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Pseudomonas aeruginosa exotoxin A: Immunochemical analysis of the proposed elongation factor 2 binding site and ADP-ribosyltransferase cross-reactive epitope

McGowan, Jean Louise, Ph.D.
The Ohio State University, 1991
PSEUDOMONAS AERUGINOSA EXOTOXIN A: IMMUNOCHEMICAL ANALYSIS OF THE PROPOSED ELONGATION FACTOR 2 BINDING SITE AND ADP-RIBOSYLTRANSFERASE CROSS-REACTIVE EPITOPE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Jean Louise McGowan, B.S.

*****

The Ohio State University
1991

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FIELDS OF STUDY

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Immunoochemical analysis of Pseudomonas aeruginosa exotoxin A using monoclonal antibodies.
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Pseudomonas aeruginosa is a gram-negative bacillus which is capable of growing in environments including soil and water that contain very low levels of nutrients. Due to the organism's ubiquitous nature and its resistance to antibiotics, it has become one of the primary causes of nosocomial infections. It is also considered a principal pathogen in cystic fibrosis. P. aeruginosa produces many cell-associated as well as extracellular products which may contribute to its pathogenicity, including phospholipase C, alkaline phosphatase, elastase, and several other proteases. Two ADP-ribosylating toxins, exoenzyme S and exotoxin A, are also produced by P. aeruginosa. The role of exotoxin A in human infections remains unclear. However, most clinical isolates produce the toxin and survivors of P. aeruginosa infections have significant levels of circulating anti-toxin antibodies (Pollack 1980; Pollack et al., 1977).

Exotoxin A production by Pseudomonas aeruginosa strains is quite variable. This variability is due in part to the presence or absence of the toxin structural gene, toxA, in the strains. In addition, at least one regulatory gene, toxR, controls toxin expression, and there is growing evidence that there may be other regulatory genes involved. Exotoxin A's production is also regulated by the level of iron in the environment. Similar to diphtheria toxin, which is produced by Corynebacterium diphtheriae, exotoxin A is maximally produced as the iron concentration decreases.
during the late stages of growth (Bjorn et al., 1978; Locke and Main, 1931). In *C. diphtheriae*, a repressor protein binds to the diphtheria toxin structural gene in the presence of iron, and inhibits its transcription. However, when iron is removed, the protein is no longer capable of binding to the DNA and the toxin gene is transcribed resulting in toxin production. No such repressor protein has yet been identified in *P. aeruginosa*. However, due to the similarity in iron repression between diphtheria and exotoxin A production, it is probable that a repressor protein also is present in *Pseudomonas*.

Exotoxin A is a member of a class of proteins called mono-ADP-ribosyl-transferases. This class also includes diphtheria toxin, cholera toxin, pertussis toxin, and *Escherichia coli*’s heat-labile toxin. *P. aeruginosa* exotoxin A transfers the adenosine 5'-diphosphate ribosyl (ADP-ribosyl) moiety of NAD⁺ to mammalian elongation factor 2. In the absence of elongation factor 2, it hydrolyzes NAD⁺ releasing nicotinamide. The purpose of the latter activity is not known; however, ADP-ribosylation of elongation factor 2 results in inhibition of protein synthesis and eventual cell death.

Prior to being enzymatically active, exotoxin A first must be "activated", which may involve the reduction of two of the toxin's four disulfide bridges (Lory and Collier, 1980). In the laboratory this usually occurs by treatment with urea and a reducing agent such as dithiothreitol, or by repeated freeze-thawing cycles. Little is known about how the toxin is processed in vivo, but proteolytic processing could occur prior to or following attachment to the cell receptor. In either case, the toxin must undergo a conformational change which allows for binding of the enzyme's substrates, NAD⁺ and elongation factor 2, in order for enzymatic activity to occur. Although intact toxin, a 66,586 dalton protein, has enzymatic activity, a 26,000 dalton fragment isolated from culture supernatants is enzymatically active. A similar fragment has been
generated by treatment of exotoxin A with chymotrypsin, suggesting that only a portion of the molecule is necessary for activity (Lory and Collier, 1980).

Exotoxin A shares a common multistep mechanism with the other ADP-ribosyltransferases; including (1) all are bound to a target cell receptor; (2) the toxin, or the catalytic moiety, is translocated across the membrane to come in contact with the cytoplasmic target protein; and (3) the toxin covalently modifies the target protein. Typically, these functions are assigned to distinct domains either in separate structural subunits (pertussis and cholera toxins) (Katada and Ui, 1982; Moss, et al., 1976), in separate proteins, or in a single polypeptide chain, such as exotoxin A (Vasil, et al., 1977). The x-ray crystallography of the exotoxin A proenzyme revealed three structural domains (Figure 1) (Allured, et al., 1986). The amino terminal domain, designated domain I, is the largest and is composed primarily of anti-parallel β-pleated sheets. Domain II, the middle and smallest domain, is composed of six alpha-helices. The carboxyl-terminal domain III, which has no distinctive secondary structure, contains a deep extended cleft. The crystallographic and biochemical data have led to the assignment of functions to each of these domains which are described below.

**Domain I: The receptor binding domain**

Domain I, the largest of the domains, is divided into two physically separated sequences, Ia and Ib. Ia contains residues 1-252 and is the receptor binding domain. Evidence of domain Ia involvement in cell binding comes from studies in which monoclonal antibodies (Mabs) specific for domain Ia protect sensitive cells from exotoxin A but do not affect its enzymatic activity, implying that they block toxin receptor interaction (Chia, et al., 1986; Galloway, et al., 1984). In order to analyze the role that domain I plays in exotoxin A activity, a toxin fragment, PE-40, which lacks all of domain I, was produced. The PE-40 fragment was not cytotoxic yet retained full
enzymatic activity (Hwang, et al., 1987). Interestingly, unlike native ETA, PE-40 fragment is enzymatically active prior to any "activation" step. This implies that domain I may impose a physical restraint on the molecule so that it cannot assume an active conformation. Removal of this structure may have the same effect on enzymatic activity as the reduction of disulfide bonds during the activation process.

Figure 1. X-ray crystallographic structure of exotoxin A (Collier, 1988). Upper panel: stereoscopic drawing of the alpha-carbon backbone of exotoxin A. Lower panel: from left to right, domains I, II, and III.

Previous data had shown that treatment of exotoxin A with iminothiolane or other substances which react specifically with lysine residues, reduces exotoxin A’s cytotoxicity while having no effect on ADP-ribosyltransferase activity (Pirker, et al., 1985). There are three lysine residues in domain III, none in domain II, and 12 in domain I (Gray, et al., 1984). Since iminothiolane treatment of exotoxin A did not
reduce the enzymatic activity, the affected lysine, or lysines, were most likely located in
domain I.

In an effort to determine which domain I residues were important, Jinno et al.
(1988) separately converted each of the 12 lysines in domain I to glutamic acid. The
only single residue substitution which had an effect on cytotoxicity was a substitution at
Lys 57. Further evidence supporting the importance of Lys 57 comes from studies in
which investigators found that a dipeptide insertion between residues 60 and 61
resulted in a mutant 500 times less toxic than exotoxin A, by reduction of its binding
ability to 1% that of native exotoxin A (Chaudry, et al., 1989). These residues have
been located on the three dimensional structure and are on one of three anti-parallel β-
sheets which line the surface of a deep concavity in domain I. Interestingly, Lys 57 is
located in the same β-sheet suggesting that the concavity may be an important structural
determinant for exotoxin A receptor binding.

The smaller portion of domain I, Ib, contains residues 365-404 that lies
between domain II and III sequences. However, the tertiary folding of the toxin
molecule brings it adjacent to domain Ia (Allured, et al., 1986). The exact role that Ib
plays in the activity of exotoxin A is not clear. It has been suggested that while the
carboxy-terminal residues of the domain are involved in the enzymatic activity, the
amino-terminal residues are responsible for the secretion of toxin from Pseudomonas
aeruginosa. Studies involving the deletion of various portions of exotoxin A revealed
that when domain III is expressed alone, no ADP-ribosyltransferase activity was
found. However, when the 20 amino acids from the carboxy-terminus of Ib and the
entire domain III were included, full enzymatic activity was retained (Hwang, et al.,
1987). This was confirmed in studies involving a chimeric protein consisting of the
exotoxin A fragment PE-40 and transforming growth factor alpha's (TGF alpha) cell
binding domain, resulting in a TGF-PE-40 fusion product (Siegal, et al., 1989). Cells
bearing epidermal growth factor receptors are sensitive to the fusion product, therefore they could be used to determine the effects of deletions made in the PE-40 fragment encoding sequence. It was found that those toxin fragments which had residues 253-399 deleted retained full enzymatic activity relative to TGF-PE40, while those missing 253-404, and 253-407 had only 38% and 2% activity, respectively. These results show the need for domain III and the carboxy-terminal residues of domain Ib for full ADP-ribosyltransferase activity in exotoxin A.

In the same study it was found that a mutant lacking residues 365-380 retained normal ADP-ribosyltransferase activity yet had increased cytotoxicity relative to the TGF alpha-PE40 protein. These results demonstrate that the amino-terminus of Ib may not be necessary for cytotoxicity of the fusion protein. Taken together, these studies indicate that domain Ib may play a role in maintaining the proper conformation of whole exotoxin A while at the same time being required for ADP-ribosyltransferase activity.

Domain II: The translocation domain

Domain II is believed to be the translocation domain which transports exotoxin A from the endocytic vesicle to the cytoplasm. This domain consists of residues 253-364, and mutants lacking the first half of this domain have full enzymatic and cell binding abilities, but are not cytotoxic (Hwang, et al., 1987; McGowan, et al., 1991). The activity of exotoxin A as a potent inhibitor of protein synthesis has been the subject of numerous investigations; however, the mechanism by which the catalytic domain crosses the membrane of the target cell to contact its cytoplasmic substrate, elongation factor 2 is still unclear. Exposure to a low pH is necessary for exotoxin A's binding, insertion and translocation, and acidification of endosomes is required for its activity (Farahbakhsh, et al., 1986; Moehring and Moehring, 1983). It has been hypothesized that when exotoxin A encounters the acidic environment in the endocytic vesicle it
undergoes a conformational change which exposes hydrophobic residues that insert into the membrane and transfer exotoxin A into the cytoplasm (Farahbakhsh, et al., 1987; Idziorek, et al., 1990). Analysis of domain II reveals that it contains six alpha helices, two of which are long enough to span a membrane (30 Å). One of these helices is connected to a region of hydrophobic residues by a short sequence of surface residues containing three arginines (274, 276, and 279) (Gray, et al., 1984). These residues have been studied because positive residues are an essential part of signal sequences, and they often flank transmembrane domains (Jinno, et al., 1989). In order to determine more precisely how domain II is involved in exotoxin A's activity, Jinno et al. (1989) substituted each arginine residue in domain II with glycine and found that Arg 276, and possibly arginines 279, 330, and 337 play a role in the translocation process. These mutants were able to ADP-ribosylate elongation factor 2 and compete with exotoxin A for binding, but were noncytotoxic, suggesting that they were unable to translocate across the membrane. In order to determine if there was a saturable step other than receptor binding, a TGF alpha-exotoxin A construct was produced for use in competitive studies. The construct had an aspartic acid substituted for glutamic acid 553 (E553D) which rendered it enzymatically inactive (Douglas and Collier, 1987). The E553D construct also did not compete with exotoxin A for cell binding due to the different receptor specificity resulting from the deletion of domain I and fusion to the transforming growth factor receptor domain (Douglas and Collier, 1987; Siegall, et al., 1989). Site directed mutagenesis was used to substitute the domain II arginines. Only exotoxin A mutants that had substitutions at Arg 276 and at Arg 279 were inhibited by the E553D construct which indicates that these residues may be involved in a specific interaction with the cell. This suggests that a second saturable step exists, and these residues play an important role in this step. It has been hypothesized that once inside the vesicle, exotoxin A binds to a intracellular site via Arg 276 and/or 279 and
undergoes a conformational change triggered by the acidic conditions. The proximity of exotoxin A and the newly exposed hydrophobic residues allows for insertion into the lipid membrane followed by translocation into the cytoplasm (Jinno, et al., 1989).

**Domain III: The catalytic domain**

The final domain, domain III, consists of residues 405-613 and has been the most intensely studied of all the domains. Unlike diphtheria toxin in which the catalytic domain is located at the amino terminus, exotoxin A's catalytic center is located in domain III at the carboxy-terminus (Gray, et al., 1984). Unlike NAD$^+$ dehydrogenase, and most NAD$^+$/NADH binding proteins, domain III of exotoxin A does not have a regular repeating alternating alpha helix, B-pleated sheet structure. This different structure may reflect the fact that NAD$^+$ dehydrogenases use NAD$^+$ as a cofactor to transfer a hydride ion, while exotoxin A binds NAD$^+$ as a substrate, cleaving it and transferring a portion of the molecule to elongation factor 2. Analysis of the crystal structure and amino acid sequence of exotoxin A reveals a deep cleft in which NAD$^+$ binding takes place. Several residues within and around this cleft have been implicated in exotoxin A's enzymatic activity (Douglas and Collier, 1987; Lukac and Collier, 1988; Wozniak, et al., 1988).

Due to the similarity between diphtheria toxin and *Pseudomonas aeruginosa* toxin activity, many attempts have been made to correlate residues known to be important in diphtheria toxin activity with residues of exotoxin A. Carroll et al. (1984) showed that when diphtheria toxin's fragment A (the active portion of diphtheria toxin consisting of residues 1-193) was exposed to ultraviolet light in the presence of $^{14}$C NAD, the radioactive labeled nicotinamide ring was specifically transferred to glutamic acid 148. This indicated that Glu 148 was either directly involved in the binding of NAD$^+$ or near the binding site. When this approach was used with exotoxin A, the
nicotinamide ring was transferred to Glu 553, which lies in the proposed NAD+ binding cleft of domain III (Allured, et al., 1986; Carroll and Collier, 1987). Site directed mutagenesis was used to replace Glu 148 in diphtheria toxin and Glu 553 in exotoxin A with an aspartic acid residue. The subsequent loss of enzymatic activity in both substituted toxins suggest a homologous role for these glutamic acid residues (Douglas and Collier, 1987; Tweten, et al., 1985).

Early attempts at aligning the amino acid sequences of diphtheria toxin and exotoxin A were unsuccessful in noting any sequence homology (Gray, et al., 1984). However, using the functionally homologous glutamic acid residues as a point of reference, Carroll et al. (1988) found a 161-amino acid segment containing 37 identical residues (23%) and 57 conservative substitutions (35%) (Figure 2). Inspection of the three dimensional structure of exotoxin A's active site cleft and amino acid sequences of the two toxins reveal that many residues near Glu 553 correspond to homologous residues in fragment A of diphtheria toxin, including His 440 (His 21); Trp 466 (Trp 50); Phe-469 (Phe 53); Tyr-470 (Tyr 54); and Tyr-481 (Tyr 65) (diphtheria toxin's corresponding residue is shown in parenthesis). The latter two residues, Tyr 470 and Tyr 481, are aromatic and project into the active site cleft.

The evolutionary relationship between exotoxin A and diphtheria toxin is unclear; however, the existence of a common precursor has been suggested based on recent reports of endogenous ADPR-transferase activities in some eukaryotic cells (Lee and Iglewski, 1984). They suggest that some cells may ADP-ribosylate elongation factor 2 as a means of regulating protein synthesis. The "capture" of the gene responsible for this activity by prokaryotes may have led to its evolution into toxins capable of ADP-ribosylating elongation factor 2. Amino acid sequence comparisons of exotoxin A and diphtheria toxin with other members of the large ADP-ribosyltransferase family have not resulted in the elucidation of significant homology.
However, differences in structure and substrate specificities of these toxins, may make it difficult to detect similarities.

Figure 2. Alignment of the catalytic domains of exotoxin A and diphtheria toxin. * indicates identical residues; † indicates conservative substitutions (Carroll and Collier 1988).

Two exotoxin A residues involved in the catalytic domain homology, Tyr 470 and Tyr 481, are aromatic and project into the active site cleft. Due to their prominence within the cleft, Tyr 470 and Tyr 481 were studied to see if they play a role in the enzymatic activity of exotoxin A. Oligonucleotide directed mutagenesis was used to change the individual tyrosines to phenylalanine. The substituted toxins then were
tested for enzymatic and binding activity (Lukac and Collier, 1988). Substitution of Tyr 470 with phenylalanine [ETA(Y470F)] did not affect exotoxin A's activity, but there was a ten-fold decrease in ADPR activity when Tyr 481 was changed. In addition, iodination of Tyr-481 completely abolished ADPR activity (Brandhuber, et al., 1988). ETA (Y481F) was able to bind NAD⁺ as well as native exotoxin A, so it was suggested that Tyr 481 may be involved in the proper orientation of elongation factor 2 for exotoxin A binding.

Additional information concerning exotoxin A's enzymatic activity comes from a nitrosoguanidine-induced mutant, *P. aeruginosa* PAO1-PR1, isolated by Cryz et al. (1980). The PR1 strain produces a mutant form of exotoxin A (CRM 66) which is immunologically indistinguishable from the native toxin, however it lacks enzymatic activity. Analysis of the CRM-66 amino acid sequence shows that there has been a substitution of a tyrosine for a histidine at position 426 (Wick and Iglewski, 1988; Wozniak, et al., 1988). A complementation experiment which replaced Tyr 426 in CRM-66 with His restored full ADP-ribosyltransferase activity (Wozniak, et al., 1988). Furthermore, an exotoxin A-specific monoclonal antibody whose epitope contains His 426 does not recognize CRM-66 (Galloway, et al., 1989). Although CRM-66 lacks ADP-ribosyltransferase activity, it is able to bind NAD⁺ as well as the native toxin, and has full NAD⁺ glycohydrolase activity (Galloway, et al., 1989; McGowan, et al., 1991). These results indicate that the His>Tyr substitution at position 426 in domain III is responsible for the loss of activity in CRM-66.

Analysis of the three-dimensional structure of exotoxin A shows that His 426 projects into a large cleft in domain III but is distal from the proposed NAD⁺ binding site. Because exotoxin A requires "activation" prior to the expression of any ADP-ribosyltransferase activity (Lory and Collier, 1980), it has been hypothesized that the activation step results in the opening of this cleft in order for elongation factor 2 to bind
to toxin (McGowan, et al., 1991). Recent studies which proposed that elongation factor 2 binding occurs between domain Ib and domain III support this model (Hwang and Chen, 1989).

