B3(Fab)-PE38M: A Recombinant Immunotoxin In Which a Mutant Form of Pseudomonas Exotoxin Is Fused to the Fab Fragment of Monoclonal Antibody B3

MuHyeon Choe, Keith O. Webber, and Ira Pastan

ABSTRACT

Recombinant immunotoxins were made by fusing the Fab domain of monoclonal antibody (MAb) B3 to PE38M, a truncated mutant form of *Pseudomonas* exotoxin (PE). The recombinant toxins were made in *Escherichia coli* by fusing genes encoding the antibody domains to a gene encoding the mutant form of PE. MAb B3 binds to a carbohydrate antigen found on many kinds of carcinomas. Immunotoxins in which MAb B3 has been chemically coupled to recombinant forms of PE have been shown to be very active cytotoxic agents. PE has also been targeted to tumor cells by replacing the cell-binding domain of PE (domain I) with a single-chain antibody to make a single-chain immunotoxin. In the current study, PE38M, a mutant form of PE, with a deletion of the cell-binding domain (amino acids 1–252) as well as mutations in domain III and some nonessential sequences in domain Ib (amino acids 365–380), was fused to the light chain of MAb B3. This protein was renatured in the presence of the Fd fragment of MAB B3 to produce a Fab-toxin fusion protein. Alternatively, the Fd fragment of MAB B3 was fused to PE38M and combined with the light chain. Both types of B3(Fab)-PE38M were just as active on target cells as previously described single-chain immunotoxins. Furthermore, the B3(Fab)-PE38M produced complete remissions of human tumor xenografts growing in nude mice. B3(Fab)-PE38M has two advantages over single-chain immunotoxins. One is that the yield of recombinant Fab-toxin is very high, with 10–22% of the starting protein recovered as cytotoxic immunotoxin after chromatographic purification. The second is that the B3(Fab)-PE38M has a much longer survival in the circulation of mice with a 1/2b of ~5 h.

INTRODUCTION

MAbs that bind specifically to cancer cells have been used to produce cytototoxic immunoconjugates by attaching the MAB to toxins or drugs (1–3). Our laboratory has isolated MAB B3 that binds to a carbohydrate epitope present on the surface of many human carcinomas including those of the colon, stomach, lung, breast, esophagus, and ovary (4). MAB B3 has been used to make immunotoxins by coupling the antibody to mutant recombinant forms of PE. PE is made up of a single polypeptide chain protein that is arranged into three structural domains (I, II, III) (5). Domain I is the cell-binding domain, domain II is the translocation domain, and domain III catalyzes the ADP ribosylation and inactivation of elongation factor 2 which inhibits protein synthesis and leads to cell death (6). Deletion of domain I produces an M4, 40,000 protein (PE40) (7) that cannot bind to target cells by itself but can be targeted to cells by attaching it to appropriate antibodies. PE40 has been further modified by removing nonessential amino acids and a cystine bridge that lie between domains II and III (aa 365–380) to produce PE38 (M, 38,000). B3-PE38 and its derivatives are very cytotoxic to cells displaying the B3 antigen and have produced complete regression of human tumors growing as xenografts in nude mice (8, 9).

The conjugation of an antibody to a toxin produces a heterogeneous mixture of reaction products because there are a number of possible sites on the toxin and also on the antibody at which the conjugation reaction can occur. The reaction products can include molecules that have been inactivated due to a chemical reaction with the binding site of the antibody or the active site of the toxin. One way to eliminate this heterogeneity is to use genetic engineering methods to fuse genes encoding the antibody and the toxin to produce a uniform protein. This has been accomplished by fusing a gene encoding a recognition function such as a single-chain antibody to a gene encoding a mutant form of PE (10). B3(Fv)-PE38 is such a molecule; it contains the variable region of the B3 antibody in a single-chain form fused to PE38. B3(Fv)-PE38 was constructed by fusing three peptide chains which consist of four functional domains. These were the VH and VL domains of the antibody and domain II and III of PE. B3(Fv)-PE38 was expressed as inclusion bodies in *Escherichia coli*, and the active form of the molecule was obtained through refolding and renaturation of the denatured and reduced protein. Although B3(Fv)-PE38 has a lower binding affinity for the B3 antigen than the native dimeric antibody, B3(Fv)-PE38 showed an increased cytotoxic activity in tissue culture over the chemical conjugates. Furthermore, in mice bearing human carcinoma xenografts, B3(Fv)-PE38 was considerably more active against the tumor. One difficulty with single-chain immunotoxins is that the final yield of properly folded and purified monomeric protein is low; it is approximately 4% of the total starting protein (11). Another characteristic of these single-chain immunotoxins is that their half-life in the circulation of mice is very short, t1/2b 15–20 min, in comparison to the chemical conjugate of whole antibody with tox (12).

