ABSTRACT

We used recombinant DNA technology to construct a mutant form of *Pseudomonas* exotoxin A (PE) called cysPE35 that contains amino acids 280–364 and 381–613 of PE. cysPE35 begins at the native PE proteolytic cleavage site and contains a single cysteine residue at position 287 that can be used to conjugate the toxin to monoclonal antibodies (MAbs). Unlike immunotoxins containing larger mutant forms of PE, such as PE40 or PE38, immunotoxins containing cysPE35 linked through a disulfide bond do not require proteolysis to generate a toxin fragment able to translocate to the cytosol. cysPE35 was conjugated to several MAbs and their activities were studied in vitro and in vivo. The concentration of toxin that inhibited protein synthesis as measured by a decrease in [3H]leucine incorporation of 50% of [3H]leucine incorporation of 50%, 200 ng/ml. An immunotoxin containing PE38 conjugated through either a disulfide or thioether bond to the MAb HB21 had a concentration of toxin that inhibited protein synthesis as measured by a decrease in [3H]leucine incorporation of 50% of 5 ng/ml, indicating that proteolysis of PE38 may be rate limiting in the action of these immunotoxins. Two other MAbs, LL2 and B3, were also conjugated through a disulfide bond to cysPE35. Both immunotoxins were also more active against cultured cells than conjugates using PE38 or PE40, and caused complete regression of human tumor xenografts growing in nude mice. In conclusion, we have constructed a mutant form of PE which must be coupled to MAbs through a disulfide bond to produce fully active immunotoxins that do not require proteolysis to generate a toxin fragment able to reach the cell cytosol.

INTRODUCTION

PE1 is a three domain bacterial toxin of 66 kDa (1). Domain Ia (amino acids 1–252) mediates cell binding. Domain II (amino acids 253–364) is responsible for translocation into the cytosol and domain III (amino acids 400–613) mediates the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and causes cell death (2). The function of domain Ib (amino acids 365–399) remains undefined and amino acids 365–380 can be deleted without loss of cytotoxic activity (3). Prior to translocation of PE, proteolytic cleavage occurs between arginine 279 and glycine 280 within a disulfide loop bounded by cysteines at 265 and 287, generating a 37-kDa carboxyl-terminal fragment (4). Reduction of the disulfide bond generates the 37-kDa toxin fragment that is translocated to the cytosol (5).

Our group has made several immunotoxins by using mutant forms of PE lacking the native binding domain which are chemically conjugated to MAbs directed at various "tumor-specific" or normal cellular antigens (6–9). Most frequently, the mutant protein PE40 (amino acids 253–613 of PE) or PE38 (amino acids 253–364 and 381–613 of PE) has been coupled to an antibody with the use of a thioether bond which is formed between the MAb and a lysine residue added near the amino-terminus of the PE mutant. In the case of PE40 or PE38, disulfide conjugation does not facilitate intracellular processing because proteolysis between amino acids 279 and 280 is still required to generate a 37-kDa carboxyl-terminal PE fragment that is able to translocate to the cytosol (Fig. 1A).

We recently reported a mutant form of PE, termed PE37, that contains a methionine at position 280 followed by amino acids 281 through 613 of native PE (Ref. 10; Fig. 2). This protein also contains a serine residue at position 287 in place of the native cysteine residue. Otherwise it is identical in sequence to the active cytosolic form of PE and therefore does not require proteolysis to generate a molecule able to translocate to the cytosol to cause cell death. PE37 can be specifically targeted to cells expressing epidermal growth factor receptors by inserting TGFα near its carboxyl terminus (11). When tested on the human epidermoid carcinoma A431 cell line, the ID₅₀ of PE37/TGFα was 0.02 ng/ml (0.46 ps). In comparison, full length PE containing TGFα near the carboxyl terminus and an inactive native binding domain, which requires proteolytic processing to generate an active carboxyl-terminal fragment, had an ID₅₀ of 0.3 ng/ml (4.2 ps; Refs. 10 and 11). These studies indicate that proteolytic processing is a rate-limiting step in the action of PE molecules directed to cells by TGFα.

In the current study we investigated whether the properties of PE37 would enable it to be a useful molecule to conjugate to antibodies to produce potent immunotoxins. We constructed a modified form of PE37, called cysPE35, which has a portion of domain Ib deleted (amino acids 365–380 of PE), and lacks the disulfide bond which connects cysteines at positions 372 and 379. cysPE35 contains a single cysteine residue at position 287, which is used to couple the toxin to MAbs via either a disulfide or thioether bond. The properties of these types of immunotoxins were compared with conjugates made with PE38 or PE40. In the immunotoxins containing cysPE35 coupled to the MAb through a disulfide bond, proteolysis should not be required to liberate a toxin fragment capable of translocating to the cytosol (Fig. 1B). Our results indicate that conjugates linked to cysPE35 through a disulfide bond have increased activity in comparison to conjugates by using either a disulfide or thioether linkage to PE38 or PE40. Furthermore, such conjugates demonstrate potent antitumor activity against appropriate human tumor xenografts growing in nude mice.

MATERIALS AND METHODS

**Cell Lines.** HUT 102 cells were a gift from T. Waldmann (National Cancer Institute). CA46 and Daudi cells were obtained from Dr. I. Magrath (National Cancer Institute). Other cell lines were from the American Type Culture Collection (Rockville, MD).