**NAD-dependent reaction of exotoxin A**

Exotoxin A must bind its substrates nicotinamide adenine dinucleotide ($\text{NAD}^+$) and elongation factor 2 prior to carrying out its ADP-ribosyltransferase activity. The binding of $\text{NAD}^+$ involves recognition of the whole molecule, with the nicotinamide ring being especially important. The pyridine nucleotide exists in both the reduced and oxidized states, NADH and $\text{NAD}^+$, respectively. As mentioned previously, $\text{NAD}^+$ is the substrate for exotoxin A and diphtheria toxin. Iglewski et al. showed that exotoxin A's inhibition of protein synthesis was dependent upon the presence of $\text{NAD}^+$, and that it is the ADP-ribose moiety of $\text{NAD}^+$ that is transferred to elongation factor 2 (Iglewski, et al., 1977). In these studies, they used the ADP-ribosyltransferase assay, which measures the amount of radioactivity transferred from $^{14}\text{C-NAD}^+$ to elongation factor 2, performed in the presence of $\text{NAD}^+$ radioactively labeled at various sites on the molecule, including the nicotinamide ring, both phosphates, the ribose ring, or the adenine ring. Analysis of the results showed that all parts of $\text{NAD}^+$, except the nicotinamide ring, were incorporated in the trichloroacetic acid-insoluble material. In addition to its major enzymatic activity, exotoxin A catalyzes the slow hydrolysis of $\text{NAD}^+$ releasing nicotinamide into solution (Leppla, et al., 1978). The glycohydrolase activity is much lower than the ADP-ribosyltransferase activity and probably does not contribute to the toxicity of exotoxin A. However, it is useful as a measure of toxin activity and implies a direct binding of $\text{NAD}^+$ by toxin. In order to measure this activity, the $\text{NAD}^+$ nicotinamide ring is radioactively labeled and incubated with exotoxin A. The amount of radioactivity detected in an ethanol extract of the mixture is a measure of glycohydrolase activity.
Other ADP-ribosyltransferase toxins

_Pseudomonas aeruginosa_ is only one member of a class of bacterial ADP-ribosyltransferases. Some of the other ADP-ribosyltransferase toxins which are similar to exotoxin A include diphtheria toxin, pertussis toxin, and cholera toxin. As in the case with exotoxin A, in the absence of specific substrates, these enzymes hydrolyze NAD$^+$ resulting in the slow release of free nicotinamide. Exotoxin A and diphtheria toxin are the most thoroughly studied toxins.

Diphtheria toxin is synthesized by _Corynebacteria diphtheriae_ strains that are lysogenic for tox$^+$ β-phage (Freeman, 1951). Thus, diphtheria toxin is produced by a phage rather than by the bacterium. This ADP-ribosyltransferase toxin is similar to exotoxin A in that it modifies elongation factor 2 resulting in the inhibition of protein synthesis (VanNess, et al., 1980). Furthermore, the stereochemistry involving the transfer of the ADP-ribose moiety to elongation factor 2 appears to be identical. Also, diphtheria toxin modifies elongation factor 2 at the same diphthamide residue as exotoxin A.

Although both exotoxin A and diphtheria toxin catalyze similar reactions, their structures are very different. Diphtheria toxin is produced as a proenzyme which after posttranslational modification has a molecular weight of 58,342 daltons. Enzymatic activity is dependent upon the reduction of its two disulfide bonds and proteolytic cleavage of the proenzyme into two fragments, A and B. The active site of the mature toxin is at the amino-terminus of fragment A, while exotoxin A's catalytic domain is at the carboxyl-terminus of the protein. Hybridization analysis of DNA from β-corynebacteriophage with _P. aeruginosa_ strain PA103 DNA showed no sequence homology (Gray, et al., 1984; Sadoff, et al., 1982). As previously described, however, Carroll et al. aligned the catalytic domains of both toxins revealing a strong
region of homology. Furthermore, cross-reactivity has been detected but only by the most sensitive methods (Sadoff, et al., 1982).

Diphtheria toxin's ability to inhibit protein synthesis was first determined in 1959, however, not until much later was the mode of action explained. It had been established that diphtheria toxin's activity was dependent upon NAD\(^+\) and resulted in the inactivation of elongation factor 2 (Goor and Pappenheimer, 1967a; Goor and Pappenheimer, 1967b). The role of NAD\(^+\) was explained when diphtheria toxin, elongation factor 2, and NAD\(^+\) radioactively labeled at various sites of the molecule were incubated together and assayed for radioactivity in the fractions of the reaction. When NAD\(^+\) was labeled on the nicotinamide ring, radioactivity was found free in solution. However, when NAD\(^+\) was labeled on the ADP-ribose moiety, elongation factor 2 became radioactively labeled indicating that inactivation of elongation factor 2 resulted from the covalent attachment of ADP-ribose (Honjo, et al., 1971).

*Vibrio cholera* produces the multi-subunit cholera toxin, which ADP-ribosylates a GTP-binding protein (G protein) inhibiting its ability to hydrolyze GTP (Moss and Vaughan, 1978). This results in the continuous stimulation of adenylate cyclase and an increase in the cytoplasmic concentration of cAMP. Elevated levels of cAMP in intestinal epithelial cells cause the fluid loss and electrolyte imbalance associated with cholera (Alberts, et al., 1983). Intact cholera toxin has a molecular weight of 84,500 daltons, and is composed of an A subunit (\(M_r = 27,000\)) and five B subunits (\(M_r = 57,500\)), which are noncovalently associated (Duffy, et al., 1981; LoSpalluto and Finkelstein, 1972). The B subunit is responsible for binding to cell receptors and is made up of five identical subunits held together by noncovalent interactions (Gill, 1976). The A subunit is composed of two chains connected by a single disulfide bond. The ADP-ribosyltransferase activity and NAD\(^+\) glycohydrolase activity reside in the A\(_1\) chain (\(M_r = 22,000\)) (Lai 1977; Moss, et al., 1976), while the function of the A\(_2\) (\(M_r =\)
5,000) chain is still unknown. Studies concerning structure/function correlation in cholera toxin have not progressed as far as the work with *Pseudomonas aeruginosa* and diphtheria toxins, however, it is known that the NAD$^+$ binding site and active site are located in the carboxy-terminal half of the A1 chain (Lai, et al., 1983; Mekalanos, et al., 1983).

*Bordetella pertussis* is the causative agent of whooping cough, but use of an inactivated whole-cell vaccine has significantly reduced the incidence of this infection. Pertussis toxin is one of the major virulence factors produced by *B. pertussis*. Its activities include the ADP-ribosylation of a regulatory component of the adenylate cyclase complex, as well as other GTP-binding proteins involved in transmembrane signaling (Collier and Mekalanos, 1980; Katada and Ui, 1982). Pertussis toxin's actions result in a decreased response to inhibitory agents of adenylate cyclase and increases the response to stimulatory agents inducing symptoms such as hypoglycemia, lymphocytosis, neurotoxicity, and increased capillary permeability (Tamura, et al., 1982). Pertussis toxin is also associated with adherence of *B. pertussis* to ciliated epithelial cells.

Typical of all A:B model enzymes, the 105,890 dalton pertussis toxin protein is composed of an A subunit, S1, where the catalytic site is located, and a B component composed of four proteins which is responsible for binding to specific cell receptors and translocating the S1 unit across the membrane (Collier and Mekalanos, 1980). The binding domain of pertussis toxin is very complex consisting of four subunits, S2, S3, S4, and S5 in a 1:1:2:1 ratio.

These ADP-ribosyltransferase toxins work by a similar mechanism which transfers the ADP-ribose moiety of NAD$^+$ to a specific target protein (X) resulting in its covalent modification (equation 1).
Eq. 1. ADP-ribosyltransferase reaction

\[
\text{NAD}^+ + X \rightarrow \text{ADPR-X} + \text{nicotinamide} + H^+ 
\]

In the absence of a target protein, the ADP-ribosyltransferase toxins catalyze the slow hydrolysis of the nicotinamide-ribose linkage of NAD\(^+\) (equation 2).

Eq. 2. NAD\(^+\) glycohydrolase reaction

\[
\text{NAD}^+ + H_2O \rightarrow \text{ADPR} + \text{nicotinamide} + H^+ 
\]

Many investigators have studied the toxins' catalytic domains in order to determine which residues are important for activity. Studies involving mutagenesis, proteolysis, and photoaffinity labeling have provided evidence which indicates that there may be a common structural relationship among the various mono-ADP-ribosyltransferases.

Recently, residues have been identified within the presumed catalytic domains of exotoxin A, diphtheria toxin, and pertussis toxin that are required for NAD\(^+\) binding activity (Carroll and Collier, 1984; Carroll and Collier, 1987; Cockle, 1989). When these toxins were mixed with NAD\(^+\) and irradiated with ultraviolet light, the nicotinamide moiety was transferred to exotoxin A's glutamic acid 553, diphtheria toxin's glutamic acid 148, and pertussis toxin's glutamic acid 129. The involvement of these residues in the toxins' enzymatic activities was confirmed using site-directed mutagenesis. The glutamic acids were replaced with aspartic acids; in each case, the substitution resulted in the loss of ADP-ribosyltransferase activity indicating
involvement of the residue in the NAD$^+$ binding site (Cockle, 1989; Douglas and Collier, 1987; Tweten, et al., 1985). No such residue has yet been identified in cholera toxin.

As mentioned previously, many NAD$^+$ binding proteins have a distinct secondary structure consisting of alternating alpha helices and $\beta$-sheets. This structural feature is also found in diphtheria toxin; however, it is not evident from the crystallographic structure of exotoxin A. The method of Chou and Fasman (1974) was used to predict the helical and $\beta$-sheet characteristics of individual cholera toxin residues to see if such a structure was apparent. The results showed residues 95-144 of cholera toxin in a region containing alternating alpha helices and $\beta$-pleated sheets, similar to that found in lactate dehydrogenase (Duffy, et al., 1985). Although there is no amino acid homology between the two proteins, they suggest that these regions may represent a convergent evolutionary structural feature important for NAD$^+$ binding.

Attempts at identifying residues involved in NAD$^+$ binding have been more successful than those studying protein substrate binding. Analysis of *Pseudomonas aeruginosa* toxin has revealed that His 426 is necessary for elongation factor 2 binding (Galloway, et al., 1989; McGowan, et al., 1991); however nothing is known about the other ADP-ribosyltransferase toxins' protein substrate binding sites. The substitution or deletion of diphtheria toxin's lysine 39, glycine 52, or glycine 128 eliminates it's ability to ribosylate elongation factor 2 (Giannini, et al., 1984; Maxwell, et al., 1987; Zhao and London, 1988). However, their role in ADP-ribosyltransferase activity is still unknown.

The ADP-ribosyltransferase toxins of *B. pertussis*, *V. cholera* and *Escherichia coli* ADP-ribosylate the G-protein alpha-subunit. Interestingly, residues 8-15 of pertussis toxin's S1 subunit contains seven residues homologous with sequences found in both cholera toxin and *E. coli*'s ADP-ribosyltransferase heat-labile toxin (Locht and
Keith, 1986). Furthermore, monoclonal antibodies produced against a pertussis peptide containing residues 6-17 of the $S_1$ subunit were able to cross-react with cholera toxin (Burns, et al., 1987). Deletion mutations produced within residues 1-22 decreased the ADP-ribosyltransferase activity of pertussis toxin, without affecting its NAD$^+$ glycohydrolase activity. Several individual residues in this region were identified as being necessary for ADP-ribosyltransferase activity, but unnecessary for NAD$^+$ binding such as Arg 9, Asp 11, and Arg 13 (Barbieri and Cortina, 1988; Barbieri, et al., 1990). These data, and the fact that pertussis toxin, cholera toxin, and $E. coli$'s heat-labile toxin have the alpha-subunit of a GTP-binding protein as a common substrate, suggest that this homologous region is involved in the transfer of the ADP-ribose moiety to the G-protein.

Obviously, there is much work to be done on the identification of enzymatically important residues in the ADP-ribosyltransferase toxins. Recent work indicates that functionally important structures may be conserved among ADP-ribosyltransferase proteins. Information regarding NAD$^+$ binding by exotoxin A, cholera toxin, diphtheria toxin, and pertussis toxin is growing rapidly; however, little is known regarding elongation factor 2 and G-protein binding by these ADP-ribosyltransferase toxins.

The determination of exotoxin A's crystallographic structure along with site-directed mutagenesis methodology has greatly enhanced our understanding of exotoxin A. These studies have allowed investigators to correlate exotoxin A's structural domains to functions such as cell binding, membrane translocation, and enzymatic activity. However, there are limitations to studies involving methods such as site-directed mutagenesis; changes or deletions of residues often result in loss of activity. Furthermore, the x-ray crystallographic structures of the other ADP-ribosyltransferase
toxins has not been completed so accurate comparisons of toxin three-dimensional structures cannot be carried out at the present time.

The use of synthetic peptides in epitope mapping

*Pseudomonas aeruginosa* exotoxin A and diphtheria toxin are similar in several respects. Not only do they catalyze identical reactions, but they modify the same target protein elongation factor 2. Due to their enzymatic similarities, researchers have sought to find homology between the two toxins using immunological approaches. However, cross-reactivity has only been detected by the most sensitive methods (Sadoff, et al., 1982). Although there is a great deal of data regarding the enzymatic activities of exotoxin A and the other ADP-ribosyltransferase toxins, little is known about the antigenic nature of the proteins.

Scientists have been trying to define protein antigenic structure for many decades. The history of such work dates back to studies on silk fibroin (1942), as well as work on serum albumin (1957). Technical advances have led to the rapid growth of this field, including the ability to define protein sites which are antigenic as well as immunogenic, often referred to as "epitope mapping."

Previous studies reveal that the antigenic structure of a protein is very complex and cannot be studied relying solely on one approach. Atassi, in 1972, attempted to define the antigenic structure of myoglobin and in so doing designed a strategy involving several different approaches, none of which by itself was considered capable of defining the full antigenic structure of a protein (Atassi, 1972). These approaches included (1) determining the role of conformation of the protein on immune response; (2) analysis of the effect of site-specific derivatization on conformation and immunochemistry; (3) analysis of proteolytic fragments and the effect of chemical derivatization; and (4) definition of identified sites using overlapping synthetic peptides.
Using this complex strategy, the full antigenic structures of myoglobin and lysozyme were defined and confirmed with synthetic peptides (Atassi, 1975; Attasi, 1978). The antigenic profiles of both of these proteins reveal that antigenic sites may consist of residues directly linked by peptide bonds (sequential epitopes) or those which are not adjacent in sequence but are brought together by virtue of protein folding (discontinuous epitopes).

The complexity of this scheme led to the design of a completely synthetic approach for the identification of the continuous antigenic sites on a protein (Kazim and Atassi, 1980). This approach includes the synthesis and examination of peptides that represent the entire protein chain. Overlapping peptides are synthesized in uniform sizes. This approach has been proven to be effective, nevertheless, it is costly and labor-intensive, especially when applied to large molecules.

An alternative approach using synthetic peptides was performed to study the binding specificities of monoclonal antibodies to the 65 Kd protein of *Mycobacterium leprae* (Anderson, et al., 1988). This approach involved analyzing the binding patterns of monoclonal antibodies to overlapping peptides by direct and indirect enzyme-linked immunosorbent assays (ELISA). The Hopp and Woods hydrophilicity plot (1981) of the protein was used to predict areas of antigenicity to aid in the selection of the peptides which were initially tested. Hydrophilic regions of a protein are usually external and are more likely to be accessible for antibody interactions. Monoclonal antibodies to a 65 Kd surface protein of *M. leprae* reacted with six of the 11 largest hydrophilic peaks, while peptides from a smaller lysine-rich peak were recognized by two monoclonal antibodies. Peptides from a non-hydrophilic region were recognized by two other monoclonal antibodies, however, this region contained several amino acids with charged side chains, believed to be important in antigen:antibody
interactions. By using this approach, epitopes were assigned to ten of the 14 different monoclonal antibodies known to recognize the 65-Kd protein.

The monoclonal antibodies analyzed in the *M. leprae* study, appear to be directed to continuous epitopes on the 65-Kd protein, however, previous reports suggest that most antibodies recognize discontinuous epitopes (Barlow, et al., 1986; Berzofsky, 1985). There are several conditions that may account for which type of epitope a monoclonal antibody will recognize. Differences in antigen preparation used for immunizations and immunization protocols play a role in determining the B and T-cell response and the resulting specificity of B-cells elicited (Anderson, et al., 1988; Meeker, et al., 1989). The preparation of the antigen can change the conformational state of the protein, and the protein may be denatured. Denaturation of a protein can lead to an increase in the number of monoclonal antibodies produced that recognize linear epitopes. Processing by macrophages will also affect which parts of an antigen are presented to B-cells and T-cells, and immune suppression may play a role in the immune response of the immunized animal. Furthermore, the method employed in the initial screening of hybridomas for antibody production can determine what type of epitope the selected monoclonal antibodies bind. For instance, proteins used in ELISA and Western blot analyses are often partially or wholly denatured, which exposes a disproportionate number of continuous epitopes while disrupting discontinuous epitopes. This results in a select group of monoclonal antibodies being chosen, as was the case with anti-65 Kd monoclonal antibodies. Thus, when analyzing the binding of monoclonal antibodies to a given antigen, it is important to recognize that all these factors may affect the success of a particular procedure. In order to detect monoclonal antibodies that have specificities for sequential as well as discontinuous epitopes, it may be useful to screen the antibodies using capture assays in which unfolding of the antigen is less likely to occur. Additionally, the production of monoclonal antibodies
to both unfolded and native antigen will increase the likelihood of obtaining antibodies directed to a variety of epitopes.

Synthetic peptides may help determine which residues play a role in antibody binding. Removal of an amino acid which results in a decrease in binding affinity indicates that the missing residue provided a significant amount of binding energy to the antibody:antigen interaction. Alternatively, the residue may stabilize a conformational epitope which disappears when the residue is deleted or replaced. Although a monoclonal antibody may be specific for a certain segment of amino acids, individual residues appear to contribute differently to each antibody's binding. Site-directed mutagenesis and deletion mutations also have been used to measure the involvement of specific amino acids in a protein's function (Cryz, et al., 1980; Douglas and Collier, 1987; Hwang, et al., 1987; Jinno, et al., 1989; Madshus and Collier, 1989); however, the results are not always easy to interpret. Changes in one region of the protein may in some circumstances alter the conformation of quite distant regions resulting in the loss of a conformationally-dependent epitope. Using peptide-specific antisera is an alternative approach to the problem of understanding the general nature and structure of antigenic determinants.

