Chimeric toxins: Toxic, disulfide-linked conjugate of concanavalin A with fragment A from diphtheria toxin
(crosslinking/affinity chromatography/protein synthesis)

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ABSTRACT A disulfide-linked conjugate of concanavalin A (Con A) and fragment A from diphtheria toxin has been synthesized and shown to be toxic for HeLa (human), Chinese hamster ovary (CHO), and SV3T3 (murine) cells. The conjugate was constructed by first coupling cystamine to Con A with a carbodiimide reagent and then reacting the modified Con A with reduced fragment A under conditions promoting disulfide interchange. The desired conjugate, obtained in nearly 50% yield relative to input of fragment A, was purified by affinity chromatography on Sephacryl S-200 and NAD-Sepharose; on analysis, it gave an average of 1.4 molecules of fragment A per tetrameric Con A molecule. The conjugate proved to be about equally active in inhibiting protein synthesis in HeLa, CHO, or SV3T3 cells in culture but was inactive relative to controls in a toxin-resistant strain of CHO cells containing altered elongation factor 2, the target protein of fragment A. With toxin-sensitive strains the conjugate was 100-1000-fold more active than controls, including fragment A, cystaminyl-Con A, and mixtures thereof, but was 1/50th to 1/500th as toxic as diphtheria toxin itself. Similar activity relative to controls was observed after intradermal inoculations in rabbits, and intravenous injections of the conjugate were lethal for mice. The activity of the conjugate in tissue culture was inhibited by Con A or α-methylmannoside but not by galactose. This and similar conjugates should be useful in studying mechanisms of entry of biologically active proteins into cells.

A central question concerning the actions of many biologically active proteins and polypeptides, such as hormones, growth factors, and toxins, is whether or not they must penetrate the plasma membrane to act. Although compelling evidence is lacking for most systems, there are certain ones in which at least partial penetration by the protein must occur. These involve toxic proteins—such as diphtheria toxin (1) and ricin and abrin (2)—and bacteriocins—such as colicin E3 (3)—that have been found to catalyze in vitro a lethal reaction known to occur within susceptible cells treated with the protein.

Diphtheria toxin (M, 60,000) contains a catalytic center that transfers the adenosine diphosphate ribose moiety (ADP-ribose) of NAD into covalent linkage with peptidyl elongation factor 2 (EF-2):

\[
\text{NAD + EF-2} \rightarrow \text{ADP-riboseyl-EF-2} + \text{nicotinamide + H}^+. 
\]

EF-2 is thereby inactivated, thus explaining the toxin's inhibition of protein synthesis in animal cells and the toxicity for animals. Because both EF-2 and NAD are cytosolic constituents, the toxin's catalytic center must somehow penetrate to the cytosol.

Although the mechanism by which the catalytic center penetrates the membrane remains unsolved for diphtheria toxin or other toxins, information relevant to this question has been obtained from studies of structure–activity relationships within these proteins. Diphtheria toxin is a single polypeptide chain (M, 60,000) containing two functionally distinct domains, both of which are required for toxic activity. The amino-terminal domain (M, 21,145), designated A for activity, carries the catalytic center responsible for the ADP-ribosylation of EF-2; the complementary domain (M, 38,000), designated B for binding, attaches the toxin to specific cell surface receptors and may also perform other functions in the entry process. The intact toxin may be converted by mild trypsinization into a "nicked" form containing the two domains connected by a disulfide bridge. The nicked form is as toxic as the intact form for whole cells. Although the ADP-ribosyltransferase activity of fragment A is expressed only after reduction of the disulfide and dissociation from fragment B, the free fragment A is virtually devoid of toxicity (≈10^-6 that of intact or trypsin-nicked toxin) (4). Hence, linkage to fragment B is required for fragment A to act on intact cells. A similar partition of function into A and B moieties is seen in many other toxic proteins.

The events that transpire after the initial binding of diphtheria toxin to specific cell surface receptors are obscure. One proposed model involves direct penetration of the plasma membrane by fragment A through a channel created by fragment B or B together with its receptor (perhaps a glycoprotein) (5). Another assumes the toxin enters attached to the membrane of some type of endocytotic vesicle, followed by disruption of the vesicle to release the toxin into the cytosol (6). At present the data are insufficient even to permit a choice between these grossly different models.

