Anti-Tac(Fv)-PE40, a Single Chain Antibody Pseudomonas Fusion Protein Directed at Interleukin 2 Receptor Bearing Cells*

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Anti-Tac(Fv)-PE40 is a chimeric single chain immunotoxin in which anti-Tac variable heavy and light chains held together by a peptide linker are attached to PE40, a truncated form of Pseudomonas exotoxin. This molecule was shown to be extremely cytotoxic for interleukin 2 (IL2) receptor-bearing cells in tissue culture (Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., FitzGerald, D. J., and Pastan, I. (1989) Nature 339, 394-397). Here we describe various forms of anti-Tac(Fv)-PE40 protein in which the order of the variable domains of anti-Tac has been switched and also three different types of peptide linkers have been used. All these proteins were purified to near homogeneity and were found to have similar cytotoxic activities against various human cells expressing the p55 subunit of the IL2 receptor. Anti-Tac(Fv)-PE40 is cytotoxic to cells expressing the p55 subunit of the human IL2 receptor (6).

The affinity of anti-Tac(Fv)-PE40 for the p55 subunit of the IL2 receptor was found to be very close to that of native anti-Tac (6). To study the structural requirements for binding and to explore the possibility of creating a more active molecule, we have made several different anti-Tac(Fv)-PE40 constructions. In these, the order of the variable heavy and light chains has been switched and the role of the peptide linker joining the variable chains has been explored by using three different linkers. In addition, the activity of anti-Tac(Fv)-PE40 was assessed on various types of normal and malignant human T cells. The cytotoxicity was compared with that of two other chimeric toxins composed of IL2 linked to different mutant forms of PE. Anti-Tac(Fv)-PE40 was found to be more active than either IL2-PE40 or IL2-PE664G (8, 9), two other chimeric toxins directed at IL2 receptor-bearing cells.

MATERIALS AND METHODS

Construction of Plasmids—Polymerase chain reaction-based methods were used to amplify, create cloning sites, and assemble the plasmids containing the variable domains of the light and the heavy chain of anti-Tat joined together by a peptide linker. These variable domains are fused to DNA sequences encoding PE40. These fusion genes are under the control of a T7 promoter and an appropriate ribosome binding site has been included.

p7018—Fig. 1 summarizes the cloning strategy employed for this construction. The starting template material was cDNA of the light and heavy chain of anti-Tac cloned as an EcoRI insert in pUC18. Oligonucleotides VK73 and VK74 were used to amplify the variable domain of the heavy chain (VH) using anti-Tac heavy chain cDNA as template. VK75 and VK76 were used to amplify the variable domain of the light chain (VL) using the anti-Tac light chain cDNA as template. The conditions of the polymerase chain reaction have been described previously (7). For VK73, 23 nucleotides at the 3′-end were the same as the 5′-sequence of the cDNA encoding the mature heavy chain of anti-Tac. VK73 also introduced an NdeI site and an initiation methionine at the 5′-end. The 3′-end of the oligonucleotide, VK74, was complementary to the 3′-end of the variable domain of the heavy chain. VK74 also encoded the first 8 anticodons of the peptide linker followed by a KpnI site and the nucleotides at its 5′-end were complementary to the sequences in the CH1 domain. VK75 carried nucleotides for a KpnI site and for the last 8 codons of a peptide linker. The last 23 nucleotides at VK75, at the 3′-end, were the same as that of the 5′-end of the mature light chain. The 3′-end of oligonucleotide VK76 contained 24 nucleotides which were complementary to the 3′-end of the variable domain of the light chain,

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† The abbreviations used are: PE, Pseudomonas exotoxin; IL2, interleukin 2; anti-Tat, monoclonal antibody to ~55 subunit of human IL2 receptor; VH, heavy chain variable region; VL, light chain variable region; kb, kilobase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PHA, phytohemagglutinin.
followed by nucleotides for a HindIII site. The 5'-end of VK76 was complementary to the constant domain of the light chain.

