the creation of a membrane leakage pore that allows equilibration of ions between the cell and the exterior. The accompanying net influx of water into the cell will result in cell swelling and eventual lysis. One prediction of this theory is that external molecules with viscometric radii too large to allow them to enter the toxin-induced leakage pore will act as

osmotic protectants by maintaining a high external osmotic pressure and preventing water influx. This phenomenon of non-specific osmotic protection forms the basis of a method for measuring the dimensions of the pore (35). Application of this method to a range of BT toxins showed that raffinose and larger molecules were able to prevent cytolysis completely, while smaller molecules delayed toxicity to an extent dependent on their size, presumably reflecting the time taken to diffuse through the pore (13,23). From these results a pore radius of 0.5-1.0 nm was estimated for BT toxins with very different specificities. This is similar in size to pores formed by other bacterial toxins believed to act by colloid osmotic lysis, e.g. 1.2 nm for Pseudomonas aeruginosa cytotoxic protein (35) and 1-1.5nm for Staphylococcus aureus a-toxin (3).

We have recently obtained data on the selectivity of the pore created by insertion of the cytA toxin into lipid bilayers (24). At alkaline pH's the toxin was found to insert spontaneously into planar bilayers where it formed cation-selective channels permeable to K^+ and Na^+ but not N-methylglucamine or Cl^-. The channels showed very fast cooperative opening and closing. Channel opening was greatly reduced in the presence of divalent cations (Ca^{2+}, Mg^{2+}) and the effect was reversed when these ions were removed. A number of diverse pore-forming agents are inhibited by divalent cations (2) including BT toxins (8).

The rate of leakage of labelled markers from susceptible insect cells treated with BT ∂-endotoxins was proportional to the marker size: ^{86}Rb leaking out first, followed by ^{3}H uridine and then ^{51}Cr (Mr 3000) (23). In other experiments (Knowles et al, this volume) the rate of glucose release from liposomes exposed to cytA toxin was directly proportional to toxin concentration, but even at high toxin doses, there was invariably a time lag before release began. This lag time doubled for a two-fold decrease in toxin concentration, a result also observed when the inhibition of insect Malpighian tubule fluid secretion caused by cytA was studied (25). Mathematical modelling of these data (25) suggested that the cytA pore may be composed of as many as 12 individual molecules, but further work is required to substantiate this. Fig 1 thus summarises our current hypothesis for toxin-membrane interaction based upon the above data . The symbol R denotes an insect specific receptor and S symbolises a segment(s) of the toxins responsible for receptor recognition. In vivo the binding of toxin to the receptor is a prerequisite for the subsequent conformational change which promotes membrane insertion of the toxin and pore formation. The latter may involve a further process of oligomerization of individual molecules.

4. STRUCTURAL BASIS FOR ∂-ENDOTOXIN ACTION

The sequences of all the ∂-endotoxins in Table 1 except cytB have been determined (for review see 19). Our proposal that toxins with quite different insect targets share a common cytolytic mechanism argues that we might expect to discover certain common structural motifs by comparing these sequences. For example in the colloid osmotic lysis mechanism we propose, different toxin domains may be responsible for the steps of receptor recognition and membrane insertion. Sequence comparisons of toxins with different specificity show a consistent excess of hydrophobic residues in the N-terminal 250 amino acids which is therefore a candidate region for promoting membrane insertion. Moreover the hydrophobic plots of this region from toxins of different insecticidal specificity are remarkably similar despite the lack of direct sequence similarity in several instances (5). This postulated role for this region is reinforced by the finding that an aizawai toxin deletion mutant encoding only the N-terminal 242 amino acids (Δ242 in Fig 2) retained the capacity to perturb lipid bilayers shown by the full length toxin (Haider, M.Z and Ellar, D.J. J.Mol. Biol. in press). Sanchis etal (28) have recently postulated that the second half of the active toxins including the conserved regions D3-D6 (see below and Fig 2) may be responsible for determining the specificity of the toxins.

In making such sequence comparisons we must take into account the fact that the active forms of these toxins produced by the action of gut proteases are significantly smaller than the protoxins. In the cases of the ~130kDa lepidopteran toxins the minimum toxic polypeptide determined by deletion mutagenesis (15,18,29), generally comprises a 55-70 KDa protease resistant fragment starting between amino acids 29 and 35 at the N-terminus and terminating between positions 590 and 620 at the C-terminus . Amino acid sequencing of the ~ 60kDa dendrolimus ∂ endotoxin activated by trypsin treatment showed that 28 residues had been cleaved from the N-terminus (26). Larval gut enzymes are therefore responsible for extensive C-terminal processing. This general scheme is shown below in Figure 2 for the

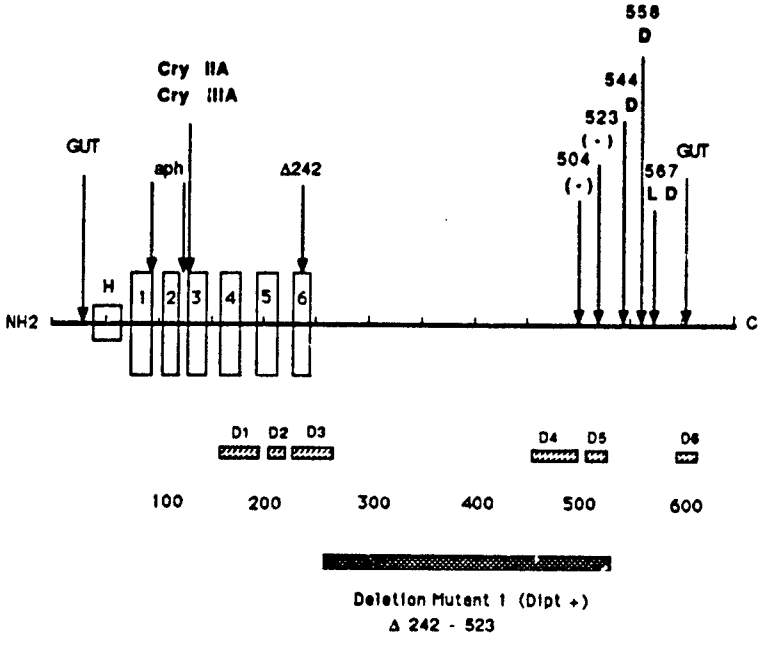


Figure 2. Generalised diagram based on the sotto cryIA(a) gene showing the approximate locations of conserved sequences (D1-D6) , potential transmembrane helices (1-5) and the hydrophobic sequence (H) exposed after protoxin activation. aph denotes potential amphiphilic helices. Toxicity for lepidoptera (L), diptera (D); Non toxic (-).For other details see text.

sotto toxin with the approximate gut enzyme cleavage points at the N and C-termini indicated by arrows. By contrast, activation of the coleopteran (cryIIIA) and dual specificity cryIIA (lepidopteran and dipteran) toxins which are approximately half the size of the monospecific lepidopteran ~130kDa protoxins (Table 1), is characterised by extensive N-terminal proteolysis (Carroll, J. et al Biochem. J. in press; Nicholls, C.N. et al; J. Bacteriol. in press.) 144 and 158 residues respectively are removed from the N-terminus of the cryIIIA and cryIIA proteins (approximate cleavage points indicated by arrows in Fig 2) with little if any proteolysis at the C-terminus. Other sequence comparisons of the toxin genes has revealed the presence of six conserved domains (D1-D6 in Fig 2). In the sotto sequence these occur between amino acids 152-183 (D1), 200-216 (D2), 225-267 (D3), 452-498 (D4) 512-523 (D5) and 591-604 (D6) (15, 28, C.Hodgman, personal communication)

Three of the highly conserved domains (D1, D2 , D3) are contained in the N-terminal 250 amino acids. Secondary structure analysis of D1 in the sotto lepidopteran toxin predicts an alpha helix between residues 162 and 169 flanked on each side by a highly hydrophobic beta segment. With the exception of the cryIVB israelensis mosquito toxin (5) the D1 region

in all the other toxins is predicted to contain an alpha helical segment. In all the toxins except for cryIB this helix is flanked on one or both sides by hydrophobic beta segments. Despite any significant sequence similarity corresponding to D1 between the 27 KDa cytA mosquitocidal protein and all the other toxins, this toxin is also predicted to contain regions with this hydrophobic beta -alpha -beta structure. We recently tested the structural importance of this alpha helix in the sotto cryIA(a) toxin by replacing the highly conserved alanine 165 with proline (W. Ahmad, unpublished). The fact that the toxicity of the mutant protein was reduced by 80% suggests that conservation of this D1 helical segment is important for toxicity. Interestingly depite the low in vivo toxicity, the capacity of the mutant toxin to bind to putative receptors on the membrane of susceptible cells was unimpaired.

