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(54) Title: TARGET-SPECIFIC, CYTOTOXIC, RECOMBINANT PSEUDOMONAS EXOTOXIN		
(57) Abstract A target-specific, cytotoxic, recombinant <i>Pseudomonas</i> exotoxin is described. Such toxins are made by inserting specific recognition molecules at specific cloning sites in at least domain III near the carboxyl terminus of the PE molecule. Various modifications of the carboxyl terminus of the PE molecule to increase cytotoxicity are set forth. Multifunctional, recombinant, cytotoxic fusion proteins containing at least two different recognition molecules are provided for killing cells expressing receptors to which the recognition molecules bind with specificity. Methods for producing novel recombinant PE molecules with specific properties are described.		

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TARGET-SPECIFIC, CYTOTOXIC, RECOMBINANT
PSEUDOMONAS EXOTOXIN

This is a continuation in part of the pending application serial number 07/459,635 filed January 2, 1990.

The present invention is related generally to the making of improved recombinant immunotoxins. More particularly, the present invention is related to the construction of a recombinant Pseudomonas exotoxin (rPE) with specific cloning sites for the insertion of recognition molecules at least at the carboxyl end of the PE to achieve target-directed cytotoxicity and for the construction of recombinant multifunctional chimeric cytotoxic proteins.

Background of the Invention

The mechanism by which protein toxins kill cells is quite complex. Many toxins bind to receptors on the surface of mammalian cells, are internalized by endocytosis, translocate to the cytosol and there exert an enzymatic activity that kills the target cell. Accordingly, these toxins have separate domains for cell binding, translocation and an enzymatic activity that inactivates an essential cellular function. Pseudomonas exotoxin A (PE) is a single polypeptide chain of 613 amino acids. X-ray crystallographic studies and mutational analysis of the PE molecule have shown that PE consists of three domains: an amino terminal cell receptor binding domain (Domain I); a middle translocation domain (Domain II); and a carboxyl terminal activity domain (Domain III). Domain III catalyzes the ADP ribosylation and inactivation of elongation Factor 2(EF-2) which inhibits protein synthesis and leads to

cell death. Mutational analysis of Domain I has revealed that lysine⁵⁷ plays a major role in receptor binding. Similarly glutamic acid⁵⁵³, Tyrosine⁴⁸¹ and histidine⁴²⁶ have been shown to be important for ADP-ribosylation activity.

5 Recently mutational analysis of domain II has shown that certain portions of this domain are absolutely required for the cytotoxicity of PE.

While constructing various chimeric toxins in which growth factors were fused to a form of PE (PE40)
10 which was devoid of domain I, it was observed that the recombinant fusion proteins, made by attaching TGF α , interleukin-2 or interleukin-4 at the carboxyl end of PE40 had poor cytotoxic activity. Hence, an examination of the role of the carboxyl terminus of the PE molecule
15 (domain III) was undertaken.

Summary of the Invention

It is, therefore, an object of the present invention to determine the role of the carboxyl terminus of the PE molecule in its cytotoxic action.

20 It is another object of the present invention to identify specific regions at the carboxyl terminus of the PE molecule for the insertion of recognition molecules for selective killing of target cells.

It is a further object of the present invention to
25 provide an improved, target-specific, cytotoxic recombinant PE molecule, wherein the improvement comprises the insertion of target-specific recognition molecule at least in domain III at the carboxyl terminus of the PE molecule.

30 A still further object of the present invention is to modify the carboxyl end of the PE to increase the potency of the chimeric toxin.

It is yet another object of the present invention to make cytotoxic PE with two recognition molecule (target ligands) wherein either the same recognition molecule is placed at two different termini, such as the amino terminus and near the carboxyl terminus, for enhanced cellular binding, or two different recognition elements are inserted, one each at two different regions of the PE molecule, to enable more effective binding of the resulting PE molecule to the cell surface having two or more different entities, such as antigens, receptors and the like to which said recognition elements could bind.

It is an additional object of the present invention to provide a recombinant PE having repeat carboxyl end sequences for enhanced cytotoxic activity.

An additional object of the present invention is to provide a multifunctional, recombinant cytotoxic chimeric protein for simultaneously killing cells expressing different types of receptors.

Various other objects and advantages will become evident from the following detailed description of the invention.

Abbreviations

Various abbreviations, symbols, terminologies and the like used herein are now set forth.

PE-40 means a PE molecule of about 40,000 (Mr. Hwang et al, 1987. Cell 48:129-136) having a deletion of domain I.

TGF α -PE40 means a chimeric protein wherein TGF α is the targeting or recognition molecule linked to PE-40. When the targeting agent is a different entity such as CD4 and the like, the chimeric protein is accordingly

designated CD4-PE40 and the like.

When a numbering system is used, such as PE-Gly609, it means that the amino acid at position 609 in the sequence of the native PE has been replaced by glycine. The same convention is used throughout the specification. The symbol means the deletion of amino acids following the symbol.

Brief Description of the Drawings

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 demonstrates cytotoxicity of PE and PE mutants on Swiss cells. Various dilutions of PE proteins were made in PBS containing 0.2% human serum albumin and added to 1×10^5 Swiss 3T3 cells in 24-well plates. Sixteen hours later the cells were pulse labeled with ^3H -leucine and TCA precipitable cell associated radioactivity was determined as a measure of protein synthesis. The results are expressed as percent of control where no toxin was added. • — • PE; o — o PE Δ 613; □ — □ PE Δ 612, 613; and Δ — Δ PE Δ 611-613. All the assays were done in duplicate and repeated twice.

Figure 2 shows the results of competition for the cellular uptake of recombinant PE. Swiss 3T3 mouse cells were incubated with 400 ng ^3H -PE (specific activity 3.5×10^5 DPM/ μg) and increasing concentrations of purified mutant proteins for one hour at 37°C. Cell monolayers were washed and cell-associated radio-activity was determined. • — • PE; $\blacktriangle$ — $\blacktriangle$ PE Δ 57; $\triangle$ — $\triangle$ PE Δ 612, 613; o — o PE Δ 613; $\blacksquare$ — $\blacksquare$ PE Δ 276; □ — □ PE Δ 609-613;

+ -- + PE Δ 609-613+598-613.

Figure 3 shows immunofluorescence detection of binding and internalization of Pseudomonas exotoxin and its recombinant variants in Swiss 3T3 cells. Swiss 3T3 cells were incubated at 37°C for 30 minutes in the absence of toxin (A) or in the presence of 10 µg/ml of native Pseudomonas exotoxin (PE) (B), recombinant PE gly⁵⁷ (C) or recombinant PE 612,613 (D). Following this incubation, the cells were fixed in formaldehyde and further incubated in the continuous presence of saponin. The cells were incubated with mouse monoclonal anti PE (M40-1) (10 µg/ml), followed by affinity-purified rhodamine-labeled goat anti-mouse IgG (25 µg/ml). (Mags - x 400; bar = 10 µm).

