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Isolation of diphtheria toxin-sensitive mouse cells from a toxin-resistant population transfected with monkey DNA

(diphtheria toxin receptor/replica plate assay/Vero cell DNA)

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ABSTRACT **Diphtheria toxin (DTX)-sensitive mouse cells were isolated from a toxin-resistant thymidine kinase (TK)-negative L-M(TK$^-$) mouse cell population that was transfected with DNA from highly toxin-sensitive monkey Vero cells. Sensitivity to DTX was screened by using a replica plate assay. The purified toxin-sensitive mouse cells were characterized with respect to their ability to bind, internalize, and translocate DTX into the cytosol. In contrast to the L-M(TK$^-$) cells, these DTX-sensitive mouse cells were able to bind and internalize radioiodinated toxin into intracellular vesicles at 37°C. Specific binding of radioiodinated toxin to their cell surface (at 4°C) could not be demonstrated. However, the following evidence for functional receptors capable of binding DTX was obtained: (*i*) when the toxin-sensitive mouse cells were first allowed to bind DTX at 4°C, followed by washing the cells and shifting the temperature to 37°C (allowing cell surface-bound toxin to enter the cells), the cells were killed; (*ii*) when cells with surface-bound DTX were exposed briefly to an acidic medium (allowing the toxin to penetrate the plasma membrane directly), protein synthesis was inhibited; and (*iii*) when cells were incubated with DTX in the presence of the CRM 197, a nontoxic form of DTX with binding properties similar to native DTX, the cytotoxic effect of DTX was markedly decreased. The results demonstrate that the toxin-sensitive mouse cells are killed by a mechanism similar to that observed in naturally occurring toxin-sensitive cell lines. The data further suggest that the transfected mouse cells express functional receptors for DTX.**

Diphtheria toxin (DTX), a protein synthesized by lysogenized strains of *Corynebacterium diphtheriae*, inhibits eucaryotic protein synthesis by catalyzing the ADP-ribosylation of elongation factor 2 (EF-2). The toxin is secreted as a single polypeptide (M_r 58,342), but it can be readily cleaved by limited proteolysis into two fragments, A (M_r 21,167) and B (M_r 37,195), which remain associated by a disulfide bond (1–4). Both fragments are required for intoxication. The B fragment is involved in the binding of the toxin to cell-surface receptors and in the subsequent translocation of the A fragment into the cytosol. The A fragment is then able to catalyze the transfer to ADP-ribose from NAD^+ to EF-2, thereby inhibiting protein synthesis. DTX enters sensitive cells via receptor-mediated endocytosis (5, 6) by: (*i*) binding to specific receptors on the cell surface, (*ii*) internalization into a vesicle, and (*iii*) translocation of the A fragment across the acidified vesicular membrane into the cytosol, where it exerts its cytotoxicity (1–4).

Mammalian cells differ considerably in their sensitivity to DTX (7, 8). Monkey kidney cells (e.g., Vero) are extremely sensitive to the action of DTX and have been extensively used to study toxin binding and entry (8–12). Human cells and Chinese hamster ovary cells are ≈2 orders of magnitude less sensitive than Vero cells, while mouse and rat cells are resistant to DTX (7). The sensitivity of Vero cells is thought to be a reflection of their greater number of specific cell-surface receptors, with reports ranging from 50,000 to 200,000 per cell (8, 10, 11). Since mouse and rat EF-2 is sensitive to DTX (1, 2), it is generally believed that mouse and rat cells are resistant to DTX because they lack functional cell-surface receptors (3, 13) or a component(s) required for DTX internalization (14–16), or both. In studies designed to isolate and characterize the DTX receptor by using a genetic approach, we have transfected L-M(TK$^-$) cells with a mixture of Vero cell DNA and plasmid DNA encoding neomycin resistance. The resultant neomycin-resistant (Neo^R) L-M(TK$^-$) cells were screened for DTX sensitivity, and a DTX-sensitive (DTX^S) mouse cell line was isolated and characterized. We propose that these cells may bear surface DTX receptors derived from Vero cells and that therefore they may facilitate the isolation of the gene(s) encoding the toxin receptor.

