Specific chemical cleavage of diphtheria toxin with hydroxylamine was performed to remove peptides of 10 and 7 kDa from the carboxyl terminus. The resulting modified proteins of 51 and 48 kDa (HA51DT and HA48DT, respectively) were purified and characterized with respect to structural and biological properties. The 51-kDa toxin binds to ATP-ρ-ribosylation, as does intact diphtheria toxin, while HA48DT does not bind to the nucleotide matrix. Neither modified toxin binds to the membranes of diphtheria toxin-sensitive cells, and, consequently, neither is toxic. However, when covalently linked to a membrane binding moiety, both HA51DT and HA48DT are toxic. Cell-killing ability during a short exposure time indicated that concanavalin A (ConA) derivatives of diphtheria toxin and HA51DT are equally toxic, ConA HA48DT being somewhat less toxic, while the conjugate of ConA to A-chain kills a small number of cells only at inordinately high concentration (1 μM). We have thus separated the cell membrane binding function of diphtheria toxin from its membrane permeation function by removing specific small peptides from the carboxyl terminus. These modified toxins may have applications in the preparation of highly potent hybrid toxins.

Diphtheria toxin is secreted from Corynebacterium diphtheriae as a single-chain protein (555 residues) which, following proteolytic processing, can be isolated as two disulfide-linked chains. One subunit, the original amino-terminal 193-residue A-chain, is an enzyme which inhibits protein synthesis by catalyzing the NAD-dependent ADP-ribosylation of elongation factor 2. The original 342-residue carboxyl-terminal B-chain displays cell surface binding properties and is involved in the translocation of the A-chain through cell membranes (Neville and Hudson, 1986).

Hybrid toxins have been developed that consist of cell recognition molecules such as antibodies or hormones conjugated to the A-chain of diphtheria toxin (Pastan et al., 1986). These conjugates are inefficient cytotoxins when toxicity is compared with the parent toxin (Columbatti et al., 1986). Several groups have observed an increase in the toxicity of hybrid toxins with the inclusion of increasing regions of the carboxyl terminus of the B-chain (Bacha et al., 1983; Columbatti et al., 1986). This has been observed through comparison of the A-chain of diphtheria toxin, CRM 45 (a mutant diphtheria toxin lacking the carboxyl-terminal 17,000 daltons of B-chain), MspSA (a genetically engineered toxin-related protein lacking the carboxyl-terminal 17,000 daltons of the B-chain), or diphtheria toxin. Although conjugates with full-length diphtheria toxin have the greatest toxicity, their use is limited by the decrease in specificity introduced by the toxin binding site.

The enhanced toxicity of hybrid proteins containing regions of the B-chain of diphtheria toxin is thought to be due to enhanced transmembrane transport of the A-chain. Diphtheria toxin B-chain has four regions of hydrophobicity postulated to be involved in the transmembrane transport of the A-chain to the cytosol (Bishai et al., 1987). Three of these domains are contained in CRM 45 and MspSA. The fourth region of hydrophobicity and the cell binding region of diphtheria toxin are contained in the 17,000-dalton carboxyl terminus of the toxin. Properties included in this region improve toxicity of hybrid toxin 100-fold over hybrids containing CRM 45 (Columbatti et al., 1986).

By genetic engineering, fusion proteins have been produced that substitute peptide hormones for the COOH-terminal 50 residues of diphtheria toxin (Murphy et al., 1986; Bishai et al., 1987; Williams et al., 1987). Results obtained with an α-MSH1 diphtheria toxin fusion protein demonstrated that a highly active selective toxin could be produced in this manner (Murphy et al., 1986). Unfortunately, this diphtheria toxin-related fusion protein, was extremely susceptible to protease activity by the expression vector; there also appear to be fewer problems with expression, possibly related to proper folding of the protein (Bishai et al., 1987; Murphy et al., 1986). Less difficulty, however, was encountered with a similar fusion protein, substituting interleukin 2 for α-MSH (Williams et al., 1987). Similar activity and selectivity was demonstrated by linking an antibody to a mutant diphtheria toxin, CRM 107, which is deficient in cell-binding activity (Greenfield et al., 1987; Johnson et al., 1988).