The ability of synthetic peptides to induce antibody which reacts with the native protein, has led to increased interest in their potential use in vaccines. Audibert et al. (1982) has been successful in producing a totally synthetic diphtheria vaccine, based upon a synthetic peptide containing residues 186-201 of diphtheria toxin linked to a synthetic carrier, and a synthetic adjuvant, N-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP). This synthetic vaccine elicited protective immune responses in immunized animals. The tetradecapeptide peptide (STDP) contains the first disulfide loop which is located at the juncture of the two functional domains in diphtheria toxin (Collier, 1975; Pappenheimer, 1977). Due to the functional importance of this region, STDP has been
the focus of several studies (Audibert, et al., 1981; Audibert, et al., 1982; Boquet, et al., 1982). Guinea-pig sera raised against the peptide (186-201) were cross-reactive with whole diphtheria toxin, and partially neutralized toxin's ADP-ribosyltransferase activity and protein synthesis inhibition in Vero cells. Although the antisera elicited by STDP wasn't completely successful at neutralizing diphtheria toxin's enzymatic activity, the results demonstrate the potential use of peptides in vaccine development.

Synthetic peptides can be very useful in the study of protein structure and activity. However, short amino acid sequences can occur in several non-related proteins and can lead to non-specific reactivity. Synthetic peptides were used to study the intracellular distribution of the transforming protein kinase of Rous sarcoma virus (RSV), p60Src (Nigg, et al., 1982). Immunofluorescent labeling studies showed that antisera produced against the hexapeptide sequence, designated src-c, specifically identified p60Src in RSV transformed cells. As the concentration of antibody was increased, however, labeling of normal cell constituents (tubulin, myosin, and vimentin) was observed. The cross-reactivity seen with anti-src-c antibodies was determined to be insignificant as far as structural or functional similarities were concerned and resulted from coincidental similarities in amino acid sequences. For example, one of the cross-reactive proteins, β-tubulin, had been sequenced and comparison of the primary sequences revealed that residues 116-120 are very similar to residues 2-5 of the src-c peptide. Non-related cross-reactivity has been reported in other studies using antibodies produced to peptides (Walter, et al., 1981). Thus, it is important to design peptides so they will likely have unique sequences. It should be noted that cross-reactivities are sometimes seen with monoclonal antibodies, but the site and nature of the site is usually unknown. In the case of anti-peptide cross-reactivity, the site is identified and the nature of the cross-reactivity may be interpreted.
The use of synthetic peptides in mapping antibody epitopes has been criticized by some who believe that the only method to determine the exact definition of an antibody's epitope involves x-ray diffraction analysis of the antigen-antibody complex (Laver, et al., 1990). In this method, a complex between the antigen and antibody Fab fragment is formed, crystallized, and its structure determined using x-ray diffraction methods. This allows for the analysis of the interaction of the molecules in their native states, while techniques relying upon synthetic peptides only study the binding of antibodies to portions of the antigen. Only five complete epitope structures have been determined using this method, including three sites on lysozyme and two on neuraminidase (Amit, et al., 1986; Padlan, et al., 1989; Sheriff, et al., 1989; Tulip, et al., 1990). Analysis of these structures reveal some common characteristics among the epitopes. In general, they are located on the surface of the molecule and are totally dependent upon the protein's conformation. Each of the epitopes is 15-22 amino acids in length, with many of these being involved in complementarity between the antibody and antigen. However, scattered throughout the epitope are five or six residues which contribute most of the binding energy. Furthermore, their findings suggest that epitopes are discontinuous, therefore linear peptides would not contain an entire epitope and would not be useful in defining epitopes. Although they agree that anti-peptide antibodies may be useful for detecting unfolded proteins, such as in Western blot analysis, they suggest that the identified epitopes lack any biological significance.

Therefore, the crystallization of the antibody:antigen complex is the most accurate method for determining the complete epitope of an antibody; however, the expense and time involved render this method impractical to many researchers. Although the use of synthetic peptides has its limitations, when used appropriately with the awareness of the possible limitations, they are useful tools for locating proteins within cells, determining binding sites of monoclonal antibodies (Anderson, et al.,
1988; McGowan, et al., 1991), and as potential immunogens in vaccines (Audibert, et al., 1982).
Projected Research Goals

A. Generate monoclonal antibodies to exotoxin A of *Pseudomonas aeruginosa*.
   1. Extend the findings of Galloway et al., (1984)
      a. Define the epitope of TC-1 antibody
      b. Define the function inhibited by the TC-1 Mab
   2. Use monoclonal antibodies to map additional B-cell epitopes and functional domains of exotoxin A.

B. Characterize the cross-reactive epitope among the ADP-ribosyltransferase toxins.
   1. Use synthetic peptides and monoclonal antibodies to identify the location of the site on exotoxin A.
   2. Generate antipeptide antiserum
   3. Evaluate possible role the cross-reactive site may have in the function of exotoxin A and other ADP-ribosyltransferase toxins.
      a. Determine ability of antiserum to cross-react with ADP-ribosyltransferase toxins.
      b. Determine ability of antiserum to inhibit the enzymatic activity of the ADP-ribosyltransferase toxins.
CHAPTER II

MATERIALS AND METHODS

Unless otherwise specified, all chemicals and biochemicals used in this study were from Sigma Chemical Co., St. Louis, Missouri. Throughout this dissertation "toxin" will is used to refer to exotoxin A unless otherwise specified.

Antibody production and utilization

Immunization of mice: Three to five week old BALB/c mice were immunized by intraperitoneal (i.p.) and subcutaneous injection (s.c.) of 250 µl of a 1:1 mixture of Freund's complete adjuvant and 20 ng of exotoxin A suspended in phosphate-buffered saline (PBS; pH 7.2). Twenty ng exotoxin A in the same solution except in Freund's incomplete adjuvant was administered intraperitoneally 30 days after the first injection, and repeated at days 48 and 72. The final injection consisted of 20 ng toxin given i.p. 4 days prior to sacrificing the mice.

Cell lines and culture conditions: X63A1 (A1) mouse myeloma cell lines were grown in RPMI 1640 (Whittaker M.A. Bioproducts, Walkersville, Maryland) supplemented to contain 100 units/ml penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate with a final concentration of 10% (v/v) heat-inactivated fetal calf serum. All cells were grown in a humidified CO₂ incubator at 37° C and 6.8% CO₂ in air.
Monoclonal antibody production: Monoclonal antibodies were generated by the method of Fazekas de St. Groth and Scheidegger (1980). Spleens from immunized mice were aseptically removed and dissociated into a single cell suspension. Spleen cell suspensions were diluted $10^8$ cells/ml in GKN saline ($8 \text{ g NaCl}, 1.42 \text{ g Na}_2\text{HPO}_4, 2 \text{ g dextrose}, 0.4 \text{ g KCl}, 0.69 \text{ g NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}, 0.01 \text{ g phenol red to one liter with pyrogen-free H}_2\text{O}, \text{ filter sterilized}$) and held at 37°C. Cells were fused with X63AI cells in prewarmed 50% polyethelene glycol (PEG 4000, Fisher Scientific Co., Fair Lawn, New Jersey) in GKN saline at cell ratios of 1:1 to 1:2 myeloma cells to spleen cells. The fused cells were resuspended in hybridoma selective media (HAT medium). Selection was carried out in complete media further supplemented to contain 100 $\mu$M hypoxanthine, 4 $\mu$M aminopterin and 2 $\mu$M thymidine (HAT). The fusions were seeded at a density of $10^5$ cells per well in 96-well microtiter plates (Corning Laboratory Sciences Co., Corning, New York) containing a macrophage feeder-layer of $1 \times 10^4$ cells/well. Every fourth day the media was aseptically removed by vacuum suction and replaced with 100 µl of fresh HAT medium until day 14 when aminopterin was removed from the media (HT). Culture supernatants were tested for anti-toxin activity by enzyme-linked immunoabsorbent assay (ELISA; described below) at 2-3 weeks. Antibody producing hybridomas were transferred to 24-well plates (Corning Laboratory) and cloned by limiting dilution. Clones were grown in culture and as ascitic tumors in BALB/c mice primed with 1 ml pristane (2,6,10,14-tetramethylpentadecane) two weeks prior to being injected i.p. with $10^6$ hybrid cells. Isotypes were determined by ELISA using the BioRad isotyping kit (Richmond, CA) as described below.

Ascites fluid was pooled and immunoglobulin was partially purified by saturated ammonium sulfate fractionation (0 to 50%). Precipitated protein was resuspended and dialyzed against 3 liters of column buffer (0.04 M sodium phosphate
buffer, pH 8.0, 0.03 M NaCl). Antibodies belonging to the IgG class were applied to a 1.5 cm x 15 cm DEAE Sephacel column at a rate of 35 ml/hour. Antibody passed through the column while contaminating proteins remained bound to the DEAE Sephacel. Antibodies belonging to the IgM subclass were purified by gel filtration on a 2.5 cm x 53 cm Sephadex G-200 (Whatman International, Ltd., Maidstone, England) column following 50% ammonium sulfate fractionation. The samples were dialyzed against the column buffer described above, and applied to the column at a rate of 65 ml/h. Four ml fractions were collected and the absorbance was monitored at 280 nm using a Beckman DU-50 spectrophotometer (Beckman, Arlington Heights, IL). The purity of each Mab was evaluated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), and anti-exotoxin A activity was measured by ELISA (described below). Pure antibody was concentrated by ultrafiltration (YM-10 filters; Amicon Corporation, Danvers, Massachusetts) and the protein concentration was determined by the Bradford method (1976). Purified monoclonal antibody was stored at -70° C in sodium phosphate buffer.

**Generation of polyclonal antiserum:** New Zealand white rabbits were initially immunized by intradermal injection using 500 µg of peptide conjugate or 50 µg toxin suspended in Freund's complete adjuvant. This was followed by three booster injections (intradermal, 10 day intervals) of 250 µg of peptide in phosphate-buffered saline (pH 7.2). Serum was collected 12 days following the last immunization.

Immunoglobulin was purified over a DEAE Affigel blue (BioRad Laboratories) column following dialysis in 0.02 M Tris-HCl (pH 8) containing 0.028 M NaCl. Immunoglobulin eluted as a single peak while contaminating proteins remained bound to the column. The purity of the IgG fraction was confirmed by SDS-PAGE. Fractions were concentrated by ultrafiltration and stored at -70°.
Enzyme linked immunosorbent assay (ELISA): Polyvinyl flat bottom 96-well microtiter plates (Immunolon-2 plates, Dynatech Laboratories, Alexandria, VA) were coated with 50 ng protein or synthetic peptide per well in 50 mM Tris-HCl (pH 8). Following overnight incubation at 37° C, plates were washed three times in 0.1% Tween 20 in PBS working buffer, and stored at 4° C until used. Culture supernatants were tested by the addition of 100 μl of supernatant to each well, while ascites and general antisera were diluted in working buffer prior to testing. Following a two h incubation at 37° C, plates were washed three times with working buffer, and 100 μl of rabbit anti-mouse immunoglobulin (diluted 1:2000; Zymed Laboratories, Inc., South San Francisco, CA) was added for one h. Subsequent incubation with 100 μl of goat anti-rabbit horseradish conjugate (diluted 1:2000; Zymed) lasted one h followed by washing with working buffer and one wash with 0.1 M citric acid pH 5.2. Citric acid buffer containing 9.2 mM o-phenylenediamine (BioRad Laboratories) and 0.3% H2O2 was added to wells and the reaction was stopped by the addition of 50 μl 12.5% H2SO4. Absorbance was read at 490 nm using a Microplate Reader MR600 (Dynatech Laboratories). Binding of rabbit antisera was detected directly using goat anti-rabbit horseradish conjugate (1:2000).

Monoclonal antibody isotypes were determined using the ELISA procedure described above with the following modifications. Following overnight incubation on toxin-coated plates, plates were washed three times in working buffer and 100 μl of isotype specific rabbit anti-mouse immunoglobulin (diluted 1:2000; BioRad Isotyping kit) was added for one h. Subsequent incubation with 100 ul goat anti-rabbit horseradish conjugate (diluted 1:2000) lasted one hour followed by washing with working buffer one wash with substrate buffer. The reaction was developed as described above. Controls included wells receiving no primary antibody and wells receiving normal mouse serum.
Antibody inhibition assays were carried out as described above with the following modifications. Equal volumes of peptide and antibody were diluted in working buffer and incubated in microtitration plates (Linbro Scientific, Hamden, CN) at room temperature (RT) for 30 minutes. Well contents were transferred to toxin-coated (50 ng/well) ELISA plates for two h at 37° C. The assay then proceeded as described above. Inhibition was determined relative to wells receiving no competing peptide.

Monoclonal antibody TC-1 was conjugated to alkaline phosphatase (described below) and used in competitive assays. Protein (either antibody, peptide, exotoxin A, or CRM-66) were mixed in equal volume with the TC-1 conjugate for 30 min. Half of the mixture was added to toxin coated plates (50 ng/well) for 2 h at 37°. The plate was washed in working buffer three times, and one time in a modified substrate buffer (0.04 M sodium carbonate, 0.1 mM MgCl2-6H2O). One hundred µl of substrate buffer containing p-nitrophenylphosphate (1mg/ml) was added to each well and the plate was incubated at 37° C. Absorbance was read at 410 nm after 30 minutes. Competition was determined relative to wells containing TC-1 conjugate and working buffer.

125I-labeling procedure: Protein was labeled with 125I (carrier-free Na-125I; ICN) by a method described by Johnston and Thorpe (1987). Capped polypropylene tubes (10.5 mm x 39 mm) were coated with 20 µg of Iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycoluril; Pierce Chemical Co., Rockford, Illinois) dissolved in chloroform and evaporated in a 37° C water bath. Ten µg of purified protein diluted in 0.25 M phosphate buffer (pH 7.4) was mixed with 200 µCi of sodium 125Iodine in the coated tube. The reaction was allowed to proceed at RT for 10 min, with mixing every minute. Free sodium 125Iodine was removed by gel filtration on a Sephadex G-25 (Pharmacia) prepacked column (1.5 cm x 5 cm) equilibrated with 0.1% gelatin in
phosphate buffer. The sample was eluted in gelatin buffer, collected in 1 ml fractions and counted in a Beckman Instruments Gamma 4000 counter. Tubes containing the highest activity were pooled and stored at 4°C. Labeling efficiency was determined by adding 10 μl of sample to 190 μl of sodium phosphate buffer containing 1% bovine serum albumin and precipitating with 100 μl 30% (w/v) trichloroacetic acid (TCA). Following a one h incubation at 4°C, samples were centrifuged at 5000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in 10% TCA and centrifuged as before. The washing procedure was performed two more times after which the pellet was assayed using a gamma counter. A 10 μl non-precipitated sample was included to determine the percent of the label on the protein.

**Dot blot assay procedure:** Dot blot procedure for analyzing antibody binding of proteins was performed according to Kazemi et al. (1990). One hundred ng of protein diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) was immobilized on nitrocellulose using a 96-well dot-blot apparatus (BioRad Laboratories). Coating buffer contained 4 M urea and 0.07 M DTT when denatured antigen was used. Non-specific binding sites were blocked at 4 °C overnight with 5% nonfat dry milk in phosphate buffered saline containing 0.05% Tween-20 (PBST). Individual strips were incubated in Mab supernatant, ascites, or antisera diluted in PBST buffer containing 0.5% milk for 2 h at RT. Incubation was followed by three washes with PBST buffer and addition of rabbit anti-mouse immunoglobulin diluted 1:2000 in 0.5% milk in PBST buffer for 2 h. Subsequent incubation with 125I Protein A (5 μCi; ICN Biomedicals, Costa Mesa, CA) for 2 h was followed by washing with PBST buffer and one wash with 0.01 M Tris-HCl, 0.015M NaCl (pH 7.2). Reactive dots were visualized on film (XAR-5 film; Eastman Kodak, Rochester, New York) following overnight exposure. Controls included preimmune antisera and samples receiving no primary antibody.
Competitive binding assay: Epitope specificities of $^{125}$I-labeled monoclonal antibodies were determined using the dot blot procedure described above with the following modifications. Exotoxin A in a modified coating buffer (TBS; 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl) was coated on to nitrocellulose using the 96-well dot blot apparatus. The nitrocellulose was blocked for 1 h with 2% nonfat dry milk in TBS buffer with continuous rocking at room temperature. The nitrocellulose was rinsed briefly with TBS buffer, cut into strips and incubated overnight with labeled monoclonal antibody alone, or with fifty-fold excess of unlabeled homologous or heterologous monoclonal antibody. The nitrocellulose was washed three times with TBS buffer and once with TBS buffer containing 0.5% NP-40, the dots were cut out and counted on a gamma counter (Beckman). In order to determine the monoclonal antibody's specificity for toxin, CRM-66, PE-40 fragment, and denatured toxin, the labeled monoclonal antibody was incubated with 50-fold molar excess of the protein prior to addition to the nitrocellulose. Toxin was denatured in 4 M urea and 0.07 M dithiothreitol in TBS buffer. Inhibition was determined relative to samples with no competing protein.

Indirect immunoprecipitation: Precipitation of $^{125}$I-labeled toxin was performed as described by Galloway et al. (1984). A series of polypropylene tubes (1.5 ml) containing 0.02 μg $^{125}$I-labeled toxin (specific activity $5 \times 10^5$ cpm/μg) in 50 mM Tris-HCl (pH 7.2), 5 mM EDTA, 0.15 M NaCl, 0.02% NaN₃, and 1 mg/ml gelatin were incubated with increasing amounts of 4 M urea dissolved in Tris buffer for 30 min at room temperature prior to the addition of 3 μg purified monoclonal antibody TC-1. Immune complexes consisting of $^{125}$I-labeled toxin bound to TC-1 were isolated by incubation with a 10% suspension of Protein A-Sepharose followed by centrifugation of the matrix. Proteins bound to the matrix were analyzed for the presence of $^{125}$I-toxin in a gamma radiation counter (Beckman). Controls containing
urea-denatured 125I-labeled toxin and non-specific antibody were included. Binding to the controls was less than 10% of that observed in the experimental samples.

**FITC-labeling procedure:** Exotoxin A and rabbit antitoxin were labeled with fluorescein isothiocyanate on celite (FITC; Calbiochem, La Jolla, CA) in order to perform cell binding studies. Five mg purified toxin (1 ml) were dialyzed against 0.15 M NaCl prior to the addition of NaCO₃ (pH 9.5) to a final concentration of 0.07 M. FITC (1.25 mg) was added to the protein and mixed by vortex. The mixture was incubated for 1 h at room temperature and the supernatant was removed following centrifugation at 2000 rpm (10 min). Free FITC was removed by gel filtration through a Sephadex G-25 column and eluted with 0.07 M NaCO₃ (pH 9.5). Column fractions were spectrophotometrically monitored for FITC-labeled protein at 495 nm and 280 nm (Beckman). Protein containing fractions with an equal fluorescence to protein ratio were pooled. Pooled protein was filter sterilized and stored at 4°C until needed. Three mg of purified rabbit anti-exotoxin A was labeled with FITC by the same procedure.