Fig. 4 schematically shows the construction of a multivalent recombinant cytotoxic fusion protein using for illustration herein TGB α and anti-Tac genes. The expression plasmid pJB370548 contains a fusion gene encoding the first 50 amino acid of TGF α , the variable domain of anti-Tac light chain (VL, first 106 amino acids of anti-Tac light chain), a 14 amino acid linker, the variable anti-Tac heavy chain (VH, first 116 amino acids of heavy chain), and amino acids 253-613 of PE. AmpR, β -lactamase gene; B, BamHI; A, AvaI. Amino acids are shown as single letter codes. The gene is under the control of the bacteriophage T7 promoter linked to a Shine-Dalgarno sequence and an initiation codon.

Detailed Description of the Invention

The above and various other objects and advantages of the present invention are achieved by a cytotoxic recombinant Pseudomonas exotoxin (rPE) having a recognition molecule inserted at least in domain III at

the carboxyl terminus of the PE for selective killing of target cells recognized by said recognition molecule without substantial cytotoxicity to other cells not recognized by said recognition molecule, and by a rPE
5 with modified "cytotoxic sequence" with increased cell killing activity. A multifunctional fusion protein having versatility, flexibility and efficacy for killing cells expressing different types of receptors is provided.

10 Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described
15 herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference. Unless mentioned otherwise, the techniques employed or contemplated herein
20 are standard methodologies well known to one of ordinary skill in the art. The materials, methods and examples are illustrative only and not limiting.

The term "recognition molecule" as defined herein means those molecules or ligands which recognize only
25 target cells desired to be killed. Examples of such recognition molecules are antibodies or portions thereof that can recognize the target cells, growth factors, lymphokines, cytokines, antigens, hormones and the like or combination thereof, which specifically bind to
30 molecules on the surface of the target cells.

The term "cytotoxic sequence" as used herein means those variety of amino acid sequences at or near the

carboxyl end of the PE, the presence of which is a prerequisite for the cytotoxic activity of the toxin and the repeat sequences of which may determine the level or cytotoxicity. The examples of such sequences are KDEL, 5 REDLK and the like as will become apparent from the various embodiments of the sequences discussed herein below.

The terms "multivalent" cytotoxic recombinant fusion protein as used herein means that the recombinant 10 fusion protein has at least two similar or different recognition molecules for receptors expressed on the target cells, to which either the first, the second or both recognition molecules bind with particular specificity. Of course, the multivalent fusion protein 15 may be made with any of a variety of cytotoxic sequences described herein.

Materials and Methods

Materials

Unless mentioned otherwise, the materials and 20 reagents used herein were obtained from commercial sources. Polymerization Chain Reaction (PCR) kit, Gene Amp Kit, was obtained from Perkin Elmer-Cetus, Norwalk, CT.

Mutants and Plasmid Constructions

25 Mutants were created by oligonucleotide directed mutagenesis using plasmid pVC45f+T as described by Jinno et al, 1988, J. Biol. Chem. 263, 13203-13207 and Jinno et al, 1989, J. Biol. Chem. 264, 15953-15959 or using polymerase chain reaction (PCR) as described below. 30 pVC45f+T carries a PE gene under a T7 promoter and also contains a T7 transcriptional terminator and a fl phage origin. The PE gene also contains an OmpA signal

sequence which is cleaved upon secretion of PE into the periplasm leaving a 3 amino acid (ala asm leu) extension at the amino terminus (Chaudhary et al, 1988, Proc. Natl. Acad. Sci. USA 85, 2939-2943). For PCR mutagenesis, two oligonucleotides and a 1.0 Kb SalI-EcoRI fragment of pVC45f+T were employed. One oligonucleotide was the same as nucleotides 2216-2236 of the PE gene (Gray et al, 1984, Proc. Natl. Acad. Sci. USA 81, 2645-2649). Other oligonucleotides were complementary to the 3' end of the coding sequence PE gene, contained desired mutations and created an EcoRI site after the stop codon. Other unique restriction sites were also created without changing amino acids to identify the mutants. A 30 cycle PCR was performed with denaturation at 94°C for 2 minutes, annealing at 55C for 1 min and polymerization at 72C for 3 min with 10 seconds extension per cycle using a gent amplification thermal cyclor (Perkin Elmer Cetus). After the PCR, amplified fragment was cut with EcoRI and BamHI, it was purified using low melting point agarose. PCR fragments were ligated with a 4.5 Kb dephosphorylated EcoRI-BamHI fragment of pVC45f+T. Mutants were identified by unique restriction sites which were created during mutagenesis and finally confirmed by sequencing by Sanger's dideoxy-chain termination procedure using Sequenase (US Biochemical Corp.).

pVC4915f+T

This plasmid contains two mutations; Codon 608, CCG and 609, CGC were changed to CCC and GGG, respectively. This mutation results in glycine at 609 in place of arginine and creates a SmaI site between codons 608 and 609. This plasmid was used to clone various carboxyl terminal fragments of PE.

pVC4975f+T: A 1 Kb BamHI-PstI fragments of pVC8 (Wozniak et al, 1988, Proc. Natl. Acad. Sci. USA 85, 8880-8884) was restricted with NarI, treated with T4 DNA polymerase to make blunt ends followed by EcoRI, and a 286bp
5 fragment was ligated to a 4.9 Kb dephosphorylated SmaI-EcoRI, fragment of pVC4915f+T. pVC4985f+T: A 1 Kb BamHI-PstI fragment of pVC8 was restricted with HinfI, treated with T4 DNA polymerase followed by EcoRI and a 237bp fragment was ligated to the 4.9 Kb SmaI-EcoRI
10 fragment of pVC45f+T. pVC4995f+T: A synthetic oligonucleotide duplex VK192/193 (not shown), containing codons 598-613 of PE with a stop codon and an EcoRI compatible 3' end, was ligated to the 4.9 Kb SmaI-EcoRI fragment of pVC4915f+T. pVC4715f+T: This plasmid was
15 created by PCR mutagenesis and contains restriction sites StuI, NdeI, SmaI, EcoRV and EcoRI within the 3' end of the PE gene and encodes amino acids RPHMPGDILK in place of PREDLK at 608 to 613. These unique sites were later used to make insertions and to attach various DNA
20 segments encoding carboxyl terminal portions of PE.

pVC47195f+T

This was created by ligating oligonucleotide duplex VK191/192 to a 4.9 Kb EcoRV-EcoRI fragment of pVC4715f+T. The carboxyl terminus of this PE mutant
25 contains amino acids RPHMPGDPDYASQPGKPPREDLK in place of amino acids 608-613 (PREDLK) of PE.

Plasmids for the insertion of receptor binding domains in the carboxyl end of PE

Plasmid pVC4715f+T consists of DNA sequences
30 encoding PE from amino acids 1 to 607 followed by a polylinker than contains StuI, NdeI, SmaI, EcoRV and AflII sites as well as encodes amino acids RPHMPGDILK.