Fab fragments of several other MAbs have been prepared by proteolytic cleavage and used to make chemical conjugates with toxins. They were all found to be active against tumor xenografts growing on nude mice. These MAbs were directed to CD22 (RF4B) (13), transferrin receptor (HB21) (14), and a pan B-cell antigen (LL2) (15). Each of these Fab-toxin conjugates manifested unique characteristics when compared to the respective IgG conjugate. However, the greater expense of making Fab-toxin conjugates compared to IgG-toxin conjugates has limited their utility. In the case of B3, the preparation of Fab from IgG by proteolytic cleavage has failed. A recombinant immunotoxin in which the Fab domain was used as a binding domain had been constructed by fusing PLC to the Fab domain of MAB anti-Tac (16). In that construction, the PLC gene was fused to the 5'-end of the VH CH1 of anti-Tac heavy chain. The PLC-VH CH1 fusion and VL CL protein were secreted into periplasm. The active proteins that were purified from periplasm were considerably less active than anti-Tac(Fv)-PE40.

In this study, we have designed and produced Fab-toxin fusion

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2 The abbreviations used are: MAB, monoclonal antibody; PE, *Pseudomonas* exotoxin A; aa, amino acids; PLC, *Clostridium perfringens* phospholipase; PCR, polymerase chain reaction; Glu, glutamic acid; Lys, lysine; ID50, the concentration needed to inhibit protein synthesis by 50%; LD50, the dose of immunotoxin that killed 50% of the animals; PBS-HSA, phosphate-buffered saline-human serum albumin; L, light chain.

3 L. H. Pai, unpublished data.
molecules in E. coli. We have chosen a mutant form of PE (PE\textsubscript{38M}) for these experiments. PE\textsubscript{38M} has a deletion of domain Ia (aa 1–252), a deletion of part of domain Ib (aa 365–380), and two of the lysine residues in domain III mutated to glutamine, and lysine 613 is deleted. This form of PE has no lysine residues and may be useful for making specific chemical derivatives (17). To make Fab-toxins, we have taken two approaches. In the first, we fused PE\textsubscript{38M} to the 3’-end of the Fd fragment of B3 and refolded it with the light chain. In the other approach, we fused PE\textsubscript{38M} to the 3’-end of the light chain and refolded it with the Fd portion of the heavy chain. The refolding yields from inclusion body protein of these Fab-toxins were quite high, ranging from 10–22% of the starting material. They have approximately the same cytotoxic activities against cultured cells as single-chain immunotoxins and produce complete regression of human tumors implanted s.c. in mice. However, they have very different pharmacokinetic properties with a greatly extended plasma half-life of \approx 5 h.

**MATERIALS AND METHODS**

**Construction of Plasmids expressing Fd-PE\textsubscript{38M} and L-PE\textsubscript{38M} Fusion Protein.** The construction of the plasmids encoding the Fd-toxin or light chain-toxin fusion proteins are presented schematically in Fig. 1. A plasmid that encodes either the chimeric Fd chain (pULI30) or chimeric light chain sequence (pULI31)\textsuperscript{4} was used as a template for each polymerase chain reaction. These plasmids were made from V\textsubscript{H} and V\textsubscript{L} of previously constructed V\textsubscript{H} and V\textsubscript{L} sequence (pUU31)\textsuperscript{4} was used as a template for each polymerase chain reaction. The molecular weight of B3(Fab)-PE\textsubscript{38M} molecule as shown in Fig. 2a. The pair were put in a 1:1 molar ratio to make a total of 50 mg in 10 ml solubilization buffer. The mixture was reduced by the addition of dithioerythritol to 0.3 M and rocking for 2 h at room temperature.