**Amplification.** Oligonucleotides C9 (5' -GAC TGG CAA GTA ATT GAT ATG-3') and C2 (5' -GGG AAC CAT TGC AGG TCC GCC CCG TGG C-3') were constructed by using a DNA synthesizer (Applied Biosystems). PCR was carried out by using 10 ng of plasmid DNA (see below) as template and reagents as per the manufacturer's
instructions (Gene Amp; Perkin-Elmer/Cetus) in the presence of 5% formamide (Fluka Chemika) and 100 pmol of primers C9 and C2. Each PCR reaction totaled 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 42°C for 90 s, and polymerization at 72°C for 2 min with a 10-s extension in each cycle. The amplified fragments were purified on 1.5% low-melting-point agarose (SeaPlaque, FMC Corp.).

**Bacterial Strains and Plasmids.** HB101 from Bethesda Research Laboratories (Gaithersburg, MD) was used for the propagation of the plasmids. BL21 (ADE3), which carries an inducible T7 RNA polymerase gene on a plasmid (12), was used as the host for protein expression. The plasmid pETD1 has been described (10). It encodes a methionine followed by amino acids 253-613 of native PE, including a cysteine at position 287 (Fig. 2). Plasmid pETD1 was made by the insertion of the 0.4 kilobase digested PCR fragment (amplified by using C9 and C2) into a dephosphorylated 3.6-kilobase fragment of plasmid DF1. It was confirmed by DNA sequencing, plasmid pETD1 encodes a protein termed cysPE35 that contains a methionine followed by amino acids 281–613 of native PE, including a cysteine at position 287 (Fig. 2).

**Expression of PE Mutants.** Expression of plasmids encoding Pseudomonas exotoxin mutants was done by using the host BL21 (ADE3) as described previously (14–16). Cells were incubated for 90 min following induction with isopropyl-β-D-thiogalactopyranoside, harvested by centrifugation, and then isopropyl-19-D-thiogalactopyranoside, harvested by centrifugation, and then incubated at 37°C for 30 min. Protein was separated from unreacted cross-linker on Sephadex G-25 (PD-10; Pharmacia). Derivatization typically introduced 0.5 mol of thiol per mol of toxin as measured by using Ellman’s reagent (23). In separate reactions, NlysPE38 or lysPE40 (6–13 mg/ml) in 0.2 M sodium phosphate (pH 7.0) containing 1 mg EDTA was derivatized with a 3-fold molar excess of SMCC (Pierce) and incubated at 22°C for 1 h. Protein was separated from reactant on Sephadex G-25. Derivatization typically introduced 0.5 reactive group per mol of toxin (24). cysPE35 was stored in 0.2 M sodium phosphate (pH 7.0) containing 1 mg EDTA and 50 mg DTT and was separated from DTT on Sephadex G-25 prior to coupling to antibody. The MAbs B3 and HB21 (4–8 mg/ml) in 0.2 M sodium phosphate (pH 7.0) containing 1 mg EDTA were reacted with a 2- and 4-fold molar excess of SMCC, respectively, and incubated at 22°C for 1 h. Derivatized MAbs was separated from reactant by using Sephadex G-25. The MAbs B3 and HB21 had 0.83 and 1.0 reactive groups measured per molecule, respectively, under these conditions (24). The MAbs B3 and HB21 (4–5 mg/ml) in 0.2 M sodium phosphate (pH 8.0) containing 1 mg EDTA was also reacted with a 2- or 3-fold molar excess of SPDP (Pierce), respectively, and incubated at 22°C for 30 min. The MAbs B3 and HB21 had 0.79 and 0.56 reactive groups measured per molecule, respectively, under these conditions (25). The MAbs LL2 and anti-Tac (6 mg/ml) in PBS containing 1 mg EDTA were reacted with a 2- to 3-fold molar excess of SPPD (Pierce), respectively, and incubated at 22°C for 30 min. The MAbs LL2 and anti-Tac (6 mg/ml) in PBS containing 1 mg EDTA were reacted with a 2- to 3-fold molar excess of SMCC and, respectively, and incubated at 22°C for 1 h. Derivatized MAbs was separated from reactant by using Sephadex G-25. The MAbs B3 and HB21 had 0.83 and 1.0 reactive groups measured per molecule, respectively, under these conditions (25). The MAbs LL2 and anti-Tac (6 mg/ml) in PBS containing 1 mg EDTA were reacted with a 2- to 3-fold molar excess of SPPD (Pierce), respectively, and incubated at 22°C for 30 min. The MAbs B3 and HB21 had 0.79 and 0.56 reactive groups measured per molecule, respectively, under these conditions (25). The MAbs LL2 and anti-Tac (6 mg/ml) in PBS containing 1 mg EDTA were reacted with a 2- to 3-fold molar excess of SMCC and, respectively, and incubated at 22°C for 1 h. 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**Construction of Immunotoxins.** NlysPE38 or lysPE40 (6–13 mg/ml) in 0.2 M sodium phosphate (pH 8.0) containing 1 mg EDTA and 50 mg DTT and was separated from DTT on Sephadex G-25 prior to coupling to antibody. The MAbs B3 and HB21 (4–8 mg/ml) in 0.2 M sodium phosphate (pH 7.0) containing 1 mg EDTA were reacted with a 2- and 4-fold molar excess of SMCC, respectively, and incubated at 22°C for 1 h. Derivatized MAbs was separated from reactant by using Sephadex G-25. The MAbs B3 and HB21 had 0.83 and 1.0 reactive groups measured per molecule, respectively, under these conditions (24). The MAbs B3 and HB21 (4–5 mg/ml) in 0.2 M sodium phosphate (pH 8.0) containing 1 mg EDTA were also reacted with a 2- or 3-fold molar excess of SPPD (Pierce), respectively, and incubated at 22°C for 30 min. The MAbs B3 and HB21 had 0.79 and 0.56 reactive groups measured per molecule, respectively, under these conditions (25). The MAbs LL2 and anti-Tac (6 mg/ml) in PBS containing 1 mg EDTA were reacted with a 2- to 3-fold molar excess of SPPD for 60 min at 37°C. Derivatized MAbs was separated from reactant by using Sephadex G-25. The MAbs B3, HB21, LL2, or anti-Tac derivatized with either SPPD or SMCC was reacted with a 2- to 3-fold molar excess of NlysPE38 or lysPE40 (that had been derivatized with 2-iminothiolane) or cysPE35 for 16 h at 22°C. Reactions were terminated by the addition of iodoacetamide (Sigma) to a final concentration of 1 mM. In addition, the MAbs LL2 and B3 were derivatized by using 2-iminothiolane. The MAbs LL2 or B3 (5–10 mg/ml) in 0.2 M sodium phosphate (pH 8.0) was reacted with a 2-fold molar excess of 2-iminothiolane at 37°C for 1 h. Derivatized antibody was separated from reactant by using Sephadex G-25. The MAbs B3 had 1.0 reactive group introduced under these conditions (23). The MAbs B3 was mixed with cysPE35 that had been derivatized with Ellman’s reagent (Pierce) as described (26). After a 2-h incubation at 22°C, the reaction was terminated by the addition of cysteine (Pierce) to a final concentration of 0.2 mM. Likewise, the MAbs LL2 and B3 that had been derivatized with 2-iminothiolane were reacted for 2 h at 22°C.
with NlysPE38 or lysPE40 that had been derivatized with SMCC. The reactions were terminated by the addition of iodoacetamide to a final concentration of 1 mM. Immunotoxins were purified by sequential use of Mono Q and TSK-250 columns using fast protein liquid chromatography as described (27).