We report a method of producing two modified diphtheria toxins by removing 54 or 82 amino acids from the carboxyl-terminal region. The method uses hydroxylamine as a proteolytic agent, to hydrolyze specifically the polypeptide chain

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1 The abbreviations used are: α-MSH, α-melanocyte-stimulating hormone; ApUP, adenylyl (3',5')-uridine 3'-monophosphate; ConA, concanavalin A; CRM 45, cross-reacting material 45; HADT, hydroxylamine-cleaved lower molecular weight forms of diphtheria toxin; HEPES, (N-2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPDP, N-succinimidyl 3-(2-pyridyldithio)propionate; PDP, pyridyl dithiopropionate; DT, diphtheria toxin; DTA, diphtheria toxin A-chain; FPLC, fast performance liquid chromatography; PBS, phosphate-buffered saline.
at asparaginyl-glycyl bonds. Diphtheria toxin has only two such bonds, which are located at residues 453/454 and 481/482 in the carboxyl-terminal region of B-chain. This report describes the reaction, purification of the products, and selected properties of the modified toxins.

**Materials and Methods**

**Reagents**

Unless otherwise noted, all reagents were obtained from Sigma. Diphtheria toxin was purchased from Connaught Laboratories and purified by DEAE-Sephacel ion-exchange chromatography. Pappenheimer Diphtheria toxin has only two pure toxin or modified toxin was obtained for each of the three proteins (Fig. 1). The toxins were stored at -80°C until further use.

**Hydroxylamine Cleavage of Diphtheria Toxin**

Hydroxylamine cleavage of the toxin was performed as modified from Bornstein and Balian (1970, 1976). A solution of 2 mM hydroxylamine, 6 mM guanidine HCl, pH 9.0 (4.5 mM LiOH as titrant) was added at room temperature to DEAE-purified diphtheria toxin so that the final concentration was 170 μM diphtheria toxin, 5.4 mM guanidine HCl, and 1.5 mM hydroxylamine. The mixture was kept at 38-40°C for 3 h with occasional stirring, and the reaction was stopped by chromatography on Bio-Gel P6-DG (2 x 18 cm) equilibrated in deionized, glass fiber-filtered 6 M urea, 0.1 M Tris-Cl, pH 7.7, containing 1 μg/ml phenylmethanesulfonfyl fluoride (renaturation buffer). The toxin was then concentrated and applied to a Pharmacia LKB Biotechnology Inc. fast performance liquid chromatography (FPLC) system using a Superose 6 column (HR 10/30, 10 x 30 mm) equilibrated in 0.05 M Tris-Cl, pH 7.7, containing 1 μg/ml phenylmethanesulfonfyl fluoride (renaturation buffer). Eluted fractions that contained monomer toxin were pooled and applied to a Pharmacia FPLC Mono-Q anion exchange column (HR 5/5, 5 x 50 mm) equilibrated in purification buffer. The proteins were eluted with a gradient of 0-0.5 M NaCl in purification buffer. As judged from chromatograms and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), fractions enriched in unreacted diphtheria toxin (HA58DT), a 51-kDa diphtheria toxin (HA51DT), and a 48-kDa diphtheria toxin (HA48DT) were pooled separately and rechromatographed over the FPLC Mono-Q column using similar conditions. A single major peak containing essentially pure toxin or modified toxin was obtained for each of the three proteins (Fig. 1). The toxins were stored at -80°C until further use.

**Urokinase Nicking and SDS-PAGE**

Diphtheria toxin and hydroxylamine-cleaved diphtheria toxin were treated with urokinase as previously described (Ceplak and Eidelis, 1987). SDS-polyacrylamide gel electrophoresis was performed on 10-15% gradient gels using a Pharmacia Phast-Gel system. Silver staining was performed on all gels.

