Expression of functional diphtheria toxin receptors on highly toxin-sensitive mouse cells that specifically bind radioiodinated toxin

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ABSTRACT Diphtheria toxin (DT), a bacterial protein exotoxin, inactivates mammalian cell elongation factor 2 after toxin internalization by receptor-mediated endocytosis. To isolate the DT receptor, we cotransfected DT-resistant wild-type mouse L-M cells with a cDNA library constructed from RNA of highly toxin-sensitive monkey Vero cells and with a neomycin-resistance gene. Stably transfected G418-resistant L-M colonies were screened for DT sensitivity in a replica plate assay. After screening of 8000 colonies, one DT-sensitive (DTs) colony was isolated. The purified DTs mouse cells are highly toxin-sensitive; they are at least 1000-fold more sensitive than wild-type L-M cells and only ∼10-fold less sensitive than Vero cells. Incubation of the DTs mouse cells with CRM 197, a nontoxic form of DT that competitively inhibits the binding of native DT to the toxin receptor, protected them from DT-mediated toxicity. More important, these DTs mouse cells express receptors on their cell surface that bind radiiodinated DT in a specific fashion, a property hitherto demonstrable only with highly toxin-sensitive cells of monkey origin. Furthermore, HA6DT, a DT fragment comprising the M, 6000 carboxyl-terminal receptor-binding domain, inhibited the binding of radiiodinated toxin to these DTs mouse cells to the same extent as unlabeled DT. With these DTs mouse cells as a source of monkey cDNA, it should be possible to clone the gene encoding the DT receptor.

Diphtheria toxin (DT), an exotoxin produced by lysogenized strains of Corynebacterium diphtheriae, is a multifunctional protein that kills susceptible mammalian cells. It is composed of two disulfide-linked fragments, both of which are required for the intoxication process (1–4). The A fragment catalyzes the ADP-ribosylation of eukaryotic elongation factor 2, thereby inhibiting protein synthesis. The B fragment is responsible for binding of the toxin to cells and is essential for facilitating the entry of the A fragment into the cytosol (1–4).

DT enters susceptible mammalian cells by receptor-mediated endocytosis (5, 6). The initial step involves the binding of DT to specific cell-surface receptors, followed by internalization of the toxin–receptor complexes into coated pits and translocation of the A fragment into the cytosol. Not all mammalian cells are equally sensitive to DT (7, 8); monkey kidney cells (e.g., Vero cells) are highly sensitive, whereas human and hamster cells are moderately sensitive and mouse and rat cells are resistant. Because elongation factor 2 of all mammalian cells demonstrates sensitivity to DT, it is generally believed that the difference in sensitivity to DT between species is due to the number of functional cell-surface receptors; i.e., Vero cells display 1–2 × 105 receptors per cell (8), whereas mouse and rat cells lack detectable receptors (3, 9).

The existence of specific cell-surface DT receptors was first demonstrated by Ittelson and Gill (10), through competitive inhibition of binding, and was later quantitatively investigated by Middlebrook et al. (8), through use of radioiodinated DT. To date, the specific receptor(s) has not been completely isolated or characterized by conventional biochemical and immunological techniques. Recently, however, our laboratory has described the isolation and characterization of a DT-sensitive (DTs) mouse cell line obtained by transfection of mouse L-M cells with monkey genomic DNA; a replica plate screen was employed that detected mouse cells that had acquired DT sensitivity and, at the same time, preserved a replica of such cells (11). These cells were shown to contain functional cell-surface toxin receptors; the intoxication of these cells occurred by a mechanism similar to that observed in other toxin-sensitive cell lines. The degree of toxin sensitivity of these DTs mouse cells resembled that of other cells of moderate sensitivity to DT; however, we were unable to detect specific binding of radioiodinated DT to their cell surfaces. This result was not completely unexpected, because specific binding of DT to cell-surface receptors has been demonstrated only with monkey kidney cells and not with cells of moderate DT sensitivity (8). Here we report the successful isolation of DTs mouse cells, obtained after transfection of L-M cells with a monkey cDNA library, that are highly sensitive to DT and that bind radiiodinated DT in a specific fashion. We propose that these cells express functional DT receptors derived from a Vero cell gene and that they will thus greatly facilitate the isolation of the specific cDNA encoding the toxin receptor.

