Journal of Supramolecular Structure 9:47–55 (1978)
Transmembrane Signaling 139–147

Studies of the Diphtheria Toxin Receptor on Chinese Hamster Cells

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Concanavalin A, wheat germ agglutinin and the ovalbumin glycopeptide are all inhibitors of the cytotoxic effect of diphtheria toxin on Chinese hamster cells. Ovalbumin glycopeptide loses its inhibitory property after treatment with β-N-acetylglucosaminidase. This demonstrates the importance of the glycopeptide structure for the mechanism of inhibition. The glycopeptide may be a toxin cell-surface receptor analogue.

Diphtheria toxin-resistant mutants were isolated in order to search for cells that might have an altered toxin receptor. One mutant was 10- to 15-fold more resistant to diphtheria toxin than wild-type cells when protein synthesis was measured as a function of toxin concentration. However, when protein synthesis was measured as a function of time at a high toxin concentration, the time before onset of inhibition was identical in the mutant and wild-type cells. We present evidence indicating that the resistance of this mutant can be accounted for by a decreased affinity of toxin for a cell-surface receptor.

Key words: diphtheria toxin, lectins, cell surface receptors, diphtheria toxin resistance, somatic cell mutants

Diphtheria toxin (DT) is a protein of molecular weight 63,000 produced by Corynebacterium diphtheriae. The cytotoxic mechanism of DT involves three steps [1, 2]. First, the toxin interacts reversibly with a specific cell surface receptor; second, at least a 24,000 molecular weight fragment of the toxin is somehow translocated from the cell exterior to the cytoplasm; and third, the cytoplasmic toxin catalyzes transfer of the adenosine diphosphate ribose portion of NAD^+ to elongation factor II, rendering it inactive. The DT model is a well-defined system for transmembrane signaling; information contained in the amino acid sequence of DT is communicated to the cytoplasm by actual transfer of a polypeptide across the plasma membrane. The mechanism by which this occurs is unknown. Nicolson [3] suggested that surface-bound ricin, a toxin similar to DT, is taken into the cell by endocytosis and released to the cytoplasm by rupture of the endocytotic

Received May 15, 1978; accepted July 26, 1978.

0091-7419/78/0901-0047$02.00

vesicle. Boquet and Pappenheimer [4] suggested that a special pore is produced in the plasma membrane through which at least a fragment of the toxin passes. Whatever the mechanism, the DT receptor is likely to be a key participant in the process, and we have focused our attention on this receptor.

Little is known about the DT receptor. Boquet and Pappenheimer obtained evidence from direct binding studies that there are about 4,000 receptor sites per HeLa cell [4]. Ittelson and Gill studied CRM197, the product of a mutated toxin gene that lacks catalytic activity and is nonlethal [5]. They found that CRM197 apparently retains receptor binding ability and is a competitive inhibitor of DT with an apparent Kd of about 10^{-8}M. Recently, we reported that Concanavalin A (ConA) and wheat germ agglutinin (WGA) are inhibitors of DT and that an oligosaccharide, the ovalbumin glycopeptide, also inhibited the toxin [6]. This suggested that the DT receptor might contain an oligosaccharide component. In this paper, we present some additional data on the inhibition of DT by lectins and by ovalbumin glycopeptide.

Mutants resistant to DT have been isolated by Moehring and Moehring [7–9]. The study of cells resistant to toxin by virtue of changes at the level of the plasma membrane might provide information about the mechanism of DT entry. We have isolated DT resistant cells in order to probe the toxin receptor with CRM197, ConA, and WGA. We have found a mutant whose resistant phenotype can be explained by a decrease in the affinity of DT for the cell surface receptor.

