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Purification and Characterization of a Shiga Toxin A Subunit-CD4 Fusion Protein Cytotoxic to Human Immunodeficiency Virus-Infected Cells

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In a previous paper, we reported that a chimeric toxin composed of the enzymatic domain of the Shiga toxin A polypeptide (StxA1) genetically fused to the human CD4 (hCD4) molecule selectively kills cells infected with human immunodeficiency virus type 1 (HIV-1). Although other hCD4-containing chimeras cytotoxic to HIV-infected cells have been developed, there is limited information regarding their receptor binding and internalization. Therefore, the goals of this study were to purify the StxA1-hCD4 fusion protein, identify the receptor(s), and investigate the cytosolic trafficking route used by the chimeric toxin. Sufficient quantities of the StxA1-hCD4 hybrid were isolated for this investigation by using the pET expression and purification system. Cos-1 cells were rendered sensitive to the StxA1-hCD4 chimera by transfection with the *env* gene, which encodes HIV-1 envelope glycoproteins. The entry and translocation pathway used by the StxA1-hCD4 hybrid toxin was investigated by assessing the protective capacities of chemical reagents which interfere with microfilament movement, acidification of endosomes, and the integrity of the Golgi apparatus. Our findings indicated that the chimera uses HIV-1 glycoprotein gp120, and perhaps gp41, as a receptor which directs its entry through receptor cycling. Uptake is pH independent, and the StxA1-hCD4 hybrid is apparently translocated to the Golgi complex as with other bipartite toxins.

Recombinant toxins must possess domains that bind to a specific receptor on the target cell, translocate the enzymatic moiety to the cytosolic site of action, and catalytically induce a lethal alteration. The human CD4 (hCD4) domain, which retains the capacity to bind glycoprotein 120 (gp120) of the human immunodeficiency virus (HIV), has been used to deliver the catalytic subunits of *Pseudomonas* exotoxin A (4), diphtheria toxin (2), ricin (30), and Shiga toxin (STX) (1) into HIV type 1 (HIV-1)-infected cells. Although these hCD4-containing toxin chimeras are all capable of selective cell killing, the mechanisms of receptor binding, entry, and translocation have not been explored. We have applied the same techniques used for the analysis of other bipartite toxins to elucidate the mechanism of cell binding and entry of the chimeric toxin composed of the StxA1 polypeptide and hCD4.

STX is internalized along with its glycolipid receptor from clathrin-coated pits (24). An essential step for intoxication by STX, as well as for that by its functional relative ricin, is the translocation of these toxins to the trans-Golgi network of the susceptible cell (25). Presumably, it is at this site that the disulfide bond linking the enzymatic A chains to the receptor-binding subunits is reduced, permitting release of the A1 polypeptides (8). The A1 polypeptides of STX and ricin bind to the acceptor site groove of ribosomes and catalytically release a specific adenine residue, causing the cessation of protein synthesis (7). The minimum domain of StxA1 which retains catalytic activity in vitro was identified (12), and fusion of this polypeptide to hCD4 formed a toxin chimera capable of selectively killing cells infected with HIV-1 (1).

HIV-1 acquires a lipid bilayer studded with the envelope glycoproteins gp120 and gp41 while budding from infected cells (19). HIV-1 binds to hCD4 through a conserved region near the C terminus of gp120, and a conformational change in a gp120-gp41-CD4 complex exposes the N terminus of gp41, leading to membrane fusion and virus entry (6, 27). Evidence supporting the role of hCD4 as the receptor for HIV-1 includes blocking studies using monoclonal antibodies and the susceptibility of $hCD4^+$ cell lines to HIV-1 infection (5, 16, 18). The extracellular 180 N-terminal amino acids of hCD4, which retain the capacity to bind gp120 (3), have been used as a vehicle to deliver the enzymatic subunits of various toxins, including STX, into HIV-infected cells.

We have purified sufficient quantities of the StxA1-hCD4 chimera to permit preliminary characterization of the intoxication process. The role of HIV-1 glycoproteins as receptors was confirmed, and intracellular trafficking of the hybrid toxin was investigated by using metabolic inhibitors which affect cytosolic pH, microfilament movement, and the integrity of the Golgi complex.