The PE\textsubscript{38M} sequence was from pRK79QA\textsuperscript{5} which consists of anti-Tac(Fv) fused to PE\textsubscript{38M}. The latter is a recombinant form of PE containing amino acids 253–612 in which lysine residues at positions 590 and 606 were changed to glutamines and a deletion of position 365–380 (14). pRK79QA\textsubscript{5} was digested with XbaI and HindIII, and the 4.1-kilobase fragment was purified. Their sequences differ from each other at just one base position, and they produced Fd-PE\textsubscript{38M} with a Glu (p; FdE-PE\textsubscript{38M}) or a Lys (k; PdK-PE\textsubscript{38M}) at the connector sequence (Fig. 2b). The T7\textsuperscript{5} promoter for the expression control of the protein (20). Purified fragments were ligated and introduced into E. coli strain DH5\textalpha. The plasmids that contain Fd-PE\textsubscript{38M} fusion are named pMC55 (FdE-PE\textsubscript{38M}) and pMC57 (FdK-PE\textsubscript{38M}), depending on the connecting amino acid sequence. The plasmids that contain light-chain toxin fusion are similarly named pMC58 (LE-PE\textsubscript{38M}) and pMC59 (LK-PE\textsubscript{38M}).

**Expression and Purification of Proteins for B3(Fab)-PE\textsubscript{38M}**

BL21(AD3) cells were transformed with the plasmids pMC56, pMC57, pMC58, and pMC59 for the production of Fd-PE\textsubscript{38M} or L-PE\textsubscript{38M} fusion proteins (Table 1), and pULI30 and pULI31 were used for the production of Fd chain and light chain, respectively. Transformed cells were grown in 1 liter superbroth containing 2% glucose-0.05% MgSO\textsubscript{4}-100 mg/ml ampicillin at 37°C. At the cell density of A\textsubscript{600} 1.5 to 2.5, the cultures were induced by 1 mM isopropyl-\beta-D-thiogalactopyranoside, and the cells were harvested after 2–3 h at a cell density of A\textsubscript{600} 4–5. The cell pastes were resuspended thoroughly in 200 ml lysis buffer (50 mM Tris, pH 8.0–20 mM EDTA-100 mM NaCl) using a tissuemizer. Lysozyme (0.65 ml of 50 mg/ml) was added to lyse the cells, and the mixture was shaken for 30 min. To digest the released DNA, 400 \mu l of D\textalpha nase (1 mg/ml) was added and shaken for an additional 30 min. The digestion mixture was spun at 27,000 \times g for 50 min at 4°C. The pellet was resuspended in 200 ml lysis buffer with a tissuemizer. Twenty-five \% Triton X-100 (20 ml) was added and shaken for 30 min. The inclusion body was pelleted by 27,000 \times g spin for 50 min at 4°C. The pellet was resuspended and washed 4 times with 200 ml lysis buffer. The purified inclusion body was stored at \approx 70°C.

**Refolding and Purification of B3(Fab)-PE\textsubscript{38M} Protein.** The purified inclusion bodies were dissolved in 10 ml solubilization buffer (6 mM guanidine-HCl-0.1 mM Tris-HCl-2 mM EDTA). The amount of protein was determined using Coomassie Plus protein assay reagent (Pierce, Rockford, IL). Fd or light chain and their PE\textsubscript{38M} fusion protein were mixed appropriately to make L-PE\textsubscript{38M}, depending on the connecting amino acid sequence. The plasmids that contain light-chain toxin fusion are similarly named pMC58 (LE-PE\textsubscript{38M}) and pMC59 (LK-PE\textsubscript{38M}).

**Cytotoxicity Assays.** The cytotoxicity of the B3(Fab)-PE\textsubscript{38M} immunotoxin was evaluated by measuring the ID\textsubscript{50} using cultured cells (8). The synthesis of protein was monitored by the incorporation of [\textsuperscript{3}H]leucine. Cells were exposed to immunotoxins for 36–48 h and labeled for 6–10 h.

