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Title of Thesis: Characterization of a Mutant Diphtheria Toxin that is Defective in Binding to Cell Membrane Receptors on Vero Cells

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ABSTRACT

Title of Dissertation: Characterization of a Mutant Diphtheria Toxin that is Defective in Binding to Cell Membrane Receptors on Vero Cells

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Mutants of corynebacteriophage β were isolated that coded for the production of mutant forms of diphtheria toxin (cross-reacting materials , CRMs) that were reduced in cytotoxicity due to defect(s) in the fragment B. A sensitive cytotoxicity assay was used to screen the supernatants of cultures of Corynebacterium diphtheriae that were infected with the mutagenized β phages. Several nontoxinogenic or hypotoxinogenic mutant phages were isolated by this procedure, plaque-purified, and used to prepare lysogenic derivatives of C. diphtheriae C7(-). A preliminary characterization of these lysogenic strains identified a new class of CRMS which were antigenically and enzymatically indistinguishable from wild-type toxin but were considerably less cytotoxic for Vero cells. Of these mutant toxins, CRM9 was the most reduced in cytotoxicity and was selected for

purification and further characterization.

Purified CRM9 was approximately 1,000-fold less toxic than diphtheria toxin in Vero cell cytotoxicity tests. The inhibition of protein synthesis in Vero cells by CRM9 and wild-type toxin proceeded at the same rate when equally toxic doses of each protein were compared. CRM9 and diphtheria toxin had identical electrophoretic mobilities in SDS-polyacrylamide gels, were antigenically indistinguishable in competitive-binding radioimmunoassays and in immunodiffusion tests, and had comparable NAD:EF2 ADPR-transferase activities after activation by trypsin treatment. ^{125}I-labeled CRM9 did not bind specifically to membrane receptors on Vero cells under conditions where specific binding of ^{125}I-labeled diphtheria toxin could be demonstrated. Competition between CRM9 and diphtheria toxin for cell membrane receptors could not be demonstrated directly in Vero cell cytotoxicity assays; however, CRM197 afforded complete protection to Vero cells from intoxication by both CRM9 and diphtheria toxin. These data indicated that CRM9 and diphtheria toxin bound to the same class of receptors on Vero cells as CRM197. Nucleotides protected Vero cells from intoxication by CRM9 or by toxin. The extent of protection was directly correlated to the number of phosphate residues in the nucleotides; however, the concentrations of nucleotides needed to protect against intoxication were lower with CRM9 than with wild-type toxin. Reconstitution experiments using the functional fragment A from CRM9 and the functional fragment B from CRM197 demonstrated that the defect in CRM9 was localized in the fragment B.

CRM9 represents a new type of mutant toxin that is defective in binding to cell membrane receptors. This mutant toxin will be a

valuable tool for studying the structural basis for the specific binding of diphtheria toxin to cell membrane receptors.

CHARACTERIZATION OF A MUTANT DIPHTHERIA TOXIN DEFECTIVE IN BINDING TO RECEPTORS ON VERO CELLS

BY

Terri Jean Phipps

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"The work, chiefly of Pappenheimer and his colleagues, in laying bare the mechanism of action of diphtheria toxin is as pretty a piece of what might be called molecular aetiology as would bring tears of pleasure to the eyes of the departed pioneers of bacteriology."

Anonymous, 1972

DEDICATION

To my parents for their continued support and encouragement throughout my education.

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INTRODUCTION

I. Early investigations.

The pathogenesis of diphtheria is complex. It was suggested nearly one hundred years ago that a toxic substance played a significant role in the severity of clinical diphtheria when Loeffler observed that injections of Corynebacterium diphtheriae into experimental animals produced both localized infections and damage to distant tissues. A second important observation was that the distant tissue lesions were found to be aseptic. Thus, Loeffler suggested that C. diphtheriae produced a toxin which was transported throughout the body (107). This hypothesis was confirmed when Roux and Yersin (168) demonstrated that cell-free filtrates from cultures from C. diphtheriae were lethal for animals (168).

Although toxinogenicity by itself is not sufficient for virulence (7, 65, 102), the importance of diphtheria toxin in the pathogenesis of the disease was demonstrated by the protection afforded to experimental animals by antitoxic antibodies (196). In addition, the subsequent development of a toxoid (162) that could be used in man led to a mass immunization program that decreased the incidence of clinical diphtheria (23). As a result of these early discoveries, diphtheria toxin has been a prototype for studying the role of toxins in bacterial pathogenesis (32, 129, 143).

Fifty years after its discovery, diphtheria toxin was isolated

and characterized chemically and physically (51, 139, 140). However, almost nothing was known of the mechanism by which diphtheria toxin damaged the tissues of a susceptible host. The physiological changes, in animals and in man, that follow intoxication by diphtheria toxin were studied extensively and many of these early studies were reviewed (73). It was generally agreed that the gross and microscopic morphological damage to tissues, the increased resistance to insulin (36), the reduced capacity to synthesize carbohydrates (38) and to metabolize lactic acid (40), all represented secondary changes which followed a rapid and irreversible primary injury (73, 142).

Pappenheimer predicted that the nature of the primary injury of diphtheria toxin in susceptible host tissues may be related to its role in the metabolism of C. diphtheriae (142). Certain observations on toxin production indicated that diphtheria toxin may be involved in oxidative mechanisms. Diphtheria toxin is produced only under strict aerobic conditions (105) and in high yields only when the iron concentration is reduced to growth-limiting levels. The addition of small amounts of iron above the optimal concentration inhibits toxin production (127, 147). In addition, a porphyrin is present in C. diphtheriae filtrates and its concentration parallels that of toxin (37, 44, 197). Porphyrin production, like toxin production, is inhibited by high concentrations of iron (147). Based on these data, Pappenheimer suggested that diphtheria toxin might be the protein moiety of the iron-containing respiratory enzyme, cytochrome b (141). Furthermore, he speculated that the primary defect produced by toxin in the host was the interference with a comparable host enzyme (142).

Diphtheria toxin was found to be active only in tissues that used the succinoxidase system for respiration (150). The effects of toxin were evident immediately during a period of growth which was dependent on progressive cytochrome synthesis and indicated that toxin interfered with cytochrome synthesis. These data were in contrast to an earlier report (154) that diphtheria toxin had no effect on enzymes involved in respiration, glycolysis, oxidations (including auto-oxidations) or phosphorous exchange in vitro. Although a defect in cellular respiration could lead to the extensive secondary effects that had been observed, the nature of the primary action of diphtheria toxin remained controversial.

The stabilization of tissue cells in culture permitted the study of uniform populations of cells. The effects of diphtheria toxin on several of these cell lines were reported (103, 156). Strauss and Hendee (182) extensively studied the effects of diphtheria toxin on many cellular processes of HeLa cells, a human epithelial carcinoma. In contrast to the studies by Pappenheimer (150) no significant effect on respiration was demonstrated. However, the synthesis of protein was significantly reduced in intoxicated cells when compared to control cells. The effects of toxin on protein synthesis were subsequently demonstrated with other cell lines (124, 125, 145, 146), with cell-free extracts (31, 35, 64, 90, 91), and in vivo (12, 61). Thus, the primary injury produced by diphtheria toxin is the inhibition of protein synthesis in eucaryotic cells.

Because early studies of diphtheria toxin were directed to understanding the mechanism of action of toxin, little was known about the structure-activity relationships of the toxin molecule. Several

observations concerning toxinogenesis in C. diphtheriae facilitated these types of studies. First, toxin production was maximal when the iron concentration of the culture medium became growth-limiting for C. diphtheriae (106, 147, 157). Increased yields of toxin were obtained following the isolation of a clinical strain, the Park-Williams No. 8 (PW8) strain (151), that produced very high yields of diphtheria toxin and the development of a semi-defined low-iron medium that was suitable for toxin production (128). Second, the structural gene for diphtheria toxin, tox, was located on the genome of corynephage β (75, 132, 187, 189) and genetic techniques for manipulating phage β were developed (75, 187, 189). The isolation of β^{tox} mutants that produced mutant toxins, or cross reacting materials (CRMs), greatly facilitated the study of diphtheria toxin structure and activity. The properties of several of these CRMs are summarized in Table 1. These aspects of toxinogenesis facilitated the detailed study of the structure-activity relationships of diphtheria toxin and are discussed in more detail below.

II. Lysogeny and toxinogenicity.

Diphtheria toxin is encoded on the genome of some corynebacteriophages. d'Herelle first reported the presence of plaque-forming corynephages in 1918 (41). Nontoxinogenic strains of C. diphtheriae were converted to toxinogenic strains by infection with bacteriophage β (56, 57). These observations were confirmed and extended in other laboratories and a relationship between toxinogenesis

Table 1. Properties of Diphtheria Toxin and Cross-reacting Materials (CRMs)[a]

Protein	Molecular Weight	Toxicity[b] (MLD/μg)	Enzyme Activity (%)[c]	Blocking Activity[d]
Toxin	62,000	25 - 30	100	Not Tested
Fragment A	21,150[e]	0	100	None
CRM1[f]	20,000	0	0	Not Tested
CRM30	30,000	0	100	Not tested
CRM45	45,000	0	100	None
CRM176	62,000	0.05-0.1	8 - 10	Not Tested
CRM197	62,000	0	0	100
CRM228	62,000	0	0	10 - 15

[a]Modified from Uchida et al (189).

[b]MLD = minimal lethal dose. One MLD is the amount of toxin that will kill a 250 gram guinea pig in 4 to 5 days.

[c]NAD:EF2 ADPR-transferase activity per mole after activation by trypsin and reduction relative to fragment A taken as 100.

[d]Ability to competitively inhibit the action of toxin on HeLa cells. The blocking activity of CRM197 is taken as 100.

[e]Determined from the amino acid sequence (42).

[f]From Holmes (74).

and lysogeny was demonstrated (11, 66, reviewed in 6, 8). Only certain strains of corynephages could convert nontoxinogenic strains of *C. diphtheriae* to toxinogenic strains (5, 67, 71, 152). The genetic determinant on these corynephages that mediated the conversion to toxinogenicity was designated *tox*$^+$ (5) and was subsequently mapped on the corynephage β genome by matings between phage β and the related nontoxinogenic phage γ (75). *Tox* mutants were isolated and the mutations were mapped on phage β to the same region as the *tox*$^+$ determinant (180). In addition, the isolation of phage β mutants that coded for the production of nontoxic CRMS (Table 1) (187, 189) and the *in vitro* synthesis of diphtheria toxin and CRM45 from DNA obtained from either phage β or the mutant phage β*tox*$^{-45}$, respectively (132), provided clear evidence that the structural gene for toxin, *tox*, was encoded on corynephage β. The *tox* gene on the vegetative map of corynephage β is located between the host range markers *h* and *h'* (75, 180). Additional mutants of phage β (100) were used to demonstrate that the prophage genetic map is a circular permutation of the vegetative genetic map, with the gene for toxin at one end of the prophage map and the gene for phage immunity, *imm*, at the other end. This cyclic permutation is in accord with the Campbell model for integration (27) if the phage attachment site is in fact located between the toxin and immunity genes on the vegetative map (100). The orientation of transcription of the *tox* gene has been determined (74, 100). More recently, the physical map of β has been constructed and the *tox* marker has been located on this map (25). In addition, the attachment site and the cohesive sites have been located. The behavior of these sites indicated that the transition of the

vegetative phage to the prophage state involves the circularization of the phage DNA and integration onto the bacterial chromosome via the attP site (24).

III. Factors which regulate toxinogenesis.

The production of diphtheria toxin is controlled by lysogenic conversion (11, 56, 57, 66). The genetic and physiologic factors that regulate toxinogenesis include the bacterium, the bacteriophage, and the concentration of iron in the bacterial medium. The mechanism by which these factors interact to control toxin production is not clear.

Strains of C. diphtheriae exhibit varying capacities for expression of the tox gene. The PW8 strain hyperproduces toxin and is lysogenic for phage P (102), a phage with some similarities to phage β (76, 102, 113). When phage P from the PW8 strain was used to lysogenize a different strain of nontoxinogenic C. diphtheriae, called C7, toxin was produced at a rate characteristic of strain C7(β) and not at the elevated rate of the PW8 strain (113). Phage were isolated from nontoxinogenic strains of C. diphtheriae that converted other nontoxinogenic strains of C. diphtheriae to toxinogenicity (68, 152). Mutants of C. diphtheriae have been described that are associated with decreased toxin yields (rev. in 179). Thus, the host bacterium has an influence on the rate and final yield of toxin synthesis.

Mutants of corynephage β have also been isolated that are

associated with reduced yields of toxin (188). The mutations affecting toxin production in these mutants are *cis* dominant (rev. in 179) and suggest that the phages are altered in a gene that regulates toxinogenesis. Phage mutants with CRM^- phenotypes have also been reported (74, 99). Thus, the bacterial host strain and the infecting bacteriophage both act to regulate toxinogenesis.

The effects of nutrients in the growth medium, of which the effect of iron is the most striking, influence the yield of *tox* gene products (106, 128, 147, 157, 178). Toxin is produced in maximal yields when the iron concentration becomes growth-limiting for the host bacterial strain (7). The addition of iron to iron-starved cultures results in rapid inhibition of toxin production (134, 189). Recent evidence suggests that regulation of toxinogenesis by iron operates at the level of transcription of the mRNA for diphtheria toxin (134).

The effects of iron on toxin synthesis were examined in *in vitro* systems programmed with mRNA from toxinogenic strains of *C. diphtheriae* (78) or with phage β DNA (132). In cell-free systems prepared from *C. diphtheriae* and programmed with mRNA, toxin production was inhibited by extracts from *C. diphtheriae* PW8 grown in iron excess (4μg Fe^{++}/ml) but not from PW8 grown in low-iron medium (0.01 μg Fe^{++}/ml) (78). When programmed with DNA from phage β, toxin was produced in extracts from *Escherichia coli* but not in extracts from nontoxinogenic *C. diphtheriae*, although other phage-encoded proteins were synthesized in both systems. The addition of iron to the *E. coli* toxin-synthesizing system had no effect on toxin production; however, the addition of *C. diphtheriae* extracts inhibited

toxin production. These data indicated that corynebacterial factors were involved in toxinogenesis (78, 132).

Mutants of C. diphtheriae that were insensitive to the inhibitory effects of iron were isolated (89). Phage β isolated from these mutants did not convert wild-type C. diphtheriae strains to iron-insensitive strains and indicated that these mutations were located in the bacterial genome. These data are consistent with the possibility that corynebacterial factors act to repress toxinogenesis. However, it is also possible that the mutations in C. diphtheriae resulted in an impaired ability to bind or transport iron to the cytoplasm.

Mutants of phage β which confer resistance to the inhibitory effects of iron on toxinogenesis have also been isolated (133, 199, 200). A mutant designated $\beta\frac{tox^+}{ctl}$ converted wild-type C. diphtheriae to a partially iron-insensitive Tox^+ phenotype (133). The toxin produced by $C7(\beta\frac{tox^+}{ctl})$ was indistinguishable from diphtheria toxin and suggested that the mutation was located outside of the structural gene for toxin. Based on these data, Murphy and Bacha (130) proposed that C. diphtheriae produced a factor, an aporepressor, that acted together with iron as a negative controlling element in the regulation of toxinogenesis in C. diphtheriae. Additional phage mutants that are insensitive to iron have been described (199, 200). One of these, designated $\beta\frac{tox\text{-}201}{}$, was more resistant to inhibition by iron than $\beta\frac{tox^+}{ctl}$. The tox-201 mutation was found to be contiguous to the tox locus on phage β and was closely linked to the proximal end of the toxin structural gene. The location and the cis-dominant expression tox-201 indicated that the most likely genetic elements to be

considered for the tox-201 site were the promoter, the operator, and the attenuator (199, 200). However, further observations on toxin production by $\beta^{tox-201}$ and β^{tox^+} indicated that additional factors were important for the regulation of toxinogenesis. Toxin was produced by C7($\beta^{tox-201}$) and C7(β^{tox^+}) only late in the growth cycle, and Welkos and Holmes postulated that toxin production was controlled, in part, by physiologic changes associated with the terminal stages of growth that were not dependent on iron deficiency (199,200).

The isolation and characterization of additional mutants of corynebacteria and corynebacteriophage that are altered in toxinogenesis, the isolation of the postulated repressor and characterization of its interactions with DNA from phage β, and further examination of the metabolism of C. diphtheriae in the terminal stages of growth may all contribute to a better understanding of the regulation of toxin production.

IV. Structure-activity relationships of diphtheria toxin

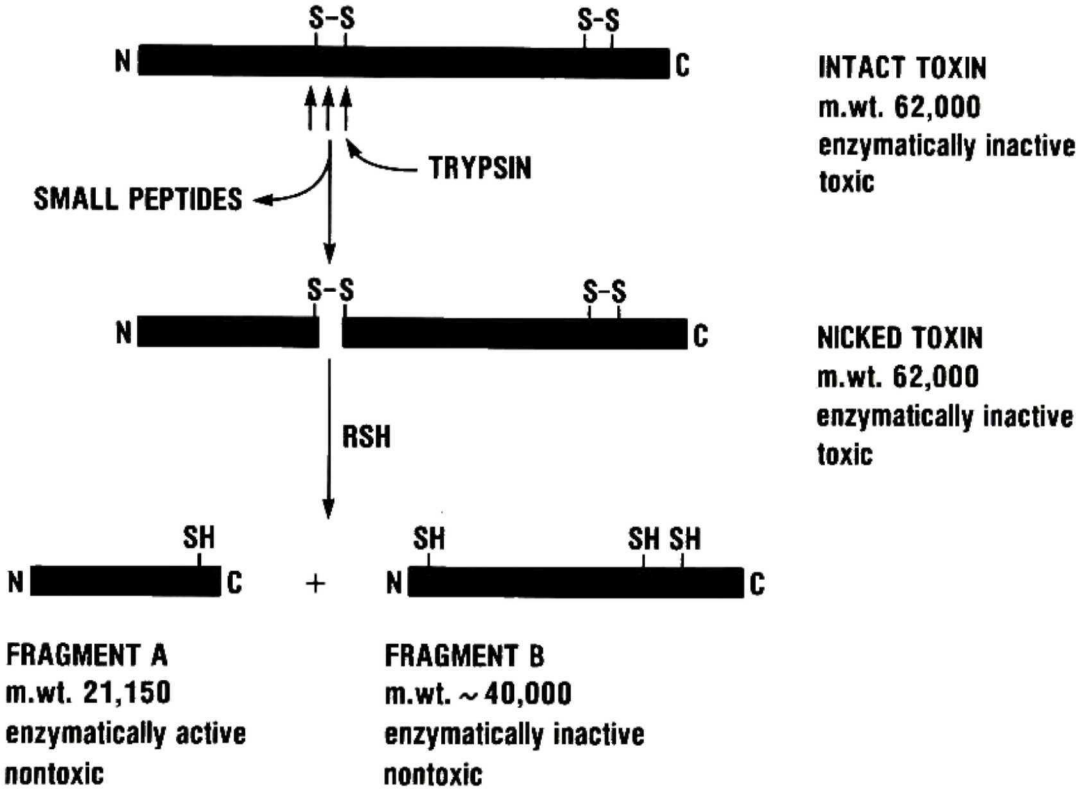
Diphtheria toxin was purified to homogeneity and the initial characterizations of structure-activity relationships were reported simultaneously by two groups of investigators (34, 59). Diphtheria toxin is secreted as a single polypeptide with a molecular weight of 62,000 (Figure 1). Three arginine residues at positions 190, 192, and 193 comprise a trypsin-sensitive region (42). When proteolytic cleavage occurs at one of these sites, three distinct forms of fragment A can be generated.

FIGURE 1

Activation of diphtheria toxin and activity of various forms and fragments of toxin[a]. Intact diphtheria toxin can be cleaved by trypsin to generate a nicked form of toxin that is joined by an interchain disulfide bond. Both of these forms are enzymatically inactive but toxic for intact cells. When toxin is treated with trypsin and a reducing agent (RSH), two fragments are formed. The amino-terminal fragment A is enzymatically active but fragment B is inactive. Neither fragment A nor fragment B, by itself, is toxic for intact cells.

[a]Illustration modified from Collier (32).

FIGURE 1


S-S
S-S
N
C
INTACT TOXIN
m.wt. 62,000
enzymatically inactive
toxic
TRYPSIN
SMALL PEPTIDES
S-S
S-S
N
C
NICKED TOXIN
m.wt. 62,000
enzymatically inactive
toxic
RSH
SH
SH
SH SH
N
C
+
N
C
FRAGMENT A
m.wt. 21,150
enzymatically active
nontoxic
FRAGMENT B
m.wt. ~40,000
enzymatically inactive
nontoxic

Diphtheria toxin contains two disulfide bonds. A cysteine residue, located near the C-terminus of fragment A, forms one half of the internal disulfide bridge that joins fragments A and B (33, 115, 143). The second disulfide bond is within fragment B. Therefore toxin can exist in an intact form or a nicked form in which the A and B fragments remain joined by a disulfide bond. Both of these forms are toxic, but they contain no enzymatic activity. When nicked toxin is reduced, the covalent linkage joining fragments A and B is disrupted and fragments A and B can be separated.

The 21,150 dalton (42, 115) amino-terminal fragment, fragment A, contains all of the enzyme activity associated with diphtheria toxin. Fragment A catalyzes the transfer of the ADP-ribose portion of NAD^+ to eucaryotic elongation factor 2 (EF2) (79, 80, 81); ADP-ribosylated EF2 is not functional in protein elongation. The resulting inhibition of protein synthesis leads to cytotoxicity.

A 39,000 dalton polypeptide is generated from the carboxyl-terminus; this fragment is called fragment B (34, 49, 59, 60). Fragment B is responsible for binding to cell membrane receptors[1] (49, 60, 187, 189) and possibly for the translocation of toxin or fragment A to the cell cytosol. The binding function of fragment B has been confirmed and is discussed in detail below.

Neither fragment A nor fragment B by itself is highly toxic for intact cells. At high concentrations, fragment A has low toxicity

[1] The term receptor will be used to describe a binding site on the cell surface that is specific for diphtheria toxin. The nature of the receptor, or receptors, that are required for the uptake of biologically active diphtheria toxin has not been determined, although several candidates have been proposed (see section V, Introduction).

whereas fragment B is nontoxic. Thus, the toxicity of diphtheria toxin requires both the enzymatic function of fragment A and the binding function of fragment B (143, reviewed in 32). The intracellular action of diphtheria toxin has been well-characterized; however, the mechanism by which diphtheria toxin binds to cell membrane receptors and traverses the plasma membrane has not been adequately explained.

The isolation of β phages that carry mutations in the tox gene and the mutant toxins (CRMs) encoded by these β phage have been useful for studying the structure-activity relationships of diphtheria toxin (74, 99, 189). The properties of some of these CRMs are outlined in Table 1. Nonsense mutations in the tox gene can give rise to CRMs containing only the amino-terminal portion of toxin. Some of these CRMs retain the enzymatic activity associated with fragment A but are nontoxic because they lack all or part of fragment B (CRM30, CRM45) (189, 190), and some are shorter than fragment A and lack enzymatic activity (CRM1) (74). In contrast, CRM176, CRM197, and CRM228 are probably the result of missense mutations in the tox gene (189, 190). These CRMs either lack part (CRM176) or all (CRM197, CRM228) of the ADPR-transferase activity of fragment A. CRM 176 retains partial ADPR-transferase activity and has a normal B fragment; thus, the specific toxicity of CRM176 is less than that of native toxin. CRM228 presumably results from two missense mutations. It has no ADPR-transferase activity and has only partial blocking activity. CRM197 has a normal fragment B but is nontoxic because it lacks the ADPR-transferase activity associated with fragment A.