MATERIALS AND METHODS

Cell lines. African green monkey kidney (Vero) cells were used for STX cytotoxicity assays (10), and the human T-cell line A3.01 and its derivative 8E5, which harbors an integrated defective HIV-1 genome (9), were used for assessing the cytotoxicity of the StxA1-hCD4 hybrid protein. The Cos-1 cell line (11) transfected with pwtEnv, which encodes HIV glycoprotein gp160 (23), was also used in cytotoxicity assays with the StxA1-hCD4 chimera.

Minimal essential medium (MEM) supplemented with 10% fetal bovine serum was used for culturing Cos-1 and Vero cells. RPMI 1640 supplemented with 10% fetal calf serum was used for culturing A3.01 and 8E5 cells. Cell culture media and sera were obtained from Sigma Chemical Co., St. Louis, Mo.

Construction of recombinant plasmid pAA58. A 1.22-kb *Bam*HI-*Xho*I fragment encoding the StxA1-hCD4 fusion protein was amplified from plasmid pFUS60-CD4 (1) by PCR and was ligated to the expression vector pET24d (Novagen, Madison, Wis.). This vector expresses proteins from the phage T7 promoter and fuses six histidine residues at the C terminus. The PCR primer ACGATAGACTTTTGGATCCAACAAAGTTAT, from position 314 to 343 of the gene sequence encoding Slt-IIA (14), introduced a *Bam*HI restriction site 5′

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TABLE 1. Recombinant plasmids

Plasmid	Gene product	Reference
pNAS10	STX	28
pFUS60-CD4	StxA1-hCD4	1
pAA58[a]	StxA1-hCD4	This study
pwtEnv	Env	23

[a] The vector for pAA58 was pET24d (Novagen).

to the gene fusion carried by pFUS60/CD4. The reverse PCR primer TAAT ACGACTCACTCGAGGGCGAATTCGAG, from position 421 to 450 of the pBS vector (Stratagene, La Jolla, Calif.), introduced an *Xho*I restriction site 3′ to the gene fusion in pFUS60/CD4. (Underlined residues were mutated from the wild-type sequences to introduce the restriction sites.) Oligonucleotides were synthesized by the Biochemistry Department Core Facility, Wayne State University Medical School, by using an Applied Biosystems model 380B DNA synthesizer with phosphoramidite chemistry.

The recombinant plasmid pAA58 (Table 1) was transformed into *Escherichia coli* BL21 lysogenized with phage DE3, which encodes LacI. BL21 carries a chromosomal copy of the phage T7 RNA polymerase gene under control of the *lacUV5* promoter (29). Expression of the recombinant protein was induced by the addition of isopropylthio-β-galactoside (IPTG) to a logarithmic-phase culture as described by the manufacturer (Novagen).

The other recombinant plasmids used in this study are listed in Table 1.

Preparation of crude toxin in periplasmic extracts. Sixty-milliliter cultures were grown overnight at 37°C with agitation, and the bacteria were collected by centrifugation at 10,000 × *g* for 10 min and washed three times with phosphate-buffered saline (PBS) (123 mM sodium chloride, 10 mM dibasic sodium phosphate, and 3 mM monobasic potassium phosphate [pH 7.4]). The bacterial pellets were resuspended in 1 ml of sterile PBS containing polymyxin B sulfate at a final concentration of 2 mg/ml and incubated on ice for 15 min. Bacteria were sedimented by centrifugation at 14,000 × *g* for 10 min, and the protein concentration in the supernatant was measured spectrophotometrically as dye complexes (Bio-Rad, Melville, N.Y.). The periplasmic extracts were filter sterilized and used for the in vitro translation and cytotoxicity assays.