In another sotto mutant we replaced Phe50 and Val51 with two aspartate residues. This has the predicted effect of introducing two charges and two turns into the conserved hydrophobic region at the extreme N-terminus (H in Fig 2). This highly hydrophobic segment is exposed by the removal of 28 residues from the N-terminus during protoxin activation in the larval gut. The toxicity of this mutant in vivo was reduced by 60% but again receptor binding was apparently unaffected. On the basis of these results we would tentatively conclude that both the N-terminal hydrophobic H-region and the D1 region are involved in the formation of the transmembrane lytic pore rather than the prior event of receptor recognition. Figure 2 also summarises some of the results from our experiments to identify segments of the aizawai IC1 cryIA(b) toxin responsible for its dual specificity (lepidoptera and diptera) (11-16; Haider,M.Z. & Ellar, D.J., J. Mol. Biol., in press). Whereas the monospecific lepidopteran protoxins are proteolytically activated to ~60kDa toxins, the aizawai IC1 toxin is processed to a 55kDa lepidopteran toxin by lepidopteran gut enzymes and a 53kDa dipteran-specific toxin by dipteran proteases. Although the dual specificity IC1 toxin differs by only three residues from the monospecific berliner cryIA(b) toxin, two of these differences are in residues linked on the C-terminal side to Arg 544 and Arg 567 (the relative positions of these residues in the aizawai toxin are arrowed in Fig 2). Computer analysis (16) suggests that these substitutions could significantly alter the environments (and hence protease sensitivity) of both arginines compared to the corresponding arginines in the berliner toxin.

Results from site-directed and deletion mutagenesis (Haider, M. and Ellar, D.J.; J. Mol. Biol. in press) indicate that cleavage of the aizawai toxin at Arg 567 by lepidopteran enzymes and at Arg 544 by dipteran enzymes generates toxins with different insect specificity. In the case of the active 60kDa berliner lepidopteran toxin, C-terminal processing is thought to proceed no further than Arg 601. These data lead us to propose a model for the folded structure of the activated ∂-endotoxin in which regions important for specificity determination close to the C-terminus associate with a hydrophobic N-terminal segment comprising all or part of the first ~250 residues (Haider, M. and Ellar, D.J.; J. Mol. Biol. in press). This model is supported by the finding that what we believe to be an aizawai IC1 fusion protein consisting of the C-terminal segment Arg 524-Arg544 linked to the N-terminal 241 residues (Deletion mutant 1 in Fig 2) exhibits dipteran toxicity, whereas a deletion mutant encoding only the 241 N-terminal residues inserts into lipid bilayers but lacks dipteran toxicity.

The possibility that the determinants for membrane insertion of these toxins reside in the hydrophobic N-terminal half is supported by a structural analysis (C. Hodgman, unpublished) which shows a remarkable conservation of 6 potential transmembrane helical segments in all the cry toxins in Table 1.(Boxes 1-6 in Fig 2). The predicted conservation of these helices despite the lack of direct sequence similarity in many instances, suggests they may play an important role in the general model of ∂-endotoxin cytolysis that we have proposed (10,13,23).

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Pertussis Toxin CRMs and New Vaccines against Whooping cough

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The cellular pertussis vaccine introduced in the forties has drastically reduced the morbidity and mortality due to whooping cough. As a consequence of the disappearance of pertussis in many countries following extensive vaccination campaigns, public fears about adverse reactions to vaccination have grown and the use of the vaccine has decreased, resulting in an increased infant mortality due to the disease. This stressed the need for a safer vaccine against whooping cough and, so far, several acellular vaccines have been proposed. Pertussis toxin (PT), a main virulence factor of Bordetella pertussis, either alone or in combination with other antigens is the main component of all acellular vaccines so far developed. Two acellular vaccines, one made of formaldehyde inactivated pertussis toxin (PT) and the other made of formaldehyde inactivated pertussis toxin and filamentous haemagglutinin (PT + FHA) have recently been used in a clinical trial in Sweden (1). Both vaccines resulted equally effective in preventing severe disease. It is therefore generally believed that future pertussis vaccines will contain inactivated pertussis toxin alone or in combination with other molecules as FHA, pili or the outer membrane protein 69k. However, there is a need for better methods to obtain complete and stable detoxification of PT because the severe complications of the cellular pertussis vaccine may be due to minute traces of active PT and the detoxified PT used in Sweden showed some reversion to toxicity (2).
We have approached the study of new acellular pertussis vaccines by using recombinant DNA techniques.

PERTUSSIS TOXIN

Pertussis toxin (PT) is an extracellular protein composed of five different subunits which have been named S1, S2, S3, S4 and S5 according to their electrophoretic mobility. Like many other bacterial protein toxins, PT can be divided into domains A and B. A (composed of subunit S1) is an enzyme which binds NAD and transfers the ADP-ribose group to a family of GTP binding proteins involved in the translocation of signals across the cellular membrane of eukaryotic cells. B (composed of subunits S2, S3, S4 and S5), binds the receptors on the surface of eukaryotic cells and facilitates the entry of the toxic subunit S1 into the cells (3, 4).

Immunization of mice with the detoxified pertussis toxin induces in vivo protection against the intracerebral challenge with virulent B. pertussis and antibodies which are able to neutralize in vitro the toxicity of pertussis toxin in the CHO assay (5, 6). Monoclonal antibodies, raised against detoxified pertussis toxin but recognizing subunit S1, are also able to neutralize the toxic activity in vitro and to protect mice in vivo (7, 8), suggesting that protection against pertussis infection can be achieved by immunizing with pertussis toxin alone.

In order to produce in E. coli large amounts of non toxic pertussis toxin, suitable for large scale vaccination, we cloned and sequenced the genes encoding the 5 subunits of pertussis toxin (9, 10). The 5 genes were found to be clustered in a segment of DNA of 3200 base pairs and to be organized in an operon. Attempts to express the PT operon in E. coli were unsuccessful (11). We therefore engineered 5 strains of E. coli which expressed each of the five subunits as fusion proteins (11). The purified fusion-proteins expressed in E. coli were then used to immunize rabbits, mice and sheep to verify whether we could induce protective immunity against whooping cough by using the recombinant subunits as antigen. To our surprise, although the recombinant subunits were able to elicit high titers of antibodies which recognized the natural subunits in Western blot, they were unable to induce any protective immunity in vitro or in vivo. This finding was unexpected, since previous reports that monoclonal antibodies against the S1 subunit were protective had suggested that polyclonal anti S1 antibodies should also be protective. Furthermore, the finding that the recombinant S1 subunit had full enzymatic activity indicated that the folding of the recombinant molecule had to be similar to the natural one.

MAPPING OF THE PROTECTIVE EPITOPE(S) OF SUBUNIT S1

With the aim of resolving the discrepancy between the fact that immunization with pertussis toxin induces protective anti S1 antibodies, while immunization with the recombinant S1 molecule does not induce protective immunity, we collected seven protective anti S1 antibodies with the intention of mapping their epitopes. In order to do so, we constructed a number of plasmids expressing a series of aminoterminal or carboxyterminal deletions (see fig. 1), and we tested the reactivity of these molecules with the seven protective monoclonal antibodies and with two non-protective monoclonals.

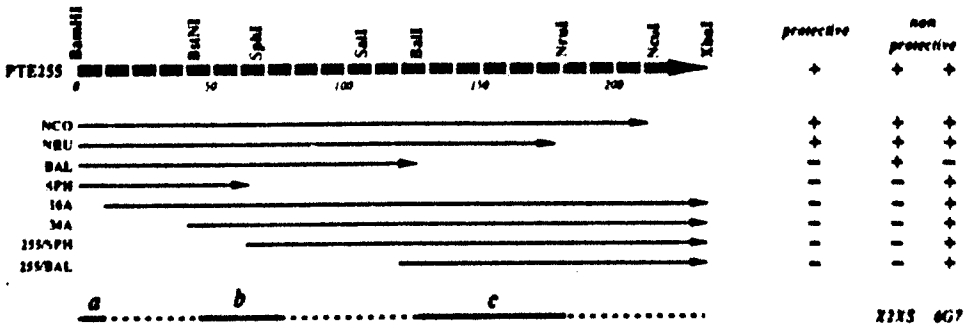


Fig. 1. Map of the S1 subunit of pertussis toxin and of the deletion mutants used in this study. On the right, the immunoreactivity (+ or -) of the seven protective monoclonal antibodies and the two non protective mAbs are reported. The three regions (A, B and C) which are required for protective antibody binding are indicated at the bottom of the figure. The numbers below the PTE255 refer to the aminoacid sequence. The restriction sites which were used to generate the deletion mutants are shown on the top of the figure.