MATERIALS AND METHODS

Diphtheria toxin was purchased (lot #D298) from Connaught Laboratories (Willowdale, Ontario, Canada) and purified to homogeneity by DE-52 chromatography. Purified CRM197 was the generous gift of Dr. A. M. Pappenheimer, Jr., Harvard University. Concanavalin A and wheat germ agglutinin were purchased from E-Y Laboratories (San Mateo, California). Jack bean β-N-acetylglucosaminidase, α-mannosidase, and purified ovalbumin were purchased from Sigma Chemical Corporation (St. Louis, Missouri). Ovalbumin glycopeptide was prepared by pronase digestion of ovalbumin by the general procedure of Huang et al [10]. Deoxyribonuclease I was obtained from Worthington Biochemical Corporation (Freehold, New Jersey).

Chinese hamster V79 cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.02 M N-2-hydroxyethylpiperazine-N′-2-ethanesulfonate buffer (HEPES), pH 7.4, penicillin, 50 units/ml, and streptomycin, 50 μg/ml. Clones resistant to DT were picked after exposure of mutagenized populations of cells to toxin. The phenotypes of resistant cells were determined by their response to increases in toxin concentration. The response of cells to DT was measured by the incorporation of [^{35}S]-methionine into trichloracetic acid-insoluble material. At 48 h before an experiment, the cells were plated in 24-well Falcon plates at 2×10^4 cells per sq/cm in normal media. Just prior to the assay, the medium was replaced with Dulbecco's modified Eagle's medium containing 1/20th the normal amount of methionine, 5 gm/liter galactose instead of glucose, and no serum. We used galactose instead of glucose to prevent interference of glucose with Concanavalin A. This had no effect on the growth rate of the cells. The assay was initiated by addition of DT, or DT mixed with an inhibitor, directly to the cells. After a 2 h incubation at 37°C, fetal bovine serum (to give a final concentration of 5%) and 0.1 μCi of [^{35}S]-methionine were added. One hour later, the cells were washed twice with phosphate-buffered saline and dissolved in 0.1 ml of a solution containing

0.05% sodium dodecyl sulfate, 1 mg/ml deoxyribonuclease I, and 1.0 mM $CaCl_2$ and $MgCl_2$. Aliquots were placed on numbered squares of Whatman 3M paper and soaked in 10% trichloroacetic acid for 30 min, followed by two washes with 95% ethanol. The papers were dried and assayed for radioactivity in a liquid scintillation counter. Schild plots were constructed as previously described [6].

RESULTS

Inhibitors of the Cytotoxic Effect of DT

The lectins ConA and WGA are inhibitors of the cytotoxic action of DT on Chinese hamster V79 cells. The antagonism of DT as a function of lectin concentration is shown in Figure 1. We have reported that the characteristics of this antagonism are consistent with a model of competitive inhibition at the level of the cell surface [6]. This suggested that