One approach to determining the role of fragment B is to crosslink fragment A to proteins that might serve as functional analogs of fragment B—that is, to various proteins known to interact with cell surfaces. This approach has been taken by Chang et al. (7, 8) in the synthesis of an artificial hybrid containing fragment A from diphtheria toxin linked through a disulfide bridge to human placental lactogen. Although it could be demonstrated that both the lactogen and fragment A retained their respective biological activities when linked together, the conjugate was not toxic when tested on explants of rabbit mammary glands.

Here we report the synthesis and characterization of an analogous conjugate in which fragment A is linked through a disulfide-containing crossbridge to concanavalin A (Con A), the well-studied lectin from jack beans. The conjugate was toxic for HeLa, Chinese hamster ovary (CHO), and SV3T3 cells in culture and in tests on whole animals.

Abbreviations: EF-2, elongation factor 2; Con A, concanavalin A; CHO, Chinese hamster ovary; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; IP3P-F, diisopropyl fluorophosphate; NaDodSO4, sodium dodecyl sulfate; IDP (24), concentration of conjugate that inhibits protein synthesis by 50% after 24-hr exposure.

5319
MATERIALS AND METHODS

Fragment A was prepared from diphtheria toxin (Connaught Laboratories) as described (9) and was heated to 80°C for 10 min to inactivate any traces of toxin present. Carboxymethyl-fragment A was prepared by the method of Wadala et al. (10) except that guanidine hydrochloride was not used. Purified Pseudomonas aeruginosa exotoxin A was the generous gift of Stephen Lory. Con A (grade IV), α-methylmannoside, glucose, glucose-free galactose, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl (EDAC), and N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES buffer) were all from Sigma. Dialyzed fetal calf serum and Hepes buffer were from Gibco. NAD-Sephase was synthesized as described (11).

Lectin activity was assayed by hemagglutination. One unit of activity is defined as that amount of lectin required to agglutinate 2 × 10⁶ human erythrocytes in 250 μl of phosphate-buffered saline. The ADP-ribosyltransferase assay was as described (12). HeLa and SV3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% antibiotic-antimycotic mixture (GIBCO). CHO-K1 cells, a cloned derivative of the CHO-K1 line of CHO cells, and CH-RE-I.22e, a diphtheria toxin-resistant variant of CHO-K1 (13), were maintained in Ham's nutrient mixture F-12, with 10% fetal calf serum and 50 μg of gentamicin per ml. Toxicity assays were performed as described (15), except that 5 mg of galactose per ml was substituted for glucose in the medium. Rabbit skin tests were performed as described (14). Lethality in mice was assayed by intravenous injection (0.1 ml) into male Swiss white mice weighing 20 ± g.

Synthesis of the Con A-SS-Fragment A Conjugate. All procedures were performed at 4°C or on ice. Con A (300 mg) was dissolved in water containing 1 mM CaCl₂, 1 mM MnCl₂, 20 mM glucose, 0.2% Na₂SO₄, 1 μg of phenylmethylsulfonyl fluoride (PMSF) per ml, and 1 μg of diisopropyl fluorophosphosphate (iP2P-F). Cystamine dihydrochloride (1.78 g) was added, and the volume was brought to 15 ml. The mixture was adjusted to pH 4.7, and cystamine coupling was initiated by addition of solid EDAC to 2 mM. The solution was maintained at pH 4.7 for 10 min, at which time it was applied to a 42 × 2.8 cm Bio-Gel P-6 column equilibrated with 20 mM TES buffer at pH 6.5 containing 1 mM CaCl₂, 1 mM MnCl₂, 0.2% Na₂SO₄, 1 μg of PMSF per ml, and 1 μg of iP2P-F per ml (TES-CM buffer, pH 6.5). Fractions containing Con A were pooled, and the extent of derivatization was determined by using 5,5'-dithiobisnitrobenzoic acid after reduction of cystaminyl-Con A with 0.1 M dithiothreitol (15). Typically, about 2.0 cystamine residues per Con A tetramer was found.

