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Enhancement of toxicity of antitransferrin receptor antibody–*Pseudomonas* exotoxin conjugates by adenovirus

(toxins/endocytosis/immunotherapy)

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ABSTRACT **Cytotoxic conjugates were constructed by chemically coupling *Pseudomonas* exotoxin to antitransferrin receptor antibodies. Toxicity of these conjugates, due to entry via the transferrin receptor, was enhanced 100- to 300-fold in the presence of adenovirus. By electron microscopy and immunofluorescence it was determined that antitransferrin receptor antibodies and the conjugates derived from them entered cells from coated pits into receptosomes. We believe that the enhanced toxicity resulted when adenovirus and the toxin conjugates were internalized into the same receptosomes. In the process of infection, adenovirus enters cells and brings about a virus-mediated disruption of receptosomes; and this disruption can liberate many more toxin molecules into the cytosol than is possible in the absence of virus.**

Many proteins, viruses, and toxins enter cells by receptor-mediated endocytosis (for review, see ref. 1). The morphological and biochemical nature of this internalization pathway has been characterized extensively for ligands such as epidermal growth factor (EGF) (2), α_2-macroglobulin (α_2M) (3), transferrin (4), low density lipoprotein (LDL) (5), triiodothyronine (T_3) (6), asialoglycoproteins (7, 8) and a few viruses (9–11). These ligands bind to cell surface receptors, are concentrated in coated pits, and finally enter cells via an uncoated vesicle we have termed "receptosome" (12). After that, the pathways diverge and various ligands are transported to different intracellular locations. Some, such as α_2M and EGF, are ultimately transferred to lysosomes; transferrin is recycled back to the cell exterior; some toxins and viruses reach the cytosol.

At present, there is great interest in the use of toxin–monoclonal antibody conjugates as potential chemotherapeutic agents (see ref. 13 for a review). Several groups have successfully coupled cytotoxic molecules to monoclonal antibodies that bind to specific cell surface determinants (14–17). However, the pathway by which these cytotoxic antibodies enter and kill cells has not been well characterized.

Pseudomonas exotoxin (PETX) is a protein that enters cells by receptor-mediated endocytosis and is cytotoxic by virtue of its ability to reach the cell cytosol and shut down protein synthesis (18). We recently have shown that human adenovirus type 2 enhances the toxicity of PETX or a hybrid of PETX conjugated to EGF (PETX-EGF) (10). Adenovirus enters KB cells by receptor-mediated endocytosis and is cointernalized with either PETX or PETX-EGF. When adenovirus escapes into the cytosol, it appears to disrupt the receptosome, thereby releasing the contents of this vesicle into the cytosol. The enhanced toxicity occurs because the toxin now has ready access to its target, EF-2.

In this paper we describe the ability of adenovirus to enhance the toxicity of a toxin–antibody conjugate. We have chosen to study a conjugate of PETX with an antibody to the transferrin receptor because this antibody is known to form cytotoxic conjugates (14).

MATERIALS AND METHODS

Cells. Cells were maintained as monolayers in Dulbecco modified Eagle medium (DME medium; GIBCO) supplemented with 10% calf serum (KB cells) or 10% fetal bovine serum (Swiss 3T3 cells) and penicillin (50 units/ml) and streptomycin (50 μg/ml). Routinely, cells were washed with DME medium containing 2 mg of bovine serum albumin per ml (DME-alb. medium) 1–2 hr before the addition of PETX or a PETX conjugate. HUT-102 cells were the generous gift of T. A. Waldmann (National Cancer Institute).

Virus. Human adenovirus type 2 was propagated in KB cells grown in suspension culture and purified as described (10, 19).

Toxin. Purified PETX was the generous gift of S. Leppla (Ft. Detrick, MD).

Monoclonal Antibodies Against the Transferrin Receptor. B3/25 was prepared as described (14). HB21 was obtained from the American Type Culture Collection, propagated as ascites in BALB/c mice, and purified by precipitation at ammonium sulfate 50% saturation and affinity chromatography on a column containing *Staphylococcus aureus* protein A. Both of these monoclonal antibodies are of the IgG1 subclass.

