Polyethylene Glycol-modified Chimeric Toxin Composed of Transforming Growth Factor α and Pseudomonas Exotoxin

Qing-cheng Wang, Lee H. Pai, Waldemar Debinski, David J. FitzGerald, and Ira Pastan

Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute, NIH, Bethesda, Maryland 20892

ABSTRACT

Modification of proteins with monomethoxy-polyethylene glycol (mPEG) has been shown to prolong circulation time and to reduce immunogenicity. To make a mPEG-modified recombinant toxin that retained cytotoxic activity but had a longer residence time in circulation, we have constructed an altered form of TGFα-PF284, a recombinant toxin composed of human transforming growth factor α (TGFα) fused to a fragment of Pseudomonas exotoxin (PE38) devoid of its cell-binding domain. In the newly designed protein, termed TGFαR29-L2-C62-PE38QQ4 (TCP), there are no lysine residues in the TGFα and PE38 portions. Human IgG4 constant region C62 and a tetradecapeptide linker, L2, are inserted between TGFα and PE38. Together, L2 and C62 contain 13 lysine residues as potential modification sites for mPEG. mPEG conjugates of TCP (PEG-TCP) were generated and the products were resolved by ion exchange chromatography. Two PEG-TCP species termed B4 and B6 retained 15 and 4% of cytotoxicity, respectively, and 26% of their receptor binding activity compared with the unmodified TCP. Both B4 and B6 had prolonged circulation times in the blood and reduced toxicity in animals. The mean residence times of B4 and B6 were 37 and 68 min, respectively, compared to 7 min for TCP. When administered i.v. to tumor-bearing mice, both B4 and B6 produced marked antitumor effects whereas the unmodified TCP had none. Also, the immunogenicity of PEG-TCP was 5–10 times less than that of TCP. We suggest that the prolonged circulating time and reduced toxicity of PEG-TCP compensate for a diminished cytotoxic activity and enlarge significantly the therapeutic window of this chimeric toxin.

INTRODUCTION

Recently, a number of recombinant toxins have been developed using gene splicing DNA techniques (1–3). In our laboratory, we have produced recombinant toxins containing growth factors, single chain antigen binding proteins and CD4 to mutant forms of PE3 (4–10). These chimeric toxins, which are very cytotoxic to target cells, can be produced in large amounts and are of uniform composition. Moreover, their properties can be modified by mutating the genes that encode them. One feature of recombinant toxins is that they have a lower molecular weight than conventional antibody-toxin conjugates (Mr approximately 195,000). This leads to a shorter survival in the circulation but may also facilitate penetration into tumors. In addition, these chimeric toxins may not be as compactly folded as natural proteins and hence be more susceptible to proteolysis. Recombinant toxins have been found to produce complete regression of some tumors and partial regression of others when tested in nude mice bearing human xenografts (9, 11, 12). There is some evidence that efficacy is enhanced if recombinant toxins survive longer in the bloodstream (12).

One way to increase the lifetime of proteins in the blood is to modify them with mPEG. This agent has been used extensively to modify various enzymes such as adenosine deaminase, asparaginase, superoxide dismutase, monoamine oxidase, immunoglobulins, interleukin 2, and also some allergens (13–22). The mPEG-modified proteins usually have longer circulating lives and reduced immunogenicity compared to unmodified proteins. In some cases, they are also more resistant to proteolytic degradation (23). Katze et al. (19) and Zimmerman et al. (20) have reported that mPEG modification of interleukin 2 increased its potency against a Meth A sarcoma. Kitamura et al. (21) have reported improved tumor localization with a mPEG-modified F(ab’)2 derived from the monoclonal antibody A7. One common feature of the proteins that have been successfully modified by mPEG is that they have a single functional domain. In contrast, recombinant toxins have several functional domains. Therefore, our goal was to design a recombinant toxin that could be modified by mPEG yet retain all of its functions.