ADP-Ribosylation Assay. ADP-ribosylation activity of protein samples was measured by the procedure of Collier and Kandel with the use of wheat germ extract enriched in elongation factor 2 (28).

Protein Synthesis Inhibition Assay. Inhibition of protein synthesis was carried out as described (29). Cells were plated 24 h prior to toxin addition at 15,000 cells/well in 96-well plates. Immunotoxins or controls, diluted in PBS containing 0.2% bovine serum albumin, were added to a final volume of 200 μl/well. After incubation at 37°C for 20 h, each well was pulsed for 2 h with 1 μCi/well of [3H]leucine (Amersham). After freezing and thawing, the cells were harvested on glass fiber filters and the incorporation of radioactivity into protein was quantitated by a Betaplate (Pharmacia, LKB) scintillation counter. The suspension cell lines CA46, Daudi, and HUT 102 were added at the same time as toxins or controls to 96-well plates. Each 100-μl well contained 10,000 cells. After incubation for 24 h, cells were pulsed for 2 to 4 h with 1 μCi/well of [3H]leucine, and [3H]leucine incorporation was determined as above. Results were calculated as a percentage of incorporated [3H]leucine of cells incubated without toxin. Competition assays were done by using immunotoxin added to cells in the presence of the respective MAb. All assays were performed in triplicate. Data shown are representative of several individual assays.

Clearance Modeling. Serum immunotoxin concentration versus time data were evaluated by an exponential curve fitting program, RSTRIP (version 5, MicroMath Scientific Software, Salt Lake City, UT), as described (30).

LD50 for B3-S-S-PE35 in Mice. Four- to 6-week-old 18-g female BALB/c mice were given injections tail vein of increasing doses of B3-S-S-PE35. The number of dead animals was determined daily for 7 days following immunotoxin administration.

Serum Levels of B3-S-S-PE35 in Mice. Six 18-g BALB/c mice were given 5 μg of B3-S-S-PE35 by tail vein. Blood from the orbital sinus was drawn 5, 30, 60, 120, 240, and 1440 min later. Blood was taken no more than three times from any one mouse and all mice resumed normal activity between and after blood draws. The level of B3-S-S-PE35 was assayed by incubating mouse serum with A431 cells and measuring its effects on protein synthesis. A standard protein synthesis inhibition curve was constructed by using purified B3-S-S-PE35 diluted in control mouse serum (31). Antitumor Activity of B3-S-S-PE35 in Nude Mice Bearing Human Epidermoid Carcinomas. A431 cells (2 × 10⁸) were injected s.c. on day 0 into 4- to 6-week-old female nude mice, and tumors approximately 60 mm³ in size developed reproducibly in all mice by day 5. Mice were given a single dose of 25 μg of B3-S-S-PE35 diluted in PBS containing 0.2% HSA, or an equimolar concentration of MAb B3 or cysPE35, or PBS containing 0.2% HSA. Each treatment group consisted of three to seven animals. Tumors were measured with caliper every 2 days and the tumor volume was calculated as:

\[ \text{Tumor volume} = 0.4 \times \text{length} \times (\text{width})^2 \]

Antitumor Activity of LL2-S-S-cysPE35 in Nude Mice Bearing Human Burkitt's Lymphomas. CA46 cells (1 × 10⁷) or Daudi cells (1.5 × 10⁷) were injected s.c. into 4- to 6-week-old female nude mice 3 days after receiving 400 rads of total body irradiation. Tumors of 100 mm³ (CA46) or 50 mm³ (Daudi) developed after 6 or 7 days, respectively, in about one-half of the animals. Mice bearing tumors of similar size were selected and randomized into groups of four or five mice and administered LL2-S-S-PE35 or controls i.v. by tail vein daily for 4 days. Tumor volume was calculated as described above.