PETX Conjugates. PETX–antibody conjugates were constructed by using a disulfide exchange reaction (20). Typically, 2 mg of PETX was treated with 26 mg of methyl-4-mercaptobutyrimidate (Pierce) at 37°C for 2 hr to introduce two thiol groups per molecule of toxin. Derivatized PETX was then treated with 1 mM dithiobis(2-nitrobenzoic acid) (Sigma). Purified antibody (2 mg) was derivatized with 1.3 mg of methyl-4-mercaptobutyrimidate for 10 min at 37°C, which introduced slightly more than one thiol group per molecule. The derivatized antibody was mixed with a 3-fold molar excess of dithiobis(2-nitrobenzoic acid)-treated toxin and allowed to incubate for 2 hr at room temperature.

PETX–antibody molecules were purified by a two-step procedure. Typically, 1 ml of conjugate at 3 mg/ml was passed over a Sepharose 6B column (9 × 59 cm). Large aggregates were in the void volume. These had low activity and were discarded. The material included on the Sepharose 6B column was passed over a 3-ml protein A column at pH 8.0. The flow-through and several washes at pH 8.0 were kept as a separate pool. A second

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Abbreviations: PETX, *Pseudomonas* exotoxin; EGF, epidermal growth factor; α_2M, α_2-macroglobulin; αTFR, antitransferrin receptor; TCD_{50}, tissue culture dose giving 50% inhibition; PETX-EGF, PETX conjugated to EGF; PETX-Cys, PETX coupled to cysteine; DME medium, Dulbecco modified Eagle medium.

pool was obtained by washing the column at pH 6.0, and a third by washing at pH 3.5. Each pool was assayed for ADP-ribosylating activity and for its ability to bind to cells via the transferrin receptor. The second pool contained material that was positive for both activities. The first pool contained mainly unconjugated derivatized PETX; the third pool contained only a small amount of protein. Each PETX molecule was coupled to two antibody molecules. PETX-EGF was synthesized as described (10).

ADP-Ribosylating Activity of Conjugates. ADP-ribosylating activity of PETX and conjugates was assayed by using [^{14}C]NAD as described (10). The coupling of antibody, EGF, or cysteine to PETX in no way diminished its ADP-ribosylating activity (Table 1).

Cell Toxicity Assays. Inhibition of protein synthesis was used as a measure of cell toxicity (10). Adenovirus was assayed for its ability to enhance toxicity by incubating cells with toxin for 1 hr at 37°C in the presence or absence of virus. The medium was then replaced with DME medium containing [^{3}H]leucine for a further 1 hr. Routinely, 1 μg of viral protein per ml, equivalent to 3×10^8 viral particles per ml, was used. At this concentration, adenovirus did not cause any reduction in protein synthesis. The quantity of adenovirus used was expressed as μg of viral protein per ml because UV treatment greatly decreased plaque formation but did not diminish adenovirus-mediated enhancement of toxicity (unpublished data).

Conjugates alone were assayed for toxicity by adding various concentrations of protein to cell monolayers at 37°C for 16–18 hr. At the end of the incubation period, the medium was replaced with DME medium containing [^{3}H]leucine. This protocol was altered slightly for testing PETX-Cys on Swiss 3T3 cells. In this case, PETX or PETX-Cys was added to precooled cells and allowed to bind for 2 hr at 4°C; then the cells were washed and incubated at 37°C for 16–18 hr. Medium containing [^{3}H]leucine was then added for a further 1 hr. All experiments were carried out with duplicate dishes and were repeated at least three or four times.

Immunofluorescence. *Specificity of PETX-HB21 uptake.* Fluorescence experiments were carried out on KB cell monolayers. PETX-HB21 (20 μg/ml) was added to cells at 4°C and allowed to bind for 1 hr. In parallel dishes, PETX-HB21 was added with either excess HB21 antibody (200 μg/ml) or excess native PETX (200 μg/ml). After binding, cells were washed extensively and incubated at 37°C for 10 min. Cells were then fixed in 3.7% formaldehyde and made permeable with 0.1% Triton X-100. The following antibodies, at 50 μg/ml, were then added in sequence in phosphate-buffered saline (containing 0.1% saponin, 1 mM EGTA, and normal goat globulin at 4 mg/ml): affinity-purified rabbit anti-PETX, fluorescein-labeled affinity-purified goat anti-rabbit IgG, and rhodamine-labeled affinity-purified goat anti-mouse IgG. The dishes were washed thoroughly with phosphate-buffered saline between antibody additions.

