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(54) Title: PYRULARIA THIONIN CONTAINING IMMUNOTOXINS AND IMMUNOTOXIN-LIKE CONJUGATES

(57) Abstract

The present invention relates to an immunotoxin comprising a mouse antihuman-CD5 monoclonal antibody conjugated to the toxin *Pyrularia* thionin (*P.*) thionin. *P.* thionin is a small, highly stable, membrane active peptide which is toxic to some procaryotic and all eucaryotic cells tested to date. The *P.* thioninimmunotoxin displays a high degree of cytotoxicity and specificity. The *P.* thionin immunotoxin was cytotoxic towards T cells which express the CD5 antigen, yet showed no reactivity towards human B cells, human or sheep erythrocytes or mouse lymphocytes which do not express the human CD5 antigen.

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Pyrularia THIONIN CONTAINING IMMUNOTOXINS AND IMMUNOTOXIN-LIKE CONJUGATES

1. FIELD OF THE INVENTION

The present invention relates to immunotoxins and immunotoxin like compounds. More particularly, the invention relates to molecules containing a protein which is specific for an antigen or ligand on the cell surface conjugated to the toxin *Pyrularia* thionin.

2. BACKGROUND OF THE INVENTION

Cancer develops when a cell in ones body undergoes uncontrolled growth. Unregulated cell proliferation is primarily a result of irreversible damage to particular classes of genes within the cell. As genetic mutations accumulate in the DNA of the cell, each successive generation of daughter cells becomes less responsive to growth inhibitory and regulatory signals. Eventually, a daughter cell will no longer respond to these signals and begins to display the signs of malignancy. The resulting cellular mass, or tumor, damages the surrounding healthy tissue. With time, the cancer can metastasize and breakdown organ tissue barriers, establishing new colonies at distant sites.

Despite the advances in anticancer therapy, metastatic cancers and malignant blood diseases remain largely incurable. The significant disadvantage of most anticancer therapies is their lack of specificity for the cancer cells. Therefore, to some degree, chemotherapy and radiotherapy contribute to the morbidity and mortality of cancer patients. Accordingly, research has focused on the development of anticancer agents with greater specificity for cancer cells, and lesser toxicity for normal cells.

Immunotoxins and immunotoxin-like compounds are anticancer agents that are highly selective for cancer cells. Immunotoxins are protein conjugates comprised of a monoclonal antibody and a toxic peptide or small protein. The monoclonal antibody portion of the immunotoxin recognizes a specific antigen expressed only, or primarily, by a particular type of cells. When the antibody binds to the antigen, it brings the

toxin in close proximity to the cancer cell were it exerts its cytotoxic effect.

Immunotoxin-like compounds derive their target specificity from growth factors that bind to a particular receptor, such as erbB2, EGF, IL2, IL4, and IL6 receptors; other antigens which are predominantly found on carcinomas; or certain oligosaccharides, such as derivatives of the Lewis Y type carbohydrate which are abundantly expressed by carcinomas.

Immunotoxins and immunotoxin-like compounds which are composed of monoclonal antibodies or growth factors coupled to plant or bacterial toxins have been shown to be highly cytotoxic to, and specific for, the targeted cancer cell. Most of these toxins rely on arresting cellular protein synthesis for their cytotoxicity. The plant toxins ricin and abrin inactivate ribosomes by cleaving a specific sequence in 60S rRNA, while the bacterial toxins diphtheria toxin and *Pseudomonas* exotoxin A inactivate the elongation factor of the protein synthesis mechanism.

Despite their promising therapeutic application, these immunotoxins and immunotoxin-like compounds are not ideal. One problem facing the art is that immunotoxins are themselves immunogenic. Upon entering the host, the immunotoxins illicit an immune response which ultimately leads to the sequestration of many of the immunotoxins before they ever reach their target cell. The immunogenicity of the immunotoxins may be reduced by producing chimeric monoclonal antibodies that contain a mouse variable region and a human constant region. This strategy, however, will not affect the immunogenicity of the conjugated toxin.

Another problem is that most of the conjugated toxins used must be internalized by receptor-mediated endocytosis in order to exert their cytotoxic effect. The bulky, sterically hindered antibody which is conjugated to the toxin results in slower internalization rates and the inability of the toxin to penetrate large tumor burdens. Moreover, many of the toxins contain two subunits, one subunit which binds to a receptor on the cell and mediates endocytosis, and another which is toxic. Some research has suggested, for example, that the absence of

the β-subunit (the binding subunit) in α-ricin immunotoxin is responsible for the immunotoxin's slow intoxication rates. Including the β-subunit, however, compromises the immunotoxin's specificity for the target cell.

It has also been shown that once the immunotoxins enter the target cell, many of them are directed to the golgi or lysosomes where they are degraded. In reality, therefore, only a small percentage of the immunotoxins actually reach their cytoplasmic target and kill the cell.

Another immunotoxin which has been developed contains the toxin purothionin, isolated from barley flour, conjugated to the monoclonal antibody 225.28S. Unlike the other toxins used to date, purothionin does not need to be internalized to be cytotoxic. Instead, purothionin binds to the cell membrane where it disrupts the phospholipid bilayer causing cell death. Thus, purothionin is never exposed to the proteases found in the cytosol and lysosomes of the host cell.

Unfortunately, conjugated purothionin is approximately 10,000 times less toxic than the ribosome inactivating toxins (1). As a result, the purothionin immunotoxin is only able to moderately suppress the growth of melanoma cells in nude mice at the early stages of growth, and thus has limited human therapeutic potential (55).

Therefore, it would be a significant advancement in the art to develop an immunotoxin that displayed a high degree of specificity and cytotoxicity. It would be a further advancement in the art to develop an immunotoxin that did not depend on receptor-mediated endocytosis for cytotoxicity. It would be yet another advancement in the art to produce an immunotoxin whose conjugated toxin was not immunogenic; was highly stable; was small; and was resistant to a wide range of proteases. In addition, it would be a significant advancement in the art if the cytotoxicity and efficacy of the toxin remained high after being conjugated to a protein, such as an antibody or growth factor.

3. SUMMARY OF THE INVENTION

Immunotoxins and immunotoxin-like molecules consist of proteins that have been chemically coupled to a toxin, usually from bacteria or plants. The protein, typically a monoclonal antibody or growth factor, is chosen such that it delivers the toxin to a selective group of cells which express the corresponding antigen or ligand. As a result, the molecules display a high degree of cellular specificity and related cytotoxicity.

The present invention relates to an immunotoxin, which in one embodiment, comprises a mouse antihuman-CD5 monoclonal antibody conjugated to the toxin *Pyrularia* thionin ("PT" or "*P.* thionin"). The anti-CD5 monoclonal antibody recognizes the antigen CD5 found on all human T-cells and activated B-cells.

PT is a small, highly basic and stable, 47 amino acid residue peptide. PT belongs to a family of peptides which, with the exception of crambin, exhibit some degree of toxicity towards animals and cultured cells including yeast, fungi, bacteria, and mammalian cells. PT, however, does not display any toxicity towards bacteria. At moderate concentrations it is toxic to cancer cells in culture and at higher concentrations it is toxic to mice. PT causes a number of damaging cellular responses including hemolysis of erythrocytes, depolarization of the cellular membrane, activation of a calcium ion channel, and activation of endogenous phospholipase A_2 enzyme.

While anti-CD5 conjugated PT was found to have the same mechanism of action as native PT, the conjugated PT was significantly more cytotoxic than native PT. In all three measures of toxicity, including trypan blue exclusion, release of radioactivity from T cells loaded with ^{51}Cr, and inhibition of mitogen-stimulated cell proliferation, the PT immunotoxin was approximately > 10^4 times more active than native PT, with an average ID_{50} of approximately 1.8 X 10^{-11} M.

The PT immunotoxin also displayed a high degree of specificity. The PT immunotoxin's specificity was shown by its inactivity towards either human or sheep erythrocytes or to mouse lymphocytes which do not express the human CD5 antigen. The anti-CD5 antibody alone was not toxic to human lymphocytes.

After treating human lymphocytes with the PT immunotoxin and guinea pig complement, only 1.5% of the remaining cells were $CD5^+$, which is specific for T cells. In contrast, 92.5% were positive for anti-human IgM-FITC, which is specific for human B cells. These data demonstrate the PT immunotoxin's specificity for $CD5^+$ cells.

This novel immunotoxin overcomes several problems encountered in the art of immunotoxins. First, PT is membrane-active, obviating the need for PT to be internalized in order to exert its cytotoxic effect. PT, therefore, is never exposed to the proteases found in the cytosol of the host cell. Second, PT is a very stable, compact peptide which is resistant to most proteases. Third, PT is not immunogenic. PT goes undetected by the host's immune system, further increasing its efficacy and cytotoxicity. Fourth, the cytotoxicity of PT does not decrease, and in fact unexpectedly increases, when it is conjugated to the monoclonal antibody. Fifth, the small size of PT facilitates the immunotoxin's entry into solid tumors. Finally, PT's cytotoxicity is lost after it is incorporated into the lipid bilayer of the host cell. Therefore, the PT immunotoxin will not produce "second round" cytotoxicity towards macrophages and other cells that come in contact with the membrane of cells containing the PT immunotoxin.