As shown in fig. 1, all the protective antibodies were able to recognize the deletion mutants NCO and NRU but did not recognize the deletion mutant BAL, indicating that the

region comprised between the NruI and BalI sites is essential for antibody recognition. Surprisingly, none of the deletion mutants at the 5' region, although containing the region between BalI and NruI, were recognized by the protective monoclonal antibodies.
Since the mutant 16A lacks only 10 aminoacids at the aminoterminus, we may conclude that two discontiguous segments containing aminoacids 1-10 (region A in fig. 1) and 124-179 (region C in fig. 1) are required for binding to the protective mAbs. In marked contrast, the non protective antibodies X2XS and 6G7 recognized an apparently contiguous epitope, since they were able to bind all the clones containing the regions 2-67 and 124-179, respectively (see fig. 1).
To test whether the two halves of the S1 molecule, which are not recognized separately by the protective antibodies, would reconstitute the epitope in vitro, the mutant proteins BAL and 255/BAL were mixed in 8M urea and allowed to refold in vitro. The mixture was then tested for binding to the mAbs by dot blot assay. As shown in fig. 2, the mixture of the two fragments is recognized by the mAbs, indicating that the two fragments are able to fold in a structure structurally identical to the native epitope (12).

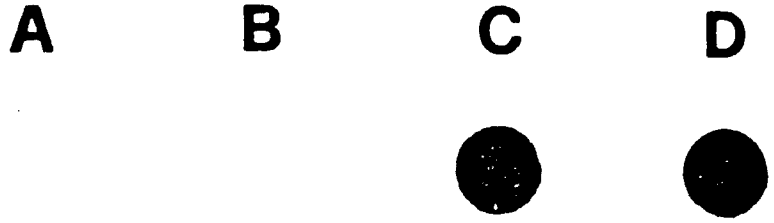


Fig. 2. Dot blot assay showing that the aminoterminal deletion mutant BAL (A) and the carboxyterminal deletion mutant 255/BAL (B) are not recognized by the protective monoclonal antibodies. However, a mixture of the two molecules, after in vitro refolding, is recognized by the protective monoclonals (C). D contains the entire S1 molecule as a positive control.

Further experiments using all the possible combinations of the aminoterminal and carboxyterminal deletion mutants showed that BAL could be complemented by all the aminoterminal deletion mutants while SPH could be complemented only by 16A and 34A. This suggested that the continuity of the aminoacid sequence spanning the SPH 1 site (region B in fig. 1) is also necessary for antibody recognition. The above data, also confirmed by sandwich and competitive radioimmunoassay, showed that regions A, B and C are necessary for protective antibody binding. Since regions B and C are adjacent in the native S1 molecule which contains a disulfide bridge linking cysteine 41 to cysteine 201, it is likely that regions B and C are part of the epitope recognized by the protective monoclonal antibodies, while region A is required for the appropriate folding of the epitope itself.

A MODEL FOR THE MECHANISM OF ACTION OF THE PROTECTIVE ANTI S1 ANTIBODIES

The mapping of the conformational epitope recognized by the protective monoclonal antibodies suggests the mechanism of action which is shown schematically in fig. 3: the S1 subunit contained in the native pertussis toxin is enzymatically inactive because the disulfide bridge does not allow the appropriate folding of the active site. Following reduction of the disulfide bridge (fig. 3, left), the active site can be formed and the S1 molecule becomes enzymatically active and therefore toxic.

In the presence of protective antibodies which bind both sides of the molecule in proximity to the disulfide bridge (fig. 3, right), the active site cannot be formed even after reduction of the disulfide bridge, and therefore the S1 molecule remains inactive.

The above model also explains why protective immunity can be achieved by immunizing with the native pertussis toxin but not with the recombinant S1 subunit. In fact, the native pertussis toxin contains the correctly folded S1 molecule and is therefore able to induce antibodies against the conformational protective epitope (fig. 4A). The recombinant S1 molecules on the other hand, being already unfolded, do not contain the protective epitope and therefore cannot induce protective immunity (fig. 4B).

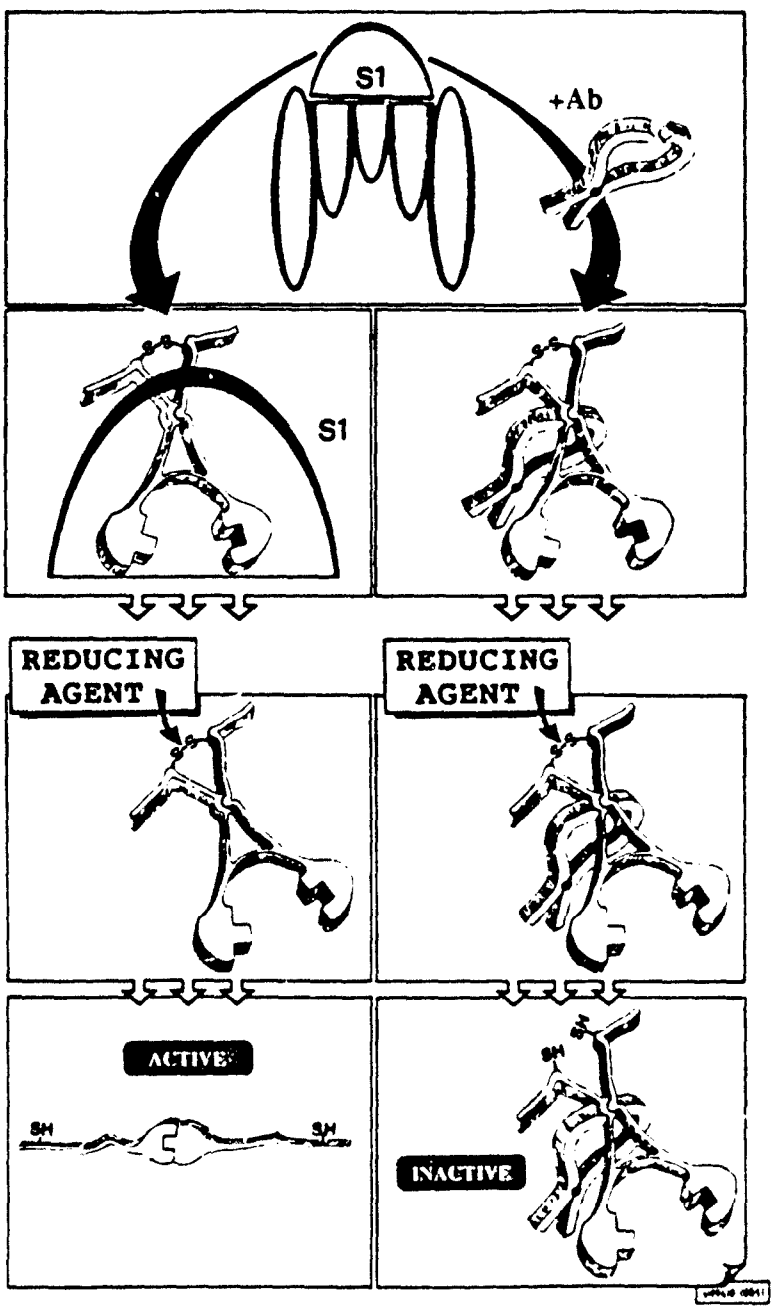


Fig. 3. Mechanism of action of the S1 subunit (left) which is enzymatically inactive when present in the pertussis toxin molecules and becomes enzymatically active following reduction of the disulfide bridge which allows the appropriate folding of the active site. When the S1 molecule has been bound by a protective antibody (right), the active site cannot be formed even after reduction of the disulfide bridge and the molecule remains inactive.