Cointernalization of PETX-HB21 and EGF. EGF-HRP was used in place of native EGF (2). EGF-HRP (200 nM) was added to cells at 4°C for 1 hr; then the cells were washed and incubated in PETX-HB21 for 1 hr at 4°C. After they were washed in the cold the cells were warmed in DME medium-alb. for 8 min at 37°C. Cells were fixed and treated as above except for the sequence of antibody additions, which was: rhodamine-labeled affinity-purified goat anti-mouse IgG and affinity-purified fluorescein-labeled rabbit anti-HRP.

Electron Microscopy. Colloidal gold (15 nm) coupled to B3/25 was prepared by standard methods as described (11). The colloidal gold was prepared by citrate reduction, and B3/25 was added in 2 mM borate buffer at pH 9.0. Free antibody was separated from the gold conjugate by centrifugation at 20,000 × g. The binding of this conjugate was specific because incubation of the gold conjugate in the presence of excess free B3/25 completely abolished gold labeling (results not shown). For incubations with KB cells, the gold conjugate (≈100 μg of antibody per ml) was added to cells in 35-mm dishes in phosphate-buffered saline with bovine albumin at 2 mg/ml; the mixture was incubated at 4°C for 3.5 hr. After washing, the cells were incubated at 37°C for 1–20 min in serum-free medium prior to fixation in 2% glutaraldehyde for 10 min at 23°C. The cells were then fixed in OsO_4, dehydrated in ethanol, and embedded in Epon 812. Thin sections were prepared with a Sorvall MT-5000 ultramicrotome and a diamond knife and, after lead citrate counterstaining, were viewed with a Hitachi HU-12A electron microscope operated at 50 kV.

Table 1. ADP-ribosylating activity of PETX and PETX conjugates

Toxin	Protein μg/ml	Protein μmol × 10^5/ml	NAD transferred, mmol × 10^{-5}/mol of toxin/30 min
PETX	15	21	0.88
	5	7	1.6
	1	1.4	1.6
PETX-EGF	7	8.4	1.8
PETX-Cys	5	7	1.8
PETX-HB21	5	1.4	2.5
PETX-B3/25	5	1.4	4.4

RESULTS

Toxicity of PETX-αTFR Conjugate. Conjugates of PETX coupled to either the B3/25 antibody or the HB21 antibody were assessed for toxicity on monolayers of KB cells by measuring levels of protein synthesis (Fig. 1). To do this, various concentrations of the toxin conjugates were added to cell monolayers at 37°C and incubated for 16–18 hr. To determine levels of protein synthesis, the toxin-containing medium was replaced with fresh medium containing [^{3}H]leucine at 2 μCi/ml (1 Ci = 3.7×10^{10} Bq) and the amount of [^{3}H]leucine incorporated into protein was determined after a further incubation of 1 hr. For purposes of comparison, the two conjugates were assayed

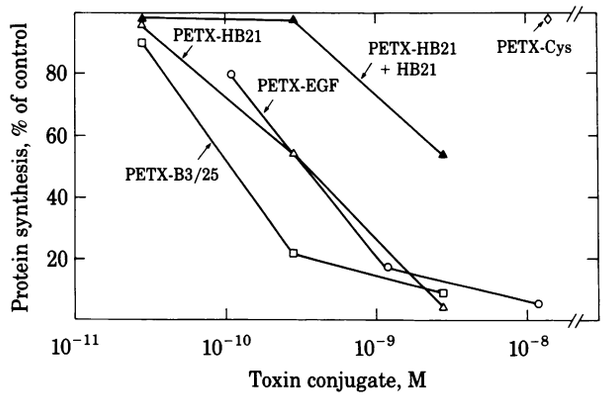

FIG. 1. Inhibition of protein synthesis by PETX conjugates. Various concentrations of hybrid toxins (◇, PETX-Cys; △, PETX-HB21; ▲, PETX-HB21 + 80 nM HB21; □, PETX-B3/25; ○, PETX-EGF) were added to KB cell monolayers incubated for 16–18 hr at 37°C. Toxin-containing medium was then replaced for 1 hr with fresh medium containing [^{3}H]leucine at 2 μCi/ml. Control incorporation into trichloroacetic acid-insoluble material was ≈25,000 cpm per dish.

in parallel with PETX-EGF, a conjugate that was known (10) to inhibit protein synthesis in KB cells. The PETX-HB21 and the PETX-EGF conjugates were of similar potency, with a TCD_{50} of approximately 0.3 nM. PETX-B3/25 was 3-fold more toxic, with a TCD_{50} of 0.1 nM.