Fragment A (3 mg/ml in 50 mM Tris-HCl buffer, pH 8.2) was reduced with 0.1 M dithiothreitol for 4 hr and then desalted on Sephadex G-25 equilibrated with TES-CM buffer, pH 6.5. Fractions containing fragment A were pooled and mixed with a 4-fold molar excess of cystaminyl-Con A. The final concentrations of fragment A and cystaminyl-Con A were 19 and 75 μM, respectively. The mixture was adjusted to pH 7.4 and, to promote disulfide exchange, was immediately dialyzed against TES-CM buffer, pH 7.4, for 24 hr. Formation of the conjugate was complete within this time. The general protocol is summarized in Fig. 1. Purification of the Con A-SS-fraction A conjugate is described in the legend to Fig. 2.

RESULTS

Synthesis of the Conjugate. Because release of free fragment A into the cytosol may be necessary for the inactivation of EF-2 in cells, we incorporated a disulfide bridge into the intermolecular linkage. We devised a method of crosslinking involving the disulfide interchange between cystamine-derivatized Con A and the intrinsic sulfhydryl group of fragment A. This method had several advantages over others tested. (i) It did not lead to formation of Con A homodimers and did not alter the ability of Con A to bind to sugar-containing resins or to hemagglutinate erythrocytes. (ii) The crosslinking was efficient relative to the amount of fragment A added. (iii) No covalent modification of fragment A was necessary prior to crosslinking.

Purification and Characterization of the Conjugate. The conjugate was purified by two steps of affinity chromatography (Fig. 2). The crude preparation was applied to a column of Sephacryl S-200. Free fragment A and disulfide-linked dimers of it emerged in the buffer wash, whereas Con A-SS-fraction A and cystaminyl-Con A were adsorbed by the interaction of Con A with glucosyl residues on the resin. The adsorbed material was then eluted with 30 mM α-methylmannoside in buffer and emerged as a single peak containing both ADP-ribosylating activity and hemagglutinating activities. When unconjugated fragment A was rapidly mixed with Con A or cystaminyl-Con A and applied immediately to the Sephacryl, no binding of the fragment was observed.

The conjugate was freed from unreacted cystaminyl-Con A by applying the eluate from the first column directly to a NAD-Sepharose column equilibrated with buffer containing α-methylmannoside. The conjugate was retained, presumably by virtue of the interaction of attached NAD with the NAD binding site on the fragment (Kₐ, 8 μM). Con A not attached to fragment A emerged at the void volume. The conjugate was eluted with buffer containing adenosine (10 mM) and NaCl (0.5 M) and emerged as a peak with coincident ADP-ribosylation and hemagglutination activities. Cystaminyl-Con A alone or after rapid mixing with either fragment A or the S-carboxymethylated fragment did not adsorb to NAD-Sepharose. The yield of conjugate was about 50% relative to the amount of input fragment A.

The purified conjugate contained an average of 1.4 molecules of fragment A per tetrameric Con A. All of the ADP-ribosylation activity present in the preparation was adsorbed to Sephacryl S-200, and 98% of this activity was released upon addition of 0.1 M dithiothreitol. Hemagglutinating activity was released only after addition of 30 mM α-methylmannoside. The hemagglutination activity of the conjugate was the same as that of nonderivatized Con A.

Analysis of the conjugate on sodium dodecyl sulfate (Na-
DodSO4/polyacrylamide gels gave the expected band patterns (Fig. 3). In the presence of mercaptoethanol the pattern was simply the sum of the patterns found with pure fragment A and Con A. In the absence of thiols, the fragment A band was missing, and another was observed corresponding to the fragment A conjugate with Con A monomer (M₄, 27,000). This preparation of Con A, like most, contained proteolytic fragments of the Con A monomer (M₄, 11,000 and 16,000), and we sometimes observed conjugates of these with fragment A.

**Activity of the Conjugate on Cultured Cells.** The effect of the conjugate on protein synthesis was monitored on monolayer cells. Cells were assayed for ability to incorporate radiolabeled amino acids into trichloroacetic acid-precipitable material after a 24-hr exposure to the conjugate. Toxicity is expressed as ID₅₀(24), defined as that concentration of conjugate that inhibits protein synthesis by 50% after the 24-hr exposure. Concentrations of conjugate are expressed in terms of the Con A moiety.

