Characterization of a Transferrin-Diphtheria Toxin Conjugate*

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We report here the synthesis and properties of a hybrid toxin prepared by covalently coupling diphtheria toxin to transferrin. The purified material contained two major hybrid protein species and was highly cytotoxic to mouse LMTK- cells in culture, reducing protein synthesis by 50% in 24 h at a concentration of 1 ng/ml. Cytotoxic activity was completely abolished in the presence of exogenous transferrin or anti-transferrin or anti-diphtheria toxin, thus demonstrating that the hybrid toxin was intoxicating cells via their transferrin receptors and that both the diphtheria toxin and transferrin components of the conjugate were necessary for activity. NH₄Cl, a drug that elevates the pH within acidic intracellular vesicles, also blocked cytotoxic activity, suggesting that a low intravesicular pH was required for activity. The inhibitory effect of NH₄Cl could be abolished by exposing toxin-treated cells to acidic culture medium, further implicating an acid-dependent step in the mechanism of the hybrid toxin action. Studies on the kinetics of intoxication also implied that endocytosis and exposure to a low pH within vesicles were necessary for cytotoxicity. Altogether, the results suggest that the transferrin-diphtheria toxin conjugate binds to transferrin receptors and is internalized into acidic endocytic vesicles. The enzymatic moiety of diphtheria toxin then apparently enters the cytosol in response to the low pH and subsequently arrests protein synthesis.

Transferrin (M₀ = 79,550), a serum glycoprotein that binds 2 mol of Fe₃⁺/mol of transferrin, is the major iron transporting protein of mammalian organisms. Transferrin delivers trivalent iron to cells by a receptor-mediated mechanism involving three major steps. 1) Transferrin binds to specific receptors on the cell surface. The receptor from humans is a dimeric glycoprotein with an apparent M₀ ~ 95,000 for the monomeric unit (1, 2), and it contains covalently attached fatty acid (3). 2) Transferrin-receptor complexes aggregate over coated pits in the plane of the membrane (4) and are internalized by endocytosis (5-7). 3) Within a prelysosomal vacuole, the transferrin is exposed to a low pH (~5) which causes the bound Fe₃⁺ to dissociate (8-12). Subsequently, the apo-transferrin-receptor complex apparently avoids entering the degradative lysosomal compartment and is recycled back out to the cell surface where the apo-transferrin is released to bind more iron and reinitiate the delivery process.

Very little is understood at the molecular level how transferrin-receptor complexes are aggregated, endocytosed, and finally exposed to a low pH within vesicles. To investigate these processes, we would ultimately like to isolate mutants deficient in various steps of the transferrin uptake pathway. To make a cytotoxic form of transferrin that should be useful in selecting mutants, we have constructed a hybrid toxin by covalently attaching diphtheria toxin to transferrin. Diphtheria toxin (M₀ = 58,342) kills cells by enzymatically inactivating elongation factor 2 in the cytosol, thus arresting protein synthesis. The catalytic site is carried by a polypeptide fragment of the toxin, called fragment A (M₀ = 21,167), that must enter the cytosol to interact with elongation factor 2. We coupled diphtheria toxin to transferrin because there are close parallels between the endocytic uptake of transferrin and the process by which fragment A of diphtheria toxin reaches the cytosol. To deliver fragment A to the cytosol, diphtheria toxin binds to cell surface receptors, is internalized via endocytosis, and is subsequently exposed to a low pH within prelysosomal vesicles (13-17). Upon encountering a low pH, the toxin undergoes a conformational change and inserts into a membrane, eventually resulting in the release of fragment A into the cytosolic space (15, 18). Thus, transferrin should carry attached diphtheria toxin into an acidic prelysosomal compartment where the low pH might then facilitate escape into the cytosol.

The transferrin-diphtheria toxin (TF-DT') conjugate proved to be highly cytotoxic to mouse LMTK- cells in culture. Moreover, the fragment A moiety of the hybrid toxin appeared to enter the cytosol by the predicted mechanism; that is, binding of TF-DT to transferrin receptors, internalization of the conjugate into acidic endocytic vesicles, and subsequent liberation of fragment A from vesicles. These results are especially interesting considering the fact that cultured cells derived from mice are normally highly resistant to the cytotoxic action of diphtheria toxin alone.