RESULTS

Construction of a 35-kDa Carboxyl-terminal Fragment of PE. We sought to determine whether a 35-kDa carboxyl-terminal fragment of PE that does not require proteolysis prior to translocation could be conjugated to MAbs to produce potent immunotoxins. To make cysPE35, the plasmid CT11 was constructed by using plasmid DF1, which encodes PE37 (see “Materials and Methods”). Plasmid CT11 contains a T7 promoter and encodes a methionine followed by amino acids 281–364 and 381–613 of native PE. The protein cysPE35 encoded by this plasmid has a single cysteine residue at position 287 (Fig. 2).

cysPE35 was expressed in BL21 (ADE3) and was found to be equally distributed between the periplasm and spheroplasts (data not shown). Purification of the protein from periplasm to >95% homogeneity was accomplished by using anion exchange, chelation, and size exclusion chromatography (see “Materials and Methods”). cysPE35 had the expected molecular mass on SDS-PAGE (35 kDa; Fig. 3) and was reactive with anti-PE rabbit sera (data not shown). The ADP-ribosylation activity of cysPE35 and activated PE were identical. The number of thiol groups in cysPE35 was found to be 1 mol/mol of protein when measured with Ellman’s reagent (23). When tested on a number of cell lines, cysPE35 had very little cytotoxic activity because it could not bind specifically to target cells (Table 1).

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**Plasmid**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF1</td>
<td>PE37</td>
</tr>
<tr>
<td>CT132</td>
<td>cysPE37</td>
</tr>
<tr>
<td>CT11</td>
<td>cysPE35</td>
</tr>
</tbody>
</table>

**Domain Ia:** aa253–364
**Domain Ib:** aa365–399
**Domain Ic:** aa400–613

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Fig. 2. Schematic diagram of PE mutants encoded by plasmids used in the study. Numbers indicate amino acid positions that span PE-encoding sequences. The single amino acid named in each PE mutant occurs at position 287 of the native PE sequence.
Design and Activity of Immunotoxins Containing MAb HB21 (Anti-Transferrin Receptor Antibody). To determine whether cysPE35 could be specifically targeted to cells it was conjugated to the MAb HB21, an antibody that recognizes the human transferrin receptor. cysPE35 was coupled by using either a thioether or a disulfide bond (Fig. 1B). We compared the activity of cysPE35-based immunotoxins with immunotoxins containing NlysPE38, which requires proteolytic processing for activity. NlysPE38 was derivatized with 2-iminothiolane to create a free sulfhydryl group, and was coupled to MAb that had been derivatized with either SMCC to produce an immunotoxin containing a thioether bond (Fig. 1A) or SPDP to produce an immunotoxin containing a disulfide bond as described in “Materials and Methods.”

Each of the four immunotoxins were purified by using anion exchange and size exclusion chromatography to >95% homogeneity. They each migrated with a molecular mass of approximately 190,000 kDa, indicating a 1:1 ratio of antibody and toxin; there was no evidence of uncoupled toxin on nonreducing SDS-PAGE of the immunotoxins (Fig. 4). Reducing SDS-PAGE produced the expected pattern of antibody and toxin fragments (Fig. 3). When the MAb HB21 conjugated to cysPE35 through a thioether bond (HB21-S-C-PE35) was reduced and subjected to SDS-PAGE it produced MAb heavy chain (50 kDa) and MAB light chain (20 kDa) as well as heavy and light chains bound to cysPE35 (corresponding to the 55- and 85-kDa bands on the gel). When the MAB HB21 conjugated through a thioether bond to NlysPE38 (HB21-S-C-PE38) was analyzed in a similar manner, it produced MAB heavy and light chains as well as heavy and light chains bound to NlysPE38. When the MAB HB21 conjugated to NlysPE38 through a disulfide bond (HB21-S-S-PE35) was reduced it produced MAB heavy and light chains as well as free toxin (38 kDa). Similarly, the MAB HB21 conjugated to cysPE35 through a disulfide bond (HB21-S-S-PE35) produced MAB heavy and light chains as well as free toxin (35 kDa) when reduced. Western blotting of the reduced immunotoxins, using anti-PE rabbit sera, confirmed the presence of free toxin (in the case of disulfide conjugates) or toxin bound to antibody heavy and light chains (in the case of thioether conjugates; data not shown).