Purification of polyhistidine-tagged StxA1-hCD4 fusion protein. The StxA1-hCD4 chimera encoded by pAA58 carries six histidine residues at its C terminus. This polyhistidine tag has a high affinity for a nickel-nitrilotriacetic acid resin (Novagen), permitting single-step purification of the fusion protein. The recombinant pAA58 was introduced into *E. coli* BL21(DE3) by transformation, and synthesis of the polyhistidine-tagged StxA1-hCD4 hybrid protein was induced by the addition of 1 mM IPTG to a log-phase culture followed by incubation at 37°C for 4 h. Bacterial pellets were collected by centrifugation at 10,000 × *g* for 10 min, and periplasmic extracts were prepared with polymyxin B as described above. The nickel-nitrilotriacetic acid resin column was washed with 20 volumes (80 ml) of a buffer composed of 50 mM sodium phosphate, 300 mM sodium chloride, and 10% glycerol at pH 6.0. The periplasmic extract was added to the column, and the polyhistidine-tagged StxA1-hCD4 recombinant protein was eluted with a 30-ml gradient of 0 to 0.5 M imidazole in wash buffer. The imidazole displaced the recombinant protein by competing for the binding to the nickel-nitrilotriacetic acid resin. The eluted protein was collected in a single fraction and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the cytotoxicity assay, and an in vitro translation assay.

In vitro assessment of enzymatic activity. The enzymatic activities of the wild-type STX encoded by the recombinant plasmid pNAS10 (28) (Table 1) and of the StxA1-hCD4 hybrid toxin were measured with a rabbit reticulocyte lysate translation assay programmed with firefly luciferase mRNA. Six microliters of the toxin-containing periplasmic extracts was incubated with 30 μl of the rabbit reticulocyte lysate at 37°C for 30 min. Following this incubation, the translation assay was performed as described by the supplier (Promega, Madison, Wis.). The capacities of the toxins to inhibit protein synthesis were measured with the luciferase enzyme assay. Ten microliters of the reaction mixture was combined with 1 ml of deionized water, and light production was measured with a scintillation counter. Enzymatic activity was measured as a reduction in luciferase production and expressed as a percentage of the activity expressed by wild-type STX.

MTT cytotoxicity assay. The number of viable 8E5 cells which had been exposed to the StxA1-hCD4 chimera or viable Vero cells exposed to STX was measured by using 3-(4,5-dimethylthiazole)-2,5-diphenyltetrazolium bromide thiazole blue (MTT) (Sigma), an indicator of oxidative metabolism (20). One hundred eighty microliters of the appropriate cell line was exposed to serial dilutions of toxin for the times indicated in Results. Twenty microliters of 5-mg/ml MTT in PBS was added to the cells, which were incubated overnight at 37°C in a 5% CO_2 atmosphere. The reaction was terminated with 10% SDS, and the A_{570} was measured with an enzyme-linked immunosorbent assay plate reader. Cytotoxicity data are expressed as the means from at least three separate experiments, and statistically significant differences between samples were assessed by Student's *t* test with a *P* value of ≤0.01.

Transfection of Cos-1 cells with pwtEnv. Cos-1 cells were transfected with the recombinant plasmid pwtEnv, which encodes the HIV-1 envelope glycoprotein gp160 and the regulatory protein Tat (23), by the lipofectamine procedure according to the manufacturer's protocol (Gibco, Gaithersburg, Md.). Briefly, cells grown to 80% confluence in 35-mm-diameter tissue culture plates were overlaid with a mixture of 5 μg of pwtEnv DNA and 10 μl of lipofectamine in 800 μl of serum-free MEM. The Cos-1 cells were incubated with the DNA-liposome complexes for 5 h before feeding with 1 ml of MEM containing 20% fetal bovine serum and overnight incubation at 37°C in a 5% CO_2 atmosphere. The cell culture medium was replaced with complete MEM on the following day, and the efficiency of transfection was measured after 48 h by the multinuclear activation of galactosidase indicator (MAGI) assay described by Kimpton and Emerman (15).