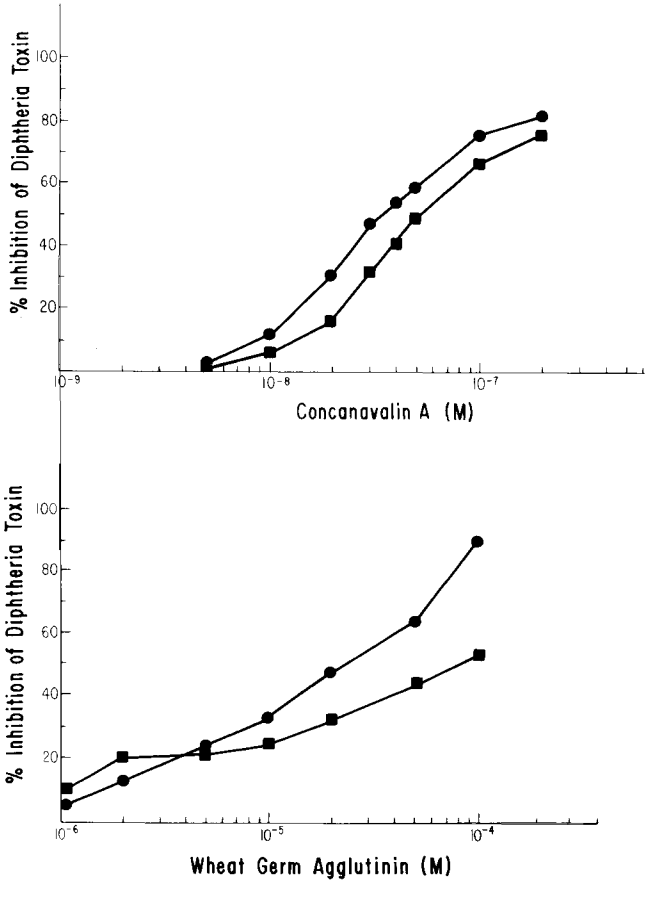

Fig 1. The inhibition by lectins of the effect of DT on wild-type cells (●) and class II mutant cells (■) as a function of ConA concentration (upper) and WGA concentration (lower). The DT concentration in the experiments with wild-type cells was 6×10^{-10}M and with mutant cells was 1.2×10^{-8}M. This concentration of toxin inhibited protein synthesis by 90% in both cases. The inhibition of the effect of DT was determined by the extent to which the lectins restored protein synthesis to control values.

DT might interact with a cell surface oligosaccharide. We screened several oligosaccharides for the ability to inhibit DT and found that ovalbumin glycopeptide shifted the toxin dose response curve of cells to higher DT concentrations. This inhibition as a function of glycopeptide concentration is shown in Figure 2. We previously proposed that the glycopeptide was a receptor analogue, and a comparative study of the inhibitory ability of several oligosaccharides suggested that terminal N-acetylglucosamine residues were important for this inhibition [6]. If this is true, it should be possible to affect the inhibitory activity of the glycopeptide by treatment with β-N-acetylglucosaminidase. We treated ovalbumin glycopeptide with either α-mannosidase or β-N-acetylglucosaminidase and tested the products for DT inhibitory activity. As seen in Figure 3, α-mannosidase treatment slightly reduced the toxin inhibitory property compared to an untreated control, and β-N-acetylglucosaminidase completely abolished all toxin inhibitory activity. This further suggests that terminal β-N-acetylglucosamine in the oligosaccharide is critical for toxin inhibition.

DT-Resistant Mutants

The isolation of DT-resistant mutants from cell populations normally sensitive to toxin has been described by Moehring and Moehring [7–9]. We found that toxin resistant cells appeared at a frequency of about 10^{-6} resistant clones per cell plated from unmutagenized populations under conditions where they were exposed to toxin during the entire period of clone formation. After treatment with 300 μg/ml of the mutagen ethyl methanesulfonate for 15 h, the frequency was increased 100-fold. We characterized the DT response of ten clones picked at random after a seven-day exposure to 10^{-9}M DT and ten

