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Single-Chain Immunotoxins Directed at the Human Transferrin Receptor Containing *Pseudomonas* Exotoxin A or Diphtheria Toxin: Anti-TFR(Fv)-PE40 and DT388-Anti-TFR(Fv)

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Two single-chain immunotoxins directed at the human transferrin receptor have been constructed by using polymerase chain reaction-based methods. Anti-TFR(Fv)-PE40 is encoded by a gene fusion between the DNA sequence encoding the antigen-binding portion (Fv) of a monoclonal antibody directed at the human transferrin receptor and that encoding a 40,000-molecular-weight fragment of *Pseudomonas* exotoxin (PE40). The other fusion protein, DT388-anti-TFR(Fv), is encoded by a gene fusion between the DNA encoding a truncated form of diphtheria toxin and that encoding the antigen-binding portion of antibody to human transferrin receptor. These gene fusions were expressed in *Escherichia coli*, and fusion proteins were purified by conventional chromatography techniques to near homogeneity. In anti-TFR(Fv)-PE40, the antigen-binding portion is placed at the amino terminus of the toxin, while in DT388-anti-TFR(Fv), it is at the carboxyl end of the toxin. Both these single-chain immunotoxins kill cells bearing the human transferrin receptors. However, anti-TFR(Fv)-PE40 was usually more active than DT388-anti-TFR(Fv), and in some cases it was several-hundred-fold more active. Anti-TFR(Fv)-PE40 was also more active on cell lines than a conjugate made by chemically coupling the native antibody to PE40, and in some cases it was more than 100-fold more active.

Immunotoxins made by chemically coupling tumor-specific monoclonal antibodies to bacterial or plant toxins, including *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT) have been shown to possess in vitro and in vivo antitumor activity (10, 19, 24). Both PE and DT kill cells by ADP-ribosylating elongation factor 2, thereby inhibiting protein synthesis. X-ray crystallographic and mutational analyses show the PE molecule to be made up of three distinct domains, namely, an amino-terminal cell-binding domain, a middle translocation domain, and a carboxyl-terminal activity domain (1, 12). DT, which is divided into two chains, A and B, linked by a disulfide bridge, is arranged functionally in the orientation opposite to that of PE. Chain B of DT, which is at the carboxyl end, is responsible for receptor binding, and chain A, at the amino end, contains the enzymatic activity (22, 23). Apparently the last 150 amino acids of DT are responsible for its cell-binding activity (21–23).

We have constructed immunotoxins by attaching antibodies either to native PE that has an M_r of 66 kDa or to truncated forms of PE that lack its cell-binding domain and have an M_r of about 40 kDa (referred to as PE40). Immunotoxins made with PE40 or LysPE40, a PE40 derivative with an extra lysine residue at its amino terminus that facilitates coupling to antibodies, have greater specificity for target cells because PE cannot bind to its cellular receptor (3, 13, 20). One such immunotoxin, anti-TFR-LysPE40, was shown to be highly cytotoxic to cells expressing the human transferrin receptor. When given intraperitoneally to mice, it caused regression of A431 tumors growing as subcutaneous xenografts (3). Immunoconjugates with low nonspecific toxicity have also been made by using modified DT with mutations in its cell-binding domain (17, 25).

Immunotoxins constructed by chemical cross-linking are heterogeneous and are difficult to produce in large amounts because the antibody and toxin must be made and purified separately and then conjugated in a reaction that often has a low yield. To address this problem, we have made two recombinant single-chain immunotoxins directed at the interleukin-2 receptor. The two are termed anti-Tac(Fv)-PE40 and DT388-anti-Tac(Fv). The Fv fragment is the smallest binding unit of an antibody which consists of a light- and a heavy-chain variable domain. Both single-chain immunotoxins consist of the variable domains of a monoclonal antibody directed at the interleukin-2 receptor arranged in a single-chain form and linked to the amino terminus of PE40 (2, 9) or to the carboxyl end of a truncated form of DT (7). Single-chain immunotoxins are constructed by starting with hybridoma RNA, using polymerase chain reaction (PCR) to amplify the DNA encoding the variable heavy and variable light chains of the antibodies, and inserting this DNA into an expression vector containing PE40 sequences (4). We used this rapid cloning technique and now report the construction of two single-chain immunotoxins, anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv). In anti-TFR(Fv)-PE40, the single-chain anti-TFR(Fv), containing the variable heavy chain of a monoclonal antibody (HB21) to the human transferrin receptor (anti-TFR) (11) linked through a peptide linker to its variable light chain, is fused to the amino terminus of PE40. In DT388-anti-TFR(Fv), the single-chain anti-TFR(Fv) is linked to the carboxyl end of the truncated DT. Both of these single-chain