The MAGI assay consisted of cocultivating the transfected Cos-1 cells with a HeLa-T4-β-gal cell line which carries the *lacZ* gene regulated by the HIV-1 long terminal repeat promoter. Expression of HIV-1 glycoproteins gp41 and gp120 by the transfected cells induced syncytium formation with the HeLa-T4-β-gal cells. Because Cos-1 cells carrying pwtEnv express a modified form of the HIV-1 Tat protein which accumulates in the nuclei of the syncytia, it transactivated the HIV-1 long terminal repeat and induced expression of LacZ, which can be visualized. Cells were fixed in 0.5% paraformaldehyde for 5 min at room temperature, washed twice with PBS, overlaid with 1 ml of the staining solution (0.2 M potassium ferrocyanide, 0.2 M potassium ferricyanide, 2.0 M magnesium chloride, 400 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml in PBS), and incubated at 37°C for 50 min. Syncytia (blue-stained cells) were counted under the light microscope at a magnification of ×100 and used as an indicator of transfection efficiency.

The MTT cytotoxicity assay was used to measure the number of viable Cos-1 and Cos-1–Env cells following exposure to the StxA1-hCD4 chimera for at least 24 h.

Protection assays with metabolic inhibitors. The metabolic inhibitors (Sigma) used in this study were 50 μg of chloroquine and 800 μg of ammonium chloride (lysosomal stabilizers) per ml, 500 ng of cytochalasin B (microfilament inhibitor) per ml, and 200 ng of brefeldin A (Golgi complex disrupter) per ml. 8E5 and Vero cells grown on 96-well plates were treated with the metabolic inhibitors at their highest noncytotoxic concentration and incubated for 30 min at 37°C in a 5% CO_2 atmosphere. The simultaneous addition of the inhibitors at the time of intoxication offered no protection. STX or the StxA1-hCD4 chimera was added to the treated cells and incubated for 48 h. The effects of the inhibitors on the uptake and translocation of the toxins were determined with the MTT cytotoxicity assay.

Golgi staining. The vital stain *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocarproyl sphingosine (C_6-NBD ceramide) accumulates in the Golgi complex, where it is converted to sphingomyelin and the fluorescent compound glucosyl cerebroside. C_6-NBD ceramide was solubilized with dimethyl sulfoxide (1 mg/ml), desiccated, and complexed to defatted bovine serum albumin as described by Lipsky and Pagano (17). Env-expressing Cos-1 cells were treated with brefeldin A overnight at 37°C in a 5% CO_2, washed twice with PBS, and incubated with C_6-NBD ceramide at 37°C for 10 min. The cells were then washed three times with PBS, incubated in 18 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer–MEM at 37°C for 30 min, observed by fluorescence microscopy, and photographed with color film. The Golgi complex appeared as a green fluorescent pattern surrounding the cell nucleus.

Autoradiographic assessment of intracellular localization. Cos-1 cells transfected with pwtEnv and Vero cells were grown on slides and treated with ^{35}S-labeled StxA1-hCD4 and STX, respectively, for 4 h at 37°C in a CO_2 atmosphere. The intoxicated cells were fixed with 0.5% paraformaldehyde and immersed in an autoradiographic emulsion (Kodak, Rochester, N.Y.). The slides were developed after 15 days at −20°C, and the cellular distribution of silver grains was visualized by light microscopy.

RESULTS

Purification of StxA1-hCD4 chimera with pET system. Because the level of StxA1-hCD4 hybrid protein expression by the recombinant plasmid pFUS60-CD4 (1) was low, we introduced the gene fusion encoding the chimera into the pET24d vector (Novagen), which expresses proteins with a C-terminal polyhistidine tag from the T7 phage promoter (29). This system permits the overexpression of proteins, which may then be easily purified by nickel-Sepharose chromatography.

The recombinant plasmid pAA58 (Table 1) was introduced into the *E. coli* host BL21(DE3), which contains a chromosomal copy of the T7 RNA polymerase gene under control of the IPTG-inducible *lacUV5* promoter. The chimeric protein was collected in the periplasmic extracts of BL21(DE3) (pAA58) and visualized by SDS-PAGE with Coomassie blue staining. Substantial quantities of a protein corresponding to