A. Structure-activity relationships of fragment A

Purified fragment A is stable to denaturing agents such as acid, alkali, and heat (59). This stability has allowed extensive characterization of its structure and activity (49, 59). Fragment A catalyzes at least three reactions involving NAD^+:

(1) $NAD^+ + EF2 \rightarrow ADPR\text{-}EF2 + nicotinamide + H^+$
(NAD^+:EF2 ADPR-transferase)

(2) $NAD^+ + H_2O \rightarrow ADPR + nicotinamide + H^+$
(NAD^+-glycohydrolase)

(3) $NAD^+ + toxin \rightarrow ADPR\text{-}toxin + nicotinamide + H^+$
(auto-ADP-ribosylation)

The NAD^+-glycohydrolase reaction and the auto-ADP-ribosylation reactions are probably not important *in vivo* (88, 143). The glycohydrolase reaction proceeds very slowly and only at high concentrations of NAD^+ (88). ADP-ribose and ribose-5-phosphate are degradation products of the NAD^+-glycohydrolase reaction and can act non-enzymatically to form Schiff's bases with many proteins (98); this may be the basis for the auto-ADP-ribosylation of toxin which occurs only at high concentrations of toxin (143). Fragment A contains a single NAD^+ binding site with an apparent dissociation constant (K_I) of approximately 8 μM. This site catalyzes both the NAD^+-glycohydrolase reaction and the NAD^+:EF2 ADPR-transferase reaction (88).

The importance of NAD:EF2 ADPR-transferase activity *in vivo* has been demonstrated by several criteria. First, CRM176 and CRM197 each contain a missense mutation in fragment A which alters the ability to catalyze the transfer of ADP-ribose to EF2 *in vitro* and *in vivo*. CRM176 retains only 8 to 10% of the enzymatic activity of wildtype toxin and is only 0.2 to 0.4% as toxic for cells; CRM197 has no enzymatic activity and is therefore nontoxic (189). Second, the restoration of EF2 activity in extracts from intoxicated cells by the addition of toxin and nicotinamide indicated that the ADPR-EF2 complex was present in intoxicated cells (62, 122, 126). Although the ADPR-EF2 complex has not been isolated from intoxicated cells, the amount of EF2 available for ADP-ribosylation in extracts of intoxicated cells decreased after exposure to toxin. Like intoxication, the rate of decrease in ADP-ribosylatable EF2 was dependent on the concentration of toxin and on the time of exposure of cells to toxin (126, 206) In addition, there was a lag period prior to the decrease in ADP-ribosylatable EF2 (126, 206); this lag time was similar to the lag time that preceded the inhibition of protein synthesis in intoxicated cells (177, 182, 191, rev. in 32). These data provided evidence that the NAD^+:EF2 ADPR-transferase activity was responsible for inhibition of protein synthesis and subsequent cytotoxicity in cells exposed to diphtheria toxin. The isolation of the ADPR-EF2 complex would provide direct evidence that protein synthesis was inhibited by this mechanism *in vivo*.

A single NAD^+ binding site (28, 88, 108) catalyzes both the NAD^+-glycohydrolase and NAD^+:EF2 ADPR-transferase reactions (88). The amino acid sequence of fragment A has been determined (42, 115), but

the amino acids that comprise the active site have not been identified. Two amino acid residues that are required for ADPR-transferase activity have been identified. Chemical modification of a single tryptophan causes complete loss or almost complete loss of this activity (32, 114) and modification of a single tyrosine (tyrosine 46) results in loss of both ADPR-transferase activity and the ability to bind NAD^+ (14, 15). In addition, CRM1 (MW 20,000) contained most of fragment A but lacked enzymatic activity; these data indicated that the C-terminal residues of fragment A are essential for ADPR-transferase activity (74).

The ADPR-transferase activity of fragment A is specific for the EF2 from eucaryotic sources (86, 104, 143, 165), but not for the analogous EF-G from most procaryotic sources or from mitochondria (165). Recent data has demonstrated that the corresponding elongation factor from the procaryotic Archaebacteria is ADP-ribosylated by diphtheria toxin (93). The striking specificity for eucaryotic EF2 as a substrate presumably results from the unique amino acid diphthamide, a modified histidine residue, which is present in EF2 and to which ADP-ribose becomes covalently attached (166, 194). Several mutant cell lines are resistant to diphtheria intoxication; these cell lines presumably do not contain the enzymes necessary for post-translational modification of histidine residues and, hence, do not contain diphthamide (124).

B. Structure-activity relationships of fragment B

Elongation factor 2 is a cytosolic protein, hence diphtheria toxin, or its fragment A, must reach the cytosol before the ADP-ribosylation of EF2 can occur. The relatively thorough understanding of the intracellular mechanism of action of diphtheria toxin is in marked contrast to our understanding of the binding of diphtheria toxin to cell membrane receptors and the entry of toxin or fragment A into the cell cytosol. It was noted relatively early that animal species varied greatly in sensitivity to diphtheria toxin (168). Rodents are relatively resistant to toxin, whereas human and guinea pigs are exceedingly sensitive (181). Similar variations in the sensitivities of cell cultures derived from different animal species were reported (58, 181), yet the protein-synthesizing systems in cell-free extracts from all eucaryotic cells are equally sensitive to the ADPR-transferase activity of fragment A (22, 31, 86). These observations suggested that sensitivity or resistance to toxin was determined at the level of the cell membrane by the exclusion of toxin from the cytosolic target.

Resistance to diphtheria toxin and therefore the mechanism by which fragment B mediates the binding and translocation of toxin into sensitive cells has been studied extensively in the last decade. Various approaches have been used to examine these steps of intoxication and each has enhanced our understanding of the initial interaction between toxin and the plasma membrane, the penetration of the hydrophobic core of the lipid bilayer, and the translocation of toxin or fragment A to the cell cytosol. These aspects of

intoxication have been studied by examining 1) the role of endocytic uptake of toxin, 2) the effects of inhibitors of cytotoxicity and binding, 3) the specific binding of toxin to cell membranes, and 4) the structure of fragment B.

1. The role of endocytosis in toxin internalization

Until recently, it was generally believed that protein molecules which reach the cytoplasm in a biologically active form did so solely by endocytosis (169). Several studies demonstrated that diphtheria toxin was internalized by endocytosis (18, 20, 121, 170). In one study, latex beads were coupled to diphtheria toxin and were incubated with resistant mouse macrophages or with sensitive guinea pig macrophages. Although both cell types were actively phagocytic, the mouse macrophages were refractory to toxin internalized by endocytosis whereas guinea pig macrophages were sensitive (170). In a second study (121), nonspecific pinocytotic internalization was stimulated by the compound poly-L-ornithine; the increase in pinocytic activity was demonstrated by the increase in lysosomal vesicles (acid phosphatase-positive vesicles) (4, 13). Poly-L-ornithine increased both pinocytosis and the sensitivity of resistant mouse L cells to diphtheria toxin, but sensitivity was not increased to the level of normally sensitive cells (121). These authors (121, 170) concluded independently that although toxin was internalized by resistant cells, it remained trapped within the endocytic vesicles and was not usually biologically expressed. Thus, the marked resistance of these cells was based on the ability of the membrane to prevent the toxin molecule from reaching the cytosol. These studies did not distinguish between

the exclusion of toxin from the cytosol due to a lack of specific receptors on the membrane or due to lack of a transport system to internalize the bound toxin.

Resistant cells are normally intoxicated only by high concentrations of toxin, fragment A, or nontoxic CRMs that retain enzymatic activity, such as CRM30 or CRM45 (see Table 1 (123, 148)). However, one molecule of fragment A, when artifically introduced into the cytosol of resistant cells, was sufficient to kill the cell (205). Protein molecules entrapped within endocytic vesicles ordinarily become degraded after fusion of the vesicles with lysosomes. At high concentrations, a sufficient number of molecules of toxin or fragment A may escape degradation by lysosomal enzymes and be released into the cytosol during the fusion of primary and secondary lysosomes (170). This mechanism of entry may explain the intoxication of resistant cells. In contrast, the exceedingly low concentration of toxin required to kill sensitive cells suggests that toxin internalized by the nonspecific endocytic route is probably not biologically expressed and is therefore not physiologically significant (20, 170).

2. Inhibitors of cytotoxicity

Factors that interfere with intoxication often provide useful information concerning the mechanism by which intoxication proceeds. The effects on cytotoxicity of several compounds, such as antitoxin (182), metabolic inhibitors (85, 207), compounds containing primary amines or phosphate groups (95, 96), and lectins (47) were examined. In addition, the effects of altering the ionic conditions of the

tissue culture medium by changing either the pH or the salt content were also studied (48, 50, 166, 171).

Sera containing antitoxin antibodies were adsorbed with the nontoxic CRM45 and were therefore specific for the 17,000 dalton C-terminus of the toxin molecule (149). Although the adsorbed antisera contained only a fraction of the toxin-precipitable antibodies present before adsorption, all of the original neutralizing activity was retained. In addition, anti-fragment A antibodies had almost no capacity to neutralize the toxicity of diphtheria toxin (149, reviewed in 32). Thus, neutralizing antibodies prepared by immunization with diphtheria toxin are directed primarily against antigenic determinants located on fragment B (149, reviewed in 32). Cells exposed to toxin in the presence of antitoxin are protected from cytotoxicity (182). At 10° C or lower, toxin bound to cells but was maintained in an antitoxin-sensitive state, presumably at the cell surface (95). The simplest explanation for these observations is that antitoxin prevented the irreversible binding of toxin to cell surface receptors or prevented subsequent conformational changes that allowed toxin to penetrate the lipid bilayer and to enter the cell cytosol.

Two general metabolic inhibitors, KCN and NaF, prevent intoxication of cells and maintain toxin in a form accessible to antibody, suggesting that toxin remains at the cell surface (85). Other inhibitors of metabolism and uncoupling agents do not (85). KCN and NaF are such broad spectrum cellular poisons that interpretation of their precise effect on intoxication is not possible.

Ammonium salts and other primary amines, such as glutamine and prolamine, inhibit cytotoxicity (95). Kim demonstrated that in the

presence of these compounds toxin remained on the cell surface in an antitoxin-sensitive state and intoxication proceeded normally when the ammonium salts were removed (96). Ammonium chloride does not inhibit the in vitro catalytic activity of fragment A nor the initial absorption of the toxin to the cell surface. These data suggested that compounds containing primary amines blocked the penetration of toxin but not the initial binding of toxin to the cell surface (95). These observations could be explained by 1) a conformational change in the toxin molecule after the initial receptor binding step that either altered or masked the antigenic determinants or 2) by an internalization step, such as receptor-mediated endocytosis, that sheltered the antigenic determinants (48).

Receptor-mediated endocytosis is a process of internalization whereby the toxin initially binds to a specific receptor and the receptor-toxin complex is internalized; the endocytosed vesicle then fuses with a lysosome (153). Fragment A, or toxin, must then traverse the membrane of the phagolysosome to exert its toxic effect on protein synthesis. The pH within lysosomal vesicles is low relative to the pH of the cell cytosol (135), thus, a mechanism of internalization such as receptor-mediated endocytosis indicates that low pH may facilitate the passage of toxin through the membrane. The effects of pH on toxicity were examined with ammonium chloride and chloroquine; both are lysosmotropic drugs which neutralize acidic intracellular pH gradients (48). At neutral pH, ammonium chloride and chloroquine protected cells against diphtheria toxin. Brief exposure of the cells to low pH (4.5-5.5) completely abolished this protection (171). When cells were exposed to both toxin and low pH, these drugs had no effect

on intoxication and suggested that an acidic environment may be required to translocate toxin to the cytosol (48, 171). The implications of these studies will be discussed below (see Model for Entry).

Regardless of whether toxin traverses the membrane at the cell surface or from within a vesicle, the initial interaction is with a specific receptor. The nature of the receptor has been studied by examining the effects of various agents on the initial adsorption of toxin. The isoelectric point of toxin is 4.1, indicating that the molecule has a net negative charge at neutral pH (203) and would be repulsed by the net negative charge of the cell surface (155, 185). An increase in pH or the presence of polyanions inhibited toxin adsorption (166). The absence of cations partially inhibited toxin adsorption to cells (50). Duncan concluded from these observations that the initial binding of toxin to the cell surface was electrostatic and involved positively charged surface groups on the toxin molecule which were neutralized at high pH or by polyanions, and that cations reduced the repulsion between the toxin molecule and the cell surface (50). Later it was reported that the C-terminal receptor-binding region of toxin contained a net positive charge (116, 144) which supported the hypothesis that the initial interaction involved a positively charged group on the toxin molecule which bound to the negatively charged cell surface.

The cell surface receptors for many plant and bacterial toxins are carbohydrate-containing molecules, either glycolipids or glycoproteins (72, 136, 193). Attempts to inhibit intoxication by diphtheria toxin with various monosaccharides were unsuccessful

(47). To determine whether toxin binding was specific for a more complex oligosaccharide, the ability of oligosaccharide-binding lectins to compete for intoxication by diphtheria toxin was examined (47). Cytotoxicity was prevented in the presence of concanavalin-A and wheat germ agglutinin and the protection was reversed by α-methly-mannoside and N-acetylglucosamine, respectively. These studies suggested that the diphtheria toxin receptor was an oligosaccharide which contained N-acetylglucosamine and mannose (47). A subsequent study showed that concanavalin-A did not inhibit the binding of radiolabeled toxin to the plasma membrane of Vero cells or BS-C-1 cells but did reduce the percentage of internalized toxin. From this evidence, it was concluded that the diphtheria toxin receptor was not a molecule containing N-acetylglucosamine or mannose (119). However, different cell lines were used in each of these studies. It is not known whether toxin binds to a similar receptor on different cell types.

Phosphate-containing compounds represent another class of agents that interfere with intoxication by diphtheria toxin (29, 117). Exogenous nucleotides protected sensitive Vero cells from the lethal effects of toxin. The protective potency was a function of the phosphate chain length, with tetraphosphates being the most inhibitory and monophosphates the least inhibitory (117). Nonhydrolyzable analogs of adenosine-5'-triphosphate (ATP) were also protective. In addition, differences were noted in the protective effect of the nucleoside triphosphates. Thymidine-5'-triphosphate (TTP), ATP, and guanosine-5'-triphosphate (GTP) were the most potent protective nucleotides, followed by uridine-5'-triphosphate (UTP) and

tripolyphosphate; the least protective nucleotide triphosphate was cytidine-5'-triphosphate (CTP). Furthermore, these nucleotides prevented the binding of radiolabeled toxin to Vero cells in a manner that correlated with the protective effect, and the inhibition of binding was competitive (117).

3. Specific binding of diphtheria toxin to the cell membrane

The specificity of the interaction between diphtheria toxin and the plasma membrane was demonstrated by the observation that CRM197 afforded complete protection to HeLa cells from intoxication by diphtheria toxin (84). A dose-ratio analysis of the protection by CRM197 showed that the protection was competitive, presumably reflecting competition between CRM197 and toxin for cell membrane receptors. The dissociation constant (K_I) for CRM197 was calculated to be approximately $1x10^8$ liters/mole (84). CRM45 did not block intoxication of HeLa cells by diphtheria toxin; therefore, the blocking activity of CRM197, or the receptor-binding activity of toxin, depended on the amino acid sequence or the conformation of the 17,000 C-terminus or both. Isoelectric focusing data indicated that the 17,000 dalton C-terminus was cationic. It was suggested that the initial reaction between toxin and the cell membrane was the binding of the cationic C-terminus of toxin with an anionic moiety on the cell membrane (189).

The uptake of ^{125}I-labeled diphtheria toxin and CRM45 by sensitive HeLa cells and resistant mouse L-cells showed that each HeLa cell contained approximately 4,000 toxin-specific binding sites; CRM45 did not bind specifically to HeLa cells. Resistant mouse L-cells

lacked toxin-specific binding sites (20). Competition experiments using CRM197 showed that the first interaction between diphtheria toxin and a specific cell membrane site was rapid and reversible and was followed by a slow, irreversible reaction during which the receptors became unavailable for interaction with additional toxin molecules. Although these experiments provided useful information about the initial interaction between toxin and the cell membrane, nonspecific uptake of labeled toxin approached 90% of the total uptake. In addition, the studies were conducted at a temperature where binding and internalization could not be differentiated. Two subsequent studies were conducted under conditions where binding was measured independently of internalization.

Twenty-one additional mammalian cell lines were examined for toxin sensitivity (116) and for the ability to bind radiolabeled diphtheria toxin (118). The low toxin concentration used in these studies reduced the nonspecific binding of toxin and incubation at 4°C prevented internalization. The number of specific binding sites per cell was directly proportional to sensitivity of the cells to toxin. Vero cells, a monkey kidney cell line, were most sensitive to diphtheria toxin and had the greatest number of specific binding sites per cell (approximately $1\text{-}2\times10^5$). The apparent association constant (K_a) was 9×10^8 liters/mole and was similar to the K_a for the toxin-receptor interaction on HeLa cell membranes (1×10^8 liters/mole, (84)). Several agents or conditions which interfered with intoxication also interfered with specific binding; 1) the specific binding was blocked by fragment B and CRM197, 2) labeled toxin-cell association was much greater at low pH (5.0), and 3) binding, like

cytotoxicity (116), was blocked by nucleotides and related phosphate-containing compounds (118). Thus, similar factors affected both binding and intoxication and suggested that these receptors were physiologically important for the uptake of biologically active toxin. The insensitive cell lines tested did not bind diphtheria toxin specifically which suggested that sensitivity was determined by the presence of a specific toxin-binding receptor on the cell surface.

Contradictory data about the ubiquity of the toxin receptor were reported (29). To examine binding independently of internalization, mammary gland or liver plasma membrane-enriched fractions were prepared from toxin-sensitive (rabbit and guinea pig) and toxin-resistant (rat and mouse) species. ^{125}I-diphtheria toxin bound specifically and reversibly to plasma membranes from both sensitive and insensitive cells. No significant differences were noted in the number of specific binding sites or in the association constants for the toxin-receptor interaction when cells from identical tissues of either sensitive or resistant animals were compared. Specific binding was blocked by nucleotides and required the presence of multivalent cations, properties that similarly affect intoxication in sensitive cells (50, 116, 117, 118). These data indicated that this specific binding was also physiologically related to the process of intoxication (29). It was concluded that resistance to diphtheria toxin was determined by a step that followed the initial binding of toxin, presumably a step in the transport process. Whether sensitivity to diphtheria toxin is determined by the binding of toxin to a specific cell membrane receptor or by the ability of the cell to

internalize toxin is not clear.

4. The hydrophobicity of fragment B

After an initial interaction with a receptor on the cell membrane, diphtheria toxin or its fragment A must traverse the plasma membrane or intracellular vesicle membrane in order to reach the cytoplasmic EF2. There is evidence that fragment B mediates the translocation of toxin to the cell cytosol.

Macromolecules which possess a hydrophobic domain can penetrate the lipid bilayer of eucaryotic cell membranes (16, 17, 43). Thus, the hydrophobicity of diphtheria toxin, CRM45, and the A and B fragments derived from them was examined. Triton X-100 replaced protein-bound phospholipids when membrane proteins were extracted into aqueous solvents; the extent of Triton X-100 binding indicated the hydrophobicity of the molecule to which it bound (30, 70). Boquet showed that CRM45, its fragment B, and fragment B from diphtheria toxin all bound equal amounts of Triton X-100. However, native diphtheria toxin did not bind Triton X-100. Thus, a hydrophobic domain was located in the N-terminal portion of fragment B and was masked in native toxin (21). Presumably, a conformational change occurred to expose this domain during the entry of toxin into the cytosol. CRM45 and the fragment B from diphtheria toxin could insert into liposomes composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. Furthermore, CRM45 retained full enzymatic activity after insertion into the artificial membranes, suggesting that insertion was unidirectional (19). The hydrophobicity of CRM45 and the fragment B of diphtheria

toxin was verified by charge-shift electrophoresis which differentiates amphiphilic and hydrophobic proteins (19). These data indicated that fragment B contained a hydrophobic domain that was located in its N-terminal portion and that could interact with the lipid bilayer of the cell membrane.

Fragment B has recently been purified to homogeneity. Five cyanogen bromide fragments from fragment B have been identified and the amino acid sequence of most of fragment B has been determined (54, 101). Two different lipid-associating domains were described. One of these lipid-associating domains corresponds to the hydrophobic domain described above; it is located in the central region of fragment B. This domain is structurally related to the membranous segments of integral membrane proteins and can interact with the hydrocarbon core of the phospholipids. These investigators proposed that this central hydrophobic domain of fragment B traversed the plasma membrane in a process of membrane penetration. In addition, a few negatively charged residues in this domain suggested that fragment B could be stabilized in the membrane by interaction with either itself, after formation of a multimeric complex, or with a preexisting integral membrane protein (92, 101). A second lipid-associating domain was highly hydrophilic and comprised the 9,000 dalton N-terminus of fragment B. This domain contains a narrow hydrophobic face surrounded by a large hydrophilic face (101) and is therefore similar to apolipopoteins which bind to the phospholipid headgroups of phosphatidylcholine molecules (176). This type of binding leads to reversible associations between proteins and surfaces of bilayers and to the formation of soluble lipid-protein complexes. Thus, fragment B

has the properties of a molecule which can penetrate the lipid bilayer of plasma membranes.

The biophysical data obtained from the amino acid sequence and the predictions of secondary structure have been a significant contribution to the study of the structure of fragment B.

V. Diphtheria toxin-receptor interaction.

Lectins and phosphate-containing compounds both inhibited cytotoxicity (see section IV, B2). These observations indicated that oligosaccharides or phospholipids, or both, on the cell surface were involved in the initial binding of toxin to cell surface receptors.

Concanavalin A and wheat germ agglutinin inhibited the cytotoxicity of toxin for Chinese hamster V79 cells and the inhibitory effects were reversed by methyl-α-mannoside and N-acetyl glucosamine, respectively. It was shown by a Schild analysis that the protection was competitive and the apparent dissociation constant (K_I) was $3x10^8$ liters/mole. The K_I for this interaction was similar to the K_I for the toxin-receptor interaction with HeLa cells ($1x10^8$ liters/mole) and indicated that Con-A and toxin competed for a common cellular target; thus, diphtheria toxin had lectin-like carbohydrate-binding properties (47). It was also possible that the binding sites for toxin and Con-A were structurally distinct and that occupation of the Con-A site sterically hindered toxin-receptor interactions. However, these studies were based on cytotoxicity and did not distinguish between binding to receptors and entry of toxin to the cell cytosol.

Subsequent studies to examine toxin binding at 4° C and toxin internalization at 37° C in Vero cells demonstrated that Con-A, or other lectins, did not block toxin receptor binding but prevented the subsequent internalization of toxin (118). However, since a different cell line was used in this study the results may not accurately reflect the effects of Con-A on other cells.

Subsequent to the report that lectins inhibited the intoxication of sensitive Chinese hamster V79 cells, Proia and collaborators (160) identified toxin-binding glycoproteins from the surface of guinea pig lymph node cells. Cell surface molecules were iodinated and a lentil lectin-binding glycoprotein fraction was prepared. One major toxin-binding component was immunoprecipitated from this fraction with diphtheria toxin and CRM197, but not with diphtheria toxoid, fragment A, or cholera toxin. Thus, specific binding was dependent on the presence of a functional fragment B from diphtheria toxin. A toxin-binding glycoprotein was not detectable in the cell membranes from toxin-resistant mouse L-cells. The toxin-binding glycoprotein represented only a small fraction of the total lentil lectin-binding glycoproteins, hence diphtheria toxin bound with greater specificity compared to classical lectins. The relevant component of the isolated glycoprotein to which the toxin bound was not identified. The specificity of the glycoprotein for fragment B from diphtheria toxin and the lack of a similar glycoprotein on resistant mouse L-cells suggested that this toxin-binding glycoprotein was physiologically relevant for intoxication (160).