**LD\textsubscript{50} Measurement.** To measure the animal toxicity of the B3(Fab)-PE\textsubscript{38M}, increasing amounts of recombinant toxin were injected through the tail vein into groups of 4- to 6-week-old female BALB/c mice. The amount of immunotoxin used was 5, 10, 15, and 20 \mu g in 200 \mu l PBS-HSA (0.2%) for each mouse (average weight, 20 g). Animals were observed for at least 10 days.
Kinetics of the Survival Activity of Immunotoxin in Mouse Blood Circulation. Immunotoxin (10 μg in 200 μl 0.2% PBS-HSA) was injected through the tail vein of BALB/c mice (6 weeks old, ~20 g). Blood samples were collected at 2, 10, and 30 min and 1, 2, 4, 8, and 12 h. The surviving activity was measured by adding serial dilutions of the serum sample into 96-well plates of A431 cells and finding out the dilution point of 50% inhibition of protein synthesis. The concentration of immunotoxin was calculated from the known ID₅₀ value. Data analysis was done using the polyesponential curve fitting program “RSTRIP,” version 5.0 (MicroMath, Inc., Salt Lake City, UT).

Antitumor Activity of B3(Fab)-PE38M in Nude Mice bearing A431 Human Epidermoid Carcinoma. A431 cells (3 × 10⁶) were injected s.c. on day 0 into female immunodeficient “nude” mice (6 weeks old, 20 g). Tumors of about 5 x 5 mm developed in all animals by day 5. Animals were treated with i.v. injections of B3(Fab)-PE38M, MAb B3 alone, or vehicle (0.2% PBS-HSA). Therapy was given on days 4, 6, and 8. Each treatment group consisted of 5 or 6 animals. Tumors were measured with a caliper every 2 days, and the volume of the tumor was calculated by using the formula

\[ \text{Tumor volume (mm}^3) = \text{Length} \times (\text{Width})^2 \times 0.4. \]

RESULTS

Construction of a Recombinant Immunotoxin That Is Composed of the Fab Domain of Monoclonal Antibody B3 and a Truncated Form of PE (PE38M). The structures of the B3(Fab)-PE38M that were constructed using the variable regions of MAb B3 are shown schematically in Fig. 2. In one case, the Fd portion of the heavy chain of MAb B3 was fused to PE38M. In the other case, the light chain of MAb B3 was fused to PE38M. A polypeptide connector (C3: ASGGPE) was inserted between the toxin and the antibody and a lysine residue (K) or a glutamate residue (E) was placed at the amino terminal of the Fab domain. The sequences of the connectors are shown schematically in Fig. 2. The Pseudomonas exotoxin derivative used in the current study (PE38M) has a deletion of domain Ia (aa 1–252), a deletion of a portion of domain Ib (aa 365–380), substitutions of glutamate (E) for lysine residues (K) at positions 590 and 606, and deletion of lysine 613. These mutations do not decrease the activity of the toxin (23). Thus, there are no lysine residues in the toxin portion of the B3(Fab)-PE38M. Plasmids encoding either the Fd-PE38M, the Fd alone, or the Fd chain alone were transfected separately into E. coli (ADE3), and each of the recombinant proteins was expressed in the form of inclusion bodies. The appropriate inclusion bodies were dissolved in guanidine and renatured using a redox shuffling method as previously described (11). This allowed, for example, the B3(Fd)-PE38M to refold and form a disulfide bond with the B3 light chain present in the same refolding solution.

The molecular weight of each B3(Fab)-PE38M calculated from the DNA sequence is 86,100 for the combination of the Fd-PE38M and the light chain (61,800 + 24,300) or light chain-PE38M and Fd (62,100 + 24,000).

Refolding of B3(Fab)-PE38M Gives a High Yield of Monomeric Active Molecules. We have previously reported that the yield of single-chain immunotoxins is low. In the case of B3(Fv)-PE38 and B3(Fv)-PE38M, the yields are about 1–4% of the protein originally present in refolding reaction solution; the low yield is largely due to aggregation that occurs during the refolding process (12). To increase the yield of active immunotoxin, we chose to fuse the whole light chain or the Fd portion of the heavy chain to the toxin (PE38M). B3(Fab)-PE38M was refolded using the same conditions as those used to prepare the single-chain immunotoxins. After dialysis, B3(Fab)-PE38M was purified first on Q-Sepharose and then on a TSK gel size exclusion column. B3(Fab)-PE38M eluted from the size exclusion column with an apparent molecular weight of 150,000 when compared to the calibration proteins (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). This indicates that the molecule is probably quite asymmetric. The yield of each form of B3(Fab)-PE38M is shown in Fig. 3. It shows that the yield of monomeric toxin is very high, ranging from 9–23%. The graph also shows that the yield is higher when PE38M is fused to the light chain than when it is fused to the Fd chain. In the case of PE38M fused to the Fd chain, the yield was 9–11%, and in the case of the light-chain fusion it was 21–23%. Therefore, the addition of a murine constant domain to a variable domain signifi-