**In Vitro Translation**

Inhibition of protein synthesis was measured using a myeloma cell (Sp2/0; American Type Culture Collection, Rockville, MD) lysate (Villemes et al., 1986). Briefly, the reaction mixture contained 1.6 ml of cell lysate, 15 mM ATP, 2.5 mM GTP, 2.4 mg of creatine phosphate/ml, 2 mg of creatine kinase/ml solution, 0.18 ml of amino acid solution (without leucine), and 65 μl of [3H]leucine. Final volume was 2 ml; 80 μl was used for measurement of protein synthesis.

**Binding of 125I-Diphtheria Toxin to Cell Membranes**

Diphtheria toxin was iodinated by the IOOD-GEN method (Franke and Speck, 1978) to a specific activity of 0.799 μCi/μg toxin. Membranes were prepared as modified from Nett et al. (1981) from human breast cancer cells (HA58DT; B, HA51DT; C, HA48DT).

**Fig. 1. FPLC Mono Q anion-exchange chromatography of partially purified hydroxylamine-cleaved diphtheria toxin.** Partially resolved proteins were applied in 50-ml volumes and chromatographed separately. The NaCl gradient (indicated by the arrow) was initiated after allowing 6 ml of equilibration buffer to elute through the column postloading of sample. For each protein, one major chromatogram peak was obtained and judged by SDS-PAGE to be essentially pure protein A, HA58DT; B, HA51DT; C, HA48DT.

**ATP Affinity Chromatography**

Nucleotide affinity chromatography (ATP (γ)-agarose, 1-ml column) was performed as previously described (Collins and Collier, 1987). Briefly, monomer toxins were individually applied to the column in 0.1 M HEPES, 0.1% bovine serum albumin (pH 7.4). Binding was measured with membranes representing 1 x 10^6 cells in 100 μl of buffer per 12 x 75-mm polypropylene tube. Iodinated toxin and unlabeled proteins were added to membranes in 50 μl of 0.025 M HEPES, 0.1% bovine serum albumin (pH 7.4). Binding was measured with membranes representing 1 x 10^6 cells in 100 μl of buffer per 12 x 75-mm polypropylene tube. Iodinated toxin and unlabeled proteins were added to membranes in 50 μl of 0.025 M HEPES, 0.1% bovine serum albumin (pH 7.4). After a 12-h incubation at 4°C and the addition of 3 ml of ice-cold (HEPES) buffer, the complex was collected by centrifugation at 30,000 X g for 10 min at 4°C. Specific binding was calculated as the amount of iodinated toxin displaced by a 1000-fold molar excess of unlabeled diphtheria toxin.

**Cytotoxicity of Diphtheria Toxin and Hydroxylamine-cleaved Toxins**

Cytotoxicity was determined using MCF-7 cells cultured as described above. Cells were plated at 5000 cells/well (200 μl) in 96-well polystyrene culture plates. After allowing 48 h for plating, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.2), and toxins were added in medium at the indicated concentrations (Fig. 5; n = 8 wells/treatment). Cells were cultured at 37°C for 36 h in the presence of toxins. Following culture, cytoxicity was determined and expressed as percent cells surviving. Cell numbers were calculated by acid phosphatase assay as previously described (Connolly et al., 1986). Following incubation, the wells were washed twice with 200 μl of PBS, and 100 μl of 0.1 M sodium acetate, 0.01 M p-nitrophenyl phosphate.
plates were then incubated at 37 °C for 4 h. At the end of the reaction, 10 μl of 1 N NaOH was added to each well, and the absorbance was determined at 405 nm. One day prior to the determination of cytotoxicity, MCF-7 cells suspended in 0.1 ml of PBS were added to 96-well plates to provide a standard curve. The number of cells added per well was: 20,000, 15,000, 10,000, 7,500, 5,000, 2,500, and 1,250.