MATERIALS AND METHODS

Materials. All tissue culture reagents were obtained from Sigma with the exception of fetal bovine serum, which was purchased from Cell Culture Laboratories (Cleveland). Genetin (G418 sulfate) was obtained from GIBCO. Sodium [125I]iodide (IMS 30; 13–17 μCi/μg; 1 Ci = 37 GBq), L-[^35S]methionine (>800 Ci/mmol), [α-32P]dCTP (3000Ci/ mmol), and L-[4,5-3H]leucine (60 Ci/mmol) were obtained from Amersham. Partially purified DT was purchased from Connaught Laboratories (lot D721) and purified further by anion-exchange chromatography according to published methods (12) with modifications (13). HA6DT was prepared by specific hydroxylamine cleavage of DT and purified as described (14). C. diphtheriae CRM 197 protein was purchased from the Swiss Serum and Vaccine Institute (Berne, Switzerland). 1,3,4,6-Tetrachloro-3a,6a-diphenylglycoluril

Abbreviations: DT, diphtheria toxin; DTs, DT-sensitive; G418®, G418-resistant.
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(iodo-Gen) was obtained from Pierce. Heps, calcium chloride, trichloroacetic acid, salicylic acid, and salmon sperm DNA were from Sigma. Urea and DNA restriction enzymes were from Boehringer Mannheim, and reagents for oligonucleotide labeling were from Pharmacia. Guanidine thiocyanate was obtained from Curtin Mathesons Scientific, Houston. Cesium chloride was obtained from Fisher. All other chemicals utilized were of the highest purity available. Polyester-PeCap HD7-17 membranes were purchased from Tekto (Elmsford, NY), and Zeta-Probe nylon membranes and ultratrap agarose, from Bio-Rad.

Cell Culture. Vero (CCL 81) and L-M(TK-)(CCL 1.3) cells were obtained from the American Type Culture Collection. Vero and L-M(TK-)(CCL 1.3) cell monolayers were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂ in air.

Construction of Monkey cDNA Library. Total RNA was obtained from Vero cells (15) and cDNA was synthesized under contract. Size-fractionated monkey cDNA inserts were ligated into the plasmid vector pCDNA1 by using nonpalindromic BlalX1 linkers. The ligated material was then used to transform competent Escherichia coli MC1061/p3 cells, yielding a monkey cDNA library in E. coli. A representative portion of this library was amplified 100-fold and the plasmids were isolated by an alkaline lysis protocol (15). The plasmids were then purified twice by cesium chloride centrifugation (15) before being used to transfect wild-type L-M cells.

Isolation of Plasmid and Genomic DNA. DNA from plasmids pS2Vneo (American Type Culture Collection) and plink760 (16) was extracted from E. coli DH5α by an alkaline lysis protocol and purified twice by cesium chloride centrifugation (15). Mouse genomic DNA was obtained from cells with an Applied Biosystems (340A) nucleic acid extractor.

Cotransfection with Monkey Cell-Derived cDNA and pS2Vneo Mouse. Mouse L-M cells (5 x 10⁵ per 100 x 15-mm dish) were transfected with a mixture of 5 µg of DNA from a library consisting of monkey cell cDNA in the pCDNA1 vector, 0.2 µg of pS2Vneo DNA, and 10 µg of mouse cell genomic (carrier) DNA by a calcium phosphate method (17). At 46 hr after transfection, the medium was replaced with a selective medium [DMEM containing 10% fetal bovine serum and the neomycin analogue Geneticin (G418 sulfate at 1 mg/ml)]. The selective medium was changed every 3-4 days, and G418-resistant (G418R) L-M colonies appeared 10-14 days after transfection.

RESULTS AND DISCUSSION

Isolation of a DT<sup>S</sup> Mouse Cell Line After Transfection with Monkey Cell cDNA. We previously reported (11) the isolation of a DT<sup>S</sup> mouse cell line obtained after transfection of toxin-resistant L-M cells with a mixture of genomic DNA from highly toxin-sensitive monkey Vero cells and plasmid DNA containing a neomycin-resistance gene. We demonstrated that these DT<sup>S</sup> mouse cells possess functional DT receptors that allow for DT intoxication via the same pathway observed with naturally toxin-sensitive cells. Although quite sensitive to DT, these cells are not as sensitive as Vero cells and are unable to bind radioiodinated toxin in a specific fashion. The absence of a full complement of Vero cell-derived DT receptors, due either to a lower level of expression of the transfected gene or to the presence of a truncated form of the gene, is the most likely explanation for this lower
sensitivity and lack of demonstrable specific binding. To increase the probability of isolating DTs mouse cells with a full complement of DT receptors and to facilitate the subsequent cloning of the DT receptor gene, we decided to transfected L-M cells with a cDNA library derived from Vero cells. We chose Vero cells as a source of RNA for the construction of the cDNA library because they are extremely sensitive to DT and possess specific DT receptors (7, 8). L-M cells were transfected with a mixture of monkey cDNA and pSV2neo DNA. Stable G418<sup>R</sup> transfectants were isolated and screened for DT sensitivity by using the previously developed replica plate system (11). After screening of ≈8000 colonies, one DTs mouse cell colony was isolated. The cells from this colony were subcultured and purified three times with the replica plating assay. The purified cell population was used for all subsequent experiments.