These and other objects and advantages of the invention will become apparent upon reading the following detailed description and appended claims, and upon reference to the accompanying drawings.

4. DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the purification profile of *Pyrularia* thionin on a Sephadex G-75 column, monitored by A_{278} and cytotoxicity. The cytotoxicity was rated between 0-4+. A score of 0 indicates that the fraction was not cytotoxic, while a score of 4+ indicates the fraction was highly cytotoxic. Both absorbance and cytotoxicity peaked at tube 34. The dashed line shows absorbance readings obtained from a solution containing cytochrome c (12.4 kDa), bovine trypsin inhibitor (6.2 kDa), and insulin (3.0 kDa).

Figure 2 illustrates the purification profile of *Pyrularia* thionin on a carboxymethyl cellulose CM52 column with a 0.1 - 1.0 M NaCl gradient, monitored by A_{278} and cytotoxicity which peaked at tube 24.

Figure 3 is an elution profile of approximately 25 μg of purified *Pyrularia* thionin from an Ultrasphere C3 column, monitored at 214 nm. Superimposed on the elution profile is a line designating the percentage of buffer B, containing 60% acetonitrile, in the developing buffer.

Figure 4 illustrates the isolation of anti-CD5-PT conjugate from the reaction mixture. Figure 4**A** illustrates the fractionation of the mixture of anti-CD5 and PT in phosphate buffer (pH 6.9, 1 mM EDTA) by cation exchange HPLC. SCX 83-C13-ETI Hydropore column was equilibrated with 0.02 M Na_2HPO_4, pH 6.9, 1 mM EDTA. A salt gradient was established with 1 M NaCl and monitored as indicated by broken line. Fraction **a** is anti-CD5, and fraction **b** is PT. Figure 4**B** illustrates the fractionation of the reaction mixture of derivatized anti-CD5 and anti-CD5 conjugated to PT upon the conditions as noted with respect to Figure 4**A**. The material eluting first at 3 minutes, designated fraction a, is unmodified anti-CD5. The material eluting at 4 minutes, designated fraction b, is anti-CD5 SPDP. The active immunotoxin eluted with a peak at 20 minutes, and is designated fraction c. This is the fraction used in the other experiments.

Figure 5 illustrates the results of SDS-polyacrylamide gel electrophoresis of fractions **a** (lane 3), **b** (lane 4), and **c** (lane 5) as described in Figure 4**B**. Lane 1 is standard high molecular weight proteins. Molecular weights are indicated in kilodaltons. Lane 2 is native anti-CD5.

Figure 6 illustrates the results of isoelectric focusing of fractions **a** (lane 3), **b** (lane 4), and **c** (lane 5) as described in Figure 4**B**. Lane 1 is standard pI reference proteins. Lane 2 is native anti-CD5.

Figure 7 illustrates the EPR spectra of 5-doxylstearic acid in oriented phospholipid-multibilayers having a spin probe/lipid mole ratio 1/100. Magnetic field parallel (solid line) and perpendicular (broken line) to the bilayer normal.

(1)- spectra from phospholipid control samples: (2)-PT/lipid mole ratio 0.075; (3)-immunotoxin/lipid mole ratio 0.075.

Figure 8 illustrates endotherms of DMPC phospholipid bilayers (upper curves) and DMPC + 5 mol% PS (lower curves) without added reagents (1), and with the addition of PT (2) and immunotoxin (3). The reagent/lipid mole ratio was 0.075.

Figure 9 illustrates the cytotoxicity of fractions **a** (open square), **b** (black squares), and **c** (black circles) of Figure 4B, as measured by trypan blue uptake, on human peripheral blood lymphocytes. Cytotoxicity is expressed as the percent of viable cells of control samples.

Figure 10 is a graph illustrating the effect of PT immunotoxin on lymphocyte proliferation as measured by ^{3}H thymidine uptake. Human lymphocytes were stimulated by pokeweed mitogen (open squares), concanavalin A (black circles), and allogenic antigens in the mixed lymphocyte reaction (open circles).

Figure 11 is a graph illustrating the cytotoxic effect of the PT immunotoxin on human lymphocytes as measured by ^{51}Cr release. Curve 1 (black circles) represents peripheral blood lymphocytes treated only with the PT immunotoxin. Curve 2 (open circles) represents cells sensitized with the defined immunotoxin conjugate concentration and then exposed to the same concentration of guinea pig complement. Curve 3 (black triangles) was run under the same conditions as curve 2 except that the PT immunotoxin was replaced with native anti-CD5 antibody.

5. DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, the present invention relates to immunotoxins and immunotoxin-like molecules. In one preferred embodiment, the invention comprises an anti-CD5 monoclonal antibody conjugated to the toxin *Pyrularia* thionin.

5.1. DEFINITIONS

Immunotoxin - is defined as a toxin coupled to a monoclonal antibody or any fragment of an antibody which confers specificity.

Immunotoxin-like molecules - is defined as a toxin coupled to a target specific molecule which confers specificity.

Target specific toxin - is defined as a toxin coupled to a target specific molecule which confers specificity.

Target specific molecule - is defined as any molecule which is capable of conferring specificity, such as growth factors, cell adhesion molecules, or carbohydrates.

Specificity - is the ability of a molecule to recognize and bind to one particular ligand on the surface of a cell in the presence of other ligands.

Conservative amino acids - are defined as natural or synthetic amino acids which have a similar size, charge, polarity or conformation, and therefore can readily substitute for one another.

5.2. TARGET SPECIFIC MOLECULE

A wide variety of target specific molecules (TSM) have been employed in the art to confer specificity to otherwise generic toxins. TSMs which are well documented in the art, and included within the scope of the present invention are described below.

5.2.1 *IgG Conjugates*

In one preferred embodiment, the present invention employs the mouse antihuman-CD5 monoclonal antibody described in United States Patent No. 4,680,383 issued to Kung et al., which is hereby incorporated by reference. CD5 is a 65-kDa membrane glycoprotein expressed on T cells and activated B cells. The glycoprotein is also expressed on most T-cell derived tumors, and on B cells in chronic B-lymphocytic leukemia.

The anti-CD5 was isolated by standard techniques well known in the art. Briefly, the anti-CD5 was obtained from a known hybridoma cell line (OKT1, ATCC CRL 8000) purchased from American Type Culture Collection, Rockville, MD. The cells were propagated by injection into pristane-primed Balb c mice. After 6-10 days the ascites fluid was collected and the antibody purified on a protein A-Sepharose column, followed by absorption chromatography on hydroxyapatite.

Similarly, other IgGs specific for an antigen expressed by a particular carcinoma could be isolated and conjugated to PT. Monoclonal antibodies such as HB21, C242, LL2, or B3 have all been successfully conjugated to plant and bacterial toxins to confer specificity.

Furthermore, the immunogenicity of the monoclonal antibodies could be reduced by producing chimeric monoclonal antibodies that contain a mouse variable region and a human constant region (41-46). Thus, a PT immunotoxin composed of a "humanized" monoclonal antibody should go virtually undetected by the host's immune system.

5.2.2. *Fab' Conjugates*

One disadvantage of IgG conjugated toxins is their size. The large molecules have difficulty penetrating large solid tumors. Preferential proteolytic cleavage of the IgG generates two Fab fragments $(Fab')_2$. Each Fab' is comprised of two heterodimers which, in turn, are comprised of a heavy chain Fd and a light chain covalently linked by disulfide bonds at their C-terminus. $(Fab')_2$ fragments can be conjugated to toxins in the same manner as IgGs.

$(Fab')_2$ fragments can be preferentially reduced to generate Fab' molecules. Like $(Fab')_2$ fragments, Fab' fragments can be conjugated to toxins in the same manner as IgGs. It has also been shown that the free mercapto group generated during reduction of the disulfide bond can be used for conjugation without affecting the Fab' fragment's full binding activity. Moreover, Fab' fragments, unlike $(Fab')_2$ fragments and IgGs, produce a homogeneous population of conjugates because the conjugation reaction occurs at a defined position. Several Fab' ricin and *Pseudomonas* exotoxin conjugates have been successfully constructed (2, 3-7).

In addition to proteolytically derived Fab's and $(Fab')_2$ fragments, these fragments could be recombinantly expressed in *E.coli*. Recombinant Fab' fragments are indistinguishable from the proteolytically derived Fab' fragments (8). Recombinant Fab' fragments, such as B3(Fab)-PE38QQD, have been used to make immunotoxins with excellent activities (9-10).

5.2.3. *Fv Conjugates*

Yet another subunit of IgGs that can be used to convey specificity are Fv fragments. An Fv is the smallest subunit of an IgG that can mediate specific binding. An Fv is comprised of a heavy and light variable region. Proteolytically derived Fv fragments cannot be obtained because they are unstable. However, recombinantly produced Fv fragments, which are stabilized by either a peptide bond (scFv) or a disulfide bond (dsFv) display good specificity and stability. The scFv fragments are produced as a single chain. The peptide bond connects the C- and N-terminal regions of the heavy and light variable regions much like a anti-parallel ß-sheet structure (11-12). scFv conjugated to bacterial toxins, such as anti-Tac(Fv)-PE40 directed at IL2 receptor, have been shown to display excellent antitumor activity in animal models (13-27).