PE consists of three major domains (24, 25). Domain Ia (amino acids 1–252) is responsible for cell binding. Domain II (amino acids 253–364) mediates the translocation of the ADP-ribosylating portion of PE into the cytosol. A subdomain termed Ib (amino acids 365–399) has no known function and most of domain Ib can be deleted without loss of activity (26). Domain III (amino acids 400–613) catalyzes ADP-ribosylation of eukaryotic elongation factor 2. PE38 consists of amino acids 252–613 of PE with a deletion of amino acids 365–380. TGFα-PE38 is a chimeric toxin in which TGFα is fused to PE38. This chimeric toxin binds to EGF receptors that are expressed in large amounts on some types of tumor cells (27–30). Because PE38 retains the ADP-ribosylating and translocation functions of PE, the chimeric toxin is extremely cytotoxic but only to the target cells. To prepare a form of TGFα-PE38 that can be modified by mPEG, we altered all of the lysine residues in TGFα and PE38 by mutations to other amino acids. We then inserted the small linking peptide L2, containing 2 lysine residues, and a human C62 domain of IgG4, consisting of 111 amino acids and containing 11 lysine residues (31), between TGFα and PE38 (Fig. 1). The novel mutant protein TGFαR29-L2-C62-PE38QQ4 is abbreviated to TCP. We anticipated that mPEG would modify primarily the lysine residues in the linker and the C62 region because the reactive group p-nitrophenyl carbonate reacts with primary amino groups in proteins. This paper reports on the properties of mPEG-modified TCP.

MATERIALS AND METHODS

Materials. Restriction endonucleases and alkaline phosphatase were obtained from Bethesda Research Laboratories (Gaithersburg, MD), United States Biochemical (Cleveland, OH), or New England BioLabs (Beverly, MA) and used under conditions recommended by the suppliers. T4 DNA ligase was purchased from Boehringer Mannheim (Indianapolis, IN). mPEG-p-nitrophenyl carbonate was obtained from Sigma Chemical Co. (St. Louis, MO). [3H]-EGF was obtained from Amersham (Arlington Heights, IL). [125I]-EGF was...
SphI fragment obtained after digestion of plasmid pWD1661 with TGFαR29) from a plasmid containing the mutant TGFα~ digested with the same enzymes. The protein encoded by pQW4 possesses two lysine residues exclusively within the sequence of L2.

The intermediate plasmids pQW1 and 2 are omitted in this scheme, bp. base pairs.

Plasmid pQW5 encodes TCP. It was constructed by inserting the C3' domain of a human IgG4 constant region between TGFαR29-L2 and PE38QQA. The fragment encoding C3'2 was cleaved by HindIII digestion of the plasmid pB387Cp3'2 encoding TGFα-C3'-PE38 and ligated to the plasmid pQW4 nicked with HindIII.

Purification of TCP. TCP was localized to the inclusion bodies, denatured, renatured, and purified using the same protocol as for the purification of B3Fv-PE38 described by Buchner et al. (33).

Preparation of mPEG-modified TCP (PEG-TCP). TCP was reacted with 30-fold molar excess of mPEG p-nitrophenyl carbonate in 0.2 M phosphate buffer (pH 8.0) containing 1 mM EDTA at 4°C overnight. The reaction mixture was dialyzed twice against 20 mM Tris- HCl (pH 7.4) containing 1 mM EDTA (buffer A) at 4°C. The crude PEG-TCP was separated from other reactants by sequential chromatography on Q-Sepharose and Mono Q with a NaCl gradient (0-0.3 M) in buffer A. Uncoupled mPEG derivatives did not bind to the Q-Sepharose column. Unmodified TCP was eluted last from both columns and measured as negative as measured by BaCl2-1 reaction (34, 35).

The SDS-PAGE profile of the crude PEG-TCP showed that there were six major bands in the gel. They were referred to as B1-B6, respectively, according to their apparent molecular weights from low to high. The fractions eluted from Mono Q containing a given species of PEG-TCP were pooled and enriched by the chromatography on Mono Q.