EXPERIMENTAL PROCEDURES

Materials

Diphtheria toxin was purchased from Connaught Laboratories (Willowdale, Ontario, Canada) and purified further by ion-exchange chromatography on DEAE-Sephacel. Human transferrin was obtained from Sigma. SPDP was purchased from Pharmacia. The abbreviations used are: Tf-DT, transferrin-diphtheria toxin; PDP, 3-(2-pyridyldithio)propionate; PDP-T, 3-(2-pyridyldithio)propionyl diphtheria toxin; PDP-Tf, 3-(2-pyridyldithio)propionyl transferrin; SDS, sodium dodecyl sulfate.

3-(2-Pyridyldithio)propionyl
diphtheria toxin

PDP, 3-(2-pyridyldithio)propionate; PDP-T, 3-(2-pyridyldithio)propionyl diphtheria toxin; PDP-Tf, 3-(2-pyridyldithio)propionyl transferrin; SDS, sodium dodecyl sulfate.

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*The abbreviations used are: TF-DT, transferrin-diphtheria toxin; SPDP, N-succinimidyl-3-(2-pyridyldithio)propionate; HEPES, N-2-hydroxyethylpiperezine-N'-2-ethanesulfonate; PDP, 3-(2-pyridyldithio)propionate; PDP-T, 3-(2-pyridyldithio)propionyl diphtheria toxin; PDP-Tf, 3-(2-pyridyldithio)propionyl transferrin; SDS, sodium dodecyl sulfate.
PA) and nitrocellulose paper was from Schleicher and Schuell. Ultragel AcA 22 was purchased from LKB Instruments (Houston, TX). All other chemicals, reagent grade or better, were obtained from either Sigma or Fisher.

**Methods**

**Cells and Assays to Measure Protein Synthesis**—Thymidine kinase-deficient mouse L cells (LMTK-) were used in all experiments. Cells were routinely grown in an atmosphere of 10% CO2 and 90% air in Dulbecco's modified Eagles medium supplemented with 5% fetal bovine serum, 10 mM HEPES, pH 7.4, 40 µg/ml l-proline, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone. Protein synthesis assays to measure the cytotoxic activity of diphtheria toxin or TF-DT were as described by Marnell et al. (16) with one addition. Prior to the start of an assay, the cells were prewashed with assay medium to remove residual serum transferrin left by the growth medium. The assay medium was the same as growth medium but without serum and contained 0.5 mg/ml bovine serum albumin and one-hundredth the normal amount of l-leucine. All experimental points were done in triplicate unless specified otherwise.

**Preparation and Purification of Antibodies**—Rabbit anti-diphtheria toxin serum was prepared with formaldehyde-treated diphtheria toxin as described previously (13). Rabbit anti-diphtheria toxin serum was prepared the same way except the formaldehyde treatment was omitted. Purified antibodies were prepared by passing immune serum over a column of Ultragel AcA 22 coupled to either transferrin or diphtheria toxin (19). Bound protein was eluted with 0.2 M HCl adjusted to pH 2.2 with glycine.

**Electrophoresis**—Polyacrylamide gel electrophoresis in the presence of SDS was according to the method of Laemmli (20). Electrophoresed proteins were visualized using either the silver staining method of Sammons et al. (21) or by Western blot analysis. For Western blotting, proteins were electrophoretically transferred from acrylamide gels to nitrocellulose paper as described by Towbin et al. (22) and then immunohchemically detected using a goat anti-rabbit IgG conjugated to horseradish peroxidase, according to Hawkes et al. (23). Protein concentrations were determined by the method of Lowry et al. (24).