These data provide evidence that diphtheria toxin binds specifically to a carbohydrate-containing compound; however, further

observations are required to demonstrate that the purified toxin-binding glycoprotein is physiologically relevant for intoxication. Although guinea pigs and mice represent toxin-sensitive and toxin-resistant species, respectively, the different sources of the cells used in these studies may not accurately reflect the presence or absence of toxin-binding glycoproteins in vivo. Guinea pig lymph node cells were obtained directly from the animals but the mouse L cells were a stabilized cell line. In view of the evidence by Chang and Neville (29) that plasma membranes isolated from rat and mouse cells did bind toxin specifically, it is possible that toxin-specific glycoproteins could be isolated from cells obtained directly from mice. In addition, the sensitivity of cells obtained from guinea pig lymph nodes was not reported and would be useful in evaluating the physiological relevance of the purified glycoprotein.

A glycoprotein with similar toxin-binding properties was subsequently isolated from hamster lymphocytes and thymocytes (158). The biochemical nature of the binding between toxin and the membrane glycoproteins was examined. Denatured toxin-binding glycoproteins were immunoprecipitated with diphtheria toxin but not with toxoid; thus, it was concluded that the secondary protein structure of the toxin-binding glycoprotein did not play a prominent role in the interaction with toxin. In addition, toxin-binding glycoproteins were immunoprecipitated with toxin following SDS-PAGE, indicating that contaminating lipids were not involved in the specific binding of toxin to the purified glycoprotein fraction.

The inhibition of cytotoxicity by the presence of exogenous nucleotides has been discussed. Middlebrook and Dorland (117)

concluded that the phosphate moiety of nucleotides prevented toxin-receptor interactions by either binding to the cell membrane receptor and sterically hindering the toxin-receptor interaction or by binding to an allosteric control site on the receptor which converted it to a form incapable of interacting with diphtheria toxin. However, more recent data indicated that the inhibition of cytotoxicity and of toxin binding to cell membranes by nucleotide triphosphates probably resulted from the inhibitor binding to the toxin molecule (110). Lory and Collier designated this phosphate-binding site the P-site (110). Based in part on the above observations, the observation that the receptor is present on toxin-resistant cells (29), and that pretreatment of cells with phospholipase C (an enzyme that removes phosphate-containing polar groups from phospholipids) decreased the inhibition of protein synthesis (120), Alving and collaborators examined the binding of toxin to phospholipids in liposomes (1). Diphtheria toxin bound to some, but not all, phospholipids (1), apparently by recognition of the phosphate groups on the phospholipids (83). Binding 1) was correlated with the degree of exposure of the phosphate, 2) did not occur with liposomes lacking a net negative charge, 3) was influenced by pH only during the initial interaction with liposomes, 4) was influenced by the composition of adjacent phospholipids that do not bind toxin, and 5) was inhibited by phosphocholine and ATP under certain conditions (1). These data indicated that the cellular receptor for diphtheria toxin may be the phosphate portion of a minor membrane phospholipid, such as phosphatidylinositolphosphate or phosphatidic acid, or some other phosphorylated membrane molecule.

Clearly, it has not been determined whether a glycoprotein, a phospholipid or phosphate-containing molecule, or both, are required for intoxication of cells by diphtheria toxin. Phosphatidylinositolphosphate and other phospholipids associate strongly with at least one intrinsic membrane glycoprotein (26, 195); thus, it is possible that both types of cell surface molecules may be required for the uptake of biologically active diphtheria toxin.

VI. Model for the entry of diphtheria toxin.

Boquet and Pappenheimer proposed a model for the process by which fragment A traverses the plasma membrane to reach the cytoplasm (20). They proposed the following sequence of events. 1) Specific cell surface receptors react reversibly with sites located on fragment B from toxin or CRM197. 2) The initial reaction is followed by a slow, irreversible process during which the receptors become inactivated, possibly by major conformational changes in the toxin molecule that allow fragment B to become embedded in the plasma membrane in association with the toxin receptor. During this process the disulfide bridge joining fragments A and B reaches the inner membrane surface. 3) Cleavage of the disulfide bridge occurs in the case of nicked toxin and fragment A is released into the cytosol. Fragment B remains embedded in the plasma membrane. In the case of intact toxin, hydrolysis of the peptide linkage may also occur during this step.

Most studies directed to examining the entry of toxin are

consistent with the basic assertions of this model. Recent evidence on the ability of toxin to bind to both a cell surface receptor and to the phosphate moiety of phosphate-containing compounds has raised questions about the nature of the receptor, or receptors, that mediate toxin entry. It is possible that the initial reversible reaction described by Boquet and Pappenheimer occurs with a toxin receptor, such as a glycoprotein, and that the major conformational changes in fragment B that facilitate insertion into the hydrophobic membrane bilayer result from interaction of fragment B with phosphate-containing compounds, such as phospholipids (109).

That the binding and entry steps of intoxication are indeed distinct steps was demonstrated by examining the properties of a toxin-related polypeptide designated 111-sup2-62 (3). This polypeptide was produced by the suppression of a nonsense mutation within the tox gene of phage $\beta^{tox-111}$ in an extragenic suppressor strain of C. diphtheriae C7 (2, 3). The specific ADP-ribosyltransferase activities of toxin and 111-sup2-62 were similar. The 111-sup2-62 polypeptide was as effective as CRM197 in blocking the intoxication of CHO cells by diphtheria toxin; however, the lag period that preceded intoxication by 111-sup2-62 was prolonged (3) when compared to that of toxin (3, 177, 191). Thus, the defect in 111-sup2-62 was found to be in a step subsequent to the specific binding to cell membrane receptors in some, but not all, sensitive cells (3).

The Boquet and Pappenheimer model is based on the assumption that the translocation of fragment A to the cytosol occurs at the plasma membrane. Recent data that the insertion of fragment B into

the membrane is enhanced at low pH (45, 87, 172) has provided evidence that fragment A may be translocated across an intracellular vesicle membrane (87). Thus, a mechanism such as receptor-mediated endocytosis may be required for intoxication. The initial toxin-receptor interaction would still occur at the cell surface and the sequence of events involved in translocation of fragment A would not necessarily be different from those steps described above. The observations that fragment A renatures at cytosolic pH (7.4) following exposure to lysosomal pH (4.7) (49) and that the fifteen minute lag period that precedes intoxication is similar to the lag period involved in receptor-mediated endocytosis (201, 202), both lend credence to this hypothesis.

VII. Diphtheria Toxin as a Model for Protein Internalization

The early discovery of diphtheria toxin (107, 168) and the development of techniques for the genetic manipulation of bacteriophage β (75, 187, 189) have made diphtheria toxin a model for the study of the action of many bacterial and plant toxins. In contrast to proteins that exert their toxic or hormonal effects through binding to the cell surface, diphtheria toxin must penetrate the membrane barrier to reach its cytosolic target (see Section IV). Two functional fragments of diphtheria toxin are required for intoxication, an enzymatically active A fragment and a B fragment that is required for the specific binding and internalization of toxin (see Section IV). Similar functional subunits have been identified for

Pseudomonas exotoxin A, cholera toxin, the plant toxins abrin, ricin, and modeccin, and the hormone insulin (reviewed in 104). Although mutant forms of diphtheria toxin have been useful for the identification of distinct functional domains within the fragment B, the nature of the toxin receptor(s) and the mechanism by which diphtheria toxin crosses the plasma membrane are not clear.

Like diphtheria toxin, the nature of the specific receptor(s) and the mechanism of internalization for some toxins or hormones is controversial. Examples of these include thyroid stimulating hormone (TSH) and the plant toxins abrin, ricin, and modeccin. Two types of molecules have been identified that are required for the action of TSH (184, 204). The nature of the physiologically relevant receptor(s) for the plant toxins is also not clear (137, 183). The mechanism of internalization for proteins is an area of extensive investigation. Diphtheria toxin (46), Pseudomonas exotoxin A (55), abrin, ricin, and modeccin (164, 173) and some hormones (reviewed in 153) all appear to undergo some form of receptor-mediated endocytosis. However, these proteins, or a subunit of the proteins, must still be translocated across the membrane to reach the cytosol. A detailed study of the structural properties of diphtheria toxin that allow penetration of the membrane may aid in our understanding of a general mechanism by which this process can occur.

Thus, the isolation of mutant forms of diphtheria toxin that are defective in binding, in entry, or in both, may enhance our knowledge of the structural properties of protein toxins and hormones that are essential for the uptake of biologically active proteins into sensitive eucaryotic cells. With advances for the manipulation of the

genes encoding other toxins or hormones, similar studies may allow the determination of their physiologic receptors and the mechanisms of protein internalization.

VIII. Summary

Several aspects of the intoxication of eucaryotic cells by diphtheria toxin have not been resolved. The nature of the toxin-binding cell membrane receptor and the relevance of the two functional binding sites on the toxin molecule remain controversial. It is not clear whether a specific receptor (such as a glycoprotein) or a phosphate-containing molecule (such as a phospholipid), or both, are involved in toxin-receptor interactions. It is also not clear whether the receptor-binding site or the phosphate-binding site on the toxin molecule, or both, are physiologically related to intoxication.

The purpose of this research project was to isolate and characterize tox mutants of phage β that coded for CRMs with amino acid substitutions that affected either binding to cell membrane receptors or entry into the cytosol, or both. This entailed the development of procedures to screen large numbers of β phages for those strains which produced antigenically intact, enzymatically active CRMs that were altered in cytotoxicity. Clearly, CRMs defective in binding or internalization would be useful for studying 1) the chemical nature of the physiologically relevant toxin receptor and 2) the structural requirements of the toxin molecule for binding to cell membrane receptors or for entry into the cytosol, or both.

MATERIALS AND METHODS

I. Reagents.

Trypsin, egg white trypsin inhibitor, bovine serum albumin (BSA), lactoperoxidase, and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) were from Sigma (St. Louis, MO). Sephadex G-100 was from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). [Adenine-2,8-^{3}H]-NAD (3.39 Ci/mmol) was from New England Nuclear (Boston, MA). Carrier-free Na^{125}I (100 mCi/ml) and L-[U-^{14}C]-leucine (300 mCi/mmol) were purchased from Amersham/Searle Corp. (Arlington Heights, IL). Tryptose, proteose peptone, and agars for the preparation of bacterial medium were purchased from Difco Laboratories (Detroit, MI). All other chemicals were reagent grade and were purchased commercially.

II. Diphtheria toxin, fragment A, and related CRMs.

Partially purified diphtheria toxin was purchased from Connaught Laboratories (Toronto, Canada), purified according to published methods, and designated Dc202 (77). Purified fragment A was prepared in our laboratory as described (39). Dc202 and purified fragment A were provided by Randall K. Holmes. A partially purified preparation of CRM197 was the generous gift of Dr. John Robbins, Bureau of Biologics, National Institutes of Health, Bethesda, MD and

was further purified as described for diphtheria toxin (77). CRM9 was produced by growing the lysogen C7(β^{h} tox-201 tox-9 h') in deferrated casamino acids/yeast extract (C-Y) medium (149). CRM9 was purified from the culture supernatant according to published methods for diphtheria toxin purification (77). Purified diphtheria toxin, fragment A, CRM197, and CRM9 were stored frozen at -70° C in 0.01 M phosphate buffer (pH 7.7) (Buffer I).

III. Antitoxin.

Hyperimmune rabbit antiserum R21 (600 A.U./ml) was prepared by repeated intramuscular (i.m.) injections of toxoid prepared from a sample of purified unnicked diphtheria toxin Jn141 (100 µg/dose) in complete Freund's adjuvant as described (39) and was used for radioimmunassays. Goat anti-diphtherial toxin pool #301-302 (450 A.U./ml) was prepared in our laboratory by repeated i.m. injections of Dc202 toxoid (39) and was used for Ouchterlony tests. Equine diphtherial antitoxin (lot no. 0862 DC, 2000 A.U./ml, Merrell National Laboratories, Cincinatti, OH) was a gift from A.N. DeSanctis and was used for rabbit skin tests and for Elek tests.

IV. Bacteria and Corynebacteriophages.

C. diphtheriae strain $C7_{s}(-)$tox- (designated C7) is nontoxinogenic and nonlysogenic, is sensitive to the toxinogenic

corynebacteriophage β and the nontoxinogenic phage γ, and is routinely used as the propagating host for these corynebacteriophages (74, 75). The selective indicator strains C7/β^{c}, C7/β^{vir}, and C7/β^{vir}/β^{hc} are resistant to wild-type phage β but sensitive to β phages that carry the host range markers, h, h', and hh' respectively, and have been described previously (74). The h and h' alleles used in this study indicate the alleles h_9 and h'_2 (74).

The corynebacteriophages β and γ were obtained from the respective lysogenic derivatives of C7. Tox mutants are designated according to the nomenclature described by Holmes (74). Phage β$^{h'}$ was used as the ancestral phage for the isolation of the mutant phage β tox-9 h'. Phage β^{h} tox-201 tox-2 is a recombinant with multiple mutant alleles (199, 200); tox-201 is a regulatory mutation that confers resistance to the inhibitory effects of iron on toxin production and tox-2 is a structural mutation that encodes a nontoxic 26,000 MW cross-reacting material (199, 200). The mixed infection method used for the construction of the hyperproducing recombinant phage β^{h} tox-201 tox-9 h' (see Results) has been described (199).

V. Media.

Strains of C. diphtheriae were routinely cultivated in the casein hydrolysate medium of Mueller & Miller (128), with the modifications of Barksdale and Pappenheimer (11). The medium was adjusted to pH 6.8 with 50% NaOH, sterilized by autoclaving and designated PGT. Sterile PGT was supplemented with 10% (v/v) of a

sterile 20% maltose solution that contained (per liter): 0.40 ml of 1% $FeSO_4 \cdot 7H_2O$ in 0.1 M HCl, 6.0 ml of 0.1% aqueous calcium pantothenate, and 30 ml of 10% aqueous calcium chloride (128). When deferrated medium was required $FeSO_4 \cdot 7H_2O$ was omitted from the maltose supplement and both the PGT medium and the maltose supplement were deferrated by coprecipitation of iron with calcium phosphate as described (128). For several purposes described below, cultures of C. diphtheriae were prepared in either low-iron PGT or high-iron PGT, or both. Deferrated PGT-maltose was supplemented with 0.075 µg of added Fe^{++}/ml or 1.0 µg of added Fe^{++}/ml as $FeSO_4$ for low-iron or high-iron medium, respectively.

Deferrated C-Y medium was used for the production of CRM9 from strain C7(β^{h} tox-201 tox-9 h'). The medium was prepared as described (149) and the solution was heated to 80° C and filtered. Two ml of solution II and 1 ml of solution III (128) were added to each liter of the sterile deferrated medium and the medium was sterilized by autoclaving.

Tryptose agar plates (1%), used for the routine cultivation of bacteria and for phage assays, were prepared with tryptose (10 g/liter), agar (10 g/liter), and NaCl (5 g/liter). Tryptose soft agar (0.5%) overlays for phage assays contained tryptose (10 g/liter) and agar (5 g/liter). Prior to use in phage assays, the molten soft agar was supplemented with 10% (v/v) of a sterile 20% glucose solution containing 0.4 ml of 1% $FeSO_4 \cdot 7H_2O$ in 0.1 M HCl, 6.0 ml of 0.1% calcium pantothenate, and 30 ml of 10% $CaCl_2 \cdot 2H_2O$ per liter of distilled water.

Deferrated agar medium (DA-1), was prepared by a modification

(198) of a published medium (97), and contained proteose peptone (20 g/liter), NaCl (8.5 g/liter.), Noble agar (10 g/liter), and Chelex 100 (10 g/liter, Bio-Rad Laboratories, Richmond, CA). The mixture was stirred at room temperature for 1.5 hours, autoclaved, and cooled to 56°C in a water bath during which time the Chelex resin settled out. The medium was decanted, without disturbing the settled Chelex, into sterile bottles for future use or into Petri dishes for use in Elek tests (199).

VI. Growth conditions for bacteria and bacteriophages.

Indicator cells of appropriate bacterial strains were used for the preparation of new lysogens, for testing for phage immunity, and for phage assays. Cultures in liquid medium were incubated in a 37°C waterbath (Model G-76, New Brunswick Scientific Co.) with rotary shaking at 240 rev/min. Growth was monitored by absorbance at 590 nm (A_{590}). To prepare lawns of indicator bacteria, cultures were used in the exponential phase of growth; C7 or C7/β^{c} at A_{590}=0.3, and C7/β^{vir} or C7/β^{vir}/β^{hc} at A_{590}=1.0. Samples of the cultures (0.1 ml) were added to 2.0 ml samples of glucose supplemented tryptose soft agar and layered onto tryptose agar plates.

New lysogenic strains were prepared by exposing lawns of the phage-sensitive C7 strain to drops of bacteria-free, plaque-purified phage stocks. Inocula from the centers of the turbid zones of confluent lysis were streaked for the isolation of surviving bacteria (75). Isolated colonies were used to prepare cultures, and lawns of

these cultures were tested for lysogenic immunity to phage β, for sensitivity to phage γ, and for either spontaneous phage release or phage release after induction by ultraviolet irradiation (75). Strains that met these criteria were considered to be lysogenic; actively growing cultures of cloned cells were prepared, mixed with an equal volume of 24% sterile glycerol, and maintained as frozen stocks at -70°C.

Plaque assays were performed by the procedure of Barksdale (10) as modified by Holmes and Barksdale (75). Phage were diluted 1/10 or 1/100 from low-titer or high-titer stocks, respectively, and 0.1 ml of the diluted sample was mixed with 0.9 ml of the indicator strain. These adsorption mixtures were incubated for 20 minutes at 37°C in a rotary shaking water bath. Adsorption mixtures were then serially diluted in PGT; 0.1 ml of dilutions of absorption mixtures and 0.1 ml of indicator cultures were added to soft agar overlays and layered into hard agar plates. The overlays were allowed to solidify and plates were incubated for 24 hours at 37°C.

Phage stocks were prepared by the procedures of Holmes and Barksdale (75) with minor modifications. Sodium citrate was added at a final concentration of 0.03 M just prior to the onset of mass lysis for the preparation of high-titer phage stocks. When high-titer stocks were to be used in recombination experiments, sodium citrate was omitted.

Strains of lysogenic C7 to be tested quantitatively for toxin or CRMs were grown in deferrated PGT-maltose for 6-8 hours in a rotary shaking water bath at 37°C. The iron-starved cultures were used to inoculate 10 ml samples of low-iron or high-iron PGT-maltose medium

and were incubated for 18 hours at 37° C (200). Culture supernatants were collected and tested for toxin antigen by radioimmunassay.

VII. Tissue culture cells and media.

African green monkey kidney cells (Vero) (82) were obtained from S. Hansen, Department of Pediatrics, Uniformed Services University of the Health Sciences, Bethesda, MD and have been maintained by S. Hansen since 1966. Chinese hamster ovary (CHO) cells were obtained from Dr. B. Ray, Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, MD (163). Cells were routinely maintained as monolayer cultures in Minimal Essential Medium (MEM, Eagle's) with Earle's salts and supplemented with 2 mM glutamine, 10% fetal calf serum (FCS), penicillin (50 units/ml) and streptomycin (50 µg/ml) (116) in 175 cm^2 flasks or 700 cm^2 roller bottles. Tissue culture medium and supplements were obtained from Grand Island Biological Co. (Grand Island, NY). For most experiments the monolayers were detached from roller bottles with phosphate buffered saline containing 0.05% ethylenediaminetetraacetic acid (EDTA); $5x10^4$ cells were seeded in each well of a 24-well multiplate (Linbro, Hamden, CT), and plates were incubated at 37° C in a humidified 5% CO_2, 95% air atmosphere until confluent monolayers developed, usually 2 to 3 days. Any changes in media that were required for the various assays are noted below.

VIII. Isolation of corynephage mutants.

Exponentially growing cultures of C. diphtheriae were infected with phage $\beta^{h'}$ (10 plaque-forming units (PFU)/colony-forming unit (CFU)) and were treated with NTG at 25 µg/ml during a single cycle of phage growth as previously described (75). The phage stocks treated with NTG were assayed with C. diphtheriae C7, and inocula picked from the centers of single phage plaques were transferred to individual wells of 96-well microtiter plates (Costar) containing 200 µl of low-iron PGT medium. After incubation at 37° C for 24 hours, the microtiter plates were centrifuged for 10 minutes at 1500 rpm. To test for the presence of toxin, we transferred 10 µl samples of culture supernatants to microtiter wells containing monolayers of Vero cells and incubated the plates at 37° C for 48 to 72 hours. Each well was examined for cytotoxicity. Phage β were recovered from rare wells showing no signs of cytotoxicity by streaking the supernatant tissue culture medium onto tryptose agar plates (1%) and overlaying with tryptose soft agar (0.5%) containing the C7 indicator strain. The isolated phage plaques were used to infect cultures of C7. Dilutions of supernatants from the phage-infected cultures were tested again for toxicity for Vero cell monolayers. Isolated phage that were nontoxinogenic or reduced in toxinogenicity were used to prepare lysogenic strains of C7. The lysogenic bacteria were grown under optimal conditions for toxin production, and the culture supernatants were tested for toxicity in Vero cell monolayers, for antigens that cross-reacted with diphtheria toxin in a competitive binding radioimmunoassay (39), and for the ADPR-transferase activity

associated with fragment A of diphtheria toxin (34). Strain C7(β^{h} tox-201 tox-9 h'), a recombinant that hyperproduced CRM9, was prepared as described in Results.

IX. Assays for toxin antigen.

(i) Recombinant phage were tested for the tox-201 regulatory marker by an Elek test (53) with the modifications of Holmes (74) and Welkos and Holmes (200). Strips of sterile filter paper were moistened with equine diphtherial antitoxin (250 A.U./ml) and were applied to the surface of freshly prepared plates containing 10 ml of DA-1 agar supplemented with either 0.075 µg Fe^{++}/ml (low-iron DA-1) or 0.12 µg Fe^{++}/ml (high-iron DA-1). Inocula from the centers of recombinant phage plaques on lawns of selective indicator cells were streaked at right angles to the filter paper strip. C7(β) and C7($\beta^{tox-201}$) were used as controls; both were positive for production of diphtheria toxin antigen on low-iron DA-1 and only C7($\beta^{tox-201}$) was positive on high-iron DA-1. Plates were incubated at 37°C for 48 to 72 hours and examined as described (74) for toxin-antitoxin precipitin lines in the agar.

(ii) Immunodiffusion tests (138) were used to detect toxin antigen in DEAE-cellulose and Sephadex column fractions and to examine the cross-reactivity between purified diphtheria toxin and purified CRM9. An agar gel containing Noble agar (1 g/100 ml) and sodium azide (1 g/100 ml) was prepared. Templates were used to cut wells in immunodiffusion slides containing 3 ml of agar (micro Ouchterlony) or

in Petri dishes (60x15 mm) containing 10 ml of agar (macro Ouchterlony). For micro Ouchterlony tests, goat antitoxin (3 µl) was placed in the center wells and 3 µl samples from each fraction to be tested were placed in the surrounding wells. Slides were incubated for 2 to 4 hours at 37°C and examined for toxin-antitoxin precipitin lines. One tenth ml volumes were added to wells for macro Ouchterlony tests. The plates were incubated for 24 hours at room temperature and then examined for precipitin lines.