These sequences are under T7 promoter control and also contain Shine-Dalgarno region and signal sequence from OmpA or *E. coli*. Plasmid pVC4715/4E f+T is similar to pVC 4715 f+T, but also contains mutations in the receptor
5 binding domain of PE (Domain I). These mutations are Lys⁵⁷ → Glu, His^{246,249} → Glu and Arg²⁴⁷ → Glu.

Plasmid pVC 47195/f+T is similar to pVC4715 f+T but contains a polylinker with StuI, NdeI, SmaI sites encoding amino acids RPHMPGI followed by the last 16
10 codons of PE that encode PDYASQPGKPPREDLK. Plasmid pVC47195/4Ef+T contains insertions of a cDNA encoding transforming growth factor α in the NdeI site of plasmids pVC 4715f+T and 4715/4Ef+T respectively. Plasmids pVC 47395f+T and pVC 4715/4Ef+T are derived from 47195f+T and
15 pVC 47195f+T by inserting TGF α sequences into the NdeI site. Plasmid pVC 47355/4Ef+T was derived from pVC47395/4Ef+T by deleting 6 amino acids, inserting TGF α and following it with 10 amino acids of the PE carboxyl end.

20 A deposit of pVC49415f+t and pVC47355/4Ef+t has been made at the ATCC, Rockville, MD, on December 28, 1989 under accession numbers 68198 and 68199, respectively. The deposit shall be viably maintained, replacing if it becomes non-viable during the life of the
25 patent, for a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of the deposit, whichever is longer, and upon issuance of the patent made available to the public without restriction in accordance with the provisions of
30 the law. The Commissioner of the Patents and Trademarks, upon request, shall have access to the deposit.

Protein Expression and Purification

Cultures of *E. coli* strain BL21 (λ DE3) containing various plasmids were grown to OD₆₅₀ of 0.6-0.8 and induced with 1 mM isopropyl-thiogalactoside for 90 minutes at 37°C. Periplasmic fractions were prepared as described by Chaudhary et al, supra. By virtue of having an OmpA signal sequence, more than 90% of each of the expressed toxin proteins were secreted into the periplasm. These proteins have a residual ala asn leu sequence at the amino end left behind after the processing of the OmpA signal sequence. Periplasmic fractions were assayed for ADP ribosylation activity and cytotoxicity. Later PE mutants were purified using a MonoQ anion exchange column (HR5/5) attached to a Pharmacia FPLC system. PE and mutant proteins eluted at NaCl concentrations of 0.22-0.26M. Upon SDS-PAGE the toxins were more than 90% pure. Protein concentration was measured by the Bradford assay Reagent (BioRad, Richmond, CA) using bovine serum albumin as a standard.

ADP Ribosylation and Cytotoxicity Assays

ADP ribosylation activity was assayed after the activation of PE and mutant proteins with 4M urea and 50 mM DTT unless otherwise stated (Collier et al, 1971, J. Biol. Chem. 246, 1946-1503). Cytotoxicity of PE mutants was determined by adding various dilutions of periplasmic proteins or purified proteins to 1×10^5 Swiss 3T3 cells in 24-well plates as described by Jinno et al, (1988) supra, and Jinno et al, (1989) supra. ADP-ribosylation and cytotoxic activities of recombinant PE and native PE (from Swiss Serum and Vaccine Institute, Berne, Switzerland) were indistinguishable.

Toxin Binding and Internalization

The ability of various mutant PE proteins to compete the binding of ^3H -labelled PE on Swiss cells as well as internalization of various mutant PE derivatives as studied by immunofluorescence has been described by Jinno et al, (1989) supra.

Preparation of Target-Specific Immunotoxins

PE expression vectors with the cloning sites in the carboxyl end of PE to produce selective cytotoxic molecules are illustrated here with TGF α , a recognition molecule which recognizes only EGF receptor bearing cells. These cloning sites were used to insert TGF α near the carboxyl end of PE which produced a very active molecule that killed EGF receptor bearing cells when the insertion was followed by the last 10 amino acids of PE (Table A). The details of the procedure are now described.

RESULTS

The role of sequences at the carboxyl end of PE was determined by making a series of carboxyl terminal deletion mutants that removed 1,2,3,7,8,11,14 and 24 amino acids. Removal of two or more amino acids eliminated cytotoxicity without affecting ADP ribosylation activity (Table 1, Fig. 1). In fact, even eleven amino acids (603-613) could be removed without any loss of ADP ribosylation activity. However, removal of 14 amino acids resulted in a protein with low, but measurable, ADP ribosylation activity (600-613) and removal of 24 amino acids (590-613) produced an enzymatically inactive protein. These results indicate that particular sequences at the carboxyl end of PE have a role in toxin action that are not required for ADP ribosylation activity.

The role of the carboxyl terminal sequences in toxin action was defined by creating a series of internal deletions and substitutions (Table 2). These mutations began at amino acid 602 so that ADP ribosylating activity would not be affected and extended to position 611. It was found that several small deletions encompassing amino acids 601-604 and 606-608 did not reduce cytotoxicity. Furthermore, two substitutions which altered amino acids 603-608 as well as two other substitutions within amino acids 606-608 of PE did not reduce cytotoxicity. Therefore, the sequence of amino acids in positions 602-608 did not appear to be important for cytotoxicity. However, deletions which removed arginine at 609 (pVC 49215 and pVC 49255) greatly reduced the cytotoxic action of PE. These results, together with the experiments in Table 1 showing that deletion of amino acids 612 and 613 abolish cytotoxicity, focused our attention on amino acids 609-613 which are situated at the carboxyl terminus of PE.

The role of arginine 609 was studied by either deleting it or replacing it with several different amino acids. Replacement of arginine at 609 with another basic amino acid, lysine, retained the cytotoxic activity of PE (Table 3). However, deleting arginine at 609 (pVC 49115) or replacing it with glycine, glutamic acid or leucine reduced cytotoxicity about 6-10-fold. Thus, a basic amino acid appears to be important at position 609.

To study the sequence specificity of the last five amino acids of PE, several other mutant molecules were then constructed. In two of these, the order of the acidic amino acids at positions 610 and 611 was reversed and lysine 613 deleted (Table 4, pVC 49415 and pVC

49425). These molecules were fully active whether or not position 609 was a lysine or an arginine. A molecule was also created with a leucine at position 609 and an arginine at 612 (pVC 49435) that was inactive.

5 Although deletion of the terminal amino acid lysine at 613 did not affect cytotoxicity, it was suspected that other mutations in this position might affect cytotoxicity in a negative manner because of the low activity of various chimeric toxins in which the
10 ligand was placed in peptide linkage at the carboxyl terminus of PE. Therefore, lysine⁶¹³ was converted to glutamine, asparagine or aspartate. All these mutations produced a less cytotoxic molecule (Table 5). Addition of 6 or 11 amino acids to the carboxyl terminus of PE
15 also produced a less cytotoxic molecule (data not shown). However, replacement of lys⁶¹³ with the basic amino acid, arginine, did not decrease cytotoxicity. Thus, positions 609 and 613 both require a basic amino acid for full cytotoxic activity. There are two other lysine residues
20 at the carboxyl end of PE; these are situated at positions 590 and 606. Both of these lysines could be converted to the uncharged amino acid glutamine without a decrease in cytotoxicity, indicating that a positively charged amino acid was not required at position 590 or
25 606 (Table 5).