**Preparation and Purification of Hybrid Conjugates**—The disulfide-linked diferric Tf-DT conjugates were prepared by reacting a diferric transferrin 3-(2-pyridyl)propionyl derivative with diphtheria toxin containing free thiol groups and allowing for intermolecular conjugation by thiol-disulfide exchange. The 3-(2-pyridyldithio)propionyl derivative of diferric transferrin was prepared using SPDP according to the method of Carlsson et al. (25). Diferric transferrin at a concentration of 5–22 mg/ml in phosphate-buffered saline pH 7.4 was derivatized with an 8 molar excess of SPDP dissolved in 95% ethanol for 30 min at room temperature. The derivatized protein was separated from unreacted SPDP by gel filtration on a Sephadex G-25 column (1.5 x 27 cm) equilibrated with 0.1 M sodium phosphate buffer containing 0.1 M NaCl, pH 7.5. Analysis of the 3-(2-pyridyl)propionyl derivative revealed that each diferric transferrin molecule contained an average of 4 to 5 residues of PDP. PDP-DT was prepared in a similar manner. Diphtheria toxin at a concentration of 10 mg/ml in phosphate-buffered saline was mixed with a 4-fold excess of SPDP. After proceeding as previously described with diferric transferrin, it was found that each molecule of diphtheria toxin contained an average of 1.2 residues of PDP. Subsequently, free thiols were generated on the PDP-DT by bringing the solution to 0.2 M dithiothreitol for 20 min at room temperature. Excess reducing agent and small molecular weight reaction products were separated from the protein by passing the mixture over a Sephadex G-25 column. Hybrid conjugates were prepared by mixing PDP-Tf at a 2–5 molar excess with the thiol-containing diphtheria toxin for 1 h at room temperature. Generally, the PDP-Tf was at a concentration greater than 3 mg/ml in the coupling reaction.

After intermolecular conjugation, any remaining free thiols were carboxyamidomethylated using iodoacetamide at 100 molar excess dissolved in 1 M NaPO4, pH 8.0, to which an equal volume of the hybrid mixture was added. After extensive dialysis against phosphate-buffered saline at 4 °C, the hybrid mixture was passed over an Ultragel AcA 22 column containing immobilized anti-diphtheria toxin antibody. Bound protein was eluted with 0.2 M HCl adjusted to pH 2.2 with glycine. After neutralization, the eluted protein was then applied to a similar column containing immobilized anti-transferrin antibodies and eluted as above. Six per cent of the initial protein was recovered after the preparation had been subjected to both immunoaffinity chromatography steps. Final purification was achieved by gel filtration through Bio-Gel P-200. Purified material was stored at 4°C in 50 mM NaPO4, pH 6.5, containing 0.1 M NaCl and retained full activity when assayed over a 4-month period. Transferrin or the hybrid conjugate were saturated with iron by incubating the protein in 5 mM NaHCO3, pH 8.1, containing 0.1 mg/ml FeCl3 at room temperature for 3 h. Excess FeCl3 was removed by extensive dialysis against phosphate-buffered saline at 4°C. Fully saturated diferric transferrin was indicated by an A280/200 nm ratio of 0.045.

**RESULTS**

Passage of the initial reaction mixture containing the conjugates over the two antibody columns, as described under "Experimental Procedures," resulted in recovery of approximately 6% of the initial protein. As shown in lane 3 of Fig. 1, the material recovered after the antibody columns contained high molecular weight conjugates as well as significant amounts of free transferrin and diphtheria toxin. To further resolve hybrid toxins, the mixture was subjected to gel filtration chromatography on a Bio-Gel P-200 column. As seen in Fig. 2, two main peaks with cytotoxic activity were recovered. Fractions comprising peak 1 and peak 2 were pooled and concentrated, as indicated in the legend of Fig. 2, and tested...
Transferrin-Diphtheria Toxin Conjugates

**FIG. 2.** Purification of Tf-DT conjugates. Following two-step immunoaffinity chromatography, as described under "Experimental Procedures," the hybrid conjugate preparation was applied to a Bio-Gel P-200 column (79 x 2.5 cm) equilibrated with 50 mM NaPO₄, pH 6.5, containing 0.1 M NaCl. Fractions were monitored for absorbance at 280 nm (●) and for cytotoxicity (○). Fractions 95–126 and 138–150 were pooled separately, concentrated, and are referred to as peak 1 and peak 2, respectively, in the text.