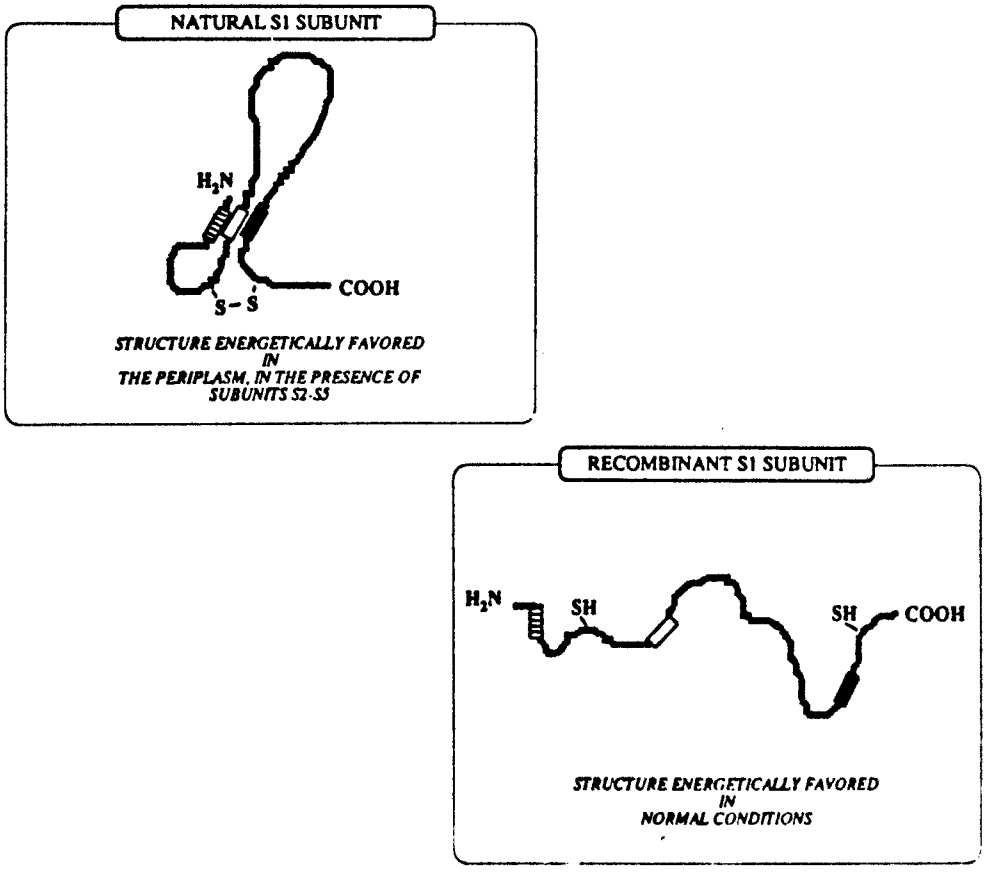


Fig. 4. Structure of the native S1 subunit (left) which contains the regions A, B and C of fig. 1 folded to form the protective epitope. The recombinant S1 subunits (right), although enzymatically active, contain mostly unfolded molecules and therefore cannot induce antibodies against the protective epitope.

ENZYMATICALLY INACTIVE PERTUSSIS TOXIN MOLECULES

The failure to induce protective immunity using recombinant pertussis toxin subunits indicates that the detoxified pertussis toxin should be the antigen of choice for vaccination against whooping cough. The pioneering work carried out with diphtheria Cross Reacting Materials (CRMs) has shown that the best way to produce a completely detoxified molecule is to engineer its gene in such a way that it will encode an immunologically active but non toxic molecule (13, 14, 15).
In order to produce such a molecule, the deletion mutants

shown in fig. 1 were tested for enzymatic activity. The region 2-179 which was found to be still enzymatically active was mutagenized by site-directed mutagenesis. We found 3 mutants where minor aminoacid changes completely abolished the enzymatic activity (16). Two of them had aminoacid changes in the region of the S1 molecule which shows homology to cholera toxin, while the third one involves substitution of glutamic acid 129, an aminoacid located in the catalytic site of the enzyme, structurally homologous to glu-148 of diphtheria toxin and glu-553 pseudomonas exotoxin A. Lysine 9 (17), Aspartic acid 11, Arginine 13 and tryptophan 26 (18) were also found by other laboratories to be essential for enzymatic activity. Mutated S1 genes encoding enzymatically inactive S1 subunits were then introduced into the PT operon and subsequently into Bordetella pertussis strains, where the wild type PT operon had been previously deleted.

These B. pertussis strains expressing mutated PT molecules where then grown in fermentors and the PT molecules purified and tested for toxicity. Most of the mutant PT molecules obtained had a reduced toxicity in CHO cells in the leukocytosis test in mices (Table I).

However, none of them had a toxicity low enough to be suitable for vaccine development. To further reduce the toxicity of the PT mutants we introduced two of the above independent mutations in a single S1 gene and we introduced them in Bordetella.

Two double mutants, one containing the Arg → Lys and Glu 129 → Gly mutations and the other containing the Arg 13 → Leu and glu 129 → Gly mutations were found to be devoid of any in vivo and in vitro toxicity (Table I) and therefore they were suitable for vaccine development, provided that they were able to induce protective immunity. Therefore we immunized rabbits and guinea pigs with the double mutants and we tested the ability of the sera obtained to neutralize the activity of pertussis toxin. Both mutants were able to induce levels of neutralizing antibodies similar to those obtained by immunizing with wild type pertussis toxin. Finally we tested the ability of these PT double mutants to protect mice form the intracerebral challenge with virulent Bordetella pertussis, a test which correlates with the protection of the cellular vaccines.

As shown on Table II, complete dose dependent protection could be achieved. We believe that the PT double mutants here described are ideal molecules for the development of new vaccines against whooping cough.

TABLE I
In vitro and in vivo toxicity of PT mutants

Mutant	% in vitro toxicity (CHO cells)	% in vivo toxicity (lymphocytosis)
Arg 9 → Lys	0.1	0.1
Asp 11 → Ser	100	n.d.
Arg 13 → Leu	25	n.d.
Trp 26 → Leu	10	n.d.
Glu 129 → Gly	5	5
Arg 9 → Lys Glu 129 → Gly	< 0.0001	< 0.001
Arg 13 → Leu Glu 129 → Gly	< 0.0001	< 0.001

TABLE II

Potency of the PT double mutant Arg 13 → Leu, 129 G → Gly as vaccines against whooping cough tested by the survival of immunized mice to the intracerebral challenge with virulent B. pertussis. The other double mutant gave similar results.

immunizing dose (ug)	survivors
30	16/16
20	15/16
15	12/16
7.5	10/16
3.75	9/16
1.8	6/16
0.9	3/16

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A Multivalent Carrier for Delivery of Epitopes and Antigens Based Upon the B-subunit Enterotoxoid of Escherichia coli

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INTRODUCTION

The B subunit toxoid is a pentamer of identical (12 kDa) subunits which bind strongly to GM1-ganglioside on the surface of mucosal cells. It is a potent immunogen, stimulating both mucosal and systemic humoural immunity. Genetic modifications to the 3'-end of the gene encoding the B subunit do not interfer with the pentameric structure or lectin-binding property of the toxoid, when a sequence of amino acid residues, Lys Leu Gly Pro Gln are attached. Here we describe a series of three plasmid vectors which allow the convenient insertion of defined epitopes and antigens onto B subunits containing this linker sequence.

MATERIALS AND METHODS

Plasmid pMMB138 (ref 1), encoding a mutant B subunit gene, etx B138 was used to insert a series of three oligonucleotide sequences which coded for the linker sequence, Lys Leu Gly Pro Gln and convenient restriction sites for insertion of other oligonucleotides or DNA sequences specifying epitopes or antigens (Fig 1).

```
                +102
pMMB68          Glu Asn ***
                GAAACTAGT
                    SpeI

pMMB138         Glu Lys Leu Ala Pro Gln Lys Arg Trp ***
                GAAAAGCTTGCCCCCCAGAAACGCTGGTGA
                   HindIII

pTRH100         Glu Lys Leu Gly Pro Gln Ala Gly Asp ***
                GAAAAGCTGGGTCCGCAGGCCGGCGACTAG
                                 NaeI
```

pTRH101 Glu Lys Leu Gly Pro Gln Gly Arg His ***
GAAAAGCTGGGTCCGCAGGGCCGGCACTAG
NaeI

pTRH102 Glu Lys Leu Gly Pro Gln Pro Alu Asp ***
GAAAAGCTGGGTCCGCAGCCGGCTGACTAG
NaeI

Fig 1 Plasmid vectors for insertion of epitopes and antigens into the NaeI site at the 3'-end of extended etxB genes. Plasmid pMMB68 encodes wild-type B subunits. The HindIII site of pMMB138 was used for insertion of oligonucleotides coding for the linker sequence amino acids.

RESULTS

The oligonucleotides shown in Fig 1, are not the complete sequences that were inserted into the HindIII site of pMMB138. Sequences distal to those above, specifying restriction endonuclease recognition sites for SpeI, BamHI and XbaI were also included. The NaeI site is located in all three reading frames in the respective plasmids pTRH100, pTRH101 and pTRH102. Expression of the B subunits is under the control of the tac promoter and the vectors (derived from plasmid pMMB66 (ref 2) contain rep genes which allow the plasmid to replicate in a wide range of Gram negative bacteria.

A hybrid protein containing the B subunit fused to the $(NANP)_3$ repetitive epitope of the malaria parasite Plasmodium falciparum was produced by inserting an oligonucleotide sequence coding for the $(NANP)_3$ epitope into the NaeI site of plasmid pTRH102. The carboxyl terminus of the fusion protein is shown below:

+102
... Glu Lys Leu Gly Pro Gln Pro Asn Ala Asn Pro Asn Ala Asp Pro Asn Ala
Asn Pro

The hybrid protein is approx 15 kDa in size, it assembles into stable pentamers that bind to GM1-ganglioside. The hybrid is recognized by both anti-B subunit and anti-NANP monoclonal antibodies.

CONCLUSION

Plasmids have been constructed which allow the production of hybrid proteins comprised of the E. coli heat-labile toxoid B subunit, a short linker sequence and an epitope or antigen. We have successfully used those vectors to generate a stable, pentameric antigen comprised of the B subunit and the $(NANP)_3$ repetitive epitope from Pl. falciparum.