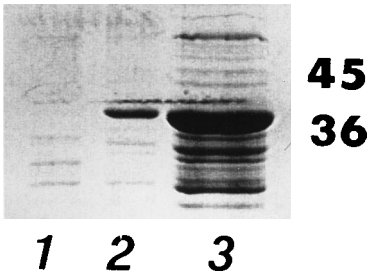

FIG. 1. SDS-PAGE analysis of periplasmic extracts from IPTG-induced BL21(DE3)(pET24d) (lane 1), uninduced BL21(DE3)(pAA58) (lane 2), and IPTG-induced BL21(DE3)(pAA58) (lane 3). The positions of two molecular mass markers are shown on the right (in kilodaltons).

the predicted molecular mass (42 kDa) of the StxA1-hCD4 chimera were observed upon IPTG induction (Fig. 1, lane 3). As expected, the chimera was not detected in extracts of the IPTG-induced vector control (Fig. 1, lane 1). Although the pET expression system is tightly regulated, the StxA1-hCD4 hybrid toxin was observed in the uninduced fraction (Fig. 1, lane 2).

Following passage of the periplasmic extracts from BL21 (DE3)(pAA58) through a nickel-Sepharose column, the purified StxA1-hCD4 fusion protein (molecular mass, 42 kDa) was eluted by using an acidic buffer (Fig. 2, lane 2). The StxA1-hCD4 hybrid toxin was not detected in column fractions collected prior to elution (Fig. 2, lane 1), demonstrating the efficiency of this purification system.

The addition of six histidine residues to the C terminus of the StxA1-hCD4 chimera did not abolish enzymatic activity or cytotoxicity. Purified StxA1-hCD4 reduced the enzymatic activity in a reticulocyte lysate by 83%, while a periplasmic extract containing STX caused a 100% reduction. A greater than 80% reduction in the 8E5 population was caused by treatment with 7 μg of the periplasmic proteins shown in Fig. 1, lane 3, while 500 ng (60 nM) of the purified StxA1-CD4 chimera shown in Fig. 2, lane 2 had the same cytotoxic effect. On the basis of these results, 500 ng (60 nM) of the purified StxA1-CD4 chimera was used in the remaining cytotoxicity assays. The specific activity of STX could not be evaluated because it was not purified with the pET system.

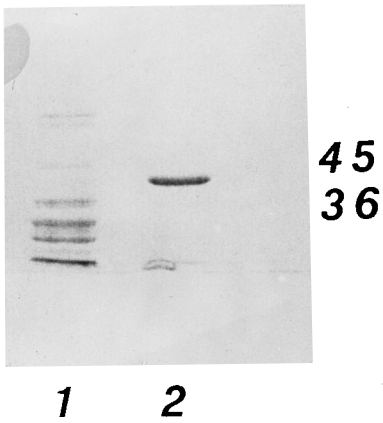

FIG. 2. Purification of periplasmic extract from BL21(DE3)(pAA58) by nickel-Sepharose chromatography. Lane 1, pooled column fractions collected prior to elution; lane 2, column fraction containing eluted StxA1-hCD4 hybrid protein. The positions of two molecular mass markers are shown on the right (in kilodaltons).

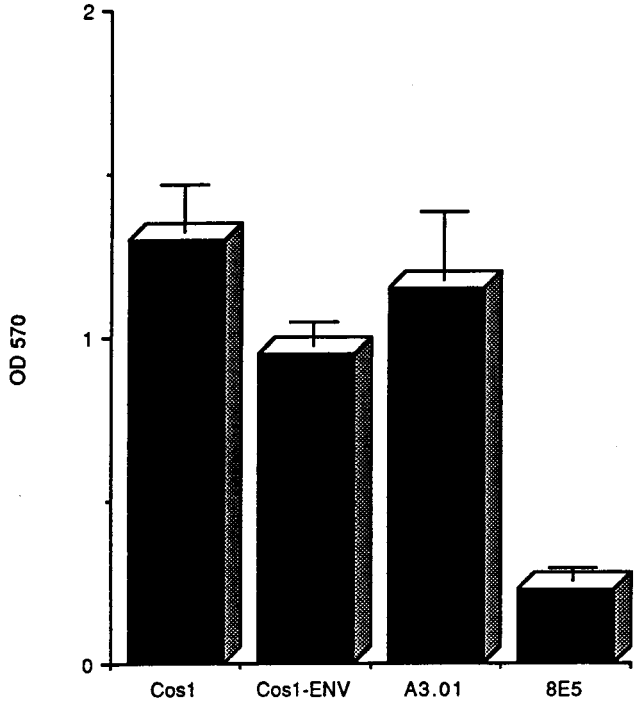

FIG. 3. Relative susceptibilities to the StxA1-hCD4 chimera of Cos-1 cells expressing HIV-1 Env proteins and of 8E5 cells. Cos-1 and A3.01 are the Env^- and uninfected parental cell lines of Cos-1 Env^+ (Cos1-Env) and 8E5, respectively. OD 570, optical density at 570 nm.