**FIG. 3.** The response of LMTK⁻ cells to peak 1 and peak 2 hybrid conjugates and diphtheria toxin and the protection afforded the cells by exogenous transferrin. The day before an experiment, 1.2 x 10⁶ cells were seeded in each well of a Falcon 24-well assay plate. Cells were incubated in the presence of the indicated concentration of protein for 24 h, the last hour of which included 2 μCi/ml of [³H]leucine. Protein synthesis is measured as trichloroacetic acid-insoluble radioactivity and is compared to controls incubated in the absence of toxin. Closed symbols represent protein synthesis in the absence of exogenous Tf while open symbols indicate protein synthesis in the presence of 0.1 μg/ml diphtheria toxin. M, □, peak 1 hybrid conjugates; ●, ○, peak 2 hybrid conjugates; and △, △, diphtheria toxin.

for cytotoxicity in the presence and absence of excess transferrin by exposing mouse L cells to the material for 24 h. The results, shown in Fig. 3, indicated that both peaks 1 and 2 contained cytotoxic material. Virtually all the cytotoxic activity in both peaks was abolished by excess free transferrin, suggesting that cytotoxic entities were operating via transferrin receptors. For comparison, it is also shown in Fig. 3 that the mouse cells are highly resistant to the cytotoxic effect of diphtheria toxin alone and that excess transferrin did not alter the response of the cells to diphtheria toxin. Moreover, the cytotoxic activity of material in peak 2 was unaffected by the addition of diphtheria toxin at a concentration (1 μg/ml) that by itself did not reduce protein synthesis (data not shown).

Material in peak 2 was used in all subsequent experiments. As seen in lane 4 of Fig. 1, peak 2 contained two major protein bands and a trace amount of free transferrin. To determine if the two major bands were heteroconjugates containing both diphtheria toxin and transferrin, samples were electrophoresed in two separate acrylamide gels with SDS and then transferred to nitrocellulose paper by electrolution. One sheet was incubated with rabbit anti-transferrin and the other with rabbit anti-diphtheria toxin. Both nitrocellulose sheets were then treated with goat anti-rabbit IgG conjugated to horseradish peroxidase, and the peroxidase was identified as described under "Experimental Procedures." As seen in Fig. 4, the two protein bands observed in lane 4 of Fig. 1 reacted with both anti-transferrin and anti-diphtheria toxin, indicating that transferrin and diphtheria toxin were present in each band.

We prepared ¹²⁵I-transferrin and measured the specific binding of the radiolabeled dipher ferric transferrin to mouse cells as a function of transferrin concentration at 4 °C. Scatchard (26) analysis of the data (not shown) revealed ~20,000 transferrin-binding sites/cell with a Kᵦ of ~2 x 10⁻⁹ M. Previous work of Didsbury et al. (27) indicated that mouse LMTK⁻ cells had ~16,000 dipheria toxin receptors/cell with a Kᵦ for dipheria toxin of ~10⁻⁹ M. We conclude from these data that the approximately 10,000-fold greater sensitivity of mouse LMTK⁻ cells to Tf-DT as compared to dipheria toxin alone cannot be explained by a proportionally increased binding of Tf-DT to transferrin receptors on the cells.

The effects of several different inhibitors on the cytotoxicity of the conjugate are presented in Fig. 5. Pretreating the conjugate with dithiothreitol to reduce the disulfide bonds connecting transferrin and dipheria toxin abolished all activity. Control experiments revealed that the reduction produced free dipheria toxin and transferrin. Thus, dipheria toxin must be attached to transferrin to achieve cytotoxicity. Treating cells with the hybrid toxin in the presence of either anti-transferrin or anti-dipheria toxin also resulted in a complete loss of cytotoxic activity, indicating that both the dipheria toxin and transferrin constituents of the conjugate were necessary for intoxication. Also shown in Fig. 5 is that...
treatment cultures with 20 mM NH₄Cl completely protected the cells from the effects of the hybrid toxin. Treating the cells with the proton ionophore monensin also protected the cells against the lethal action of the conjugate (data not shown). Because both NH₄Cl and monensin raise the pH inside acidic intracellular vesicles, the observation that these two drugs inhibited the effects of the hybrid toxin suggests that it is important for the conjugate to encounter a reduced pH to introduce fragment A into the cytosol, as is known to be the case with diphtheria toxin.