 Having shown the importance of particular amino acids at the carboxyl terminus of PE, it was determined that the five carboxyl terminal amino acids could be separated from the ADP ribosylation domain to regenerate
30 an active toxin. As shown in Table 6, a fully active cytotoxic molecule could be generated from PE 609-613 (which is not cytotoxic) by the addition of amino acids

551-613, 567-613 or 598-613 of an intact PE to the carboxyl terminus of PE 609-613. Thus, the distance between the ADP ribosylation domain which ends around amino acid 600 and the essential amino acids at positions 5 609-613 was not critical and could be substantially increased without a decrease in cytotoxicity. Also shown in Table 6 is a PE molecule with the carboxyl terminus of RPHMPGDILK in place of PREDLK. This molecule, in which arg⁶⁰⁹ and asp⁶¹¹ were altered, was not cytotoxic. But 10 attaching the last 16 amino acids of an intact PE molecule to give a carboxyl terminus of RPHMPGDPDYASQPGKPPREDLK restored cytotoxicity to this molecule.

Furthermore, constructs were made in which a cDNA 15 TGF was inserted at the carboxyl end of PE with an inactive carboxyl terminus (Table A pVC 47315/4Ef+T) and an active carboxyl terminus (Table A, pVC 47355f+T and pVC 47395f+T). The constructs with good carboxyl termini were more than 50 times as cytotoxic to cells with EGF 20 receptors (TGF α binds to the EGF receptor) as the ones with the bad carboxyl ends. This clearly indicates that for the highest cytotoxic activity, a suitable carboxyl end is an essential requirement.

Altogether the data presented herein demonstrate 25 that the cytotoxic activity of a PE molecule that is inactive due to a deletion or modification within the carboxyl end can be restored by attaching an intact carboxyl end. Hence, it is now possible to create active chimeric molecules by inserting a binding ligand such as 30 TGF α at 608 within the carboxyl end of PE thus retaining the last five amino acids as REDLK.

Although it has been previously demonstrated that domain I of PE is the region responsible for cell binding, it was important to show that the mutations at the carboxyl end of PE that decreased cytotoxicity did not also somehow decrease cell binding. To test this, the ability of various mutant forms of PE to compete for the uptake of [³H]-PE was evaluated. As shown in Figure 2, several PE mutants that had decreased cytotoxicity due to mutations at the carboxyl terminus of PE were just as able to compete for the uptake of [³H]-PE as authentic wild type of PE. In this competition assay, PE40 which has a deletion of domain I and PEglu⁵⁷ were inactive as previously described (Jinno et al, supra).

These uptake results were confirmed using a fluorescence assay that measured the internalization of PE and various mutant PE molecules (Figure 3). In this assay, cells are incubated with various toxins for 30 minutes to allow binding and internalization into endocytic vesicles. Molecules with a point mutation in domain I (PEglu⁵⁷) or PE40 were not internalized. In contrast, all the other PE molecules, whether or not they contained mutations at the carboxyl end of domain III, were found to have bound and internalized into endocytic vesicles and other elements in the trans-Golgi system in the perinuclear area of the cells (Figure 3, Panel B and D). These results clearly show that decreased cytotoxicity of carboxyl terminal mutants is not due to decreased receptor binding or cellular uptake of PE molecules.

In summary, the results presented herein clearly show that mutations at the carboxyl end of PE and particularly in the last five amino acids of PE result in

a molecule with full ADP ribosylation activity, but greatly reduced cytotoxicity. The data shown that the amino acid sequence at the carboxyl end of PE is Arg, Glu, Asp, Leu, Lys (REDLK, Table 2). The arginine at 609
5 can be replaced by lysine but non basic amino acids cannot be tolerated (Table 3). Lysine at 613 is not essential and can be deleted without loss of cytotoxic activity (Table 1), but it cannot be replaced with a non-basic amino acid (Table 5). Thus, having either
10 ArgGluAspLeu or LysGluAspLeuLys at the carboxyl terminus produced a fully cytotoxic molecule (Table 4). A search of the literature for similar sequences that were present in other molecules and performed a specific biological function revealed that the sequence which retains newly
15 formed proteins within the endoplasmic reticulum is LysAspGluLeu (Munro et al, 1987, Cell 48, 899-907). Therefore, several other mutant molecules were constructed, one of which contained the exact sequence previously described as being responsible for the
20 retention of the protein in the lumen of the endoplasmic reticulum (Table 4). It was found that a molecule ending with LysAspGluLeu (KDEL) was fully cytotoxic. Also a molecule ending in ArgAspGluLeu (RDEL) but not LeuAspGluArg(LDER) was fully active. These findings
25 indicate that the successful entry of PE into the cytosol from an endocytic compartment requires interaction with the similar cellular component that helps retain proteins made by the cells within the endoplasmic reticulum. These findings also suggest that the sequence at the
30 carboxyl end of PE acts as some type of recognition sequence to assist translocation of PE from an endocytic compartment into the cytosol. Other sequences that

perform the same function would likewise increase the activity.

Of further significance was the finding that because the cell targeting ligands can be inserted at two
5 cloning regions in the PE molecule (at the amino terminus as previously described or near the carboxyl end as described herein), the same or different targeting ligands can be inserted at these two regions thereby increasing either cell binding, cytotoxicity or both.
10 Different targeting molecules at each of the two cloning regions would enable the chimeric toxin to bind to two different types of receptors present on the same cell. This is important because some antigens on target cells do not internalize well and are, therefore, poor targets
15 for immunotoxins. But, if the chimeric toxin or immunotoxin also binds to another antigen that is well internalized, specific cell killing is increased greatly.

It was further discovered during the modificational study of the carboxyl end of the PE that
20 if the REDLK (single letter amino acid code) sequence is replaced with KDEL, the resulting molecule is about two fold more active. Even more spectacular was the finding that a molecule with three repeats of KDEL in place of REDLK, was three times as active (Table B) indicating
25 that by adding KDEL or equivalent repeat sequences, chimeric toxins with enhanced cytotoxicity can be produced.

In short, the present invention for the first time shows that:

- 30 1. An appropriate carboxyl end sequence is absolutely required for cytotoxicity of the PE;

2. Deletion of as few as two amino acids from the carboxyl end of PE yields a molecule that contains full ADP ribosylation and receptor binding activity, but is nontoxic to target cells (Table 1);
- 5 3. Mutational analyses indicate that PE should possess a positively charged amino acid at 609, negatively charged amino acids at 610 and 611 and a leucine at 612;
4. Lysine at 613 can be deleted but cannot be substituted with several other amino acid residues;
- 10 5. Addition of random amino acid residues at the carboxyl end of PE produce relatively inactive molecules (data not shown).
6. Addition of at least 10 carboxyl end amino acids of PE to the PE molecules that are not cytotoxic due to
- 15 mutations in the carboxyl end, restores full cytotoxic activity (Table 4);
7. Different targeting ligands at different ends (amino and carboxyl) provide the flexibility of producing better binding and cytotoxic PE molecules; and
- 20 8. Repeat "cytotoxic sequences" multiply the cytotoxicity in appropriate cases.