Cytotoxicity to HIV-1 *env*-transfected Cos-1 cells. We intoxicated Cos-1 cells transfected with the recombinant plasmid pwtEnv (Table 1) (23) to establish the requirement for HIV-1 gp120 and gp41 as receptors for the StxA1-hCD4 chimera. Surface expression of the HIV-1 glycoproteins on the transfected Cos-1 cells was established by using the MAGI assay described in Materials and Methods. Under standard culture conditions, an average of 18% of the pwtEnv-transfected Cos-1 cells expressed HIV-1 glycoproteins on the cell surface. Cytotoxicity was measured with the MTT cell viability assay (20).

The susceptibility of the gp120- and gp41-expressing Cos-1 cells to the toxin chimera was compared with that of the nontransfected cells (Fig. 3). Intoxication with the StxA1-hCD4 chimera caused a 27% reduction in the number of viable pwtEnv-transfected Cos-1 cells compared with the Cos-1 control. In contrast, the toxin chimera reduced the number of viable 8E5 cells by 80% in comparison to the parental T-cell line A3.01, which is not infected with HIV-1. This reduced susceptibility of Env^+ Cos-1 cells in comparison to 8E5 cells is probably due to the relatively low percentage of Cos-1 cells which are expressing gp120 and gp41 in the total transfected cell population.

Kinetics of cell killing. Time courses of cytotoxicity for Vero cells intoxicated with STX and for 8E5 cells intoxicated with the StxA1-hCD4 chimera were determined to compare the kinetics of receptor binding, entry, and translocation for these molecules. Table 2 shows that STX reduced the total number of Vero cells by 67% (3.0×10^5 to 1.0×10^5 cells per ml) following a 6-h intoxication and caused a 93% reduction (3.0×10^5 to 2.0×10^4 cells per ml) after 12 h. In contrast, the StxA1-hCD4 fusion protein intoxicated 8E5 cells at a lower rate. The chimera caused a 30% reduction (2.3×10^6 to 1.6×10^6 cells per ml) in viable cell counts in 6 h and an 84% reduction (2.3×10^6 to 3.6×10^5) in the number of HIV-

TABLE 2. Time course of cell killing[a]

Time (h)	10^4 Mean no. of cells (SE)	
	STX treatment	StxA1-hCD4 treatment
0	30 (3.0)	230 (10)
6	10 (2.0)	160 (14)
12	2 (0.2)	36 (2)
18	<1	4 (1)
24	<1	<1
36	<1	<1

[a] Cell numbers were measured by trypan blue staining. STX was assessed with Vero cells; StxA1-hCD4 was assessed with 8E5 cells.

infected cells sampled 12 h after the addition of toxin (Table 2). Both STX and the StxA1-hCD4 hybrid toxin reduced the total population of susceptible cells to undetectable levels following 24 h of incubation (Table 2).

Although different cell lines were used to assess the time courses of cell killing by STX and the StxA1-hCD4 chimera, the findings shown in Table 2 indicate that the binding, entry, and translocation kinetics of the hybrid toxin were slower than those of native STX.

Effect of metabolic inhibitors on intoxication. Vero cells and 8E5 cells were treated with the highest nonlethal concentrations of metabolic inhibitors which disrupt cellular processes involved in ligand entry and translocation prior to intoxication with STX and the StxA1-hCD4 chimera. The metabolic inhibitors used in this study were (i) ammonium chloride and chloroquine, which neutralize endosomes and subsequently inhibit toxin entry by receptor-mediated endocytosis; (ii) cytochalasin B, which inhibits microfilament movement required for receptor-ligand trafficking; and (iii) brefeldin A, which disrupts the Golgi apparatus.