The dsFv fragments are generated by recombinant expression of the variable heavy and light chains separately in *E. coli* and combined during refolding. While the dsFv display similar properties as the svFv fragments, they typically give higher yields and are more resistant to thermal and chemical denaturation. Examples of dsFv immunotoxins are B3(dsFv)-PE38, B3(dsFv)-PE38KDEL, anti-Tac(dsFv)-PE38KDEL, e23(dsFv)-PE38KDEL (28-29).

5.2.4. *Growth Factors and Other Ligands*

Growth factors make good TSMs because many of the receptors for growth factors are overexpressed in carcinoma cells. Growth factor conjugates are constructed under the same principle as scFv fragments, as a single chain fusion protein. Because many of the growth factors have been cloned, growth factor PT conjugates can be constructed using basic recombinant DNA technology. Indeed, many growth factor fusions display a high degree of specificity and cytotoxicity (30-40).

For example, TGFα-PE40 binds specifically to EGF receptors overexpressed in many carcinomas. Another example is CD4-Pe40 in which the HIV1-gp41 binding portion of CD4 is selectively cytotoxic to HIV1 infected cells.

5.3 **LINKAGE**

In one preferred embodiment, the immunotoxin of this invention employs a cleavable disulfide linker well known in the art. Specifically, in one embodiment the PT was conjugated to the anti-CD5 antibody by treating PT with 2-Iminothiolane-HCl to provide sulfhydryl groups, and anti-CD5 with N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) to provide pyridyl disulfide residues. The general reaction used was:

Step 1: anti-CD5-NH_2 + SPDP → anti-CD5-NH-CO-CH_2-CH_2-S-S-PY

Step 2: anti-CD5-NH-CO-CH_2-CH_2-S-S-PY + PT-SH →
anti-CD5-NH-CO-CH_2-CH_2-S-S-PT + PYSH

Other linkages which are commonly utilized and expected to be useful for conjugating PT to target specific molecules are imminothiolane/succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate and carbodiimide linkages. Many other linkages for conjugating proteins of varying lengths, stability, flexibility and chemical reactivity are well known in the art and in many cases are commercially available.

5.4 *P.* **THIONIN**

5.4.1. *Purification and Recombinant Expression of PT*

PT was purified from nuts of *Pyrularia pubera* that were ground in cold 0.1 M phosphate buffered saline (PBS) as described previously (53). The insoluble matter was centrifuged and the supernatant was fractionated with ammonium sulfate. The majority of the biologically active material precipitated between 45% ammonium sulfate and saturation as determined by the cytotoxicity of the fractions. Residual ammonium sulfate was removed by dialysis against 0.01 M PBS.

The PT containing fractions were then further purified by gel filtration on a Sephadex G-75 column (Figure 1). The PT was eluted with 0.02 M NaCl/0.02 M phosphate buffer. The biologically active fractions were then subjected to ion exchange chromatography with a carboxymethyl cellulose CM52 column (Figure 2). PT was eluted with a linear gradient of 0.1 to 1.0 M NaCl. The resulting biologically active fraction was

greater than 95% pure as judged by SDS-PAGE (data not shown) and reverse-phase HPLC (Figure 3).

Recombinant PT could also be used to make PT immunotoxin. A nucleic acid sequence which codes for the amino acid sequence of PT containing an initiator methionine at the amino terminus and a stop codon at the carboxyl terminus could be synthesized. The double stranded nucleotide sequence could then be subcloned into a eucaryotic or procaryotic expression vector which is commercially available. Systems which allow proteins to be overexpressed in, and purified from, bacteria, baculovirus or mammalian cells are well known in the art.

In one preferred embodiment, a synthetically engineered PT gene was subcloned into plasmids capable of expressing the gene in *E. coli* using techniques well known in the art. Briefly, two synthetic primers, each comprising slightly over half of the PT gene, were synthesized and used to create a full length PT gene by polymerase chain reaction. The nucleotide sequence of the synthetic PT gene (SEQ ID NO.1) contains an initiator methionine immediately preceeding the first amino acid of PT, and a stop codon immediately following the last amino acid of PT, both which are Lys. The gene also contains a *Nde* I restriction endonuclease site (CATATG) flanking the initiator methionine, and a *Bam* HI restriction site (GGATCC) just 3′ of the stop codon.

The PT gene was then directionally cloned into the *Nde* I and *Bam* HI sites of the expression vectors pET21 (Novagen Inc.) and pRE1 (Life Sciences, Inc.). pET21/ptox consists of the PT gene under the control of the T7 promoter. It can be expressed in any bacterial cell that contains the T7 RNA polymerase gene under the control of an inducible promoter, such as BL21DE3 and BL21DE3pLysS.

pRE1/ptox consists of the PT gene under the control of the lambda PL promoter. pRE1/ptox is expressed in the *E. coli* strain CJ347 which expresses the CI857 gene, a temperature sensitive lambda repressor. pRE1/ptox is repressed at 30° C and derepressed (or induced) at 42° C. Typically, PT is expressed by growing the bacteria at 30° C until they reach mid-log phase, and then at 42° C for thirty minutes. Finally, the bacteria are

grown at 37° C for approximately two to five hours. SDS or tricine polyacrylamide gel electrophoresis can be used to visualize the recombinant PT peptide.

5.4.2. *Amino Acid Sequence*

PT is a 47 amino acid, heat stable, highly basic peptide. It has a calculated molecular weight of 5280. The amino acid sequence of PT (SEQ ID NO. 2) as determined by chemical sequencing is shown below:

Lys-Ser-Cys-Cys-Arg-Asn-Thr-Trp-Ala-Arg-Asn-Cys-Tyr-Asn-Val-Cys-Arg-Leu-Pro-Gly-Thr-Ile-Ser-Arg-Glu-Ile-Cys-Ala-Lys-Lys-Cys-Asp-Cys-Lys-Ile-Ile-Ser-Gly-Thr-Thr-Cys-Pro-Ser-Asp-Tyr-Pro-Lys

Unlike the viscotoxins that contain 6 cysteine residues, PT and the cereal thionins contain 8 conserved cysteine residues which form four disulfide bonds. A comparison of PT with several other members of the thionin family is shown below.

TABLE 1

Peptide	
Pyrularia	KSCCRNTWARNCYNVCRLPGTISREICAKKCDCKIISGTTCPSDYP-K
Viscum A3	KSCCPNTTGRNIYNACRLTGA-PRPTCAKLSGCKIISGSTCPS-YPDK
Viscum B	KSCCPNTTGRNIYNTCRLGGG-SRERCASLSGCKIISASTCPS-YPDK
Viscum A2	KSCCPNTTGRNIYNTCRFGGG-SREVCASLSGCKIISASTCPS-YPDK
Phoradendron	KSCCPTTTARNIYNTCRFGGG-SRPVCAKLSGCKIISGTKCDSGW-NH
Crambe	TTCCPSIVARSNFNVCRLPGT-SEAICATYTGCIIIPGATCPGDYAN-
Wheat beta	KSCCKSTLGRNCYNLCRARGA-QK-LCANVCRCKLTSGLSCPKDFP-K
Wheat alpha 1	KSCCRSTLGRNCYNLCRARGA-QK-LCAGVCRCKISSGLSCPKGFP-K
Wheat alpha 2	KSCCRTTLGRNCYNLCRSRGA-QK-LCSTVCRCKLTSGLSCPKGFP-K
Barley alpha	KSCCRSTLGRNCYNLCRVRGA-QK-LCAGVCRCKLTSTGSCPKGFP-K
Barley beta	KSCCRSTLGRNCYNLCRVRGA-QK-LCANACRCKLTSGLSCPKGFP-K

The one-letter code for amino acids used is: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Dashes indicate gaps inserted to improve alignment. The shaded regions indicate the amino acids that are conserved between thionins.

5.4.3. *Protein Modification*

Included within the scope of the present invention are protein modifications of PT. Protein modifications can be subdivided into three general classes: substitutions, additions and deletions. These general groups apply to both the nucleic acid and amino acid sequence of the PT immunotoxin. While protein modifications may occur naturally, most often protein modifications are deliberately engineered into the nucleic acid sequence that codes for the protein. Protein modification techniques such as site-directed mutagenesis are well known in the art and in many cases are commercially available as kits complete with instructions. Kits of this type are available from, for example, Amersham and Bethesda Research Laboratories.

It is well known in the art that some amino acid substitutions may be made in a protein without significantly altering the protein's function. Substitutions, as defined herein, are modifications made to the nucleic acid or amino acid sequence of the protein which produce a protein with a different amino acid sequence than the native protein without significantly altering the major properties or function of the toxin. The most favorable substitutions occur when amino acids are substituted by "conserved" amino acids. Conserved amino acids are natural or synthetic amino acids which because of size, charge, polarity and conformation can be substituted into the protein without significantly affecting the structure and function of the protein.