To confirm that these DTs mouse cells contained Vero cell cDNA, Southern blotting analysis was performed. DNA from these cells was digested with an array of restriction enzymes, and the fragments were separated in an agarose gel, transferred to a nylon membrane, and probed with a <sup>32</sup>P-labeled cytomegalovirus immediate early promoter DNA sequence (16) under very stringent hybridization conditions (Materials and Methods). The DNA hybridization pattern obtained revealed that these cells contained vector sequences (data not shown), a result consistent with the interpretation that the observed DTs phenotype is most likely due to the presence of a specific monkey cDNA in these cells.

Toxin Sensitivity. There is great variability in sensitivity to DT among different mammalian cell lines (7, 8). For example, the previously isolated DTs mouse cells (obtained after transfection with monkey cell genomic DNA), although significantly more sensitive than wild-type L-M cells, were ≈100-fold less sensitive than Vero cells (11); the concentration of DT required to inhibit protein synthesis by 50% (IC<sub>50</sub>) in those DTs mouse cells was similar to that observed with cells of moderate toxin sensitivity [e.g., human and hamster cell lines (7, 8)]. To determine the degree of toxin sensitivity of the DTs mouse cell cDNA transfectants, we used an in vitro cytotoxicity assay. After incubation of the cells with DT at 37°C, it was observed that these cells were at least 1000-fold more sensitive than wild-type L-M cells; an IC<sub>50</sub> of 3.1 × 10<sup>-3</sup> mg/ml was obtained with the DTs cDNA transfectant mouse cells, in contrast to an IC<sub>50</sub> of >10<sup>-2</sup> mg/ml for the L-M cells (Fig. 1A). When the IC<sub>50</sub> of DTs mouse cells was compared with that of Vero cells, the Vero cells were found to be ≈10-fold more sensitive (Fig. 1B). These results show that the DTs mouse cells obtained by transfection with monkey cDNA are considerably more sensitive to DT than wild-type L-M cells and only slightly less sensitive than Vero cells.

Protection of DTs Mouse Cells by CRM 197. CRM 197 protein is a nontoxic analog of DT that has an inactive A fragment and a normal B fragment (10, 21) that binds to cell-surface toxin receptors and hence competes with active DT. If these DTs mouse cells bear specific DT receptors, CRM 197 should recognize them and compete with DT for binding. When these DTs mouse cells were incubated at 37°C with DT in the presence of CRM 197, these cells were protected in a dose-related fashion from the cytotoxic effects of DT (Fig. 1A). The dose–response curves all had the same shape and were shifted progressively to the right, as expected for a competitive inhibitor (10, 20, 22, 23). These observations strongly suggest that the DTs mouse cells do possess specific cell-surface DT receptors. Moreover, Schild analysis (10, 20, 22) of the CRM 197 competition data (obtained from Fig. 1A) demonstrated that the DT receptors on the DTs mouse cells bind DT with high affinity; the Schild analysis yielded an apparent dissociation constant of 1.1 × 10<sup>-8</sup> M, a value which is very similar to that reported for other naturally occurring toxin-sensitive cells (10, 23) and which differs only by a factor of 2 from the apparent dissociation constant of 5.3 × 10<sup>-9</sup> M calculated for Vero cells (from data of Fig. 1B). The slope obtained from the Schild plot was 1.20 for the DTs mouse cell data and 1.31 for the Vero cell data, values that are in good agreement with an expected slope of 1.0 for a competitive inhibitor (22).

Specific Binding of DT to the DT Receptor on DTs Mouse Cells. The results shown in Fig. 1A suggest that specific functional DT receptors on the surface of the DTs mouse cells bind DT and mediate the cytotoxic process. The presence of such receptors can be further demonstrated by isolating the initial binding step in the in vitro cytotoxicity assay from the subsequent steps. Thus, DT (10<sup>−6</sup>–10<sup>−3</sup> mg/ml) was first allowed to bind to the DTs mouse cells at 4°C (5 hr), a condition under which endocytic uptake is inhibited (8). The cells were then washed to remove any unbound toxin, and the temperature was shifted to 37°C to allow cell surface-bound

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** DT-mediated toxicity and protection by CRM 197 of DTs mouse cells and Vero cells. Various amounts of DT were added to cells without competitor (●, ▼) or with CRM 197 protein at 1 μg/ml (●), 5 μg/ml (▲), or 10 μg/ml (▲). The cells were incubated for 1.5 hr at 37°C and subsequently assayed for [3H]leucine incorporation into trichloroacetic acid-precipitable material. Results are expressed as the percentage of control protein synthesis in the absence of toxin (DTs mouse cells, 15,611 cpm; DT-resistant L-M cells, 33,654 cpm; Vero cells, 11,352 cpm). The experiments shown in A and B were performed at the same time. (A) DTs mouse cells (●, ▲, △, □) and DT-resistant wild-type L-M cells (▼). (B) Vero cells.
DT to be internalized and translocated. Protein synthesis was inhibited ($IC_{50} = 3.7 \times 10^{-5} \text{ mg/ml}$) in the DT$^5$ mouse cells, whereas control wild-type L-M cells were unaffected. This experiment demonstrates that DT did indeed originally bind, at 4°C, to functional cell-surface receptors.