Preparation and Cytotoxicity of Hybrid Proteins Containing Hydroxylamine-cleaved Diphtheria Toxin

**Protein**—Diphtheria toxin (ApUp-free monomer), diphtheria toxin A-chain, and hydroxylamine-cleaved toxins were equilibrated in PBS (pH 7.5). ConA was brought into solution in PBS from lyophilized stocks.

**Conjugation to ConA**—A procedure modified from Guillemot et al. (1985) for derivatization and conjugation of ConA to diphtheria toxin with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) was used for all proteins, as described in Table I.

**Cytotoxicity Assays**—Mouse Leydig tumor cells (MLTC 1) were maintained in RPMI culture media buffered with 0.025 M HEPES, 0.025 M sodium bicarbonate, with 250 μg/ml gentamycin sulfate and 5% fetal calf serum, pH 7.7. The cells were grown in 75-cm² flasks in a atmosphere of 5% CO₂ (37 °C) and mechanically harvested on day 3 post-transfer for all studies. On the day prior to addition of toxins the cells were plated in 96-well plates at 5000 cells/well in 200 μl of media. After allowing 24 h for plating, the cells were washed twice with PBS prior to the addition of hybrid proteins or toxins in the medium. For a determination of cytotoxicity cultures were incubated for 36 h with the ConA conjugates. An indication of killing speed was obtained by a short-term exposure to the conjugates. After 2 h in the presence of conjugates, cells were washed twice with media to remove toxins and cultured for the remainder of 36 h in the absence of conjugates. Cell numbers was determined by acid phosphatase assay. Differences in toxicity between proteins were evaluated by analysis of variance.

**RESULTS**

**Hydroxylamine Cleavage**—The main products of the hydroxylamine reaction are two peptides of the predicted size (HA51DT and HA48DT) and unconjugated toxin (HA58DT, Fig. 2). In control reactions in which the hydroxylamine was omitted, no proteins corresponding to either HA51DT or HA48DT were observed. Minor, apparently nonspecific, degradation of diphtheria toxin occurred during the reaction with and without hydroxylamine. Urokinase nicking of renatured hydroxylamine-cleaved diphtheria toxin produced peptides of the size expected for carboxyl-terminal truncated B-chains and full-length A-chains. Urokinase is specific for arginine-valine bonds, and as previously noted by Cieplak and Eidels (1987), results in a highly specific nicking between diphtheria toxin A- and B-fragments. Treatment of isolated A-chain with hydroxylamine did not result in proteolysis. These results indicate that the reaction with hydroxylamine took place, as expected, exclusively in the B-chain. The preceding observations, taken together with the biological data presented below, indicate that hydroxylamine cleavage is indeed specific for the removal of the predicted carboxyl-terminal peptides from diphtheria toxin. A typical final yield of the proteins from the starting concentration of toxin was 8.9% for HA58DT, 9.2% for HA51DT, and 14.3% for HA48DT. The great majority of the protein loss occurred in the concentration steps during purification. After final separation, each of the proteins was monomeric.

**Properties**—Treatment of diphtheria toxin with denaturing reagents followed by molecular sieve chromatography releases endogenous bound nucleotide (ApUp). Consequently, ApUp-free diphtheria toxin was used for all comparisons of structural and biological properties. No differences in structural parameters were observed at any time between HA58DT (unreacted full-length diphtheria toxin) and monomeric ApUp-free diphtheria toxin, indicating that the reaction conditions caused no irreversible change in toxin structure. Further, only minor differences are noted in the circular dichroism and Fourier transform infrared spectra of ApUp-free monomeric diphtheria toxin and the two truncated toxins. Fluorescence spectroscopy at pH 7.7 produced no major differences in the wavelength maxima or the spectral profiles of diphtheria toxin and HA51DT. However, a small red shift occurred in the wavelength maximum of HA48DT, compared with diphtheria toxin, suggesting that some higher order structural changes may occur when as many as 82 amino acids are removed from the carboxyl-terminal end of diphtheria toxin. Diphtheria toxin, HA51DT, and HA48DT all aggregate following treatment at pH 4.5 for 2 h at 23 °C, indicating that