MATERIALS AND METHODS

Materials. All chemicals utilized were of the highest purity available. All tissue culture reagents and Geneticin (G418 sulfate) were obtained from GIBCO with the exception of fetal bovine serum, which was purchased from Hazelton Biologics, Inc. (Lenexa, KS). $Na^{125}I$ (IMS 30; 13–17 μCi/μg; 1 Ci = 37 GBq), L-[4,5-^{3}H]leucine (60 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), and L-[^{35}S]methionine (>800 Ci/mmol) were obtained from Amersham. Partially purified DTX was purchased from Connaught Laboratories (lot D721) and further purified by anion-exchange chromatography according to published methods (17) with modifications (18); the purified DTX was mostly unnicked (18) and was used throughout, except where noted. Limited proteolysis (nicking) of DTX to generate disulfide-linked A and B fragments was carried out with trypsin as described (19). *C. diphtheria* CRM 197 (nontoxic analog of DTX) was purchased from the Swiss Serum and Vaccine Institute (Berne, Switzerland); DNA restriction enzymes, from Boehringer Mannheim Biochemicals; and reagents for oligonucleotide labeling, from Pharmacia. 1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril (Iodo-Gen) was obtained from Pierce. Trypsin (type XI), soybean trypsin inhibitor (type 1-S), Hepes, calcium chloride, trichloroacetic acid, salicylic acid, and sonicated salmon sperm DNA were obtained from Sigma. Polyester-PeCap HD7-17 membranes were purchased from Tetko (Elmsford, NY); glass beads, from Scientific Products; and Zeta-Probe nylon membrane and ultrapure agarose, from Bio-Rad.

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Abbreviations: DTX, diphtheria toxin; EF-2, elongation factor 2; Neo^R, neomycin resistant; DTX^S, diphtheria toxin sensitive; PBS/Ca/Mg, phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$; IC_{50}, concentration of DTX required to inhibit protein synthesis by 50%; TK, thymidine kinase.

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Cell Culture. Vero cells (CCL 81) and thymidine kinase (TK)-negative L-M(TK$^-$) (CCL 1.3) cells were obtained from the American Type Culture Collection. Vero and L-M(TK$^-$) cell monolayers were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 μg/ml).

Isolation of Plasmid and Genomic DNA. DNA from plasmid, pSV2-neo (American Type Culture Collection) was extracted from *Escherichia coli* DH5α as described (20), and genomic Vero cell DNA was obtained as described (21).

Transfection with Plasmid and Genomic DNA. Mouse L-M(TK$^-$) cells (5×10^5 cells per 100 × 15 mm dish) were transfected with a mixture of 18 μg of Vero DNA and 0.13 μg of *Eco*RI-digested pSV2-neo DNA (22) as described (23). Forty-six hours after transfection, the medium was replaced with "selective medium" [DMEM containing 10% fetal bovine serum and Geneticin (1 mg/ml)]. The selective medium was changed every 2–3 days, and Neo^R L-M(TK$^-$) colonies appeared 10–14 days after transfection.

Isolation of DTX^S Mouse Cells. Neo^R L-M(TK$^-$) colonies were screened for DTX sensitivity by using the replica plating protocol described by Robbins and Roff (24) with modifications. Briefly, 100–150 Neo^R L-M(TK$^-$) colonies per 100 × 15 mm tissue culture dish were overlaid with a stack of three polyester-PeCap HD7-17 filter membranes and a layer of glass beads. The cells were allowed to grow through the filter membrane stacks for 7 days before they were screened for DTX sensitivity. The stack of filters was carefully removed from the master dish and transferred to another dish containing selective medium. The medium in the master dish was replaced with fresh selective medium, and the cells were further incubated at 37°C. The bottom filter, the one in direct contact with the cells, was incubated with 2 μg of DTX per ml for 18 hr at 37°C, and the remaining two filters were maintained together at 37°C in a separate dish containing fresh selective medium. The DT-treated Neo^R L-M(TK$^-$) colonies were washed with Mg^{2+}/Ca^{2+}-containing phosphate-buffered saline (PBS/Ca/Mg; 8.8 mM Na_2HPO_4/1.2 mM KH_2PO_4/140 mM NaCl/10 mM KCl/1.0 mM $CaCl_2$/0.5 mM $MgCl_2$, pH 7.4) and incubated in 5 ml of DMEM lacking L-methionine and supplemented with 20 mM Hepes for 1.5 hr at 37°C; afterwards, L-[^{35}S]methionine (1×10^6 cpm/ml), diluted in the methionine-deficient medium, was added, and the colonies were incubated for an additional 1.5 hr at 37°C. The filters were washed with PBS/Ca/Mg, and the cells were treated with ice-cold 15% CCl_3COOH. The filters were then washed, incubated at ambient temperature in 1 M sodium salicylate (pH 6.25) for 30 min (25), dried, and exposed to XAR-5 film for 16 hr at −80°C. After autoradiography, these same filters were stained with Coomassie G-250, and the stained colonies were compared with the corresponding areas on the autoradiograms. Those colonies that appeared blue on the filter but had not been able to incorporate radiolabeled methionine in the presence of DTX, as indicated by a clear zone on the autoradiogram, were isolated by cutting out the corresponding individual colonies from the middle membrane filter. The DTX^S mouse cells were amplified and rescreened twice with this replica plate protocol.