Fig. 4 shows the effect of the conjugate on HeLa cells. The ID₅₀(24) of the conjugate was about 2 nM, compared with values of 1 mM for free fragment A or cystaminyl-Con A. When reduced fragment A was S-carboxymethylated and incubated with cystaminyl-Con A under conditions normally used for conjugate formation, the mixture was no more toxic than cystaminyl-Con A alone. Reduction and alkylation of the conjugate abolished its toxicity. Diphtheria toxin had an ID₅₀(24) of 4 pM and was therefore about 500-fold more toxic for HeLa cells than the conjugate.

Cells from mice are resistant to diphtheria toxin due to a defect in toxin binding or transport (their EF-2 is fully sensitive), and we therefore tested the conjugate on mouse SV3T3 cells (Fig. 5). The conjugate showed about the same activity as on HeLa cells [ID₅₀(24) = 6 nM], whereas diphtheria toxin, as expected, inhibited protein synthesis only at very high concentrations [ID₅₀(24) > 300 nM]. This result eliminates the possibility that the conjugate's toxicity on HeLa cells might be due to contaminating diphtheria toxin. Fragment A, cystaminyl-Con A, reduced and alkylated conjugate, and a mixture of carboxymethylated fragment A and cystaminyl-Con A all were negligibly toxic for the mouse cells. However, these cells were highly sensitive to exotoxin A from *P. aeruginosa*, a toxin that ADP-ribosylates EF-2 but, unlike diphtheria toxin, is highly toxic for mice.

Table 1 shows the effects of the conjugate and diphtheria toxin on HeLa cells in the presence of antagonists of Con A binding. Con A and α-methylmannoside protected cells from the action of the conjugate, whereas galactose had little or no effect. Studies with radiolabeled conjugate (data not presented) showed that the protection by Con A or α-methylmannoside correlated with inhibition of binding of the conjugate. Serum served a slight protective function, perhaps due to binding of the conjugate to serum glycoproteins. The toxicity of diphtheria toxin was unaffected by α-methylmannoside or galactose, but was slightly reduced by Con A, a result consistent with recent findings by Draper et al. (16). Similar tests with antagonists on SV3T3 cells gave virtually identical results.

The conjugate was also tested on CH-RE1.25c, a translational variant of CHO cells (13) containing EF-2 incapable of acting...
as substrate of ADP-ribosyl transfer. This variant is resistant to both diphtheria toxin and \textit{P. aeruginosa} exotoxin A. As shown in Fig. 6, the variant was also markedly insensitive to the conjugate, although the parental CHO-K1 cells were as sensitive as HeLa cells. The slight activity observed on CH-RE1.22c was attributable to the toxicity of the Con A moiety. This provides strong evidence that the ADP-ribosyl transferase activity of the fragment A moiety of the conjugate was responsible for the inhibition of protein synthesis observed in cell lines with normal EF-2.

**Toxicity Tests in Animals.** Toxicity for cultured cells correlated with the effect of the conjugate in rabbit skin tests. About 0.1 ng of conjugate induced a visible lesion; cystaminyl-Con A or fragment A alone was no more than 1/1000th as toxic. A mixture of cystaminyl-Con A and carboxymethylated fragment A was not toxic in doses up to 10 ng. Diptheria toxin was 200-fold more toxic than the conjugate and gave a response at doses of 0.5 µg.

The LD$_{50}$ of the conjugate injected intravenously in mice was 80 µg, whereas diphtheria toxin and \textit{P. aeruginosa} exotoxin A had LD$_{50}$ of 10 and 0.5 µg, respectively. Cystaminyl-Con A or fragment A alone showed no effect at doses up to 150 µg, and a mixture of cystaminyl-Con A and fragment A was not toxic in doses up to 100 µg. The reason for the difference in the relative toxicities of the conjugate and diphtheria toxin on mice as compared with those on cultured mouse cells is not clear.