The cytotoxic activities of the conjugates were then tested on human epidermoid carcinoma A431 and human breast carcinoma MCF7 cell lines. On A431 cells, HB21-S-S-PE35 had an IDso of 1.0 ng/ml (Table 1; Fig. 5). NlysPE38 conjugates were 5-fold less active on A431 cells than HB21-S-S-PE35, and displayed nearly identical cytotoxicity regardless of the method of conjugation (IDso = 5.0 ng/ml). The thioether conjugate made by using cysPE35 was more than 100-fold less active than HB21-S-S-PE35 (IDso = 200 ng/ml). On MCF7 cells, conjugates using a disulfide bond to cysPE35 (IDso = 1.2) or either a thioether or disulfide bond to NlysPE38 (IDso of 1.2 and 2.0, respectively) were nearly equally active (Table 1). The thioether conjugate to cysPE35, however, was much less active (IDso = 30 ng/ml).

Mouse L929 cells, which do not express the human transferrin receptor, were resistant to the toxic effects of all immunotoxins containing the MAB HB21 at concentrations up to 1000 ng/ml. Furthermore, cytotoxicity on the A431 cell line was inhibited by 10 μg/ml of MAB HB21, indicating that the immunotoxins were binding specifically to the human transferrin receptor (data not shown).

Design and Activity of Immunotoxins Containing MAB B3. To determine whether cysPE35 could be specifically targeted to cells expressing a tumor-associated antigen, it was conjugated to the MAb B3, an antibody that recognizes a polysaccharide antigen expressed by many human cancers (18). In these studies, cysPE35 was activated with Ellman’s reagent and coupled through a disulfide bond to the MAB B3 that had been derivatized with 2-iminothiolane to produce a free sulfhydryl group (see “Materials and Methods”). For comparison,
an immunotoxin made by coupling NlysPE38 (that had been derivatized with SMCC) through a thioether bond to the MAb B3 (that had been derivatized with 2-iminothiolane) was used.

The immunotoxin containing a disulfide bond to cysPE35 (B3-S-S-PE35) was 3 times more active on MCF7 cells than the immunotoxin containing the MAb B3 coupled to NlysPE38 (B3-S-C-PE38) (ID$_{50}$ of 1.0 versus 3.2 ng/ml). In addition, B3-S-S-PE35 was slightly more active than B3-S-C-PE38 on A431 cells (ID$_{50}$ of 4.7 versus 6.0 ng/ml; Table 1; Fig. 6). KB cells, which lack the B3 antigen, were resistant to the toxic effects of B3-S-S-PE35 up to a concentration of 1000 ng/ml. As well, the cytotoxic activity of B3-S-S-PE35 on MCF7 cells was completely inhibited by 900 µg/ml of MAB B3, indicating that the immunotoxin was binding specifically to the B3 antigen.

**Design and Activity of Immunotoxins Containing MAB LL2.**

To test whether the pan-B-cell MAB LL2 could, like the MAbs HB21 (ID$_{50}$ of 1.0 ng/ml) and B3 (ID$_{50}$ of 2.4 ng/ml) and B3-S-S-PE35 was coupled through a thioether bond to the MAb LL2 that had been derivatized with SPDP (see “Materials and Methods”). For comparison, an immunotoxin made by coupling lysPE40 through a thioether bond to the MAb LL2 was used. An immunotoxin containing the MAb anti-Tac (that had been derivatized with SPDP) conjugated to reduced cysPE35 was used to determine that the immunotoxin was binding specifically to the B3 antigen.

![Diagram](image_url)

**Table 2. Cytotoxic activities of immunoconjugates to the MAbs LL2 and anti-Tac**

<table>
<thead>
<tr>
<th>Immunoconjugate</th>
<th>CA 46 (ng/ml)</th>
<th>Daudi (ng/ml)</th>
<th>HUT 102 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL2-S-S-PE35*</td>
<td>8.0</td>
<td>3.5</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>LL2-S-C-PE40</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Anti-Tac-S-S-PE35*</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

*Immunotoxin in which the MAB was derivatized with SPDP and coupled to reduced cysPE38 or lysPE40 that had been derivatized with 2-iminothiolane.

**Pharmacokinetics and Antitumor Experiments in Mice.** To test the pan-B-cell MAB LL2 could, like the MAbs HB21 and B3, direct cysPE35 to appropriate target cells, we conjugated the MAB LL2 to cysPE35 to produce LL2-S-S-PE35. In these studies, reduced cysPE35 was coupled through a disulfide bond to the MAB LL2 that had been derivatized with SPDP (see “Materials and Methods”). For comparison, an immunotoxin made by coupling lysPE40 through a thioether bond to the MAB LL2 was used. An immunotoxin containing the MAB anti-Tac (that had been derivatized with SPDP) conjugated to reduced cysPE35 was constructed to serve as a negative control for in vitro and in vivo experiments.

![Diagram](image_url)

The activities of immunotoxins containing the MAbs LL2 and anti-Tac were then assayed on Burkitt’s lymphoma cell lines (Table 2). The ID$_{50}$ of LL2-S-S-PE35 on the Burkitt’s lymphoma CA46 cell line was 8.0 ng/ml. In comparison, the ID$_{50}$ of a thioether conjugate between MAB LL2 and lysPE40 (LL2-S-C-PE40) was 38 ng/ml. Daudi cells were about twice as sensitive to both conjugates. Both Burkitt’s lymphoma cell lines were resistant to anti-Tac coupled by a disulfide bond to cysPE35 (anti-Tac-S-S-PE35), indicating that their sensitivity to LL2-S-S-PE35 was not due to nonspecific toxin internalization. LL2-S-S-PE35 was not cytotoxic toward HUT 102 cells, which contain high levels of the interleukin 2 receptor and are very sensitive to anti-Tac-S-S-PE35 (ID$_{50}$ = 4.0 ng/ml). As well, the activity of LL2-S-S-PE35 on both Burkitt’s lymphoma cell lines was inhibited by 20 µg/ml of the MAB LL2, indicating that the immunotoxin was binding specifically to the LL2 antigen.