Protein Assay. The protein concentration of TCP and PEG-TCP species was determined using Pierce Coomassie Plus Protein Assay Reagent. Bovine serum albumin was used as the standard protein.

Cytotoxicity and Binding Assays. A431 cells expressing more than 2 x 10⁶ receptors/cell were used as target cells. The cytotoxicity of TCP and PEG-TCP was measured as described previously (25). The binding assay was based on the competition of 125I-EGF. The assay was carried out basically as the experiment described by Kreitman et al. (36).

LD₅₀ in Mice. TCP and PEG-TCP species of different molecular weights were diluted in 0.2% human serum albumin-phosphate-buffered saline and BALB/c mice (6 weeks old, 20-22 g) received injections into the tail veins of 0.2-ml aliquots of various doses. Animals were observed for 10 days for signs of toxicity or death.

Pharmacokinetics of TCP and PEG-TCP in Mice. BALB/c mice (6 weeks old, 20-22 g) were given a single dose i.v. of TCP (250 μg/kg) or PEG-TCP species (500 μg/kg). Blood samples were collected at 2, 10, 30, 45, 60, 90, 120, 180, and 240 min. Each time point represents the mean results obtained from three animals. The concentration of the toxin was determined by incubating the serum with A431 cells and by measuring its ability to inhibit protein synthesis. A standard curve was used to determine the concentration of toxin in each sample.

Antitumor Activity of TCP and PEG-TCP in Nude Mice Bearing a Human Epidermoid Carcinoma. A431 cells (3 x 10⁶ cells) were injected s.c. on day 0 into female athymic nude mice (4-6 weeks old, 18-20 g). Tumors measuring about 5 x 5 mm developed in all animals by day 5. Treatment with TCP and PEG-TCP was started on day 5 after tumor implantation. Each treatment group consisted of five or six animals. Tumors were measured with a caliper every second day and the volume of the tumor was calculated as described (37).

Detection of Anti-PE Antibody. An ELISA was used to determine the presence of mouse anti-PE antibodies in the serum of treated and control animals. Microtiter 96-well plates (Dynatech Laboratories, Alexandria, VA) were coated with PE38QQA (50 ng/well) and incubated overnight at 4°C. Each time point represents the mean results obtained from three animals. The concentration of the toxin was determined by incubating the serum with A431 cells and by measuring its ability to inhibit protein synthesis. A standard curve was used to determine the concentration of toxin in each sample.

Chimeric Toxin Neutralization by Serum Containing Anti-PE Antibodies. Serum from animals immunized with TCP and PEG-TCP and control serum were diluted with TCP at a final concentration of 0.1, 1, 10, 100, and 1000 ng/ml and then incubated for 30 min at 37°C prior to the protein synthesis assay.

a J. Butz and I. Pustan, unpublished work.

Linker 2: 5'-CCGACCTCTGCTAGTAACTTCGTAGCCTGGTTCAGAATCTACACACTCA-3' 3'-GTACCGGGAGGAGCCAGTCTCAAGTGGAGAGTTGAGTTGAGA-5'
RESULTS

Preparation of TCP and PEG-TCP. To prepare a chimeric toxin that could be derivatized with mPEG in such a manner that cell binding, translocation, and ADP-ribosylation activities would be retained, we engineered an altered form of TGFα-PE38 in which potential reactive lysine residues were located only in positions not involved with any of these three essential processes. To this end, the single lysine residue in TGFα was converted to an arginine (TGFαR29), the two lysine residues within PE38 at positions 590 and 606 were converted to glutamines, and lysine 613 was deleted (PE38QQA). In addition, a lysine-rich domain was placed between TGFα and PE38. This lysine-rich domain consists of a CH2 domain derived from a human IgG4 constant region preceded by a small peptide containing two lysine residues (L2). The construction of the plasmid used to express TCP is shown in Fig. 1. The chimeric toxin accumulated in E. coli within inclusion bodies and was purified as previously described (33). This molecule is designed so that, despite derivatization with mPEG, it could be normally processed within target cells and could release a Mr 37,000 fragment that has no mPEG attached to it.