Vero cells incubated with sublethal concentrations of ammonium chloride (800 μg/ml), cytochalasin B (500 ng/ml), and brefeldin A (200 ng/ml) prior to treatment with STX were partially protected from intoxication (30% restoration of cell viability [Fig. 4, upper panel]). These findings support the previous reports suggesting that STX is internalized through coated pits by receptor-mediated endocytosis and is transported to the Golgi complex (24, 25). 8E5 cells were also partially protected from intoxication by the StxA1-hCD4 fusion protein when they were treated with cytochalasin B and brefeldin A but not when they were treated with ammonium chloride (50% restoration of cell viability [Fig. 4, lower panel]). Neither 8E5 nor Vero cells were protected by 50 μg of chloroquine per ml.

Because cells treated with brefeldin A were protected from intoxication by native STX and the StxA1-hCD4 chimera, it seems likely that toxin translocation to the Golgi apparatus is an essential step for intoxication, considering the effect of brefeldin A on this organelle (31). Therefore, we visualized disruption of the Golgi complex by brefeldin A by using the Golgi stain C_6-NBD ceramide (17). Prominent green fluorescent staining of the Golgi apparatus surrounding the dark-centered nucleus was seen in cells treated with C_6-NBD ceramide. However, the pattern of fluorescence staining in cells treated with brefeldin A was dispersed, obscuring the cell nucleus (data not shown).

Autoradiography. Vero cells and Env^+ Cos-1 cells were treated with ^{35}S-labeled STX and the StxA1-hCD4 chimera, respectively. Intoxicated cells were fixed on slides and immersed in a radiographic emulsion to permit visualization by light microscopy. Examination of the intoxicated cells revealed high concentrations of both labeled STX and the StxA1-hCD4 chimera diffused throughout the whole cells. There were no distinct foci or significant differences between the autoradiographic patterns of the two toxins (data not shown).

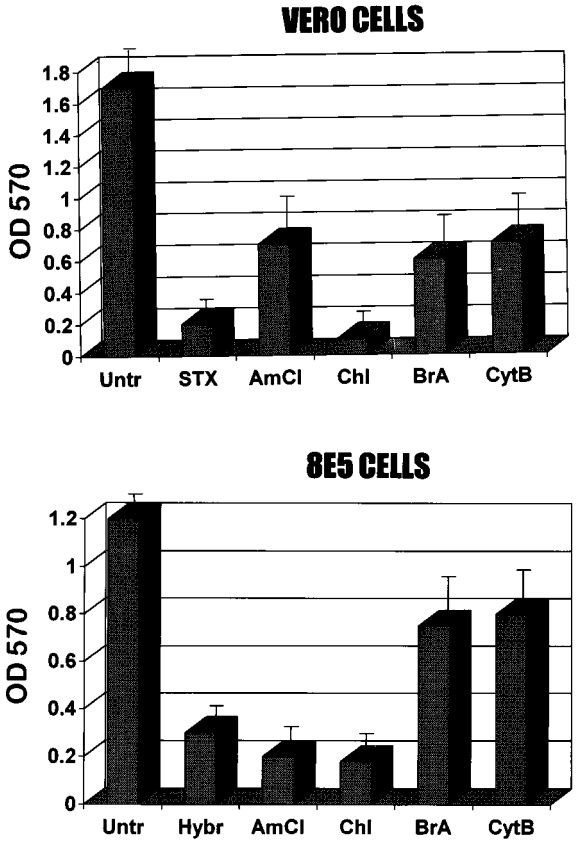

FIG. 4. Protection studies using sublethal concentrations of ammonium chloride (AmCl), chloroquine (Chl), brefeldin A (BrA), and cytochalasin B (CytB). Vero cells were intoxicated with STX, and 8E5 cells were intoxicated with the StxA1-hCD4 chimera (Hybr). Untr, untreated; OD 570, optical density at 570 nm.