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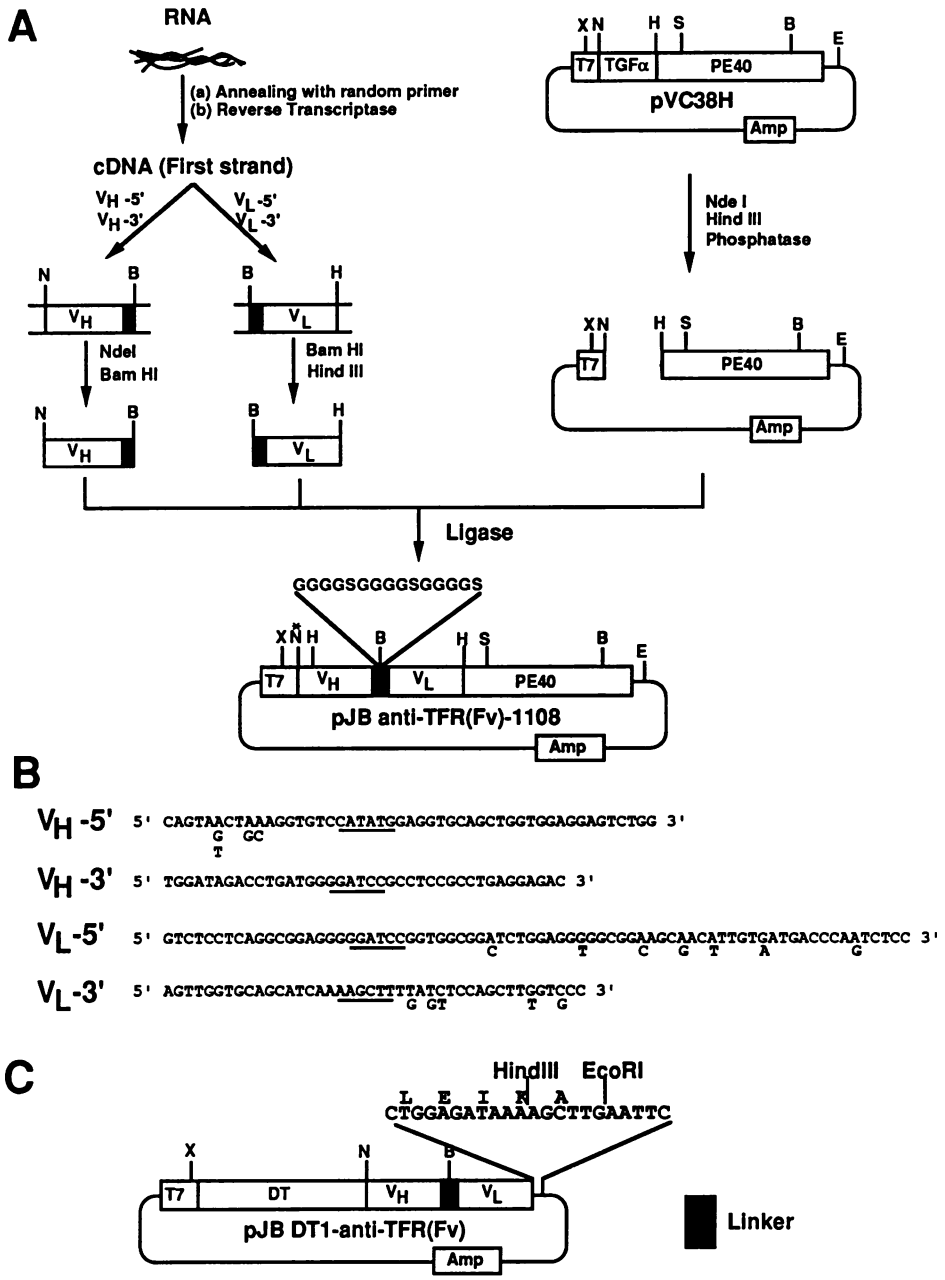