(iii) Competitive binding radioimmunossays for diphtheria toxin and fragment A have been described (39). All reagents were diluted in assay buffer containing NaCl (150 mM), tris(hydroxymethyl)aminomethane-HCl (Tris) buffer (50 mM, pH 7.4), EDTA (5 mM), NaN_3 (0.02%), Nonidet-P-40 (0.05%, Accurate Chemical and Scientific Company, Hicksville, NY), bovine serum albumin (BSA, 1 mg/ml), and NaI (5 mM) (94). Diphtheria toxin or fragment A was labeled with $Na^{125}I$ by the lactoperoxidase method of Haustein (69). Specific activity ranged from 1-10 µCi/µg of protein (39). Reaction mixtures for this radioimmunossay were 0.5 ml and contained assay buffer, radiolabeled antigen (10,000 CPM), nonradioactive antigen (either purified or from culture supernatants), and a sufficient quantity of R21 antiserum to bind approximately 50% of the radiolabeled antigen in the absence of competing antigen (39). The reaction mixtures were incubated for 15 minutes at room temperature prior to the addition of 75 µl of heat-killed Staphylococcus aureus Cowan strain I (94); the reaction mixtures were then incubated an additional 5 minutes. The staphylococci containing the adsorbed immune complexes were collected by centrifugation, washed twice with

1.0 ml of assay buffer, and radioactivity was counted in a Searle 1185 gamma counter. Bound radioactivity was expressed as a fraction of total radioactivity and was designated B. The data were plotted as 1/B versus the concentration of nonradioactive competing antigen (52).

X. Toxicity assays.

Cytotoxicity was determined both qualitatively and quantitatively. First, tissue culture cells were exposed to toxin or CRM9, diluted as required in growth medium, incubated for 48 to 72 hours at 37° C, and each well was examined by phase contrast microscopy for a qualitative determination of cytotoxicity. Two methods were used to measure cytotoxicity quantitatively. Monolayers were washed twice with Hanks' balanced salt solution (HBSS) to remove detached cells. The remaining cells were solubilized in 0.5 ml of 0.1 N NaOH. An aliquot of each adherent fraction was removed for total cell protein determination by the method of Lowry (111). In a second quantitative assay the effect on protein synthesis was examined. Following a 3 hour exposure to toxin or CRM9, 0.25 μCi of [^{14}C]-leucine was added to each well. The cells were incubated for one hour, washed with HBSS, and the cells were then detached by trypsinization and filtered. The incorporation of [^{14}C]-leucine into TCA-precipitable protein was determined as described in section XI, Methods (131).

The effects of competing ligands and of nucleotides on

intoxication were examined using the protein synthesis assay. Dilutions of the intoxicating proteins and the competing ligands were prepared in MEM (2% FCS) and were added simultaneously to the wells. The effects on protein synthesis were determined as described (131). The effects of nucleotides on intoxication were similarly tested except that dilutions were prepared in 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, pH 7.4 (117).

Toxicity in vivo was determined by a rabbit skin test according to published methods (9).

XI. Assay for NAD:EF2 ADPR-transferase.

ADPR-transferase activity was assayed according to published methods (34) with some modifications. Wheat germ EF2 was prepared (186) and aliquots were frozen at -70°C. The optimal dilution of EF2 required to detect enzyme activity was determined by titration. Each reaction was carried out in a 12 x 75 mm glass tube. Concentrated assay buffer contained 0.05 M Tris (pH 8.2), 0.1 mM EDTA, and 0.04 M dithiothreitol (DTT). The total volume of the reaction mixtures was 64 µl and each contained 25 µl of concentrated assay buffer, 25 µl EF2, 10 µl of purified toxin, purified CRM, or culture supernatant to be tested, and 4 µl of [^{3}H]-NAD$^+$ (12.5 pmoles); samples were cooled to 0°C until all reagents were added. Reaction mixtures were incubated at room temperature for 15 minutes, cooled to 0°C, and precipitated with an equal volume of chilled 10% TCA. Five ml of chilled 5% TCA were added to each tube and the samples were collected on Whatman GF/A

filters. Tubes were rinsed one additional time and the filters were washed twice with chilled 5% TCA, dried, and placed in glass scintillation vials containing toluene/liquiflor scintillation cocktail. The precipitated tritiated protein was counted in a Beckman LS7000 scintillation counter.

XII. Binding studies.

The toxin or CRM9 used for binding studies was radiolabeled by the chloramine-T method (167). One and one half mCi of $Na^{125}I$ in 0.015 ml was added to an equal volume of 0.2 M monosodium phosphate. Sixty micrograms of toxin or CRM were added (5 µl or 20 µl, respectively) followed by two 10 µl doses of chloramine-T (30 µg/ml in 0.3 M NaH_2PO_4) at intervals of approximately 30 seconds. Each sample was incubated for 2 minutes, and the reaction was stopped by the addition of 20 µl of sodium bisulfite (40 µg/ml). Each sample was diluted to 1 ml with PBS/BSA and aliquots were removed for specific activity determinations by the precipitation of protein in the iodinated samples onto glass fiber filters using 5% TCA. Specific activity was usually 10 to 15 µCi/µg of protein. The remainder of each sample was dialyzed overnight against PBS at 4° C and was fractionated by gel filtration chromatography on Sephadex G-100, and fractions corresponding to the peak of radiolabeled monomeric toxin or CRM were pooled.

For binding studies, monolayers were grown as described in section VII (Materials and Methods). Prior to the binding assay, the

growth medium was replaced with 0.9 ml of M199 medium supplemented with 10% FCS and the plates were chilled to 4°C, and 0.1 ml doses of each dilution of ^{125}I-toxin or CRM9 to be tested were added to triplicate wells. The cells were incubated for 14 hours at 4°C, washed four times with chilled HBSS, and dissolved in one ml of 0.1 M NaOH. Each sample was then transferred to a 12x75 plastic tube and ^{125}I-toxin or CRM9 was counted in a Searle 1185 Gamma counter.

XIII. Reconstitution of diphtheria toxin from nontoxic mutant proteins.

The protein, or equimolar concentrations of the proteins, to be tested were added in 1.0 ml of 0.01M phosphate buffer (pH 7.8) to give a total concentration of approximately $1x10^{-5}$ M. Aliquots (0.4 ml) were removed to assay the toxicity of each mixture before treatment with trypsin and DTT. Trypsin was added from a concentrated stock solution (1 mg/ml) to give a final concentration of 6 µg/ml. To each sample 0.6 ml of 20 mM DTT was added and the samples were incubated for 18 minutes in a 37°C waterbath. The reaction was stopped by the addition of 18 µg/ml of soybean trypsin inhibitor from a stock solution (1.5 mg/ml). Aliquots were removed for SDS-PAGE to determine the extent of nicking of the toxin or CRM in each sample. The remainder of each sample was dialyzed against 0.01 M phosphate buffer (pH 7.8) for 24-48 hours to remove the DTT. SDS-PAGE in the absence of DTT was used to show that the A and B fragments had rejoined to form 62,000 MW proteins. Samples were assayed for

cytotoxicity in the protein synthesis assay described above.

XIV. Polyacrylamide gel electrophoresis.

Proteins were boiled for 2 minutes in a sample treatment mix that contained 6 M urea and 1% sodium dodecylsulfate (SDS), with or without 0.2% 2-mercaptoethanol (112). Polyacrylamide slab gels (10%) containing 0.1% SDS were electrophoresed for 4 hours at 20 milliamperes per gel in the discontinuous buffer system described by Maizel (112). Following electrophoresis, the gels were stained with Coomassie brilliant blue.

RESULTS

I. Isolation of corynebacteriophage mutants.

CRMs that are defective in binding to cell membrane receptors or in entry to the cell cytosol have been difficult to isolate for several reasons. First, the *tox* gene on corynephage β is not an essential gene for replication, hence positive selection methods are not available for the isolation of *tox* mutants. Large numbers of mutagenized β phage must therefore be screened. Second, for the purpose of studying the structural requirements of toxin that mediate binding or entry, we wished to be able to detect CRMs which were altered in these processes but remained antigenically and enzymatically indistinguishable from native toxin. Thus, rapid *in vitro* assays based on antigen cross-reactivity or on ADPR-transferase activity could not be used to distinguish CRMs defective in binding or entry from other nontoxic CRMs. The only easily tested property of these types of CRMs that should be significantly different from native toxin is toxicity. Thus, a screening procedure based on biological activity was required.

Diphtheria toxin is produced when corynephage β is present in *C. diphtheriae* as a prophage, a vegetative phage, or as a nonreplicating exogenote (63). Because toxin is produced during the lytic infection of *C. diphtheriae* with phage β, the extracellular toxin is present in plugs of agar excised from phage plaques (131). These findings provided the rationale for the biological assay to

screen for tox mutants of phage β. The assay was initially designed to test the cytotoxicity of plugs of agar excised from plaques formed by NTG-treated phage. Several parameters were chosen to facilitate the isolation of tox mutants and the subsequent purification of tox gene products from the mutants of interest. First, Vero cells were chosen for the cytotoxicity assays because they are exceedingly sensitive to toxin due to a high number of specific toxin receptors (116). Second, β$^{tox-201}$ was chosen as the ancestral phage. Significant amounts of toxin are produced by C. diphtheriae infected with β$^{tox-201}$ even under high-iron conditions (199). Thus, regardless of the iron concentration of the agar used for phage assays significant amounts of toxin would still be produced. In addition, C7(β$^{tox-201}$) produces almost 4 times more toxin than wild-type C7(β) under low-iron conditions (199). Strains lysogenic for newly isolated tox mutants that also carried the tox-201 marker would therefore produce tox gene products in high yield which would facilitate the subsequent purification of mutant toxins. In initial experiments, plugs of agar from plaques of NTG-treated β$^{tox-201}$ were transferred to wells of a microtiter plate containing monolayers of sensitive Vero cells. The plates were incubated and were examined for wells that showed no signs of cytotoxicity after 48 or 72 hours.

Several problems were encountered using this procedure. The results of several trial experiments are summarized in Table 2. In four separate experiments, plugs of agar from isolated plaques of NTG-treated β$^{tox-201}$ assayed on tryptose agar were transferred to Vero cell monolayers. All phage isolates tested were toxinogenic. Based on the frequency with which tox mutants of phage β have been isolated

Table 2. Effects of Ancestral Phage Genotype and Type of Agar on the Development of a Screening Procedure to Detect Nontoxinogenic or Hypotoxinogenic Phage Mutants

Ancestral Phage	Type of Agar for Phage Assay	Number of Phage Plaques Tested	Percent Toxinogenic
β tox-201	Tryptose[a]	3,260	100
β	Tryptose	1,080	100[c]
$\beta^{h'}$	DA-1[b]	1,030	100

[a]Tryptose agar was not deferrated and was considered to be high-iron.

[b]DA-1 agar was deferrated and contained 0.075 µg/ml of added Fe^{++} (low-iron conditions).

[c]Approximately 15% were recovered as presumptive tox^- isolates but secondary screening demonstrated that all were tox^+.

(74, 99, 189), similar nontoxinogenic mutants should have been detected among the number of phage isolates screened. The frequency of clear mutants of phage β isolated under the same conditions indicated that the NTG mutagenesis was effective. To rule out the possibility that the tox-201 marker interfered with the detection of nontoxinogenic mutants, wild-type phage β were treated with NTG and samples from isolated phage plaques were tested in this assay. Approximately 15% of the phages appeared to be nontoxinogenic. However, when these presumptive nontoxinogenic mutants were retested under low-iron conditions, all were found to be as toxinogenic as wild-type phage β for Vero cells. The large number of false nontoxinogenic phage isolates probably resulted from the inhibitory effects of iron present in the tryptose agar used for the phage assay. For the next mutagenesis, phage $\beta^{h'}$ was used as the ancestral phage. Low-iron DA-1 agar was used for the isolation of NTG-treated phage plaques to increase the amount of toxin produced in the phage assay. The h' host range marker in the genome of the ancestral phage would facilitate the subsequent construction by recombination of a nontoxinogenic or hypotoxinogenic mutant that produced the tox gene product in high yield. Low-iron DA-1 agar was used for the isolation of NTG-treated phage plaques to increase the amount of toxin produced in the phage assay. As seen in Table 2, all of the phage tested under these conditions were toxinogenic.

Taken together, the above observations indicated that there were problems inherent in the screening procedure which prevented the detection of either nontoxinogenic or hypotoxinogenic phage mutants. It was possible that the combinations of phage genotypes and the agar

media used affected the yields of toxin produced in the phage assays and therefore affected the apparent toxinogenicity of the phage isolates tested. In the case of either $\beta^{tox-201}$ assayed on tryptose agar or $\beta^{h'}$ assayed on low-iron DA-1 agar, toxin would be produced at the level of wild-type C7(β) under optimal conditions. In contrast, when wild-type phage β was assayed on tryptose agar, toxin production might be inhibited and many of the samples of agar transferred to Vero cell monolayers would appear to be nontoxic.

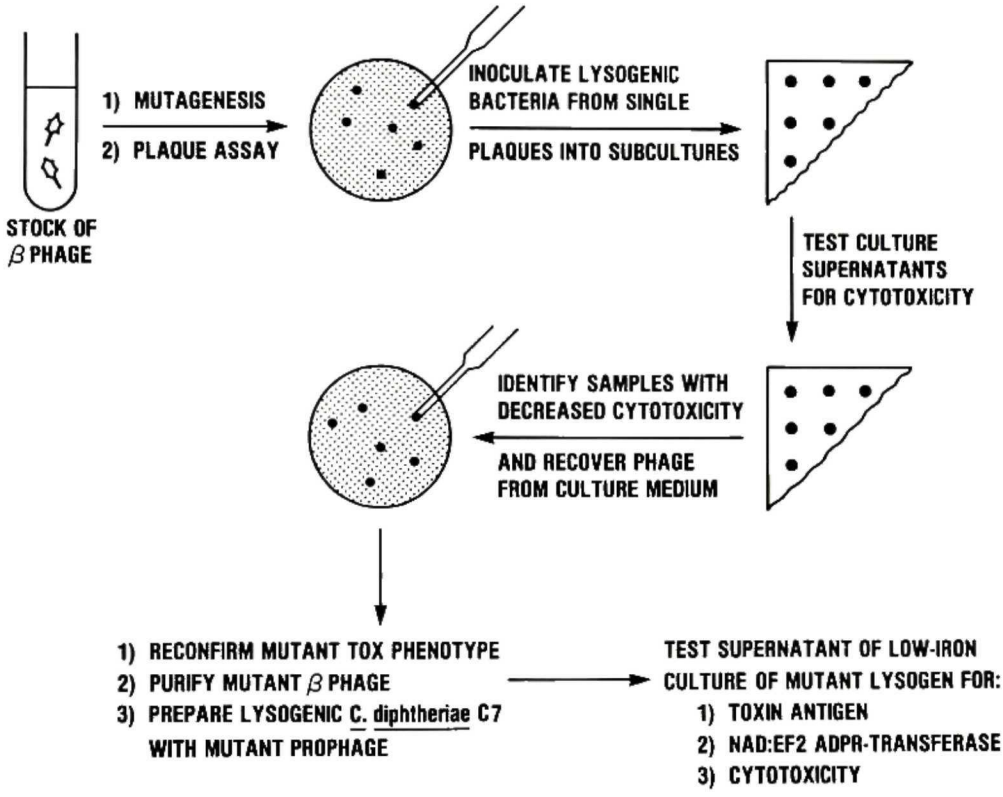
To test this hypothesis, $\beta^{h'}$ was assayed on tryptose agar and on DA-1 agar containing varying concentrations of iron. Plugs of agar surrounding well-isolated phage plaques were tested for toxicity for Vero cell monolayers. Variations in the iron concentration of the media tested had little effect on the number of toxic samples. All samples of agar within a radius of at least 1.5 cm from isolated plaques on DA-1 agar were toxic and 90% of the samples taken from tryptose agar plates were toxic. Thus, the failure to detect nontoxinogenic mutants of the NTG-treated phage apparently resulted from the diffusion of toxin from the plaques produced by tox^+ phages into the surrounding agar.

To eliminate the problem of toxin diffusion, the screening assay was modified to test supernatants from individual cultures of *C. diphtheriae* C7 which were infected with NTG-treated phage $\beta^{h'}$. The screening procedure used for the isolation of the *tox* mutants of corynephage β described below is illustrated in Figure 2. Several classes of *tox* mutants were identified using this procedure, and the CRMs produced by these mutants were categorized by antigenic cross-reactivity with toxin in competitive binding radioimmunoassays, by

FIGURE 2

Isolation of *tox* mutants of corynephage $\beta^{h'}$. Phage $\beta^{h'}$ was treated with the mutagenic agent nitrosoguanidine. Bacteria were recovered from single plaques and subcultured into microtiter wells containing low-iron PGT medium with 0.075 μg of added Fe^{++}/ml and were incubated for 24 hours. The microtiter plates were centrifuged and 10 μl samples of culture supernatants were transferred to the wells of a second microtiter plate that contained monolayers of Vero cells. Presumptive *tox* mutants were identified by the absence of cytotoxicity, and the phage were recovered. The toxinogenicity of these isolates was tested as described in Materials and Methods.

FIGURE 2


STOCK OF
β PHAGE
1) MUTAGENESIS
2) PLAQUE ASSAY
INOCULATE LYSOGENIC
BACTERIA FROM SINGLE
PLAQUES INTO SUBCULTURES
TEST CULTURE
SUPERNATANTS
FOR CYTOTOXICITY
IDENTIFY SAMPLES WITH
DECREASED CYTOTOXICITY
AND RECOVER PHAGE
FROM CULTURE MEDIUM
1) RECONFIRM MUTANT TOX PHENOTYPE
2) PURIFY MUTANT β PHAGE
3) PREPARE LYSOGENIC C. diphtheriae C7
WITH MUTANT PROPHAGE
TEST SUPERNATANT OF LOW-IRON
CULTURE OF MUTANT LYSOGEN FOR:
1) TOXIN ANTIGEN
2) NAD:EF2 ADPR-TRANSFERASE
3) CYTOTOXICITY

ADPR-transferase activity, and by cytotoxicity for Vero cells (Table 3).

Many of the newly isolated *tox* mutants were similar to previously described classes of mutants (74, 99, 189). CRM12 did not compete with toxin in a competitive-binding radioimmunoassay but did compete with fragment A and indicated that only the antigenic determinants found on purified fragment A were present on CRM12 (data not shown). CRM12 retained wild-type ADPR-transferase activity and was nontoxic for Vero cells. Thus, it appeared that β^{tox-12} *h'* contained a chain-terminating mutation or a deletion in the toxin structural gene and coded for a mutant protein that was antigenically similar to the amino-terminal portion of toxin. Therefore CRM12 resembles CRM30 (Table 1).

Three of the mutants, β^{tox-14}, β^{tox-11}, and β^{tox-15}, were similar to a class of *tox* mutants that contained missense mutations in the portion of the toxin structural gene corresponding to fragment A. CRM11, CRM14, and CRM15 were antigenically indistinguishable from toxin in a competitive-binding radioimmunoassay using toxin as the competing ligand. The culture supernatants from these isolates lacked ADPR-transferase activity and were nontoxic for Vero cells. These CRMs resemble CRM197 (Table 1).

CRM13 was antigenically indistinguishable from toxin in a competitive-binding radioimmunoassay and had no detectable *in vitro* NAD:EF2 ADPR-transferase activity. However, culture supernatants from C7(β^{tox-13} *h'*) were slightly toxic for Vero cells, indicating that residual enzyme activity was present. The failure to detect enzyme activity was probably a function of the insensitivity of the *in vitro*

Table 3. Preliminary Characteistics of Mutant Toxins (CRMs)

Phage Strain	*tox* Gene Product	Toxin Antigen[a] (μg/ml)	ADPR-Transferase Activity[b]	Vero Cell Cytotoxic Dose[c] (pg)	Phenotype
β*tox*$^+$	Toxin	5.4	+	1.0	Wild-type
β*tox*-9 *h*'	CRM9	4.9	+	1875	Reduced in toxicity for Vero cells
β*tox*-10 *h*'	CRM10	5.3	+	74	"
β*tox*-16 *h*'	CRM16	2.9	+	8.5	"
β*tox*-12 *h*'	CRM12	0	+	Nontoxic	Lacks Fragment B determinants
β*tox*-14 *h*'	CRM14	0.6	-	Nontoxic	No ADPR-transferase activity
β*tox*-11 *h*'	CRM11	6.8	-	Nontoxic	"
β*tox*-15 *h*'	CRM15	6.5	-	Nontoxic	"
β*tox*-13 *h*'	CRM13	4.8	-	390	Reduced in ADPR-transferase activity

[a]The concentration of toxin antigen in lysogenic culture supernatants was determined by competitive-binding radioimmunoassays using ^{125}I-toxin as described in Materials and Methods.

[b]The ADPR-transferase activity in lysogenic culture supernatants was determined *in vitro* before and after activation with trypsin, as described in Materials and Methods. (+) ≅ wild-type levels. (-) = not detectable.

[c]The cytotoxicity of lysogenic culture supernatants was determined by incubating Vero cell monolayers in microtiter plates with dilutions of culture supernatants for 48 to 72 hours. Each well was examined microscopically for signs of cytotoxicity.

assay. This CRM may resemble CRM176 (Table 1).

In addition, a new class of tox mutants was identified. Each of these tox mutants, β^{tox-9}, β^{tox-10}, and β^{tox-16} produced a CRM that was antigenically indistinguishable from wild-type toxin in a competitive binding radioimmunoassay. The ADPR-transferase activity associated with each of these CRMs was similar to the ADPR-transferase activity associated with wild-type toxin. However, each CRM was significantly less toxic for Vero cells than native toxin. Based on these observed properties, CRM9, CRM10, and CRM16 were candidates for mutant toxins which were altered in binding to cell membrane receptors or in entry into the cytosol, or both.

II. Purification of CRM9

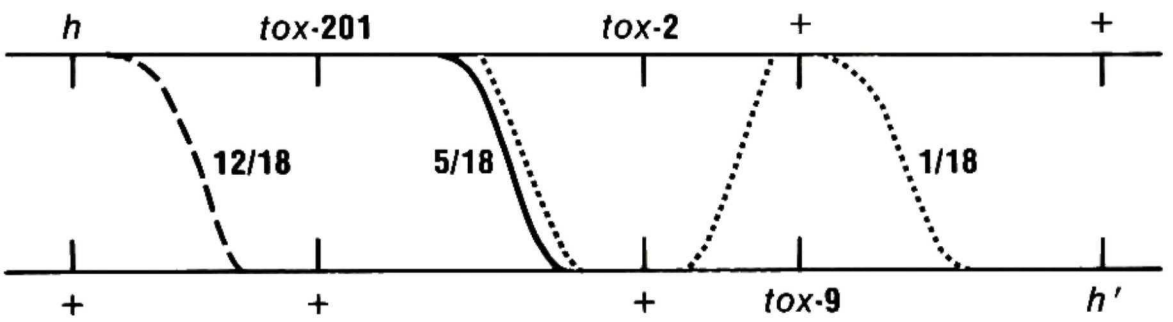
CRM9, which was most reduced in cytotoxicty when compared to toxin, was selected for purification and further characterization. To increase the quantity of CRM9 produced in culture supernatants, a hyperproducing strain was constructed by recombination (Figure 3).

Recombinant phage that contained both the h and h' markers were selected by using lawns of the C7/β^{vir}/β^{hc} indicator strain for phage assays (Figure 3). Lysogens from the centers of the selected recombinant phage plaques were screened by a modified Elek test (see Methods) for the ability to produce toxin antigen. Isolates that produced toxin antigen in this test were tested for the tox-201 marker by using competitive-binding radioimmunoassays to compare the yields of toxin antigen in supernatants from phage-infected C7 cultures grown in low-iron and high-iron PGT medium (0.075 µg or 1.0 µg of added Fe^{++}/ml, respectively). From eighteen hh' recombinant phages that

FIGURE 3

Recombination to construct a strain that hyperproduces CRM9: $_{\beta}$h tox-201 tox-2 x $_{\beta}$tox-9 h'. The genotypes of the parental phages are shown in the upper and lower lines, and some of the possible crossovers within the hh' segment of the genetic map are illustrated. Although the precise location of the tox-9 marker is not known, the indicated location in the distal portion of the tox gene is consistent with the observed results. Fractions indicate the number of observed recombinants with the genotypes indicated divided by the total number of CRM^+ hh' recombinants tested. The recombinants of interest have the genotype h tox-201 tox-9 h' and were presumably generated by the single crossover represented by the solid line.