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Gene Fusions to the E. coli and V. cholerae Enterotoxins

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INTRODUCTION

Cholera toxin and the closely related heat-labile enterotoxin of E. coli are composed of five copies of a receptor binding B subunit (CTB or LTB) and one copy of an ADP-ribosylating A subunit (CTA or LTA). Gene fusions to the non-toxic immunogenic CTB or LTB may permit their use as carriers for diverse foreign epitopes. The use of CTB or LTB is more attractive by the fact that when expressed in V. cholerae they or their fusion products are excreted to the cell medium from where they can be conveniently purified.
Fusions to the A subunits might, on the other hand, yield hybrids which could be used to direct the ADP-ribosylating activity of the protein to selected targets in a similar fashion the diphteria toxin fusion proteins(1). Here we report on fusions of the STa heat-stable enterotoxin of E. coli to the amino (NH2) and carboxy (COOH) ends of CTB and on the fusion of human interleukin-2 (IL2) to the COOH end of LTA.

MATERIALS AND METHODS

The STa gene was fused to the NH2 end of CTB as synthetic oligonucleotides compatible with the single SacI and XmaI sites in plasmid pJS152 (2). Fusion of these same oligonucleotides at the COOH end of CTB were made possible by first introducing at the CTB COOH end synthetic oligonucleotides containing several restriction sites including SacI, XmaI and SpeI. For purification of the hybrid proteins by GM1 affinity chromatography the recombinant plasmids were transferred to V. cholerae JS1559 (2). IL2 was fused to LTA by joining the single SphI site at the start of mature IL2 to the single EcoRI at the COOH end of LTA via an ad hoc synthetic linker.

RESULTS

Gene fusion of STa at the NH2 end of CTB gave a hybrid with all 19 aa in STa followed at its COOH end by Pro and Gly (encoded by the linker) and Tyr Ala His and Gly originating in the truncated CTB precursor. At its NH2 end STa was preceded by alanine (potential cleavage point of the functional leader peptide). The fusion of STa to the CTB COOH end gave a fusion protein containing all 103 aa in CTB Gly and Ala preceding the 19 aa in STa and at the COOH end a Pro Gly and Asn tail. The presence of both STa-CTB (NH2 fusion) and CTB-STa (COOH fusion) were initially followed by GM1 ELISA and their hybrid nature fully confirmed by immunoblots (Fig. 1). Preeliminary assays of the purified STa-CTB and CTB-STa in the infant mouse test showed them to bear about 1-2% of the expected STa-associated toxicity.

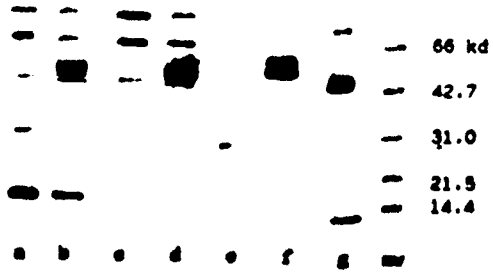


Fig. 1. Immunoblot analyses of STa-CTB and CTB-STa. Samples of purified hybrid proteins were separated on a 15% SDS polyacrilamide gel and electrotransferred to nitrocellulose. Lanes a-d CTB-STa, lanes e and f STa-CTB, lane g a reference decapeptide-CTB (3). Samples were either boiled (lanes a,c and e) or not boiled (lanes b,d,f and g). Lanes a,b and g reaction with anti-STa lanes c-f with anti-CTB. A biotinylated molecular weight standard is shown (mw).

Fusion of IL2 to LTA gave a hybrid with IL2 joined to aa 236 in LTA (total length 240 aa)(Fig. 4). For its detection the LTA-IL2 hybrid was trans-complemented by LTB. Sonic extracts of E. coli cells expressing LTA-IL2 and LTB were then assayed in GM1 ELISA using anti-LTA or anti-IL2 antibodies.

```
          236  *   *   *   *   *  -1  +1  +2
           Ile Leu Ser Met His Ala His Ala Pro
     A ATT CTA TCG ATG CAT GCC CAT GCG CCT
```

Fig 2. Fusion of IL2 to the LTA carboxy end. Aa numbering corresponds to positions in LTA(236) or to the leader peptide-mature IL2 junction. The aa denoted by asterisks are those encoded by the synthetic linker.

DISCUSSION

The STa toxin is a very poor immunogen unless coupled to a carrier protein. We have described a CTB hybrid carrying a non-toxic decapeptide analogous to STa with key substitutions on its cysteine residues (3). Upon rabbit immunizations the antisera obtained proved unable to neutralize STa. In efforts to prepare hybrids that give neutralizing antibodies we have now fused genetically the entire STa molecule to CTB. To increase our possibilities the STa was joined at both the NH2 and COOH ends of CTB so as to have freely rotating NH2 and COOH STa termini. Both the NH2 and COOH fusion hybrids were secreted by V. cholerae to the cell culture medium from where they were purified. Studies are in progress to determine if the hybrid proteins are able to generate good neutralizing antibodies.
The gene fusion of IL2 to LTA provided with an LTA-IL2 hybrid which apparently associates with LTB to give LTA-IL2/LTB complexes that bind to GM1 in ELISA. With the current antibodies positive yet weak signals have been observed in this test. Further immunological characterization of the hybrid is in progress. Expression of the LTA-IL2/LTB complexes in V. cholerae is also planed to determine if they are secreted by this host so as to initiate their purification by GM1 affinity chromatography.

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Novel Cytotoxic Agents Made by the Fusion of Cell Recognition and Pseudomonas Exotoxin Genes

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INTRODUCTION:

Conventionally specific cytotoxic agents have been produced by chemically attaching antibodies and growth factors to different protein toxins to selectively kill cells bearing appropriate antigen or receptors (1,2). These molecules are heterogenous and yields are often poor. With the understanding of toxin structure and the availability of the genes encoding toxins and cell recognition molecules, it has been possible to produce chimeric proteins as single polypeptide chains by gene fusion in *E. coli* (3). Here we describe expression of a recombinant single chain immunotoxin which consists of variable domains of an antibody to the Tac peptide of the human IL2 receptor attached to PE40 (a modified form of PE missing the binding domain). The hybrid protein, anti-Tac(Fv)-PE40 was toxic to cells bearing the p55 subunit of human IL2 receptor without affecting cells devoid of the human p55 subunit.

MATERIALS AND METHODS:

A plasmid pVC70108 was assembled and expressed in *E. coli* strain BL21λ(DE3) as described earlier (4). Upon IPTG induction, a 65 kd protein was produced as insoluble aggregates which was extracted using guanidine HCl denaturation followed by rapid dilution in PBS to renature the protein. Renatured monomeric protein was successively purified on Mono Q ion exchange and TSK-250 gel filtration columns. The cytotoxic activity of the purified protein was tested on various cell lines by measuring the incorporation of [^{3}H]-leucine in TCA precipitable material.

RESULTS AND DISCUSSION:

Figure 1 shows the hybrid protein consisting of the variable domain of the heavy chain (VH) joined through a 15 amino acid linker to the amino terminus of the variable domain of the light chain (VL), which in turn is joined to PE40 amino acids 253-613 of PE. As shown in Table I, antiTac(Fv)-PE40 was tested on various cell lines with and without human IL2 receptors. The hybrid protein was very cytotoxic to all the cell lines which had the p55 subunit of the human IL2 receptor (low affinity) with an ID50 of 0.15 - 2.7 ng/ml depending upon the cell line. Due to the specific cytotoxic activity, this hybrid protein may be useful in eliminating cells which express large numbers of the p55 subunit of human IL2 receptor.

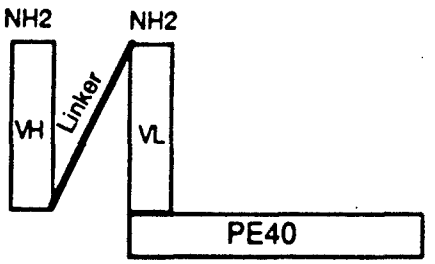


FIGURE 1: Diagramatic Representation of Hybrid Protein, Anti-Tac(Fv)-PE40.

Table I: Cytotoxicity of AntiTac(Fv)-PE40 on Various Cell Lines

	IL2 Receptors Per Cell		
Cell Lines	Low Affinity (p55)	High Affinity	ID50 ng/ml[a]
HUT 102[b]	94,000	3,800	0.15
CrII.2[b]	12,000	350	2.7
MT-1[b]	100,000	<20	0.51
MLA 144[b,c]	<20	6,500[d]	>1000
CEM[b]	<20	<20	>1000
OVCAR3	-	-	>1000
KB	-	-	>1000
A431	-	-	>1000

HUT102, CrII.2, CEM are human leukemia T cell lines. OVCAR3 is a human ovarian cancer cell line. KB and A431 are human epidermoid carcinoma cell lines.
[a]ID_{50} is the concentration of hybrid toxin to inhibit protein synthesis by 50% of control where no toxin is added.
[b]Cell lines were washed once with serum-free medium before adding hybrid protein.
[c]MLA 144 - a gibbon ape cell line, carries only the p75 subunit.
[d]Number of the p75 subunit.