FIG. 1. (A) Scheme for construction of plasmid pJB anti-TFR(Fv)-1108 encoding anti-TFR(Fv)-PE40. N, *Nde*I; B, *Bam*HI; H, *Hin*d III; X, *Xba*I; S, *Sal*I; E, *Eco*RI. Fifteen amino acids of the linker are shown in single-letter code. (B) Structure of oligonucleotides used for PCR. (C) Expression plasmid pJBDT1-anti-TFR(Fv).

immunotoxins were expressed in *Escherichia coli* and purified to near homogeneity. Anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv) were very cytotoxic to cell lines bearing human transferrin receptors, but large and unexpected differences in the activities of these molecules on target cells were detected.

MATERIALS AND METHODS

Plasmid constructions. The scheme for construction of expression plasmid pJB-anti-TFR(Fv)-1108 encoding anti-TFR(Fv)-PE40 is shown in Fig. 1A. Isolation of RNA, cDNA first-strand synthesis, and PCR conditions have been described previously (4). Primers used to amplify the variable domains are shown in Fig. 1B. The purified PCR products were digested with *Nde*I and *Bam*HI for the fragment encoding V_H and part of the linker at its 3′ end or with *Bam*HI and *Hin*dIII for V_L and the rest of the linker at its 5′ end. Expression vector pVC38H was digested with *Nde*I and *Hin*dIII and dephosphorylated with calf intestinal phosphatase. The DNA fragments were purified on Sea-Plaque agarose gel. A three-fragment ligation was set up with 3.6-kb dephosphorylated vector and V_H and V_L fragments. The recombinants were screened with *Xba*I, *Sal*I, *Hin*dIII,

and *Bam*HI. Several positive clones were checked for protein expression in BL21 (λDE3).

Figure 1C shows the plasmid used for the expression of DT388-anti-TFR(Fv). To construct this plasmid, PCR with pJB-anti-TFR(Fv)-1108 as template was used to amplify DNA for anti-TFR(Fv) such that it had *Nde*I at the 5′ end and *Eco*RI at the 3′ end. The PCR-amplified fragment was restricted with *Nde*I and *Eco*RI and ligated to pVC-DT-IL2 (6, 7) digested with *Nde*I and *Eco*RI. The recombinants were screened with *Hin*dIII, *Bam*HI, and *Nde*I. pVC-DT1-IL2 contains the first 388 codons of DT with the initiator methionine fused to cDNA encoding human interleukin-2 (6).

Protein expression and purification. BL21 (λDE3) cells were transformed with the plasmids pJB anti-TFR(Fv)-1108 or pJB DT388-anti-TFR(Fv). The cells were grown in superbroth containing 100 μg of ampicillin per ml at 37°C. At an A_{650} of 0.8, cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested 90 min later, and the location of the fusion protein was determined as described elsewhere (5). The fusion protein was isolated from the inclusion bodies by denaturation in 7 M guanidine HCl and renaturation by rapid dilution in phosphate-buffered saline. After dialysis, the renatured protein was applied to an 8-ml Q-Sepharose column. Proteins were batch eluted from the column with 0.1, 0.35, and 1 M NaCl. The pool containing the desired fusion protein (0.35 M salt eluate) was diluted and loaded onto a Mono Q 10/10 fast protein liquid chromatography column. The fusion protein was eluted by a linear gradient of 0 to 0.5 M NaCl in 0.02 M Tris (pH 7.4). Further purification was achieved by gel filtration chromatography on a TSK 250 column.