FIGURE 3



produced toxin antigen in Elek tests, six of these were refractory to the inhibitory effects of iron indicating that the tox-201 marker was present. Serial dilutions of the culture supernatants from these six isolates were tested in a Vero cell cytotoxicity assay. Five of the isolates were reduced in toxinogenicity in a manner similar to βtox-9 h' and one isolate was as toxinogenic as wild-type $\beta^{h'}$. A phage isolate that contained both the tox-201 and tox-9 markers was purified by repeated phage isolation and used to prepare lysogenic C. diphtheriae C7. The lysogen, designated C7(β^{h} tox-201 tox-9 h') was grown under the low-iron conditions necessary for optimal toxin production, in deferrated C-Y medium with 0.01 µg of added Fe^{++}/ml.

CRM9 was purified from culture supernatants of C7(β^{h} tox-201 tox-9 h') according to standard methods for the purification of diphtheria toxin (Figure 4). Twelve liters of culture supernatant containing CRM9 were collected, partially concentrated by membrane filtration, and fractionated with ammonium sulfate. The material that precipitated between 35% and 70% of saturation was dialyzed against 0.01 M phosphate buffer (pH 7.7), applied to a DEAE-cellulose column, and eluted with a linear gradient of NaCl (Figure 5, panel A). Toxin antigen was recovered between 0.07 and 0.08 M NaCl. Every other fraction was tested for toxin antigen by micro Ouchterlony assays and fractions 198-226 that contained toxin antigen were pooled and dialyzed against Buffer I. The dialyzed sample was concentrated to approximately 10% of the original volume by batch DEAE-cellulose concentration. The sample containing toxin antigen was incubated with 4 grams of DEAE-cellulose and centrifuged. The adherent toxin antigen was eluted with 0.15 M NaCl and was monitored by the A_{280}. The

FIGURE 4

Purification of diphtheria toxin and cross-reacting materials.

FIGURE 4

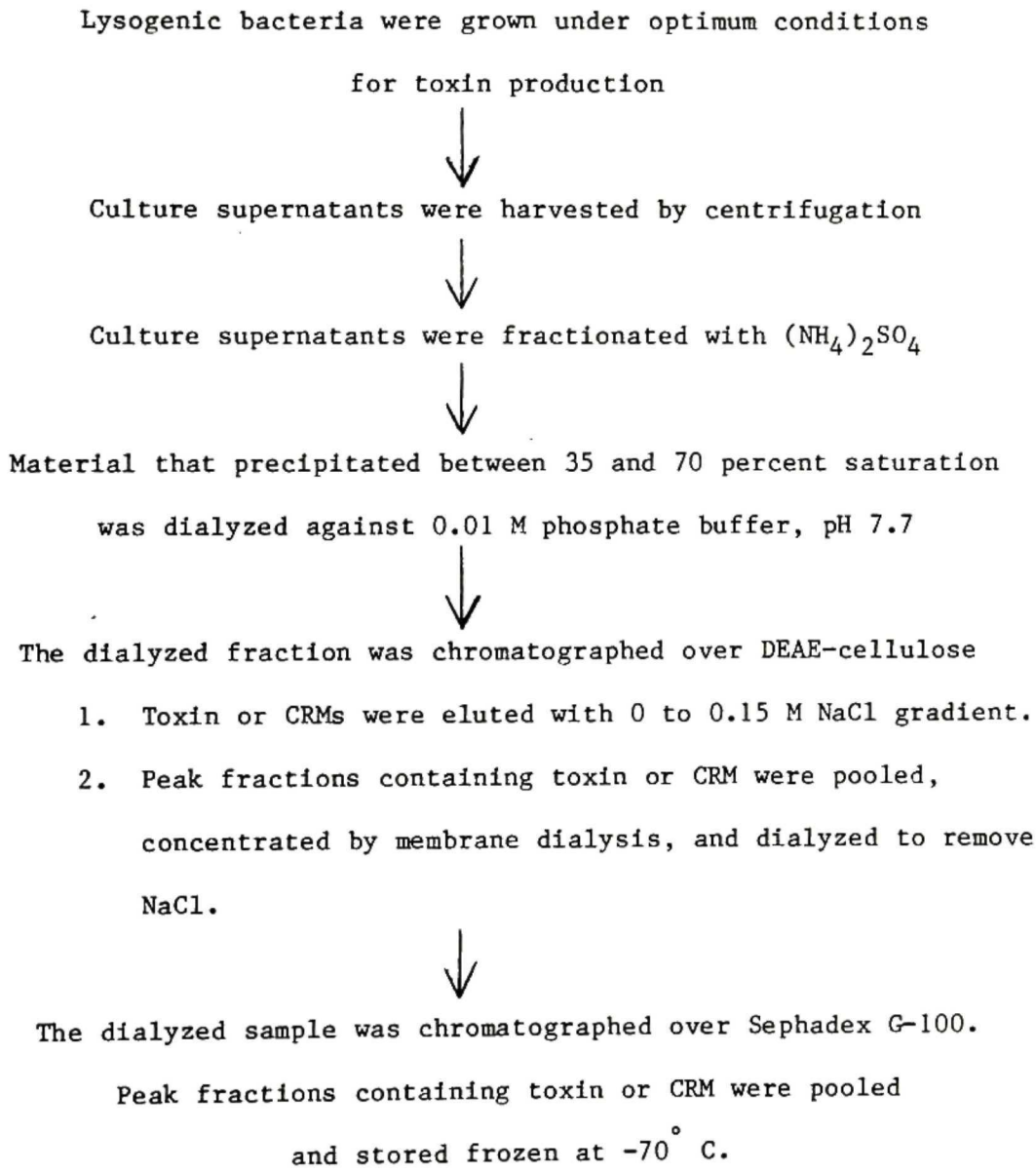

Lysogenic bacteria were grown under optimum conditions for toxin production
Culture supernatants were harvested by centrifugation
Culture supernatants were fractionated with $(NH_4)_2SO_4$
Material that precipitated between 35 and 70 percent saturation was dialyzed against 0.01 M phosphate buffer, pH 7.7
The dialyzed fraction was chromatographed over DEAE-cellulose
1. Toxin or CRMs were eluted with 0 to 0.15 M NaCl gradient.
2. Peak fractions containing toxin or CRM were pooled, concentrated by membrane dialysis, and dialyzed to remove NaCl.
The dialyzed sample was chromatographed over Sephadex G-100.
Peak fractions containing toxin or CRM were pooled and stored frozen at -70° C.

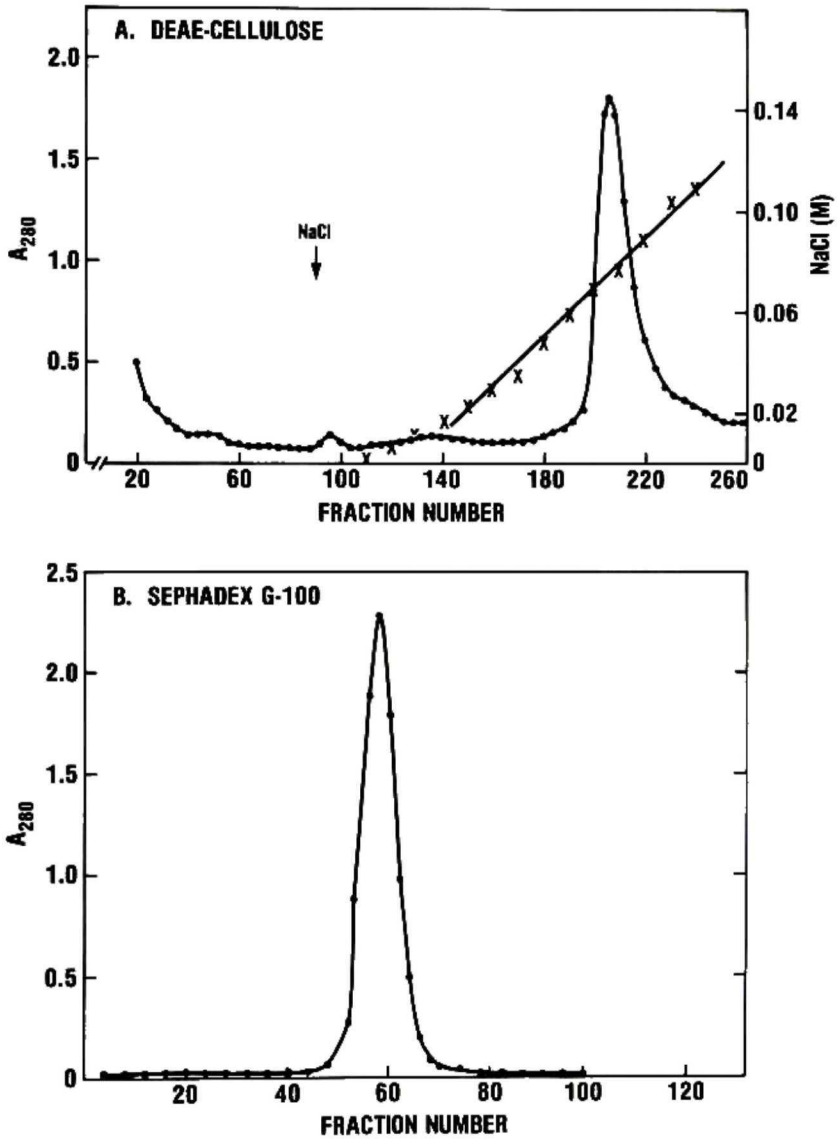
FIGURE 5

Purification of CRM9.

Panel A: Material that was precipitated between 35 and 70% saturation of ammonium sulfate was dialyzed against Buffer I and applied to a DEAE-cellulose column (2 x 45 cm) equilibrated with Buffer I. The column was washed extensively with Buffer I and the adsorbed material was eluted with a 150 ml linear gradient of NaCl (0 to 0.15 M). Fractions were collected in 6.5 ml volumes. The A_{280} and conductivity of alternate fractions were measured, and samples of these fractions were removed for micro Ouchterlony tests as described in Materials and Methods. Peak fractions were analyzed by SDS-PAGE, pooled (fractions 198-226), and dialyzed against Buffer I.

Panel B: The concentrated and dialyzed sample obtained from DEAE-cellulose chromatography was applied to a Sephadex G-100 column (2.5 x 74 cm) equilibrated in Buffer I. Three ml fractions were collected. The A_{280} of alternate fractions was determined and samples were removed for micro Ouchterlony tests and SDS-PAGE as described in Materials and Methods.

FIGURE 5


A. DEAE-CELLULOSE
A_{280}
NaCl
NaCl (M)
FRACTION NUMBER
B. SEPHADEX G-100
A_{280}
FRACTION NUMBER

centrifugation and elution steps were repeated until the A_{280} remained constant. The sample was further concentrated by membrane dialysis and then chromatographed on Sephadex G-100 (Figure 5, panel B). Every other fraction was tested for toxin antigen as described above. Samples of the fractions containing toxin antigen were electrophoresed in SDS-polyacrylamide gels, and minor amounts of contaminating proteins were shown to be present. Fractions 52-66 were pooled and rechromatographed over DEAE-cellulose and eluted as described. A single protein peak was eluted, and the peak fractions (162-168, total volume 12.5 ml) were pooled. The protein concentration of the pooled fractions (2.5 mg/ml) was determined, and toxin antigen was determined by a competitive-binding radioimmunoassay as equivalent to 2.4 mg/ml of reference Dc202 toxin. Analysis of 2 µg samples of purified CRM9 by SDS-PAGE revealed a single band of protein (Figure 6, panel C). Immunoprecipitation of ^{125}I-CRM9 demonstrated that all radioactivity was precipitated with antitoxin serum. Approximately 50 mg of purified CRM9 was recovered from 12 liters of the crude culture supernatant that contained approximately 1.5 grams of toxin antigen. CRM197 was purified by similar methods with the following modifications: 1) a partially purified preparation of CRM197 was obtained, dialyzed against Buffer I, and applied directly to a DEAE-cellulose column, 2) pooled fractions from the DEAE-cellulose column were concentrated only by membrane dialysis, and 3) the pooled Sephadex G-100 fractions were not rechromatographed over a second DEAE-cellulose column.

III. Characterization of purified CRM9.

Several characteristics of purified CRM9 were examined (Figure 6). CRM9 and toxin competed equally well with ^{125}I-toxin for binding to antitoxin antibodies in a competitive-binding radioimmunoassay (Figure 6, panel A). In an immunodiffusion test using diphtheria antitoxin, a line of identity was produced between CRM9 and toxin (Figure 6, panel A, inset). Thus, CRM9 and toxin were antigenically indistinguishable by these criteria.

The ADPR-transferase activities associated with the fragment A from CRM9 and from toxin were assayed before trypsin treatment and after treatment with trypsin. The activated, trypsin-treated proteins had similar ADPR-transferase activities; however, before treatment with trypsin only toxin was active. Diphtheria toxin (Dc202) contained both intact and nicked molecules (Figure 6, panel C, lane 1), whereas purified CRM9 contained only intact molecules (Figure 6, panel c, lane 2). The presence of the nicked population of toxin molecules accounted for the ADPR-transferase activity of toxin present before trypsin treatment. SDS-PAGE demonstrated that toxin and CRM9 had similar electrophoretic mobilities, as did the A fragments or B fragments derived from toxin and CRM9. Therefore, the apparent molecular weights of intact toxin, fragment A and fragment B were identical with those of the corresponding polypeptides derived from CRM9.

These data indicated that CRM9 and toxin were antigenically indistinguishable, had similar ADPR-transferase activities, and had the same apparent molecular weights.

FIGURE 6

Comparison of properties of purified CRM9 and diphtheria toxin

Panel A: Competitive binding radioimmunoassay with ^{125}I-toxin. Reaction mixtures contained ^{125}I-toxin, nonradioactive toxin (•) or CRM9 (o), and R21 antiserum. The samples were processed as described in Materials and Methods. The reciprocal of the fraction of ^{125}I-toxin bound (1/B) was plotted as a function of the concentration of the nonradioactive competing antigen.

Panel A insert: Comparison of CRM9 and toxin by gel immunodiffusion. The lower well contained goat antitoxin serum (pool #301-302, diluted 1:5), and the upper wells contained toxin (DT) or CRM9 at 100 µg/ml.

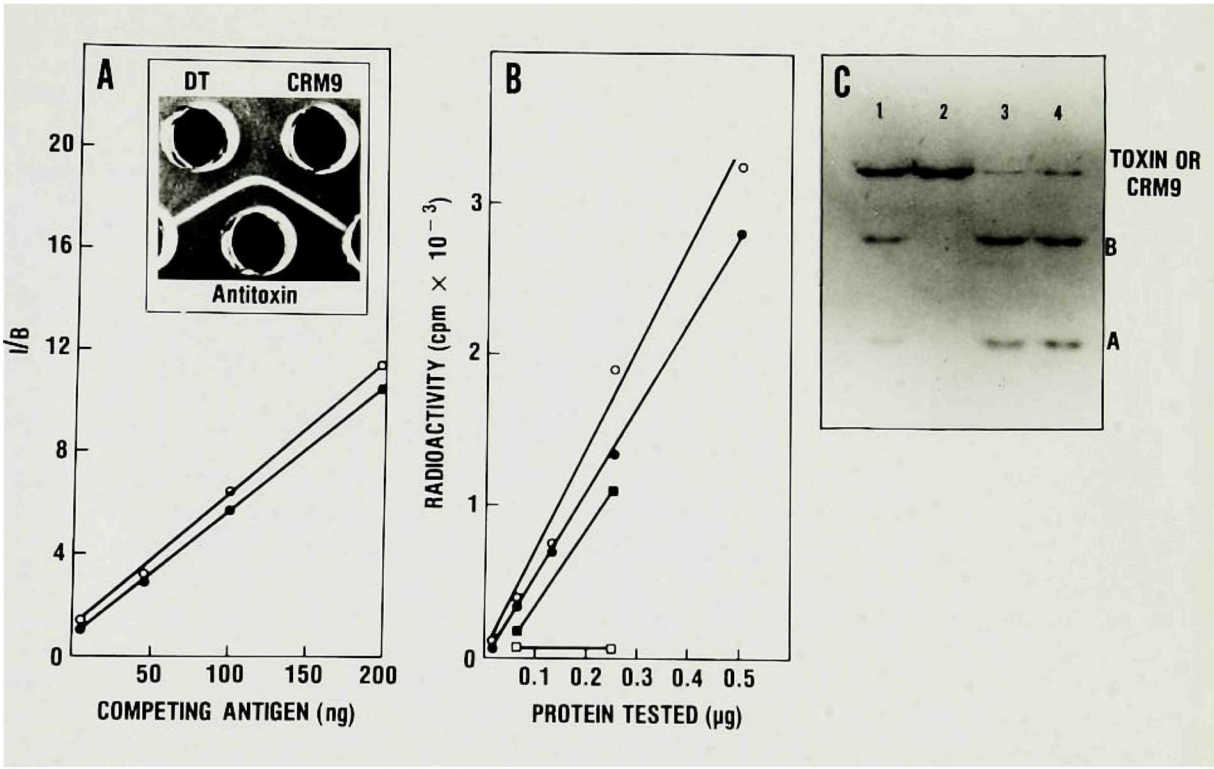
Panel B: In vitro NAD:EF2 ADPR-transferase activities of CRM9 and toxin. CRM9 and toxin (100 µg/ml) were assayed before (□, CRM9; ■, toxin) and after (o, CRM9; •, toxin) treatment with trypsin (1 µg/ml at 37° C for 10 minutes) for the ability to transfer the ADP ribose moiety of ^{3}H-NAD to wheat germ elongation factor 2 as described in Materials and Methods. The radioactivity precipitated with 5% TCA was plotted as a function of the concentration of CRM9 or toxin.

Panel C: Comparison of toxin and CRM9 by SDS-PAGE. Samples (2 µg) were incubated with 5% 2-mercaptoethanol with and without prior trypsin treatment.

Lane 1: toxin
Lane 2: CRM9
Lane 3: trypsin-treated toxin
Lane 4: trypsin-treated CRM9

The positions of stained bands corresponding to toxin or CRM9, fragment B and fragment A are indicated.

FIGURE 6


A
DT
CRM9
Antitoxin
I/B
20
16
12
8
4
0
50
100
150
200
COMPETING ANTIGEN (ng)
B
RADIOACTIVITY (cpm × 10⁻³)
3
2
1
0
0.1
0.2
0.3
0.4
0.5
PROTEIN TESTED (µg)
C
1
2
3
4
TOXIN OR CRM9
B
A

IV. Cytotoxicity of CRM9

Purified CRM9 was tested for toxicity using three different toxicity assays. In quantitative comparisons of the cytotoxicities of CRM9 and toxin, CRM9 was significantly less toxic for Vero cells (Figure 7) than toxin. With assays to determine total cell protein in monolayers after exposure to toxin or CRM9 (Figure 7, panel A) or with assays to determine the effects of toxin or CRM9 on protein synthesis (Figure 7, panel B), CRM9 was approximately 1,000-fold less cytotoxic for Vero cells than toxin. The cytotoxicity of toxin and CRM9 has also been demonstrated in CHO cells (Figure 7, panel B). Although higher concentrations of toxin and CRM9 were required to produce responses on CHO cells than on Vero cells, toxin was much more toxic than CRM9 for CHO cells.

The time courses for the inhibition of protein synthesis in Vero cells by toxin and CRM9 were compared. Monolayers of Vero cells were inhibited with varying concentrations of toxin or CRM9 and the extent of protein synthesis inhibition was determined at the times indicated (Figure 8). As the concentration of either toxin or CRM9 was increased, the rate of inhibition of protein synthesis was also increased. The ratio of the the concentrations of toxin and CRM9 required to give similar rates of inhibition was consistent with the differences in their toxicity in the cytotoxicity tests described previously.

In addition to the in vitro cytotoxicity assays, the toxicity of toxin and of CRM9 was tested in rabbit skin tests. The minimal reactive dose (MRD) was defined as the smallest dose required to produce a 10 millimeter lesion. The MRD of toxin was 1.25 ng whereas

FIGURE 7

Quantitative comparisons of the toxicities of purified CRM9 and diphtheria toxin. Panel A: Cytotoxicity was measured by the detachment of Vero cells from a tissue culture well. The monolayers were incubated with toxin or CRM9 for 72 hours, washed, and solubilized for the determination of protein in the adherent cells as described in Materials and Methods. The dose response curves compare the protein in untreated controls (c) to protein in intoxicated cultures (c'). Response is given by ((c-c')/c).

Panel B: Vero cell monolayers and CHO cell monolayers were incubated with toxin or CRM9 for 3 hours, followed by a one hour pulse with ^{14}C-leucine (0.25 μCi). Samples were harvested for the determination of protein synthesis as described in Materials and Methods. The dose response curves compare the incorporation of radioactivity into protein in untreated controls (c) and in intoxicated cells (c'). Response is given by ((c-c')/c). Vero cells and toxin (●) or CRM9 (o); CHO cells and toxin (■) or CRM9 (□).