Cytotoxicity Assay. Tissue culture dishes (48 well) were seeded with 2.5×10^4 cells per well and grown to confluency. Culture media containing 20 mM Hepes (pH 7.4) and various amounts of DTX were added to cell monolayers, and the cells were incubated at 37°C for the indicated times. The monolayers were washed and further incubated for 1 hr at 37°C in leucine-deficient DMEM. [^{3}H]Leucine (4 μCi) was added for a final hour, and incorporation into Cl_3COOH-precipitable material was measured as described (26, 27). All assays were performed in triplicate, and variation from the mean was 5–10%.

DTX Internalization Assay. Culture medium containing 20 mM Hepes and 20 mM methylamine (pH 7.4; refs. 28 and 29) was added to the cell monolayers, and the cells were incubated for 30 min at 37°C. Radiolabeled DTX (50–200 ng/ml; 1–2 × 10^7 cpm/μg) (18), with or without a 100-fold excess of unlabeled DTX, was added to the cells, and the incubation was continued for 14 hr at 37°C. The cells were washed with PBS/Ca/Mg to remove unbound DTX and were solubilized in 0.2 M NaOH before the cell-associated radioactivity was measured (8). Nonspecific association is defined as the amount of radioactive DTX that remains associated with the cells when the radiolabeled DTX incubation is performed in the presence of a 100-fold excess of unlabeled DTX. All assays were performed in triplicate, and variation from the mean was 5–10%.

Southern Analysis. Digestion with the restriction enzyme *Hin*dIII was performed under the conditions described by the manufacturer. Restriction enzyme-digested DNA was analyzed by 0.7% (wt/vol) agarose gel electrophoresis (30). DNA was transferred from the agarose gel to a Zeta-Probe membrane by the method of Southern (31). Total *Hin*dIII-digested Vero DNA was labeled with [α-^{32}P]dCTP to a specific activity of 1–2 × 10^8 cpm/μg. Hybridization was for 18 hr at 68°C with 10 ng of radiolabeled DNA and 100 μg of sonicated salmon sperm DNA per ml in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 0.01 M EDTA, 5× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), and 0.5% sodium dodecyl sulfate. After hybridization, filters were washed as described (30). Autoradiography was for 7 days at −80°C.

RESULTS AND DISCUSSION

Isolation of a DTX^S Mouse Cell Line. Mouse and rat cells are very resistant to DTX intoxication; however, human cells are sensitive, and monkey kidney cells (e.g., Vero) are extremely sensitive (7, 8). Several groups (32–35), using mouse–human cell hybrids, have clearly demonstrated that the human DTX-sensitivity gene(s) can be expressed in a mouse genetic background; thus, we assumed that the Vero DTX-sensitivity gene(s) could also be expressed in mouse cells. We chose Vero cell DNA because these cells have a large number of toxin receptors, and specific binding of radioiodinated DTX can be measured readily. We isolated a DTX^S mouse cell line by transfecting L-M(TK$^-$) cell monolayers with a mixture of total genomic Vero DNA and linear plasmid DNA containing a neomycin-resistance gene (22). After transfection, a selective medium containing Geneticin was added, and after 2 weeks, ≈120 Neo^R colonies were obtained per 5×10^5 L-M(TK$^-$) cells.