Middlebrook et al. (8) have demonstrated that DT binding to Vero, BS-C-1, and MK-2 cells can be measured directly. With the exception of these cell lines (all of which are of monkey kidney origin), specific binding of radioiodinated DT to other cells has not been clearly demonstrated. To test directly for the presence of specific DT cell-surface receptors, the DT$^5$ mouse cells were incubated at 4°C for 5 hr with radioiodinated DT alone or with radioiodinated DT and a 200-fold excess of unlabeled DT. In a typical binding experiment, specific binding of radioiodinated DT to cell-surface receptors on the DT$^5$ mouse cells could be readily detected (Fig. 2A); specific binding to Vero cells was assayed in parallel (Fig. 2B). The extent of binding and the percentage

- **Fig. 2.** Specific binding of $^{125}$I-labeled DT to DT$^5$ mouse cells (A) and Vero cells (B). Cells were incubated at 4°C with $^{125}$I-DT alone or with $^{125}$I-DT and a 200-fold excess of unlabeled DT in binding medium. After 5 hr the cells were washed with ice-cold PBS/ Ca/Mg to remove unbound toxin, and the radioactivity associated with the cells was assayed. Specific binding (*) was determined by calculating the difference between the total binding with $^{125}$I-DT ($\lambda$) and the nonspecific binding obtained with $^{125}$I-DT in the presence of excess unlabeled DT ($\circ$). The experiments shown in A and B were performed at the same time.

- **Fig. 3.** Inhibition of $^{125}$I-DT binding to DT$^5$ mouse cells by HA6DT. DT$^5$ mouse cells were incubated for 5 hr at 4°C in binding medium with $^{125}$I-DT (200 ng/ml, 3.4 nM) and various amounts of unlabeled competitor protein ($\lambda$, insulin; $\circ$, DT; $\bullet$, HA6DT). The cells were washed to remove unbound ligands, and cell-associated radioactivity was assessed. The average radioactivity bound in the absence of any competitor protein (100% control) was 1247 cpm.
of specific binding obtained with the DT<sup>S</sup> mouse cells (ranging from 40% to 70% of the total binding in a number of experiments) are quite high and readily demonstrable, whereas it is difficult to demonstrate such binding with all other toxin-sensitive cell lines except those of monkey origin (8, 11). Although reproducible and readily measured, specific binding was not saturable at the concentrations employed. Therefore, the data were not amenable to accurate Scatchard analysis and the number of toxin-binding sites on the DT<sup>S</sup> mouse cells could not be determined.

**Prevention of Radioliodinated DT Binding to DT<sup>S</sup> Mouse Cells by HA6DT.** This laboratory has previously demonstrated (14) that the peptide HA6DT, the Mr 5982 carboxyl-terminal receptor-binding domain of DT (obtained by hydroxylamine cleavage of DT), protects Vero cells from the lethal action of DT. This protection was shown to be due to inhibition of DT binding to the Vero cell-surface toxin receptor (14). To characterize further the DT receptor on the DT<sup>S</sup> mouse cells, we tested whether HA6DT—the smallest DT receptor-binding peptide so far described—affected the binding of radioliodinated DT to these cells. In a typical experiment, HA6DT inhibited the binding of radioliodinated DT to the DT<sup>S</sup> mouse cells as effectively as unlabeled DT; in contrast, insulin, an unrelated peptide with a molecular weight similar to that of HA6DT, had no effect on the binding (Fig. 3). This result demonstrates not only that the toxin-binding site of the receptor on the DT<sup>S</sup> mouse cells is specific for DT but, more precisely, that it is specific for that region of DT involved in receptor binding.

Our results are entirely consistent with the notion that the DT<sup>S</sup> mouse cells, obtained after transfection with Vero cell-derived cDNA, bear toxin receptors with properties very similar to those widely acknowledged to be characteristic of the Vero cell DT receptor. (i) These are functional DT receptors whose presence results in cells being highly sensitive to the toxin (Fig. 1). (ii) CRM 197 protein and HA6DT peptide compete with DT for binding to these receptors (Figs. 1 and 3). (iii) These receptors bind DT in a highly specific fashion (Fig. 2). Use of these DT<sup>S</sup> mouse cells should make it possible to isolate the specific monkey cell cDNA and to clone the gene, presumably the DT receptor gene, that is responsible for these properties.

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