FIGURE 7

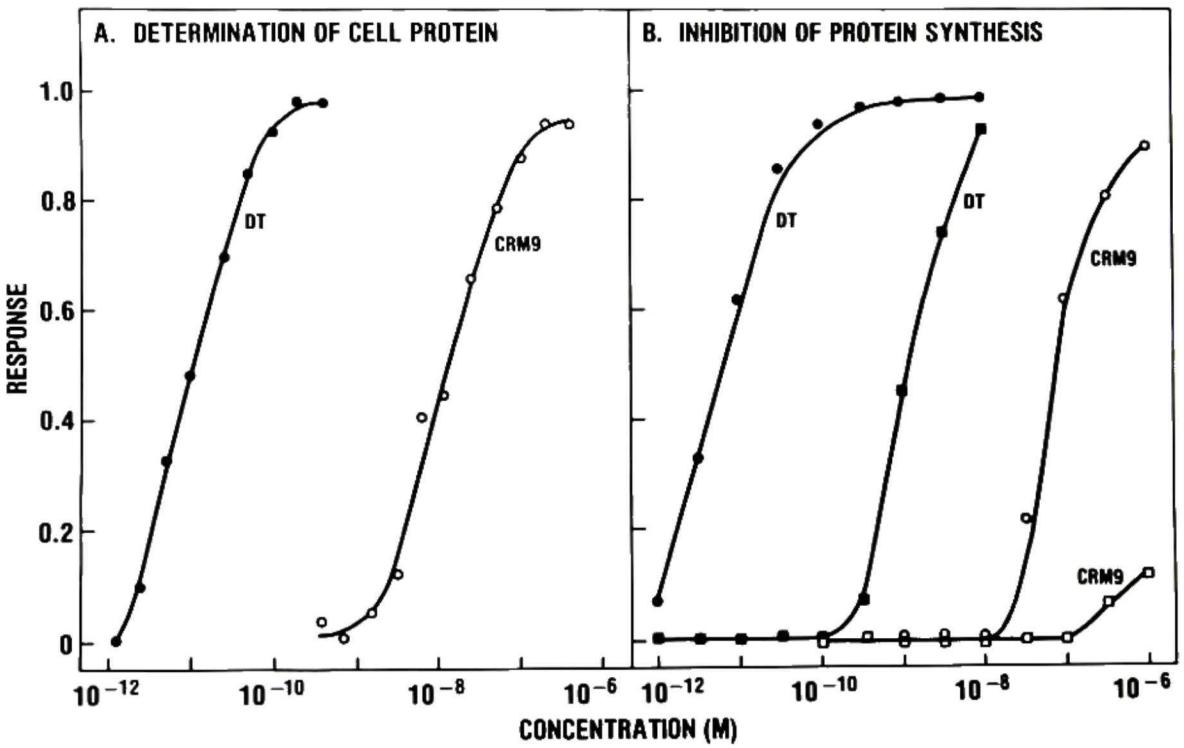
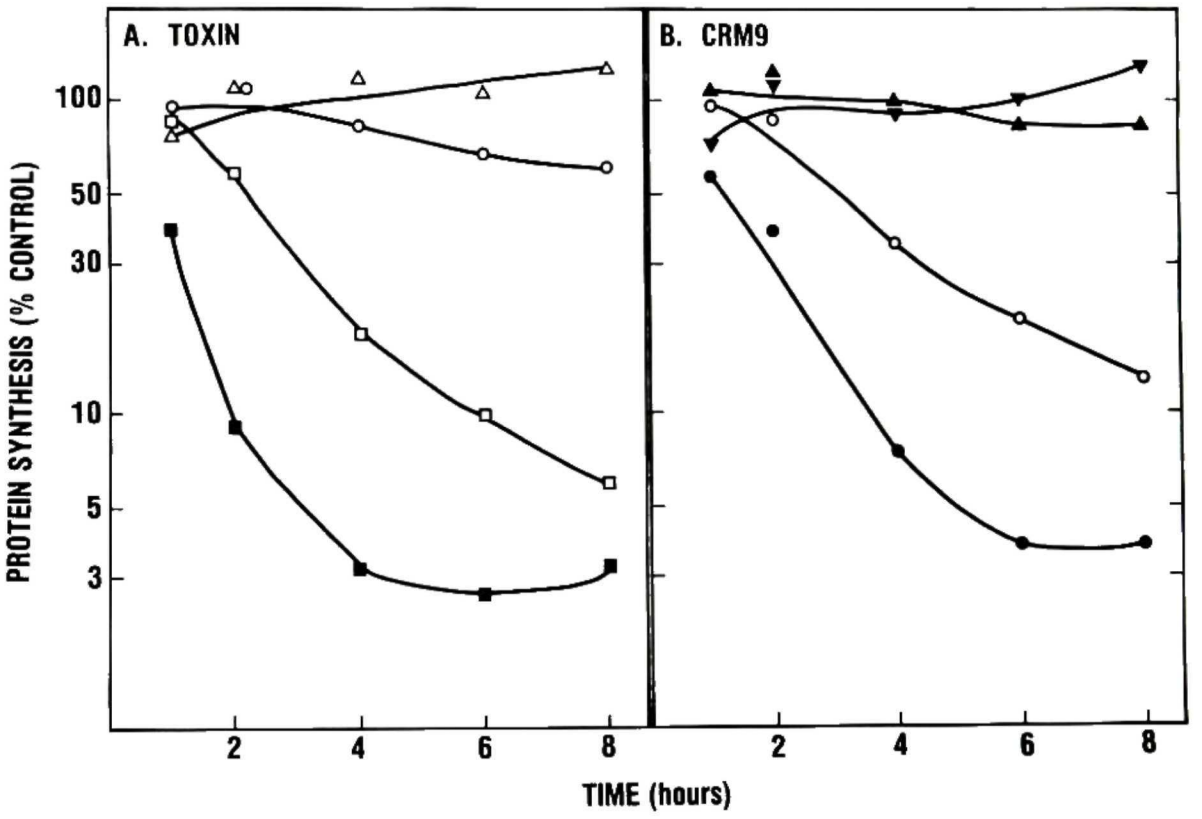

A. DETERMINATION OF CELL PROTEIN
B. INHIBITION OF PROTEIN SYNTHESIS
RESPONSE
1.0
0.8
0.6
0.4
0.2
0
DT
CRM9
DT
DT
CRM9
CRM9
10^{-12}
10^{-10}
10^{-8}
10^{-6}
10^{-12}
10^{-10}
10^{-8}
10^{-6}
CONCENTRATION (M)

FIGURE 8

Time course for the inhibition of protein synthesis by toxin and CRM9 in Vero cells. Monolayers of Vero cells in MEM (2% FCS) were treated with increasing concentrations of toxin (Panel A) or CRM9 (Panel B) for 3 hours followed by a one hour pulse with ^{14}C-leucine (0.25 μCi). Protein synthesis is expressed as a percent of the rate of protein synthesis in untreated control cells. Panel A: $1x10^{-9}$ M (■), $1x10^{-10}$ M (□), $1x10^{-11}$ M (o), $1x10^{-12}$ M (Δ) toxin. Panel B: $1x10^{-6}$ M (●), $1x10^{-7}$ M (o), $1x10^{-8}$ M (▲), $1x10^{-9}$ M (▼) CRM9.

FIGURE 8


A. TOXIN
B. CRM9
PROTEIN SYNTHESIS (% CONTROL)
100
50
30
10
5
3
2
4
6
8
TIME (hours)

the MRD of CRM9 was 625 ng (Figure 9). Passive immunization of the rabbit with 1,000 units of antitoxin intravenously at 30 minutes before administration of the intracutaneous test dose did not completely protect against the lesion produced by CRM9. The most likely explanation for this failure to neutralize CRM9 completely is that the dose of CRM9 was too large an amount of toxin antigen to be neutralized by the amount of antitoxin present at the site of the intracutaneous injection of toxin. By these three toxicity assays, CRM9 was approximately 1,000-fold less toxic than wild-type toxin.

V. Binding of CRM9 to Vero cell membranes.

It was possible that the decrease in the toxicity of CRM9 resulted from a defect in binding to cell membrane receptors or in entry into the cytosol, or both. To examine these possibilities, the binding of radiolabeled toxin or CRM9 was examined.

Middlebrook et al. (118) demonstrated the specific binding of toxin to cell membrane receptors on Vero cells under conditions where toxin was not internalized. The ability of CRM9 to bind to Vero cell membrane receptors was compared with toxin. ^{125}I-toxin and ^{125}I-CRM9 were first tested for cytotoxicity to ensure that iodination did not eliminate the cytotoxicity of either toxin or CRM9 (Figure 10). The dose response curves before and after iodination of these proteins were similar and indicated that toxicity for Vero cells was not destroyed. The binding of ^{125}I-toxin to Vero cell membranes reached a maximum after 8 to 10 hours of incubation at 4° C and remained constant for up to 24 hours (Figure 11). For subsequent binding experiments, cell monolayers were incubated with ^{125}I-toxin or

FIGURE 9

Comparison of toxin and CRM9 in rabbit skin tests. Samples of toxin and CRM9 (0.1 ml) were injected into the depililated backs of rabbits 3.5 hours before administration of diphtheria antitoxin (upper row) and 0.5 hour after intravenous injection of 1,000 units of antitoxin (lower row). Panels a, 1.25 ng of toxin; panels b, 0.25 ng of toxin; panels c, 625 ng of CRM9; panels d, 125 ng of CRM9. (Magnification = 2.2)

FIGURE 9

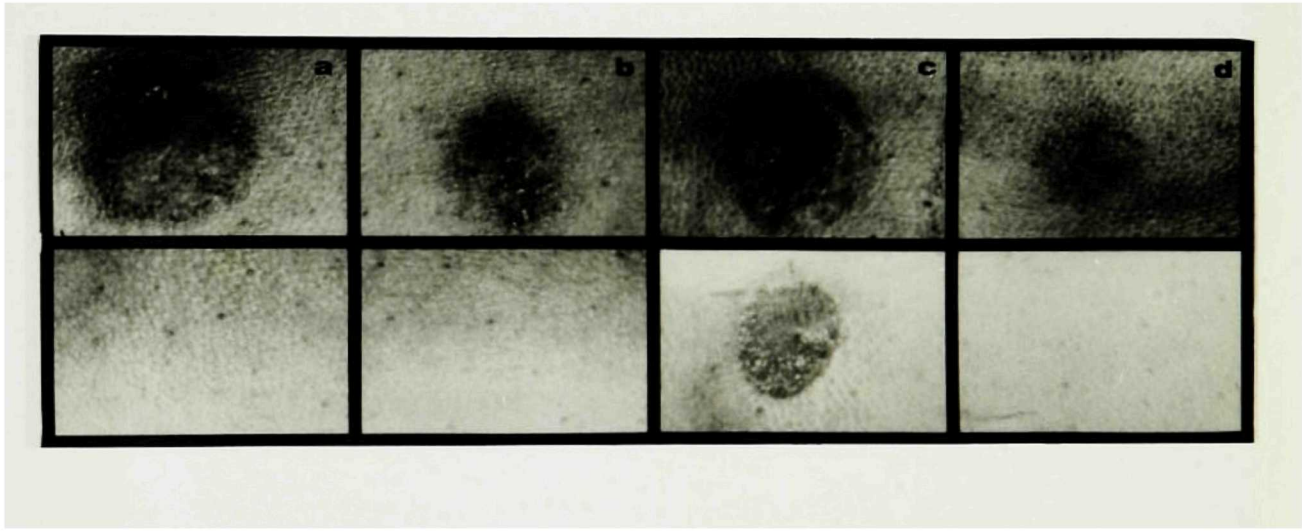


FIGURE 10

Comparison of the cytotoxicities of radioactive and nonradioactive toxin and CRM9. Toxin and CRM9 were iodinated by the chloramine-T method described in Materials and Methods. Dilutions of ^{125}I-toxin (■), unlabeled toxin (●), ^{125}I-CRM9 (□), and unlabeled CRM9 (o) were added to monolayers of Vero cells in multiwell plates. The cells were incubated for 72 hours, washed, and assayed for total cell protein in the adherent fraction of cells as described in Materials and Methods. The dose response curves compare the protein in untreated controls (c) to protein in intoxicated cultures (c'). Response is given by ((c-c')/c).

FIGURE 10

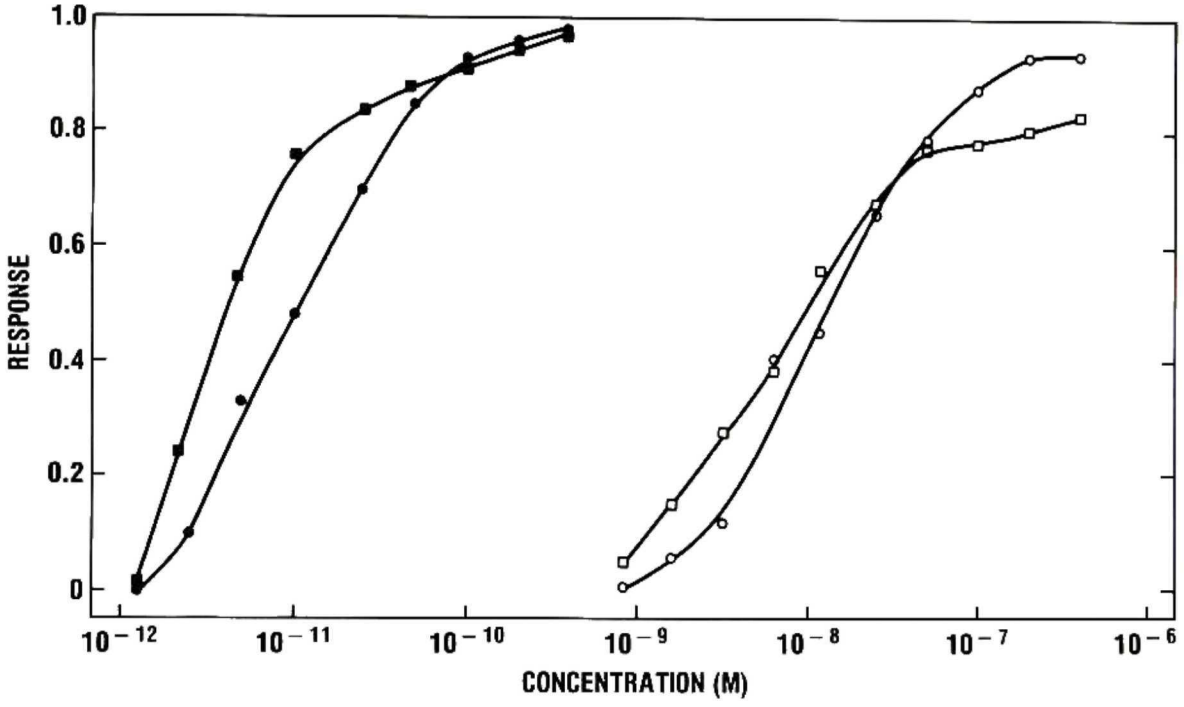
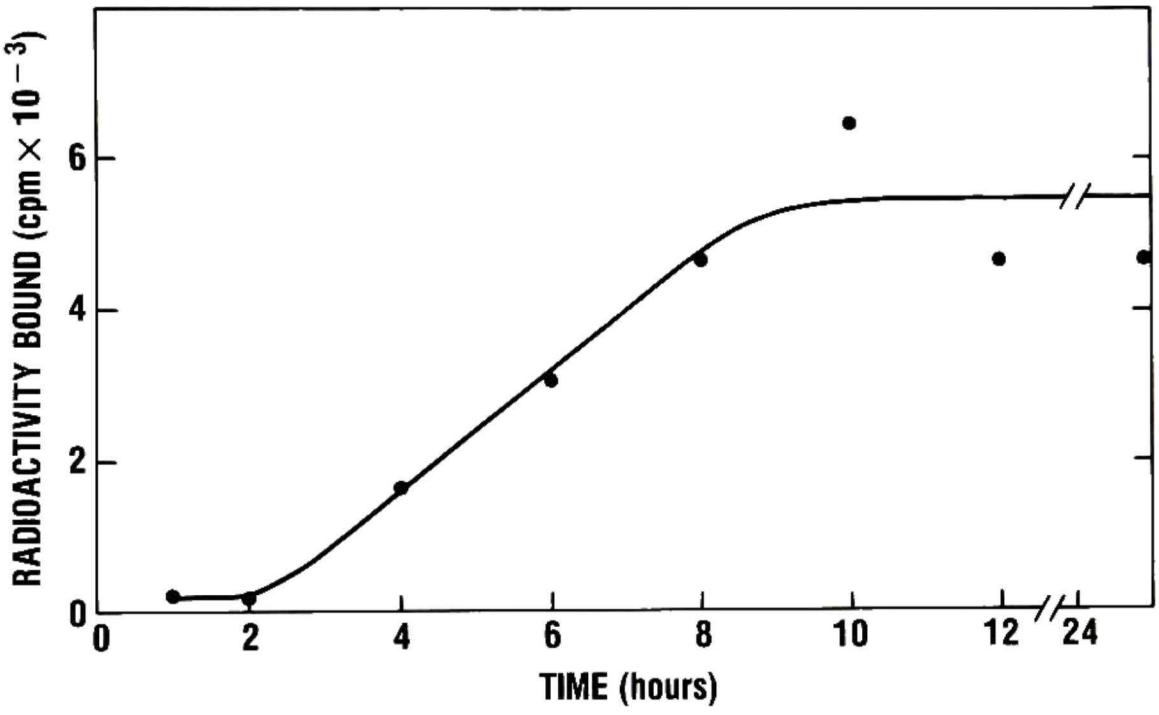

1.0
0.8
0.6
0.4
0.2
0
RESPONSE
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
CONCENTRATION (M)

FIGURE 11

Time course for the binding of ^{125}I-toxin to Vero cells. Monolayers of Vero cells in M199 (2% FCS) were chilled to 4° C. ^{125}I-toxin (25 ng/ml) was added to each well. The cells were incubated at 4° C for the times indicated on the abcissa. At each time point duplicate samples were harvested as described in Materials and Methods.

FIGURE 11



CRM9 for 14 hours. Binding of ^{125}I-toxin was blocked by the presence of a large excess of unlabeled toxin, demonstrating that specific binding of ^{125}I-toxin occurred (Figure 12, panel A). Under the same conditions, specific binding of ^{125}I-CRM9 to Vero cells was not observed (Figure 12, panel B). Based on a Scatchard plot of the specific binding of toxin (Figure 13), the affinity constant, determined from the slope, was 1.4×10^9 liters/mole, and there were approximately 7,700 specific binding sites/cell (calculated from the X-intercept and the number of cells per well). In four different experiments the affinity constants ranged from 1.4×10^9 to 2×10^9 and the number of binding sites/cell ranged from 3,200 to 7,700. A similar analysis was not possible for the binding of CRM9 since specific binding was not detected. The binding of ^{125}I-CRM9 was not blocked by an excess of unlabeled CRM9 (data not shown). Unlabeled CRM9 was less effective than comparable concentrations of unlabeled toxin in competing with ^{125}I-toxin for binding sites on Vero cell membranes (Figure 12, panel C).

VI. Competition for cell membrane receptors.

It has been shown that CRM197, which has a normal fragment B, competed with toxin for cell membrane receptors and blocked the intoxication of HeLa cells (84, 189). Similarly, the abilities of CRM9 and CRM197 to block the intoxication of Vero cells by toxin were tested (Figure 14, panels A and B). As expected, CRM197 blocked the intoxication of Vero cells by toxin (Figure 14, panel A). CRM9 failed to block intoxication by native toxin. At concentrations that were

FIGURE 12

Binding of ^{125}I-toxin and ^{125}I-CRM9 to Vero cells. Monolayers of Vero cells in M199 medium (10% FCS) were chilled for 1 hour prior to each assay. Dilutions of radiolabeled toxin ± unlabeled toxin (Panel A), radiolabeled CRM9 ± unlabeled toxin (Panel B), and radiolabeled toxin ± unlabeled CRM9 were added to triplicate wells, incubated for 14 hours at 4° C, and harvested as described in Materials and Methods. Specific binding (Δ) is the difference between the curves for label alone (•) and label + unlabeled protein (o). Panel A: (•), ^{125}I-toxin; (o), ^{125}I-toxin + unlabeled toxin (20 μg/ml); (Δ), specific binding. Panel B: (•), ^{125}I-CRM9; (o), ^{125}I-CRM9 + unlabeled toxin (20 μg/ml). Panel C: (•), ^{125}I-toxin; (o), ^{125}I-toxin + unlabeled CRM9 (20 μg/ml); (Δ), specific binding.

FIGURE 12

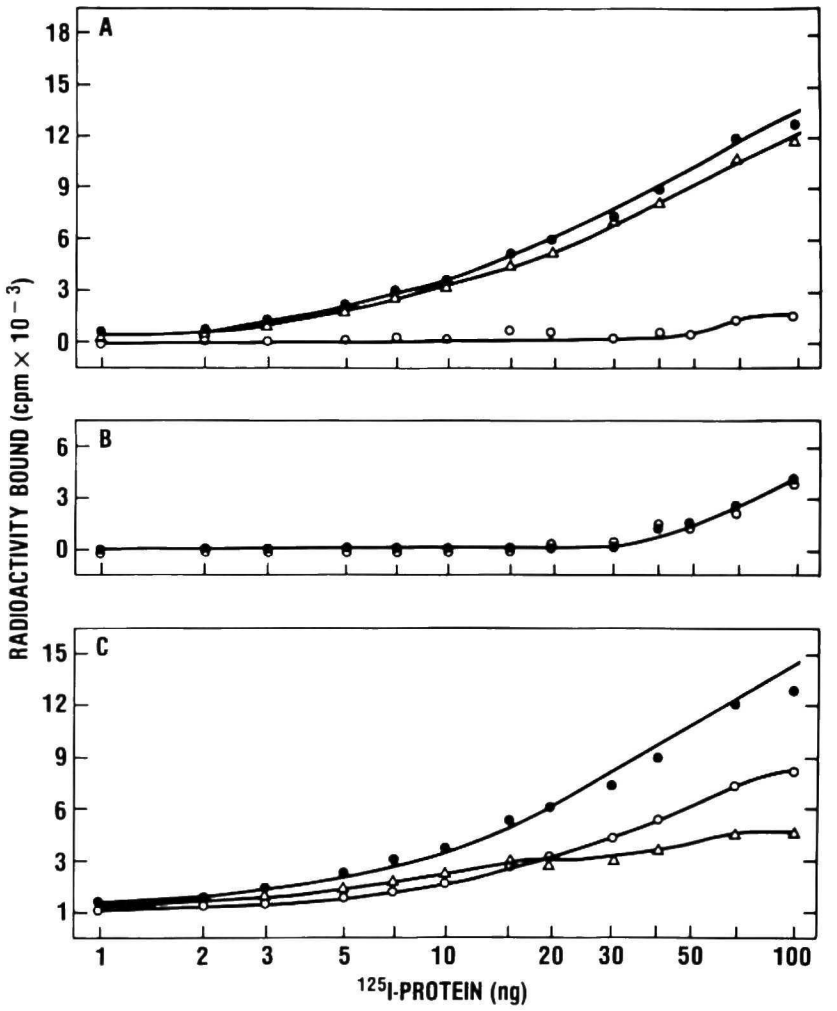

A
B
C
18
15
12
9
6
3
0
6
3
0
15
12
9
6
3
1
1
2
3
5
10
20
30
50
100
RADIOACTIVITY BOUND (cpm × 10⁻³)
¹²⁵I-PROTEIN (ng)

FIGURE 13

Scatchard analysis of the specific binding of toxin to Vero cells. Specific cell-associated counts were plotted by the method of Scatchard (174). Bound counts (B) are plotted on the abcissa and bound/free (B/F) on the ordinate. The line was calculated for best fit by linear regression analysis.