To isolate those Neo^R L-M(TK$^-$) cells that had acquired the DTX-sensitivity gene(s), we used a replica plate protocol that allowed us to screen for those cells whose protein synthesis is inhibited upon exposure to DTX and, at the same time, preserve a "replica" of those cells. The Neo^R L-M(TK$^-$) cell colonies were overlaid with a stack of polyester filter membranes, and the cells were allowed to grow through the membranes. The Neo^R L-M(TK$^-$) cell colonies, immobilized on one filter, were treated with DTX and later assayed for their ability to incorporate [^{35}S]methionine into protein. Those L-M(TK$^-$) cells that acquired the DTX-sensitivity gene(s) should be unable to incorporate [^{35}S]methionine in the presence of DTX and therefore would appear as a clear zone on the autoradiogram with a corresponding blue colony on the stained polyester filter, while those L-M(TK$^-$) cells resistant to DTX would appear as a dark colony on the autoradiogram and as a blue-stained colony on the filter. After screening 12,000 Neo^R L-M(TK$^-$) colonies, one DTX^S Neo^R colony was isolated (Fig. 1). This DTX^S colony was amplified and rescreened twice with this replica plating

protocol. This purified primary transfectant cell population was utilized for all subsequent experiments.

Southern Analysis of the DNA Obtained from DTX^S Mouse Cells. To determine whether the DTX^S mouse cells contained Vero cell DNA, Southern blotting analysis was performed. DNA from DTX^S mouse cells was digested with *Hin*dIII and probed with ^{32}P-labeled total genomic Vero cell DNA. Fig. 2 shows that the ^{32}P-labeled Vero cell DNA hybridized with DTX^S mouse DNA (lane B), whereas it did not hybridize with L-M(TK$^-$) cell DNA (lane A). The hybridization pattern observed with DTX^S mouse cell DNA (lane B) is typical of that seen in other primary transfectant cell populations and is most likely due to the incorporation of large amounts of donor DNA into the transfectant cell genome (36–38).

Toxin Sensitivity. To assess the degree of DTX sensitivity of the DTX^S mouse cells, we used an *in vitro* cytotoxicity assay to determine the concentration of DTX required to inhibit protein synthesis by 50% (IC_{50}). After incubation of the cells with DTX, it was observed that the DTX^S mouse cells are at least a 1,000-fold more sensitive to DTX than are the wild-type L-M(TK$^-$) cells: an IC_{50} of 1.8×10^{-5} mg/ml was obtained with the DTX^S mouse cells compared with an IC_{50} of $>10^{-2}$ mg/ml for the L-M(TK$^-$) cells (Fig. 3*A*). When the IC_{50} of DTX^S mouse cells and Vero cells is compared, the Vero cells are ≈100-fold more sensitive to DTX than the DTX^S mouse cells (Fig. 3*B*). From these data it appears that the DTX^S mouse cells are of intermediate DTX sensitivity because their IC_{50} is similar to that obtained with human and hamster cell lines (7, 8). More importantly, the DTX^S mouse cells have acquired toxin sensitivity well above that observed with L-M(TK$^-$) cells.

Prevention of DTX-Mediated Cytotoxicity by the *C. diphtheriae* CRM 197 Analog. CRM 197 is a nontoxic analog of DTX with an inactive A fragment and a normal B fragment that can bind to toxin cell-surface receptors and hence compete with active DTX for receptor binding (10). If these DTX^S mouse cells contain functional DTX receptors, then CRM 197 should compete with DTX for binding to these cell-surface receptors. When the DTX^S mouse cells were incubated with DTX in the presence of CRM 197, it was observed that CRM 197 afforded considerable protection to both the DTX^S mouse (Fig. 3*A*) and the Vero cells (Fig. 3*B*). This protective effect is dose dependent, since increasing concentrations of CRM 197 shifts the sensitivity curves progressively to the right. These data demonstrate that the DTX^S mouse cells, in a manner similar to other toxin-sensitive cells (10), allow CRM 197 binding to similar functional receptors as native DTX.

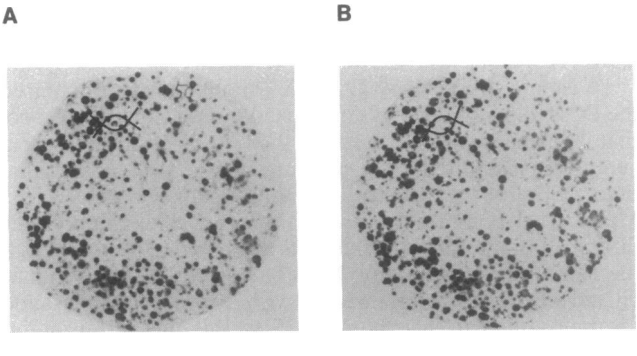

FIG. 1. Autoradiographic detection of DTX^S mouse cells immobilized on a polyester membrane. A polyester membrane containing Neo^R mouse cells was treated with DTX for 18 hr at 37°C. The cells were then measured for their ability to incorporate [^{35}S]methionine into protein followed by autoradiography as described. (*A*) Polyester membrane containing Coomassie blue-stained DTX-treated mouse cell colonies. (*B*) Corresponding autoradiogram of the mouse cell colonies after a 16-hr exposure. The marked area indicates the position of a colony that was unable to incorporate [^{35}S]methionine into protein in the presence of DTX.