**Fluorescence-activated cell sorter (FACS) procedure:** Mouse fibroblast cells (L929) were harvested by gently hitting the tissue culture flask following a 10 min incubation with phosphate buffered saline (PBS) containing 5 mM EDTA. Cells were pelleted by centrifugation at 2000 rpm for 10 min and washed two times with phosphate-buffered saline containing 1% fetal calf serum (FCS/PBS). A total of 1 ml of suspended cells (1 x 10⁶ L929 cells) was dispensed into glass test tubes, fixed for 10 min in methanol at -20°C, and washed with FCS/PBS two times. FITC-exotoxin A (0.75 µg) was added to the cells in 100 µl for 1 h on ice, with occasional gentle stirring. For the determination of blocking of toxin binding to cells, antibody was preincubated with 10 µl of FITC-exotoxin A (0.75 µg) for 1 h at RT prior to incubation with cells. Following incubation of toxin with cells, two ml of FCS/PBS was added to the samples and the cells were washed three times in buffer. Cells were resuspended in
0.5 ml FCS/PBS and kept on ice until analyzed by either fluorescent microscopy or FACS. Controls included cells receiving no FITC-exotoxin A or samples incubated with FITC-exotoxin A preincubated with anti-pyocin antibody.

Binding of exotoxin A to cells was also measured using FITC-labeled anti-exotoxin A antibody as follows. Cells were harvested, washed and fixed in methanol as described above. Increasing amounts of toxin were incubated with the cells for 1 h on ice and washed two times in FCS/PBS. FITC-labeled antibody was added for 1 h on ice then washed three times and analyzed. Controls included cells incubated with FITC-labeled antibody with no prior exotoxin A incubation.

Alkaline phosphatase conjugation: Purified monoclonal antibody TC-1 was conjugated to alkaline phosphatase according to a previously published procedure (Voller and Bartlett, 1976). Alkaline phosphatase (Type VII-S: orthophosphoric-monoester phosphohydrolase; EC 3.1.3.1) was added to pure antibody in a ratio of 2:1, enzyme to antibody and dialyzed overnight at 4°C in PBS. Glutaraldehyde (25%) was added to a final concentration of 10% (v/v) and incubated for 2 h at room temperature with constant rocking. The mixture was then dialyzed overnight against 2 L phosphate-buffered saline at 4°C followed by overnight dialysis against borate-buffered saline at 4°C. The conjugated antibody was concentrated using a YM-10 Amicon filter and stored at -20°C until used.

Coupling of peptides to thyroglobulin: Peptides were coupled to thyroglobulin (TGG) using the heterobifunctional cross-linking reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce). Twenty mg MBS was dissolved in 2 ml dimethylformamide and added to 40 mg TGG to achieve a molar ratio of 1000:1 of MBS:TGG. After stirring for 1 h, the free MBS was removed by gel filtration through a Sephadex G-50 (Pharmacia) column and eluted with 100 mM phosphate buffer (pH 6). Peptide corresponding to amino acids 453-477 in toxin was synthesized with an
added cysteine residue at the carboxyl-terminus to facilitate covalent coupling to TGG. Ten mg of the synthetic peptide was added with stirring to activated carrier protein for 4 h and the free peptide was removed by gel filtration. Peptides 427-438 and 419-432, which lacked terminal cysteines, were chemically coupled to TGG using gluteraldehyde. Briefly, 10 mg of peptide were mixed with 10 mg of TGG in 100 mM sodium bicarbonate (pH 7.4). Twenty-five percent glutaraldehyde was added to a final concentration of 3% (v/v) and this was incubated for 3 h at RT. Free peptide was removed by gel filtration through a Sephadex G-50 column eluted with 100 mM sodium phosphate buffer (pH 6). Column fractions were spectrophotometrically monitored for conjugated peptide at 280 nm (Beckman Instruments).

Protein purification and activity assays

The following procedures were used to purify proteins and evaluate their purity and enzymatic activity.

Bacterial strains: The studies described below were performed with proteins purified from the following strains of Pseudomonas aeruginosa. Strain PA01 is a wild-type prototrophic strain (Holloway, et al., 1979) upon which the Pseudomonas genetic map is based. PA01-PR1 is a nitrosoguanidine-generated mutant of strain PA01 which produces CRM-66, a 66,583 dalton cross-reactive toxin that is enzymatically inactive due to a His to Tyr substitution at amino acid 426 (Cryz, et al., 1980; Wozniak, et al., 1987). Strains PA103-29 (Ohman, Sadoff et al. 1980) and PA01-PR1 transformed with plasmid pGW28 containing the toxR gene (Wozniak, et al., 1987) were used for toxin production. Transformation with plasmid pGW28 results in strains which hyperproduce their respective toxins for purification according to published procedures (Leppla, 1976). Due to difficulties growing strain PA01 to high densities, strain
PA103A (toxA⁻) transformed with plasmid HB101/pHG105 containing the PAO1 toxA gene was used for the production of PAO1 toxin.

**Toxin purification:** Exotoxin A from three *Pseudomonas aeruginosa* strains were purified according to the procedure described by Leppla (1976) with the following modifications. Cultures were grown in trypticase soy broth dialysate (TSBD) for 18 h at 250 rpm, and maintained at 32°C. All of the following procedures were carried out at 4°C. The supernatant was collected by centrifugation (10,000 rpm for 10 min) and diluted in chilled distilled water. DEAE-cellulose beads (Whatman) equilibrated in 0.01 M Tris-HCl (pH 8.1; buffer A) were added and continuously stirred for two hours, then allowed to settle for two hours. The supernatant fraction was poured off and the cellulose beads were washed extensively with buffer A. Protein was eluted from DEAE-cellulose beads with buffer A containing 0.15 M NaCl and 2-mercaptoethanol was added to a final concentration of 5 mM. The protein sample was then fractionated by the addition of 70% saturated ammonium sulfate and allowed to settle for 2 h.

Following centrifugation, the protein pellet was resuspended in buffer A containing 2 mM 2-mercaptoethanol (ANM buffer) and dialyzed against this buffer. This preparation was separated using a DEAE-sephacel column and eluted with a continuous gradient of ANM buffer containing 0.3 M NaCl. Fractions were tested for the presence of toxin using the dot blot assay previously described. Rabbit antitoxin was used to detect toxin containing fractions which were pooled, concentrated and diafiltered into 0.005 M potassium phosphate buffer containing 0.05 M NaCl and 2 mM 2-mercaptoethanol (column buffer) using a YM 10 Amicon filter. The toxin sample was separated using a hydroxylapatite (BioRad) column and eluted with a continuous gradient of column buffer and 0.1 M potassium phosphate buffer containing 0.05 M NaCl and 2 mM 2-mercaptoethanol. Fractions were tested for the presence of toxin as described. The purity of the toxin samples was analyzed by sodium dodecyl sulfate
polyacrylamide gel electrophoresis; and the presence of toxin was confirmed by Western blot analysis (described below). PA103 toxin and CRM-66 were aliquoted and stored at -70° C. PAO1 toxin was filter sterilized and stored at 4 °C. Unless specifically stated throughout this study, "toxin" refers to exotoxin A from strain PA103. Several purifications were carried out during this study; however, one protein sample was used throughout the study for each of the toxins described.

**Protein determination:** Protein concentrations were determined by the Bradford method (1976). In addition, estimates of protein concentration were calculated using equation 3 (Warburg and Christian, 1941):

\[
\text{Eq. 3: } 1.45 (\text{OD}_{280}) - 0.74 (\text{OD}_{260}) = \text{mg/ml}
\]

**Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis:** Solubilized proteins were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). Samples were diluted in a ratio of 1:5 in sample buffer (60 mM Tris-HCl, pH 6.8, 2% β-mercaptoethanol, 10% glycerol, 0.02% bromphenol blue) and heated for three minutes at 100° C. Gels were cast in a Hoeffer SE 600 vertical apparatus (Hoeffer Scientific Instruments, San Francisco, CA). The resolving gel contained 10% acrylamide, 0.4 M Tris-hydrochloride, pH 8.8, 1% ammonium persulfate, 0.1% sodium dodecyl sulfate (SDS), and 0.1% N,N,N,N-tetra-methylethylenediamine (TEMED; BioRad). The stacking gel consisted of 4% acrylamide, 0.12 M Tris-hydrochloride, pH 6.8, 1% ammonium persulfate, 0.1% SDS, and 0.1% TEMED. Running buffer was 0.05 M Tris (pH 8.3) containing 0.4 M glycine and 0.1% SDS. Proteins were electrophoresed with a constant current of 35 mA for 1.5 mm gels. Gels were stained in 0.3% Coomassie blue R-250 (BioRad) prepared in 45% methanol and 10% acetic acid for 30 min and destained in a solution.
containing 20% methanol and 10% acetic acid. Low molecular weight standards containing phosphorylase B (92,000 D), bovine serum albumin (66,200 D), ovalbumin (45,000 D), carbonic anhydrase (31,000 D), soybean trypsin inhibitor (21,500 D), and lysozyme (14,400 D) also were electrophoresed on the gel.

**Gel preparation for fluorography:** Polyacrylamide gels were prepared for fluorography by the method of Bonner (1974). Proteins were fixed in SDS-PAGE gels in a solution of 20% (v/v) methanol and 10% (v/v) acetic acid for 1 h at 25°C, followed by two 30 min incubations in dimethylsulfoxide (DMSO). DMSO saturated gels were then incubated in 20% (v/v) 2,5-diphenyloxazole (PPO) in DMSO for 3 h with constant rocking, followed by two 30 min washes in distilled water. Gels were dried on a DryGel Sr. slab gel dryer (Model SE1160; Hoeffer Scientific Instruments) at 65°C for 90 min and exposed to film (XAR-5) overnight at -70°C.

**Immunoblotting of proteins to nitrocellulose:** For immunoblotting, protein was resolved using a 12.5% acrylamide gel followed by electrophoretic transfer to nitrocellulose sheets as described by Towbin (1979). Pre-soaked nitrocellulose sheets and gels containing 200 ng of purified protein (per lane) were sandwiched in a Hoeffer TE42 electrophoretic transfer unit filled with six liters of Tris-glycine methanol blotting buffer containing 0.1% SDS. Proteins were transferred to nitrocellulose in 80 min, with increasing amperage from 0.6 A to 1.2 A. The nitrocellulose sheets were blocked using 20 mM Tris-hydrochloride, 0.5 M NaCl (pH 7.2; TBS) containing 3% gelatin. After 1 h, the sheets were rinsed using 0.1% Tween 20 in TBS buffer (TTBS) and incubated overnight at room temperature with diluted hybridoma supernatant, ascites fluid or rabbit antisera containing 1% gelatin in TBS buffer. The sheets then were washed three times in TTBS buffer, and rabbit anti-mouse antibody diluted 1:2000 in buffer containing 1% gelatin was added. The sheets were incubated for 2 h with constant shaking. The washing procedure was repeated and horseradish peroxidase
conjugated goat anti-rabbit immunoglobulin (1:2000) was added. Rabbit antisera was
detected directly using goat anti-rabbit horseradish peroxidase. After 2 h, the sheets
were washed in TTBS buffer followed by one rinse in TBS buffer prior to visualizing
the immunoreactive bands by the addition of hydrogen peroxide and 4-chloro-1-
naphthol (BioRad). Autoradiographs of the results were prepared in a similar manner,
however the antibody conjugate was substituted with $^{125}$I-Protein A and exposed to
XAR-5 film for 16 h.

**ADP-ribosylation assay:** ADP-ribosyltransferase assays were performed as
previously described (Galloway, et al., 1989) using nicotinamide (U-14C) adenine
dinucleotide (302 mCi/mmol; Amersham Corp., Arlington Heights, IL) and a crude
extract of wheat germ elongation factor 2 prepared according to Chung et al. (Chung
and Collier 1977). Activation of toxin was accomplished by incubating equal volumes
of toxin and 8 M urea, 2% dithiothreitol (DTT), and 0.2 mg/ml bovine serum albumin
(Fraction V) for 15 minutes at 25° C. Alternatively, toxin was activated following three
cycles of rapidly freezing in liquid nitrogen and thawing in a 37° C waterbath. Briefly,
activated toxin was incubated with $^{14}$C-NAD$^+$ and elongation factor 2 for 15 min at 25°
C and the reaction stopped by the addition of 10% trichloroacetic acid. The precipitate
was collected by vacuum suction onto 934-AH glass microfiber filters (Whatman),
washed with 5% (w/v) trichloroacetic acid, dried, and counted using liquid scintillation
on a Beckman LS6800 scintillation counter. For the determination of neutralizing
activity, purified antibody was preincubated with 5 μl of toxin (50 ng/μl) for 1 h at 25°
C. To analyze the specific formation of the ADP-ribose-EF-2 complex, duplicate ADP-
riboseyltransferase assay samples were inactivated by the addition of an equal volume of
SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% 2-β-mercaptoethanol, 10%
glycerol, 0.01% bromphenol blue) and boiled for three min. The entire sample was
then resolved using a 10% SDS-polyacrylamide gel and visualized by fluorographic analysis performed as described above.

**NAD\(^+\) glycohydrolase assay:** NAD-glycohydrolase activity was determined according to a method described by Barbieri et al. (1989). Three μg of toxin were incubated in 25 μl of reaction mixture consisting of 100 mM (Trizma base; pH 8) containing 20 mM dithiothreitol, egg albumin (1 mg/ml) and 1.83 nmol of (carbonyl-\(^{14}\)C)NAD (41 mCi/mmol; Amersham). Following incubation at 37°C for 4 h, the reaction was stopped by the addition of 10 μl of boric acid (1 M). Two-hundred fifty μl of water-saturated ethyl acetate was added to each sample, which was then vortexed and centrifuged for 10 minutes at 14,000 rpm. NAD-glycohydrolase activity was determined by measurement of the \(^{14}\)C nicotinamide extracted in 100 μl of the ethyl acetate phase and counted by liquid scintillation. Assays measuring inhibition of activity by immunoglobulin included controls containing 10 μg of egg albumin or 10 μg IgG preincubated with toxin for 1 h at room temperature prior to the addition of (\(^{14}\)C) NAD. Samples including either antibody and NAD\(^+\) or NAD\(^+\) alone were included as controls.

**Cytotoxicity assay:** Cytotoxicity of non-activated toxin and CRM-66 were measured using Chinese hamster ovary cells (CHO). Cells were cultured in RPMI 1640 medium (Whittaker) supplemented with 10% (v/v) fetal calf serum in 96-well microtiter plates at a concentration of 2 x 10\(^4\) cells/well. Purified toxin or CRM-66 were added to the CHO cell cultures in increasing quantities and the plates were incubated at 37°C in 5% CO\(_2\) for 3-4 h. Following incubation, \(^3\)H-thymidine (DuPont New England Nuclear, Boston, MA) was added at a concentration of 0.8 μCi/well, and the cells were incubated for an additional 18 h. Cells were washed three times in phosphate-buffered saline, lysed in 0.1 M NaOH, and collected on filters on a Mash II
unit (Whittaker). The uptake of $^3$H-thymidine provided an index of the inhibition of DNA synthesis as a measure of cytotoxicity.

**Protein synthesis inhibition assay:** Inhibition of protein synthesis was measured by an *in vitro* procedure using mouse fibroblast cells (L929) as described above with the following modifications. Following a 1 h incubation with toxin or CRM-66, cells were washed three times and incubated with leucine-free RPMI media for 1 h. Protein synthesis was measured following a 1 h pulse with 0.3 μCi/well of $^3$H-leucine (ICN). Cells were washed and harvested as described. Inhibition was determined relative to $^3$H-leucine incorporated by control cells receiving media alone.
CHAPTER III

RESULTS

Exotoxin A purification and analysis

In order to perform structure/function analysis of Pseudomonas exotoxin A (ETA), it was necessary to purify the toxin from Pseudomonas aeruginosa strains PA103, PAO1, and PAO1-PR1. The two toxins from wild-type strains PA103 and PAO1, have similar ADP-ribosyltransferase activities although they differ in amino acid sequence at four specific residues (Table 1). Strain PAO1-PR1 is a nitrosoguanidine generated mutant of strain PAO1 (Cryz, et al., 1980). The critical difference between the mutant toxin from strain PAO1-PR1 (CRM-66) and the other toxins is the substitution of a histidine for a tyrosine at position 426. This single amino acid substitution is the only difference between the PAO1 and PAO1-PR1 toxins, and results in a loss of ADP-ribosyltransferase activity in the CRM-66 toxin (Wozniak, et al., 1988).

Toxins from these strains were purified from the culture supernatant fraction of cells grown in trypticase soy broth dialysate according to a procedure described by Leppla (1976). Exotoxin A from strain PA103 was purified from a 80 liter culture, yielding one g of purified toxin. Exotoxin A from strain PAO1 was purified from a three liter culture, yielding three mg. CRM-66 was purified from a six liter culture and yielded 11 mg of pure protein. The purity of the toxins was analyzed following electrophoresis through a 10% sodium dodecyl sulfate polyacrylamide gel.
stained with Coomassie blue. The toxins from these strains have the same molecular weight (66,583 daltons), and appear as a single band (Figure 3). Western blot analysis of the toxins shows that they are indistinguishable by antitoxin rabbit serum (Figure 4).

Table 1. Amino acid sequence differences in exotoxin A from *P. aeruginosa* strains PA103, PAO1, and PAO1-PR1

<table>
<thead>
<tr>
<th>Exotoxin A</th>
<th>Domain I</th>
<th>Domain II</th>
<th>Domain III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>aa 179</td>
<td>aa 364</td>
<td>aa 407</td>
</tr>
<tr>
<td>PA103</td>
<td>Thr</td>
<td>Asn</td>
<td>Val</td>
</tr>
<tr>
<td>PAO1</td>
<td>Ala</td>
<td>Ser</td>
<td>Ile</td>
</tr>
<tr>
<td>PAO1-PR1</td>
<td>Ala</td>
<td>Ser</td>
<td>Ile</td>
</tr>
</tbody>
</table>

*a Amino acid comparison is from Wick et al. (1988).