The derivatization procedure with polyethylene glycol was carried out as described in “Materials and Methods.” Analysis of the derivatized products by SDS-PAGE showed that six major species with different molecular weights were present and referred to as B1–B6, respectively. To separate these species, the mixture of derivatized molecules was applied on a Mono Q column and eluted with a gradient of NaCl as shown in Fig. 2. It is evident that the higher molecular weight proteins eluted from the column before the lower molecular weight species. However, this single chromatographic step did not adequately resolve species of different molecular weights. To obtain better separation, selected column fractions were pooled and rechromatographed on Mono Q columns. The apparent molecular weights of B1–B6 are 72,000, 81,000, 87,000, 103,000, 122,000, and 135,000, respectively.

Cytotoxicity and Binding Activity of PEG-TCP. Each fraction (B1–B6) was tested for its ability to inhibit protein synthesis using A431 cells and for its ability to compete for the binding of 125I-EGF to the EGF receptor on A431 cells. The data are summarized in Table 1 and Fig. 4. Generally, we found that as the molecular weight of PEG-TCP increased, reflecting the degree of derivatization by PEG, the binding activity and cytotoxic activity decreased. Fractions B2 and
B4-B6, retained between 15 and 4% of their cytotoxicity, though they differ in molecular weight. The higher molecular weight B3 have similar cytotoxicities and similar binding properties, even though they differ in molecular weight. The higher molecular weight TCP concentration at time 0. High molecular weight mPEG derivatized molecules to 67 min for the most highly derivatized molecules (B6). Clearance rates changed accordingly. The major changes were evident in the $t_{1/2}$ of the clearance curves (Table 2). Fig. 5 shows the relative serum concentrations of chimeric toxins as a percentage of TCP concentration at time 0. High molecular weight mPEG derivatized TCP molecules (B4 and B6) had a much longer survival in the plasma than less modified (B2) or unmodified TCP.

**Animal Toxicity Studies.** To determine the amount of TCP that could be administered to animals for therapeutic studies, increasing amounts of the parental molecules and the modified mPEG molecules were injected into mice and the $LD_{50}$ was obtained by noting the doses that caused death. As shown in Table 3, a significant increase in $LD_{50}$ was observed upon "PEGylation." The unmodified TCP had an $LD_{50}$ of approximately 50 $\mu$g/kg, whereas the most derivatized mPEG-TCP had an $LD_{50}$ of 400 $\mu$g/kg.

**Antitumor Activity.** The data in Fig. 6 compare the antitumor activity of PEGylated molecules with that of unmodified TCP. In these experiments, A431 cells (3 $\times$ 10^6 cells) were injected s.c. into athymic mice on day 0. Therapy with TCP or PEG-TCP was delivered on days 4, 5, and 6, when the tumor reached 0.5 x 0.5 cm. Animals received various doses of TCP (1.25, 2.5, 5, or 10 $\mu$g/kg/day for 3 doses) or PEG-TCP species B2, B4, or B6 (50, 100, 150, 200, or 250 $\mu$g/kg/day for 3 doses). The control group received the phosphate buffer solution. Fig. 6 shows the effect of TCP and B4, B5, and B6 when given at the maximum tolerated doses (the highest dose which does not produce weight loss or death). The maximum tolerated dose for TCP is 2.5 $\mu$g/kg/day for 3 doses; for B4, B5, and B6 maximum tolerated doses are 100, 150, and 200 $\mu$g/kg/day for 3 doses, respectively. As shown in Fig. 6, high molecular weight derivatized chimeric toxin molecules (B5 and B6) are better tolerated and are capable of causing tumor regression in nude mice when given by i.v. bolus injection. In contrast, no antitumor effect was noted with the unmodified molecule, even near or at the MTD.