The amplified DNA containing Vκ was digested with NdeI and KpnI, while that containing Vλ was digested with KpnI and HindIII. The digested fragments were purified on 1.8% low melting point agarose (Sea Plaque GTG agarose, FMC). Separately, plasmid pVC20 was digested with NdeI and HindIII and dephosphorylated. pVC20 encodes a PE derivative of 43 kDa termed PE43 (10). A three fragment in-gel ligation was set up using two polymerase chain reaction-amplified, enzyme-digested fragments containing Vκ and Vλ with a 3.75-kb fragment of pVC20 carrying PE43 and promoter sequences. Correct clones were identified by restriction enzyme analysis. One of the right clones, pVC701, was digested with KpnI followed by T4 DNA polymerase to remove the 4-base overhang and ligated. The clones were screened for the loss of the KpnI site. These clones (pVC701020) produced a protein of approximately 66 kDa reacting with antibodies to PE. pVC701020 was digested with HindIII and BamHI and dephosphorylated. Separately, pVC38H (7) was digested with HindIII and BamHI to isolate a 0.8-kb fragment which was ligated with the HindIII- and BamHI-restricted 2.9-kb fragment of pVC701020. The resulting clones contained Vκ, linker Ll, Vλ, and DNA sequences for PE40 under the control of a T7 promoter (Fig. 2). The clones were checked by restriction enzyme digestions and, finally, by protein expression. One of the correct clones, pVC7018, which expressed a 63-kDa protein reactive with the antibodies to PE, was selected for large scale expression and purification of the protein.
peptide linker at the 5'-end of Vκ, and a HindIII site at the 3'-end of Vκ. Other steps were the same as described above for the construction of p7018.

p70538—This plasmid is similar to p70528 but contains a 16-amino acid peptide linker termed L2 which has a different amino acid composition (Fig. 2). ~70538 was derived from ~70528 using two oligonucleotides, VK94 and VK89, which introduced a 14-amino acid linker termed L3 (Fig. 2).

The DNA sequences in the peptide linker region were confirmed by dideoxy sequencing.

Expression and Purification of Proteins—To express and localize anti-Tac(Fv)-PE40, E. coli BL21 (DE3) cells were transformed with the appropriate plasmid and cultured at 37 °C in super broth supplemented with ampicillin (100 μg/ml). At an OD600 of 0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was further incubated for 90 min. Cells were harvested and protein was localized as described previously (11). The fusion proteins were mostly contained in the inclusion bodies so these were used for further purification. Proteins purified in this manner were used as the source for further purification. Proteins were detected by SDS/PAGE and cytotoxic activity on HUT102 cells, Cr11.2, MT-1, EIL5, and EL4.3.4. Cells were harvested and resuspended in the assay medium as described above.

To determine the effect of anti-Tac(Fv)-PE40, lymphoblasts were aliquoted into 96-well plates, and serial dilutions of the toxin were added along with rIL2 (10 units/ml). The culture was incubated for 2 days at 37 °C, pulsed for 6 h with [3H]leucine. Cells were harvested using a microplate harvester on glass fiber filters and counted.

RESULTS

We have previously shown that a single chain anti-Tac(Fv)-PE40 molecule composed of the heavy and light chain variable regions of anti-Tac connected to PE40 makes a very active immunotoxin that inhibits protein synthesis in HUT102 cells with an ID50 of ~0.15 ng/ml (6). To determine if the structure of the recombinant immunotoxin could be altered and its activity retained or improved, we made several new constructions in which the order of the Vκ and Vκ chains was reversed, or in which the amino acid sequence of the linking peptide was altered. The structures of these new constructions are illustrated in Fig. 2 and described under “Materials and Methods.” All the chimeric proteins were expressed in E. coli using the T7 expression system, extracted from inclusion bodies by guanidine hydrochloride, and, after renaturation, purified to near homogeneity by successive ion exchange and size exclusion chromatography. In all cases, a highly purified chimeric toxin was obtained (Fig. 3), and the yields of toxin were approximately equal.

