Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 9491–9494, December 1990
Medical Sciences

A recombinant single-chain immunotoxin composed of anti-Tac variable regions and a truncated diphtheria toxin

(interleukin 2 receptor/leukemia/protein engineering/antibody/cancer)

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ABSTRACT **To kill human or primate cells expressing the p55 subunit of the interleukin 2 receptor, we have constructed a single-chain immunotoxin. DNA sequences encoding the first 388 amino acids of diphtheria toxin (DT) were fused to DNA elements encoding the antigen-binding portion (variable region or Fv) of the anti-Tac monoclonal antibody. The antigen-binding portion consists of 116 amino acids of the heavy-chain variable region connected by a 15-amino acid linker to 106 amino acids of the variable region of the light chain. The single-chain immunotoxin DT388–anti-Tac(Fv) was expressed in *Escherichia coli* and found in inclusion bodies. The monomeric form was then purified to near homogeneity with a high yield (3–5 mg/liter). Monomeric DT388–anti-Tac(Fv) was highly cytotoxic to cell lines bearing the p55 subunit of the human interleukin 2 receptor but not to cells without this subunit. DT388–anti-Tac(Fv) was also very effective in killing proliferating human T cells produced in a mixed leukocyte reaction.**

Both bacterial and plant toxins, including *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT), have been employed in developing targeted toxins as reagents for the treatment of cancer, AIDS, and immunological disorders (see refs. 1–3 for review). Initially, chemical conjugation procedures were used to couple antibodies or growth factors to toxins (4–6). More recently, recombinant DNA techniques have been employed to synthesize chimeric toxins in *Escherichia coli*; these chimeric toxins are composed of growth factors or lymphokines linked to modified forms of PE or DT (7–12). These fusion proteins selectively kill cells bearing appropriate growth factor receptors or antigens. Both chemically conjugated and recombinant products have been successfully used in animal models (13–15). Several of these are now being evaluated in animal models and in clinical trials (16–18).

The immunogenic nature of these reagents currently prohibits their repeated and long-term or prophylactic use due to the production of antibodies to the chimeric toxin. Since the toxin portion is highly immunogenic, it is desirable to prepare reagents that have the same binding portion, but different toxin moieties, so that in the event of development of antibodies to one toxin a similar reagent with a different toxin can be used.

Recently, we have described a recombinant single-chain immunotoxin, anti-Tac(Fv)–PE40, that consists of the variable domains (Fv) of the light and the heavy chains of the anti-Tac monoclonal antibody linked to PE40, a truncated form of PE (9). Anti-Tac recognizes and binds tightly to the p55 subunit of the human interleukin 2 (IL-2) receptor; PE40 is a truncated form of PE that is devoid of its cell binding domain (19, 20). Anti-Tac(Fv)–PE40 selectively killed cell lines that express large numbers of the p55 subunit of the human IL-2 receptor. This chimeric protein was also very effective in killing human cells activated in a mixed leukocyte reaction (MLR) as well as human phytohemagglutinin-stimulated blasts (21). We have also prepared a fusion protein DT388–IL2 that consists of the first 388 amino acids of DT fused to human IL-2 (V.K.C., D.J.F., and I.P., unpublished results). This protein was also very cytotoxic to cells bearing the human as well as the mouse high-affinity IL-2 receptors.

In anti-Tac(Fv)–PE40, the toxin is placed at the carboxyl end of the Fv. To determine if a toxin can be placed at the amino end of the single-chain antibody and also to try and circumvent the problem of high titer antibodies to PE40 that may arise upon repeated treatment with anti-Tac(Fv)–PE40, we have now constructed a recombinant immunotoxin [DT388–anti-Tac(Fv)] using a truncated form of DT that is devoid of its own receptor binding site. DT388–anti-Tac(Fv) was expressed in *E. coli* and was purified to near homogeneity. DT388–anti-Tac(Fv) was very cytotoxic to cell lines bearing the p55 subunit of the human IL-2 receptor. This fusion protein was also very effective in killing proliferating human T cells produced in a human MLR.