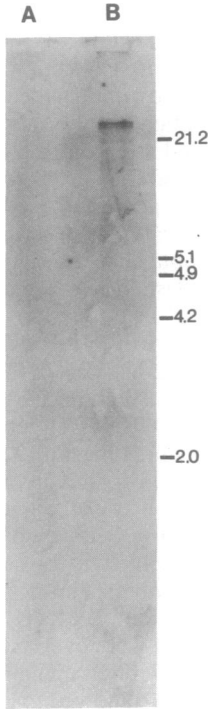

FIG. 2. Southern blot-hybridization analysis of the DNA obtained from DTX^S mouse cells and DTX-resistant L-M(TK$^-$) cells. Mouse cell DNA from DTX^S mouse cells or L-M(TK$^-$) cells (12 μg) was digested with *Hin*dIII, separated on an agarose gel, and transferred to a nylon membrane as described. Vero cell DNA was digested with *Hin*dIII and labeled with [α-^{32}P]dCTP for use as a probe. Hybridization conditions, posthybridization washes, and autoradiography were performed by the method of Maniatis *et al.* (30). Lanes: A, *Hin*dIII-digested DNA from L-M(TK$^-$) cells; B, *Hin*dIII-digested DNA from DTX^S mouse cells. Positions of standards (in kb) are indicated at the right.

Binding and Internalization of DTX. The initial step in DTX-mediated intoxication of sensitive cells involves toxin binding to specific cell-surface receptors (8, 9). *Specific binding* of DTX to cell-surface receptors can be demonstrated readily with Vero cells but not with such cells of intermediate DTX sensitivity as human or hamster cells (8). When the DTX^S mouse cells were incubated overnight at 4°C with radioiodinated DTX alone or with radioiodinated DTX and a 100-fold excess of unlabeled DTX, specific binding of radioiodinated DTX to specific cell-surface receptors could not be detected. This result may be a consequence of the intermediate sensitivity of the DTX^S mouse cells, presumably as a consequence of having fewer functional cell-surface receptors than Vero cells (8).

Since it was not possible to detect specific binding of radioiodinated DTX at 4°C with the DTX^S mouse cells, we measured the specific association of DTX at 37°C in the presence of methylamine. Methylamine does not interfere with the initial binding and internalization of DTX but rather inhibits the subsequent translocation of DTX and its intracellular breakdown, thereby allowing the detection of accumulated radioiodinated DTX in cells of intermediate toxin sensitivity (28, 29). When these DTX^S mouse cells and L-M(TK$^-$) cells were incubated with radioiodinated DTX and methylamine, the DTX^S mouse cells accumulated a significant amount of radioactive DTX, whereas the L-M(TK$^-$) cells did not (Fig. 4). These experiments are consistent with the notion that DTX intoxication of the DTX^S