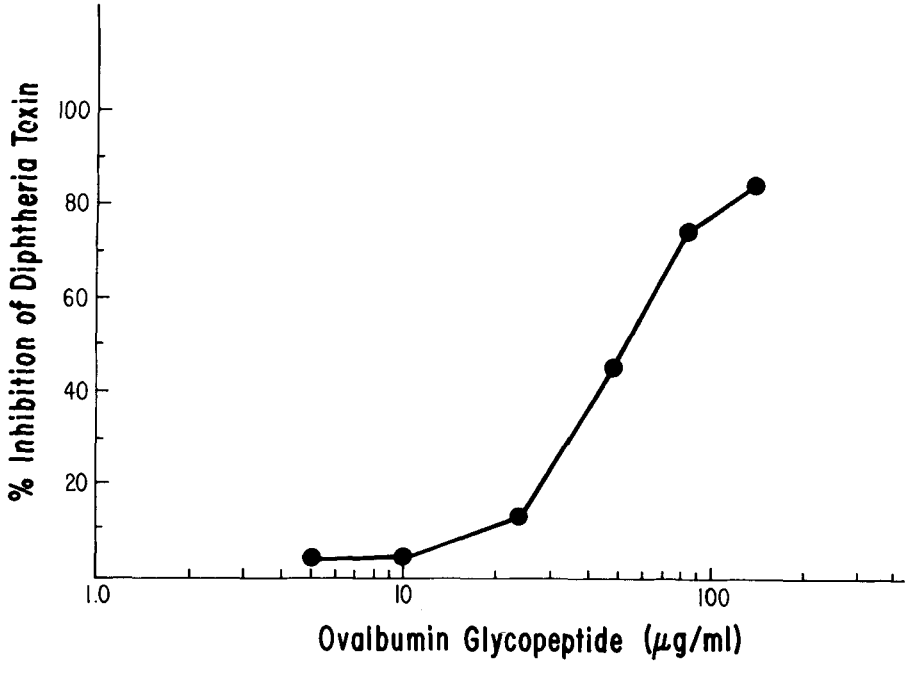

Fig 2. The inhibition of DT as a function of ovalbumin glycopeptide concentration. The DT in this experiment inhibited protein synthesis by 90% in the absence of glycopeptide.

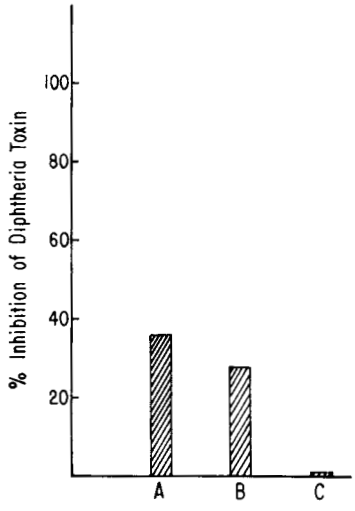

Fig 3. The effect on the DT antagonistic property of ovalbumin glycopeptide after treatment with glycosidases. No treatment, A; α-mannosidase treatment, B; β-N-acetylglucosaminidase treatment, C. 120 μg of glycopeptide was incubated for 5 h at 25°C in a volume of 100 μl with no enzymes, or with 5 units of α-mannosidase in 0.05 M citrate buffer, pH 4.5, or with 5 units of β-N-acetylglucosaminidase in 0.05 M citrate buffer, pH 5.0. The reaction was stopped by boiling the samples. The final concentration of glycopeptide in the assay was 46 μg/ml. The DT inhibited protein synthesis by 50% in the absence of glycopeptide. The inhibition of DT caused by the glycopeptide was determined by the degree to which protein synthesis was restored to normal.

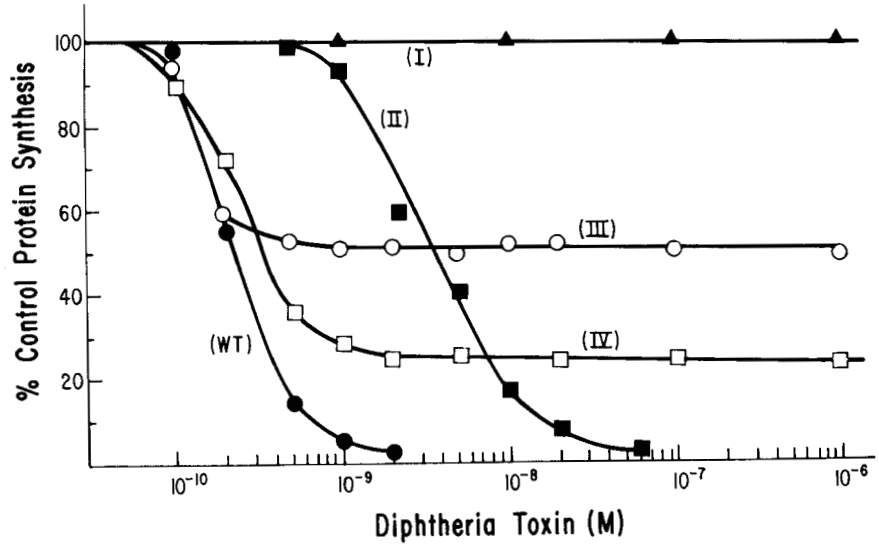

Fig 4. The DT resistant phenotypes of toxin-resistant mutants. Wild-type (wt), ●; Class I, ▲; Class II, ■; Class III, ○; Class IV, □.