**TABLE I**

| Protein | Concentration (mg/ml) | Molar ratio (SPDP:protein) | PDP/ligand (mol/mol) | Ligand/toxin |
|---------|-----------------------|---------------------------|----------------------|--------------|
| ConA    | 1.0                   | 2                         | 2.01                 |              |
| DT      | 1.0                   | 2                         | 1.12                 | 1.06         |
| HA51DT  | 0.5                   | 2                         | 0.9                  | 0.87         |
| HA48DT  | 0.5                   | 2                         | 1.31                 | 1.25         |
| DTA     | 1.0                   | 2                         | 0.91                 |              |

![FIG. 2. Hydroxylamine cleavage of diphtheria toxin. Lane A, molecular weight standard proteins (66, bovine serum albumin; 45, ovalbumin; 29, carbonic anhydrase; 14, lysozyme); lane B, diphtheria toxin; lane C, hydroxylamine-cleaved diphtheria toxin (58.5, HA58DT; 51.4, HA51DT; 47.7, HA48DT).](image)
Specific Cleavage of Diphtheria Toxin

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the low pH-induced conformation change, characteristic of diphtheria toxin, is not altered by the removal of these carboxyl-terminal peptides.

The inhibition of in vitro translation by reduced diphtheria toxin, hydroxylamine-treated A-chain, and untreated A-chain was similar, indicating that the hydroxylamine reaction does not significantly alter enzymic activity.

ATP and Cell Membrane Binding; Cytotoxicity—HA48DT does not bind an ATP-agarose matrix (Fig. 3), in contrast to HA51DT and diphtheria toxin. Diphtheria toxin binding to MCF-7 cell membranes was saturable and displaceable in a concentration-dependent manner by unlabeled diphtheria toxin (100-fold molar excess for full displacement). However, HA48DT and HA51DT do not displace labeled toxin from MCF-7 cell membranes even at 1000-fold molar excess (Fig. 4).

Neither modified toxin killed DT-susceptible cells at concentrations up to 100 nM (Fig. 5). Both toxins decreased the number of surviving cells (86 and 80% of control for HA48DT and HA51DT, respectively) at a concentration of 1 μM (p ≤ 0.05). HA58DT was as toxic to these cells as ApUp-free diphtheria toxin monomer.

Cytotoxicity of Hybrid Toxins—Murine cells, such as the MLTC1 line, are insensitive to the effects of diphtheria toxin but have fully susceptible elongation factor 2. Therefore, these cells provide an excellent model for comparing toxicity of hybrids containing toxin or modified toxins. Toxicity was expressed as percentage of cells surviving, determined by the acid phosphatase assay. MLTC1 cells were resistant to diphtheria toxin except at the highest concentration tested (1 μM).

Over a 36-h culture in the presence of hybrid toxins, the ConA conjugates to diphtheria toxin or HA51DT were equally toxic (ED₅₀ = 21 and 25 PM, respectively) (Fig. 6a). The conjugate of ConA to HA48DT was somewhat less toxic (ED₅₀ = 270 PM; p < 0.1) than conjugates of ConA to HA51DT or diphtheria toxin. The conjugate of ConA to DTA was least toxic, being 400-fold less toxic than that of ConA diphtheria toxin (ED₅₀ = 8.4 nM; p < 0.01). When MLTC1 cells were exposed to conjugates for a short time (2 h) (Fig. 6b), conjugates of ConA to diphtheria toxin and HA51DT were again equally toxic (ED₅₀ = 940 and 740 PM, respectively). The conjugate of ConA to HA48DT was again less toxic (ED₅₀ = 3.5 nM; p < 0.1) than the conjugates to diphtheria toxin or HA51DT. The conjugate of ConA to A-chain was only slightly toxic at 1 KM, being approximately 5000-fold less toxic than conjugates of ConA to diphtheria toxin or HA51DT.