**DISCUSSION**

The conjugate described above appears to act as a functional analog of diphtheria toxin—that is, toxicity depends on the functional contributions of two physically linked protein moieties. The fact that the inhibitory effects of sugars on the toxicity of the conjugate correlates with the known specificity of Con A indicates that binding of the conjugate to saccharide-containing residues on the cell surface is an essential step in its action. Similarly, the fact that a mutant cell line containing EF-2 that does not act as substrate for ADP-ribosyl transfer is markedly resistant to the conjugate as well as to diphtheria and \textit{P. aeruginosa} toxins implies an essential role of the fragment A moiety. That physical linkage of Con A and fragment A is required for toxicity is clear from the facts that (i) the actions of unlinked Con A and fragment A were not synergistic when the two were mixed, and (ii) reduction and carboxymethylation of the conjugate abolished its activity.

### Table 1. Effect of serum, sugars, and Con A on conjugate toxicity

<table>
<thead>
<tr>
<th>Additions*</th>
<th>Incorporation, % of control†</th>
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<tr>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td>Serum</td>
<td>16</td>
</tr>
<tr>
<td>Galactose</td>
<td>8</td>
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<tr>
<td>α-Methylmannoside</td>
<td>9</td>
</tr>
<tr>
<td>Con A</td>
<td>21</td>
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</table>

* Final concentrations: dialyzed fetal calf serum, 10%; sugars, 30 mM; Con A, 100 nM.
† Monolayers were washed twice with 2 ml of phosphate-buffered saline; 1 ml of the medium lacking glucose but containing 50 mM Hepes buffer plus sugars, serum, or Con A as indicated was added to designated vials, together with 10 µl of appropriate concentrations of diphtheria toxin or conjugate. After a 2-hr incubation the medium was replaced with normal medium, and the vials were incubated for 22 hr and processed. The toxin and the conjugate were used at concentrations (0.1 and 5 nM, respectively) that gave 10% of control incorporation of $^{14}$C-labeled amino acids after a 24-hr incubation on HeLa cell monolayers.
Conclusions from these studies concerning the mechanism of entry of proteins into cells are limited at present. One might argue that the model of penetration involving formation of a channel by fragment B is incorrect, inasmuch as it would seem unlikely that a structurally dissimilar molecule such as Con A would function in entry like fragment B of toxin. However, the entry mechanism of the conjugate may well be different from that of the toxin. Given the low toxicity of the conjugate compared with that of the toxin, it is conceivable that the conjugated fragment A enters by a mechanism of low efficiency, perhaps endocytotic in nature, and that the Con A moiety simply serves to concentrate fragment A at the cell surface. Also, it is noteworthy that Con A contains a hydrophobic binding site and can interact strongly with artificial membranes lacking saccharides (17, 18). Thus, the Con A moiety of the conjugate might, after initial binding to saccharides on the cell surface, undergo a secondary interaction with the membrane, resulting in direct membrane penetration by the attached fragment A. Such a role has been postulated by Boquet et al. (5) for fragment B of diphtheria toxin.

Inasmuch as we do not know the distribution of the conjugate on various types of cell surface receptors, it is difficult to speculate on the role of receptors in mediating entry of the fragment A moiety. We initially chose Con A as a potential analog of fragment B because it was a well-studied cell surface ligand with defined specificity toward certain saccharides. After synthesis and initial testing of the conjugate were complete, Draper et al. (16) reported evidence suggesting that the diphtheria toxin receptors on cells contained an oligosaccharide moiety essential for recognition. Among the evidence put forth were the findings that Con A, wheat germ agglutinin, and certain oligosaccharides inhibited the action of diphtheria toxin in cultured cells. Thus, the diphtheria toxin receptors may be a subset of the receptors to which Con A binds. If this is the case, it is conceivable that the low toxicity of the conjugate relative to that of the toxin is due to the low percentage of bound conjugate that is attached to the toxin receptors. (We have found, by using radiolabeled conjugate or toxin, that the percentage of added protein that is bound to cells is similar for both.)

Despite the fact that definitive conclusions can not be drawn from the data regarding the mode of entry of fragment A, the general approach taken here should be pursued. Attachment of fragment A or similar moieties from other toxins to other more specific cell surface ligands (for example, see ref. 19) such as hormones or antibodies may permit one not only to define entry mechanisms but also to delineate classes of cell surface receptors that promote entry of their ligands. Recent work of Thorpe et al. (20) has shown that the toxicity of whole diphtheria toxin is enhanced by crosslinking to cell-specific antibody; thus, the possibility that one may be able to target the action of fragment A to specific classes of cells in a similar fashion should not be overlooked.

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