clones picked after a 3 h exposure to 10^{-8}M DT. The response of these mutants when measured by the inhibition of protein synthesis after a 3 h exposure to increasing DT concentrations fell into one of four phenotypic classes, as shown in Figure 4. The number of clones belonging to each class from both selections is shown in Table I. Class I was totally resistant up to 10^{-5}M DT. Class II has a dose-response curve parallel to the wild-type but shifted ten- or twenty-fold to higher toxin concentrations.

TABLE I. The Number of Clones Belonging to Different Toxin-Resistant Phenotype Groups*

	Phenotypic class				
	wt	I	II	III	IV
Number of clones from selection 1	0	7	0	2	1
Number of clones from selection 2	2	4	2	1	1

*The phenotypic classes are shown in Figure 4. Cells were exposed to 10^{-9} M DT for 7 days in selection 1 and 10^{-8} M DT for 3 h in selection 2.

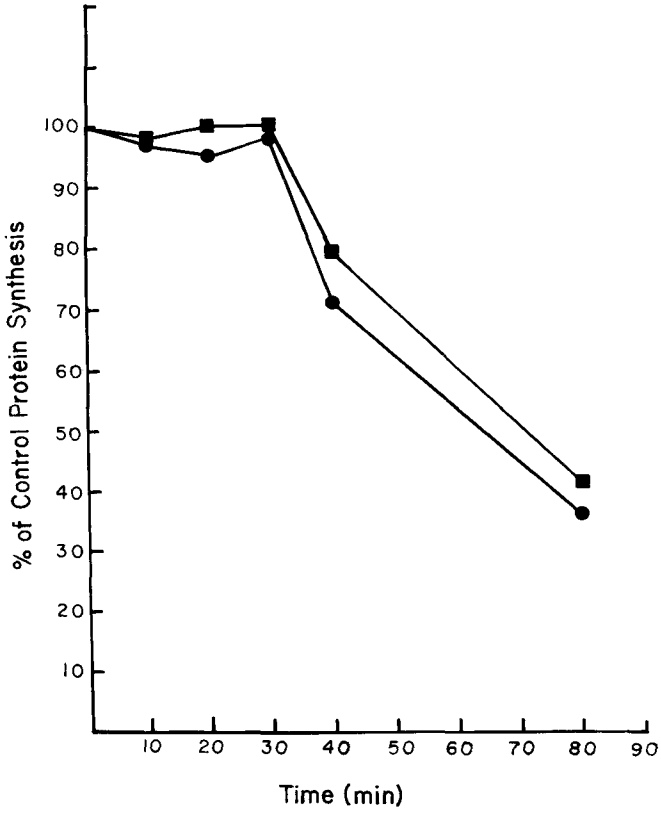

Fig 5. The inhibition of protein synthesis as a function of time for wild-type cells, •, and a class II mutant, ▪, exposed to 10^{-6} M DT. The experiment was initiated by addition of [^{35}S]-methionine and 10^{-6}M DT to the cells. At the indicated times, protein synthesis was compared between toxin treated and untreated cells.

Classes III and IV showed initial sensitivity similar to wild-type cells but then maintained either 50% or 25% of normal synthesis up to the highest toxin concentration. All of these phenotypes were stable after prolonged culture in the absence of DT and after recloning.