**Detection of Anti-PE Antibodies.** Other researchers have demonstrated that derivatization of proteins, such as bovine serum albumin and liver catalase, with mPEG makes these molecules less immunogenic to animals (23, 39). To study the immunogenicity of TCP and PEG-TCP, three experiments were performed. First, we administered similar doses of TCP and conjugates B4 and B6 to BD2 F1 mice (6-8 weeks old) and compared the antibody response to that of PE. Animals received daily i.p. injections of 2.5 or 5 $\mu$g/kg of TCP or PEG-TCP species B4 or B6 for 7 days. Blood samples were collected on day 10 and mouse anti-PE antibody levels were determined by ELISA. At 2.5 $\mu$g/kg/day, no antibody was detected in the animals that received B4 or B6 of PEG-TCP. At 5 $\mu$g/kg/day, anti-PE was present in the TCP group (Fig. 7) but not in the animals that received either B4 (not shown) or B6. We next administered higher doses of B6 conjugate (25, 50, or 100 $\mu$g/kg/day for 7 doses) and compared its immunogenicity with TCP, 5 $\mu$g/kg/day for 7 doses. Higher doses of chimeric toxins

<table>
<thead>
<tr>
<th>Protein</th>
<th>LD_{50} (mg/kg)</th>
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<tbody>
<tr>
<td>TCP</td>
<td>50</td>
</tr>
<tr>
<td>PEG-TCP (B2)</td>
<td>75</td>
</tr>
<tr>
<td>PEG-TCP (B4)</td>
<td>200</td>
</tr>
<tr>
<td>PEG-TCP (B6)</td>
<td>400</td>
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</table>

Table 3 LD_{50} of TCP and PEG-TCP species B2, B4, and B6 in mice

The calculations of MRT, clearance, $t_{\alpha}$, and $t_{\beta}$ were based on the data shown in Fig. 5. Pharmacokinetic parameters were calculated using the exponential curve fitting program RSTRIP (MicroMath Scientific Software, Salt Lake City, UT).

![Fig. 5. Pharmacokinetic profile of PEG-TCP species B2, B4, and B6 in mice. The toxin concentration was determined as described in "Materials and Methods." The relative serum concentration is exhibited as percentage of the toxin concentration at time 0. (*) TCP, (o) B2, (:) B4, (c) B6.](image)

![Fig. 6. Effect of TCP and PEG-TCP on the growth of A431 tumors in nude mice. Mice received injections of 3 $\times$ 10^6 A431 cells on day 0 and were treated i.v. on days 4, 5, and 6 with TCP (■) and PEG-TCP species B4 (□), B5 (△), and B6 (▲). Control group received PBS (○).](image)
was used to detect the antibody response. At 5 and 25 μg/kg, the antibody response was measured on day 10 by ELISA. Results are reported as absorbance (OD) values of serum specimens diluted 1:100. Fig. 7 shows a histogram of mouse immunoglobulin response following immunization. Native PE mPEG derivatized TCP was weakly immunogenic. At higher doses, however, the mPEG-conjugated protein was as immunogenic as TCP.

To determine if the antibody produced in response to mPEG-TCP had neutralizing activity, serum from animals immunized with TCP and mPEG-TCP and control serum were mixed with TCP at a final concentration of 0.1, 1, 10, 100, and 1000 ng/ml; incubated for 30 min at 37°C; then tested for inhibition of protein synthesis. As shown in Fig. 8, sera from animals immunized with 5 μg of TCP or 50 and 100 μg of B6 were capable of neutralizing up to 10 ng/ml of TCP, while serum from animals immunized with 5 (not shown) and 25 μg/kg of B6 were not.

DISCUSSION

In this study, we have constructed an altered form of TGFα-PE38 that can be derivatized with polyethylene glycol yet still maintains its cytotoxic activity. We found that PEGylated conjugates, with increasing amounts of PEGylation (B2, B4, and B6), were active and had extended lifetimes in the blood. Moreover, B4 and B6 exhibited markedly enhanced antitumor activities when compared to unmodified chimeric toxin. Thus, we achieved our goal of preparing an active PEGylated chimeric toxin that had a prolonged lifetime in the blood and enhanced antitumor activity.