MATERIALS AND METHODS

The sources of the reagents used have been described (7–9).

Plasmid Construction. Plasmid pVCDT1-anti-Tac(Fv) was constructed by ligating a 0.7-kilobase *Nde* I–*Hin*dIII fragment from p7018 (21) to a dephosphorylated 4.0-kilobase *Nde* I–*Hin*dIII fragment of pVCDT1-IL2 (22). p7018 is similar to pVC70108 (9), but the junction between the antibody Fv portion and PE40 is different due to the incorporation of a *Hin*dIII restriction site. pVCDT1-IL2 contains the first 388 codons of DT with an initiator methionine fused to cDNA encoding human IL-2 (22). Details of the plasmid pVCDT1-anti-Tac(Fv) are shown in Fig. 1.

Protein Expression and Purification. *E. coli* strain BL21 (λDE3) containing the plasmid pVCDT1-anti-Tac(Fv) was used for the expression of the fusion protein at 37°C as described (8–10). Since the protein was localized in the inclusion bodies, guanidine hydrochloride extraction followed by rapid dilution in phosphate-buffered saline (PBS) was employed to extract and renature the fusion protein. A highly purified monomeric form of DT388–anti-Tac(Fv) was obtained after a three-step purification procedure that involved Q Sepharose, Mono Q, and gel-filtration column chromatography.

Cytotoxicity Assay. Cytotoxic activities of DT388–anti-Tac(Fv), anti-Tac(Fv)–PE40, and DT388–IL2 on various cell lines were evaluated by assaying the inhibition of protein synthesis as measured by [^{3}H]leucine incorporation (9).

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Abbreviations: MLR, mixed leukocyte reaction; Fv, antibody fragment consisting of two variable domains; DT, diphtheria toxin; PE, *Pseudomonas* exotoxin; IL-2, interleukin 2.

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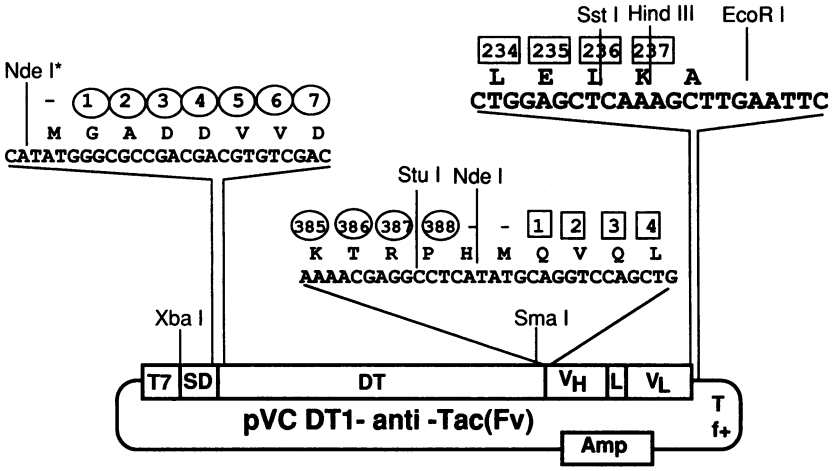

FIG. 1. Expression plasmid for DT388–anti-Tac(Fv). Plasmid pVCDT1-anti-Tac(Fv) contains a gene fusion between the DNA sequences encoding the first 388 amino acids of native DT and DNA elements encoding the variable domains of the heavy and the light chains of the anti-Tac monoclonal antibody linked together by a 15-residue peptide linker (9, 21). An initiation methionine has been added, and codon 386 of DT has been changed to AGG to create a unique *Stu* I restriction site. The numbers in circles are of DT and the numbers in the boxes are of anti-Tac(Fv). A dash indicates an extra amino acid. The 5′ *Nde* I site was destroyed due to the loss of a thymidine residue. V_H, variable domain of the heavy chain; L, $[(Gly)_4Ser]_3$ linker; V_L, variable domain of the light chain; T7, T7 promoter; SD, Shine–Dalgarno box; f+, phage F1 origin; T, T7 transcription terminator; Amp, β-lactamase gene.