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<td>PrLK</td>
<td>CCCCGGAAGCTT ACTGCAACCCCTGACGAGA</td>
<td>P2</td>
<td>pmCS9</td>
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Table 1 Sequences of primers used in PCR fragment generation

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Introduction of a negatively (Glu) or positively (Lys) charged residue right after the Fd fragment or the light chain before the peptide (C3) connecting the antibody to the toxin had very little effect on the yield of toxin. As shown in Fig. 3, the refolding yield of both Fd-PE38M fusions was about 9—11%. Similarly, both fusions to the light chain PE38M gave 2-fold higher yield than Fd-PE38M fusions. These yields are 2- to 10-fold higher than the single-chain Fv-PE38M fusion protein. Four kinds of constructs are shown: B3(Fab)-FdK-PE38M (FdK), B3(Fab)-LE-PE38M (LE), and B3(Fab)-LK-PE38M (LK). Columns (bars), means (±SE).

The purified B3(Fab)-PE38M molecules were analyzed by SDS-PAGE, and the stained gels are shown in Fig. 4. The samples were analyzed under both nonreducing (Fig. 4A) and reducing (Fig. 4B) conditions. The reducing gels show that only two bands were present in each protein preparation and these had apparent molecular weights of about 62,000 and 24,000 as expected. The nonreducing gel shows that the two chains are mainly associated together as a disulfide linked dimer, and because of its abnormal branched molecular shape it migrated in the position of 130,000 relative to the size marker (Amersham International, Amersham, United Kingdom). In the case of PE38M fused to the light chain, the cysteine needed for the disulfide bond formation between heavy and light chain was between two bulky amino acids, Glu and Lys. Probably because of the difficulties in disulfide bond formation, a small portion of the purified protein was nondisulfide bonded and gave a faint band that migrated near the Mr 62,000 marker. This band was about one-tenth the intensity of the higher molecular weight band. The Mr 62,000 band was B3 light chain-PE38M; the Fd fragment could not be detected by staining.

Cytoxicity Test of B3(Fab)-PE38M. All four B3(Fab)-PE38M molecules were tested for their cytotoxic activity using several tumor cell lines. The incorporation of [3H]leucine into protein was measured because it is a good indicator of cell viability (6). The activities of the four different B3(Fab)-PE38M constructs on A431 cells, which express large amounts of the B3 antigen, are shown in Fig. 5. It is evident that all of the molecules have similar activities with an ID50 of about 2.5 ng/ml regardless of whether they have PE38M fused to the Fd or light chain. Also, the presence of a lysine or glutamate in the connector sequence did not affect cytotoxic activity. Because all of the B3(Fab)-PE38M had similar activities on A431 cells, they were next evaluated on a panel of cell lines with different amounts of B3 antigen that had been used in studies with other types of B3-containing immunotoxins (4, 8). The cell lines used were A431, MCF7, CRL 1739, LNCaP, KB, and HUT102. Fig. 6 shows cytotoxicity measurements of the form of B3(Fab)-FdK-PE38M in which the toxin is fused to the Fd fragment. Consistent with previous data with the B3(Fv)-toxin from which the antigen-binding domain was taken to make the B3(Fab)-PE38M molecules (12), A431, MCF7, and CRL 1739 cells were very sensitive to the immunotoxin, whereas KB and HUT102 cells were much less affected. A summary of the activity of all four B3(Fab)-PE38M molecules is shown in Table 2. The pattern of activity of all constructs was very similar. They were very active on A431, MCF7, CRL 1739, and LNCaP cells. Furthermore, compared to the chemical conjugate of the B3 antibody with the truncated form of PE and the single-chain Fv fusion construct [B3(Fv)-PE38M], the cytotoxic potency of B3(Fab)-PE38M is similar on a molar basis.

Antitumor Activity. The antitumor activity of one of the constructs, B3(Fab)-FdK-PE38M, was assessed in nude mice bearing tumors composed of human A431 carcinoma cells. To determine how much immunotoxin could be given to the mice for therapeutic studies, the maximum tolerated dose of both forms of B3(Fab)-PE38M was determined. The LD100 of a single dose was determined to be 750 μg/kg for B3(Fab)-FdK-PE38M and B3(Fab)-LK-PE38M (data not shown).