Table 2. Enzymatic activities of *Pseudomonas aeruginosa* exotoxin purified from strains PA103, PAO1, and PAO1-PR1

<table>
<thead>
<tr>
<th>Exotoxin A</th>
<th>(14C)ADPR-EF-2a (pmol/h/µg)</th>
<th>NAD+ hydrolysis (pmol/h/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activated</td>
<td>Non-activated</td>
</tr>
<tr>
<td>PA103</td>
<td>720</td>
<td>308</td>
</tr>
<tr>
<td>PAO1</td>
<td>808</td>
<td>52</td>
</tr>
<tr>
<td>PAO1-PR1</td>
<td>8</td>
<td>44</td>
</tr>
</tbody>
</table>

*a Activated samples were assayed in 4 M urea and 70 mM DTT. Results indicate pmole of NAD+ detected in precipitable material per µg of exotoxin tested.

*b Results indicate pmole of nicotinamide detected in ethanol extract per hour per µg of exotoxin tested. Samples included approximately 3 µg of toxin and 1.83 nmoles NAD+.
Figure 3. 10% SDS-polyacrylamide gel showing purified toxins stained with Coomassie blue. Lane 1, Low molecular weight standards: phosphorylase B (92,500); bovine serum albumin (66,200); ovalbumin (45,000); carbonic anhydrase (31,000); lysozyme (14,400); lane 2, 5 µg PA103 exotoxin A; lane 3, 5 µg PAO1 exotoxin A; lane 4, 4 µg CRM-66.
Figure 4. Western blot analysis of purified toxins from *Pseudomonas aeruginosa* strains PA103 (lane 1), PAO1 (lane 2), and PAO1-PR1 (lane 3). 100 ng samples of purified toxin were electrophoresed through a 10% SDS-PAGE gel and detected with purified rabbit anti-exotoxin A and goat anti-rabbit horseradish peroxidase conjugate.

A comparison of the ADP-ribosyltransferase (ADPRT) and NAD$^+$ glycohydrolase activities of the purified toxins is provided in Table 2. In order to measure ADPRT activity, it is first necessary to activate toxin. In this laboratory this is accomplished by diluting toxin in buffer containing 4 M urea and 0.07 M dithiothreitol (DTT), or by repeated cycles of freeze-thawing. As shown in Table 2, when PA103 toxin is activated, its ADP-ribosyltransferase activity increases approximately two-fold. However, PAO1 toxin ADP-ribosyltransferase activity increases 16-fold. This difference is apparently due to the manner in which the toxins were stored before use.
The PAO1 toxin used in this study was recently purified and had never been frozen, whereas the PA103 toxin had been stored at -70° C. Thus, the PA103 toxin preparation was partially activated as a result of the storage process. This has been shown to be due to the generation of a 26 kD ADP-ribosyltransferase active fragment upon prolonged storage (Chung and Collier, 1977). In agreement with a recent study, CRM-66 appears to have some ADP-ribosyltransferase activity which is lost upon activation (Wick and Iglewski, 1988). However, its level of activity is substantially lower than wildtype toxin.

Inhibition of protein synthesis was confirmed in vitro using mouse L929 fibroblast cells (Figure 5). As the results in Figure 5 illustrate, as little as 0.75 ng of the PA103 and PAO1 toxins inhibit protein synthesis by 80%, whereas 12 ng of CRM-66 inhibits synthesis by only 23%. Inhibition of protein synthesis observed in CRM-66 (12 ng) may be due to residual ADPRT activity (Table 2). Non-activated CRM-66 has low levels of ADPRT activity which is lost upon activation; however, the mechanism involved in in vivo activation is not understood and CRM-66 may retain its activity in vivo. Recently, a graduate student in our laboratory, Xiang-Yang Han, has shown that CRM-66 expresses ADPRT activity when the ADPRT assay is extended from 25 min to two hours. This suggests that CRM-66 may retain some ADP-ribosyltransferase activity, however, the ability to carry out this activity is diminished due to a decrease its efficiency of elongation factor 2 binding. The protein synthesis assay involves incubation of mouse fibroblast cells in the presence of CRM-66 for one hour which may be long enough for CRM-66 to express sufficient ADPRT activity to result in the death of some cells.

The role of toxin's glycohydrolase activity is not fully understood; however, it is useful for measuring the ability of toxin to bind NAD+. The assay measures toxin's ability to bind and cleave ¹⁴C-labeled NAD+, releasing radioactively-labeled
nicotinamide. Analysis of NAD$^+$ glycohydrolase activities show that the three toxins have equal activity (Table 2); therefore, the substitution of His 426 to Tyr does not appear to affect toxin's ability to bind NAD$^+$. This finding has been substantiated by measuring fluorescence quenching due to NAD$^+$ binding which revealed that both PA103 and CRM-66 toxins are equally proficient at NAD$^+$ binding (Galloway, et al., 1989). CRM-66's inability to ADP-ribosylate appears to be due to its ability to bind its other substrate, EF-2. Thus, we have proposed that His 426 is not involved in NAD$^+$ binding but in the binding of EF-2 and that without this critical residue the ability of toxin to bind its protein substrate is reduced (Galloway, et al., 1989).

![Graph](image)

**Figure 5.** Inhibition of protein synthesis in mouse L929 cells using purified exotoxin A from strains PA103, PAO1, and PAO1-PR1. Toxins were tested in increasing amounts to inhibit incorporation of $^3$H-leucine by mouse fibroblasts. Results represent the averages of triplicate samples.
Immunoochemical analysis of exotoxin A

Production of monoclonal antibodies Three fusions of X63A1 mouse myeloma cells and toxin primed spleen cells (Balb/c) were performed (Fazekas de St. Groth and Scheidegger, 1980) and 361 hybridomas were tested by ELISA for production of anti-exotoxin A antibody. Specific efficiencies of each fusion are shown in Table 3. From thirty-three positive wells, nineteen hybridomas were cloned resulting in 97 clonal cell lines which produced monoclonal antibodies specific for exotoxin A. Monoclonal antibodies produced in the first fusion subsequently stopped producing antibody. Only one monoclonal antibody, T2, was derived from fusion series two and all other monoclonal antibodies were derived from fusion three.

Table 3. Fusion specific efficiencies

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Ratio of spleen to A-1 cells</th>
<th>Number of wells inoculated</th>
<th>Number of hybridomas tested</th>
<th>Clones produced</th>
<th>Specific efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1</td>
<td>192</td>
<td>7</td>
<td>2</td>
<td>28.6%</td>
</tr>
<tr>
<td>2</td>
<td>1:1</td>
<td>192</td>
<td>30</td>
<td>7</td>
<td>23.3%</td>
</tr>
<tr>
<td>3</td>
<td>2:1</td>
<td>480</td>
<td>324</td>
<td>24</td>
<td>7.4%</td>
</tr>
</tbody>
</table>

a Specific efficiencies are calculated as the ratio of specific antitoxin hybridomas to total hybridomas X 100. Immunizing antigen for each fusion was exotoxin A.

Antibody purification and specificity Monoclonal antibody was generally purified from ascites fluid by ammonium sulfate fractionation and column chromatography as described. The ability of the clones to grow as ascitic tumors was quite variable. As a result, the amount of antibody produced and the success of production was dependent upon each clone. Hybridoma cells were also grown in tissue culture. Cells were centrifuged and the supernatant collected after 72 h of
growth. Some supernatant fractions had high antibody titers so there was no need to isolate purified antibody for subsequent analysis. If purified antibody was used in an assay it will be indicated, otherwise it will be stated as the ascites or culture supernatant fraction.

Seven cell lines, as well as other anti-exotoxin A monoclonal antibodies were selected for a more detailed analysis (Table 4). Monoclonal antibody TC-1 was produced by Galloway et al. (1984), and monoclonal antibodies 38-B1 and 19-B1 were sent to our laboratory by the NeoRx Corporation (Seattle, Washington). Subtyping analysis of the monoclonal antibodies by ELISA revealed that five belong to IgG1, one to IgG3, while the other four belong to the IgM heavy chain class. All of the monoclonal antibodies express the kappa light chain.

Table 4. Summary of monoclonal antibodies used in this study and their characteristics.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Ig Subtypea</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>IgG3</td>
</tr>
<tr>
<td>T4</td>
<td>IgM</td>
</tr>
<tr>
<td>T8</td>
<td>IgG1</td>
</tr>
<tr>
<td>T9</td>
<td>IgG1</td>
</tr>
<tr>
<td>T13</td>
<td>IgG1</td>
</tr>
<tr>
<td>T19</td>
<td>IgG1</td>
</tr>
<tr>
<td>T20</td>
<td>IgM</td>
</tr>
<tr>
<td>TC-1</td>
<td>IgG1</td>
</tr>
<tr>
<td>19-B1</td>
<td>IgM</td>
</tr>
<tr>
<td>38-B1</td>
<td>IgM</td>
</tr>
</tbody>
</table>

aHeavy chain was determined by ELISA using the BioRad typing kit.
Monoclonal antibodies were further categorized by their ability to recognize native toxin and CRM-66 (H426Y). The ability of monoclonal antibodies to bind these proteins indicated the general location of their epitopes. As shown in Table 1, CRM-66 has a single amino acid substitution at position 426 as compared to PAO1 toxin. A monoclonal antibody that binds exotoxin A produced by strains PAO1 and PA103, but not CRM-66 has a specificity for domain III. Reactivity of monoclonal antibodies was determined by ELISA (Table 5) and in some cases confirmed by Western blot analysis (Figure 6).

Table 5. Specificities for exotoxin A and CRM-66 by ELISA

<table>
<thead>
<tr>
<th>Clone</th>
<th>ETA</th>
<th>CRM-66</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.) TC-1</td>
<td>0.300</td>
<td>0.035</td>
</tr>
<tr>
<td>T9</td>
<td>0.213</td>
<td>0.039</td>
</tr>
<tr>
<td>2.) T2</td>
<td>0.266</td>
<td>0.289</td>
</tr>
<tr>
<td>T8</td>
<td>0.335</td>
<td>0.376</td>
</tr>
<tr>
<td>T19</td>
<td>0.271</td>
<td>0.236</td>
</tr>
<tr>
<td>T20</td>
<td>0.468</td>
<td>0.498</td>
</tr>
<tr>
<td>19-B1</td>
<td>0.271</td>
<td>0.205</td>
</tr>
<tr>
<td>38-B1</td>
<td>0.172</td>
<td>0.185</td>
</tr>
<tr>
<td>T13</td>
<td>0.504</td>
<td>0.513</td>
</tr>
<tr>
<td>Anti-pyocin</td>
<td>0.023</td>
<td>0.022</td>
</tr>
</tbody>
</table>

*aReadings reflect the average of three wells.
Domain specificities were determined by accumulated data from several experiments (Figure 7). Domain I assignments were made to monoclonal antibodies which did not bind a toxin fragment, PE-40, which is missing domain I residues 1-252 (data not shown). Monoclonal antibodies which protected cells in CHO cell or protein synthesis assays, but did not inhibit the ADP-ribosyltransferase activity of toxin were also given domain I assignments. The binding of T13 to CRM-66 is equivalent to that of toxin; however, its binding to the PE-40 fragment is reduced (data not shown).

The binding specificities of the monoclonal antibodies were confirmed by Western blot analysis (Figure 6), T13's binding specificity could not be confirmed by this method due to its inability to bind unfolded toxin. As shown in Figure 6, T20 reacts with several protein bands in the toxin sample. This may be due to the generation of toxin fragments that are reactive with T20. Domain III monoclonal antibodies were grouped according to their ability to inhibit the ADP-ribosyltransferase activity of toxin. Interestingly, two monoclonal antibodies failed to bind to CRM-66 with a H426Y mutation. Additional domain III-specific monoclonal antibodies showed cross-reactivity with diphtheria, pertussis and cholera toxin by ELISA analysis (data not shown). Synthetic peptides were used to investigate the specificity of this binding and revealed that a cross-reactive epitope exists within domain III. This will be discussed later. Monoclonal antibodies grouped in II and III recognized the PE-40 fragment and CRM-66 and neutralized toxin in the protein synthesis assay, but did not affect the ADPRT activity of toxin.
<table>
<thead>
<tr>
<th>T19</th>
<th>TC-1</th>
<th>T20</th>
<th>Anti-pyocin</th>
<th>T9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 7. Domain assignments of monoclonal antibodies as determined by ELISA, Western blot analysis and toxin activity assays. [ ] Represents domain I.

**Competitive binding studies.** A more detailed analysis of binding specificity of monoclonal antibodies for epitopes on the toxin molecule was performed by competition dot blots. The ability of one antibody to inhibit the binding of another antibody to toxin indicates either common specificity or steric hindrance of the binding site by the blocking antibody. Conversely, monoclonal antibodies that do not inhibit the binding of another monoclonal antibody are most likely directed to different sites on the molecule. A competitive binding assay between monoclonal antibody T13, and monoclonal antibodies against either domain II/III (T8) or domain III (TC-1, T4, T9, and T20) was performed (Table 6). As expected, domain III-specific monoclonal antibodies TC-1 and T20 do not inhibit binding of $^{125}$I-labeled T13 to toxin. Previous ELISA results (data not shown) indicate T13 binds to the amino-terminus of the toxin.

<table>
<thead>
<tr>
<th></th>
<th>Ia</th>
<th>II</th>
<th>Ib</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_2$</td>
<td></td>
<td></td>
<td></td>
<td>COOH</td>
</tr>
<tr>
<td>1</td>
<td>252</td>
<td>365</td>
<td>405</td>
<td>613</td>
</tr>
<tr>
<td>T13</td>
<td>T2</td>
<td>T2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T8</td>
<td>T4</td>
<td>T8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T9</td>
<td>T19</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T20</td>
<td>TC-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>38-B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>19-B1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
molecule and TC-1 binds in domain III (Table 5) near the carboxy-terminus. It is presumed, therefore, that one monoclonal antibody should not interfere with the binding of the other. These results (Table 6) support that finding. Other monoclonal antibodies specific for domains II and III inhibited T13's binding to varying degrees (T8 and T4).

Table 6. Competitive binding of 125I-labeled T13 with unlabeled homologous and heterologous Mabs

<table>
<thead>
<tr>
<th>Clone</th>
<th>% Inhibition^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>T13</td>
<td>100</td>
</tr>
<tr>
<td>TC-1</td>
<td>0</td>
</tr>
<tr>
<td>T4</td>
<td>17</td>
</tr>
<tr>
<td>T8</td>
<td>40</td>
</tr>
<tr>
<td>T20</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Three ng of 125I-labeled T13 and 900 ng of unlabeled antibody were used in each competitive assay. Results are from three separate experiments.
^b Inhibition was determined relative to T13 samples incubated without competing antibody.

Table 7 shows that binding of labeled T9, a domain III-specific antibody, is completely inhibited by unlabeled homologous T9 and heterologous domain III-specific monoclonal antibodies T4, TC-1 and T2. Monoclonal antibody T20, another domain III-specific antibody, and monoclonal antibody T8 inhibit binding of T9 to a lesser extent.
Competitive dot blot assays were performed using $^{125}I$-labeled T9 and T13 monoclonal antibodies against toxin, CRM-66, the PE-40 fragment, and unfolded toxin to confirm data obtained by ELISA (Table 5). In this assay, antibody is incubated with the competing protein prior to the addition to toxin-coated nitrocellulose. Inhibition of antibody binding indicates that the antibody binds the competing protein and therefore, is not available to bind to nitrocellulose-bound toxin. As Table 8 illustrates, the binding of T13 to toxin was inhibited by exotoxin A but not by the PE-40 fragment or denatured toxin. CRM-66 inhibited T13 to the same extent as toxin (data not shown). These results confirm previous findings that suggest that T13 is specific for a domain I epitope which is composed of a discontinuous sequence (data not shown). Table 9 shows that the binding of T9 was inhibited by toxin, unfolded toxin and the PE-40 fragment; however, CRM-66 did not inhibit its binding. The inability of CRM-66 to inhibit T9 binding in this assay supports the results obtained by Western blot analysis and ELISA (Figure 6 and Table 5).

Table 7. Competitive binding of $^{125}I$-labeled T9 with unlabeled homologous and heterologous Mabs$^a$

<table>
<thead>
<tr>
<th>Clone</th>
<th>% Inhibition$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T9</td>
<td>100</td>
</tr>
<tr>
<td>TC-1</td>
<td>100</td>
</tr>
<tr>
<td>T2</td>
<td>100</td>
</tr>
<tr>
<td>T4</td>
<td>100</td>
</tr>
<tr>
<td>T8</td>
<td>29</td>
</tr>
<tr>
<td>T20</td>
<td>45</td>
</tr>
</tbody>
</table>

$a$ Three ng of $^{125}I$-labeled T9 and 900 ng of unlabeled antibody were used in each competitive assay. Results were taken from three experiments.

$b$ Inhibition was determined relative to T9 samples incubated without competing antibody.
Table 8. Competitive binding of $^{125}$I-labeled T13 against exotoxin A, PE-40 fragment and unfolded exotoxin A$^a$

<table>
<thead>
<tr>
<th>Exotoxin</th>
<th>% Inhibition$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exotoxin A</td>
<td>100</td>
</tr>
<tr>
<td>PE-40 fragment</td>
<td>0</td>
</tr>
<tr>
<td>Unfolded exotoxin A</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Each well received 100 ng toxin and 3.6 µg of protein were included as competitor.

$^b$ Inhibition was determined relative to T13 samples incubated with no free protein.

Table 9. Competitive binding of $^{125}$I-labeled T9 against exotoxin A, PE-40 fragment, CRM-66 and unfolded exotoxin A$^a$

<table>
<thead>
<tr>
<th>Exotoxin</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exotoxin A</td>
<td>100</td>
</tr>
<tr>
<td>PE-40 fragment</td>
<td>100</td>
</tr>
<tr>
<td>CRM-66</td>
<td>14</td>
</tr>
<tr>
<td>Unfolded exotoxin A</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$ Each well received 100 ng toxin and 3.6 µg of protein were included as competitor.