FIGURE 13

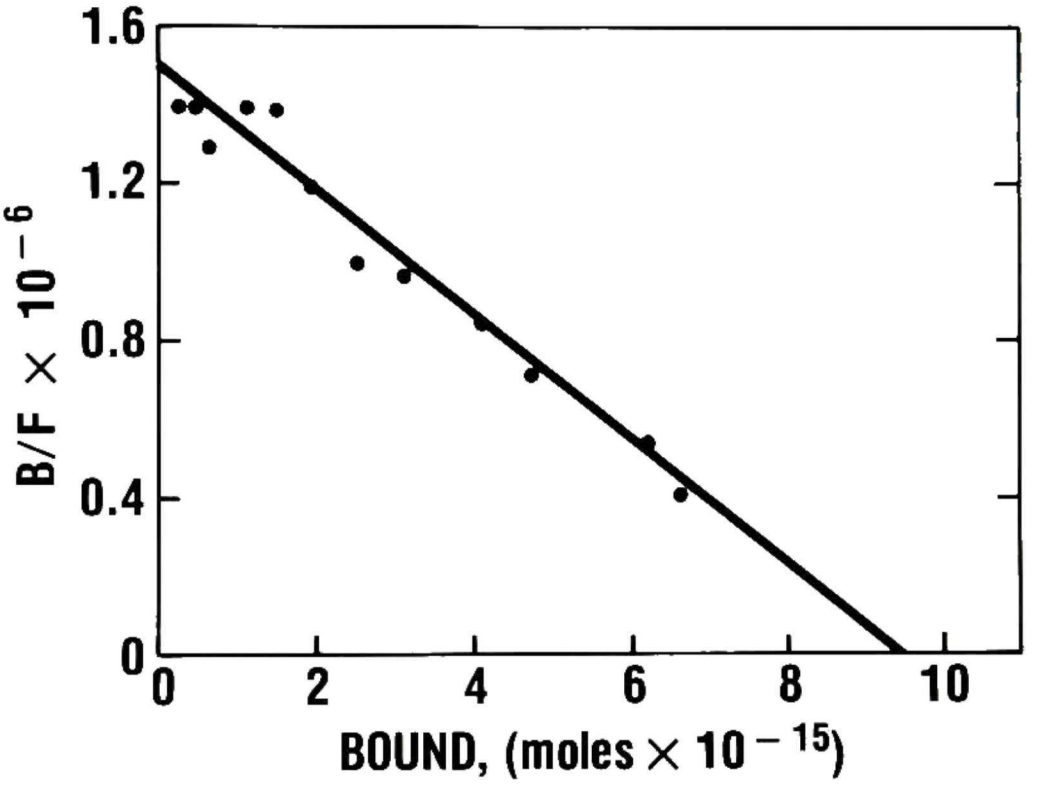


FIGURE 14

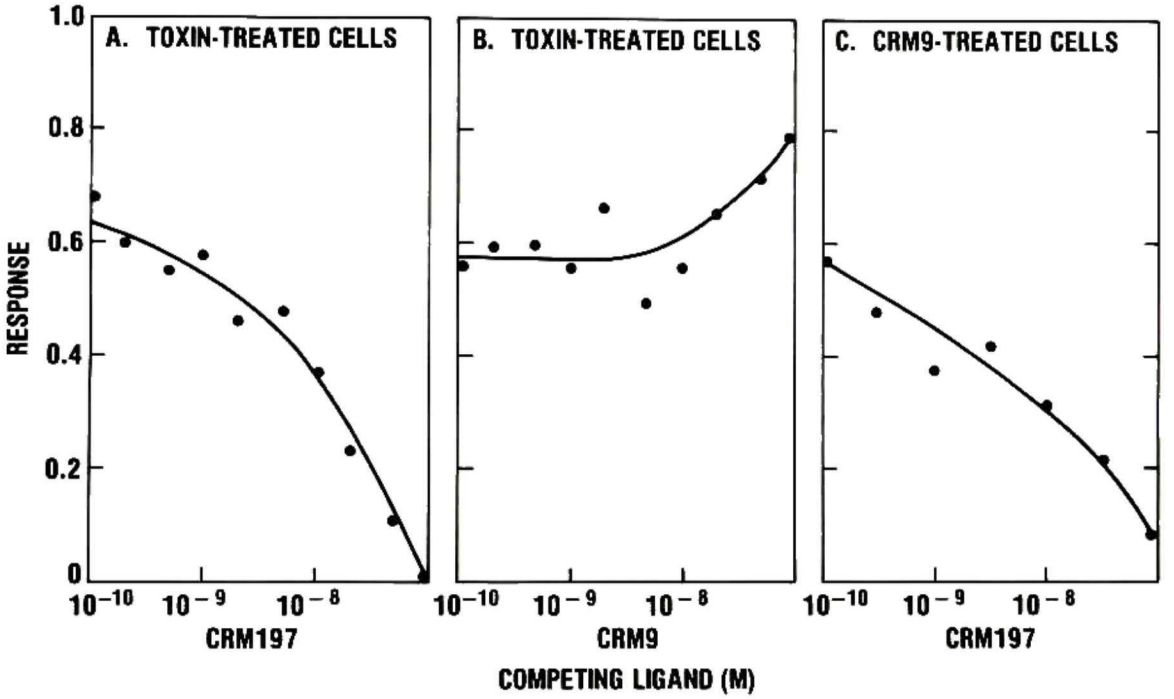
Ability of CRM197 and CRM9 to inhibit intoxication of Vero cells .

Panel A: Monolayers of Vero cells were treated with a concentration of toxin that produced a response of 0.6 plus various concentrations of CRM197. Each monolayer was tested for protein synthesis as described in Materials and Methods. Response is given by ((c-c')/c) (see Figure 7).

Panel B: Monolayers of Vero cells were treated with a concentration of toxin that produced a response of 0.6 plus various concentrations of CRM9. Each monolayer was harvested as described for panel A.

Panel C: Monolayers of Vero cells were treated with a concentration of CRM9 that produced a response of 0.6 plus increasing concentrations of CRM197 and were harvested as described for panel A.

FIGURE 14


A. TOXIN-TREATED CELLS
B. TOXIN-TREATED CELLS
C. CRM9-TREATED CELLS
RESPONSE
1.0
0.8
0.6
0.4
0.2
0
10^{-10} 10^{-9} 10^{-8}
CRM197
CRM9
CRM197
COMPETING LIGAND (M)

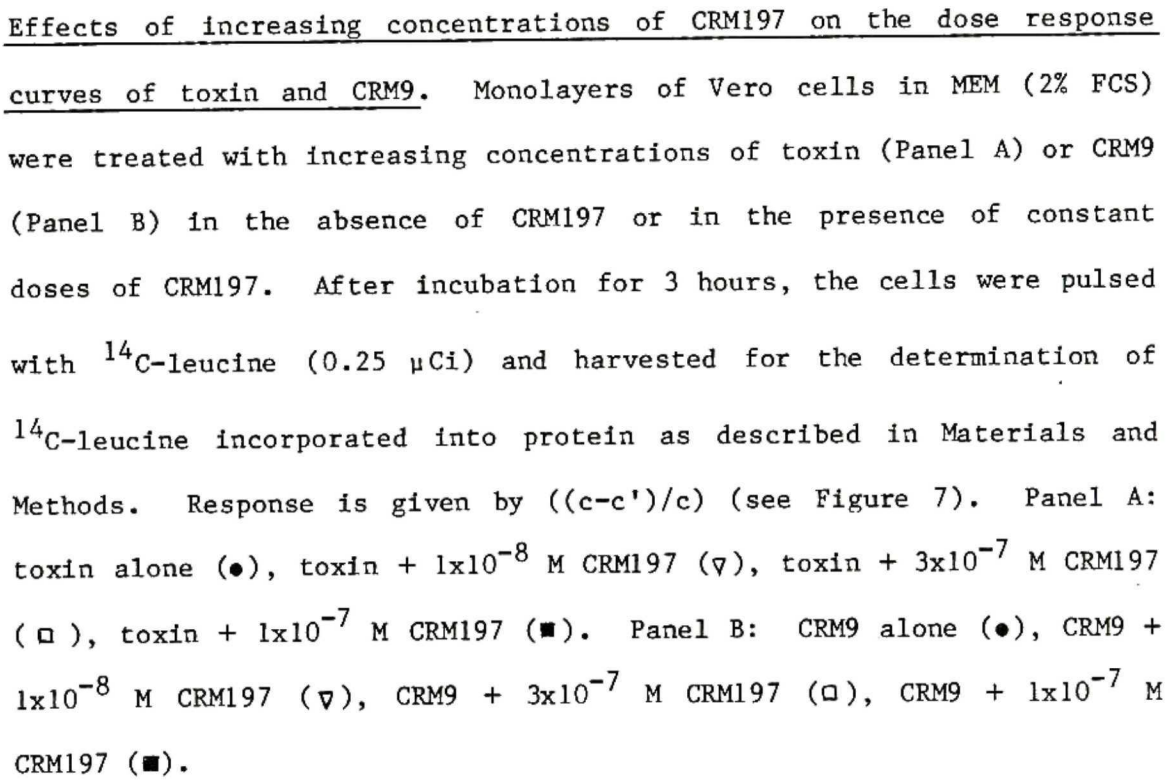
found to be protective when CRM197 was the competing ligand, CRM9 was inherently toxic and increased the toxic response (Figure 14, panel B). In a similar experiment the ability of CRM197 to block intoxication by CRM9 was tested (Figure 14, panel C). CRM197 afforded complete protection to Vero cells from intoxication by CRM9. The protective effect of CRM197 suggested that CRM197 and CRM9 competed for a common cellular target on Vero cells.

Next, the apparent dissociation constant (K_I) for CRM197, in the presence of either toxin or CRM9, was determined. Dose response curves for toxin in the presence or absence of CRM197 were generated (Figure 15, panel A). Similar dose response curves for CRM9 were generated (Figure 15, panel B). Each series of curves was plotted by the method of Schild (175, Figure 16, panels A & B). The K_I for CRM197 is indicated by the intercept. The K_I values for CRM197 were $5x10^{-9}$ M and $1x10^{-8}$ M, respectively, in the experiments with toxin and with CRM9. The slopes of these lines on the Schild plots were 0.76 for CRM197 in the presence of toxin and 1.3 for CRM197 in the presence of CRM9.

VII. Effects of nucleotides on intoxication of Vero cells.

The effects of nucleotides on the intoxication of Vero cells by CRM9 or by toxin were examined. The effectiveness of nucleoside triphosphates as inhibitors of cytotoxicity was demonstrated (Figure 17, panels A and B). ATP was the most potent inhibitor, followed by GTP, UTP, and CTP. In addition, the potency of the adenine nucleotides in preventing intoxication by either toxin or CRM9 was directly correlated with the number of phosphate residues; the order

FIGURE 15



Effects of increasing concentrations of CRM197 on the dose response curves of toxin and CRM9. Monolayers of Vero cells in MEM (2% FCS) were treated with increasing concentrations of toxin (Panel A) or CRM9 (Panel B) in the absence of CRM197 or in the presence of constant doses of CRM197. After incubation for 3 hours, the cells were pulsed with ^{14}C-leucine (0.25 μCi) and harvested for the determination of ^{14}C-leucine incorporated into protein as described in Materials and Methods. Response is given by ((c-c')/c) (see Figure 7). Panel A: toxin alone (●), toxin + $1x10^{-8}$ M CRM197 (▽), toxin + $3x10^{-7}$ M CRM197 (□), toxin + $1x10^{-7}$ M CRM197 (■). Panel B: CRM9 alone (●), CRM9 + $1x10^{-8}$ M CRM197 (▽), CRM9 + $3x10^{-7}$ M CRM197 (□), CRM9 + $1x10^{-7}$ M CRM197 (■).

FIGURE 15

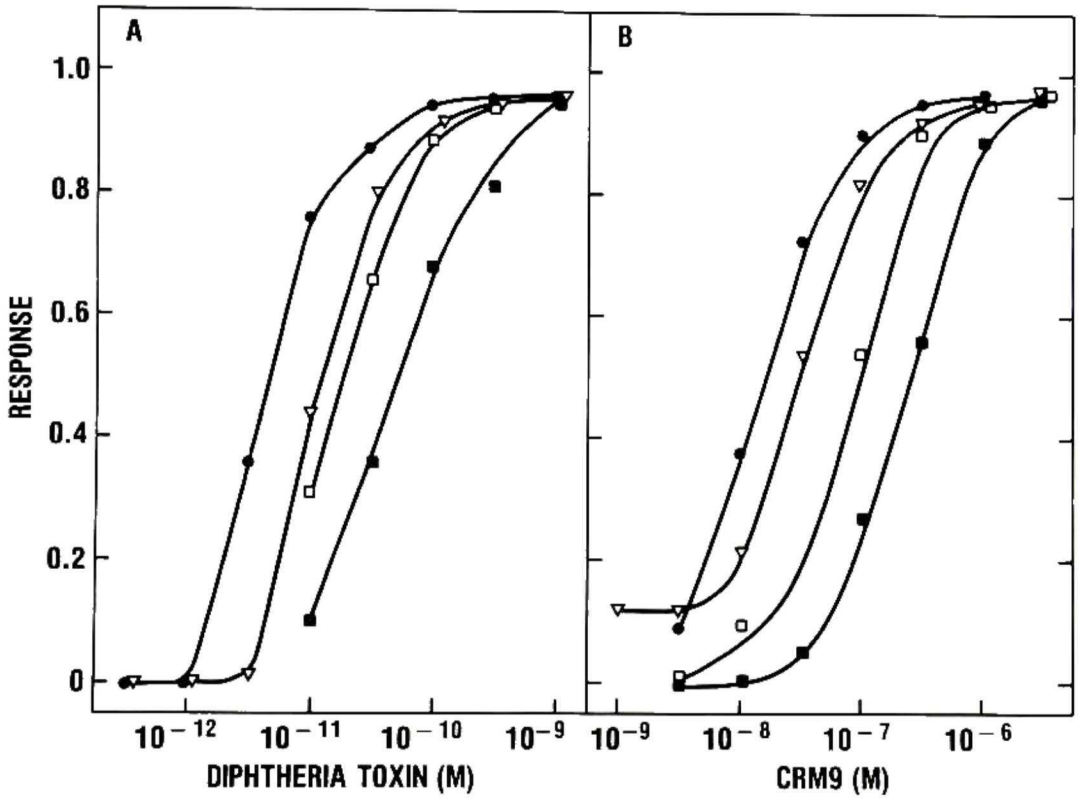

A
B
1.0
0.8
0.6
0.4
0.2
0
RESPONSE
10^{-12}
10^{-11}
10^{-10}
10^{-9}
DIPHTHERIA TOXIN (M)
10^{-9}
10^{-8}
10^{-7}
10^{-6}
CRM9 (M)

FIGURE 16

Schild plot of the competition between CRM197 and toxin (Panel A) and CRM197 and CRM9 (Panel B) in Vero cells. I= molar concentration of CRM197, T= molar concentration of intoxicating ligand to give a response of 0.5 in the absence of CRM197, T' = molar concentration of intoxicating ligand to give a response of 0.5 in the presence of I concentration of inhibitor. Response is a measure of protein synthesis and was determined as described in the legend for Figure 15.

FIGURE 16

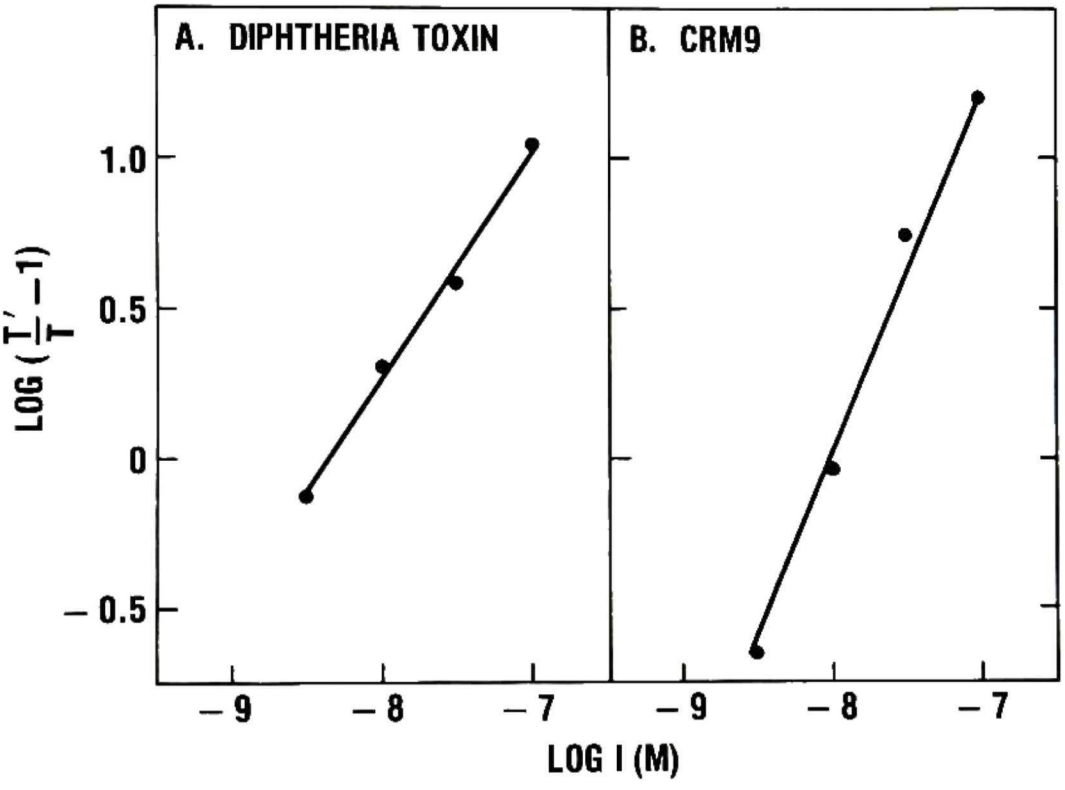
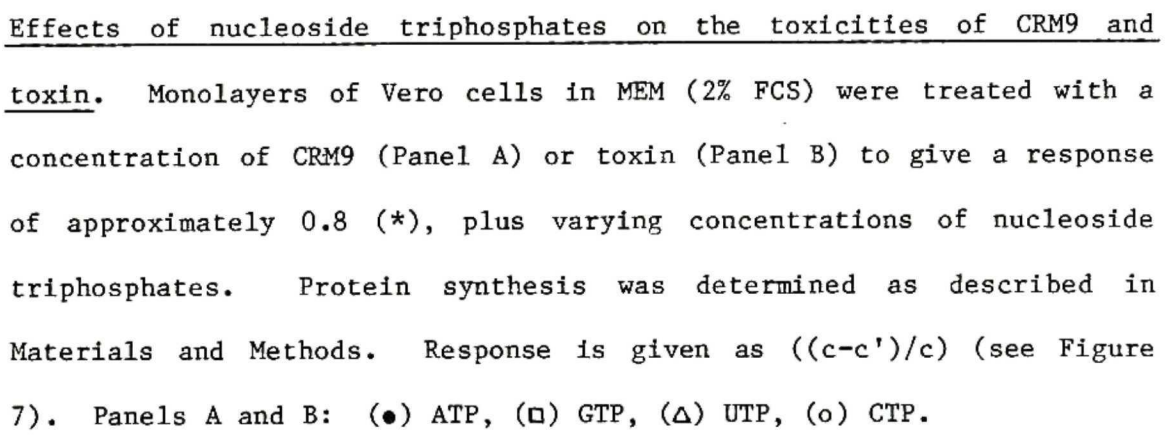
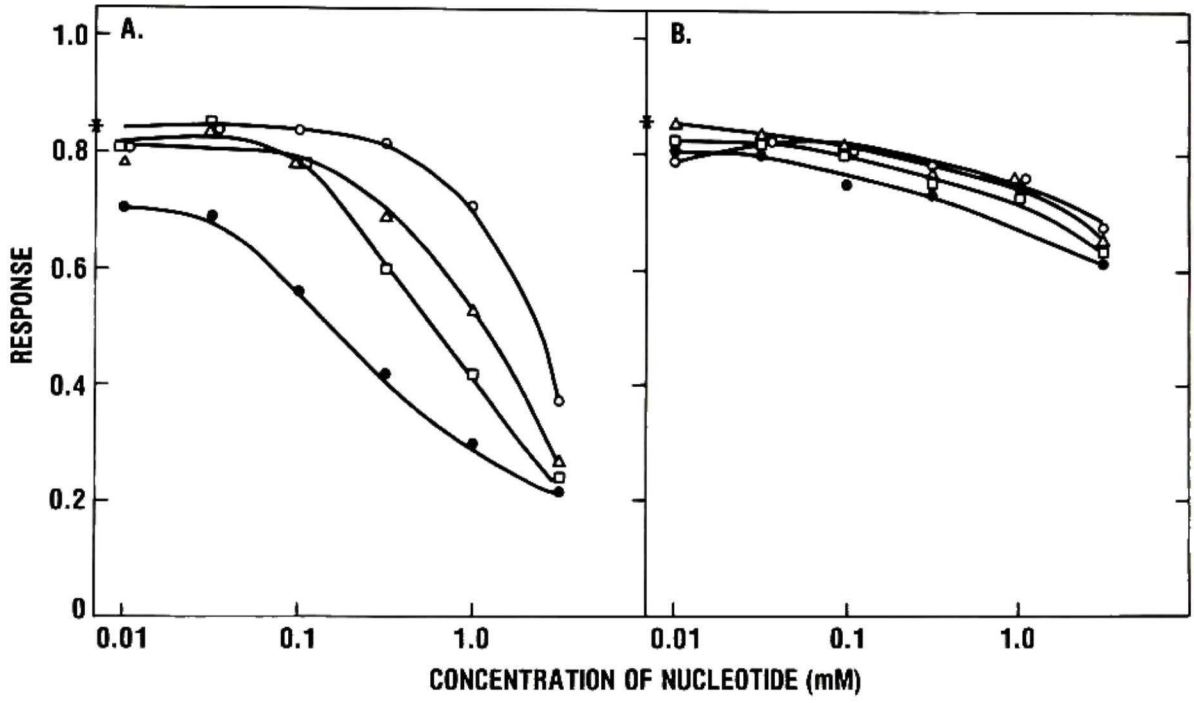


FIGURE 17



Effects of nucleoside triphosphates on the toxicities of CRM9 and toxin. Monolayers of Vero cells in MEM (2% FCS) were treated with a concentration of CRM9 (Panel A) or toxin (Panel B) to give a response of approximately 0.8 (*), plus varying concentrations of nucleoside triphosphates. Protein synthesis was determined as described in Materials and Methods. Response is given as ((c-c')/c) (see Figure 7). Panels A and B: (●) ATP, (□) GTP, (△) UTP, (o) CTP.

FIGURE 17


A.
B.
1.0
0.8
0.6
0.4
0.2
0
RESPONSE
0.01
0.1
1.0
0.01
0.1
1.0
CONCENTRATION OF NUCLEOTIDE (mM)

of potency was adenosine tetraphosphate > ATP > ADP > AMP (Figure 18, panels A and B). In both assays, the protection afforded to Vero cells by nucleotides was greater with CRM9 than with toxin.

VIII. Reconstitution of native toxin from CRM9 and CRM197.

Finally, the location of the defect in CRM9 was identified. Uchida and coworkers (192) demonstrated that when the normal fragment A from the nontoxic CRM45 and the normal fragment B from the nontoxic CRM197 were combined, toxicity was restored to the level of native toxin. In similar reconstitution experiments, samples containing either toxin, toxin-related proteins, or mixtures of these proteins, were treated with trypsin and DTT to separate the A and B fragments. The subsequent removal of DTT allowed reoxidation of sulfhydryl groups and the concomitant rejoining of fragments A and B. The cytotoxicity of each sample mixture was assayed before and after treatment (Table 4). In control experiments, when mixtures of CRM197 and purified fragment A from wild-type toxin were treated, cytotoxicity was restored to the level of wild-type toxin (Table 4). In contrast, when CRM9 and purified fragment A from toxin were treated in the same sample mixture, the level of cytotoxicity did not change (Table 4). Before treatment, samples containing both CRM9 and CRM197 were nontoxic for Vero cells but after treatment, cytotoxicity was restored to the level of wild-type toxin (Figure 19 and Table 4). In this sample, some of the reconstituted molecules contained the fragment A from CRM9 and the fragment B from CRM197. From these data, we inferred that the fully toxic reconstituted molecules contained a normal fragment A from CRM9 and a normal fragment B from CRM197.

FIGURE 18

Effects of adenine nucleotides on the toxicities of CRM9 and toxin. Monolayers of Vero cells in MEM (2% FCS) were treated with a concentration of CRM9 (Panel A) or toxin (Panel B) to give a response of approximately 0.8 (*), plus varying concentrations of adenine nucleotides in 0.01 M Hepes buffer. Protein synthesis was determined as described in Materials and Methods. Response is given as ((c-c')/c) (see Figure 7). Panels A and B: (●) A-tetra-P, (Δ) ATP, (□) ADP, (o) AMP.