DISCUSSION

The StxA1-hCD4 chimera was overexpressed by using the pET system, which fuses six histidine residues to its C terminus. The specific activity of the hybrid toxin was increased in comparison to that of crude periplasmic extracts following purification by nickel-Sepharose chromatography. These findings indicate that addition of the C-terminal polyhistidine tag to the StxA1-hCD4 chimera did not abolish activity and permitted preliminary characterization of the receptor binding and cytosolic trafficking processes.

Transfection of otherwise toxin-resistant Cos-1 cells with an Env-expressing recombinant plasmid rendered these cells susceptible to killing, establishing the requirement for gp120 and/or gp41 as a receptor(s) for the StxA1-hCD4 chimera. However, the transfected Cos-1 cells were not as susceptible to the hybrid toxin as were the 8E5 cells, although autoradiography revealed high concentrations of the ^{35}S-labeled chimera bound to individual cells. There are two possible explanations for these disparate susceptibilities: (i) only a small portion of the entire Cos-1 cell population was efficiently transfected and was expressing the HIV glycoprotein receptor, or (ii) expression of hCD4 on the target cell surface may promote internal-

ization of the chimera-receptor complex through oligomerization of the hCD4 molecules, and therefore, Cos-1 cells were less susceptible because they are $hCD4^-$. While the MAGI assay supported the hypothesis that the transfected Cos-1 cells were less susceptible to intoxication because of low numbers of receptor-bearing cells, we are currently assessing the susceptibilities of $hCD4^+$ and $hCD4^-$ cells expressing gp120 and gp41 to the StxA1-hCD4 chimera.

Our cytotoxicity experiments demonstrated that the kinetics of cell killing by the StxA1-hCD4 hybrid toxin were slower than those by STX. However, because it was necessary to use two distinct cell lines, 8E5 for StxA1-hCD4 and Vero for STX, we cannot rule out possible differences in receptor cycling efficiencies, translocation rates, and receptor-ligand affinities as explanations for the lower intoxication rate of the chimera. Therefore, reagents which disrupt lysosome-endosome fusion, microfilament movement, and the integrity of the Golgi apparatus were also used to evaluate the entry and translocation pathway of the StxA1-hCD4 hybrid toxin.

Chloroquine and ammonium chloride inhibit receptor-mediated endocytosis by increasing the endosomal pH, thereby preventing dissociation of receptor-ligand complexes and receptor reuse (21). In this study, treatment of 8E5 cells with these reagents had no effect on cytotoxicity, indicating that the StxA1-hCD4 hybrid toxin enters target cells in a pH-independent manner. In contrast, ammonium chloride protected Vero cells from STX, which is endocytosed by receptor-mediated endocytosis through coated pits (24). Cytochalasin B protected cells from STX and the StxA1-hCD4 chimera, demonstrating the essential role for microfilament movement in receptor-ligand uptake and translocation. These chemical protection experiments support our hypothesis that the StxA1-hCD4 hybrid toxin is internalized into HIV-infected cells by means of glycoprotein cycling. Many membrane-associated viral glycoproteins cycle between the cell surface and the endosomal compartment (13). Therefore, gp120 may provide a productive route for the StxA1-hCD4 chimera to enter HIV-1-infected cells through this cycling process.

Translocation to the Golgi complex appears to be a common pathway for toxins internalized by receptor-mediated endocytosis (25). Our brefeldin A protection experiments and C_6-NBD ceramide staining results suggested that hCD4-containing hybrid toxins are also translocated to the Golgi complex. However, in contrast to the ease for other naturally occurring toxins such as STX, the StxA1-hCD4 chimera contains no known amino acid sequence subject to proteolytic nicking and reduction, which permit release of the enzymatic subunit. Perhaps a cryptic processing site exists in the chimera and the STX A1 polypeptide is released at the Golgi apparatus. We are currently constructing additional StxA1-hCD4 fusion proteins containing an endoplasmic reticulum retention signal (22) and an internal HIV protease site (26) in an attempt to enhance the cytotoxic capacity of the chimera. These modified hybrid toxins and more-refined studies of the StxA1-hCD4 chimera translocation pathway with electron microscopy may foster the development of therapeutic reagents with high specificities and potencies.

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