Various dilutions of fusion proteins were added to 1 ml of freshly washed cells (4×10^5/ml) in 24-well plates for 16–20 hr. The cells were then pulsed for 90 min with 2 μCi (1 Ci = 37 GBq) of [^{3}H]leucine, and the trichloroacetic acid-precipitable cell-associated radioactivity was determined. The results are expressed as the percent of the control where no toxin was added.

MLR. Purified peripheral blood mononuclear cells, prepared from the blood of normal volunteers, were used to set up a two-way MLR, and inhibition of protein synthesis by various chimeric toxins was determined as described (21).

Other Methods. Protein concentration was measured by the method of Bradford (22) using a Bio-Rad protein assay reagent with bovine serum albumin as a standard. SDS/PAGE on 10% gels was performed as described (9, 21).

RESULTS

Plasmid Construction, Protein Expression, and Purification. Plasmid pVCDT1-anti-Tac(Fv) was constructed from plasmids pVCDT1-IL2 and p7018 as shown in Fig. 1. This plasmid, upon isopropyl β-D-thiogalactoside induction in BL21 (λDE3) cells, expressed large amounts of DT388–anti-Tac(Fv), which has a molecular mass of about 66 kDa. This protein is the major component of spheroplasts (Fig. 2, lane 1). DT388–anti-Tac(Fv) was localized in the 100,000 × *g* pellet of the sonicated spheroplast (Fig. 2, lane 3). The fusion protein in the inclusion bodies was denatured in 7 M guanidine hydrochloride and renatured by rapid dilution in PBS. Then a successive three-step procedure was used to obtain a highly purified monomeric preparation of DT388–anti-Tac(Fv). Stepwise elution from a Q Sepharose column removed contaminating nucleic acid fragments (Fig. 3*A*). A Mono Q step separated monomers from multimers of DT388–anti-Tac(Fv) (Fig. 3*B*). The peak that eluted at about 0.2 M NaCl consisted of monomers and was very cytotoxic to HUT102 cells. The multimers that eluted at higher ionic strength (0.3 M) did not inhibit protein synthesis on HUT102 cells (data not shown). Finally, gel filtration on a TSK-250 column separated the monomeric form from small amounts of contaminating dimers and small peptide fragments (Fig. 3*C*). After this purification procedure, a fusion protein of greater than 90% purity was obtained (Fig. 2, lane 5). The total yield after the gel-filtration column was about 2.5 mg of protein from a 500-ml culture induced by isopropyl β-D-thiogalactoside at an OD_{650} of 0.8. The amino-terminal sequence of DT388–anti-Tac(Fv) was found to be Met-Gly-Ala-Asp-Asp-Val-Val-Asp-Ser-Ser-Lys-Ser.