In general, the non-polar amino acids Gly, Ala, Val, Ile and Leu; the non-polar aromatic amino acids Phe, Trp and Tyr; the neutral polar amino acids Ser, Thr, Cys, Gln, Asn and Met; the negatively charged amino acids Lys, Arg and His; the positively charged amino acids Asp and Glu, represent conservative groups of amino acids. This list is not exhaustive. For example, it is well known that Ala, Gly, Ser and sometimes Cys

can substitute for each other even though they belong to different groups.

Conservative amino acid substitutions are not limited to naturally occurring amino acids, but also include synthetic amino acids. Commonly used synthetic amino acids are ω amino acids of various chain lengths and cyclohexyl alanine which are neutral non-polar analogs; citulline and methionine sulfoxide which are neutral non-polar analogs, phenylglycine which is an aromatic neutral analog; cysteic acid which is a positively charged analog and ornithine which is a negatively charged amino acid analog. Like the naturally occurring amino acids, this list is not exhaustive, but merely exemplary of the substitutions that are well known in the art.

Whether an amino acid can be substituted at all, or whether it can only be substituted by a conserved amino acid is best determined by comparing PT with other members of the thionin family. Amino acids that are identical in all the members of a protein family usually cannot be substituted. This is the case with the 8 cysteine residues which are absolutely conserved between thionins. Except for cysteines which form essential disulfide bonds, amino acids which are conserved between members of the same family of proteins can usually be substituted by other conserved amino acids without significantly affecting the protein's function. Thus, the basic amino acids which are required for binding to the cell membrane, including the terminal lysine residue of PT, could likely be substituted only by other basic amino acids. Finally, amino acids which are not conserved within a family can usually be freely substituted. One exception is the Trp at position number 8 which is not conserved between other thionins, but has been shown to be essential for activity. Trp 8, could probably only be substituted with Phe, Tyr or a large non-polar, non-aromatic amino acid.

Equally important is the comparison with the amino acid sequence of *Crambe* which displays a high degree of homology but is not cytotoxic. Amino acids that are not conserved between *Crambe* and other thionins can be expected to be critical for biological activity. Accordingly, one skilled in the art could

reasonably anticipate which amino acids in PT could be substituted without significantly altering its biological activity.

Also, included within the scope of the present invention are additions. Additions, as defined herein, are modifications made to the nucleic acid or amino acid sequence of the protein which produce a protein containing at least one amino acid more than the native amino acid sequence of the protein without significantly altering the function of the toxin.

In the present invention, for example, the mature protein lacks an initiator methionine which may be included if the toxin is recombinantly expressed. The addition of a methionine to the amino terminus of the PT, as well as the additions of other amino acids which facilitate the expression of the protein such as stop codons, are not expected to significantly affect the function of the toxin.

Protein modifications may also occur through deletions. Deletions, as defined herein, are modifications made to the nucleic acid or amino acid sequence of the protein which produce a protein containing at least one amino acid less than the native amino acid sequence of the protein without significantly altering the function of the toxin.

A comparison of thionins reveals that several single amino acids deletions have naturally occurred throughout the protein without the loss of cytotoxicity. Likewise, thionins with small deletions within the coding region of the peptide which do not interrupt stretches of highly conserved amino acids are expected to be biologically active and included within the scope of the present invention.

5.4.4. *Biological Activity*

PT belongs to a family of peptides which, with the exception of crambin, exhibit some degree of toxicity towards animals and cultured cells including yeast, fungi, bacteria, and mammalian cells (54). PT, however, does not display any toxicity towards bacteria.

Purified PT is cytotoxic for all the cell lines tested in tissue culture to date. The ID_{50} of several of these cell lines is shown in Table 2 below:

TABLE 2

	CELL LINE	ID_{50} ug/ml
HUMAN	HELA	17
	MRC-5	0.46
MOUSE	B16	3.0
	P388	0.62
	L1210	3.9
MONKEY	VERO	9.6

As discussed above, unlike most immunotoxins developed to date that require internalization by receptor-mediated endocytosis, thionins act on the cell membrane. Without being bound by theory, it is presently believed that PT's action on the cell membrane can be divided into four distinct phases: (1) binding of PT to the cell membrane by electrostatic forces; (2) perturbation of the phospholipid structure of the bilayer; (3) depolarization of the membrane and opening of a calcium channel; and (4) activation of endogenous phospholipase A_2 (PLA_2).

The binding phase, which has been studied extensively in erythrocytes, most likely involves PT binding to phosphatidylserine (PS). This is probably accomplished by electrostatic binding of the basic amino acid residues of PT with the acidic headgroup of PS (47).

After binding, PT perturbs the membrane by inserting Trp number 8 into the lipid bilayer. This changes the membrane fluidity of the cell and leads to depolarization of the cellular membrane and rapid changes in membrane permeability. All these responses are rapid, proceeding without any appreciable delay.

The third phase involves membrane depolarization and the opening of a calcium channel with an influx of calcium. This has been shown with mouse P388 cells and with rat anterior pituitary cells in which the calcium channel blockers methoxyverapamil and dopamine inhibited PT release of growth hormone from the cell.

The final phase, which is the activation of endogenous PLA_2, displays a distinct 20 minute time lag. PT-induced PLA_2

activity has been demonstrated in NIH 3T3 fibroblast cells by the release of radiolabled arachidonic acid at concentration above 10μg/ml (about 2μM). The enzyme hydrolyzes the fatty acid at the SN-2 position of the phospholipid, liberating arachidonic acid which serves as a precursor of prostaglandins. Thus, PT is able to influence cellular second messenger systems without entering the cell.

5.5. *P.* THIONIN IMMUNOTOXIN

5.5.1 *Immunotoxin Preparation*

In one preferred embodiment, the PT immunotoxin was prepared by linking PT to mouse antihuman-CD5 through an artificial disulfide bond. 2-Iminothiolane-HCl was used to provide PT with sulfhydryl groups and N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) served to provide the anti-CD5 with pyridyl disulfide residues to react with thiols of the derivatized PT. PT was incubated with 2-Iminothiolane-HCl in phosphate buffer. Anti-CD5 was incubated with SPDP in phosphate buffer. Derivatized PT and anti-CD5 were conjugated by incubating the derivatized PT and anti-CD5 together overnight at room temperature in phosphate buffer.

Unreacted PT was removed by dialysis against water and the reaction mixture concentrated with a Centricon 30 microconcentrator. Unreacted anti-CD5 was eliminated by cation exchange HPLC as shown in Figure 4. Fractions were examined for anti-CD5 and PT immunotoxin by a slot-blot procedure utilizing anti-mouse IgG-horseradish peroxidase as a secondary antibody. Figure 5 illustrates the integrity of the fractions as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, while Figure 6 illustrates the integrity of the fractions by isoelectric focusing (IEF). The higher pI for fraction c, the immunotoxin, is consistent with the conjugation of the very basic PT to anti-CD5.

5.5.2. *Biological Activity*

The immunotoxin composed of PT and anti-CD5 antibody interacts with phospholipid bilayers in a manner similar to native PT. Previous research has shown that the thionin inter-

acts with phospholipid bilayers consisting of phosphatidylcholine and small amounts of phosphatidylserine by binding to the phosphatidylserine, thus perturbing the order of the phospholipid bilayer. This was shown by EPR (electron paramagnetic resonance) measurements on phospholipid bilayer which have the spin probe 5-doxylstearic acid incorporated into the bilayer. Figure 7 shows such data obtained with phosphatidylcholine (PC) bilayers containing 0 and 30 mole % phosphatidylserine (PS) and a spin probe/lipid mole ratio of 1/100. The experiment was performed with the magnetic field parallel (solid line) and perpendicular (dashed line) to the bilayer normal. Curve 1 shows the spectra obtained with phospholipid bilayer alone, while for curve 2 PT was added at a PT/lipid mole ratio of 0.075, and for curve 3 immunotoxin was added at a immunotoxin/lipid mole ratio of 0.075. Complete spectral isotropy was found for the PC + PS membranes treated with PT. Similar membranes treated with immunotoxin retained some spectral anisotropy, but the same general response was shown for both the immunotoxin and PT.

Another method of measuring changes in the order and structure of phospholipid bilayers involves the use of calorimetry. The results of such measurements, presented in the thermograms, are shown in Figure 8. The upper set of curves represents the data obtained with a bilayer composed of dimyristoylphosphatidylcholine (DMPC) and the lower set of curves represents data obtained with a bilayer composed of DMPC and 5 mole % PS. The sharp peak in curve 1 of each set indicates the temperature at which the bilayer undergoes the major transition from the gel to fluid state in the absence of added substance. Curve 2 represents similar data obtained after adding PT and curve 3 represents data obtained after adding the PT immunotoxin at a protein/lipid mole ratio of 0.075. As expected, the greatest effect of the added material was seen in the bilayer consisting of DMPC and PS since PT reacts preferentially with PS in the bilayer. The responses of PT and the PT immunotoxin were very similar, suggesting that native PT and conjugated PT interact with the lipid membrane via similar mechanism.