We were interested in the effects of derivatizing agents on immunotoxin activity. The immunotoxin B3-S-C-PE38, in which the antibody had been derivatized with 2-iminothiolane and the toxin had been derivatized with SMCC, was compared to a similar conjugate made by using the identical proteins but reversing the order of derivatizing agents. Interestingly, B3-S-C-PE38 containing the MAB B3 that had been derivatized with SMCC was 5-fold less active on A431 cells (ID$_{50}$ of 32 versus 6.0 ng/ml) and 4-fold less active on MCF7 cells (ID$_{50}$ of 12 versus 3.2 ng/ml) than the identical immunotoxin in which the antibody had been derivatized with 2-iminothiolane. Also, B3-S-S-PE35 was 4- to 5-fold less active on A431 cells (ID$_{50}$ of 19 versus 4.7 ng/ml) and MCF7 cells (ID$_{50}$ of 5.5 versus 1.0 ng/ml) when the MAB B3 had been derivatized with SPDP than when the antibody had been derivatized with 2-iminothiolane. We did not observe a significant effect of derivatizing agents on the activity of immunotoxins containing the MAB HB21 (see “Discussion”).

**Table 2. Cytotoxic activities of immunoconjugates to the MAbs LL2 and anti-Tac**

<table>
<thead>
<tr>
<th>Immunoconjugate</th>
<th>CA 46 (ng/ml)</th>
<th>Daudi (ng/ml)</th>
<th>HUT 102 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL2-S-S-PE35*</td>
<td>8.0</td>
<td>3.5</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>LL2-S-C-PE40</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Anti-Tac-S-S-PE35*</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

*Immunotoxin in which the MAB was derivatized with SPDP and coupled to reduced cysPE38 or lysPE40 that had been derivatized with 2-iminothiolane.

The activities of immunotoxins containing the MAbs LL2 and anti-Tac were then assayed on Burkitt’s lymphoma cell lines (Table 2). The ID$_{50}$ of LL2-S-S-PE35 on the Burkitt’s lymphoma CA46 cell line was 8.0 ng/ml. In comparison, the ID$_{50}$ of a thioether conjugate between MAB LL2 and lysPE40 (LL2-S-C-PE40) was 38 ng/ml. Daudi cells were about twice as sensitive to both conjugates. Both Burkitt’s lymphoma cell lines were resistant to anti-Tac coupled by a disulfide bond to cysPE35 (anti-Tac-S-S-PE35), indicating that their sensitivity to LL2-S-S-PE35 was not due to nonspecific toxin internalization. LL2-S-S-PE35 was not cytotoxic toward HUT 102 cells, which contain high levels of the interleukin 2 receptor and are very sensitive to anti-Tac-S-S-PE35 (ID$_{50}$ = 4.0 ng/ml). As well, the activity of LL2-S-S-PE35 on both Burkitt’s lymphoma cell lines was inhibited by 20 µg/ml of the MAB LL2, indicating that the immunotoxin was binding specifically to the LL2 antigen.
period of observation following remission and animals showed no signs of toxicity for the duration of the study. In contrast, neither the MAb B3 nor cysPE35 demonstrated antitumor activity. Thus, the MAb conjugated to cysPE35 through a disulfide bond can cause complete and sustained remission of 100% of human tumor xenografts growing in nude mice at tolerable doses.

LL2-S-S-PE35 was tested for antitumor activity on Burkitt’s lymphoma xenografts. Nude mice were given injections of $1 \times 10^7$ CA46 cells s.c., and 6 days later visible tumors were treated by four daily i.v. doses of LL2-S-S-PE35. Fig. 9 shows that 5 and 10 lag of LL2-S-S-PE35 per day for 4 days demonstrated a significant antitumor effect. Ten pg of LL2-S-S-PE35 represents 20% of the multiple dose LD$_{50}$, and was nontoxic to the mice. The complete remission rate was 25% at the 5-µg dose and 50% at the 10-µg dose, suggesting a dose-dependent response. The MAb LL2 given at 8 µg/day for four doses did not cause tumor regression, indicating that the antitumor effect of LL2-S-S-PE35 required the toxin moiety. By Fisher’s $t$ test, the effect of the 5-lag dose was significant at the 0.05-level by day 9 and at the 0.01-level by day 13. The effect of the 10-µg dose was significant at the 0.05-level by day 8 and at the 0.01-level by day 10. LL2-S-S-PE35 had similar antitumor activity on Daudi cell xenografts growing in nude mice (data not shown). Anti-Tac-S-S-PE35 administered at 10 µg i.v./day had no antitumor effect on the Burkitt’s lymphoma xenografts, indicating that the antitumor effect of LL2-S-S-PE35 on these cells was not due to the toxin portion of the immunotoxin alone (data not shown).