The purified toxins were tested on several different cell lines. These included HUT102 and CrlII.2 which contain both

FIG. 2. Expression vector for various anti-Tac(Fv)-PE40 proteins. A. p7018 showing the basic plasmid used for expression of the chimeric proteins. B, order of variable domains in various constructions and linkers. Vκ, variable heavy; Vλ, variable light; L, linker; B, BamHI; A, AvaI; Amp, β-lactamase gene.

FIG. 3. SDS-PAGE of purified anti-Tac(Fv)-PE40 proteins. 10% SDS-polyacrylamide gels were run and stained with Coomassie Blue. Lane 1, 7018; lane 2, 70528; lane 3, 70538; lane 4, 70548. Molecular weight markers are shown × 106.
IL2 Receptor-specific Toxin

subunits of the human IL-2 receptor (p55 and p75), MT-1 which contains only human p55, ELT5 which has human p55 and mouse p75, and EL4J3.4 which has mouse p55 and p75. As shown in Fig. 4 and Table I, all four chimeric toxins were very active in inhibiting protein synthesis in HUT102 cells with ID$_{50}$ values ranging from 0.10 to 0.15 ng/ml. The agents also had similar action against the other cell lines which contained the human p55 subunit (CrII.2, MT-1, and ELT5) but not on cells containing the mouse p55 subunit reflecting the species specificity of the anti-Tat antibody.

Effect of Anti-Tac(Fv)-PE40 on Human T Lymphoblast Proliferation—One possible use of anti-Tac(Fv)-PE40 is in the treatment of autoimmune diseases or allograft rejection. Therefore, we tested the activity of one of the recombinant immunotoxins on human lymphoblasts which were activated either by the mitogen, PHA, or in a mixed lymphocyte reaction. The stimulated lymphoblasts express functional IL2 receptor on their surface. For this purpose, we used the protein encoded by plasmid p/018 (Fig. 2). As shown in Table II, anti-Tac(Fv)-PE40 inhibited the protein synthesis of both PHA and mixed lymphocyte reaction-stimulated human lymphoblasts with ID$_{50}$ values of 0.13 ng/ml and 0.05 ng/ml, respectively. As a control, we tested the toxin on K562 cells which do not express the IL2 receptors and found that high levels of the toxin were required to intoxicate these cells. The activity of anti-Tac(Fv)-PE40 on activated T lymphoblasts was compared with IL2-PE40 and IL2-PE664G', chimeric toxins that also bind to the IL2 receptor. IL2-PE40, although previously shown to be very active against mouse splenocytes (14), had very little activity toward the human cells (Table II). IL2-PE664G', a chimeric toxin in which the ability of domain I to bind to the Pseudomonas exotoxin receptor has been inactivated by mutations at positions 57, 246, 247, and 249, had more activity than IL2-PE40 against the activated human cells, but was nevertheless much less potent than anti-Tac(Fv)-PE40 (Table II). Clearly, anti-Tac(Fv)-PE40 was the most cytotoxic of the three molecules tested.

Blood Level and Toxicity of Anti-Tac(Fv)-PE40 in Mice—The extremely potent in vitro cytotoxicity of anti-Tac(Fv)-PE40 on IL2 receptor-bearing cells makes it a promising molecule for testing in vivo against IL2 receptor-bearing tumors or for immunosuppression. Accordingly, various amounts of anti-Tac(Fv)-PE40 were given intraperitoneally to mice to assess its toxicity. The LD$_{50}$ of a single dose was found to be between 10 and 25 μg (Table III). To determine the blood level of anti-Tac(Fv)-PE40, BALB/c female mice were injected intraperitoneally with 10 μg of protein. Blood was drawn at various times after the injection and assayed on HUT102 cells for immunotoxin activity. A peak blood level
of 1.9 µg/ml was obtained 2 h after the injections (Fig. 5). Detectable levels of toxin were present for up to 8 h, but by 12 h, toxin could no longer be found in the blood (Fig. 5).