Of course, other target-specific immunotoxins are made similar to the method described herein supra by using appropriate recognition molecules, toxins and

25 cytotoxic sequences including such modified recombinants as TGF α -PE40, CD4-PE40 and the like (See Table C). An example of a bifunctional toxin, cytotoxic for cells expressing two different receptors is now provided in accordance with the present invention to illustrate such

30 constructions. It may be noted in this respect that conventional immunotoxins and chimeric toxins usually made in bacteria are ordinarily directed to only one

receptor or antigen on target cells. The successful construction of an active chimeric toxin molecule containing more than one target recognizing entities, opens the possibility of producing chimeric molecules of greater versatility, flexibility and efficacy. Figure 4 shows the schematic construction of pJB370548 to produce a multipurpose chimeric protein containing two recognition molecules, TGF α and anti-Tac (Fv) for binding to cells expressing either EGF, IL2 or both. Table D shows a comparison of the cytotoxic activity of this novel multifunctional recombinant fusion protein compared to monofunctional entities tested against appropriate cells easily suggested to one of ordinary skill in the art.

A deposit of pJB370548 has been made at the ATCC, Rockville, MD., on May 4, 1990 under accession number 68318. The deposit shall be viably maintained, replacing if it becomes non-viable during the life of the patent, for a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of deposit, whichever is longer, and upon issuance of the patent made available to the public without restriction in accordance with the provisions of the law. The Commissioner of the Patents and Trademarks, upon request, shall have access to the deposit.

Of course, a method of preparing a cytotoxic, recombinant PE in accordance with the present invention, comprises the steps of utilizing the plasmid described herein without alteration or modifying said plasmid to contain a desired DNA sequence and then functionally inserting said plasmid in an expression vector so that a desired cytotoxic recombinant PE is produced and then

recovering the desired PE in a substantially pure form.
It is noted that the procedures for modifying, expressing
and obtaining the desired PE from the plasmids are quite
standard in the art and easily suggested to one of
5 ordinary skill, given the teachings contained herein.

It is understood that the examples and embodiments
described herein are for illustrative purposes only and
that various changes and modifications in light thereof
will be suggested to persons skilled in the art and are
10 to be included within the spirit and purview of this
application and scope of the appended claims.

Table 1: Deletion analysis of the carboxyl terminus of PE

Mutants amino acids present	amino acids deleted	Cytotoxicity	ADP ribosylation activity
1-589	590-613	<0.1	0
1-599	600-613	<0.1	20
1-602	603-613	<0.1	100
1-605	606-613	<0.1	100
1-606	607-613	<0.1	100
1-610	611-613	<0.1	100
1-611	612,613	<0.1	100
1-612	613	100	100
1-613		100	

Legend to Table 1. Mutant PE proteins were expressed in E. coli using T7 promoter based vector (Studier and Moffatt, 1986) and purified from the periplasm. All proteins contain a 3 amino acid (ala asn leu) extension at the amino terminus remaining after the processing of the OmpA signal sequence. These amino acids were not considered when assigning residues-numbers to the above mutant proteins. Cytotoxicity was determined by assaying inhibition of protein synthesis on Swiss 3T3 mouse cells. All results are expressed as percent of the Activity obtained with recombinant full length PE molecules. All the assays were done in duplicate and at least 2 separate clones were tested.

TABLE 2: Internal deletions and substitutions within the carboxyl terminus of PE

Plasmid pVC	Location of the amino acids in PE																		Cytotoxicity
	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	
45	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100
49215	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	<0.1
49235	1	2	3	4	5	6	7	8	9	0	1	1	1	1	1	1	1	1	100
49245	A	S	Q	P	G	K	P	P	R	E	D	L	K	K	K	K	K	K	100
49255	A	S	Q	P	G	K	P	P	R	E	D	L	K	K	K	K	K	K	100
4935	A	S	Q	P	G	K	P	P	R	E	D	L	K	K	K	K	K	K	100
4955	A	S	Q	P	G	K	P	P	R	E	D	L	K	K	K	K	K	K	100
49315	A	S	Q	P	G	K	P	P	R	E	D	L	K	K	K	K	K	K	100

Mutant PE proteins were expressed in E. coli and purified from the periplasm.

ADP ribosylation activities of all the mutants were indistinguishable from the full length PE.

Amino acids within the carboxyl end of PE (601-613) are shown as single letter code. The substitutions have been underlined.

Table 3: Mutations at position 609 of PE

Plasmid (pVC)	Mutant Proteins	Cytotoxicity (% of PE)
49115	PE 609	12
49125	PELys ⁶⁰⁹	100
4915	PEgly ⁶⁰⁹	10
49135	PEglu ⁶⁰⁹	16
49155	PEleu ⁶⁰⁹	15

Mutant PE proteins were expressed in E. coli and purified from the periplasm. Substitutions are shown as replacement amino acids (also see Tables 1 and 2).

Table 4: Sequence specificity of last 5 amino acids of PE

Plasmids (pvc)	Location of the amino acid in PE					Cytotoxicity (% of PE)
	609	610	611	612	613	
45	R	E	D	L	K	100
49125	K	E	D	L	K	100
4215	R	E	D	L		100
49415	K	D	E	L		100
49425	R	D	E	L		100
49435	L	D	E	R		<0.03

For details see legend to Tables 1 and 2.

Table 5: Mutation of the lysines residues 590, 606 and 613 in the carboxyl terminal domain of PE

Mutants	Cytotoxicity (% of PE)	ADP-ribosylation (% of PE)
PE ⁶¹³	100	100
PEarg ⁶¹³	100	100
PEgln ⁶¹³	1	100
PEglu ⁶¹³	1	100
PEasn ⁶¹³	1	100
PEgln ⁶⁰⁶	100	100
PEgln ⁵⁹⁰	100	100
PEgln ^{590,606,613}	1	100
PEgln ^{590,606} arg ⁶¹³	100	100

Analyses were performed as described in Tables 1 and 2.

Table 6: Addition of various portions of PE carboxyl terminus to PE 609-613

Plasmid (pVC)	Mutant Proteins	Cytotoxicity (% of PE)	ADP-ribosylation activity (% of PE)
4905	PE 609-613	<0.1	100
4975	PE 609-613+551-613	100	100
4985	PE 609-613+567-613	100	
4995	PE 609-613+598-613	100	
4715	PE 609-613 RPHMPGDILK	<0.1	100
47195	PE 608-613 RPHMPGD+598-613	50	100

Legend to Table 6. A plasmid pVC4915 with a SmaI site between codons 608 and 609 of PE was created and various portions of the carboxyl terminus were attached after codon 608. pVC4995 was constructed using synthetic oligonucleotides. The last 16 amino acids (598-613) of PE consists of PDYASQPGKPPREDLK (also see Table 1 and 2). means the deletion of amino acids following the symbol.