Next, the ability of B3(Fab)-FdK-PE38M to cause tumor regression was evaluated, and the results are shown in Fig. 8. Tumor-bearing mice were treated starting on day 4 when the tumors measured approximately 50 mm3. Animals were treated on days 4, 6, and 8. Complete disappearance of the tumors occurred after treatment with 100 μg/kg i.v. B3(Fab)-FdK-PE38M. At this dose level all of the animals appeared healthy, and no weight loss or other toxicity was apparent. Significant tumor regression was also observed at doses of 25 and 50 μg/kg given every other day × 3. In animals treated with MAb B3 alone or PBS-HSA (0.2%), no inhibition of tumor growth was noted. These animals were killed on days 14—16 when the tumors had grown to 1 x 1.5 cm.

DISCUSSION

We have used MAb B3 (4) which binds to many human cancers and a recombinant form of Pseudomonas exotoxin to make several types of B3(Fab)-toxins in E. coli. These Fab-toxins are very cytotoxic to target cells in vitro and in vivo and have several properties which make them attractive molecules for cancer therapy.
A high refolding yield of Fab-PE38<sup>M</sup>. The first type of recombinant immunotoxin constructed was a single-chain molecule in which the variable regions of light and heavy chains of an antibody were joined together by a polypeptide linker and the toxin was fused to the carboxyl terminus of the single-chain antibody. These molecules are produced in large amounts in E. coli, where they accumulate in inclusion bodies. To prepare soluble proteins, the inclusion bodies are dissolved in guanidine and renatured using a redox buffer that con-
tains arginine to prevent aggregation. Nevertheless, the yield of single-chain immunotoxins is low, approximately 4%, because the molecules strongly aggregate during the renaturation process. Analysis of the kinetics of the refolding process showed that the variable domains renature more slowly than the toxin (19), indicating that the folding pathway of the variable domain is more complex than that of the toxin. To increase the yield of properly folded molecules, several different approaches were taken. One approach was to alter the refolding protocol (11). Another was to insert different sequences between the antibody and the toxin (19). Other attempts involved utilizing purified E. coli chaperones during the refolding process (24) or trying to secrete the single-chain immunotoxin and allow refolding to take place in the periplasm in a physiological environment (6). However, none of these changes were able to increase the yield >5%.

Fab molecules have been made previously in E. coli, and the yield of the refolded active molecules was reported to be as high as 40% (22). Utilizing this information, we designed several recombinant Fab immunotoxins. Our strategy was to attach the toxin moiety, PE38M, to the carboxyl terminus of the light chain or the carboxyl end of the Cβ domain of the heavy chain (i.e., the Fd fragment) (16). Both strategies worked to increase the yield of active monomers. However, the yield was best (approximately 20%) when PE38M was attached to the end of the light chain. One obvious change produced by adding the constant domain is that the unnaturally exposed surface of Fv domains, which contain hydrophobic Leu residues at positions H11, H11, and L105, are not accessible for interaction with other hydrophobic residues because they are covered by their normal partner residues in the constant domains. Previous attempts to replace these
residues with nonhydrophobic residues did not lead to Fv-toxins with significantly improved activity or refolding yields. Another factor may be that the normal folding pathway by which antibody domains assemble is disrupted in the Fv-containing molecules.

The fusion of PE38KDEL to light chain resulted in a 2-fold higher yield of monomeric active protein than the fusion to Fd chain. This is an interesting result which was unexpected. We thought that attaching PE38KDEL to the Fd chain, which could be viewed as a substitution of PE38KDEL for the Cj2 and Cj3 domains of the heavy chain, would be a better design than attaching PE38KDEL to the light chain, which normally has no flanking peptide chain in the antibody structure. This observation strongly suggests that the increased yield of properly refolded protein is a result of the characteristics of each component.