FIGURE 18

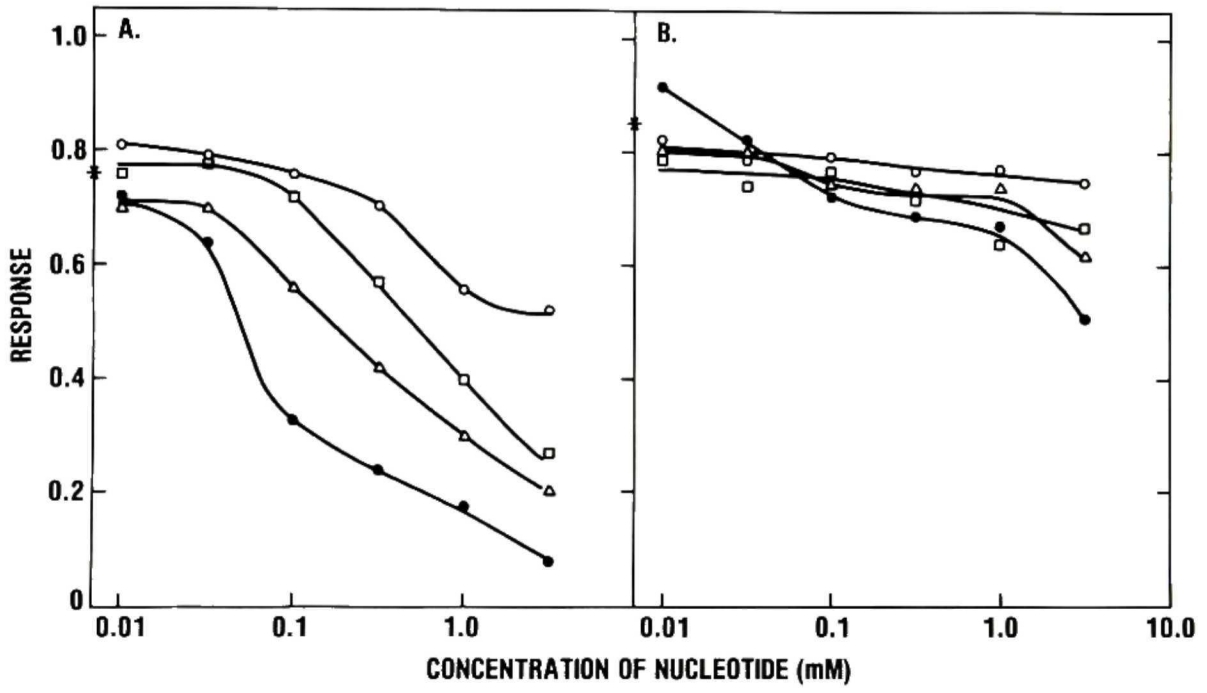

A.
B.
1.0
0.8
0.6
0.4
0.2
0
RESPONSE
0.01
0.1
1.0
0.01
0.1
1.0
10.0
CONCENTRATION OF NUCLEOTIDE (mM)

Table 4. Reconstitution of Diphtheria Toxin from CRM9 and CRM197

Sample Treated[a]	Dose to Give a Response of 0.5	
	Before Treatment	After Treatment
Diphtheria Toxin	7×10^{-11} M	7×10^{-11} M
CRM9 + CRM197	Nontoxic	7×10^{-11} M
CRM197 + Fragment A	1×10^{-7} M	7×10^{-11} M
CRM9 + Fragment A	4×10^{-8} M	4×10^{-8} M
CRM9	4×10^{-8} M	4×10^{-8} M
CRM197	Nontoxic	Nontoxic
Fragment A	1×10^{-7} M	1×10^{-7} M

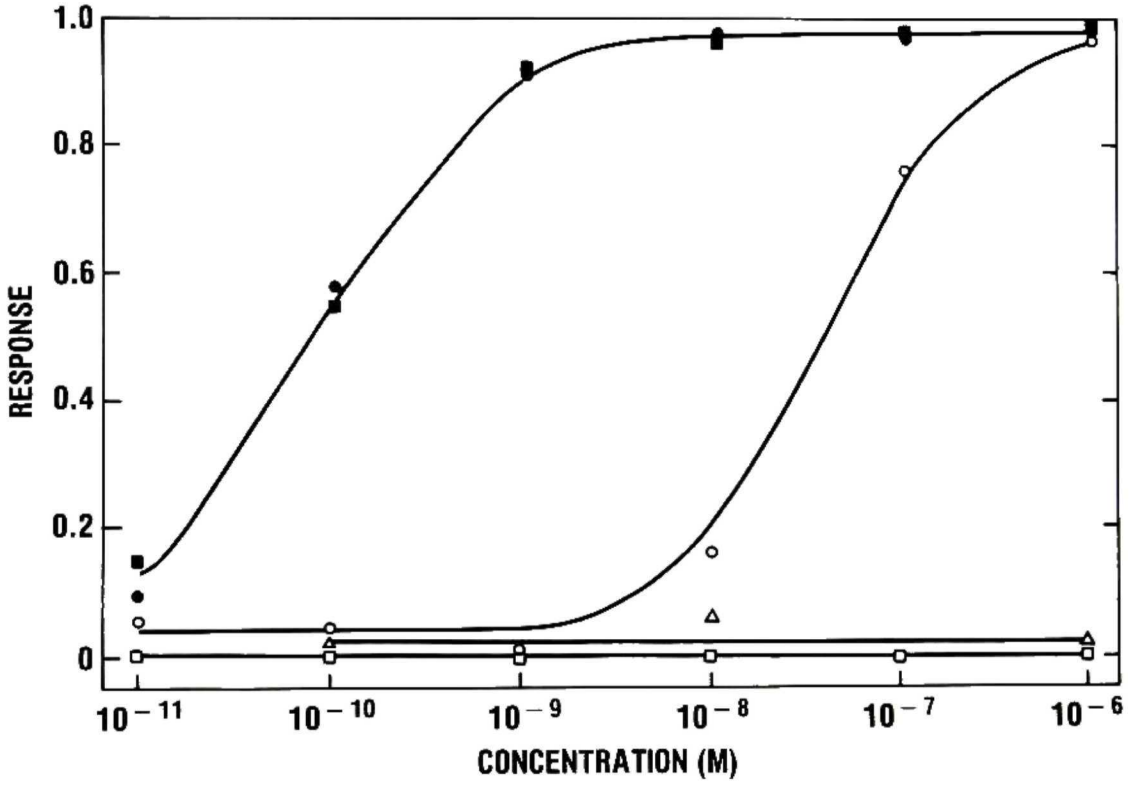
[a]See Materials and Methods for a description of the concentrations of samples tested, treatment with trypsin and dithiothreitol, and measurement of dose response.

FIGURE 19

Reconstitution of biologically active toxin from CRM9 and CRM197.

The protein, or equimolar concentrations of the proteins, to be tested were treated as described in Materials and Methods. Monolayers of Vero cells in MEM (2% FCS) were tested before and after treatment for protein synthesis as described in Materials and Methods. Response is given as ((c-c')/c) (see Figure 7). (●) toxin; (■) CRM9 plus CRM197, after treatment; (□) CRM9 plus CRM197, before treatment; (o) CRM9; (Δ) CRM197.

FIGURE 19


1.0
0.8
0.6
0.4
0.2
0
RESPONSE
10 − 11
10 − 10
10 − 9
10 − 8
10 − 7
10 − 6
CONCENTRATION (M)

Furthermore, the defect in CRM9 that caused hypotoxicity for Vero cells was located in its fragment B.

IX. Summary of the properties of CRM9.

In summary, $\beta^{tox-9\ h'}$ represents a new type of tox mutant of corynebacteriophage β. The C7($\beta^{h\ tox-201\ tox-9\ h'}$) produced a mutant protein called CRM9 that was defective in cytotoxicity for sensitive tissue culture cells and in toxicity in rabbit skin tests. Purified CRM9 was biochemically indistinguishable from diphtheria toxin in assays that measured antigenic cross-reactivity, ADPR-transferase activity associated with fragment A, and apparent molecular weight as determined by SDS-PAGE. Specific binding of CRM9 to Vero cells was not demonstrated under conditions where specific binding of toxin could be demonstrated; however, competition studies indicated that toxin or CRM9 competed with CRM197 for a common cellular target. Finally, reconstitution experiments demonstrated that the defect in CRM9 was located within fragment B. Thus, the decreased toxicity of CRM9 presumably resulted from a decreased affinity for binding to cell membrane receptors on sensitive cells.

DISCUSSION

The intracellular mechanism of action of diphtheria toxin has been well-characterized; however, several aspects of intoxication have not been adequately explained. These include 1) the structural requirements of the toxin molecule that mediate the binding to specific cell membrane receptor(s) and the translocation of toxin or fragment A across the cell membrane and 2) the chemical nature of the cell membrane receptor. The goal of this research project was to isolate tox mutants of phage β that produced CRMs which were defective in binding to cell membrane receptors or in entry into the cytosol, or both. These types of CRMs would be useful for examining the structure-activity relationships of fragment B and would contribute to a better understanding of the interactions of the toxin with its physiologic receptor(s).

The difficulty in isolating this type of mutant arises from the requirement that a biological assay be used for the initial screening. Vero cells in tissue culture were used for these studies as an extremely sensitive assay for cytotoxicity (116). Under the conditions of the screening assay that was developed, using a small number of cells per monolayer and a prolonged incubation period (48 to 72 hours), one picogram of toxin was sufficient to cause cytotoxicity (Table 3). Preliminary experiments (Table 2) demonstrated the desirability of screening for mutants by testing individual culture supernatants rather than samples taken from individual plaques on phage assay plates. This procedure eliminated the problem of testing samples from phage plaques that appeared to be toxinogenic because

small amounts of toxin from adjacent plaques had contaminated the samples.

Approximately 4,000 phage-infected cultures were screened for toxinogenicity by the method illustrated in Figure 2, and 1% of these were selected as presumptive hypotoxinogenic mutants. When the *tox* gene products encoded by these mutants were retested quantitatively for cytotoxicity, only eight were confirmed to be nontoxinogenic or hypotoxinogenic. The incomplete reliability of the preliminary screening procedure can probably be explained by variable toxin production in phage-infected cultures of *C. diphtheriae* in microtiter plates. In control experiments, competitive-binding radioimmunassays were used to demonstrate that the concentration of toxin antigen in supernatants from individual wells of a microtiter plate varied greatly under these conditions (data not shown). Thus, the secondary screening step was required to confirm the hypotoxinogenic phenotype of presumptive nontoxinogenic mutants.

Of the eight *tox* mutants isolated, four were hypotoxinogenic for Vero cells (β*tox*-9 *h'*, β*tox*-10 *h'*, β*tox*-16 *h'*, β*tox*-13 *h'*). The phenotypes of these mutants were determined by *in vitro* assays. Competitive binding radioimmunoassays using ^{125}I-toxin as the labeled ligand demonstrated that the CRMs produced by the mutant phages had the same antigenic determinants as wild-type toxin. However, *in vitro* ADPR-transferase assays demonstrated that only CRM9, CRM10, and CRM16 had wild-type levels of ADPR-transferase activity (Table 3). These three isolates, β*tox*-9 *h'*, β*tox*-10 *h'*, β*tox*-16 *h'*, represented a new class of *tox* mutants of phage β with genetic defects that could potentially affect the binding of toxin to cell membrane receptors or

entry of toxin into target cells, or both. CRM9 was most reduced in cytotoxicity and was selected for purification and further characterization.

Purified CRM9 and toxin were antigenically indistinguishable by competitive-binding radioimmunoassays and by immunodiffusion tests. The specific ADPR-transferase activities of fully activated CRM9 and toxin were also identical as were the apparent molecular weights. When cytotoxicity was compared in microtiter plates containing monolayers of Vero cells, the toxicity per unit of antigen in crude culture supernatants containing CRM9 (Table 3) was similar to the specific toxicity of purified CRM9 (data not shown). Thus, the culture supernatant did not contain factors which may have prevented the toxicity of CRM9 from being expressed. In quantitative comparisons, however, the cytotoxicity of CRM9 was less than that of toxin by approximately 1,000-fold.

The binding of CRM9 to Vero cells was examined using two different assays. ^{125}I-CRM9 did not bind specifically to Vero cell membranes under conditions where specific binding of ^{125}I-toxin could be demonstrated (Figure 12). As the concentration of radiolabeled toxin was increased above 100 ng per ml, nonspecific binding accounted for a significant proportion of total binding (data not shown). From a Scatchard analysis of the specific binding of toxin, an association constant of 1.4×10^9 liters/mole was calculated. This value is within the range of previously reported association constants for the binding of toxin to Vero cells (118). The Scatchard analysis indicated that there were 7,500 specific receptor sites per Vero cell. Although this number of binding sites was significantly lower than previously

reported values ($1-2x10^5$ binding sites/cell, (118)), our results were consistent for four separate experiments (see Results). The difference in the number of receptor sites per cell and the difference in susceptibility of Vero cells to toxin might reflect differences in these two sublines of Vero cells.

At 20 μg/ml ($3x10^{-7}$ M), CRM9 was less effective than toxin in blocking the binding of ^{125}I-toxin (Figure 12, compare panels A and C). At a concentration of $3x10^{-7}$ M CRM9 is toxic for Vero cells (Figure 7). The decreased binding of ^{125}I-toxin in the presence of a fully toxic dose of CRM9 suggested that toxin and CRM9 might be capable of binding to a common cell membrane receptor, although with different affinities.

We wished to determine if the intoxication of Vero cells by high concentrations of CRM9 reflected a nonspecific pathway for uptake and internalization of CRM9 or if intoxication was dependent on the same specific pathway involved in the receptor-mediated binding and internalization of wild-type diphtheria toxin. CRM197 was previously shown to block the intoxication of sensitive cells by toxin, and this inhibition has been interpreted by many investigators as a competition between toxin and CRM197 for binding to toxin receptors on the plasma membrane. We tested the ability of CRM197 to inhibit intoxication of Vero cells by high concentrations of CRM9. If the uptake of CRM9 occurred by a nonspecific pathway, then no competition by CRM197 would be expected. In contrast, we found that CRM197 could provide complete protection against the intoxication of Vero cells by CRM9 (Figure 14).

A series of dose response curves for toxin and for CRM9 were generated in the presence of increasing concentrations of CRM197. The

dose response curves for toxin and for CRM9 in the presence or absence of CRM197 were parallel and reached the same maxima which indicated that CRM197 acted as a competitive antagonist (Figure 15). Although the K_I's for these plots were similar, the deviation of the slopes from unity suggested that the inhibition of toxin or CRM9 toxicity by CRM197 may be more complex than a simple competition for binding of these two ligands to a common cell membrane receptor. The measured response was the inhibition of protein synthesis; thus the assay depends not only on the binding of toxin to the cell surface but also on additional steps involved in the endocytosis of toxin and the translocation of biologically active fragment A to the cytosol. There is a considerable lag period which precedes the inhibition of protein synthesis, and the events which transpire during this period are not completely clear. Methods based on direct binding assays are independent from steps in the intoxication process subsequent to binding and should provide the most accurate means of assessing toxin-receptor interactions. From the partial inhibition of binding of ^{125}I-toxin by CRM9 and from the complete inhibition of toxin or CRM9 toxicity by CRM197, however, we infer that CRM9 and toxin bind to a common class of cell membrane receptors that are essential for the uptake of toxin or CRM9 by Vero cells. The decreased toxicity of CRM9 appears to be due to a decreased affinity of binding to toxin receptors to a level that precludes direct measurement of binding of ^{125}I-CRM9 to Vero cells in the assay system that was used for our experiments.

Since the binding of toxin is mediated by fragment B, it would be expected that the mutation in $\beta\underline{tox\text{-}9}\ \underline{h'}$ would alter the fragment B

of CRM9. To determine the location of the tox-9 mutation, we treated samples containing mixtures of toxin, fragment A, or CRMs under conditions that allowed separation and random reassociation of the A and B fragments. In control experiments, purified fragment A from wild-type toxin was combined with fragment B from CRM9 and the dose response curves were not changed. In contrast, when fragment A from toxin was combined with fragment B from CRM197, fully toxic molecules were formed. Finally, the fragments derived from CRM9 and CRM197 could reassociate to form the fully active toxin. From these data, we concluded that fragment A from CRM9 and fragment B from CRM197 were similar to the A and B fragments from wild-type toxin and that the defect that caused hypotoxicity in CRM9 was located in the fragment B.

The binding function(s) associated with fragment B of toxin are located in the 17,000 dalton carboxyl-terminus of the molecule (189). The chemical nature of the cell surface receptor(s) is not clear. It is also not clear whether this specific binding is the result of interactions with one or more sites on the toxin molecule. Recent evidence demonstrated that CRM197 did not bind to P-site ligands (109) and led to the hypothesis that toxin may bind to two types of cell surface molecules, a specific receptor and a phosphate-containing molecule. It was also proposed that two sites on the toxin were involved, the receptor-binding site and the P-site, respectively (109).

It has been demonstrated that P-site ligands, such as nucleotides, inhibit both the binding of toxin to cell membranes (29, 118) and to phospholipid-containing liposomes (1) and also inhibit the intoxication of sensitive cells (117).

For these reasons, preliminary studies were designed to compare the effects of phosphate-containing compounds on the intoxication of Vero cells by toxin and by CRM9. The nucleotides tested inhibited intoxication both by toxin and CRM9 (Figure 17). The inhibitory effects were enhanced by an increase in the number of phosphates (Figure 18). The reason for the difference in the extent of inhibition of toxin and CRM9 by nucleotides is not yet clear.

It has been suggested that reversible binding of toxin to specific cell membrane receptors may be followed by interaction of the P-site with phosphate-containing cell surface molecules (109). If cooperative binding is involved in intoxication, it is possible that CRM9 binds normally to phosphate compounds and is altered in its specific binding to receptors. This could result if the alteration in CRM9 were located in the receptor-binding site or caused conformational changes that altered the receptor-binding site. If the increased effect of nucleotides as inhibitors of the intoxication by CRM9 versus toxin is repeated and confirmed then the tox-9 mutation might also be considered to have a direct or indirect effect on the P-site.

The mechanism by which P-site ligands inhibit cytotoxicity is believed to be by the direct binding of the phosphate moiety to the P-site in fragment B (109, 161). The P-site has been localized to the 8,000 dalton carboxyl-terminus of fragment B (161). Affinity labeling studies similar to those described (109, 161) would be useful for directly examining the ability of CRM9 to bind polyphosphates. Flow dialysis was used by Lory and coworkers (109) to determine the apparent dissociation constant between toxin and ATP. Measurements

such as these would allow comparisons of the dissociation constants for the interaction of toxin and CRM9 with polyphosphates which are independent of the complex interaction(s) which occur at the cell membrane.

A second possibility for the difference in nucleotide-binding by toxin and CRM9 might be based on toxin heterogeneity (110). Toxin was separated into two fractions by chromatography on an ATP-Sepharose column. Treatment of one of these fractions with urea revealed that a tightly bound, 260 nm-absorbing (nucleotide-like) compound was released. These fractions, designated DTN_b (nucleotide-bound form of diphtheria toxin) and DTN_f (nucleotide-free form of diphtheria toxin), respectively, differed in their abilities to bind to P-site ligands (159). The direct binding of DTN_b to Vero cells was reduced at 4°C compared to toxin (159). However, both forms of the toxin were equally cytotoxic at 37° C (110, 159) and this cytotoxicity was inhibited by ATP (110). These data suggested that the endogenous nucleotide slowly dissociated from the toxin during long incubation periods (i.e. 24 hours) and resulted in full toxicity. It does not seem likely that the binding of endogenous nucleotide caused the reduced toxicity of CRM9 for the following reasons. First, CRM9 is significantly less toxic than toxin for Vero cells at 37°C and 72 hours of incubation. Second, the toxicity of CRM9 was inhibited by nucleotides. Finally, the $A_{280/260}$ ratios for toxin and for CRM9 were identical (1.3/1), indicating that the proportions of DTN_f/DTN_b in each preparation were similar.

In contrast to the hypothesis that toxin binding to the cell surface involves two distinct sites on the toxin molecule, Proia and

coworkers (161) have proposed that nucleotides and a solubilized toxin-binding glycoprotein bind to a common site on the toxin molecule. This hypothesis is based on the following indirect evidence: 1) DTN_b does not bind to the solubilized glycoprotein (161), 2) nucleotides inhibit toxin-receptor interactions (118), 3) CRM45 which lacks the receptor-binding region of toxin does not bind nucleotides, and 4) DTN_b does not bind to Vero cell membrane receptors.

Preliminary experiments were designed to examine the binding of CRM9 and toxin to a purified ^{125}I-glycoprotein fraction isolated by the method of Proia (160) from the surface of Vero cells (data not shown). CRM9 and antitoxin immunoprecipitated approximately half as much radioactivity from the ^{125}I-glycoprotein fraction as was precipitated by CRM197 and antitoxin. These data suggested that CRM9 did bind to the ^{125}I-glycoprotein fraction but with a lower affinity than CRM197. However, further characterization of this fraction is required to demonstrate whether the immunoprecipitated fraction contained a single glycoprotein and whether this glycoprotein is involved in the physiologic binding of toxin to the cell surface.

The relationship, if any, between the ability of toxin to bind to phosphates and the insertion and translocation of toxin into the cell cytosol is not known. It has been demonstrated that the hydrophobic segments of fragment B (92, 101) have properties that would allow insertion into the hydrophobic core of the cell membrane and that fragment B does insert into and form channels in artificial membranes (45, 87) and liposomes composed of phospholipids (1). Thus, it seems likely that toxin interactions with phospholipids are

involved in the translocation of fragment A into the cytosol. Experiments to determine the ability of CRM9 to insert into membranes, in conjunction with studies to examine the ability of CRM9 to bind to phosphate-containing compounds, would be useful for studying the structural requirements of the toxin molecule for entry into the cell cytosol.

At present, the precise location of the tox-9 mutation on the genetic map of phage β is not known. The orientation of transcription of the tox gene in prophage β (100) and with vegetative phage β (74) has been determined. The order of sites tox-1, tox-2, tox-30, and tox-45 has also been determined (74, 100). The mutation which alters fragment B from CRM9 may be located between the tox-45 site and the distal end of the tox structural gene, in the segment corresponding to the 17,000 dalton C-terminal polypeptide that is missing from CRM45. The tox-9 marker could be mapped relative to tox-45; however, no markers more distal than tox-45 are currently available for the fine mapping of tox-9. It is also possible, however, that the binding defect in CRM9 is the result of a mutation in the portion of the tox gene corresponding to the proximal portion of the B fragment between tox-1 and tox-45. Localization of tox-9 to this region would provide evidence that the proximal portion of fragment B is either a part of the receptor-binding site or is essential for determining the conformation of the receptor-binding site. Recently, fragment B has been purified to homogeneity (54), five cyanogen bromide fragments from fragment B have been identified (54), and the amino acid sequence of fragment B has been partially determined (101). It should ultimately be possible, therefore, to purify fragment B from CRM9,

identify the CNBr fragment that is altered by the tox-9 mutation and determine the sequence of the altered peptide. These data would reveal the altered sequence associated with the putative receptor-binding site, or one of the lipid-associating domains, or some other region of the altered CRM9 polypeptide.

DNA sequencing would also allow the precise localization of the tox-9 mutation to be determined. Buck and coworkers (25) have recently constructed a physical map of restriction endonuclease cleavage sites on the DNA of corynebacteriophage β and have identified the specific BamHI fragment that contains the toxin structural gene. The DNA sequence of this fragment of DNA from wild-type β and from $_{\beta}$tox-9 h' could be compared to identify nucleotide substitutions in the mutant tox gene.

Determining the precise location of the tox-9 mutation, in conjunction with examining the nature of the specific receptor-binding properties, would enhance our understanding of the location and the physiological relevance of the functional domains of fragment B.

SUMMARY

A screening procedure using toxin-sensitive cells in culture was developed to identify mutants of corynephage β that were altered in toxinogenicity. Using this procedure, several classes of *tox* mutants were isolated, including a new type of mutant that was defective in binding to cell membrane receptors. Phage β*tox*-9 *h'* coded for a mutant toxin (CRM9) that was antigenically and enzymatically indistinguishable from wild-type diphtheria toxin but was significantly less toxic than wild-type toxin. The hypotoxicity of CRM9 was due to a decreased affinity for the toxin receptor. The defect that altered the binding properties of CRM9 was localized to fragment B. Two additional mutants were isolated that coded for hypotoxic CRMs. Together with CRM9, these mutant toxins will be valuable tools for studying the structural basis for the specific binding of diphtheria toxin to specific plasma membrane receptors on eucaryotic target cells.

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