Table A: Cytotoxic activity on A431 cells of various carboxy terminal insertions of TGF α into PE.

<u>Plasmid</u>	<u>Protein^a</u>	<u>ID₅₀(ng/ml)^b</u>
pVC47315/4E(f+)T	PE1-607RPHMA (TGF α) <u>AHMPGDILK</u>	>25
pVC47395/4E(f+)T	PE1-607RPHMA (TGF α) <u>AHMPGIPDYASQPGKPPREDLK</u>	0.5
pVC47355/4E(f+)T	PE1-607RPHMA (TGF α) <u>AHMPGKPPREDLK</u>	0.5

^a Fusion proteins were partially purified from periplasm. SDS-PAGE indicated that the fusion proteins were 20-30% pure. Residues normally present in PE are underlined.

^b ID₅₀ is the concentration of fusion protein (estimated as total protein concentration) that is required to inhibit protein synthesis by 50 percent as compared to control where no toxin was added. Protein synthesis was measured by 3H-leucine incorporation.

Table B
Cytotoxic activity on Swiss 3T3 cells of various PE derivatives

Plasmid	Protein ^a	ID ₅₀ (ng/ml) ^b
pVC 45f+T	PE1-608REDLK	1.6
pVC 49415f+T	PE1-608KDEL	0.76
pSS 49445f+T	PE1-608KDELKDELKDEL	0.55

^a PE proteins were purified on Mono Q column and were approximately 90% pure.

^b Same as Table A.

Table C
I. ACTIVITY OF TGF α -PE40 AND KDEL DERIVATIVES (ID₅₀)
ON CELLS WITH EGF RECEPTORS.

	A431	KB	OVCAR3	HUT 102
	ng/ml	ng/ml	ng/ml	ng/ml
TGFA-PE40	.35 .44	.96	5.4	>312
TGFA-PE40 KDEL*	.048 .034	.37	.84	>312
TGFA-PE40 (KDEL) ₃ **	.076 .022	.12	1.1	>312
* TGF α -PE40 (253-609 KDEL)				
** TGF α -PE40 (253-609 KDEL KDEL KDEL)				

This table shows that replacing the last 5 amino acids of TGF α -PE40 with KDEL or (KDEL)₃ increases its activity 3 to 10 -fold.

Table CII. CYTOTOXICITY OF CD4-PE40 DERIVATIVES ON ENV-5 CELLS
THAT EXPRESS gp120 OF HUMAN IMMUNODEFICIENCY VIRUS.

PROTEIN	ID ₅₀ (ng/ml)
CD4-0E40+REDLK	2.5
CD4-PE40+KDEL	0.5
CD4-PE40+(KDEL) ₃	0.65

Increased cytotoxicity of CD4-PE40 on target cells expressing HIV gp 120 produced by replacing the last 5 amino acids of CD4-PE40 with KDEL or (KDEL)₃. ENV-5 cells express gp 120.

Table D

Comparison of cytotoxic activity of bifunctional and monofunctional recombinant fusion proteins.

	ID ₅₀ ^a (ng/ml)	
	HUT102	A431
TGF α -anti-Tac(Fv)-PE40	7.8	12.0
Anti-Tac(Fv)-PE40	2.3	~500
TGF α -PE40	>500	0.5

^a ID₅₀ is the concentration of the fusion protein that gave 50% inhibition of protein synthesis.

WHAT IS CLAIMED IS:

1. A cytotoxic Pseudomonas exotoxin (PE) having a first recognition molecule inserted in domain III near the carboxyl terminus of the PE, the resulting PE being
5 selectively cytotoxic only to target cells recognized by said recognition molecule.
2. The PE of claim 1 wherein a second recognition molecule is inserted in a region other than said carboxyl terminus.
- 10 3. The PE of claim 2 wherein said first and second recognition molecules are the same or different.
4. The PE of claim 1 wherein said recognition molecule is an antibody or a portion of an antibody which recognizes said target cell.
- 15 5. The PE of claim 1 wherein said recognition molecule is selected from the group consisting of a growth factor, a lymphokine, a cytokine, a receptor, an antigen, a hormone and a combination thereof.
6. The PE of claim 5 wherein said recognition molecule
20 is TGFa or CD4.
7. The PE of claim 1 wherein said recognition molecule is inserted after amino acid 607, the numbering sequence being that of the native PE.
8. The PE of claim 1 wherein said carboxyl terminus has
25 only the last 10 amino acid residues of PE following insertion at 607 and deletion of 608-613.
9. The PE of claim 1 wherein the amino acid after residue 608 comprises a sequence KDEL, REDLK or repeat thereof.
- 30 10. The PE of claim 9 wherein the amino acid after residue 608 comprises a sequence KDELKDEL.

11. The PE of claim 9 wherein the sequence after residue 608 is KDELKDELKDEL.
12. The PE of claim 9 wherein the repeat sequence yields a rPE molecule with enhanced cytotoxicity compared to a molecule without the repeat sequence.
13. A composition, comprising a cytotoxic amount of the PE of claim 1 and a pharmaceutically acceptable carrier.
14. A method for killing target cells, comprising contacting cells targeted to be killed, with a cytotoxic amount of the PE of claim 1.
15. A method for producing a target-specific cytotoxic PE molecule, comprising inserting a first target-specific recognition molecule at least in domain III near the carboxyl terminus of the PE molecule.
16. The method of claim 15 further comprising the step of replacing the amino acid residues after amino acid number 608, with the sequence KDEL or repeats thereof.
17. The method of claim 16 further comprising the step of inserting a second recognition molecule in a region other than said carboxyl terminus of the PE molecule.
18. The method of claim 17 wherein said second recognition molecule is the same as or different from said first recognition molecule.
19. The method of claim 18 wherein the second recognition molecule is inserted near the amino terminus of the PE molecule.
20. A multifunctional, cytotoxic, recombinant fusion protein containing at least two different recognition molecules for killing cells expressing receptors to which said recognition molecules bind.
21. The multifunctional protein of claim 20 wherein said recognition molecule is selected from the group

consisting of an antigen, an antibody or a fraction thereof, a growth factor, a cytokine, a receptor, an antigen and a combination thereof.

22. The multifunctional protein of claim 21 wherein said
5 recognition molecules are TGF α and anti-Tac.

23. A composition comprising a cytotoxic amount of the multifunctional protein of claim 20 in a sterile, nontoxic carrier.

24. A method of killing targeted cells without
10 substantial toxicity to non-targeted cells, comprising the step of contacting cells targeted to be killed, with a cytotoxic amount of the multifunctional protein of claim 20 wherein said multifunctional protein has recognition molecules which have specific binding
15 affinity only to cells targeted to be killed.