The specificity of toxicity of the conjugates with the transferrin antibodies was tested in two ways. In the presence of excess unconjugated antibody (28 nM), the toxicity of PETX-HB21 was decreased to 1/10th the value without the excess (Fig. 1). Presumably, this decrease was due to competition for antibody binding sites. Also, to determine how much activity was due to toxin entry separate from that mediated by antibody binding, PETX-Cys was assayed for its potency on cells. PETX-Cys was 0.1–0.2% as active as PETX-αTFR or PETX-EGF.

Mouse cells are very sensitive to PETX (21). To evaluate the toxicity of PETX-Cys more stringently, Swiss 3T3 mouse fibroblasts were used. PETX-Cys or native PETX was added to Swiss 3T3 cells at 4°C for 2 hr; then the cells were washed extensively, warmed to 37°C, and incubated overnight. PETX-Cys at 0.14 μM was not toxic, whereas PETX at 0.14 nM induced a 40% reduction of protein synthesis. Thus, when a binding step at 4°C was included in the assay, PETX-Cys was no more than 0.1% as active as native PETX.

Toxicity of PETX-αTFR in the Presence of Adenovirus. PETX-αTFR was added to KB cells at 37°C for 1 hr in the presence or absence of human adenovirus type 2. After 1 hr the medium was removed from the cells and replaced with fresh medium containing [^{3}H]leucine at 2 μCi/ml. Adenovirus alone (1 μg of viral protein per ml) did not decrease the level of protein synthesis. During this short assay period, neither PETX-αTFR at 2.8 nM nor PETX-EGF at 12 nM affected protein synthesis (Fig. 2). However, when adenovirus and PETX-αTFR (2.8 nM) were added together, 70–80% inhibition of protein synthesis was noted. In various experiments, adenovirus at 1 μg/ml enhanced the toxicity of PETX-αTFR 100- to 300-fold. The addition of PETX-Cys (14 nM) and adenovirus did not result in a significant reduction in protein synthesis.

The interaction of adenovirus with mammalian cells has been studied most extensively in KB cells. To determine the generality of the adenovirus effect, other human cell lines were assayed for their response to various toxin conjugates in the presence and absence of adenovirus (Table 2). Three other human cell lines were sensitive to viral enhancement of toxicity.

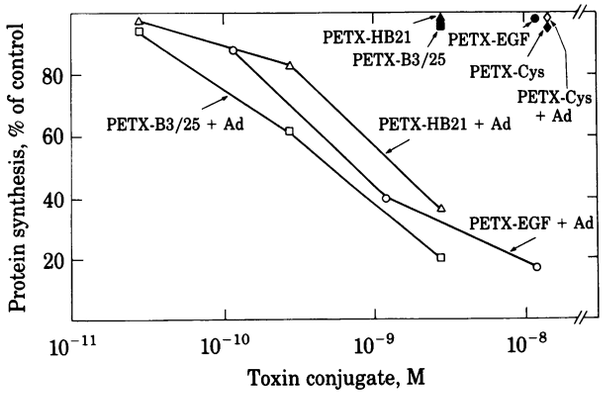

FIG. 2. Enhancement of toxicity by adenovirus (Ad). Various concentrations of hybrid toxins (◇, PETX-Cys + adenovirus; ◆, PETX-Cys; △, PETX-HB21 + adenovirus; ▲, PETX-HB21; □, PETX-B3/25 + adenovirus; ■, PETX-B3/25; ○, PETX-EGF + adenovirus; ●, PETX-EGF) were added to KB cell monolayers incubated for 1 hr at 37°C in the presence or absence of 3×10^8 viral particles per ml. At the end of 1 hr, the medium was replaced for a further hour with fresh medium containing [^{3}H]leucine at 1 μCi/ml.