**DISCUSSION**

We have shown that anti-Tac(Fv)-PE40, a single chain immunotoxin, is very active in killing cells bearing the human p55 subunit of the IL2 receptor. In this type of chimeric toxin, the variable regions of the heavy and light chain are held together by a linking peptide. We have utilized several different peptide linkers containing 14, 15, or 16 amino acids and found all of them to produce active immunotoxins with about the same activity. Furthermore, we have changed the order of the variable regions without affecting activity (Table I). Stimulation of the binding of Ward *et al.* (15) that a single variable region can bind antigen, we made a chimeric toxin composed of the Vh of anti-Tac fused to PE40, but found it to be inactive (data not shown). Apparently, Vh by itself does not have a high affinity for the p55 subunit. However, it may be possible to make active immunotoxins with single variable regions with selected antibodies.

Anti-Tac(Fv)-PE40 has much greater activity on human cells than IL2-PE40, a molecule which also binds to the IL2 receptor (Table II). There are several reasons that may account for this difference which are related to the number of p55 subunits on the target cells and the relative affinities of the two ligands for p55. Anti-Tac binds to p55 with a KD ~ 10^-8 M, whereas the affinity of IL2 for p55 is ~ 10^-4 M (16). Only when p55 is associated with p75 is the affinity increased to ~10^-11 M. In MT-1 cells where there are 100,000 p55 molecules, the ID50 of anti-Tac(Fv)-PE40 is <1 ng/ml and the ID50 of IL2-PE40 is >100 ng/ml. This probably reflects the affinities of the two ligands. On HUT102 cells, where there are many p55 subunits and a moderate number of p75 subunits, IL2-PE40 and anti-Tac(Fv)-PE40 are about equally active. It has been shown previously that the high affinity IL2 receptor composed of p55 and p75 is rapidly internalized when occupied by IL2 (or anti-Tac), whereas the low affinity p55 receptor is internalized much more slowly (17). Nevertheless, it appears that this slow entry route is compensated for by the fact that there are many more p55 molecules than high affinity receptors on the surface of target cells and also because the binding of anti-Tac to these molecules is much stronger than the binding of IL2 to p55.

Anti Tac(Fv) PE40 is very active on HUT102 cells that express both the low and high affinity human IL2 receptor. Moreover, the fusion protein is also highly active on cells with moderate or low numbers of receptors (Table I). In a mixed leukocyte (leukocyte) reaction, which is an in vitro model system for alloreactive responses, anti-Tac(Fv)-PE40 inhibited the protein synthesis of T-lymphoblasts very efficiently. It has been shown that IL2 receptor-bearing cells play a very crucial role in graft rejection and the administration of anti-IL2 receptor antibody prolongs allograft survival (18–20). We have earlier made a chimeric toxin containing IL2 and PE40, which is active on mouse IL2 receptor-bearing cells (8, 14) and has been shown to significantly prolong the survival of vascularized heart allografts in mice (21). However, IL2-PE40 has low activity on human activated T cells. Anti-Tac(Fv)-PE40, being enormously more active, might prove to be a potent immunosuppressive agent in humans.

Another objective of creating chimeric toxins is their potential use as anti-tumor agents. Since anti-Tac(Fv)-PE40 appears to be a very active protein in *vivo*, we performed several experiments to investigate its activity in *vivo*. The LD50 of anti-Tac(Fv)-PE40 in mice was determined to be between 10 and 25 µg. The protein appears to be slightly more toxic than some other chimeric toxins produced in our laboratory (LD50 ~ 20–50 µg). However, because of the extremely high activity of anti-Tac(Fv)-PE40, it may not be required to use very high doses in *vivo*. Anti-Tac(Fv)-PE40 when injected intraperitoneally in mice above a peak of activity in the blood after 2 h and detectable levels are present up to 8 h.

In summary, anti-Tac(Fv)-PE40 is a chimeric toxin which has shown to be extremely cytotoxic to IL2 receptor expressing cells in tissue culture and in mixed lymphocyte reaction. The agent warrants evaluation as an anti-tumor and immunosuppressive agent in humans.

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**REFERENCES**