We observed that the chimeric toxin with the lowest amount of PEGylation, such as the B2 or B3 conjugate, had higher cytotoxicity and cell-binding ability than molecules with more polyethylene glycol chains added, i.e., B4 and B6 conjugates. However, the decrease in cytotoxicity with increasing derivatization was not parallel to the observed decrease in cell binding. For example, B4, B5, and B6 PEG-TCPS have quite similar binding activities, yet B6, which has the highest molecular mass of 135 kDa, retains only 4% of its original cytotoxicity. These results suggest that the decrease in binding to the EGF receptors is not the sole reason for the reduction in PEG-TCP cytotoxicity. The mPEG chains probably interfere with some other steps in toxin function.

The lifetime of the B2 conjugate in the plasma was only slightly prolonged, whereas B4 and B6 survived much longer than the parent protein. At the 90-min point, 4.5% of B4 and 13% of B6 were left in the circulation while only 0.13% of the parent protein was present (Fig. 5). In addition, the toxicity to mice of B4 and B6 was 4-8 times lower than that of the parent protein. The extended plasma half-life and the reduced toxicity of B4 and B6 conjugates in mice were enough to compensate for their reduced cytotoxic activity to target cells and made these molecules more therapeutically effective. Kitamura et al. (21, 40) found that PEGylated F(ab')2 of the monoclonal antibody A7 had less uptake in the liver and kidney and had a higher tumor:normal tissue ratio when compared with the parent F(ab')2, thus achieving a more specific localization of the F(ab')2. Also, less uptake of PEGylated tissue plasminogen activator by the liver was found by Bergeret et al. (41). Thus, we speculate that in addition to the reduction in cytotoxicity, the reduction of the animal toxicity of PEG-TCP may partly be due to less liver uptake of PEG-TCP. The improved antitumor effect of PEG-TCP may be attributed to a higher tumor:normal tissue ratio of PEG-TCP and a higher tolerance of the mice to it.

The results shown in Figs. 7 and 8 show that the more heavily PEGylated TCP has lower immunogenicity in comparison with the less PEGylated material. It is plausible, as hypothesized by Shohon (16), that down-regulation of the primary antibody response was due in part to activation of antigen-specific suppressor T-cells (16, 42). It is also possible that PEGylation of antigens influences their degradation in macrophages or that the PEG chains shield some epitopes of the peptides derived from the antigens after the degradation. We believe it is likely that some portion of PE is released by proteolysis in vivo, thereby eliciting an immune response. Therefore, it will be necessary in the future to couple PEG chains to appropriate sites on PE to reduce the immunogenicity of PE-containing chimeric toxins further.

A number of enzymes and immunoglobulins have been highly modified by mPEG. For instance, adenosine deaminase coupled with dozens of flexible mPEG chains still retains about 60% of its original enzyme activity (43). Because of its relatively large size, mPEG couples only to residues on the surface of protein molecules and usually does not block the active site of enzymes which are usually buried deeper within the protein structure. Because the mPEG-masked surface remains permeable to low molecular weight substrates, the enzymes can continue to operate despite extensive modifications.

Fig. 7. Histogram of mouse IgG + IgM response to PE following 7 daily doses of TCP (5 μg/kg/day) and PEG-TCP (B6) (5, 25, 50, and 100 μg/kg/day). Antibody response was measured on day 10 by ELISA. Results are reported as absorbance (OD) values of serum specimens diluted 1:100. Bars, SD.

Fig. 8. Serum from mice immunized with multiple doses of TCP, 5 μg/kg/day (●), and PEG-TCP (B6), 25 μg (●), 50 μg (○), and 100 μg/kg/day (□) and control sera (□) were incubated with TCP and tested for neutralizing activity in A431 cells.