5.5.3. *Immunotoxin Cytotoxicity*

PT immunotoxin cytotoxicity was determined by the trypan blue exclusion assay, lymphocyte proliferation, and ^{51}Cr release. Trypan blue is excluded by normal healthy cells, but is readily taken up by cells with perturbed or disrupted membranes. As expected, fraction **c** of Figure 4B containing the PT immunotoxin was highly toxic to cells, fraction **b** which likely contains the anti-CD5 SPDP derivative was only slightly toxic, and fraction **a** containing unconjugated antibody was not toxic. (Figure 9). The ID_{50} value for conjugated PT was 4×10^{-11} M, while native PT had an ID_{50} value of approximately 1×10^{-6} M.

The influence of cell proliferation was determined by $^{3}[H]$ thymidine uptake after stimulation with mitogen or in a mixed lymphocyte reaction using standard procedures. Human lymphocytes were stimulated with either concanavalin A or mixed lymphocyte reaction, which only stimulate T cells, or pokeweed mitogen, which stimulates both B and T cells. Stimulated cells were then pulsed with $^{3}[H]$ thymidine and incorporated $^{3}[H]$ thymidine determined by liquid scintillation counting.

Figure 10 demonstrates that 1×10^{-9} M of PT immunotoxin completely suppressed stimulation by concanavalin A and mixed lymphocytes. Since pokeweed mitogen stimulates both B and T cells in the lymphocyte preparation, and because B cells do not contain CD5, the PT immunotoxin was unable to completely inhibit cell proliferation in the presence of pokeweed mitogen. The ID_{50} value for conjugated PT was approximately 5×10^{-12} M, while native PT had an ID_{50} value of approximately 1×10^{-7} M.

Cytotoxicity was also measured by the release of ^{51}Cr from human lymphocytes loaded with chromate ion containing ^{51}Cr. The experiments were performed using the anti-CD5 immunotoxin alone and also with PT immunotoxin and anti-CD5 antibody, both in the presence of guinea pig complement which lyses cells in the presence of antibody independently of the action of the PT immunotoxin. The data obtained are shown in Figure 11. The PT immunotoxin alone was able to lyse about 50% of the cells. Half-maximal activity of the PT immunotoxin alone was observed at about 1×10^{-11} M. The immunotoxin conjugate in the presence of complement was more effective in terms of lysis, with a ID_{50}

of 1 X 10^{-12} M. At concentrations below 10^{-11} M of antibody or immunotoxin conjugate, added complement was synergistic to the toxic effect of PT immunotoxin alone. This indicates that perturbation of the membrane bilayer by the PT contained on the immunotoxin facilitated the action of complement on the membrane.

These results were unexpected based on logic and the prior art. One skilled in the art would not predict that the toxin would display greater cytotoxicity when conjugated to a bulky antibody which would likely create stearic hinderance around the toxin's binding site. This is especially true given the contradictory data for the purothionin immunotoxin.

5.5.4. *Immunotoxin Specificity*

The specificity of PT immunotoxin for $CD5^+$ cells was assessed by immunofluorescence. For lymphocytes treated with the PT immunotoxin only, 1.5 ± 0.11 % of the cells tested positive for the CD5 antigen, while 92.5 ± 4.8 % of the cells were positive for anti-human IgM-FITC which is specific for human B cells. In control samples, 75.8 ± 3.9 % of the cells were positive for CD5, while 19.2 ± 1.3 % of the cells tested positive for anti-human IgM-FITC. These data demonstrate that the PT immunotoxin is specific for cells that express the CD5 antigen, killing only the $CD5^+$ T cells.

5.5.5. *Treatment of T Cell Leukemias*

The PT immunotoxin of the present invention may be used for the treatment of T cell leukemias *in vivo*. Recent studies have demonstrated that anti-CD5 monoclonal antibodies conjugated to both single and double chain ribosomal inactivating proteins are useful for treating T-cell leukemias. One such study employed an anti-CD5 momordin conjugate (48). The potency of the immunotoxin was high against the T-cell leukemia cell line Jurkat, with an ID_{50} of 1-10 pM. The immunotoxin also significantly reduced tumor development in nude mice bearing the Jurkat leukemia. Thus, given that this invention and the latter study use the same monoclonal antibody, and that they

display similar ID_{50} values, the PT immunotoxin is also expected to be effective against T cell leukemias *in vivo*.

In addition to the treatment of T cell leukemias *in vivo* by direct application of PT immunotoxin, the use of a cocktail of immunotoxins and immunotoxin-like molecules should be more cytotoxic and more effective for killing tumor cells. The other immunotoxins would be those containing ribosomal inactivating proteins (RIP) toxins conjugated to the same anti-CD5 antibody, or could be ribosomal inactivating proteins toxins conjugated to other target specific molecules directed toward other antigens on the same cell. The advantage of using a cocktail of immunotoxins is that the two types of conjugated toxins would react with, and kill, the cell by different mechanisms. The RIP immunctoxins would enter the cytosol and inhibiting protein synthesis, while the PT immunotoxins would disrupt the cell membrane and initiate second messenger systems. Further, the second immunotoxin in the cocktail could be an antibody-drug conjugate containing a drug such as doxorubicin, or doxorubicin alone, whose effectiveness would be increased by the membrane perturbation caused by PT.

Another approach to increasing the effectiveness of the PT immunotoxin would be to prepare double conjugates which contain both PT and a ribosomal inactivating protein conjugated to the same TSM. The two toxins could be joined together using basic recombinant technology to form a double toxin, and then conjugate this double toxin to the TSM by a single linker. Alternatively, both toxins could be joined directly to the TSM with linkers of sufficient length to allow both toxins to interact with the cell membrane and exert their toxic effects.

5.5.6. *Graft-versus-host Disease*

It is also expected that the PT immunotoxin of the present invention may be used for the *in vitro* manipulation of donor cells used in tissue and organ grafts, blood transfusions, and bone marrow transplants. One area of particular concern is when the donor and the recipient have different histocompatibility. When the donor's mature immunocompetent T cells are transfused, the allogeneic recipient is unable to identify and

reject the T cells. As a result, the donor's T cells react against the tissues of the host in a graft-versus-host response. This response triggers many responses in the host, including skin, gut and liver damage which often leads to death.

Conventional treatments attempt to suppress the donor cell's immune reaction with drugs such as steroids and cyclosporin. The major drawback of these drugs is that steroids and immunosuppressants are not specific for the donor's T cells. Using immunotoxin technology, however, it is possible to kill the donor's T cells using the PT immunotoxin before the tissue is grafted or the blood is transfused into the recipient. Thus, the recipient receives the tissue or blood from the donor free of T-cells.

Immunotoxins directed at T-cells have been previously used to treat graft-versus-host disease (49). The immunotoxin used in the above cited study employed the ricin A chain toxin conjugated to a antihuman-CD5 antibody. The treatment resulted in a rapid reduction of peripheral blood T lymphocytes which persisted for more than a month. Other studies have used T101 Fab fragment (50), anti-CD3 (51) and a combination of all three (52). It is expected that strategies similar to those used to treat T cell leukemias would be effective for graft-versus-host disease.

6. EXAMPLES

EXAMPLE 1

Plant Material: *Pyrularia pubera* was collected in the areas of Clemson, South Carolina, and Cullowhee, North Carolina. The initial experiments were performed with leaves collected during the month of June. Later experiments used nuts collected during August, the seeds being separated from the fruit, which was discarded.

EXAMPLE 2

Extraction and ammonium sulfate precipitation: Five hundred grams of fresh or frozen *Pyrularia* leaves, or 300 g of fresh or frozen *Pyrularia* nuts were ground for approximately 1

minute in 500 ml of 0.1 M phosphate buffer, pH 7.0, in a chilled blender, and the homogenate was frozen overnight. Upon thawing, the homogenate was ground again for 15 seconds in the blender and the resulting homogenate was centrifuged at 1000*g* for 10 min to remove debris, which was extracted again with 100 ml of buffer solution. To each 100 ml of supernatant fluid was added successively 20, 10, 10, 10, and 10 g of $(NH_4)_2SO_4$. After each addition, the suspension stood for 10 min at 25°C and was then centrifuged at 20,000*g* to remove the precipitated protein. The last 10 g of ammonium sulfate did not completely dissolve at room temperature, since the solution was >95% saturated. The sedimented protein from each stage was dissolved in 50 ml of 0.05 M phosphate buffer, pH 7.2, to give fractions which we designate as I, II, III, IV, and V, respectively. The fractions were dialyzed overnight against 5 mM phosphate buffer, pH 7.2, and tested for cytotoxicity on mouse B16 melanoma cells. The higher-toxicity fractions were used for the further purification.

EXAMPLE 3

Gel filtration on Sephadex G-75: After dialysis against 0.01 M phosphate buffer, pH 7.0, fractions IV and V were further purified by gel filtration on Sephadex G-75 (2 X 38 cm) (Figure 1). The column was first washed with a solution 0.02 M in NaCl and 0.02 M in phosphate buffer, pH 7.2. A 15-ml portion of the ammonium sulfate fraction was loaded on the column and eluted with the buffer solution described above. Three-milliliter fractions were collected for absorbance readings at 278 nm.