If the conjugate is endocytosed and encounters a low pH within vesicles, then toxin inside the vesicles should be sequestered from either anti-diphtheria toxin or anti-transferrin added to the extracellular fluid. To test this prediction, cells were pretreated with NH₄Cl and incubated with the conjugate. Under these conditions, the toxin should be internalized into vesicles, but fragment A should be unable to enter the cytosol because of the elevated pH. The cells were then chilled and exposed to either anti-diphtheria toxin or anti-transferrin to neutralize any accessible toxin. The NH₄Cl and the antibodies were removed by washing, and the cells were placed at 37°C to monitor any cytotoxic response. As seen in Fig. 6, the cells were intoxicated when the NH₄Cl was removed, implying that a lethal dose of toxin had been endocytosed and was protected from extracellular anti-diphtheria toxin or anti-transferrin.

When the NH₄Cl was removed, enabling vesicles to reacidify, neither fragment A could apparently pass into the cytosol, as is known to be the case with diphtheria toxin.

To further assess the role of a low pH in the cytotoxic process of the TF-DT conjugate, we investigated the effect of acidic medium on cytotoxicity. Cells were incubated with ammonium chloride to increase the pH within vesicles and then exposed to the conjugate. The pH of the medium was reduced to 4.6 for 10 min and then returned to pH 7.4. As seen in Table I, the protective effect of NH₄Cl was completely abrogated by the acidic medium. When either exogenous

### Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Acidic medium</th>
<th>Protein Synthesis, % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF-DT</td>
<td>-</td>
<td>106</td>
</tr>
<tr>
<td>TF-DT</td>
<td>+</td>
<td>9</td>
</tr>
<tr>
<td>TF-DT plus exogenous Tf</td>
<td>+</td>
<td>105</td>
</tr>
<tr>
<td>TF-DT plus anti-DT</td>
<td>+</td>
<td>104</td>
</tr>
</tbody>
</table>
transferrin or anti-diphtheria toxin was present, in addition to Tf-DT, the acidic medium did not potentiate cytotoxicity, demonstrating that the effect on protein synthesis was specific to the conjugate. Similar results have been reported for diphtheria toxin with diphtheria toxin-sensitive cells (13, 14) and are interpreted to suggest that acidic culture medium substitutes for a low pH normally encountered by the toxin within vesicles. Thus, the Tf-DT conjugate appears to respond to acidic culture medium as does diphtheria toxin alone in other cell systems. We also found (data not shown), as have others (14, 27), that diphtheria toxin alone does not respond to a low pH and efficiently intoxicate mouse cells under conditions similar to those described in Table I.

Data on the kinetics of intoxication are presented in Fig. 7. The addition of Tf-DT to cells at 37 °C was followed by a dose-dependent, first order decline in the rate of protein synthesis, as is shown in Fig. 7A. Even at high concentrations of the conjugate, there was a minimum lag period of 20-30 min before protein synthesis began to decline. In Fig. 7B, we measured the time required for Tf-DT on the cell surface to be endocytosed and subsequently respond to a low pH. Cells were chilled to 4 °C and exposed to a high concentration of the conjugate (1 μg/ml) to saturate surface receptors. The medium was then replaced with medium containing 1 μg/ml of Tf-DT at 37 °C. At various times after the addition of warm medium, the cells were treated with either anti-diphtheria toxin or NH₄Cl and incubated for 24 h before measuring protein synthesis. Control experiments revealed that the maximum effect of the hybrid toxin on protein synthesis was complete within 24 h. Anti-diphtheria toxin should not inhibit the action of the conjugate once it has been endocytosed whereas NH₄Cl should be unable to inhibit the conjugate after it has been endocytosed and has responded to a low pH within vesicles. As seen in Fig. 7B, the hybrid toxin first became insensitive to anti-diphtheria toxin within a few minutes at 37 °C and about 5 min later became insensitive to NH₄Cl. These results are fully consistent with a model of toxin entry involving endocytosis and subsequent exposure to a low pH. Also shown in Fig. 7B is that the lag time before onset of protein synthesis inhibition under these experimental conditions was about 35 min. Thus, there was no reduction in protein synthesis until about 25 min after the Tf-DT conjugate first became insensitive to inhibition by NH₄Cl. A similar interval between acidification and the first effect on protein synthesis has previously been noted to occur during the intoxication of Vero cells by diphtheria toxin (17).