The variation of positive charge and negative charge in the sequence connecting the antibody and toxin domains did not affect the refolding yield or the activity of the molecule. In the connection peptide, ASGGPKE, the initial four amino acids, ASGG, give a flexible structure, and the proline at the end introduces a kink that stops structural propagation of structure from domain II of PE which refolds more rapidly than Fab fragments of antibodies (19, 22). In the front of this connector, the variation in charged amino acid (K or E) does not influence the folding of the Fd or light chain domains. This indicates that the refolding is mainly dictated by the motifs in the domains, and other residues outside the domains may not be involved in determining the folding pathway unless they strongly interfere with space restriction (19) or some other strong residue-residue interactions.

The B3(Fab)-LE-PE38KDEL and B3(Fab)-LK-PE38KDEL molecules contained a small amount of nondisulfide-bonded heterodimers when analyzed by nondeducing SDS-PAGE. These nondisulfide-bonded molecules represented about 10% of the total recombinant toxin, and their presence did not appear to affect the overall activity of the toxin preparation. One possible explanation of the diminished formation of the disulfide bond between the Fd and light chains of these molecules is that the lysine residue adjacent to the cysteine sterically interferes with disulfide bond formation. In the case of B3(Fab)-Fde-PE38KDEL or B3(Fab)-Fdk-PE38KDEL molecules there is a glycine residue before the lysine that provides sterically free space for the interaction of the two cysteine residues to form a disulfide bond.

Increased Half-Life of Fab Toxin. When injected i.v. into mice, B3(Fab)-Fdk-PE38KDEL and B3(Fab)-LK-PE38KDEL showed a much longer half-life in the circulation than the Fv molecule. For the Fab-PE38KDEL molecules, the t1/2a was ~35 min and the t1/2b was ~5 h. In contrast, single-chain immunotoxins have a t1/2a of 1 min and t1/2b of 15–20 min (12). Furthermore, the half-lives of PE and PE40 in mice are very short; they are ~15 min for PE and ~5 min for PE40 (25). We would expect that the half-life of PE38KDEL would be similar to that of PE40 because it is only slightly smaller. The reason why B3(Fv)-PE38 and PE disappear rapidly from the circulation is unknown. Compared to these molecules, the chemical conjugate of the B3 antibody with PE38 had a long half-life with a t1/2 of 5–6 h, which is similar to that of the B3(Fab)-PE38KDEL molecule. This indicates that the Cj2 and Cj3 regions might have an important role in the half-life of these molecules. Although the biochemical explanation for this finding is not clear, one possible explanation is that it might be more stable in plasma. Studies with intact antibodies containing mutations in different regions of the heavy chain have shown that several domains contribute to increased half-life (26).

Antitumor Experiments. Because all four of the B3(Fab)-PE38KDEL molecules had similar activities on target cells, and two of them had similar pharmacokinetics and toxicity in mice, we chose only one of these for antitumor experiments. The data of Fig. 8 shows that B3(Fab)-Fdk-PE38KDEL caused complete remissions of s.c. tumors when given at 100 µg/kg and partial regressions at 50 µg/kg. This is about the same activity as the corresponding single-chain molecule, B3(Fv)-PE38KDEL. This result was unexpected, because we assumed, because of the prolonged half-life of the Fab-toxin, it would penetrate the tumor better and be more active. The failure to observe increased antitumor effect can be explained by the results of the competition binding assay which showed a 7–8-fold decrease in binding of B3(Fab)-PE38KDEL compared to B3(scFv)-PE38 (data not shown). The activity of the toxin part of these molecules, which was measured by ADP-ribosylation assay, was the same for all tested molecules within experimental error (data not shown). This excludes the possibility of a defect in the ADP-ribosylation domain of the B3(Fab)-PE38KDEL molecule.

Activities and Stabilities. We find that the B3(Fab)-PE38KDEL is more resistant to degradation and survives longer than B3(Fv)-PE38KDEL in all conditions tested, but it has less binding affinity toward the B3 antigen. The stability compensates for low binding and results in the same cytotoxic activity and antitumor activity. In the cytotoxic assay the test cells were cultured at 37°C in media which contained fetal calf serum for about 20 h. B3(Fv)-PE38 aggregates at 37°C, whereas, B3(Fab)-PE38KDEL does not. Furthermore, a protease in the serum would attack the chimeric toxin. B3(Fv)-PE38 is more vulnerable to proteolytic attack because the molecule is not in the form of stable dimer of Vj and Vd domain. In contrast, B3(Fab)-PE38KDEL has a disulfide bond between the heavy and light chain and is in the form of stable dimer. This makes B3(Fab)-PE38KDEL more resistant to proteolytic attack. Similar antitumor activities of B3(Fab)-PE38KDEL and B3(Fv)-PE38KDEL can be explained in the same way, although the B3(Fab)-PE38KDEL has a much longer half-life in mouse blood circulation.