EXAMPLE 4

Chromatography on carboxymethyl cellulose CM52: Ion-exchange chromatography with a carboxymethyl cellulose CM52 column gave significant further purification (Figure 2). The column was washed extensively with 0.05 M phosphate buffer, pH 7.0, before adding 15 ml of dialyzed fraction V. A linear gradient of 0.1 to 1.0 M NaCl was applied to elute protein from the column. PT eluted as the major band which was greater that 95% pure as determined by HPLC (Figure 3).

EXAMPLE 5

Toxicity assay: For L1210 and P388 cells, test fractions were dissolved in growth medium (RPMI) supplemented with 10% fetal calf serum and 20 mM Hepes buffer) at twice the desired final concentration. Ordinarily, compounds were tested over a 3-4 log dilution range. Cells were adjusted to $1x10^5$ cells/ml in growth medium and dispensed into 13x100 mm test tubes (1 ml/tube). One ml of test compound was added to each tube with duplicate tubes run at each dose level. Cells were incubated at 37°C for 48 h. Growth was determined by cell count using a Coulter counter. At each dose level, growth was expressed as a percentage of growth in the untreated control.

For Hela, B16, MRC-5, and Vero cells, cells were adjusted to $2.5x10^4$ cells/ml in growth medium (Eagle's MEM supplemented with 10% FCS and 20 mm Hepes buffer) and plated in 24 well tissue culture plates (1 ml/well). Plates were incubated for 24 h at 37°C in a 5% carbon dioxide atmosphere. Medium was removed and replaced with 1 ml/well of test compound dissolved in fresh growth medium (duplicate wells at each dose level). Cells were grown 72 h at 37°C in 5% carbon dioxide. The medium was then removed and the plate was washed twice with normal saline (1 ml/well). Growth was assessed by a Lowry protein determination, and growth at each dose level was expressed as a percentage of growth in the untreated controls.

EXAMPLE 6

Monoclonal antibody: Hybridoma cells (OKT1, ATCC CRL 8000) that produced a monoclonal antibody specific for the CD5 antigen on human lymphocytes (anti-CD5) were purchased from the American Type Culture Collection (Rochville, MD). The cells were injected into pristane-primed mice to propagate monoclonal antibody production. Six to ten days later, ascites fluid was collected from the peritoneal cavity and the antibodies were purified on a protein A-Sepharose column (Pharmacia, Uppsala, Sweden) followed by adsorption chromatography on hydroxyapatite (Bio-Rad, Hercules, CA). The purity of the monoclonal antibody was determined by the standard techniques of SDS-polyacrylamide gel electrophoresis and isoelectric focusing.

EXAMPLE 7

Immunotoxin preparation: The PT immunotoxin was prepared by linking PT to anti-CD5 through an artificial disulfide bond. 2-Iminothiolane-HCl (Pierce, Rockford, IL) was used to provide PT with sulfhydryl groups and N-Succinimidyl 3-(2-pyridyl-dithio) propionate (SPDP) (Pierce, Rockford, IL) served to provide the anti-CD5 monoclonal antibody with pyridyl disulfide residues. PT at 10^{-3} M was incubated at 37°C for 45 min with 6 x 10^{-3} M of 2-Iminothiolane-HCl in 0.02 M Na_2HPO_4 (pH 7.0), 1 mM EDTA. Anti-CD5 at 10^{-4}M was incubated at 37°C for 1 hr with 5 X 10^{-4} M of SPDP in 0.02 M Na_2HPO_4 (pH 8.0), 1 mM EDTA. The derivatized proteins were separated from the reactants by dialysis against 0.02 M Na_2HPO_4 (pH 8.0), 1 mM EDTA. Derivatized PT and anti-CD5 were incubated together overnight at room temperature in 0.02 M Na_2HPO_4 (pH 8.0), 1 mM EDTA. Unreacted PT was removed by dialysis (14,000 MW cutoff) against water. The reaction mixture was then concentrated with a Centricon 30 microconcentrator (30,000 MW cutoff; Amicon, Danvers, MA). Aggregated material was removed by centrifugation. Unreacted anti-CD5 was eliminated by cation exchange HPLC with a SCX 83-C13-ETI Hydropore column (Rainin Instrument Company, Inc., Woburn, MA) equilibrated with 0.02 M Na_2HPO_4 (pH 6.9), 1 mM EDTA. A salt gradient was established with buffer containing 1 M NaCl (Figure 4). Fractions **a**, **b** and **c** were examined for anti-CD5 and PT immunotoxin by a slot-blot procedure utilizing anti-mouse IgG-horseradish peroxidase (Sigma, St. Louis, MO) as secondary antibody. The integrity of fractions was determined by the standard techniques of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions (Figure 5) and by isoelectric focusing (Figure 6). Samples for SDS-PAGE were dissolved in buffer (20 mM Tris-HCl, 2 mM EDTA, 5% SDS, 0.01% Bromophenol Blue) and applied to an 8-25% acrylamide gradient gel. IEF was done in isogel agarose gels with a pH gradient from 3.0 to 10.0.

EXAMPLE 8

Cytotoxicity - Trypan blue exclusion: Cytotoxicity was determined by trypan blue uptake of PT immunotoxin treated

cells (Figure 9). Lymphocytes from human blood and from spleens of adult BALB/c mice were separated by density gradient centrifugation on LSM solution (Bionetics, Kensington, MD). Cells (1 X 10^6) in hepes buffered saline solution (HBSS) containing 2% (by volume) heat-inactivated human serum (56°C for 30 min) were incubated for different time periods at 37°C with a specified concentration of fraction **a**, **b**, or **c** of Figure 4**B**. Anti-CD5 was used as a control. Each data point reported is the mean of three experiments, each performed in triplicate. The standard deviation was always within ± 6% of means. The lytic activity of the fraction was also tested on human and sheep red blood cells. Hemolysis was determined by OD at 541 nm.

EXAMPLE 9

Cytotoxicity - ^{3}[H] Thymidine uptake: The influence of the PT immunotoxin on cell proliferation was determined by mitogen stimulation or by the mixed lymphocyte reaction (Figure 10) following methods described previously (48). Briefly, lymphocytes suspended in RPMI 1640 supplemented with 2% (by volume) heat-inactivated human serum, 2 mM L-glutamine and 10 μg/ml gentamicine were seeded in 96-well plates (10^5 cells/well). Cell proliferation was induced either by 0.1 mg/ml Concanavalin A (Con A; Difco Lab., Detroit, Michigan) or by 0.1 mg/ml Pokeweed Mitogen (PWM; Sigma, St. Louis, MO). The cells were incubated for 48 hr in a 5% CO_2 humidified atmosphere at 37°C, then pulsed with ^{3}[H] thymidine (1 μCi/well). The cells were harvested 5 hr later. In the mixed lymphocyte reaction 10^5 responder cells were mixed in each well with 10^5 stimulator cells. Stimulator cells had been treated with mitomycin C (25 μg/10^7 cells) for 20 min at 37°C followed by washing three times in RPMI 1640. The cells were incubated for 120 hr and then pulsed with ^{3}[H] thymidine (1 μCi/well) 5 hr prior to harvesting. The test wells were incubated with specified concentrations of PT immunotoxin or anti-CD5, and control wells without PT immunotoxin or anti-CD5. The incorporated radioactivity was measured by liquid scintillation counting. Data are expressed as means of two separate experiments, each performed in triplicate. The standard deviation was always within ± 5% of means.

EXAMPLE 10

Cytotoxicity - ^{51}Cr release: The cytotoxic activity of the PT immunotoxin with and without added guinea pig complement was examined by a ^{51}Cr release assay (Figure 11). Cells suspended in Dulbecco's Minimal Essential Media (MEM) were labled with ^{51}Cr (100 μCi ^{51}Cr per 10^7 cells). Radioactivity released from the lysed cells was detected by liquid scintillation spectroscopy. Cytolytic activity was calculated using the formula:

$$\text{Cytolytic activity} = \frac{\text{Experimental cpm - background release cpm}}{\text{Total release cpm - background release cpm}} \times 100\%$$

EXAMPLE 11

Immunotoxin specificity: Specificity of the PT immunotoxin was determined by immunofluorescent staining of human blood lymphocytes. Cells (10^7) suspended in HBSS containing 2% (by volume) heat inactivated human serum were incubated with 10^{-7} M of PT immunotoxin for 2 hr at 37°C followed by incubation with 10^{-7} M of anti-CD5 for an additional 2.5 hr. In control samples, 10^7 cells were incubated for 2 hr without PT immunotoxin, then incubated for an additional 2.5 hr with 10^{-7} M of anti-CD5. Viable cells were separated on LSM solution and then incubated with either anti-mouse IgG (Fc specific)-FITC to label the T cells that bound anti-CD5, or anti-human IgM (μ-chain specific)-FITC to label the B cells. The results obtained were expressed as the percent of labeled cells to the total number of cells in the samples. Data were obtained from four separate experiments, each performed in triplicate. The standard deviation was always within ± 7% of means.