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Oral Immunization of Mice with a Recombinant Yersinia enterocolitica 0:9 Strain Producing the Cholera Toxin B Subunit

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INTRODUCTION.

The enteropathogen Yersinia enterocolitica of serogroup 9 causes a benign enteritis in human and animals. It enters its host via the oral route and has a tropism for lymphoid tissue. A 70 kb plasmid contributes to the virulence of Y. enterocolitica. At 37°C in absence of Ca^{2+}, this plasmid directs the synthesis and secretion of high amounts of several proteins called Yops. Strong promoters govern the expression of the yop genes and a transactivator thermoregulates their transcription (1). Since these Yop proteins are produced in the course of infection and stimulate the immune system, we assumed that insertion of foreign genes downstream from yop promoters could also allow the production of the relevant antigens and a specific immune response. In this work, we investigated the potentiality of Y. enterocolitica to act as a live oral carrier for the cholera toxin B subunit (CT-B).

RESULTS.

EXPRESSION OF CT-B IN Y. ENTEROCOLITICA. We first constructed plasmid pMS41 by inserting the ctxB gene behind the promoter of the yop51 gene. This plasmid was introduced by conjugation into Y. enterocolitica W22703(pGC1256). The cholera toxin B subunit as well as Yops were produced when recombinant bacteria were incubated at 37°C in a medium deprived of Ca^{2+}. However, the B subunit was not secreted in the culture medium.

In a second type of constructions, the ctxB gene was inserted in frame behind the yop51 gene. When the recombinant plasmid pMS44 was introduced in Y. enterocolitica, Yop51-B subunit hybrid proteins were efficiently secreted in the surrounding medium by incubation of bacteria in conditions of Yops expression.

IMMUNIZATION OF MICE. In pYV mutant pGC1256, a mini-Mu d*lac* element is inserted in the gene yop25, preventing the production of Yop25. LD_{50} of the strain carrying this plasmid is at least 20 fold higher than that of the wild type parental strain (2). This mutant was selected as the host strain for vectors pMS41 and pMS44.
BALB/c mice treated with desferrioxamine B and iron dextran were orally inoculated twice with about 5 10^9 bacteria of strains W22703(pGC1256) and W22703(pGC1256)(pMS41). Immunoblots showed that antibodies against the cholera toxin B subunit were present in sera of mice inoculated with the strain harboring pMS41. Only the polymeric form of the B subunit was detected.
The antibody response induced by strain W22703(pGC1256)(pMS41) was also tested in C57BL/6 mice inoculated only once by gastric intubation with about 1.5 10^{10} bacteria. Immunoblot analysis of the sera revealed an antibody response against the polymeric form of the cholera toxin B subunit. GM1-ELISA showed that all the mice inoculated had antibodies against the cholera toxin B subunit.
No antibody response was observed after oral inoculation of mice with recombinant Y. enterocolitica producing Yop51-B subunit hybrid proteins. This can result from the inability of the chimeric protein to assemble into the polymeric form of the cholera toxin B subunit or from the fact that the Yop51 moiety of the hybrid proteins masks the epitopes of CT-B.

CONCLUSION.

Y. enterocolitica of serogroup 9 has a low natural virulence. However after penetration by the oral route, this pathogen has the ability to invade the host intestinal tissue and to stimulate the immune system. Hence, Y. enterocolitica can be converted into an immunogenic live carrier for delivery of various protective antigens.
In this report, we show that a Y. enterocolitica strain producing the cholera toxin B subunit from a yersinial yop promoter is able to elicit an antibody response against the polymeric form of this antigen, after one oral inoculation to mice.

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A Semi-Synthetic Vaccine Involving CRM 197 as Carrier for H. Influenzae Type B Capsular Oligosaccharides

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CRM 197 is a non toxic derivative of diphtheria toxin (DT). A single aminoacidic substitution in the position 52 (GLY-GLU), results in the loss of the enzimatic activity: NAD:EF2 ADP-ribosyl transferase (1). Neverthless CRM 197 shows immunochemical identity with DT and is able to elicit antitoxic antibodies in animal models. These two properties make CRM 197 a very attractive molecule in view of the development of a new antidiphtheria vaccine. Together with this possibility we wondered whether CRM 197 could also be a good carrier for carbohydrate antigens which need the conjugation to immunogenic proteins to increase their potency in infants (2). To ask these questions we prepared a conjugate (CRM-HIB) between CRM 197 and small oligosaccharides derived from the capsular polysaccharide of Haemophilus influenzae type b [-→3)-D-Ribf-(1→1)-ribitol-5→ PO_4^-] , and we studied its immunogenic properties, for both the antigenic moyeties, in animals and in human beeings. To prepare CRM-HIB we utilized a four steps method involving: 1) Hydrolysis of the Hib polysaccharide to get oligomers of about 4 repeats; 2) Introduction of a primary amino group on the end-reducing group of the oligomers by reductive amination in the presence of $NaBH_3$ CN and NH_4Cl; 3) Derivatization of the amino groups by reaction with the succinimido diester of adipic acid; 4) Coupling to CRM 197.

The resulting product contained about 3 moles of oligosaccharide per mole of protein.

The immunological properties of CRM-HIB were studied first in rabbits (an animal model which does'not respond to purified polysaccharides). The conjugate was able to induce specific antibodies toward the two antigenic moyeties of the molecule with a clear booster effect after a second administration.

After pharmacotoxycological studies which shown that CRM-HIB is acceptable for human administration, twelve adult volunteers were vaccinated with a single dose of conjugate containing 3 ug of oligosaccharide linked to 45 ug of CRM 197

adsorbed on 1 mg of $Al(OH)_3$. Nine of the vaccinated had a significative increment of anti Hib and antidiphtheria toxin antibodies (Fig. 1) and none of them shown adverse reactions to the product.

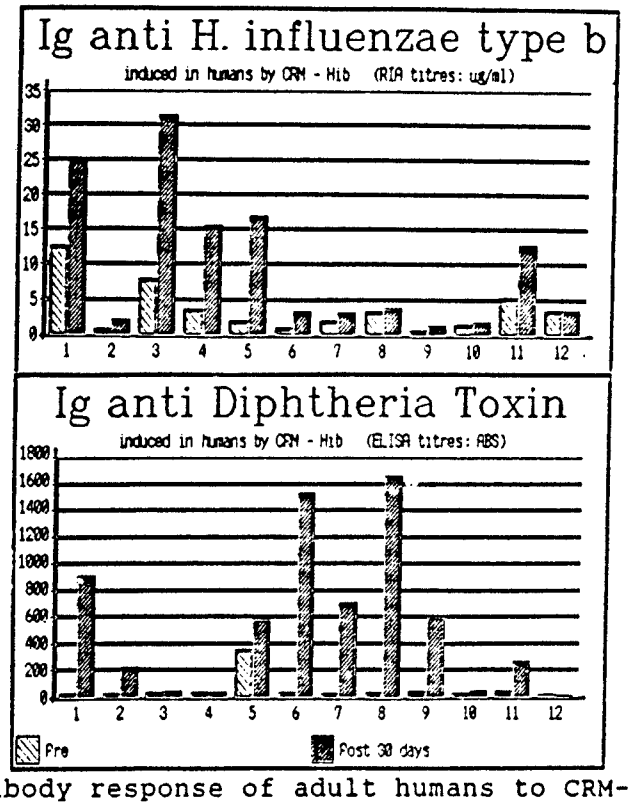


Fig. 1. Antibody response of adult humans to CRM-HIB

These results indicated the possibility of using glycoconjugates between CRM 197 and bacterial capsular oligosaccharides as better carbohydrate vaccines and as a new antidiphtheria one. In the next phase II clinical trials, variables such as the substitution degree of the oligosaccharide on the protein, the length of the carbohydrate oligomers and the booster effect, after a second administration, will be studied on children.

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Synthetic Pertussis Toxin Peptides in Mice and Guinea Pigs

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INTRODUCTION

Future developments of vaccines against whooping cough may include synthetic peptides resembling immunogenic epitopes of some major Bordetella pertussis protective antigens (1,3).
Regarding protective antigens pertussistoxin (Ptx) has been recognized to play a central role. The S1-subunit of Ptx carry the enzymatic active parts of the molecule and by the aid of monoclonal antibodies, it was assumed that the S1 carry also protective epitopes (4). S1 was therefore subject for analysis via two synthetic peptides.