**DISCUSSION**

Hydroxylamine can be used as a specific reagent to cleave asparaginyl-glycyl peptide bonds, or more accurately, the high pH-induced cyclic imide derivative of this bond (Bornstein and Balian, 1976). Proteins are usually reduced, carboxymethylated, and denatured to increase exposure of the asparaginyl-glycyl bonds to the nucleophile. Our modification of this procedure omits not only the usual carboxymethylation

**Fig. 3.** ATP-agarose affinity chromatography of diphtheria toxin, HA51DT, and HA48DT. Bound toxin was eluted from the column with 0.5 M NaCl (arrow). 0.5-ml fractions were collected.

**Fig. 4.** Displacement of bound ¹²⁵I-diphtheria toxin from membranes prepared from MCF-7 cells by diphtheria toxin, HA51DT, and HA48DT. Each point represents an average of three observations. Pooled standard errors (SE) are given.

**Fig. 5.** Cytotoxicity of diphtheria toxin, HA58DT, HA51DT, and HA48DT for MCF-7 cells. Each point represents an average of eight observations. Pooled standard error (SE) is given.
Specific Cleavage of Diphtheria Toxin

![Diagram](image)

**FIG. 6.** a, cytotoxicity of conjugates of ConA to diphtheria toxin, HA51DT, HA48DT, or A-chain for MLTC 1 cells. Cells were cultured in the presence of toxins for 36 h. Each point represents an average of eight observations. Pooled standard error (SE) is indicated. b, short exposure toxicity of ConA conjugates. Cells were cultured for 2 h in the presence of conjugate and subsequently for 36 h without conjugate. Each point represents an average of eight observations. Pooled standard error (SE) is given.

of protein prior to hydroxylamine cleavage but also disulfide reduction. Initial experiments using the earlier reaction conditions produced unsatisfactory results, with difficulty experienced in renaturation.

Cleavage of diphtheria toxin with hydroxylamine resulted in the appearance of proteins of 51 and 48 kDa plus smaller fragments of 10, 7, and 3 kDa. These fragments are those expected from the cleavage of asparaginyl-glycyl bonds 453/454 and 481/482. Based on 1) previous observations of the specificity of hydroxylamine treatment of proteins; 2) the generation of proteins of appropriate molecular weight following treatment of diphtheria toxin with hydroxylamine; 3) lack of cleavage of diphtheria toxin A-chain by hydroxylamine; and 4) truncated B-chains of appropriate size following urokinase treatment, we conclude that, under the reported conditions, diphtheria toxin reacts with hydroxylamine primarily at the two asparaginyl-glycyl residues in the carboxyl-terminal region of the toxin. Two biological characteristics of the truncated toxins taken together with previously obtained information about diphtheria toxin support the above conclusion. Those characteristics are: 1) the lack of binding and consequently toxicity of the truncated toxins toward diphtheria toxin-sensitive cells; and 2) the exhibition of full toxicity when the truncated toxins are linked to a cell-binding moiety such as ConA.

As indicated by cytotoxicity, enzyme activity, and spectrophotometric analysis of unreacted diphtheria toxin (HA68DT) recovered from the reaction mixture, the reaction conditions do not lead to nonspecific damage of the toxin. Structural analysis (CD, Fourier transform infrared, and fluorescence spectroscopy) of the modified proteins revealed no major perturbations in secondary structure. This, as well as the biological activity data, indicates that the toxin folds properly during renaturation regardless of the removal of carboxyl-terminal peptides (82 or 54 residues). The truncated toxins, as well as diphtheria toxin, aggregate upon exposure to low pH. Aggregation of diphtheria toxin is considered to be caused by the exposure to the aqueous environment of previously sequestered hydrophobic regions in the B-chain. A low pH environment upon internalization of toxin is necessary for expression of toxicity (Sandvig and Olsnes, 1980); at low pH, the toxin undergoes insertion into lipid bilayers and can transfer A-chain enzymatic activity to the trans-side of liposomes (Donovan et al., 1985). This insertion is, therefore, considered to be a property of the B-chain and necessary for translocation of the A-chain to the cytosol (Neville and Hudson, 1986). The ability of the modified toxins to respond to low pH is, therefore, an indication of retention of one structural aspect that is associated with translocation of the A-chain and may explain in part why truncated toxins lacking the fourth region of hydrophobicity (CRM 45 and MSPA 1) are not as efficient cytotoxins as intact toxin.