EXAMPLE 12

Immunotoxin membrane interaction - electron paramagnetic resonance: The ability of the PT immunotoxin to interact with lipid membranes was analyzed by EPR of spin probes (Figure 7). Egg yolk L-α-phosphatidylcholine (PC), bovine brain L-α-phosphatidylserine (PS), and dimyristoylphosphatidylserine (DMPS)

were used to prepare lipid membranes. Membranes prepared for EPR studies were composed of PC or PC + 30 mol% PS. Lipids were hydrated in 10 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA. 5-doxylstearic acid was used as a spin probe. Oriented multibilayers were prepared by squeezing 50 μl of hydrated lipid (5 mg/ml) between two glass plates as previously described. Aripov et al. (1986) Akad Nauk SSSR 288:728. The lipid to probe molar ratio was 100/1. The EPR spectra of spin probes were recorded with a Varian E-4 spectrometer at modulation amplitudes not exceeding 2 x 10^{-4} T and a resonator input power not exceeding 20 mW.

EXAMPLE 13

Immunotoxin membrane interaction - calorimetry: The ability of the IT to interact with lipid membranes was analyzed by differential scanning microcalorimetry (Figure 8). Multilamellar lipid dispersions for calorimetric studies were prepared from dimyristoylphosphatidylcholine (DMPC) or DMPC + 5 mol% phosphatidylserine (PS). The low proportion of PS used was due to the fact that PS (as any lipid with unsaturated hydrocarbon chains) appreciable disturbs the ordered packing of synthetic saturated lipids, thus notably reducing the degree of transition cooperativity. Lipids were dissolved in chloroform/ethanol/methanol (6/1/3 by volume) solvent. Organic solvent was removed with a flux of helium, followed by exposure to vacuum for 1.5 hr and then, after addition of buffer (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA), mixing with a Vortex mixer for 15 min. Multilamellar dispersions were incubated in a helium atmosphere for 2 hr at 30°C. Calorimetric measurements were performed at a recording rate of 1°C per min using a differential scanning microcalorimeter (DASM-4, Pushino, USSR).

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Vernon, Leo P., Rael, Eppie D. and Gasanov, Sardar E.

(ii) TITLE OF THE INVENTION: *Pyrularia* thionin containing immunotoxins and immunotoxin-like compounds

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: MADSON & METCALF

(B) STREET: 950 First Interstate Building, 170 South Main Street

(C) CITY: Salt Lake City

(D) STATE: Utah

(E) COUNTRY: USA

(F) ZIP: 84101

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 Mb storage

(B) COMPUTER: IBM

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: WORDPERFECT 5.1

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: L. CRAIG METCALF

(B) REGISTRATION NUMBER: 31,398

(C) REFERENCE DOCKET NUMBER: 1771.2.1a

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (801) 537-1700

(B) TELEFAX: (801) 537-1799

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTIC:

(A) LENGTH: 156

(B) TYPE: nucleotides

(C) STRANDEDNESS: double

(D) TOPOLOGY: unknown

(ii) MOLECULAR TYPE: synthetic

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE:

(v) FRAGMENT TYPE:

(vii) IMMEDIATE SOURCE:

(B) CLONE: pBCsk+/ptox7

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CAT ATG AAA AGT TGC TGC CGT AAC ACG TGG GCT AGA    36
    Met Lys Ser Cys Cys Arg Asn Thr Trp Ala Arg
    1               5                   10

AAC TGC TAC AAC GTT TGC CGC CTG CCT GGT ACC ATC    72
Asn Cys Tyr Asn Val Cys Arg Leu Pro Gly Thr Ile
            15                  20

TCC CGT GAA ATC TGC GCA AAA AAA TGT GAT TGT AAA   108
Ser Arg Glu Ile Cys Ala Lys Lys Cys Asp Cys Lys
    25                  30                  35

ATC ATA AGT GGC ACC ACC TGC CCT AGT GAC TAT CCT   144
Ile Ile Ser Gly Thr Thr Cys Pro Ser Asp Tyr Pro
                40                  45

AAA TAAGGATCC                                     156
Lys
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTIC:

(A) LENGTH: 47

(B) TYPE: amino acids

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULAR TYPE: peptide

(iii) HYPOTHETICAL:

(iv) ANTI-SENSE: no

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Pyrularia pubera*

(F) TISSUE TYPE: leaves and nuts

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Vernon, Leo P., Evett, Gary E., Zeikus, Regina D. and Gray, William R.

(B) TITLE: A Toxic Thionin from *Pyrularia pubera*: Purification, properties, and Amino Acid Sequence

(C) JOURNAL: Archives of Biochemistry and Biophysics

(D) VOLUME: 238

(E) ISSUE: 1

(F) PAGES: 18-29

(G) DATE: April 1995

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Lys Ser Cys Cys Arg Asn Thr Trp Ala Arg Asn Cys Tyr Asn Val
1 5 10 15

Cys Arg Leu Pro Gly Thr Ile Ser Arg Glu Ile Cys Ala Lys Lys
20 25 30

Cys Asp Cys Lys Ile Ile Ser Gly Thr Thr Cys Pro Ser Asp Tyr
35 40 45

Pro Lys

CLAIMS:

1. A target specific toxin having a formula:

A - X - B

wherein A is a molecule capable of binding with specificity to the surface of a cell;

B is a toxin comprising the amino acid sequence SEQ ID NO: 2 or a functional derivative or fragment thereof which is cytotoxic; and

X is a covalent bond linking A and B.

2. The molecule of claim 1 wherein A is a monoclonal antibody or any fragment of an antibody which binds to the surface of eucaryotic cells with specificity.

3. The molecule of claim 1 wherein A is a monoclonal antibody or any fragment of an antibody which binds with specificity to T cells and activated B cells.

4. The molecule of claim 1 wherein A is the monoclonal antibody anti-CD5.

5. The molecule of claim 1 wherein the primary amino acid sequence of B has been modified by the addition, deletion, or substitution of at least one amino acid, which based on comparison with other members of the thionin family is expected to be, and is, cytotoxic.

6. The molecule of claim 1 wherein the amino acid sequence of B has been modified by the addition of an initiator methionine and a stop codon, and is cytotoxic.

7. The molecule of claim 1 wherein the linkage X is a divalent covalent bond comprising a disulfide group or a thioester group.

8. A target specific toxin having a formula:

A - X - B

wherein A is an antibody or fragment of an antibody which is capable of binding with specificity to the surface of a eucaryotic cell;
B is a toxin comprising the amino acid sequence SEQ ID NO: 2 or a functional derivative or fragment thereof which is cytotoxic; and
X is a covalent bond linking A and B.

9. The immunotoxin of claim 8 wherein said antibody contains a foreign variable region and a human constant region.

10. The molecule of claim 8 wherein A is the monoclonal antibody anti-CD5.

11. The molecule of claim 8 wherein the primary amino acid sequence of B has been modified by the addition, deletion, substitution of at least one amino acid which based on comparison with other members of the thionin family is expected to be, and is, cytotoxic to eucaryotic cells.

12. The molecule of claim 8 wherein the linkage X is a divalent covalent bond comprising a disulfide group or a thioester group.

13. A target specific toxin having a formula:

A - X - B

wherein A is an antibody or any fragment of an antibody which binds with specificity to T cells and activated B cells.
B is a toxin comprising the amino acid sequence SEQ ID NO: 2 or a functional derivative or fragment thereof which is toxic to eucaryotic cells; and
X is a covalent bond linking A and B.

14. The immunotoxin of claim 13 wherein A comprises an antibody containing a foreign variable region and a human constant region.

15. The molecule of claim 14 wherein A is the antibody anti-CD5.

16. The molecule of claim 13 wherein A is the antibody anti-CD5.

17. The molecule of claim 13 wherein the primary amino acid sequence of B has been modified by the addition, deletion, substitution of at least one amino acid which based on comparison with other members of the thionin family is expected to be, and is, cytotoxic to T cells and activated B cells.

18. The molecule of claim 1 wherein the amino acid sequence of B has been modified by the addition of an initiator methionine and a stop codon, and is cytotoxic.

19. The molecule of claim 13 wherein B is the toxin *Pyrularia* thionin.

20. The molecule of claim 13 wherein the linkage X is a divalent covalent bond comprising a disulfide group or a thioester group.

21. The molecule of claim 13 wherein X comprises a N-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) linkage.

22. A method of killing selected undesirable cells to which *P.* thionin is generally cytotoxic, comprising the steps of:

a. selecting a target specific molecule;

b. attaching to said molecule the toxin *P.* thionin, or any functional derivative or fragment thereof which is cytotoxic to said cells such that said toxin and said target specific molecule form a target specific toxin;

c. administering a cytotoxic dose of said target specific toxin to said cells.

23. A method as defined in claim 22 wherein *P.* thionin is toxic to said cells to the extent that the ID_{50} is between about 1 X 10^{-6} M and about 1 X 10^{-12} M.

24. A method of preparing a target specific toxin comprising steps of preparing a toxin having the formula:

A - X - B

wherein A is a molecule capable of binding with specificity to the surface of a cell;

B is a toxin comprising the amino acid sequence SEQ ID NO: 2 or a functional derivative or fragment thereof which is cytotoxic; and

X is a covalent bond linking A and B.