**DISCUSSION**

We have constructed potent immunotoxins with the use of a carboxyl-terminal fragment of PE that, after internalization into cells, is able to translocate to the cytosol without requiring proteolysis. PE-based immunotoxins originally used native PE and more recently have used PE mutants lacking domain Ia (amino acids 1–252) (6–9). Within the target cell, immunotoxins containing PE, PE40 (amino acids 253–613 of PE), or PE38 (amino acids 253–364 and 381–613 of PE) require two essential steps to liberate a carboxyl-terminal toxin fragment capable of reaching the cytosol: (a) proteolysis between amino acids at positions 279 and 280, and (b) reduction of a disulfide bond spanning amino acids at positions 265 and 287 of PE (4, 5). We designed cysPE35 so that it could be easily conjugated to MAbs through its cysteine residue and would not require intracellular proteolysis for activity.

NlysPE38 conjugates made by using either a disulfide or thioether linkage to the MAb HB21 had similar cytotoxic activities. Regardless of the method of conjugation, immunotoxins containing NlysPE38 require both proteolysis and reduction of a disulfide bond to generate a carboxyl-terminal toxin fragment capable of translocating to the cell cytosol (Fig. 1). In contrast, HB21-S-S-PE35 was 5-fold more active on A431 cells than the NlysPE38 conjugates. The toxin fragments of HB21-S-S-PE35 and of the HB21 conjugates to NlysPE38 that are translocated to the cytosol are nearly identical (amino acids 280–364 and 381–613 of PE). The single difference is a methionine substitution for glycine at position 280 that is known not to affect toxin activity (4). cysPE35 and the processed form of NlysPE38 should therefore be able to translocate to the cytosol equally well. Thus the decreased activity of NlysPE38 conjugates should be due to a rate-limiting step that occurs prior to translocation of the active cytosolic form of PE.

Several factors may account for the differences in cytotoxic activities of conjugates containing cysPE35 and NlysPE38. We have shown
previously that proteolytic processing is rate limiting in the action of a derivative of PE directed to A431 cells by TGFα (10). If proteolysis between amino acids 279 and 280 but not reduction of a disulfide bond is rate limiting within the cell, disulfide conjugates to cysPE35 might be more active than disulfide or thioether conjugates containing NlysPE38. Cell lines may have a range of proteolytic efficiencies. One would therefore expect the enhanced activities of cysPE35 disulfide conjugates to be less apparent on certain cell lines. The conjugates HB21-S-S-PE35, HB21-S-S-PE38, and HB21-S-C-PE38 had nearly identical activities on MCF7 cells, indicating that this cell line may efficiently process NlysPE38.

Alternatively, chemical modification of the toxin, which is unnecessary for cysPE35, may reduce the activity of immunotoxins containing NlysPE38 or lysPE40. For instance, SMCC, SPDP, or 2-iminothiolane may react with one of the three lysine residues that occur in domain III of PE38 or PE40. Such derivatization may directly impair the intracellular trafficking or ADP-ribosylation activity of the toxin. As well, immunotoxins conjugated through lysine residues that occur on the carboxy-terminal side of the native proteolysis site are likely inactive since proteolysis and disulfide bond reduction do not release a carboxy-terminal toxin fragment able to translocate to the cytosol. It is possible that coupling through improper lysines to NlysPE38 accounted for the decreased activity of HB21-S-S-PE38 and HB21-S-C-PE38 in comparison to HB21-S-S-PE35 on A431 cells. However, HB21-S-S-PE35 did not exhibit increased cytotoxicity in comparison to HB21 conjugates containing NlysPE38 on MCF7 cells. If the decreased activity of NlysPE38 conjugates were due to coupling through improper lysine residues, we would have expected these immunotoxins to have been less active than HB21-S-S-PE35 on both MCF7 and A431 cells. Thus, our data are insufficient to prove the mechanism that limits the toxicity of NlysPE38 conjugates on certain cell lines, and both proteolysis as well as the effects of toxin derivatization may play important roles.

Interestingly, the enhanced activity of B3-S-S-PE35 in comparison to B3-S-C-PE38 was more apparent on MCF7 cells rather than on A431 cells. In contrast, the enhanced activity of HB21-S-S-PE35 in comparison to HB21-S-C-PE38 was more apparent on A431 rather than MCF7 cells. It is unclear why the metabolism of NlysPE38 appears to be more rate limiting on A431 cells in the case of HB21 conjugates and also more rate limiting on MCF7 cells in the case of B3 conjugates. Clearly, the ability of specific cell lines to process immunotoxins containing NlysPE38 depends on a certain degree on properties inherent to the MAb and the conjugation chemistry.

The immunotoxin containing cysPE35 linked to the MAb HB21 through a thioether bond (HB21-S-C-PE35) requires proteolysis to separate the toxin moiety from the MAb, so that toxin translocation can occur. Because cysPE35 starts at amino acid 280 of native PE, it does not contain the native proteolysis site between amino acids 279 and 280 recognized by mammalian cells that process PE (4). Hence, the cysPE35 thioether conjugate to the MAB HB21 (HB21-S-C-PE35) had low activity. However, some residual activity was detected. We have shown previously that mutants of PE37 containing deletions of the amino terminus retain some activity (10). As well, a mutant of PE37 containing the amino acids MPQ prior to amino acid 280 of PE is very active. Thus, carboxy-terminal fragments of PE may be translocated to the cytosol even if proteolytic cleavage occurs at a site other than the one between amino acids 279 and 280. We believe that the small degree of activity observed with HB21-S-C-PE35 can be attributed to proteolytic processing occurring at other sites within the MAb or cysPE35 and inefficient translocation of the resulting fragments.