25. A plasmid selected from the group consisting of pVC49415f+t, pVC47355/4Ef+t and pJB370548.

26. The plasmid of claim 25 being pVC49415f+t deposited at the ATCC under accession number 68198.

20 27. The plasmid of claim 25 being pVC47355/4Ef+t deposited at the ATCC under accession number 68199.

28. The plasmid of claim 25 being pJB370548 deposited at the ATCC under accession number 68318.

29. A method of preparing a cytotoxic, recombinant PE,
25 comprising the step of utilizing the plasmid of claim 25 without alteration or modifying said plasmid to contain a desired DNA sequence and then functionally inserting said plasmid in an expression vector so that a desired cytotoxic recombinant PE is produced and then recovering
30 the desired PE in a substantially pure form.

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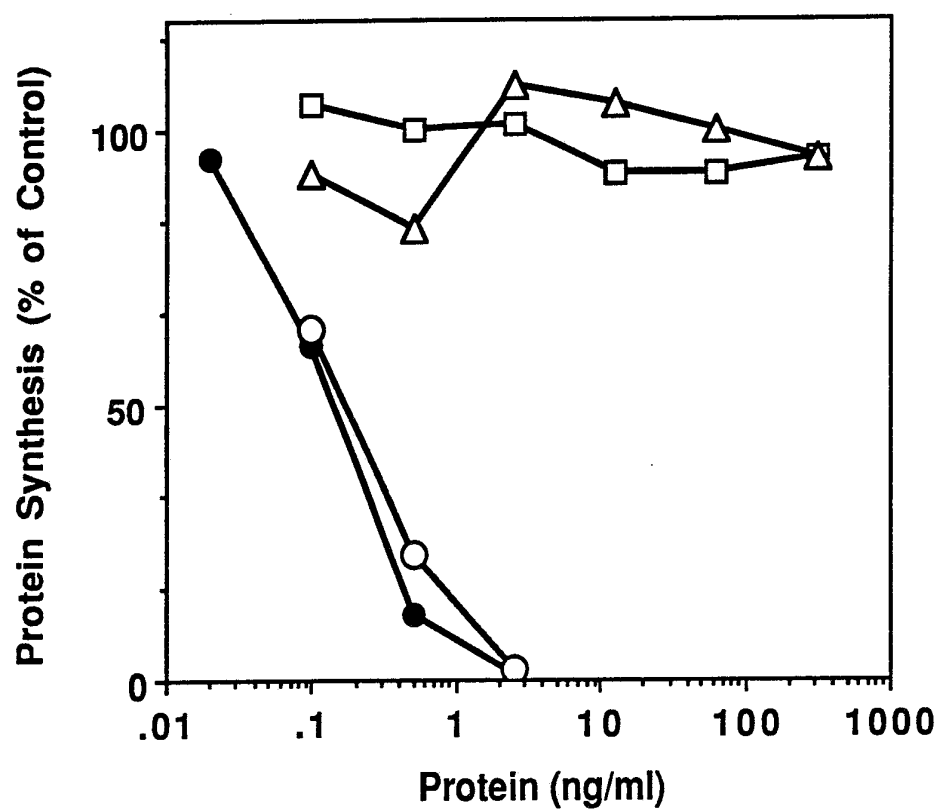


FIG. 1

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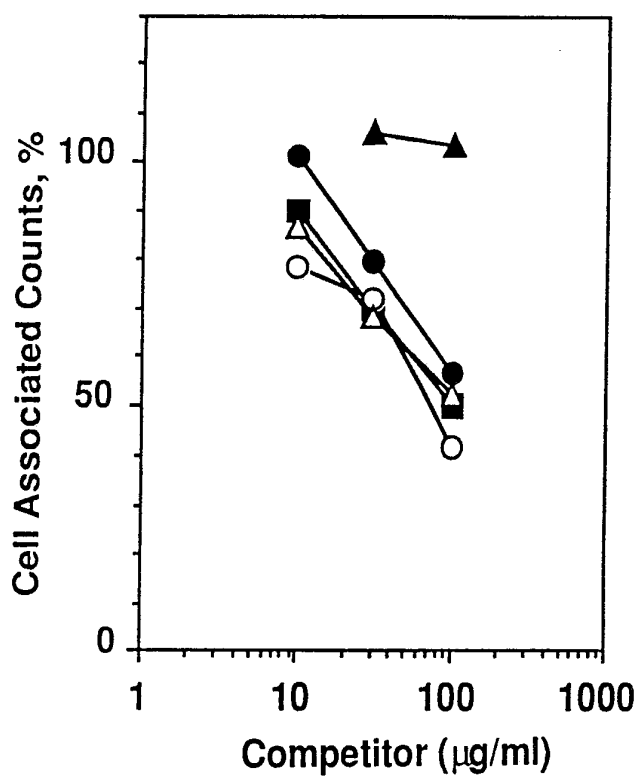


FIG. 2A

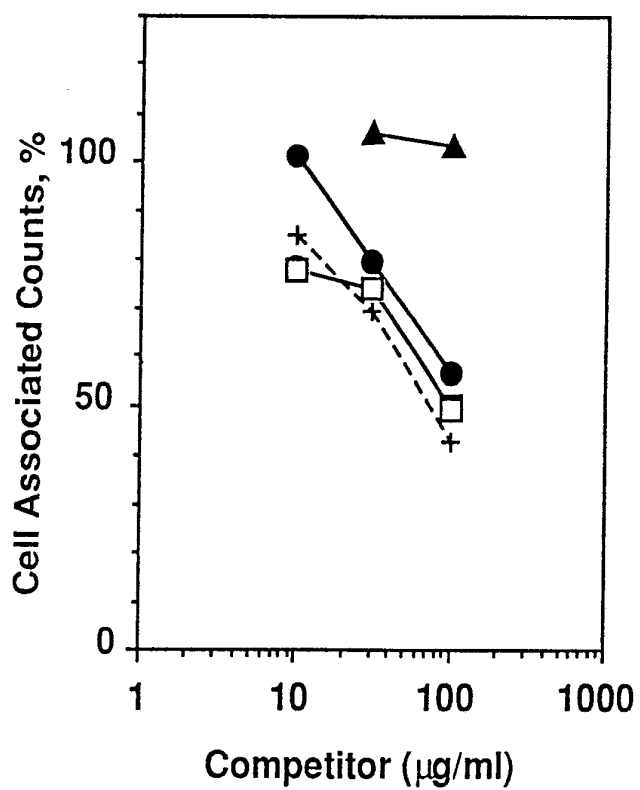


FIG. 2B

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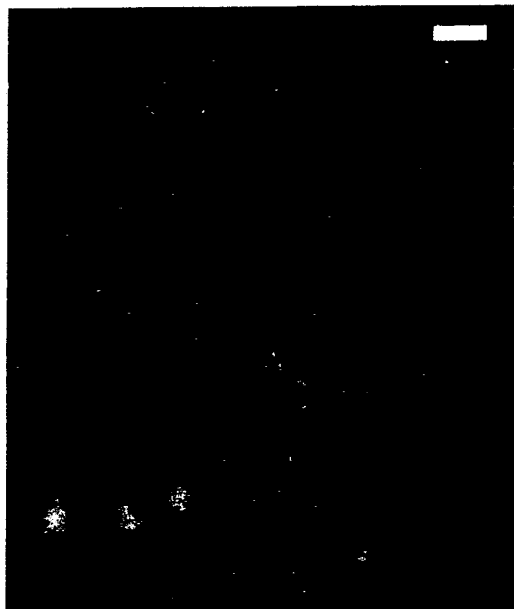