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However, the same scenario is not applicable to chimeric toxins. In the case of TGFα-PE38, its cytotoxicity is dependent on several different interactions. For example, TGFα must bind to its receptor, and domain III of PE must interact with NAD+ and elongation factor 2. In addition, there must be a proteolytic cleavage between arginine 279 and glycine 280 that generates a M, 37,000 peptide fragment that must be translocated across a membrane into the cytosol. Therefore, we had to prepare a recombinant toxin that would still undergo these interactions after PEGylation. We used TGFα-P because Defeo-Jones et al. (44) reported that a conservative substitution of arginine for lysine 29 of human TGFα had little effect on cell-binding or mitogenesis. Chaudhary et al. (45) found that deletion of Lys613 from PE and the substitution of glutamine for Lys590 and Lys606 had no effect on the cytotoxicity of PE. Also, Debinski and Pastan5 found that PE40QQD retained full activities of PE40. Finally, Kasturi et al.6 found that a C42 domain of IgG4 can be inserted in CD4-PE40 between the CD4 and PE40 domains without loss of cytotoxic activity. On the basis of these observations, we designed TCP, expecting that it could be PE- Gylated and would retain its cytotoxicity. The mPEG chains should couple mainly to the L2 and C2 regions of TCP, although the NH2-terminal methionine residue was available for PEGylation. Using this strategy, we produced a series of active PEGylated conjugates of TCP. We found that PEGylation often resulted in a considerable loss in cytotoxicity, as discussed above. To explain this, we suggest that the unfolded, flexible mPEG chain that has a backbone of about 150 atoms may sterically interfere with TGFα binding or with functions of domains II or III of PE38. The NH2 terminus of TGFα is exposed on the surface of the molecule (46). Therefore, we cannot exclude the possibility that a mPEG chain may also be coupled to the NH2 terminus and in this way interfere with the binding of TGFα to its receptor.

mPEG has only one hydroxyl group in each polymer chain and this can be transformed to an activated form. Three major reactive derivatives of mPEG have been studied. These are cyanuric chloride activated mPEG (39), mPEG-succinimidyl succinate (47), and mPEG-NPC (48). The cyanuric chloride derivative is toxic and may also react with amino acid residues other than lysine (47). The ester linkage between mPEG and the succinic acid residue is labile in aqueous solution (22, 49). The reaction of mPEG-NPC with proteins yields urethane-linked proteins that are stable under physiological conditions (48). Therefore, we chose to use mPEG-NPC to modify our chimeric toxins. Because the lysine residues of TCP were modified by mPEG-NPC, the resulting conjugates were more acidic than the parent protein. Yet contrary to expectations, the less PEGylated toxins eluted later from the Mono Q column than the heavily PEGylated molecules, and the unmodified toxin eluted last. To account for this behavior, we suggest that the mPEG chains may shield the charges of the TCP molecule or hamper the binding of the negatively charged functional groups of the molecule to the Mono Q column. Similar phenomena have been noted by Davis et al. (50) and Somack et al. (51).

In the presence of 2-mercaptoethanol, the ester linkage between PEG and p-nitrophenyl carbonate is not completely stable at high temperature. Therefore, some PEG chains may be released when heating the PEG-TCP samples for SDS-PAGE, generating lower molecular weight PEG-TCP species, and even free TCP. Using nonreducing SDS-PAGE, B5 and B6 were quite pure and not contaminated with free TCP, and B4 was contaminated with a small amount of free TCP (data not shown). Since the pharmacokinetic data were measured by cytotoxicity assays and unPEGylated TCP was cleared very rapidly in vivo, we cannot attribute the prolonged half-life of PEG-TCP to the presence of free TCP. For a similar reason, it is very unlikely that the enhanced antitumor effects shown by PEG-TCP are due to the contamination with free TCP because free TCP was much too toxic to mice and ineffective at its MTD.

REFERENCES


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RECOMBINANT TOXINS

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