Interestingly, B3-S-C-PE38 made by using SMCC to derivatize the antibody and 2-iminothiolane to derivatize the toxin was less active than the same immunotoxin made in which the antibody was derivatized with 2-iminothiolane and NlysPE38 was derivatized with SMCC. While both SMCC and 2-iminothiolane react with amino groups, they differ in polarity (2-iminothiolane > SPDP > SMCC). We suspect that the nonpoly reactive SMCC derivatized a lysine residue that interfered with a critical property of the MAB B3, such as antibody binding, that contributes to immunotoxin activity. In addition, B3-S-S-PE35 made by using 2-iminothiolane to derivatize the MAB B3 was 4- to 5-fold more potent than one using SPDP to derivatize the antibody. We also believe the more polar molecule, 2-iminothiolane, reacts with an amino group in the MAB B3 at a site that does not affect immunotoxin activity. We did not observe any difference in immunotoxin activity by using different methods to derivatize the MAB HB21. Hence, we conclude that the ability of certain derivatizing agents to impair the potency of immunotoxins can be an antibody-specific phenomenon.

When administered i.v., B3-S-S-PE35 had a β half-life of 6.3 h. This value is similar to the β half-life (8.3 h) reported for an immunotoxin containing the MAB 0X7 linked through a disulfide bond to deglycosylated ricin A chain (32). Thorpe et al. have shown that 4-succinimidylidoxycarbonyl-α-methyl-α-(2-pyridyldithio)-toluene can be used to derivatize MAbs to produce immunotoxins containing hindered disulfide bonds. In the case of the MAB 0X7, conjugation through a hindered disulfide bond to deglycosylated ricin A chain produced an immunotoxin with an increased β half-life of 20.9 h that also demonstrated an improved therapeutic index (33). Our results indicate that an immunotoxin (B3-S-S-PE35) containing an unhindered disulfide bond to cysPE35 causes complete regression of 100% of human tumors growing in nude mice at tolerable doses. The therapeutic index of immunono conjugates containing cysPE35, however, may benefit similarly by a hindered disulfide linkage.

We found that the pan-B-cell monoclonal antibody LL2 can target cysPE35 to human B-cell lymphoma in tissue culture and in nude mice. Disulfide conjugates using translocatable forms of other toxins have also shown efficacy against such cells in vivo. For example, ricin A chain can be targeted to Daudi cells growing in SCID mice by the anti-CD22 antibody RFB4 or its Fab' fragment (34). Moreover, in clinical trials, remissions were induced in a high percentage of patients with CD22-positive tumors (35). Ricin A chain, pokeweed antiviral protein, saporin, and momordin have been conjugated to the anti-CD19 antibody-B43 to target B-lineage leukemia cells (36). B43-PAP showed antitumor activity in SCID mice bearing human CD19-positive leukemia cells (37). At this time it is unclear how the in vivo activity of LL2-S-S-PE35 compares with that of these agents or with that of thioether conjugates of other PE mutants with LL2.4

Among many toxin fragments, ricin A chain and diphtheria toxin A chain share with cysPE35 the ability to arrest protein synthesis. Also they are the final processed forms of their respective whole toxins. Each of these catalytic fragments are portions of whole toxins that, like PE, have the ability to bind to mammalian cells and to translocate a catalytic fragment into the cytosol to arrest protein synthesis. Furthermore, each of the whole toxins is encoded by a single gene and is subsequently cleaved into two fragments that are joined by a disulfide bond (28, 38). While the three toxins are remarkably similar in these respects, the location of proteolytic cleavage is different for each toxin. Ricin is cleaved into two fragments within the endoplasmic reticulum of the castor oil plant seeds that encode the protein (39). Diphtheria toxin is cleaved by an

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3 C. Theuer and I. Pastan, unpublished results.

extracellular protease prior to endocytosis by target cells (40). PE is cleaved within the target cell (4, 5). For all three toxins, reduction is the final event required to generate a catalytic fragment that is translocated to the cell cytosol. Hence, immunotoxins containing only the catalytic fragments of either of these toxins must use a disulfide linkage in order to release an active toxin fragment.

In conclusion, we have constructed a mutant form of PE, termed cysPE35, that is able to translocate to the cytosol without requiring proteolysis. cysPE35 can be easily conjugated through a disulfide bond to MAbs to produce immunotoxins of high potency. On some cell types, these immunotoxins are more potent than immunotoxins made with NlysPE38 or lysPE40. MAbs conjugated to cysPE35 through a disulfide bond have potent antitumor activities and have the potential to be useful clinical agents.

ACKNOWLEDGMENTS

We thank B. Y. for assistance with protein purification, E. Lovelace and A. Harris for cell culture assistance, A. Jackson and E. Gott for editorial assistance, and M. Rauschenberg and her staff for their care of the animals used in the study. We thank Charlotte Frying for supplying the MAb HB21, Lee Pai for supplying the MAb B3, John Hakimi of Hoffmann-La Roche for supplying the MAb anti-Tac, and Hans Hansen of Immunomedics for supplying the MAB L12.

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Immunotoxins Made with a Recombinant Form of *Pseudomonas* Exotoxin A That Do Not Require Proteolysis for Activity

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