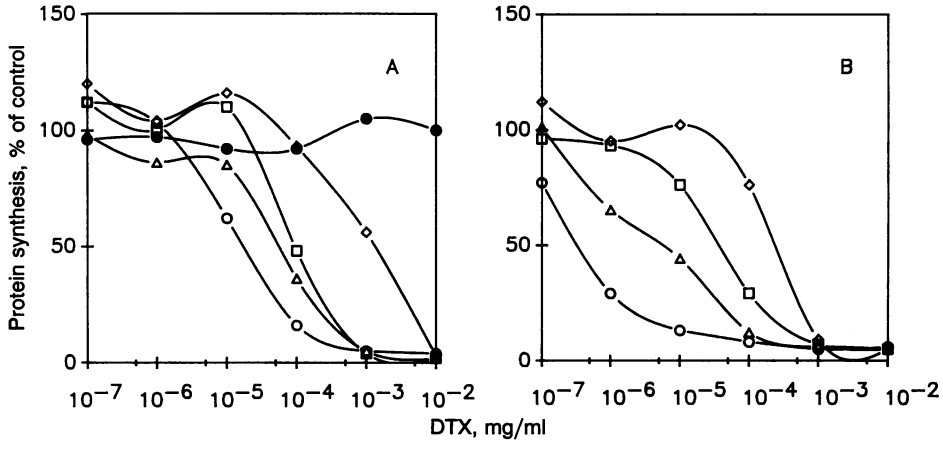

FIG. 3. Differential toxin sensitivity of DTX^S mouse cells, L-M(TK$^-$) cells, and Vero cells and the effect of CRM 197 on toxin sensitivity. Increasing amounts of DTX were added to cells in the absence or presence of various concentrations of competitor CRM 197 protein. The cells were then incubated for 1.5 hr at 37°C and subsequently assayed for [^{3}H]leucine incorporation into CCl_3COOH-precipitable material as described. Results are expressed as the percentage of control protein synthesis in the absence of toxin (DTX^S mouse cells, 34,912 cpm; Vero cells, 35,071 cpm; L-M(TK$^-$) cells, 34,138 cpm). (*A*) Open symbols, DTX^S mouse cells; closed symbols, DTX-resistant L-M(TK$^-$) cells. (*B*) Vero cells only. ○ and ●, Without CRM 197; △, 0.1 μg of CRM 197 per ml; □, 1.00 μg of CRM 197 per ml; ◇, 10.0 μg of CRM 197 per ml.

mouse cells involves DTX binding to cell-surface receptors and its subsequent translocation from acidified vesicles.

To test more rigorously for the presence of functional receptors on the cell surface of the DTX^S mouse cells, DTX was first allowed to bind to these cells for 4 hr at 4°C, conditions under which endocytic uptake is inhibited (8). The cells then were washed to remove unbound DTX, and the temperature was shifted to 37°C to allow cell surface-bound toxin to be internalized. Protein synthesis in the DTX^S mouse cells was inhibited, whereas the L-M(TK$^-$) cells were resistant to DTX (Fig. 5). These experiments show that DTX was originally bound to functional DTX^S mouse cell-surface toxin receptors before it was internalized and translocated into the cytosol.

Acid-Shock Receptor-Dependent Penetration of DTX. To kill mammalian cells, DTX bound to specific receptors must be endocytosed and then encounter a low pH within intracellular vesicles (13, 39, 40). The low-pH environment of the endocytic vesicles enables DTX to penetrate the vesicular membrane, resulting in the translocation of the A fragment into the cytosol of the cell (39, 40). NH_4Cl is known to raise the pH within acidic vesicles, resulting in protection of DTX^S cells from DTX cytotoxicity (39, 40). Since we had demonstrated that the DTX^S mouse cells were sensitive to DTX (Figs. 3*A* and 5) via a receptor-mediated endocytosis pathway, it was of interest to determine whether NH_4Cl would block the action of DTX. The data of Fig. 5 show that in the presence of 10 mM ammonium chloride, the DTX^S mouse cells are indeed protected against DTX cytotoxicity. This result supports the notion that DTX prebound to the cell surface of the DTX^S mouse cells is endocytosed into vesicles before being translocated into the cytosol, as is the case with other DTX^S cells (13, 39, 40).

The low-pH environment of the endocytic vesicles can be mimicked by acidifying the culture medium surrounding the

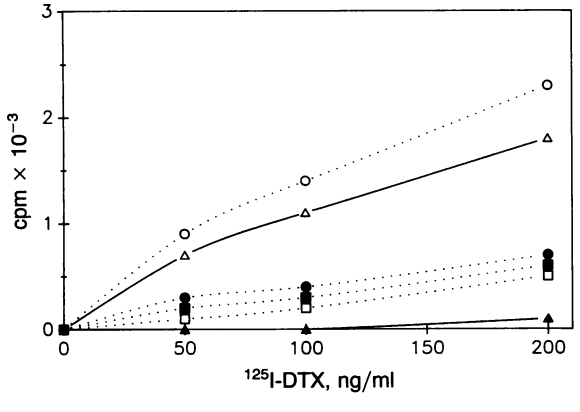

FIG. 4. Specific association of radioiodinated DTX (^{125}I-DTX) with DTX^S mouse cells. Cells were incubated with 20 mM methylamine for 30 min at 37°C before ^{125}I-DTX with or without a 100-fold excess of unlabeled DTX was added. After 14 hr at 37°C, the cells were washed with PBS/Ca/Mg to remove any unbound toxin, and the radioactivity associated with the cells was determined as described. Open symbols, DTX^S mouse cells; closed symbols, DTX-resistant L-M(TK$^-$) cells; ○ and ●, ^{125}I-DTX; □ and ■, ^{125}I-DTX and excess unlabeled DTX; △ and ▲, the difference between ^{125}I-DTX and ^{125}I-DTX in the presence of excess unlabeled DTX (specific association).