Cytotoxic Activity of DT388–anti-Tac(Fv) on Various Cell Lines. Since anti-Tac binds to the p55 subunit of the human IL-2 receptor, DT388–anti-Tac(Fv) was tested on a leukemic T-cell line, HUT102, and its cytotoxicity was compared to previously described IL-2 receptor-targeted chimeric toxins, DT388–IL2 and anti-Tac(Fv)–PE40 (9, 12, 22). DT388–anti-Tac(Fv) inhibited protein synthesis on HUT102 cells in a dose-dependent manner, with an ID_{50} of 0.3–0.4 ng/ml. Anti-Tac(Fv)–PE40 and DT388–IL2, in the same assay, had, in several experiments, ID_{50} values of 0.15–0.2 and 0.2–0.4 ng/ml, respectively (Fig. 4). The cytotoxic effect of DT388–anti-Tac(Fv) was blocked by an excess of anti-Tac, showing the specificity of the cytotoxic protein (Fig. 4). Two other constructs of DT fused to IL-2 have been described (12, 23). One of these, DAB389–IL2, exhibited an ID_{50} similar to that of DT388–IL2 prepared in our laboratory. DT388–IL2 and DAB-389–IL2 have a few differences at the junction of DT and IL-2, which do not seem to affect cytotoxic activity. On another leukemic human T-cell line, Cr II.2, which has fewer IL-2 receptors, DT388–anti-Tac(Fv) was also cytotoxic, with an ID_{50} of 1.2 ng/ml, which was comparable to anti-Tac(Fv)–PE40 (Table 1). On these cells, DT388–IL2 was severalfold more active than either of these proteins. On ELT5 cells, which have the mouse p75 and the human p55 subunit of the IL-2 receptor, DT388–anti-Tac(Fv) was also quite cytotoxic, with an ID_{50} of 0.5 ng/ml. This cell line was produced by transfecting the cDNA of the human p55 subunit into a mouse thymoma cell line bearing only the p75 subunit of mouse IL-2

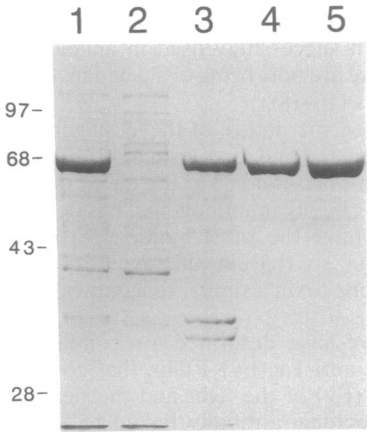

FIG. 2. SDS/PAGE of DT388–anti-Tac(Fv) at various stages of purification. The protein samples were boiled with Laemmli sample buffer for 3 min at 110°C, and samples containing 3–5 μg of protein were applied on a 10% polyacrylamide gel. The protein bands were visualized with Coomassie blue R250. Lanes: 1, spheroplasts; 2, cytosol; 3, 100,000 × *g* pellet of the sonicated spheroplasts (inclusion bodies); 4, pool after Mono Q column; 5, pool after TSK-250 gel-filtration column. Molecular size markers (in kDa) are indicated at left.