Two of these were of epithelial origin (A431 and HeLa); the other was a T-cell leukemia line. The degree of enhancement and the amount of the toxin conjugate necessary to decrease protein synthesis to <50% of normal varied from cell line to cell line. The reasons for these variations are not understood but are probably related to such properties as the number of receptors for the conjugate, the number of viral receptors, and the rate of uptake. 3T3, a mouse cell line, did not respond to adenovirus; this is because the adenovirus strain used infects human but not mouse cells.

It had been shown previously that adenovirus disrupts receptosomes, releasing the contents of the vesicle into the cytosol. When the contents of the receptosome included adenovirus and PETX-EGF or PETX, the inhibition of protein synthesis due to these toxins was markedly enhanced. In the present study, adenovirus enhanced the toxicity of PETX-αTFR conjugates which also enter cells in receptosomes (see below).

Trowbridge and Domingo (14) had shown that it was possible to couple ricin A chain chemically to the B3/25 antitransferrin receptor antibody and thus generate a cytotoxic conjugate. We confirmed their finding by using ricin A chain and the HB21 antibody (results not shown). The availability of ricin A-HB21 provided a second, biochemically distinct, toxin to be tested in the system. As with the PETX conjugates, when ricin A-HB21 and adenovirus were added to KB cells, there was enhanced toxicity compared with the addition of the conjugate alone (Table 2).

Detection of Internalized PETX-αTFR by Immunofluorescence. To identify the intracellular location of the toxic conjugate, PETX-αTFR (HB21) at 20 μg/ml was added at 4°C to monolayers of KB cells and allowed to bind for 1 hr. The cells were then washed with DME medium/albumin and warmed to 37°C for 10 min to allow internalization of the bound hybrid toxin. A 10-min incubation was chosen because at this time most ligands internalized by receptor-mediated endocytosis are in receptosomes (1). Cells were fixed, made permeable, and treated with affinity-purified antibodies to locate intracellular PETX-αTFR. When cells were incubated with PETX-αTFR alone, a coincident punctate pattern of brightly labeled receptosomes was seen on both the fluorescein (PETX) and rhodamine (antibody) channels (Fig. 3 *A* and *A'*). In the presence of excess unconjugated antibody (200 μg/ml), the fluorescein signal was abolished (Fig. 3*B*), indicating that entry of the toxin-αTFR conjugate was abolished. Instead, only unconjugated αTFR antibody was detected within cells (Fig. 3*B'*). The added PETX (200 μg/ml) did not compete with the uptake of PETX-αTFR as indicated by a punctate fluorescent pattern on both channels (Fig. 3 *C* and *C'*). These results indicate that PETX-αTFR was internalized when bound to the transferrin receptor and not when bound to the PETX receptor.

The punctate fluorescent pattern of PETX-αTFR indicates that it is in the pattern characteristic of the distribution of re-

Table 2. Enhancement of toxicity in various cell lines by adenovirus

Cell line	Conjugate Kind	Conjugate Conc., μg/ml	% reduction of protein synthesis Without adenovirus	% reduction of protein synthesis With adenovirus
HUT-102	PETX-HB21	1	17	65
HeLa S-3	PETX-EGF	0.01	0	88
A431	PETX-EGF	0.01	20	80
A431	PETX-HB21	1	27	78
Swiss 3T3	PETX-EGF	0.1	58	67
KB	Ricin A-HB21	1	15	86