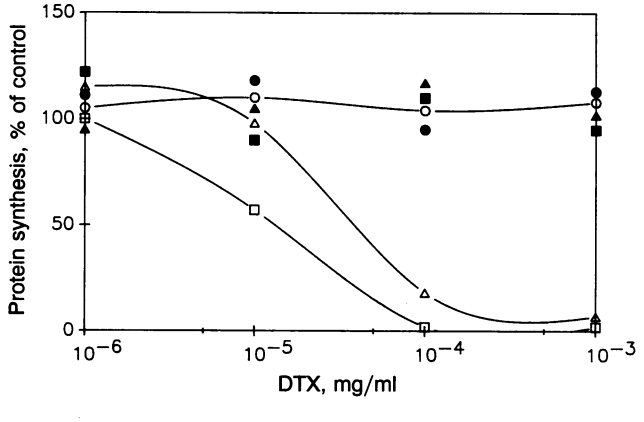

FIG. 5. Cytotoxic activity of receptor-bound DTX after exposure of DTX^S mouse cells to acidic medium. Cells were bound with nicked DTX for 4 hr at 4°C before being washed with PBS/Ca/Mg to remove unbound toxin. The treated cells [DTX^S mouse cells (open symbols) and DTX-resistant L-M(TK$^-$) cells (closed symbols)] were incubated for 10 min at 37°C in the presence (○ and ●) or absence (△ and ▲) of 10 mM NH_4Cl at pH 7.2 or in the presence (□ and ■) of 10 mM NH_4Cl at pH 4.5. Cells were then washed and further incubated at 37°C for 14 hr in a leucine-deficient DMEM with (○, ●, □, and ■) or without (△ and ▲) NH_4Cl. The cells were subsequently assayed for [^{3}H]leucine incorporation into CCl_3COOH-precipitable material as described. The results are expressed as the percentage of control protein synthesis in the absence of toxin (DTX^S mouse cells, 59,740 cpm; L-M(TK$^-$) cells, 11,667 cpm).

cells to pH 4.5 (39, 40). Under these conditions receptor-bound DTX is believed to penetrate directly through the plasma membrane into the cytosol (39, 40), bypassing the protective effect of NH_4Cl. Fig. 5 shows that after a brief shift of the culture medium pH from 7.2 to 4.5 (in the presence of NH_4Cl), receptor-bound DTX is able to enter the DTX^S mouse cells and inhibit protein synthesis. Moreover, under these acid-shock conditions, the L-M(TK^-) cells are still resistant to DTX intoxication (Fig. 5). The results presented in Fig. 5 confirm that DTX molecules prebound to cell-surface receptors on toxin-sensitive cells can be directed into the cytosol via the acid-shock procedure and that this procedure does not result in the inhibition of protein synthesis in toxin-resistant cells—i.e., L-M(TK^-) cells.

The data presented herein are consistent with the idea that the DTX^S mouse cells now possess functional cell-surface DTX receptors that are capable of binding toxin and directing its internalization and translocation into the cytosol. Since with these DTX^S mouse cells we were not able to demonstrate specific binding of radiolabeled DTX, a property unique to Vero cells (8), we can conclude that the transfected cells do not possess a full complement of Vero DTX receptors. Several possibilities exist to explain the presence of functional DTX receptors on the transfected DTX^S mouse cells. One possibility is that these transfected mouse cells contain the Vero cell gene for the DTX receptor. The expression of the gene may be altered because of the particular fragment of DNA present or the nature of the integration site of the introduced Vero DNA. Another possibility is that a limited number of constitutive L-M(TK^-) cell proteins have been modified by a transfected Vero cell gene(s) product in such a way as to lead to the expression of functional mouse DTX receptors. A final, though the least likely, possibility is that the replica plate screen enabled us to isolate a spontaneous DTX^S cell and that the Vero DNA contained within these cells does not encode DTX sensitivity.

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