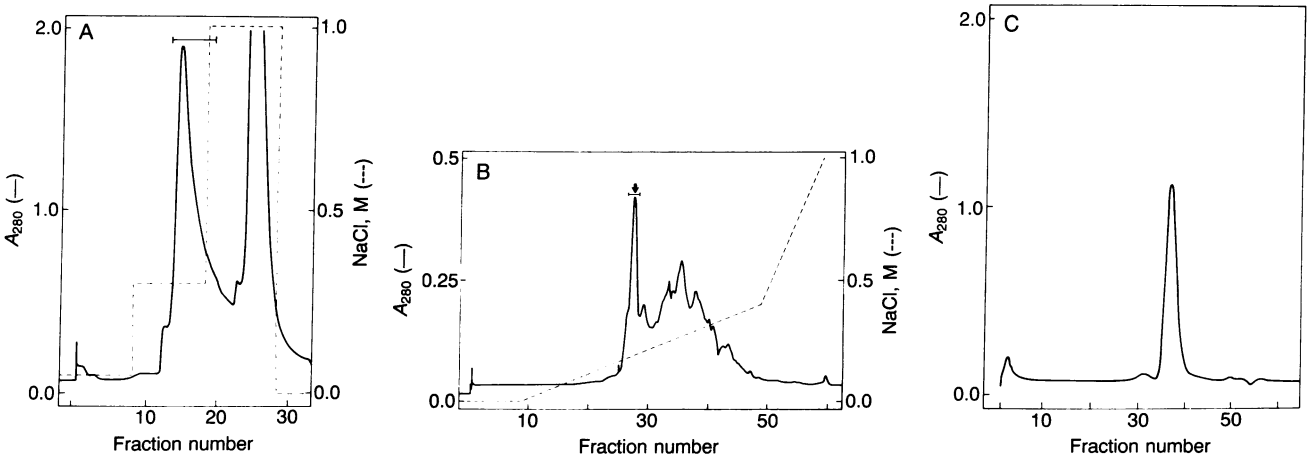

FIG. 3. (*A*) Q Sepharose column chromatography of DT388–anti-Tac(Fv). After denaturing inclusion bodies from 500 ml of culture in 7 M guanidine hydrochloride, the extract was rapidly diluted in strongly stirring PBS to renature the proteins, followed by dialysis against 20 mM Tris (pH 7.4) (7–9). The renatured material was centrifuged at 100,000 × *g* for 15 min and filtered through a 0.45-μm filter and applied at a flow rate of 4 ml/min on a 8-ml Q Sepharose column (HR 10/10) that was prequilibrated in 20 mM Tris·HCl (pH 7.4) (buffer A). The column was attached to a Pharmacia FPLC column and washed with 20 ml of buffer A containing 100 mM NaCl (flow rate of 4 ml/min), and fractions of 2 ml were collected. The material was eluted with buffer A containing 300 mM NaCl, and fractions of 2 ml were collected. Finally the column was washed with 1 M NaCl in buffer A. Fractions 13–19 were pooled and diluted in 100 ml of 10 mM Tris·HCl (pH 7.4). (*B*) Mono Q column chromatography of DT388–anti-Tac(Fv). The eluate (120 ml) from a Q Sepharose column was applied on a Mono Q column (HR 10/10) at 4 ml/min. The column was washed with 20 ml of buffer A and then eluted with a 80-ml linear gradient of NaCl (0–0.4 M) in buffer A (flow rate of 3 ml/min). Fractions of 2 ml were collected. The fractions were analyzed by SDS/PAGE and for cytotoxicity on HUT102 cells (not shown). Fractions 27 and 28 (marked by a horizontal bar) were pooled and concentrated to 1 ml in a Centricon-30. (*C*) TSK-250 gel-filtration column chromatography of DT388–anti-Tac(Fv). The Mono Q pool (1 ml) containing 3–4 mg of protein was applied to a TSK-250 (0.7 × 60 cm) column attached to a Pharmacia FPLC. The column was developed with PBS at 0.7 ml/min, and 0.5-ml fractions were collected and analyzed by SDS/PAGE. Fractions 35–39 were pooled and used for cytotoxicity assays.

receptor (24). ELT5 cells make tumors in nude mice and will be useful to evaluate the antitumor efficacy of IL-2 receptor-targeted toxins. DT388–anti-Tac(Fv) was cytotoxic to MT-1 cells, which contain only the p55 subunit of the human IL-2 receptor, and the ID_{50} was comparable to that of anti-Tac(Fv)–PE40 (Table 1 and ref. 21). However, DT388–IL2 was much less effective on these cells. This is probably due to the higher affinity of anti-Tac toward the p55 subunit than toward IL-2. On EL4J 3.4 cells, a mouse thymoma line (24) that has mouse p55 and p75 subunits, anti-Tac(Fv)-containing chimeric toxins were not cytotoxic, even at 312 ng/ml, showing the specificity of the anti-Tac(Fv) fusion proteins toward cells that have the human p55 subunit. However, DT388–IL2 killed EL4J3.4 cells with an ID_{50} of 0.18 ng/ml (Table 1). Neither of the fusion proteins was cytotoxic to CEM cells, which do not have IL-2 receptors, confirming that the cytotoxic effects of these proteins are mediated through IL-2 receptors.