Cytotoxicity assay. Cytotoxic activities of anti-TFR(Fv)-PE40, anti-TFR-LysPE40, and DT388-anti-TFR(Fv) were determined by assaying the inhibition of protein synthesis as measured by [^{3}H]leucine incorporation into total cell protein (9). The results are expressed as percentage of control, to which no toxin was added. For competition experiments, 20 μg of antibody was added per well prior to the addition of the toxin (9).

Binding studies. ^{125}I-labeled HB21 (20 μCi/μg) was added as a tracer at 1.5 ng per assay with various concentrations of competitor and 4×10^5 HUT102 cells or 5×10^5 A431 cells in 0.2 ml of binding buffer (RPMI 1640 with 10% fetal bovine serum, 100 μg of human immunoglobulin per ml, and 0.1% sodium azide) and incubated at room temperature with shaking for 2 h. At the end of the incubation, cells were washed three times with the binding buffer and counted in a gamma counter (9).

Other methods. Anti-TFR-LysPE40 was constructed as described elsewhere (3). Protein was assayed by Bradford's method with Bio-Rad protein assay reagent (3a). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Laemmli (14).

RESULTS

Plasmid construction, protein expression, and purification. The structure of a plasmid encoding anti-TFR(Fv)-PE40 is shown in Fig. 1A. The assembled gene is under the control of a bacteriophage T7 promoter. The plasmid expresses the first ~110 amino acids of anti-TFR heavy chain, a 15-amino-acid linker containing $(Gly_4Ser)_3$, the first ~100 amino acids of anti-TFR light chain, and amino acids 253 to 613 of PE. The expression plasmid pJB-DT388-anti-TFR(Fv) shown in Fig. 1C encodes the first 388 amino acids of DT fused to the variable heavy domain of anti-TFR antibody, which is connected to its variable light chain through the $(Gly_4Ser)_3$ linker. Anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv) were expressed in *E. coli* (BL21 λDE3), and after induction with IPTG, the fusion proteins were contained primarily in the inclusion bodies. The fusion protein from the inclusion bodies was denatured in 7 M guanidine HCl and renatured by rapidly dilution in phosphate-buffered saline. Purification was performed by chromatography on a Q-Sepharose anion-exchange column and then on a Mono Q fast protein liquid chromatography column. The pool containing the active protein was finally purified by gel filtration on a TSK 250 column. The purity of anti-TFR(Fv)-PE40 and DT388-anti-TFR(Fv) at each purification step is shown in Fig. 2A and B, respectively. The protein after gel filtration chromatography appeared to be >90% pure and was used for all subsequent studies.

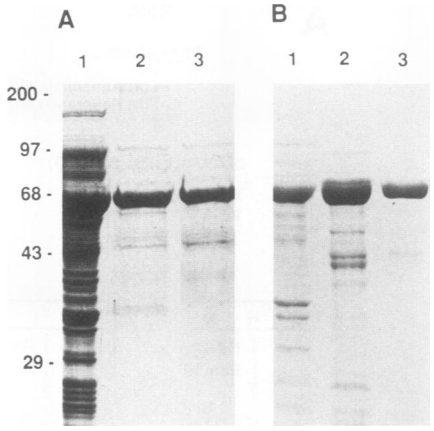

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of anti-TFR(Fv)-PE40 (A) and DT388-anti-TFR(Fv) (B) at different steps of purification. Lane 1, Inclusion bodies; lane 2, Mono Q pool; lane 3, TSK gel filtration pool. Gel was stained with Coomassie blue. Numbers at left are molecular sizes in kilodaltons.