FIG. 3A



FIG. 3B

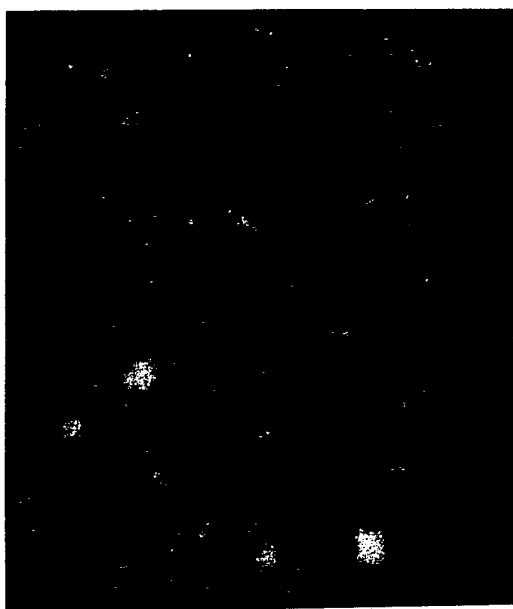


FIG. 3C

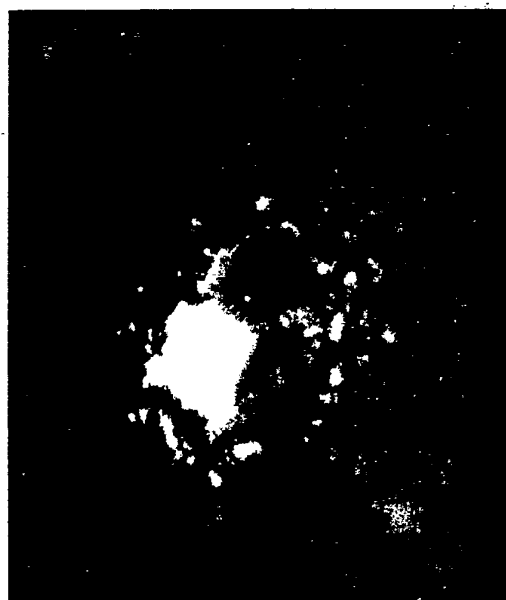


FIG. 3D

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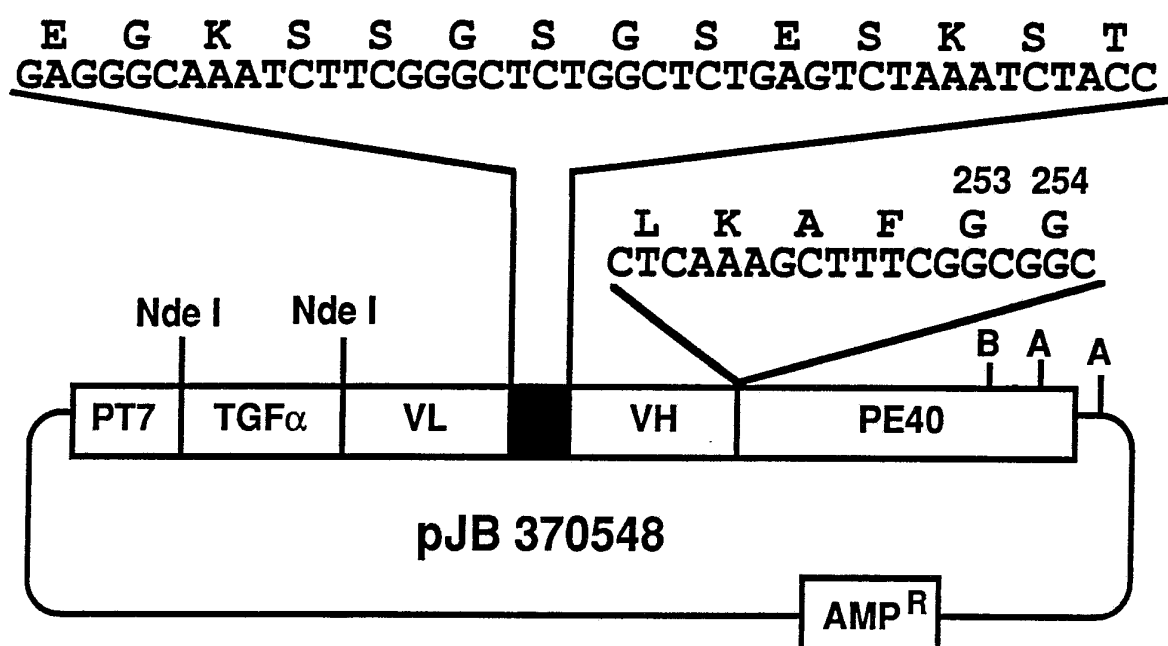


FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03121

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/31
U.S. CL.: 424/85.91

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷	
Classification System	Classification Symbols
U.S.	435/69.7; 69.4; 69.5 - 69.52; 530/351; 424/85.91

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

Automated Patent System File: USPAT
DIALOG Files: BIOSIS, Pascal, EMBASE, CA, Biotech Abstracts e
Search Terms attached

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Nature, vol. 335, issued September 1988, Chaudhary et al., "Selective killing of HIV-infected cells by recombinant human CD4- <u>Pseudomonas</u> exotoxin hybrid protein", pp. 369-372, see entire document.	1-7,14-19
Y	Cell, vol 48, issued January 1987, Hwang et al, "Functional Domains of <u>Pseudomonas</u> Exotoxin identified by deletion analysis of the gene expressed in <u>E. Coli</u> ", pp 129-136, see entire document.	1-7,14-19
Y	Proceedings of the National Academy of Sciences, USA, vol. 87, issued January 1990, Chaudhary et al., " <u>Pseudomonas</u> exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity", pp 308-312, see entire document.	1-7,14-19

^{*} Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Δ" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

26 August 1991

Date of Mailing of this International Search Report

12 SEP 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

T. Michael Nisbet

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-7, 14 and 15-19
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	ICSU Short Reports, vol. 8 issued 1988. Pastan et al, "Oncotoxins: genetically engineered toxins as agents for cancer treatment - recombinant transforming growth factor- and <u>Pseudomonas</u> exotoxin hybrid production, purification and characterization", page 225, see abstract No 89-11355.	1-7,14-19
Y	Proceedings of the National Academy of Sciences, vol. 85, issued December 1988. Seigall et al, "Cytotoxic activity of an interleukin 6- <u>Pseudomonas</u> exotoxin fusion protein on human myeloma cells", pp 9738-9742, see entire document.	1-7,14-19