$^b$ Inhibition was determined relative to T9 samples incubated with no free protein.
Exotoxin A Neutralizing Antibodies: The ability of the monoclonal antibodies to protect cells from the cytotoxic activity of toxin was determined \textit{in vitro} using a protein synthesis assay and Chinese hamster ovary cell cytotoxicity assay. The protein synthesis assay measures the incorporation of $^3$H-leucine into protein, while the Chinese hamster ovary cell assay measures cellular proliferation. Because toxin inhibits protein synthesis and is cytotoxic, both assays may be used to determine whether toxin cytotoxicity has been inhibited by antibody. Although some monoclonal antibodies may protect tissue culture cells from the cytotoxic effects of toxin, these assays do not reveal how the toxin is neutralized. One obvious possibility involves blocking toxin binding to its cell receptor. However, it may also be possible to protect cells by inhibiting ADP-ribosyltransferase activity. Toxin has two substrates, elongation factor 2 and NAD$^+$; therefore, neutralizing monoclonal antibodies may act by blocking either the elongation factor 2 binding site or the NAD$^+$ binding site. Alternatively, the monoclonal antibody could bind the toxin in such a way that the conformation of the protein is altered rendering it inactive. Table 10 shows a summary of the results of protein synthesis assays, Chinese hamster ovary cell assays, and ADP-ribosyltransferase assays. A "+" sign indicates that the ADPRT activity or the cytotoxicity of exotoxin A was inhibited by 50% in the presence of antibody relative to the activity of exotoxin A tested alone. Those antibodies that did not inhibit exotoxin A activity by 50% are indicated by a "-" sign. Monoclonal antibodies have been classified into three groups depending upon their effect on toxin in these assays. Monoclonal antibody T13 protects cells from toxin and binds to domain I which suggests that it may block cell receptor binding. Domain II/III specific monoclonal antibodies T2 and T8, protect cells from toxin but do not interfere with ADP-ribosyltransferase activity indicating that they may interfere with the translocation of toxin across the cellular
membrane or some other function. Monoclonal antibodies T9, T19, T20, and TC-1 inhibit ADP-ribosyltransferase activity but do not protect cells from toxin.

It should be noted that although a monoclonal antibody may neutralize toxin's enzymatic activity it may not necessarily protect cells from toxin in the *in vitro* assay used in this study. As previously mentioned, it is necessary to "activate" toxin in order to measure its ADP-ribosyltransferase activity. This activation generates an enzymatically active 26 kD toxin fragment that is not cytotoxic (Chung and Collier, 1977). Furthermore, toxin that has been "activated" by repeated cycles of freeze-thawing, is not cytotoxic to mouse L929 fibroblast cells (data not shown). Presumably, loss of cytotoxicity results from the dissociation of the active fragment from domain I. Hence, neutralizing monoclonal antibodies that are specific for epitopes within the domain III cleft require the unfolding of toxin prior to antibody binding, and thus the toxin's cytotoxic effects would be abolished.

The ability of monoclonal antibody T13 to protect Chinese hamster ovary cells from the cytotoxicity activity of toxin, and the antibody's inability to bind the PE-40 fragment suggests that this monoclonal antibody may be interfering with the binding of toxin to its cell receptor. Purified toxin was labeled with fluorescein isothiocyanate (FITC) to measure the binding of toxin to cells and analyzed by fluorescent microscopy and fluorescence-activated cell sorter. Inhibition of binding of toxin to cell-receptors was measured following a one hour incubation of antibody with FITC-labeled-toxin prior to the addition to cells. Binding of toxin to the cells was detected by both methods (data not shown), however, the binding could not be blocked by monoclonal antibody or rabbit-antiexotoxin A. The binding of toxin to cells appeared reduced in the presence of T13 and antitoxin, however, neither antibody could completely block the association. Results from this laboratory (data not shown) suggest that under some circumstances, toxin molecules may associate with each other resulting in a toxin...
complex being formed. In support of this finding, fluorescence-activated cell sorter analysis of samples in which unlabeled toxin was used to block the binding of FITC-toxin to cells reveal that toxin binding was enhanced (data not shown). The formation of a toxin-complex may result in the masking of particular epitopes so that antibody can no longer bind. Furthermore, saturable binding has never been demonstrated, which suggests that toxin may associate with cells by other means than domain I-receptor interaction. Thus, if toxin is binding to cells via a domain other than domain I and a complex is formed, it would be impossible to block toxin binding with a single monoclonal antibody. Purified rabbit-antiexotoxin A was also fluorescently-labeled to indirectly measure binding of toxin to cells. Samples were analyzed as before with similar results (data not shown).

Table 10. Summary of effects of monoclonal antibodies on exotoxin A activity

<table>
<thead>
<tr>
<th>Clone</th>
<th>ADPRT Inhibition(^a)</th>
<th>Cell Protection(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.) T13</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2.) T2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>T8</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3.) T9</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T19</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T20</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>TC-1</td>
<td>+</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>19-B1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>38-B1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) "+" indicates 50% inhibition of toxin activity in the presence of antibody relative to toxin tested alone. "-" indicates that the activity of toxin (ADPRT or cytotoxicity) was not inhibited by 50% in the presence of antibody.

\(^b\) Protein synthesis and CHO cell assays were used to measure exotoxin A's effect *in vitro*.

\(^c\) ND= not determined.
Additional studies investigating the binding of T13 to toxin involved the use of synthetic peptides. Domain I specific peptides were tested for their reactivity with T13 in direct and competitive ELISA. Competitive binding studies show that monoclonal antibody T13 is partially inhibited by peptides representing amino acids 40-64, 53-77, and 66-90 (Figure 8). Peptides representing other sequences in the toxin molecule did not show any reactivity with T13 (data not shown). Domain I is composed of several anti-parallel B-pleated sheets which may contribute to the binding site of T13 (Figure 1). Due to the conformational nature of this domain, many non-sequential amino acids may be involved in T13's epitope making it difficult to map using synthetic peptides.

![Graph showing inhibition of monoclonal antibody T13 binding to toxin-coated plates by increasing amounts of peptide.](image)

**Figure 8.** Competitive ELISA showing inhibition of monoclonal antibody T13 binding to toxin-coated plates by increasing amounts of peptide. Peptide and antibody were incubated together for 30 min prior to addition to plates coated with 50 ng toxin. Inhibition was determined relative to wells receiving no peptide.
Monoclonal antibody T9 partially neutralizes toxin's ADP-ribosyltransferase activity; however, if the toxin is activated prior to incubation with the monoclonal antibody, inhibition is increased (Figure 9). This indicates that the epitope is masked in the native molecule and unfolding toxin exposes the site. The inability of T9 to bind CRM-66 by Western blot analysis (Figure 7), ELISA (Table 6) and competitive assays (Table 9) suggests that the antibody binds the region containing His 426 and this data supports this conclusion.

![Figure 9. Exotoxin A's ADP-ribosyltransferase activity measured in the presence of monoclonal antibody T9. Five hundred ng of toxin was incubated with increasing amounts of T9 ascites fluid either following activation with 4 M urea and 0.07 M dithiothreitol or prior to activation.](image)

To determine whether the neutralizing monoclonal antibodies were blocking the binding of elongation factor 2 or NAD⁺, the NAD⁺ glycohydrolase assay was performed. In this assay the hydrolysis of NAD⁺ is measured by the release of ¹⁴C-
labeled nicotinamide in the presence or absence of monoclonal antibody. None of the monoclonal antibodies in this study were able to inhibit this activity (data not shown), indicating that the ADP-ribosyltransferase inhibiting monoclonal antibodies may be blocking elongation factor 2 binding or affecting the catalytic function of the enzyme. Furthermore, to determine if the monoclonal antibodies were interfering with the ability of toxin to form a complex with elongation factor 2 or to carry out the catalytic reaction, toxin's ADP-ribosylation reaction was analyzed by gel electrophoresis. As Figure 10 illustrates, in the presence of neutralizing monoclonal antibody T20, as well as neutralizing antibodies TC-1, T9, 19-B1, and T19, (data not shown) there is no ADPR-elongation factor 2 end-product. However non-neutralizing monoclonal antibody 38-B1 does not interfere with this reaction.
Figure 10. Autofluorographic profile of a 10% SDS-polyacrylamide gel illustrating the covalent modification of wheat germ elongation factor 2 with \(^{14}\text{C}\) ADP-ribose. All samples were run as described using 0.25 \(\mu\)g purified toxin in the presence of 5 \(\mu\)m \(^{14}\text{C}\) NAD\(^+\) and wheat germ elongation factor 2. The bands correspond to \(^{14}\text{C}\) ADP-ribose-EF-2. Lane 1, toxin + EF-2; lane 2, toxin + EF-2 following incubation with monoclonal antibody T20; lane 3, toxin + EF-2 following incubation with monoclonal antibody 38-B1.

Monoclonal antibody specificity for ADP-ribosyltransferase toxins

Because both diphtheria toxin and exotoxin A catalyze an ADP-ribosyltransferase reaction that results in the modification of elongation factor 2, we wanted to determine if the exotoxin A monoclonal antibodies would react with diphtheria toxin. In addition, it was important to determine if other ADP-ribosylating
toxins shared binding specificities with exotoxin. The ability of monoclonal antibodies to bind to ADP-ribosyltransferase toxins, diphtheria (DT), cholera toxin (CT), pertussis toxin (PT), and ETA was tested by ELISA and Western blot analysis (Table 11 and Figure 11). The monoclonal antibodies either showed cross-reactivity with all of the ADPRT toxins, or they showed no detectable cross-reactivity. No monoclonal antibodies were found to cross-react with some of the toxins but not others. The domain III specific monoclonal antibodies T19, T20, 19-B1, and 38-B1 were able to bind to these toxins. Significantly, ADPRT-neutralizing monoclonal antibody T20 reacted with all the toxins in both ELISA and Western blot analyses (Figure 11). However, monoclonal antibodies TC-1 and T9, although domain III-specific antibodies, showed no binding to diphtheria toxin, cholera toxin, or CRM-66 (data not shown). Because not all domain III-specific Mabs recognize other ADP-ribosyltransferase toxins, this indicates that only a segment of domain III contains a cross-reactive epitope.

Although there are several reports of domain I specific antibodies (Chia, et al., 1986; Galloway, et al., 1984) and domain I-specific T13 was isolated in this study, the majority of antibodies analyzed have been assigned domain III specificities (Figure 7). Preliminary studies involving the binding of rabbit-exotoxin A antibody to trypsin-digested exotoxin A suggests that there may be one immunodominant site on toxin (data not shown). Presently, the T-cell response to exotoxin A is being carried out using synthetic peptides, CRM-66, and whole toxin in order to more fully understand the immune response to exotoxin A. The existance of an immunodominant site in domain III would explain the large number of domain III-specific antibodies.

Following the domain assignments and preliminary characterization of the exotoxin A monoclonal antibodies, additional studies were performed to further investigate the structure/function relationship of exotoxin A. As described, T13 was
used to analyze the binding of toxin to cell receptors; however, the results were inconclusive. Monoclonal antibodies T19, T20, 19-B1 and 38-B1 are cross-reactive with the ADP-ribosyltransferase toxins diphtheria toxin, cholera toxin, pertussis toxin, and exotoxin A. Initial screening of these monoclonal antibodies using synthetic peptides indicated that all of these antibodies recognize the same peptides (data not shown). Additional studies to further investigate the cross-reactive site, therefore, was limited to the use of one antibody T20. Monoclonal antibodies T9 and TC-1 appear to share an epitope specificity that involves His 426. TC-1 was chosen for further analysis of the role of His 426 in the ADP-ribosyltransferase activity of toxin.

Table 11. Cross-reactivity of antitoxin monoclonal antibodies

<table>
<thead>
<tr>
<th>Clone</th>
<th>Cross-reactivitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>-</td>
</tr>
<tr>
<td>T8</td>
<td>-</td>
</tr>
<tr>
<td>T9</td>
<td>-</td>
</tr>
<tr>
<td>T13</td>
<td>-</td>
</tr>
<tr>
<td>T19</td>
<td>+</td>
</tr>
<tr>
<td>T20</td>
<td>+</td>
</tr>
<tr>
<td>TC-1</td>
<td>-</td>
</tr>
<tr>
<td>19-B1</td>
<td>+</td>
</tr>
<tr>
<td>38-B1</td>
<td>+</td>
</tr>
</tbody>
</table>

a Anti-pyocin antibody was included as a negative control and subtracted as background from the samples' readings. Cross-reactivity was confirmed by Western blot analysis.
Figure 11. Western blot analysis illustrating reactivity of antitoxin monoclonal antibody T20. One μg of protein was electrophoresed through a 12% SDS-polyacrylamide gel and detected with T20. Lane 1, cholera toxin; lane 2, pertussis toxin; lane 3, diphtheria toxin; lane 4, exotoxin A. Controls included antipyocin monoclonal antibody which showed no reactivity.

Immunological analysis of the His 426 epitope

As previously mentioned, several residues have been implicated in the enzymatic activity of exotoxin A (Carroll and Collier, 1987; Galloway, et al., 1989; Wozniak, et al., 1988). One of these residues is the histidine at position 426. A tyrosine substitution at this site results in loss of ADPRT activity yet does not affect NAD⁺ glycohydrolase activity (Table 2). Monoclonal antibody TC-1 inhibits the ADP-
ribozyme transferase activity of toxin and does not bind CRM-66 (Table 5). Therefore, this monoclonal antibody is an ideal tool for investigating the His 426 site more thoroughly.

It has previously been shown that TC-1 cannot immunoprecipitate native toxin in solution which indicates that TC-1 binds to a site that becomes exposed following the unfolding of the molecule (Galloway, et al., 1984) and binds to a linear epitope. In order to determine if TC-1 binds to a previously unexposed site, 125I-exotoxin A was incubated with increasing concentrations of urea prior to immunoprecipitation with TC-1. Figure 12 shows that as the urea concentration increases, the ability of TC-1 to precipitate the 125I-labeled toxin is enhanced. When the concentration of urea exceeded 0.25 molar, the binding began to decrease. This was most likely due to interference with monoclonal antibody binding by higher concentrations of urea.

![Graph](image)

Figure 12. Indirect immunoprecipitation of urea-treated 125I-toxin by monoclonal antibody TC-1. Two hundred ng of 125I-toxin were preincubated with urea from 0.025 to 0.25 molar for 30 min prior to addition of 3 μg of TC-1. The ability to precipitate toxin was measured as described under Materials and Methods. Each point represents 125I-toxin precipitated minus background resulting from any non-specific binding.
Role of His 426 in enzymatic activity

Although it was previously shown that TC-1 was able to inhibit ADP-ribosyltransferase activity, it was not known whether the antibody interferes by blocking the binding of elongation factor 2 or by blocking the interaction between toxin and NAD⁺. To resolve this, the NAD⁺ glycohydrolase assay was used to determine if NAD⁺ binding occurs in the presence of TC-1. In this assay, the activity of both activated and non-activated toxin was measured in the presence and absence of TC-1 (Table 12). These results indicate that TC-1 does not block binding of NAD⁺. Furthermore, TC-1 enhanced the NAD glycohydrolase activity of toxin indicating that it may stabilize the molecule in a conformation which makes NAD⁺ binding more efficient. Kandel et al. (1974) reported increased glycohydrolase activity with activated toxin which supports our finding.

Table 12. Exotoxin A-catalyzed hydrolysis of NAD⁺

<table>
<thead>
<tr>
<th>Exotoxin A Sample</th>
<th>NAD⁺ hydrolysis (pmol/h)a</th>
<th>+ Activationb</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 µg ETA</td>
<td>1.88</td>
<td>3.58</td>
</tr>
<tr>
<td>3 µg ETA + TC-1</td>
<td>7.57</td>
<td>6.75</td>
</tr>
<tr>
<td>3 µg ETA + Anti-(453-477)</td>
<td>0.98</td>
<td>4.19</td>
</tr>
</tbody>
</table>

a Samples contained 3 µg toxin, 10 µg antibody, and 1.83 nmole NAD⁺.
b Activated toxin was produced by freeze-thawing three times in the presence of 0.07 M dithiothreitol.

Previous studies have shown that TC-1 binds to a linear epitope requiring His 426 (Galloway, et al., 1984). To determine more accurately where TC-1 binds, overlapping synthetic peptides representing the entire toxin molecule were made by D. Anderson (NeoRx Corp.) as part of a collaborative study. The 25-mer peptides
overlapped by 13 residues so each adjacent peptides had a common sequence with the previous and subsequent peptides. Table 13 shows four of the representative peptides used in this study.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Residues^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>LGDGGDVSFSTRGTQNWTVERRLLQA</td>
</tr>
<tr>
<td>81</td>
<td>GTQNWTVERLLQAHRQLEERGYSFV</td>
</tr>
<tr>
<td>82</td>
<td>RQLEERGYSFVGYHGTFLEAAQSTV</td>
</tr>
<tr>
<td>83</td>
<td>YHGTFLEAAQSVFGGVR</td>
</tr>
</tbody>
</table>

^a Bold letter indicates the site of His 426. Peptide 81 (413-438) is the only peptide containing this residue.

These peptides were used to more accurately map the TC-1 epitope. Attempts at demonstrating direct binding of TC-1 to peptides were not successful; therefore, competitive binding assays were performed. Peptides representing sequences within domain III as well as domains I and II were tested for the ability block TC-1 binding to intact toxin in an ELISA format. Synthetic peptides containing residues 413-438 (peptide 81) and 427-451 (peptide 82) were able to block the binding of TC-1 to toxin (Figure 13). Not surprising, TC-1 specific peptide 413-438 contains His 426 while non-inhibiting peptides 401-425 and 439-464 do not. The ability of residues 427-451 to partially block TC-1 binding indicates that this peptide includes part of the TC-1 epitope.

From the data described above, the binding of TC-1 may be explained in the following manner. Analysis of the three dimensional structure of toxin, reveals that residues 419-432 form an alpha helical segment in domain III (405-613) (Figure 14).
The His 426 residue extends out from this helix into a cleft, which makes it inaccessible when the molecule is in its native state. However, when the molecule becomes unfolded during activation His 426 becomes accessible. This supports our findings that TC-1 does not bind native toxin, and also leads us to speculate that activation of toxin results in the exposure of His 426.

![Figure 13](image)

**Figure 13.** Competitive ELISA showing 50% inhibition of monoclonal antibody TC-1 binding to toxin-coated plates. Plates were coated with 50 ng toxin and peptides were tested in amounts ranging from 3.6 pmoles to 58 pmoles. Peptides not showing 50% inhibition are indicated by the maximum level (58 pmoles) tested.

To more precisely define TC-1's epitope, additional peptides were produced, including the helix sequence 419-432 and shorter peptides. As Table 14 shows, the 11-residue peptide containing residues 422-432 (LLQAHRQLEER) appears to contain the key residues involved in TC-1 binding. As expected, there are several other
residues in addition to His 426 which make up the TC-1 epitope, including Leu 422, Leu 423, and Gln 424.

Figure 14. Domain III of exotoxin A showing the alpha helix and residues His 426 and Glu 553.

We have shown that TC-1's binding to toxin requires the presence of His 426 (McGowan, et al., 1991). To confirm that TC-1 binding to the His 426 site inhibits ADPRT activity, site-specific antisera was tested for inactivation of toxin (Figure 15). Antisera against whole toxin, peptide 419-432 and peptide 427-438 inhibited ADP-ribosyltransferase activity. However, only antibody against whole toxin inhibited the NAD\(^+\) glycohydrolase activity of toxin (data not shown). This corroborates data which suggests that TC-1's epitope containing His 426 is involved in elongation factor 2
binding rather than NAD$^+$ binding. A graduate student in this laboratory, Sean Kessler, has preliminary evidence which suggests that site-specific antisera against 419-432 blocks the binding of elongation factor 2 by exotoxin A in an ELISA format (data not shown).