We selected a clone from phenotype class II for further study. One characteristic feature of DT intoxication is the minimum time before inhibition of protein synthesis begins when cells are exposed to high toxin concentrations [1, 2]. It is believed this lag time reflects the time required for toxin to enter the cells. This lag time is compared for a class II mutant and wild-type cells in Figure 5. The two cell types are identical with respect to the time required before a decrease in protein synthesis is evident. This minimum time of 30 to 40 min could not be shortened by exposure to higher concentrations of DT. This suggests that this mutant might have a change at the level of the cell-surface receptor. We used CRM197 as a competitive inhibitor of toxin to probe the surface receptor. The apparent Kd of CRM197 for wild-type and mutant cells was determined by a Schild plot, as seen in Figure 6. The apparent Kd for wild-type cells is 1.2×10^{-8}M and for mutant cells is 1.6×10^{-7}M. This suggests that the affinity of DT for the receptor has been decreased about 13-fold in this mutant

We measured the ability of ConA and WGA to inhibit the effect of DT on this mutant to determine if any change in this characteristic had occurred. As shown in Figure 1, there is only little, if any, difference in the effect of these lectins on the mutant and wild-type cells.

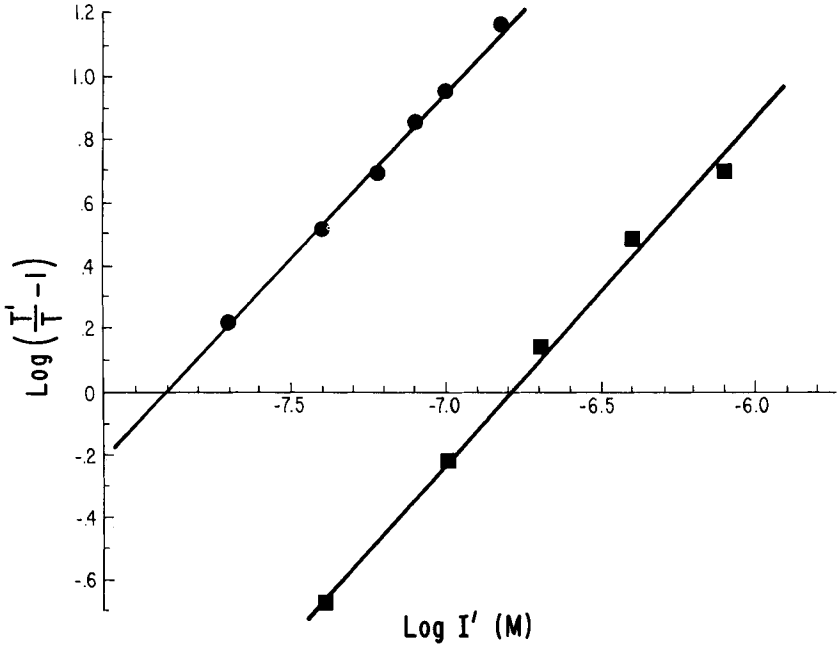

Fig 6. Schild plots for determination of the apparent Kd of CRM197 for the toxin receptor of wild-type cells, • and a class II mutant, ■. These plots were constructed from sets of dose response curves as described in Refs. 5 and 6. T is the concentration of DT required to give a response of 0.5. T′ is the concentration of DT required to give the same response in the presence of CRM197. I′ is the concentration of CRM197 used at each point for calculation of the ratio T′/T.

DISCUSSION

Our experiments demonstrate that certain lectins are potent inhibitors of DT. If it is assumed that binding of DT and ConA to cells has reached equilibrium, then a Schild plot may be used to test for competitive inhibition [6]. The results of this experiment suggest that DT and ConA compete for a common target at some time during the intoxication process. This might be explained by three models. First, the structural determinants that direct lectin binding and DT binding may be, at least in part, the same. This model predicts that the receptor is an oligosaccharide-containing molecule and that DT has oligosaccharide-binding properties. Second, the binding sites may be structurally distinct but adjacent such that occupation of one site blocks occupation of the other. This model makes no prediction about the binding specificity of DT. Third, the apparent competitive relationship between DT and ConA may be complicated. For example, it is possible that the DT-receptor complex must interact with some other membrane component before penetration can occur, and ConA might interfere with this interaction. The fact that ovalbumin glycopeptide inhibits DT is relevant to these possibilities. The glycopeptide may function by interacting with the cells or by interacting with DT. Pretreatment of cells with the glycopeptide and exposure to DT without glycopeptide in the media has no effect on the activity of toxin [6]. This suggests that the glycopeptide is not forming a stable complex with the cells and that it may be interacting with the toxin. However, we have not directly demonstrated such an interaction. The ovalbumin glycopeptide is a complex mixture of at least seven separate oligosaccharide structures [11, 12], some of which contain terminal N-acetylglucosamine residues susceptible to enzymatic removal. We have demonstrated that treatment of the glycopeptide with N-acetylglucosaminidase abolishes its antagonistic property. This is consistent with earlier work on the comparative inhibition of DT by different oligosaccharides [6].