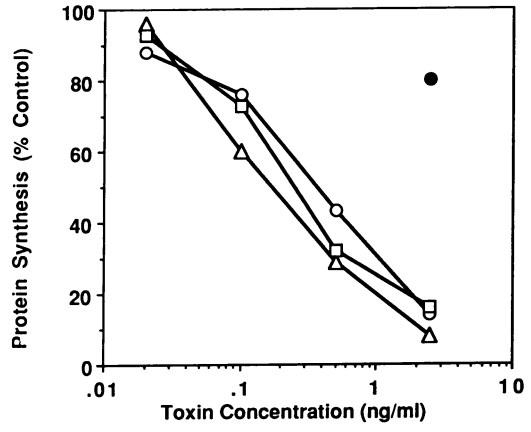

FIG. 4. Cytotoxicity of IL-2 receptor-targeting toxins on HUT102 cells. HUT102 cells were washed with RPMI-1640 containing 10% fetal bovine serum and seeded at 4 × 10^5 cells per well in medium containing 10% fetal bovine serum in 24-well plates. Various dilutions of DT388–anti-Tac(Fv), anti-Tac(Fv)–PE40, and DT388–IL2 were made in PBS containing 0.2% human serum albumin, and 20 μl was added to each well for 16–20 hr. The cells were then pulsed for 90 min with 2 μCi of [^{3}H]leucine. Total cells were collected and trichloroacetic acid-precipitable radioactivity was determined. The results are expressed as a percent of the control without any toxin. ○, DT388–anti-Tac(Fv); △, anti-Tac(Fv)–PE40; □, DT388–IL2; ●, DT388–anti-Tac(Fv) plus anti-Tac at 2 μg/ml.

Effect of DT388–anti-Tac(Fv) on Activated Human T Lymphocytes. Since one possible use of IL-2 receptor-based therapy is in the treatment of autoimmune diseases and allograft rejection, we tested the activity of DT388–anti-Tac(Fv) on human lymphocytes that were activated in a MLR. The activated lymphoblasts contain large numbers of IL-2 receptors on their surface. As shown in Fig. 5, DT388–anti-Tac(Fv) inhibited protein synthesis of MLR-stimulated human lymphoblasts with an ID_{50} of 0.07 ng/ml, which is very similar to that of anti-Tac(Fv)–PE40 (Table 2 and ref. 21). In the same assay, DT388–IL2 was 2-fold less active. These toxins were also tested on the individual donor lymphocytes and found to have no effect

Table 1. Cytotoxicity of IL-2 receptor-targeted chimeric toxins of various cell lines

Cell line	IL-2 receptor subunit present		ID_{50}, ng/ml		
	p55	p75	DT388–anti-Tac(Fv)	anti-Tac (Fv)–PE40	DT388–IL2
HUT102	H	H	0.37	0.17	0.24
CrII.2	H	H	1.2	0.6	0.17
ELT5	H	M	0.5	0.96	0.90
EL4J 3.4	M	M	>312	>312	0.18
CEM	—	—	>312	>312	>312
MT-1	H	—	0.6	0.3	>100

ID_{50}, protein concentration required to inhibit protein synthesis by 50% in a 16- to 20-hr assay; H, human; M, mouse; —, no receptor subunit.