Table 14. Inhibition of TC-1 binding to toxin-coated plate by different peptides

<table>
<thead>
<tr>
<th>Residues</th>
<th>Synthetic Peptide</th>
<th>ID$_{50}^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>401-425</td>
<td>LGDGGDVSTRGQTQNLQVVERLLQA</td>
<td>&gt;80</td>
</tr>
<tr>
<td>413-438</td>
<td>GTQNWTVERLLQAHQLEERGYVVFV</td>
<td>13</td>
</tr>
<tr>
<td>419-432</td>
<td>VERLLQAHQLEER</td>
<td>5</td>
</tr>
<tr>
<td>420-432</td>
<td>ERLLQAHQLEER</td>
<td>5</td>
</tr>
<tr>
<td>421-432</td>
<td>RLLQAHQLEER</td>
<td>5</td>
</tr>
<tr>
<td>422-432</td>
<td>LLQAHQLEER</td>
<td>5</td>
</tr>
<tr>
<td>423-432</td>
<td>LQAHQLEER</td>
<td>10</td>
</tr>
<tr>
<td>424-432</td>
<td>QAHRQLEER</td>
<td>20</td>
</tr>
<tr>
<td>425-432</td>
<td>AHRQLEER</td>
<td>&gt;80</td>
</tr>
<tr>
<td>426-432</td>
<td>HRQLEER</td>
<td>&gt;80</td>
</tr>
<tr>
<td>427-438</td>
<td>RQLEERGYVVF</td>
<td>&gt;80</td>
</tr>
</tbody>
</table>

Epitope$^b$ LLOQAHQLEER

$^a$ Picomoles of peptide required for 50% inhibition of TC-1 binding to exotoxin A.

$^b$ Probable size of epitope as estimated from inhibition data; critical residues are underlined.
Figure 15. Exotoxin A's ADP-ribosyltransferase activity alone or in the presence of antisera. Five hundred ng of activated toxin were incubated with 10 μl antisera for 1 h at 25°C prior to assaying for enzymatic activity. Controls included samples receiving no antisera and samples incubated with preimmune antisera. Antisera are indicated by their immunogen. ETA: antitoxin; 419: 419-432; 427: 427-438. Preimmune sera is indicated by R1 and R0, respectively.

Cross-reactive epitope analysis

Several anti-exotoxin A monoclonal antibodies were identified that recognize diphtheria toxin, cholera toxin and pertussis toxin (Table 11). However, monoclonal antibody T20 was selected for a more in depth study due to its ability to inhibit ADP-ribosyltransferase activity of exotoxin (Table 10 and Figure 10) and its reactivity with ADPRT toxins by Western blot analysis (Figure 11). None of the cross-reactive monoclonal antibodies tested were able to neutralize the enzymatic activity of diphtheria toxin (data not shown). The immunoblot shown in Figure 11 shows bands representing diphtheria toxin (58 kD), cholera toxin's A1 chain (24 kD), pertussis toxin (28 kD and 25 kD) and exotoxin A (66 kD) as detected by monoclonal antibody T20. The reaction seen with the pertussis subunits is surprising in that two subunits (S1 and
S2) are detected by the monoclonal antibody. The catalytic domain is in S1 and would be expected to show cross-reactivity if there is a conserved site within the ADP-ribosyltransferase toxins. However, subunits S2, S3, S4 and S5 are involved in cell receptor binding. We have found no explanation for this result, however, Drusilla Burns and colleagues at the FDA in Bethesda, MD have often observed non-specific reactivity among these subunits (personal communication).

**Peptide screening.** Analysis of the Kyte and Doolittle hydrophilicity plot of toxin was used in determining which peptides should initially be screened with monoclonal antibody T20 (Figure 16) (Kyte and Doolittle, 1982). Peptides which represented hydrophilic residues and would therefore most likely be antigenic were selected for direct binding assays. Although the background in the assays was very high (data not shown), it was apparent that T20 and the other cross-reactive monoclonal antibodies were binding more strongly to peptides containing residues 413-477. According to the hydrophilicity plot, this region corresponds to a hydrophilic peak. The binding specificity of non-specific reactivity T20 was verified in a competitive assay in which peptides representing residues in domains I, II and III, were incubated with the monoclonal antibody prior to addition to toxin-coated plates. As Figure 17 illustrates, residues 413-451 were able to inhibit the binding of T20 to ETA. The peptide representing domain I residues (40-64) did not compete with T20 for binding.

Interestingly, residues involved in the cross-reactive epitope were also involved in the binding of TC-1, yet monoclonal antibody TC-1 does not bind to the other toxins. A competitive assay was performed to determine if the Mabs TC-i and T20 compete for toxin binding (Figure 18). Increasing amounts of purified antibody were mixed with TC-1 conjugated to alkaline phosphatase before adding the antibody mixture to a toxin-coated plate. As the graph shows, 0.08 pmole of purified unlabeled TC-1 inhibits TC-1 conjugate binding 50%, however, 0.66 pmole of purified T20
inhibits TC-1 by 20%. As stated earlier, residues 419-432 form a long alpha helix in domain III (Figure 14). These two monoclonal antibodies appear to bind residues that are near each other in the protein's primary sequence yet on opposite faces of the structure formed by the 419-432 sequence.

![Hydrophilicity plot of P. aeruginosa exotoxin A](image.png)

**Figure 16.** Hydrophilicity plot of *P. aeruginosa* exotoxin A. This plot was generated by the method of Kyte and Doolittle (1982).

![Competitive ELISA showing inhibition of monoclonal antibody T20 binding to toxin-coated plates by increasing amounts of peptide](image.png)

**Figure 17.** Competitive ELISA showing inhibition of monoclonal antibody T20 binding to toxin-coated plates by increasing amounts of peptide. Peptide and antibody were incubated together for 30 min prior to addition to plates coated with 50 ng toxin. Inhibition was determined relative to wells receiving no peptide.
Figure 18. Competitive assay showing inhibition of alkaline phosphatase TC-1 binding to toxin-coated plates by unlabeled TC-1 (solid circles). TC-1 binding in the presence of T20 is indicated by hollow circles.

Site-specific antiserum studies  Antisera produced against peptides 427-453, 419-432, 427-438, and 453-477 were tested for cross-reactivity by ELISA (Table 15), Western blot analysis (data not shown) and dot blot analysis (Figure 19).

Table 15. Antisera reactivities with ADP-ribosyltransferase toxins as measured by ELISA

<table>
<thead>
<tr>
<th>Antisera</th>
<th>ETA</th>
<th>DT</th>
<th>CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-419-432</td>
<td>0.321</td>
<td>0.387</td>
<td>0.395</td>
</tr>
<tr>
<td>Anti-427-438</td>
<td>0.288</td>
<td>0.286</td>
<td>0.322</td>
</tr>
<tr>
<td>Anti-453-477</td>
<td>0.289</td>
<td>0.039</td>
<td>0.044</td>
</tr>
<tr>
<td>Anti-ETA</td>
<td>0.898</td>
<td>0.029</td>
<td>0.034</td>
</tr>
</tbody>
</table>

a Results represent the average of three wells. Preimmune sera were included as controls.
Western blot analysis showed cross-reactivity with antisera to peptides (419-427) and (427-438), but serum against peptide (453-477) only reacted with exotoxin A and CRM-66 (data not shown), confirming previous ELISA results. Dot blot analysis of anti-peptide (419-432) shows reactivity with the ADP-ribosyltransferase toxins (Figure 19). Lanes 1 and 2 are controls which show the specificity of antitoxin and anti-diphtheria antibodies for their respective immunogens. Antipeptide 453-477 also shows specificity for ETA (lane 7). Preimmune sera were included as controls for the antipeptide sera (lanes 4 and 6). Antitoxin showed no reactivity for peptides containing residues 413-477, so no cross-reactivity would be expected (Table 16). Although antidiaphtheria did not show cross-reactivity by dot blot analysis, the serum antibodies did bind peptide 413-438 by direct ELISA (Table 16).

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Figure 19. Dot blot analysis of antisera binding to ADP-ribosyltransferase toxins. One hundred ng of protein were coated using a 96-well microtiter apparatus (BioRad). Antisera reactivity was detected using 125I-Protein A. Strip 1, anti-ETA; strip 2, anti-DT; strip 3, anti-pertussis peptide (21-35); strips 4 and 5, preimmune and anti-peptide (419-432); and strips 6 and 7, preimmune and anti-peptide (453-477).
Antidiphtheria antiserum was tested for binding to native exotoxin A, diphtheria toxin, and cholera toxin as well as the unfolded toxins by ELISA (Table 17). Although binding was not strong, there was an increase in the reactivity of the serum antibodies to unfolded exotoxin A and cholera toxin. This implies that there may be a conserved sequence among the ADPRT toxins which is accessible on native diphtheria toxin but is hidden in native exotoxin A and cholera toxin.

Table 16. Antisera reactivities with exotoxin A, diphtheria and exotoxin A synthetic peptides by direct ELISA$^a$

<table>
<thead>
<tr>
<th>Protein</th>
<th>Anti-exotoxin A</th>
<th>Anti-diphtheria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exotoxin A</td>
<td>0.627</td>
<td>0.070</td>
</tr>
<tr>
<td>Diphtheria toxin</td>
<td>0.029</td>
<td>1.136</td>
</tr>
<tr>
<td>401-425</td>
<td>0.031</td>
<td>0.060</td>
</tr>
<tr>
<td>413-438</td>
<td>0.029</td>
<td>0.209</td>
</tr>
<tr>
<td>427-451</td>
<td>0.025</td>
<td>0.091</td>
</tr>
<tr>
<td>439-464</td>
<td>0.020</td>
<td>0.095</td>
</tr>
<tr>
<td>453-477</td>
<td>0.019</td>
<td>0.066</td>
</tr>
<tr>
<td>466-490</td>
<td>0.006</td>
<td>0.054</td>
</tr>
</tbody>
</table>

$^a$ Protein binding was determined by direct ELISA. Fifty ng of protein was coated in wells on Immulon II plates. Background was determined by wells receiving no primary antibody and subtracted from readings.

The ability of the toxin-peptides to inhibit anti-diphtheria's binding to diphtheria toxin was tested in a competitive ELISA. As Figure 20 shows, the 25-residue peptides representing amino acids 413-438 and 427-451 were able to partially inhibit anti-diphtheria binding to diphtheria toxin. The peptides containing amino acids 387-412 and 401-425 did not affect binding of anti-diphtheria to diphtheria toxin coated plates. Furthermore, smaller peptides containing residues 419-432 and 427-438 were also able to inhibit the binding of anti-diphtheria to diphtheria diphtheria (Figure 21).
Table 17. Antisera reactivities with native toxins and unfolded toxins

<table>
<thead>
<tr>
<th>Antisera</th>
<th>ETA Native</th>
<th>ETA DTT</th>
<th>DT Native</th>
<th>DT DTT</th>
<th>CT Native</th>
<th>CT DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ETA</td>
<td>0.627</td>
<td>0.550</td>
<td>0.029</td>
<td>0.066</td>
<td>0.035</td>
<td>0.056</td>
</tr>
<tr>
<td>Anti-DT</td>
<td>0.035</td>
<td>0.178</td>
<td>1.143</td>
<td>1.071</td>
<td>0.54</td>
<td>0.199</td>
</tr>
</tbody>
</table>

*a* ELISA plates were coated with toxin or toxin treated with 0.07 M dithiothreitol. Background included wells receiving no primary antibody and was subtracted from the readings.

Figure 20. Competitive ELISA showing inhibition of anti-diphtheria binding to diphtheria by exotoxin A peptides 413-438 and 427-451. Anti-diphtheria and peptides were incubated at 25° C for 30 min prior to being added to plates coated with 50 ng diphtheria toxin. Inhibition was determined relative to wells receiving no competitor.
Figure 21. Competitive ELISA showing inhibition of anti-diphtheria to diphtheria toxin-coated plates by exotoxin A peptides 419-432 and 427-438. Peptides were incubated at 25° C with antiserum for 30 min prior to adding to plates coated with 50 ng of diphtheria toxin. Inhibition was determined relative to wells receiving no competitor.

ADP-ribosyltransferase neutralizing antisera As described previously, the sera raised against peptides 419-432 and 427-438 were not able to protect cells from toxin in the protein synthesis assay; however, they were able to neutralize its ADP-ribosyltransferase activity (Figure 15). Although none of the cross-reactive monoclonal antibodies inhibited the ADP-ribosyltransferase activity of diphtheria toxin, we wanted to test the cross-reactive antisera for this ability. As Figure 22 illustrates, anti-peptide sera against 419-432 and 427-438 were able to inhibit ADP-ribosyltransferase activity of exotoxin A and diphtheria toxin, while anti-toxin and anti-diphtheria antibodies only inhibited their respective toxin activities. These antisera were not able to inhibit NAD+ glycohydrolase activity of exotoxin A, but anti-toxin was able to block this activity (data not shown). Interestingly, the glycohydrolase neutralizing serum, anti-peptide 453-477, did not inhibit ADP-ribosyltransferase activity of toxin (data not shown).
Toxin is "activated" in order to measure its ADP-ribosyltransferase activity. The change in toxin's conformation that occurs during this process may eliminate the site-specific antibodies' epitope. As Table 12 shows, the ability of anti-453-477 to inhibit the glycohydrolase activity of toxin is lost upon activation of toxin.

![Bar graph showing ADP-ribosyltransferase activity of exotoxin A and diphtheria toxin in the presence of antisera.](image)

**Figure 22.** ADP-ribosyltransferase activity of exotoxin A and diphtheria toxin in the presence of antisera. Five hundred ng of toxin and 10 μl sera were incubated at 25°C prior to assaying. Antisera is indicated by its immunogen. ETA: anti-exotoxin; DT: anti-diphtheria; 419: 419-432; 427: 427-438.

**Analysis of pertussis toxin peptides**

Synthetic peptides representing the entire S1 subunit of pertussis toxin were sent to us from R. D. Sublett (The R. W. Johnson Pharmaceutical Research Institute, San Diego, CA) to further characterize the cross-reactive site among ADP-ribosyltransferase toxins. The cross-reactive antiserum raised against toxin peptides 419-432 was tested for its reactivity with pertussis toxin peptides using the competitive ELISA assay. As Figure 23 illustrates, pertussis peptides containing residues 51-70...
are able to block binding of anti-419-432 to pertussis toxin. The assay was also performed measuring the binding of anti-419-432 to toxin in the presence of pertussis peptides, with the result that pertussis toxin peptide 51-70 interfered with the binding of the site-specific antibody to exotoxin A (Figure 24). Analysis of pertussis toxin residues 51-77 reveals that some homology exists between exotoxin A and pertussis toxin in the proposed cross-reactive sequence 419-438 (Figure 25). The ability of the cross-reactive antibodies to neutralize ADP-ribosyltransferase activity of pertussis toxin has not been performed yet; however, the ability of pertussis toxin residues 51-70 to react with the site-specific antibody suggests that these residues may be involved in a conserved sequence between exotoxin A and pertussis toxin.

Figure 23. Competitive ELISA showing inhibition of anti-peptide 419-432 to pertussis toxin-coated plates by pertussis toxin S1 subunit peptides 51-65 and 56-70. Peptides were incubated at 25°C with antiserum for 30 min prior to adding to plates coated with 30 ng of pertussis toxin. Inhibition was determined relative to wells receiving no competitor.
Figure 24. Competitive ELISA showing inhibition of anti-peptide 419-432 to exotoxin A-coated plates by pertussis toxin S₁ subunit peptides 51-65 and 56-70. Peptides were incubated at 25° C with antiserum for 30 min prior to adding to plates coated with 30 ng of exotoxin A. Inhibition was determined relative to wells receiving no competitor.

Exotoxin A 422-440: LLQAHHQ LEERGYVFVGYH COOH
• •  * •
Pertussis toxin 66-80: VYLEHRMQE AveAERAGR GC OOH

Figure 25. Amino acid sequence comparison of exotoxin A domain III residues involved in cross-reactivity and pertussis toxin S₁ subunit residues. • indicates homologous amino acids.
CHAPTER IV
DISCUSSION

Mapping of exotoxin A domains by monoclonal antibodies

*Pseudomonas aeruginosa* exotoxin A catalyzes the transfer of the adenosine 5'-diphosphate ribosyl moiety (ADPR) of NAD⁺ to elongation factor 2 (EF-2) which inhibits protein synthesis in susceptible cells. Cytotoxicity of exotoxin A is dependent upon the processes of receptor binding, membrane translocation, and covalent modification of the target protein elongation factor 2. A library of monoclonal antibodies was produced in order to analyze these events and define sites on toxin involved in each function. The advantage of monoclonal antibodies as site specific probes helps to overcome some of the limitations of polyclonal antisera, which have more than one specificity.

The ten antibodies described in this report have different binding specificities and can be grouped accordingly (Figure 7). Interestingly, monoclonal antibodies belonging to two separate specificity groups inhibit ADP-ribosyltransferase activity while one monoclonal antibody with a different specificity blocks cytotoxicity of toxin *in vitro*. None of these antibodies neutralize NAD⁺ glycohydrolase activity, although rabbit antiserum to exotoxin A and anti-peptide 453-477 inhibit this activity.

One antibody with domain I specificity, T13, neutralizes toxin's cytotoxicity and inhibits protein synthesis *in vitro*, but does not affect the ADP-ribosyltransferase activity, suggesting that this monoclonal antibody inhibits the receptor-binding function of toxin. Recognition of toxin by T13 is dependent upon the conformation of toxin as shown by its inability to bind denatured toxin (Table 8). Domain I has a defined

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secondary structure consisting mostly of B-pleated sheets (Allured, et al., 1986) which may be involved in the formation of the T13 epitope. A further indication of domain I specificity for monoclonal antibody T13 is its inability to bind the PE-40 toxin fragment (Table 8), which is missing domain I.

Preliminary experiments (Figure 8) using synthetic peptides indicate that part of the T13 epitope is within residues 40-77. Several studies suggest that lysines in domain I play an important role in cell-receptor binding (Jinno, et al., 1988; Pirker, et al., 1985). Using site-directed mutagenesis, each lysine in domain I was separately substituted with glutamic acid. Cytotoxicity of toxin was only affected by a substitution at position 57 (Jinno, et al., 1988). In another study, Chaudry et al. (1989) produced several toxin variants containing dipeptide insertions within the domain I sequence. One variant containing an insert between residues Ile 60 and Gln 61 was 500-fold less cytotoxic than toxin yet had full enzymatic activity. Three-dimensional analysis of toxin reveals that a deep concavity exists on the surface of domain I (Allured, et al., 1986). Residues Lys 57, Ile 60 and Gln 61 reside on one of three B-pleated sheets that line this concavity implying a role for this site in receptor binding. Thus, these findings support the results of this study showing that T13 binds to a site within residues 40-77 and interferes with toxin cell receptor interaction.