MATERIALS AND METHODS

Two peptides were synthesized on account of hydrophilicity-plotting of the aminoacid sequence og Ptx-S1-subunit (Fig.1) (2). These peptides were coupled to a carrier, tuberculin purified protein derivative (PPD), at the N-terminal end (L-SMCC and K-SMCC) or in an unoriented manner (L-glut and K-glut). Mice and guinea pigs were immunized four times, over a period of 14 to 16 weeks, using these peptides. Seven to fourteen days after each immunization the animals were bled and the sera were tested for antibodies against individual peptide-conjugates in ELISA.

DSRPPEDV	Peptide K
VYRYDSRPPEDV	Peptide L

Fig.1 Aminoacid-sequence of the synthetic Ptx-peptides.

RESULTS AND CONCLUSIONS

All combinations of peptide were found to be immunogenic and cross-reacting antibodies were developed in varying amounts depending on the peptide-couple and on the animal source.
Peptide L coupled to a carrier (PPD) in an oriented manner elicited more antibodies reactive to the intact pertussis toxin than any of the other antigen preparations.
This applied both in mice and guinea pigs, allthough the mice apparently are better responders to both the coupled peptide and to the carrier itself (Fig.2).
Taking this into consideration other peptides will be and have been synthesized and administered in mice in future. Th new peptides will be chosen in the light of hydrophlilicity-plots and especially surface-plots.

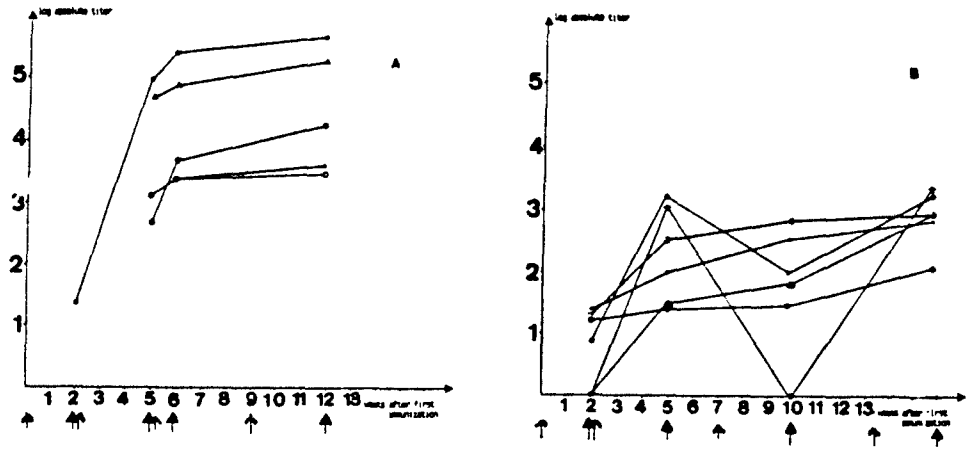


Fig.2 Sera in mice (A) and guinea pigs(B) immunized with peptide L-SMCC. Reactions to L-glut (○), K-glut (★), L-SMCC (✱), K-SMCC (▲), PPD (●) and intact Ptx (☆).
(↟) = Immunization, (↟) = bleeding.

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Epitope-Mapping of Pertussis toxin.

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INTRODUCTION

The main purpose of this investigation is to formulate/produce a non-toxic pertussis vaccine consisting of one or more synthetic peptides imitating the major protective epitopes of Bordetella pertussis antigens.

MATERIALS AND METHODS

Monoclonal antibodies (Mabs) against PT-subunits S2, S3 and S4 were produced by immunization of BALB-C mice with PT (3). Mabs against S1 and S5 were raised by immunization with S1 and S5, respectively. The subunit specificities of the Mabs were determined by Western blotting and ELISA (3) using purified S1, S2, S4, S5 subunits and S3+S4, S2+S4 dimers, PT and the B-oligomer.
Inhibition of PT-induced biological effects in hemagglutination (HA), lymphocytosis promoting (LP) activity, histamine sensitization (HS), Chinese Hamster Ovary (CHO) cell clustering and intra-cerebrally (IC) challenge assays was tested as described previously (3). The intra-nasal (IN) challenge test was performed by the method of Sato et al (2).

RESULTS

Immunization of mice with purified PT resulted in the production of twelve Mabs with different specificity for the S2, S3 and S4 subunits and three Mabs (21.2, 21.3 D12 and 22.2) which recognized intact PT only. Mabs 18.3, 20.6, 47.2 and 47.4 reacted with both S3 and S4; Mab 22.4 G2 with S2; Mabs 20.5, 22.4 G7, 47.3 C4, 47.3 G3, 21.3 D9 and 21.3 D11 recognized S2 and in part S3 whereas 47.1 reacted with S3 only. Five anti-S5 Mabs were produced out of which only three reacted with S5 in Western blotting. Nine anti-S1 Mabs were produced which all reacted with S1 in Western blotting.
The PT-neutralizing properties of the Mabs with specificity for the B-oligomer were studied. None of the the S5-specific Mabs showed any PT-neutralizing effect. Moreover, seven of the twelve S2, S3 and/or

S4-specific Mabs were inable to neutralize PT in any of the assays used, whereas 20.6 and 21.3 D12 showed a high level of PT neutralizing-activity in the LP, HS, CHO, IC challenge and IN challenge tests ($\geq$ 75%). Mab 47.4 effectively inhibited LP, and CHO cell clustering activity ($\geq$ 75%) and to a lower degree influenced HS activity and IC challenge assays (25-50%). In addition to some inhibition of LP-activity and CHO cell clustering (50-75%) Mabs 21.3 D9 and 21.3 D11 revealed a strong inhibition of deaths after IN-challenge ($\geq$ 75%) and inhibition of HA activity (50-$\leq$75%). Mab 47.1 showed a weak inhibition of HS-activity (25-50%) and in resemblance to 47.2 some inhibitory effect on LP-activity and CHO-cell clustering (50-$\leq$75%).

DISCUSSION

The specificity of a panel of Mabs against PT has been studied by means of ELISA and Western blotting. The results indicate that mice immunized with native PT gave rise to three Mabs that reacted with intact PT only and that the remaining twelve Mabs recognized either S2, S2+S3, S3 or S3+S4. The reasons for the lack of S1 and S5 specific Mabs derived from these fusions are not obvious but may suggest that these subunits are not immunodominant in mice.

Several Mabs showed specificity for more than one subunit. It is not obvious why some Mabs have specificity for S3 and S4 as no homology in amino-acid sequences between these subunits has been described. In contrast, the cross-reactivity between S2 and S3 is more comprehensible as these two show about 70% homology.

By comparing the subunit specificities and the PT-neutralizing effect of the Mabs it is possible to get an indication of the importance of the subunits in search of protective epitopes. The most interesting Mabs are 20.6, 21.3 D12, 47.4 and to a certain extent 47.2 because of their marked PT-neutralizing abilities. So far, it is impossible to get any detailed information about the fine subunit specificity of 21.3 D12 because of its reactivity with intact PT only. Apparently, the presence of S1 is of importance as this Mab not even react with the B-oligomer. The three other Mabs, however, recognized S3 and S4.

From these data we made the assumption that S4 in particular might possess epitopes of biological importance and decided to try to localize these epitopes. Six epitopes - based on amino-acid sequences showing high levels of hydrophilicity, accessibility and flexibility (1) - were selected and peptides copying these sequences are presently being synthesized. In the near future these peptides will be studied further in animal models as candidates for a new, synthetic pertussis vaccine.

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Mutant Strains Producing Pertussis Toxin CRMS

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INTRODUCTION

Pertussis toxin (PT) is a major virulence factor of B. pertussis and is one of the main protective antigens in vaccines against whooping cough. Each subunit either separated from PT or produced by recombinant techniques in E. coli, however, showed little protective immunogenicity in mice in both active and passive immune systems although monoclonal antibodies against S1, S2 or S3 protected mice in both ic and aerosol or in aerosol challenge systems (1, 2, 3 and unpublished data). These facts led us to a fundamental study on the mechanisms of production and antigenicity of PT composed of oligomeric subunits. For this purpose, we isolated and characterized mutant strains producing PT CRMs from B. pertussis, strain Tohama, phase I by NTG treatment.