Cytotoxicity and specificity of anti-TFR(Fv)-PE40. As shown in Fig. 3, anti-TFR(Fv)-PE40 was extremely active and inhibited the protein synthesis of A431 cells in a dose-dependent manner with a 50% inhibitory dosage (ID_{50}) of 0.02 ng/ml, which is equivalent to 0.2 pM (Table 1). The cytotoxic effect was blocked by competition with excess

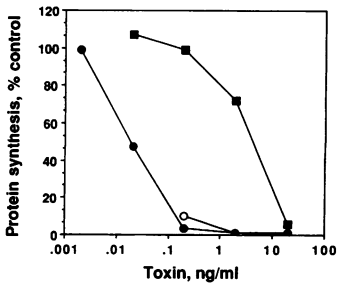

FIG. 3. Cytotoxicity of anti-TFR(Fv)-PE40 on A431 cells. Cells were incubated with the toxin for 16 to 20 h and pulsed with [^{3}H]leucine, and radioactivity was measured in the trichloroacetic acid-precipitable protein. Results are shown as percentage of control, to which no toxin was added. Symbols: ●, anti-TFR(Fv)-PE40 alone; ○, anti-TFR(Fv)-PE40 plus 20 μg of OVB3; ■, anti-TFR(Fv)-PE40 plus 20 μg of HB21. HB21 is a monoclonal antibody to the human transferrin receptor produced by the hybridoma used as the source of RNA for cloning the variable domains.

TABLE 1. Activity of anti-TFR(Fv)-PE40 on various human cell lines

Cell line	Origin	ID_{50} (pM)		Relative activity[a]
		Anti-TFR(Fv)-PE40	Anti-TFR-LysPE40	
A431	Epidermoid carcinoma	0.18	20	111
KB	Epidermoid carcinoma	0.10	75	750
MCF7	Breast carcinoma	0.14	13.5	96
OVCAR3	Ovarian carcinoma	8.0	1,000	125
HUT102	Adult T-cell leukemia	37.0	50	1.3
HT29	Colon carcinoma	45.0	160	3
PC3	Prostate carcinoma	58.5	280	5
LNCAP	Prostate carcinoma	2.6	10	4
DU145	Prostate carcinoma	92.0	400	4
L929	Fibroblast (mouse)	>30,000	>10,000	
Swiss 3T3	Fibroblast (mouse)	>30,000	>10,000	

[a] Ratio of activity of anti-TFR(Fv)-PE40 to activity of anti-TFR-LysPE40 on the same cell line.

anti-TFR, whereas OVB3, a control antibody, did not block the cytotoxicity, demonstrating the specificity of anti-TFR(Fv)-PE40 (Fig. 2) for the transferrin receptor. Also, the fusion protein at 2 μg/ml (30 nM) did not inhibit protein synthesis in either mouse Swiss 3T3 or mouse L929 cells, reflecting the species specificity of the antibody (Table 1).

Comparison of cytotoxic activities of recombinant immunotoxin and chemical conjugate. The activity of anti-TFR(Fv)-PE40 was assayed on a variety of human cell lines and compared with that of anti-TFR-LysPE40, the chemical conjugate composed of the native antibody and PE40 (3). As shown in Table 1, anti-TFR(Fv)-PE40 inhibited protein synthesis in all the human cell lines studied, with ID_{50}s ranging between 0.10 and 92 pM. The chemical conjugate anti-TFR-LysPE40 was also toxic to these cells. However, ID_{50}s were up to 750-fold lower than that of the recombinant single-chain immunotoxin. Both molecules were inactive on mouse cells, demonstrating their specificity for human transferrin receptor (Table 1).

Binding of anti-TFR(Fv)-PE40 to the transferrin receptor. To compare the binding activity of anti-TFR(Fv)-PE40 with that of the native antibody, competition binding analyses were performed with HUT102 and A431 cells (Fig. 4). In these studies, the abilities of various chimeric toxins to compete for the binding of ^{125}I-HB21 (anti-TFR) to A431 cells was determined. Anti-TFR(Fv)-PE40 was shown to compete for binding to the human transferrin receptor very efficiently; its binding affinity was similar to that of HB21 on A431 cells and approximately twofold lower on HUT102 cells. In the same experiments, the chemical conjugate anti-TFR-LysPE40 was tested and found to compete much less well than native HB21. The binding affinity of anti-TFR-LysPE40 appears to be about 10-fold lower than that of the native anti-TFR antibody (Fig. 4).