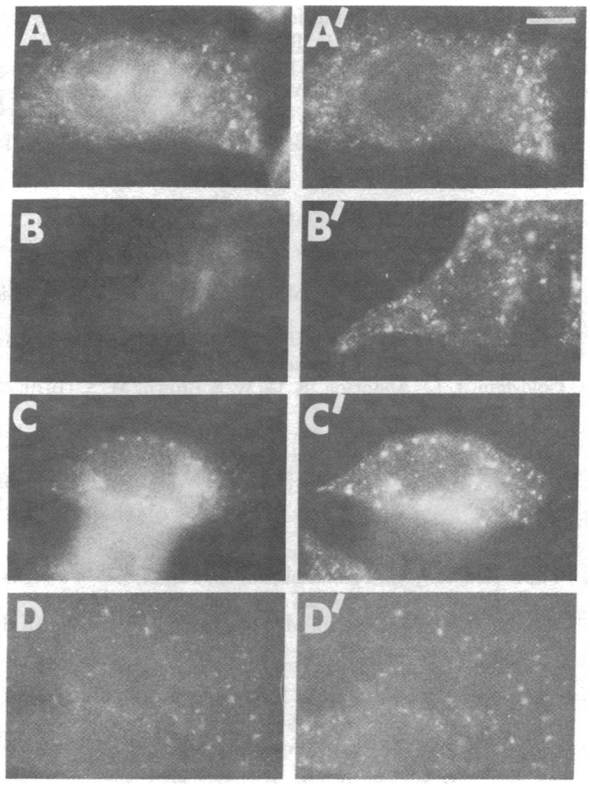

FIG. 3. Immunofluorescence detection of PETX-HB21 internalization. (*A*) PETX-HB21 (20 μg/ml) was added to KB cells at 4°C for 1 hr. The cells were washed and warmed by the addition of DME medium-alb. (37°C) for 10 min. Cells were then fixed and made permeable, and the following affinity-purified antibodies were added in sequence: rabbit anti-PETX, fluorescein-labeled goat anti-rabbit IgG, and rhodamine-labeled goat anti-mouse IgG. (*B*) Same as *A* but with the addition of HB21 antibody at 200 μg/ml. (*C*) Same as *A* but with the addition of native PETX at 200 μg/ml. (*D*) EGF-HRP and PETX-HB21 were bound to the same cells, each for 1 hr at 4°C; then, the cells were washed and warmed to 37°C for 8 min. The cells were fixed and made permeable, and the following affinity-purified antibodies were added: rhodamine-labeled goat-anti-mouse IgG and fluorescein-labeled rabbit anti-HRP. *A–D*, fluorescein channel; *A'–D'*, rhodamine channel (×700; bar = 10 μm).

ceptosomes. To confirm that PETX-αTFR was entering cells in receptosomes, a cointernalization experiment with EGF (EGF-HRP) and PETX-αTFR was carried out. It had been shown (1) that, at 8 min after entry, EGF is found almost exclusively in receptosomes. After an 8 min warm-up EGF and PETX-αTFR were seen as a coincident punctate pattern (Fig. 3 *D* and *D'*), indicating that EGF and PETX-αTFR were internalized in the same receptosomes.

Table 3. Distribution of B3/25-gold particles in various organelle compartments in KB cells

Time after warming to 37°C, min	Distribution, %		
	Plasma membrane	Surface coated pits	Receptosomes
1	63.2	36.5	0.2
5	19.6	7.9	69.0

Total gold particles counted: at 1 min, 734; at 5 min, 1,227. Each time point represents ≈30 cell profiles. "Plasma membrane" includes free cell surface, microvilli, and surface caveolae. Other organelles include lysosomes and transreticular Golgi elements which represented 0% and 3.5% of the total label at 1 and 5 min, respectively. The percentage is calculated from the number of gold particles in each compartment divided by the total number of gold particles seen in all compartments.

Characterization of Antitransferrin Receptor Entry by Electron Microscopy. Monoclonal antibody B3/25 was adsorbed to 15-nm gold particles (αTFR-Au) and its entry into KB cells was examined. To do this, αTFR-Au was added to cell monolayers at 4°C for 3.5 hr; after this, the cells were warmed to 37°C for 1–20 min prior to fixation. After 1 min at 37°C, αTFR-Au was seen often in coated pits; one typical example is shown in Fig. 4*A*. By 5 min after warming to 37°C, αTFR-Au was mostly found in receptosomes; a typical example is shown in Fig. 4*B*. Quantitation of the distribution of this gold conjugate in different compartments is shown in Table 3. More than one-third of the label could be found in coated pits at 1 min after warm-up, the rest being distributed elsewhere on the cell surface. By 5 min after warming, more than two-thirds of the label was found in receptosomes.