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REFERENCES


7 R. Kreitman, M. Gallo, and I. Pastan, unpublished data.
B3(Fab)-RECOMBINANT IMMUNOTOXIN


CALENDAR OF EVENTS

Fifth Conference on DNA Topoisomerases in Therapy, October 3–6, 1994, New York University Medical Center, New York, NY. Contact: NYU Medical Center, Post-Graduate Medical School, 530 First Ave., New York, NY 10016. Telephone: (212) 263-5295.


Consensus Conference on Follow-Up of Breast Cancer Patients, October 6–8, 1994, Bari, Italy. Contact: Dr. Mario De Lena or Dr. Angelo Paradiso, Oncology Institute, Via Amendola 209, Bari, Italy 70126. Telephone: (39) 80-5555560; FAX: (39) 80-5555559.

Multiple Myeloma: From Biology to Therapy—Current Concepts, October 24–26, 1994, Mulhouse, France. Contact: Francine Morgenthaler, Institut de Recherche en Hématologie et Transfusion, Hôpital du Hasenrain, 87 Avenue d’Altkirch, 68051 Mulhouse Cedex, France. Telephone: (33) 89-64-74-18; FAX: (33) 89-64-71-87.

Fifth Workshop on Mouse Liver Tumors, November 7–9, 1994, Crystal Gateway Marriott Hotel, Arlington, VA. Contact: Ms. Gretchen Bretsch, ILSI Health and Environmental Sciences Institute, 1126 Sixteenth St., N.W., Washington, DC 20036. Telephone: (202) 659-0074; FAX: (202) 659-3859.


Uro-Oncology Update: 1995, January 7, 1995, Ritz-Carlton Hotel, Boston, MA. Contact: Dept. of Continuing Medical Education, Boston University School of Medicine, 80 East Concord St., Boston, MA 02118-2394. Telephone: (617) 638-4605.


Fifth International Congress on Hormones and Cancer, September 17–20, 1995, Quebec Congress Center, Quebec City, Canada. Contact: Congress Secretariat, Fifth International Congress on Hormones and Cancer, Laval University Medical Center, 2705 Boulevard Laurier, Sainte-Foy, G1V 4G2 Quebec, Canada. Telephone: (418) 654-2129; FAX: (418) 654-2714.

International Symposium on Recent Advances in Diagnosis and Treatment of Prostate Cancer, September 21–23, 1995, Quebec Hilton, Quebec, Canada. Contact: Congress Secretariat, International Symposium on Recent Advances in Diagnosis and Treatment of Prostate Cancer, Laval University Medical Center, 2705 Boulevard Laurier, Sainte-Foy, G1V 4G2 Quebec, Canada. Telephone: (418) 654-2129; FAX: (418) 654-2714.

Seventeenth Annual Pharmacy Symposium on Cancer Chemotherapy, October 8–10, 1995, J. W. Marriott Hotel, Houston, TX. Contact: Coni Tierney, Conference Services, Box 131, 1515 Holcombe Boulevard, Houston, TX 77030-4095. Telephone: (713) 792-2222; FAX: (713) 794-1724.

Forty-eighth Annual Research Symposium: Genetic Mechanisms of Cancer, October 17–20, 1995, J. W. Marriott Hotel, Houston, TX. Contact: Coni Tierney, Conference Services, Box 131, 1515 Holcombe Boulevard, Houston, TX 77030-4095. Telephone: (713) 792-2222; FAX: (713) 794-1724.


Erratum

In the article by Choe et al., which appeared in the July 1, 1994 issue of Cancer Research (pp. 3460–3467), an author was omitted from the author line. The authorship of this paper should read as follows: MuHyeon Choe, Lee H. Pal, Keith O. Webber, and Ira Pastan.
B3(Fab)-PE38M: A Recombinant Immunotoxin in Which a Mutant Form of *Pseudomonas* Exotoxin Is Fused to the Fab Fragment of Monoclonal Antibody B3

MuHyeon Choe, Keith O. Webber and Ira Pastan


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