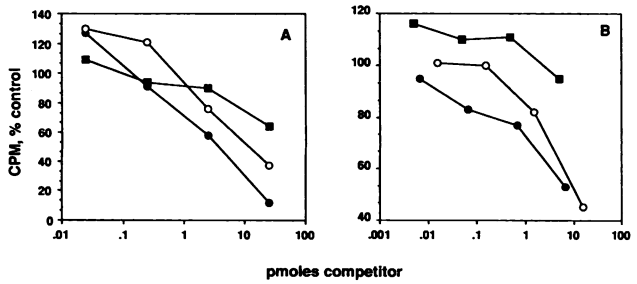

FIG. 4. Competition binding analysis of anti-TFR antibody, HB21, versus anti-TFR(Fv)-PE40. Competition of anti-TFR(Fv)-PE40 (○), HB21-LysPE40 (■), and native HB21 (●) with ^{125}I-labeled tracer HB21 on HUT102 (A) and A431 (B) cells.

TABLE 2. Activity of DT388-anti-TFR(Fv) on various human cell lines

Cell line	Toxin ID_{50} (pM)	Ratio of DT-anti-TFR(Fv) to anti-TFR(Fv)-PE40[a]
A431	88	0.002
KB	82	0.001
MCF7	59	0.002
OVCAR3	34	0.2
HUT102	10	3.7
HT29	16	2.8
L929	15,000	

[a] Ratio of ID_{50}s of DT-anti-TFR(Fv) to those of anti-TFR(Fv)-PE40 on the same cell line.

Cytotoxicity and specificity of DT388-anti-TFR(Fv). DT388-anti-TFR(Fv) was also prepared, tested, and found to be cytotoxic to several human cell lines, with ID_{50}s ranging between 10 and 100 pM (Table 2). Its cytotoxic activity was also blocked by excess antibody, demonstrating the specificity of DT388-anti-TFR(Fv) for the transferrin receptor (data not shown). Furthermore, DT388-anti-TFR(Fv) was inactive on mouse L929 cells, demonstrating its specificity for human cells (Table 2). When compared with anti-TFR(Fv)-PE40, the DT immunotoxin was 400- to 800-fold less active on A431, KB, and MCF7 cells but 2- to 3-fold more active on HUT102 and HT29 cells.

DISCUSSION

We have constructed two new single-chain immunotoxins directed at the human transferrin receptor. One of these is anti-TFR(Fv)-PE40, which consists of the variable regions of HB21, a monoclonal antibody to the human transferrin receptor, joined to PE40, a truncated form of PE devoid of its binding domain. This fusion protein was expressed in *E. coli* and purified to near homogeneity. Anti-TFR(Fv)-PE40 was found to be very cytotoxic to cells bearing human transferrin receptors. The cytotoxicity was specific, as shown by competition with excess native antibody. To demonstrate that it should be possible to make single-chain immunotoxins that have the same antigen-binding domain but a different toxin and to compare their activities on different cell types, we constructed a second single-chain immunotoxin, DT388-anti-TFR(Fv), containing a truncated form of DT lacking DT binding activity. This fusion protein also was found to be cytotoxic to cells expressing the human transferrin receptor. Unexpectedly, large differences in the activities of the two single-chain immunotoxins were observed. On some cell lines (A431, KB, and MCF7), anti-TFR(Fv)-PE40 was at least 100-fold more active than DT388-anti-TFR(Fv). On two cell lines (HUT102 and HT29), DT388-anti-TFR(Fv) was about threefold more active. However, in no case was DT388-anti-TFR(Fv) much more active than the PE-containing immunotoxin, whereas the reverse was observed. The basis of these differences in activities remains to be explored. The mechanism by which immuno-

toxins kill cells is complex and involves binding to the receptor, internalization via coated pits into endocytic vesicles, and proteolytic processing of the toxin to a form which can be translocated into the cytosol. It seems unlikely to us that the difference in the activities is related to binding or the rate of internalization; instead, the difference probably arises during the proteolytic processing or translocation steps.