Mutants resistant to DT would be expected either to present a barrier to toxin entry or to contain protein synthetic machinery that is resistant to inactivation by toxin. Moehring and Moehring have described DT-resistant cells that fall into both of these categories [7–9]. It would be informative to categorize further and study mutants that do not allow toxin entry. For example, some mutants might be defective in toxin binding, whereas others might bind but not transfer it to the cytoplasm. Identification of these mutants requires measurement of toxin binding to cells. This has been a difficult problem. There are few DT receptors per cell [4], and radio-iodinated DT is difficult to work with. Ittelson and Gill used CRM197 with a Schild plot to determine an apparent Kd for this toxin analogue [5]. This technique does not rely on radio-iodinated DT, and it should be possible to apply it to mutants, provided they have some residual toxin sensitivity. This method gives no information about the number of binding sites, but it is free from problems of nonspecific binding often encountered with radio-iodinated proteins since only functional receptor–toxin complexes are measured.

We searched for mutants partially resistant to DT that would be amenable to examination by this technique. We found four distinct resistant phenotypes, all of which were stable after recloning. Two phenotypes appeared in clones selected after prolonged exposure to DT. When the exposure time was decreased, two additional phenotypes were discovered. Mutant Classes III and IV were sensitive to DT at concentrations that normally affected wild-type cells; however, they continued to synthesize a significant and constant amount of protein independent of increases in toxin dose. The defect in these cells is unknown, but they do not appear to present a barrier to toxin penetration. It is possible

they may have some type of partially resistant protein synthetic apparatus. Class I mutants were totally resistant to toxin during a three-hour exposure and were unsuitable for our purposes. Class II mutants provided a phenotype we could further study.

There was no difference in the time before onset of protein synthesis inhibition at very high toxin levels between wild-type cells and a Class II mutant. This suggests a difference in some step of toxin entry that can be saturated at high toxin doses to give wild-type behavior. The apparent Kd of CRM197 for the mutant as determined from a Schild plot indicated a 13-fold decrease in affinity of toxin for the mutant receptor. This is sufficient to account for the toxin resistance of this mutant. It is possible that the change responsible for reducing the affinity of toxin for the recptor might alter the inhibition of toxin by ConA and WGA. We found only a slight difference, if any, in the effect of these lectins between mutant and normal cells. The change responsible for the relatively small 13-fold decrease in affinity of CRM197 for the receptor may not have significantly altered parameters specifying lectin binding.

In this study we examined a relatively small number of DT-resistant mutants. By screening a larger number of cells, it may be possible to isolate other toxin-resistant cells with enough residual sensitivity to determine the apparent Kd of CRM197 for the toxin receptor. We have found this technique far easier to use than radio-iodinated DT. The study of such mutants should provide information about the DT receptor, about the relationship between the toxin receptor and the lectin binding sites, and possibly about the mechanism by which DT is transferred from outside the cell to the cytoplasm.

ACKNOWLEDGMENTS

We thank Dr. Immo Scheffler for valuable discussion and D. Eurey-Owens for technical assistance. This work was supported by California Research Coordinating Committee grant (CRCC-77SD11). R.D. is supported by a National Institutes of Health postdoctoral fellowship (USPHS-GM-07199); and D.C. and L.S. are supported by a National Institutes of Health predoctoral training grant (USPHS-GM-7313).

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