**DISCUSSION**

Tf-DT is an extremely potent protein toxin with LMTK⁻ cells. Under conditions where the toxin was allowed to saturate surface receptors at 4 °C, and the cells were then raised to 37 °C, protein synthesis declined, after a lag period of ~35 min, as a first order function of time with a τₒ of about 12 min (calculated from the slope of Fig. 7B). Diphtheria toxin alone under similar conditions of prebinding at 4 °C reduces protein synthesis in Vero cells, a cell line that is extremely sensitive to the action of diphtheria toxin (28), with a τₒ of about 12 min, following a 20-min lag (17). There are, however, about 1.6 × 10⁹ receptors/cell for diphtheria toxin on Vero cells (28), compared to 2 × 10⁹ transferrin receptors/LMTK⁻ cells. When Vero cells are treated with subsaturating concentrations of diphtheria toxin, the τₒ of the subsequent first order decline in protein synthesis is increased. This implies that, on a per receptor basis, fragment A of Tf-DT enters the cytosol of LMTK⁻ cells more efficiently than fragment A of diphtheria toxin enters the cytosol of Vero cells.

We suggest the following explanation for the apparent high intoxication efficiency of Tf-DT with LMTK⁻ cells as compared to diphtheria toxin with Vero cells. Diphtheria toxin is known to be degraded within lysosomes of Vero cells (29), and it is believed that there is an interval, after endocytosis but before entry into lysosomes (15, 30), during which fragment A must enter the cytosol or else the toxin reaches lysosomes and is destroyed. Transferrin, on the other hand, apparently does not enter lysosomes during its cycle through the cell (5-12). Assuming that Tf-DT also bypasses the lys-
somal compartment, then the fragment A portion of Tf-DT might have a better chance for entering the cytosol because it would not be subjected to destruction within lysosomes. This emphasizes that the potency of hybrid toxins might be improved in general by targeting the hybrid toxins not only to specific receptors, but also to the appropriate component of the vacuolar compartment.

We are aware of one other report describing a hybrid toxin prepared by attaching a protein toxin to transferrin. Raso and Basala (31) attached the A chain of the plant toxin ricin to transferrin to produce a cytotoxic form of transferrin that operated via transferrin receptors; however, the mechanism by which the ricin A chain reached the cytosol from toxin-receptor complexes on the cell surface was unknown. We have focused here on how fragment A of the Tf-DT conjugate reaches the cytosolic compartment. Our data support the feasibility of a genetic approach to investigating transferrin endocytosis; however, the mechanism appears identical to that by which fragment A readily entered cells or their membranes because fragment A readily entered the cytosol of mouse cells is not some general property of mouse sensitive to the enzymatic activity of fragment A, but also to the appropriate component of the vacuolar compartment.

We intend to use the Tf-DT conjugate to try and isolate mutants that are defective in transferrin endocytosis. The feasibility of a genetic approach to investigating transferrin endocytosis has been demonstrated by the recent work of Raso and Basala (31) who isolated cell variants that apparently have modified transferrin receptors by culturing the cells for several months in the presence of transferrin conjugated to ricin A chain. The high potency of the Tf-DT conjugate described here should lend itself to single-step selections for cells that have acquired resistance to Tf-DT.

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REFERENCES