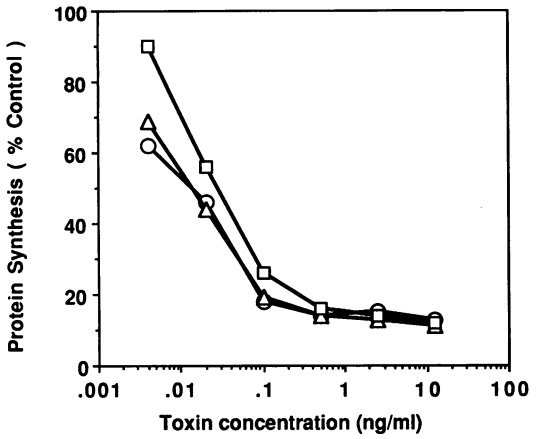

FIG. 5. Cytotoxic activity of DT388–anti-Tac(Fv) on human lymphoblasts produced in MLR. Human peripheral blood mononuclear cells were prepared from two donors and used in a two-way MLR as described (21). Approximately 1×10^5 cells in 200 μl of RPMI with 10% fetal bovine serum from a mixed culture or from an individual donor were plated in each well of 96-well plates, and 10 μl of serial dilutions of chimeric toxins was added and incubated for 2 days. Then the cells were pulsed for 6 hr with 2 μCi of [^{3}H]leucine per well. Cells were harvested and assayed as described in Table 2. ○, DT388–anti-Tac(Fv); △, anti-Tac(Fv)–PE40; □, DT388–IL2.

(Table 2). The total incorporation of [^{3}H]leucine in these donor cells was only 5–10% of the MLR-stimulated lymphoblasts (data not shown).

DISCUSSION

We have constructed a plasmid, pVCDT1-anti-Tac(Fv) (Fig. 1), that expresses an active single-chain immunotoxin, DT388–anti-Tac(Fv). The yields of the purified monomeric chimeric toxin were 3–5 mg per liter of culture, which is severalfold better than the previously described recombinant single-chain immunotoxin anti-Tac(Fv)–PE40 (9). This is probably because under the conditions employed the DT fusion protein folds better than the PE40-containing protein; however, other refolding conditions could increase the yields of both single-chain chimeric toxins. DT388–anti-Tac(Fv) was selectively cytotoxic to cell lines that bear the p55 subunit of the human IL-2 receptor; its cytotoxic activity was comparable to anti-Tac(Fv)–PE40. DT388–anti-Tac(Fv) was also very cytotoxic to human lymphoblasts activated in a MLR. The toxicity of DT388–anti-Tac(Fv)–PE40 has not yet been evaluated in animals. Because mice are resistant to DT, toxicity studies are probably best performed in primates.

Table 2. Cytotoxic activity of IL-2 receptor-targeted chimeric toxins on human lymphoblasts

Protein	ID_{50}, ng/ml		
	Mixed cells	Donor 1	Donor 2
DT388–anti-Tac(Fv)	0.07	>12.5	>12.5
anti-Tac(Fv)–PE40	0.06	>12.5	>12.5
DT388–IL2	0.15	>12.5	>12.5

Human lymphoblasts were prepared in a two-way MLR. Various dilutions of the chimeric toxins were added for 48 hr, followed by a 6-hr [^{3}H]leucine pulse. Cell-associated radioactivity was collected on a glass fiber filter mat using an automatic 96-well plate cell harvester (TomTec, Orange, CT) and assayed in a LKB scintillation counter.

The high cytotoxic activity of DT388–anti-Tac(Fv) shows that the amino terminus of an antibody Fv fragment can be associated in peptide linkage with a much larger protein. We have previously shown with anti-Tac(Fv)–PE40 that the order of the heavy- and light-chain variable domains can be switched and that several different peptide linkers with various lengths and compositions can be used to link together these variable domains (21). In the case of DT388–anti-Tac(Fv), the toxin is placed at the amino terminus of the antibody and is connected directly to the first amino acid of the heavy-chain variable region of anti-Tac. Based on the results with anti-Tac(Fv)–PE40, the order of the light and heavy chains should be able to be reversed. Finally, it should also be possible to make a recombinant immunotoxin such as DT388–anti-Tac(Fv)–PE40 that has two different toxin moieties, one on each side of the antibody variable regions. Such chimeric molecules may be much more active on target cells and be able to kill cells with very few antigen-binding sites or receptors.

We thank Mark Lively for amino-terminal sequencing, Betty Lovelace for cell culture, Althea Gaddis for secretarial help, and Steve Neal for photography.

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