**Role of His 426 in exotoxin A activity**

This dissertation describes the production of monoclonal antibodies and site-specific antisera which inhibit the enzymatic activities of exotoxin A from *Pseudomonas aeruginosa*. Comparative amino acid sequence analysis of toxins from mutant and wild-type strains reveals that the mutant toxin CRM-66 (H426Y) differs from PA103 toxin at five residues; three of these residues are located in catalytic domain III (405-613). However, PAO1 toxin and CRM-66 differ only at histidine 426 (Table 1) (Wick...
and Iglewski, 1988). Previous studies in this laboratory have shown that CRM-66 lacks ADP-ribosyltransferase activity due to the substitution of Tyr at position 426 (Wozniak, et al., 1988). However, it was not established whether loss of ADP-ribosyltransferase activity is due to an inability to bind elongation factor 2, or NAD+, or to some other function. Purified toxins were used in this study to demonstrate that PA103 and PA01 toxins have equivalent ADP-ribosyltransferase and NAD+ glycohydrolase activities (Table 2). However, CRM-66, while lacking ADP-ribosyltransferase activity retains full NAD+ glycohydrolase activity. Studies performed in this laboratory indicate that NAD+ is bound by CRM-66 as efficiently as by wild-type toxin (Galloway, et al., 1989), using protein fluorescence quenching as an indirect measure of NAD+ binding. Three-dimensional analysis of toxin reveals that the proposed NAD+ binding site resides within a deep cleft in domain III (Allured, et al., 1986; Brandhuber, et al., 1988). On the other hand, the side chain of His 426 extends into a separate domain III cleft that is quite distant (25 Å) from the proposed NAD+ binding site (Figure 14). We proposed that substitution of a tyrosine at position 426 renders CRM-66 ADP-ribosyltransferase inactive (McGowan, et al., 1991) due to the reduced ability of CRM-66 to bind elongation factor 2.

Two groups of ADP-ribosyltransferase-neutralizing monoclonal antibodies bind near His 426 but do not compete for the same epitope. ADP-ribosyltransferase activity of toxin is inhibited by monoclonal antibodies belonging to both groups while NAD+ binding is not affected (Table 12). The first group includes monoclonal antibodies TC-1 and T9. These monoclonal antibodies do not bind CRM-66 suggesting that their epitopes contain His 426. Alternatively, a difference in conformation may have been caused by the H426Y substitution resulting in the loss of the epitope shared by these antibodies. Results from other studies in this laboratory and the crystallization of CRM-66 indicate that the substitution at position 426 in CRM-66 does not affect the
conformation of the protein, suggesting involvement of His 426 in the antibodies' epitope. Competitive binding assays indicate TC-1 competitively inhibits monoclonal antibody T9 binding, indicating a shared epitope (Table 7). This study shows that TC-1 binds to a sequential epitope containing His 426, which is normally hidden when toxin is in the proenzyme conformation (Table 14 and Figure 13). These results show that activation of toxin opens the cleft bordered by the A and E helices (Figure 1) in domain III thereby exposing the His 426 residue.

Previous studies have shown that toxin requires activation for enzymatic activity. Treatment of toxin with increasing amounts of urea (which is included in the in vitro activation step) enhances TC-1 binding (Figure 12). The PE-40 fragment, which is missing domain I, does not require activation in order for ADP-ribosyltransferase activity to be expressed (Hwang, et al., 1987). Presumably, removal of domain I allows domain III to assume an ADP-ribosyltransferase active conformation. TC-1 binds to the PE-40 fragment suggesting that the His 426 residue is accessible in PE-40 (McGowan, et al., 1991). This confirms that the epitope containing His 426 is normally buried in the proenzyme conformation and becomes accessible upon activation of toxin. Recent work by Hwang and Chen (1989) supports these findings. Using domain-specific antisera, their results suggested that elongation factor 2 binding by toxin occurs between domains Ib and III. The work presented here further narrows the binding site to include His 426. This present study suggests that domain III residue His 426 is near or within the elongation factor 2 binding site of toxin.

Synthetic peptides were used to further define and confirm the TC-1 epitope. Competitive assays were used to block TC-1 binding using peptides representing sequences in domains I, II and III. A sequential epitope was indicated by the ability of residues 413-438 and 427-451 to inhibit TC-1 binding (Figure 13). In order to more
precisely define the TC-1 epitope, shorter peptides were produced and tested for TC-1 binding. As shown in Table 14, a peptide containing residues 422-432 contained the TC-1 epitope. In the toxin molecule these residues form part of a long alpha helical segment that borders a domain III cleft. The location of this helix in the carbon-nitrogen backbone structure of toxin is shown in Figure 14. The proposed NAD\(^+\) binding site is also designated and is distant from residues 422-432.

Site-specific antipeptide sera was produced to confirm that binding of TC-1 to the His 426 epitope inhibits enzymatic activity. Antisera against peptides 419-432 and 427-438 recognize toxin and CRM-66 (data not shown). Furthermore, both antisera inhibit ADP-ribosyltransferase activity (Figure 15) but do not affect NAD\(^+\) glycohydrolase activity (data not shown). These results confirm that His 426 is not involved in NAD\(^+\) binding. The ability of antisera generated against residues 453-477 to block glycohydrolase activity supports this finding.

Therefore, a model is proposed in which His 426 is located in domain III where it is inaccessible in the native exotoxin A conformation. When toxin is activated, domain III undergoes a conformational change that opens the cleft exposing the His 426 residue so that it is available for substrate binding. The ability of CRM-66 to bind NAD\(^+\) as efficiently as does toxin supports the suggestion that His 426 is involved in the binding of elongation factor 2.

Cross-reactivity among ADPRT toxins

Monoclonal antibodies belonging to the second ADPRT-neutralizing group (T19, T20, 19-B1 and 38-B1) recognized diphtheria toxin, cholera toxin, pertussis toxin, CRM-66 and exotoxin A. Mabs T19, T20 and 19-B1 also inhibit ADP-ribosyltransferase activity of exotoxin A. Although the binding of monoclonal antibody T20 inhibits the activity of toxin, it does not bind to the same site as T9 and TC-1.
(Table 7 and Figure 18). The ability of two distinct groups of monoclonal antibodies which bind to the same peptide sequence and inhibit the ADP-ribosyltransferase activity of toxin suggests that two overlapping epitopes exist within the A helix, and are near the proposed elongation factor 2 binding site. Furthermore, these results suggest the cross-reactive epitope may be conserved among ADP-ribosyltransferase toxins and important to toxin function.

Additional studies regarding the nature of the cross-reactive epitope were performed using monoclonal antibody T20. The ability of cross-reactive monoclonal antibodies to neutralize ADP-ribosyltransferase activity suggests that the epitope could be near His 426 since this site has been shown to be critical to ADP-ribosyltransferase activity. Synthetic peptides were used to determine if the cross-reactive site included His 426 and adjacent residues in the A helix (419-432). Peptides including residues 413-438 and 427-451 bind cross-reactive Mab T20 (Figure 17). The location of these sequences in the toxin molecule and their role in ADP-ribosyltransferase activity of toxin has already been shown (Figure 14 and Figure 15). ADPRT-neutralizing monoclonal antibody TC-1 does not cross-react with these toxins even though it recognizes the same peptide fragment as T20. Competitive binding assays show that T20 does not compete with TC-1 for binding to toxin indicating that the two antibodies bind to distinct epitopes the A helix sequence (Figure 18).

Antipeptide sera against residues 419-432 and 427-438 inhibit the ADP-ribosyltransferase activity of toxin (Figure 15). Additionally, these antibodies are able to recognize diphtheria toxin, cholera toxin and pertussis toxin as well as exotoxin A and CRM-66 (Figure 19 and Table 15). The ability of neutralizing antisera to cross-react with several ADP-ribosyltransferase toxins suggests a common epitope associated with these toxins perhaps associated with either substrate binding (NAD+ or elongation factor 2) or catalysis. Furthermore, a homologous site between exotoxin A and
diphtheria toxin would not be unexpected due to similarities in their substrates and enzymatic activity. Diphtheria toxin's ADP-ribosyltransferase activity was measured in the presence of cross-reactive antisera to determine if the cross-reactive site was associated with ADP-ribosyltransferase activity of diphtheria toxin as well as exotoxin A. Figure 22 illustrates that antisera specific for exotoxin A sequences 419-432 and 427-438 inhibits ADP-ribosyltransferase activity of both exotoxin A and diphtheria toxin. Due to the antibodies' inability to neutralize the NAD$^+$ glycohydrolase activity of toxin (data not shown), it was not unexpected that their site-specific antisera did not affect this activity in diphtheria toxin (data not shown). These results differ from previous results reported by Sadoff et al. (1982). They detected cross-reactivity between exotoxin A and diphtheria toxin using rabbit antisera against intact exotoxin A. However, their antibody inhibited exotoxin A's ADP-ribosyltransferase activity but did not affect the enzymatic activity of diphtheria toxin fragment A. Studies involving the antigenicity of diphtheria toxin suggest that there are at least two independent antigenic regions on diphtheria toxin fragment A with one located in or near the active site of the molecule (Rittenbery, et al., 1976). The cross-reactivity detected by Sadoff et al. (1982) may be directed to a different antigenic site. Thus, this would explain the inability of their antisera to neutralize the ADP-ribosyltransferase activity of the A fragment of diphtheria toxin.

In the present study, antiserum against diphtheria toxin was able to neutralize the ADP-ribosyltransferase activity of diphtheria toxin although it did not affect the activity of exotoxin A (Figure 22). Anti-diphtheria toxin serum was tested for reactivity with toxin peptides to determine whether it recognized a similar sequence. As shown in Table 16 and Figure 20, anti-diphtheria toxin react with peptides 413-438 and 427-451. Additional studies using smaller peptides revealed that toxin peptides 419-432 and 427-438 partially block binding of anti-diphtheria to the homologous protein toxin (Figure
21). These results indicate the existence of a cross-reactive epitope on both diphtheria toxin and exotoxin A that may be involved in binding of elongation factor 2. Interestingly, previously reported cross-reactivity was detected between whole exotoxin A and trypsin-treated diphtheria toxin, suggesting a site that is accessible on exotoxin A but hidden in diphtheria toxin (Sadoff, et al., 1982). This supports the existence of two distinct sites on exotoxin A and diphtheria toxin that may be involved in cross-reactivity.

The ADP-ribosyltransferase proteins cholera toxin and *Escherichia coli* heat-labile toxin share a high degree of amino acid sequence homology. These toxins and pertussis toxin catalyze the ADP-ribosylation of a GTP-binding protein which results in increased cellular cAMP levels. Recently, cross-reactivity was detected between cholera toxin, *E. coli*'s heat-labile toxin and pertussis toxin by antisera against residues 6-17 of pertussis toxin's catalytic domain (Burns, et al., 1987). Previous reports show that substitutions in this region reduce ADP-ribosyltransferase activity of pertussis toxin but do not affect NAD⁺ glycohydrolase activity (Barbieri and Cortina, 1988). This suggests that the cross-reactive site may be involved in binding the G-protein component. However, a conserved structure does not necessarily indicate conserved amino acid sequences. Sequence analysis of several NAD⁺ dehydrogenases predicts a common secondary structure although no apparent sequence homology exists (Rossman, et al., 1974). This structure is found in NAD⁺ binding proteins such as lactate dehydrogenase, cholera toxin and diphtheria toxin. However, analysis of the three-dimensional structure of exotoxin A does not identify such a structure. Results from the present study and those of Sadoff et al. (1982) indicate that two cross-reactive sites exist on exotoxin A and diphtheria toxin. Antibody against toxin peptides 419-432 and 427-438 recognize diphtheria toxin, cholera toxin, and pertussis toxin although amino sequence homology has only been detected in exotoxin A and diphtheria toxin
(Carroll and Collier, 1988) (See Appendix A for amino acid sequences of ADP-ribosyltransferase toxins). Synthetic peptides representing the entire S₁ subunit of pertussis toxin were tested for reactivity with antisera against toxin peptides 419-432 (Figure 23 and Figure 24). Pertussis peptides containing residues 51-77 partially block the binding of the anti419-432 to pertussis toxin and exotoxin A in a competitive ELISA format. The MacVector protein analysis program has been used to analyze the predicted secondary structure of the pertussis S₁ subunit. This program provides the secondary structures as predicted by both the Chou and Fasman method (1974) and Robson and Garnier method (1978). The resulting graph (Figure 26) shows the secondary structures predicted by both methods. Interestingly, pertussis residues 65-75 are predicted to be in an alpha helical configuration. Analysis of pertussis S₁ residues 66-80 reveals that there is some sequence homology with toxin residues 422-440 (Figure 26). Additionally, the hydrophilicity plot determined by Kyte and Doolittle (not shown) shows that these residues are hydrophilic. This suggests that pertussis residues 51-77 may be involved in a similar structure as the exotoxin A residues 419-432. This cannot be confirmed until the three-dimensional structure of pertussis toxin and the other ADP-ribosyltransferase toxins is determined.

Figure 26. Secondary structure of the pertussis toxin S₁ subunit predicting residues 65-75 in an alpha helix.
The involvement of His 426 in ADP-ribosyltransferase activity of toxin has been described in this work. Interestingly, the same helix has been shown to contain a cross-reactive epitope which exists among ADP-ribosyltransferase toxins. Monoclonal antibody T20 recognized these toxins and neutralizes ADP-ribosyltransferase activity of toxin (Figure 6 and Figure 13). Using synthetic peptides, part of the T20 epitope has been defined in the sequence 427-432 (Figure 15). Monoclonal antibody TC-1 binds to an epitope contained within the 422-432 sequence but does not cross-react with the other ADP-ribosyltransferase toxins. The three dimensional structure of toxin shows that residues 419-432 form a long alpha helical segment in domain III (Figure 14); TC-1 and T20 appear to bind on opposite sides of the helix, or opposite ends. This is supported by the ability of cross-reactive antisera to bind CRM-66 as well as toxin. The entire helix, made up of residues 419-432, may be an important structure involved in positioning elongation factor 2 for binding to toxin. This model supports the role of His 426 in elongation factor 2 binding and suggests that sequence 427-432 (RQLEER) is involved in a conserved structure found in ADP-ribosyltransferase toxins. No homologous sequence has been found in the amino acid sequence analysis of diphtheria toxin, cholera toxin and pertussis toxin (Appendix A). However, the existence of a conserved structural feature among NAD⁺ binding proteins which lack amino acid homology lends support to the possible existence of a conserved structural feature among ADP-ribosylating toxins.
APPENDIX A

Amino acid sequences of ADPRT toxins

Exotoxin A (1-613) (Gray, et al., 1984)

AAEAFDLWNECACKACVLDLKDGVSSRRMSVDPAIADTNQ
QGVLHYSVLEGNDALKLAIDNALSITSDGLTIRLEGGV
EPNKPVRYTSYTRQARGWSLWLVPIHEKPSNIKVFHE
LNAGNQLSHMSPIYTIEMGDELLAKLRDATFFVRAHESN
EMQPTLAISHAGVSVVMAQTQPRRRERWSEWASGKVCLLLD
PLDGVYNYLAQQRCLNDDTWEGKRYVLAGNPAKHDLIK
PTVISYRFEPGLSLAALTHTQACRLPLETFTRHRQPRG
WEQLEQCGYPVQRVLYLAARLSWNQVDQVIRNALASPG
SGGDLGEAREIQPEQRALALTAAAESERFVRQGTGNDK
GAANADVVSTECTPVAAGECAGPADSGLALLERNYPTGAE
LDGGDVSTFRGTQNWTRLQAHRCLEEYVFVGYH
GTFLEAAQSIVFGGVTRARRSDLDAITRGFYIAAGDPAAYG
YAQDQEPDARGRIRNGALLRYYVPSSLPFGYRTSLTLAA
PEAAAGEVERLGHPLPELRDLAIGPEEGGGRLETILGWPL
AERTVVIPSAIPTDPRNVGGLDPSSIPDKEQAISALPDY
ASQPGKPPREDLK

* Amino acid sequence involved in the cross-reactive epitope (427-432) is indicated in bold print.
Diphtheria fragment A (1-194) (Greenfield, et al., 1983)

GADDVVDSSSFVMENFSSYHGTTKPGYVDSIQKGIEMKPS 40
GTQGN¥DDWKGYSTDYDAAGYSVDNEPLSGKAGGV 80
VKVTPGTLKVLAKVDNAETIKKELGLSLTEPLMEQVGT 120
EEFIKRGDGASRVLGPLFAEHSSSVEYINWQAKALS 160
VELEINFETRGKRRGNDAMYEYMAMQACAGNVRSS 194

*Diphtheria fragment A's sole histidine that has been implicated in NAD+ binding is indicated in bold print.

Pertussis toxin S1 (1-234) (Locht and Keith, 1986)

DDP¥ATVYRYDSRPPEDVFQNGFATWGNNDNVLDHLTGRS 40
CQVGSSNSAFVSTSSRRYTEVLYERMQEAVEAERAGRG 80
TGHFIGYTYEVRADNNFYGAASSYFYVDYVGDNRILA 120
GALAT¥QSEYLAHRRIPPNIRVRVHYNGITGETITTE 160
YSNARYVSQL®TRANP¥TSRRSVASIVGTLLVHGAGDSAC 200
MARQAESSEAMAAWSERAGEAMVLYVYESIAYSF 234

*Eight residues involved in amino acid sequence homology with cholera toxin and E. coli heat-labile toxin is indicated by bold print.

Cholera toxin A chain (1-238) (Mekalanos, et al., 1983)

DKLYRADSRPPDEIKQSGLMPRGQSEYFDRTGQMNINLY 40
DHTARGQTGVRHDGYVSTISLRSALVGQTLVGHT 80
YYT¥VIATAPNMFINVDGAYSPHPDEQVESALGGIPYS 120
Q¥YGW¥VRVHFGVLDHQHRNGYRD¥YSNLIAEDY 160
GLAGF¥PPEHRAWREPIHAPPGCGNAPRSSLNTCDEK 200
TQSLG¥KFLDEYQSKVRQIFS¥QSDIDTHNRIKDE¥ 238

*Eight residues involved in amino acid sequence homology with pertussis toxin and E. coli heat-labile toxin is indicated by bold print.
REFERENCES