RESULTS AND DISCUSSION

Strains producing higher PT tend to show higher ic virulence although it is not necessarily parallel to the amount of active PT in the culture supernatant. No correlation between other factors and the virulence was found as shown in Table 1. Table 2 shows the antigenic or toxic properties of PT or PT CRMs produced by the mutant strains: antigenic PT calculated from its binding activity to MAbs against different subunits (A) and construction of active PT by addition of S1 (A protomer) to the culture supernatants (B). Most CRMs were less reactive to anti-S1, 1B7, and much more reactive to anti-S4, 1H2, which had more reactivity with the dissociated S4 than with native PT. Each mutant showed specific binding against many distinct MAbs which recognized various primary and/or tertiary structures of PT (data not shown). The results suggested that the mutants must produce CRMs in diverse forms with different antigenicity. When CRMs produced by 69D, 74E or 79G were mixed with purified A protomer, the activity assayed by clustering of CHO-cells increased significantly (Table 2B), but not when mixed with B oligomer (not shown). These CRMs may be composed of defective S1 and almost native S2, S3, S4 and S5 proteins. CRM of 35C was activated with neither A protomer nor B oligomer. Molecular sizes of PT CRMs were analyzed by sucrose density gradient centrifugation. The sizes of the CRMs were in the range of 10-Kd to 200-Kd, which must be composed of mixtures of various combinations of subunits, but the biological activity of PT was detected at

the same molecular size as native PT. The majority of the CRM could be released into culture medium or be accumulated in the cell without completion of assembly of subunits to form the hexamer in PT-form. Base sequence analysis of the S1 gene of 79G showed that one point mutation occurred from G to A at the 730th base from the Eco RI site of the PT gene. Replacement of Cys-41 with Tyr-41 must have resulted from this mutation. This mutation was confirmed by disappearance of the digestion site with Bgl I in the PT gene of 79G. The other mutants had no such mutation as 79G.

Table 1. Production of pathogenic factors and virulence of the mutant strains derived from B. pertussis strain Tohama phase I.

Strain	Sero type	PT (ng/ml) Sup CHO	PT (ng/ml) Sup ELISA	PT (ng/ml) Cell CHO	PT (ng/ml) Cell ELISA	FHA (ug/ml) Sup HA	FHA (ug/ml) Sup ELISA	ACase (nmol/ml) Sup cAMP	ACase (nmol/ml) Cell cAMP	Virulence LD_{50}/mouse ic
Tohama	124	3,000	2,500	300	260	27	32	1.36	35.71	$6.3x10^2$
19H	124	3,000	2,600	170	320	1	0.3	ND	ND	$1.0x10^4$
13G	1	2,280	3,000	67	150	1	0.04	2.50	2.86	$>2.5x10^7$
35C	124	80	150	6	65	0.3	0.8	5.18	4.64	$>2.5x10^7$
69D	124	3	950	<4	800	15	32	ND	ND	$>2.5x10^7$
74E	124	<0.2	340	<0.2	230	27	45	0.78	21.43	$3.5x10^6$
79G	124	<0.2	300	<0.2	230	18	28	1.69	35.71	$>2.5x10^7$
34B	124	<0.2	<2	<0.2	<8	1.7	24	ND	ND	$>2.5x10^7$

Table 2. PT antigens detected by anti-PT ELISA (A) and construction of active PT by addition of isolated S1 (B)

Strain	A (anti-PT-ELISA) PT in culture sup (ng/ml) PT (M6)	S1 (1B7)	S2 (9G8)	S3 (7E10)	S23 (11E6)	S4 (1H2)	B (CHO-cell cluster) Active PT (ng/ml) No S1	With S1
Tohama	1,500	1,300	1,400	1,300	1,100	1,500	ND	ND
19H	2,600	1,600	1,600	2,100	1,900	1,600	ND	ND
13G	3,000	2,700	2,600	2,700	2,600	1,700	ND	ND
35C	170	29	3	120	61	460	150	100
69D	950	48	340	1,100	360	7,800	1.7	27
74E	340	44	72	220	130	1,300	<0.2	27
79G	300	13	62	220	130	1,500	<0.2	27
34B	<2	<2	<2	<2	<2	<2	0.3	0.3

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The Cellular and Humoral Immune Response to Pertussis Toxin and its Subunits in four Pertussis Vaccinated Humans

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INTRODUCTION

Cellular immunity against B. pertussis and T cell immune responses against pertussis toxin (Ptx) is gradually coming into focus and has drawn more attention in recent years. The aim of the present study has been to investigate in humans the in vitro proliferative response of lymphocytes to Ptx and its subunits. Lymphocytes isolated from peripheral blood from pertussis vaccinated adult human donors have been studied.

MATERIALS AND METHODS

Four adult human volunteers became immunized by vaccination; two persons received the traditional danish whole-cell pertussis vaccine (W-V), and two the japanese experimental component pertussis vaccine (C-V) JNIH 3, containing detoxified Ptx and filamentous haemagglutinin. Two non-immunized donors were used as controls. Lymphocytes were isolated from peripheral venous blood 2 times before and a total of 8 times at different days following the vaccination. The lymphocytes were stimulated in vitro with Ptx and the isolated Ptx subunits S1, S2-S4, S3-S4 and S5. The lymphocyte proliferative response was measured by incorporation of tritiated thymidine. Sera collected at the time of the lymphocyte isolation, were furthermore analysed for antibodies against these antigens using an ELISA method.

RESULTS

Ptx induced a proliferative response of lymphocytes from all six donors, and antibodies against Ptx were detected in sera from the four vaccinated donors. The subunits S1, S2-S4, S3-S4 and S5 induced a proliferative response of lymphocytes from the two C-V vaccinated donors, and from one

of the W-V vaccinated donors. Antibody titers against subunit S2-S4 and S3-S4 rose to high levels, especially in sera from the C-V vaccinated donors, and these donors also had the highest antibody titer against subunit S1. The antibody response against subunit S5 was very weak, compared to the response against the other subunits.

DISCUSSION

The proliferative response of lymphocytes from both vaccinated and non vaccinated donors to stimulation with the intact Ptx is not surprising, since Ptx is known as a potent mitogen for T-cells (2). The subunits S1, S2-S4, S3-S4 and S5 were found all to be able to stimulate an in vitro proliferative response of lymphocytes from the two C-V vaccinated donors and one of the W-V vaccinated donors. This means that 1) pertussis vaccination induces a specific polyclonal T-cell response to Ptx and that 2) the monomeric subunits S1 and S5 and the dimeric subunits S2-S4 and S3-S4 contain T-cell epitopes, as also suggested by others (1,3). The lack of an in vitro proliferative response of one of the W-V vaccinated donors, can be explained by a generally low in vitro lymphocyte proliferative response of this donor, as revealed by stimulation by other mitogens and antigens. The component vaccine as well as the whole-cell vaccine induced an antibody response against subunit S1, S2-S4 and S3-S4 in all four donors. The titers were highest in the sera from C-V vaccinated donors, which may be due to the higher quantity of Ptx in the component vaccine, as compared to the whole-cell vaccine. The antibody response to subunit S5 was weak, compared to the other subunits, whereas the in vitro lymphocyte proliferative response to subunit S5 equaled the proliferative response to the other subunits. This can probably be explained by the hidden position of the S5 subunit in the theoretic three dimensional structure of Ptx, where few or none of the S5 subunit B-cell epitopes can be expected to be accessible. When Ptx is processed and exposed on an antigen presenting cell the S5 subunit can be expected to be uncovered and presented to specific T-cells.
In conclusion the results of this study shows that pertussis vaccination activates a polyclonal T-cell response against several T-cell epitopes on various subunits of Ptx. This may have important implications for the design of a synthetic vaccine against pertussis.

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Import into K562 cells of anti-tetanustoxin F (ab') $_2$ conjugated to transferrin with homobifunctional maleimide linkers

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We have shown previously (Erdmann et al. 1981) that intrathecally injected anti-tetanus F(ab')2 fragments intercepted tetanus toxin in the extracellular space between neurons but were not taken up into nerve cells. As a consequence, intrathecally injected F(ab')2 prevented the development of tetanus symptoms but did not reverse existing symptoms. In an attempt to import F(ab')2 fragments into neurons we linked them to helper molecules for which a carrier system in the neuronal plasma membrane is known. Here we show that 125I-F(ab')2 linked to transferrin with either the acid-stable bis-maleimidohexane (BMH) or the acid-labile bis-(maleimidoethoxy)-propane (BMEP, Srinivasachar and Neville 1989) is translocated into K562 erythroleukemia cells. More radioactivity was found in the cells incubated with the BMEP conjugate than in those incubated with the BMH conjugate. The uptake could be prevented both with excess diferric transferrin and with a combination of inhibitors (NaN3, KCN, desoxy-D-glucose). The intracellular degradation of the BMH conjugate remained small, the intracellular hydrolysis of the BMEP conjugate was considerable. The cells released more than 50% of both the BMEP and the BMH intact conjugate within 30 min, probably through the transferrin pathway. However, the radioactive species formed by degradation of the BMEP conjugate were retained by the cells.

The F(ab')2-BMH-transferrin conjugate may be used to bind mobile antigen inside the cells, and a fast elimination of the complex antigen&F(ab')2-BMH-transferrin could occur. F(ab')2 freed by intracellular hydrolysis of the BMEP conjugate could reach additional cell compartments and may neutralize mobile and sessile antigens.

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