The ATP or polyphosphate binding region of diphtheria toxin is localized in a region of dense positive charge near the intrachain disulfide loop in diphtheria toxin B-chain (Carroll et al., 1986). It has been suggested that this site on diphtheria toxin is identical to the cell membrane binding site (Eidels et al., 1982). Our observation of the inability of HA51DT to bind to membranes from, or to exhibit lethality toward, sensitive cells while retaining ATP-binding properties demonstrates that these two functional sites are distinct. Alving et al. (1980) proposed that the ATP site on the protein may be involved in a low affinity association of diphtheria toxin to phospholipids in cell membranes. This proposition is consistent with the somewhat greater toxicity of HA51DT than HA48DT to MCF-7 cells. Also, the lack of ATP binding by HA48DT may
account for the somewhat lower toxicity of hybrid toxins prepared with this modified toxin, if one assumes that a low affinity association with phospholipids contributes to the translocation of A chain.

As discussed earlier, the region of diphtheria toxin containing receptor binding properties is located in the 17-kDa carboxyl-terminal region of the B-chain. However, this region also contains groupings involved in the translocation of the A-chain of diphtheria toxin. More recently, the properties of a fusion protein in which the final 50 residues of diphtheria toxin are replaced by α-MSH have reduced to a smaller region the area of the toxin responsible for binding. However, direct data on receptor binding and translocation have not been reported for this fusion protein.

A major drawback in the development of immunotoxins has been the slow kinetics of killing offered by the current available A-chain conjugates. The A-chain hybrids appear to be sufficiently selective, but cytotoxic potency appears to be too low for in vivo applications. Injections of up to 1 mg/kg body weight of ricin A-chain immunotoxin resulted in a 96% decrease in tumor burden for a mouse lymphoma (Fulton et al., 1987). This amount of tumor cell killing is insufficient to cure the animal. Using mice, which are relatively insensitive to the effects of diphtheria toxin, injection of 1 μg of diphtheria toxin/animal is capable of completely regressing a solid human tumor without harm to the animal (Pastan et al., 1986). The latter result indicates that a toxin with similar kinetics of action to diphtheria toxin but lacking the intrinsic binding properties of the toxin, when linked to the appropriate cell recognition moiety, may produce cures in vivo. Our results indicate that modified toxins HA48DT and HA51DT possess those two properties, retention of translocation and lack of intrinsic binding, required for effective use in the development of highly efficient hybrid toxins for use in vivo.

One consideration related to the potential usefulness of modified diphtheria toxins results from the fact that most adults in the U.S. population have been immunized with diphtheria toxoid. Indeed, sera from U.S. adults apparently contain neutralizing antibodies to CRM 107 immunotoxins. However, Zucker and Murphy (1984) observed that only those antibodies which prevent toxin from binding to its receptor are neutralizing. Further, using diphtheria toxoid-immunized mice, Kelley et al. (1988) found no difference in in vivo effectiveness of an interleukin 2 diphtheria toxin fusion protein as a function of the presence of antitoxin antibodies. The fusion protein, of course, lacked the carboxyl-terminal region of diphtheria toxin, since that is the portion of diphtheria toxin for which interleukin 2 was substituted. Several interpretations of these results (Kelley et al., 1988) are possible, one of which is that diphtheria toxins, such as those described in this report, which lack the carboxyl-terminal region of the toxin, may be applicable to toxoid-immunized humans. Further experimentation will be required to clarify this issue.

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