DISCUSSION

In the present study we have shown that conjugates of PETX with two different antitransferrin receptor antibodies are toxic for KB cells and that their toxicity is enhanced by the presence of adenovirus. Enhancement of toxicity by adenovirus was shown previously when PETX or PETX-EGF and adenovirus were internalized together in the same receptosome (10). We have provided both biochemical and morphological evidence that αTFR antibodies enter cells via coated pits and receptosomes. The internalization of PETX-αTFR conjugates into receptosomes made them subject to virus-mediated enhancement of toxicity because these conjugates enter in the same vesicles as adenovirus.

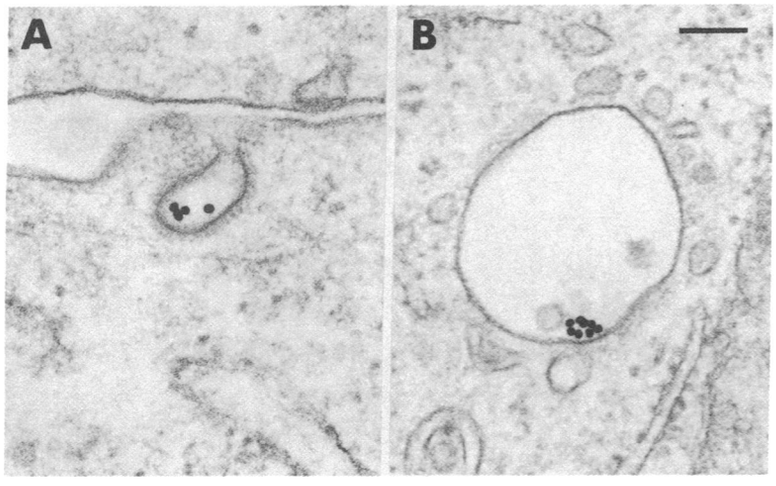

FIG. 4. Electron microscopic detection of the internalization of B3/25 conjugated to colloidal gold. KB cells were incubated with the B3/25–colloidal gold conjugate at 4°C and then warmed to 37°C for 1 min (*A*) or 5 min (*B*). Colloidal labeling can be seen in a cell surface coated pit in *A* at 1 min and in a receptosome (R) at 5 min (*B*). Quantitative data from such images are shown in Table 3. (×90,000; bar = 0.1 μm.)

To carry out these studies it was necessary to couple PETX with antibody molecules to form cytotoxic conjugates. These conjugates enter cells only by antibody recognition properties because treatment of the toxin with methyl-4-mercaptobutyrimidate prevents its binding to the PETX receptor. PETX-αTFR was shown to enter cells via the transferrin receptor because excess antibody, but not excess PETX, competed for the uptake of the toxin–antibody conjugate. Trowbridge and Domingo (14) reported that ricin A chain coupled to the B3/25 antitransferrin receptor antibody is cytotoxic.

To investigate the possible consequences of reduction of the disulfide bond joining PETX to the antibody outside the cell with the release of derivatized toxin, a conjugate was made in which cysteine was substituted for the antibody. PETX-Cys at 140 nM was assayed for toxicity on KB cells in the presence and absence of adenovirus and was found not to inhibit protein synthesis. However, PETX-Cys at 140 nM caused a 35–45% reduction in protein synthesis in the presence, but not in the absence, of adenovirus in a 2-hr assay, and a 40% reduction was noted in the 16-hr assay. To determine what portion of this toxicity was due to binding and entry via the PETX receptor, PETX-Cys was assayed in parallel with native PETX on Swiss 3T3 cells, which have many more PETX receptors than human cells do (21). To provide more stringent conditions for toxin binding and less chance of uptake by fluid-phase endocytosis, a 2-hr binding step at 4°C was used for this comparison. In this type of assay, PETX-Cys proved to be no more than 0.1% as toxic as PETX. The diminished toxicity is due in large part to loss of PETX-binding function after derivatization. The decreased toxicity of PETX-Cys provides further justification for the use of PETX in construction of immunotoxins and other toxin hybrids because the reduction of a PETX hybrid to its component molecules would result in a modified toxin with little toxicity for nontarget tissues.

It has been shown that adenovirus enhances the toxicity of a growth factor–PETX conjugate, an antibody–PETX conjugate, and PETX itself. The active component of adenovirus appears to be a capsid protein. It may be possible to isolate this protein and use it in combination with immunotoxins to construct more effective agents for use in cancer treatment.

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