Recently, we have shown that PE is processed by a cellular protease to produce an N-terminal 28-kDa and a C-terminal 37-kDa fragment. Only the 37-kDa fragment is translocated to the cytosol (18). The proteolytic clip occurs at or near Arg-279 in domain I of PE. Production of the 37-kDa fragment appears to be essential for toxicity, since mutant PE molecules PEgly^{276}, PEgly^{279}, and PEhis^{274,276,279} that do not produce this fragment are unable to kill target cells (18). The amino acid sequence at the carboxyl end of PE is REDLK, and changing it to REDL or KEDLK does not affect the cytotoxic activity of the toxin (8). Mutant PE molecules ending with KDEL or RDEL were fully active, whereas LDER at the carboxyl end resulted in an inactive molecule, even though the mutant PE molecule ending with LDER was processed normally, generating the 28- and 37-kDa fragments (18). This observation suggests that the sequence at the carboxyl end of PE acts as a recognition sequence to assist translocation of the toxin from an endocytic compartment to the cytosol (8). Thus, at least two specific recognition events occur within the cells, one within domain II that leads to processing and one at the carboxyl end of domain III. Olsnes and colleagues, using a system in which whole cells are exposed to a low-pH environment, have presented strong evidence that a fragment of DT closely resembling the entire A chain is the final processed form that reaches the cytosol (15). DT contains a trypsin-sensitive region with three closely spaced arginines at positions 190, 192, and 193. Cleavage of the toxin to produce A and B fragments appears to occur in a stochastic manner after either of these arginine residues (16). The site of cleavage appears to be one of the factors affecting translocation, since only A fragments lacking both Arg-192 and Arg-193 were found to be translocation competent (16). DT does not contain a terminal sequence resembling REDLK, indicating that it probably has a mechanism of cellular entry different from that of PE. From the present study, it appears that despite identical ADP ribosylation activities, both PE and DT are probably processed or translocated or both by different mechanisms, resulting in the differential sensitivity of the cells towards these toxins.

Competition studies showed the binding affinity of anti-TFR(Fv)-PE40 to be very similar to that of the native bivalent antibody on A431 cells and HUT102 cells. Since anti-TFR(Fv)-PE40 is monovalent, this was a somewhat surprising result. Previously, we had found that anti-Tac(Fv)-PE40 bound threefold less well than anti-Tac to the human interleukin 2 receptor-bearing cells, HUT102. On comparing the cytotoxic activity of anti-TFR(Fv)-PE40 with that of a chemical conjugate, anti-TFR-Lys(PE40), the recombinant protein was found to be much more active on all the human tumor cells investigated. This difference was in part explained by the diminished binding of the chemical conjugate to the transferrin receptor, probably as a result of treatment of the antibody with the modifying 2-iminothiolane during the preparation of the immunotoxin (3). However, other steps in immunotoxin action must be differentially affected as well.

One objective of creating chimeric toxins is their potential usefulness as antitumor agents. Our previous study shows that anti-TFR-LysPE40 has a very potent in vivo antitumor activity against A431 epidermoid carcinomas. Because anti-TFR(Fv)-PE40 is very cytotoxic to A431 and KB cells in culture (Table 1), we plan to test anti-TFR(Fv)-PE40 in animals with these tumors to determine the in vivo antitumor activity of this single-chain immunotoxin.

In summary, we have shown in the present study as well as in our earlier report (20) that the single-chain immunotoxins made by the fusion of antigen-binding and toxin domains can retain the binding affinity of the native antibody and are generally more active than the chemical conjugates. Also, active single-chain immunotoxins can be made with different toxin moieties, thus placing antigen-binding portions either at the amino terminus or the carboxyl terminus as required. With this information in hand, it should be possible to make active single-chain immunotoxins from the wide variety of toxins (plant, bacterial, and animal) that are now being made by chemical coupling methods (10, 19, 24).

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