25. A method for the chemotherapeutic treatment of cancer in a warm blooded mammal, consisting of injecting into such mammal a chemotherapeutic effective quantity of a target specific toxin comprising the following formula:

A - X - B

wherein A is a molecule capable of binding with specificity to the surface of a cell;

B is a toxin comprising the amino acid sequence SEQ ID NO: 2 or a functional derivative or fragment thereof which is cytotoxic; and

X is a covalent bond linking A and B.

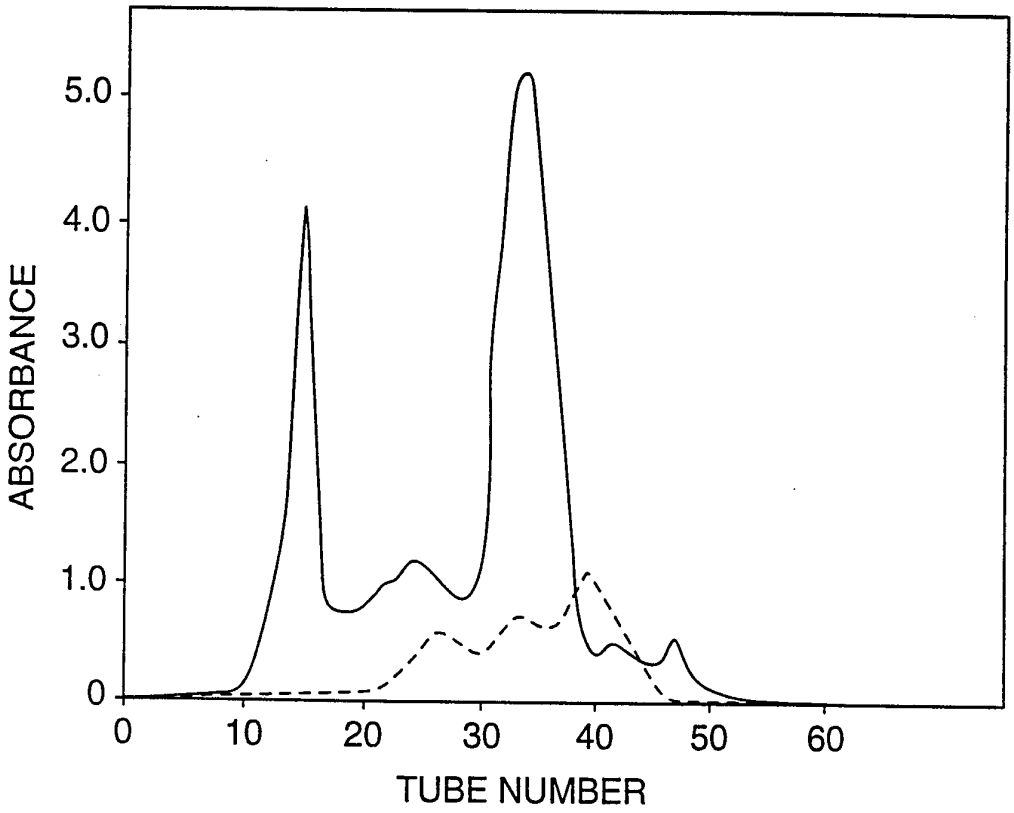


FIG. 1

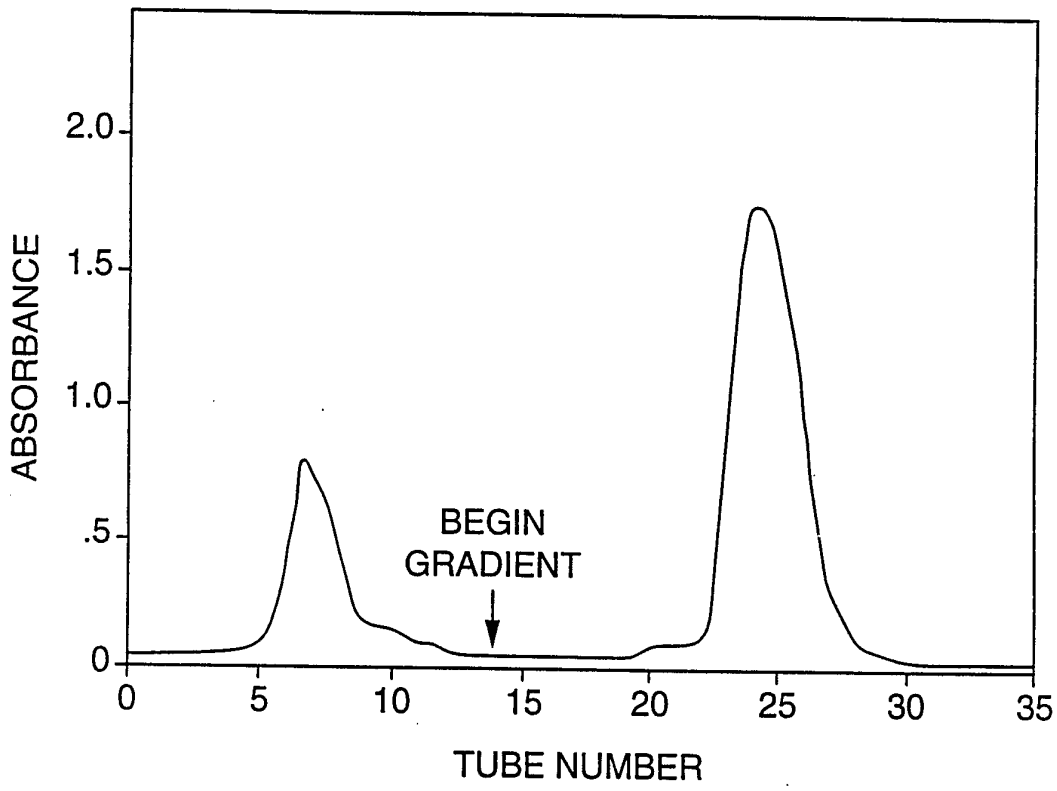

ABSORBANCE
2.0
1.5
1.0
.5
0
BEGIN
GRADIENT
0
5
10
15
20
25
30
35
TUBE NUMBER

FIG. 2

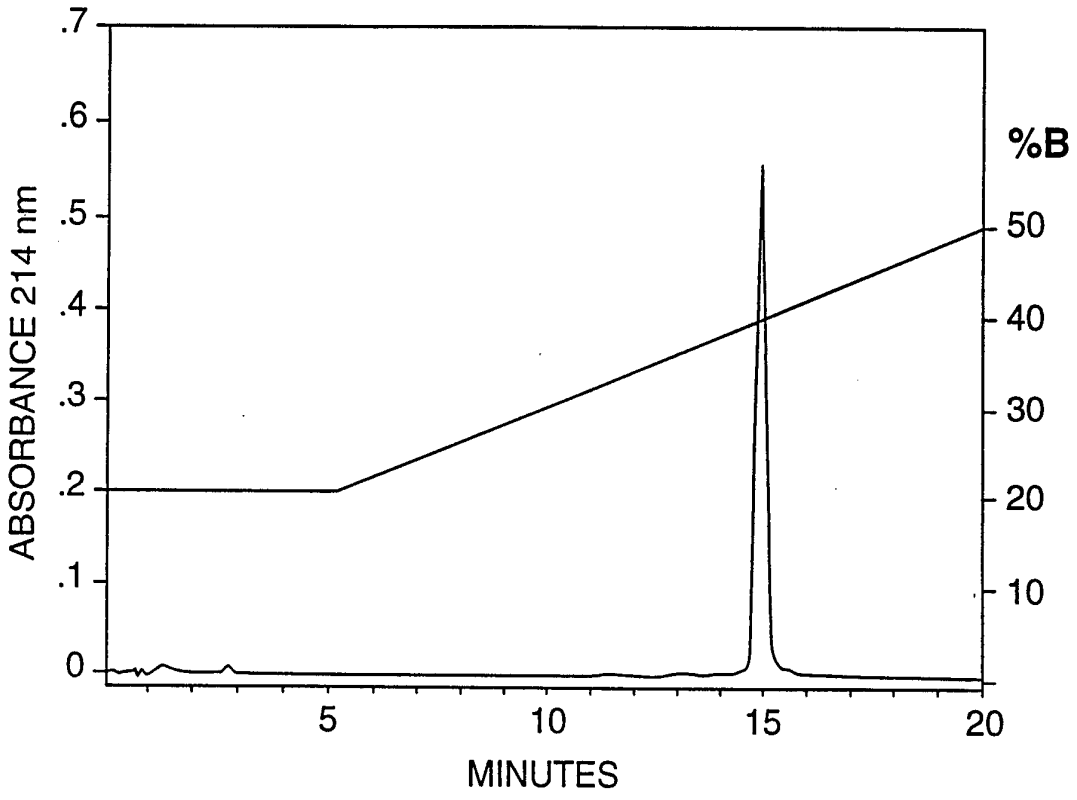
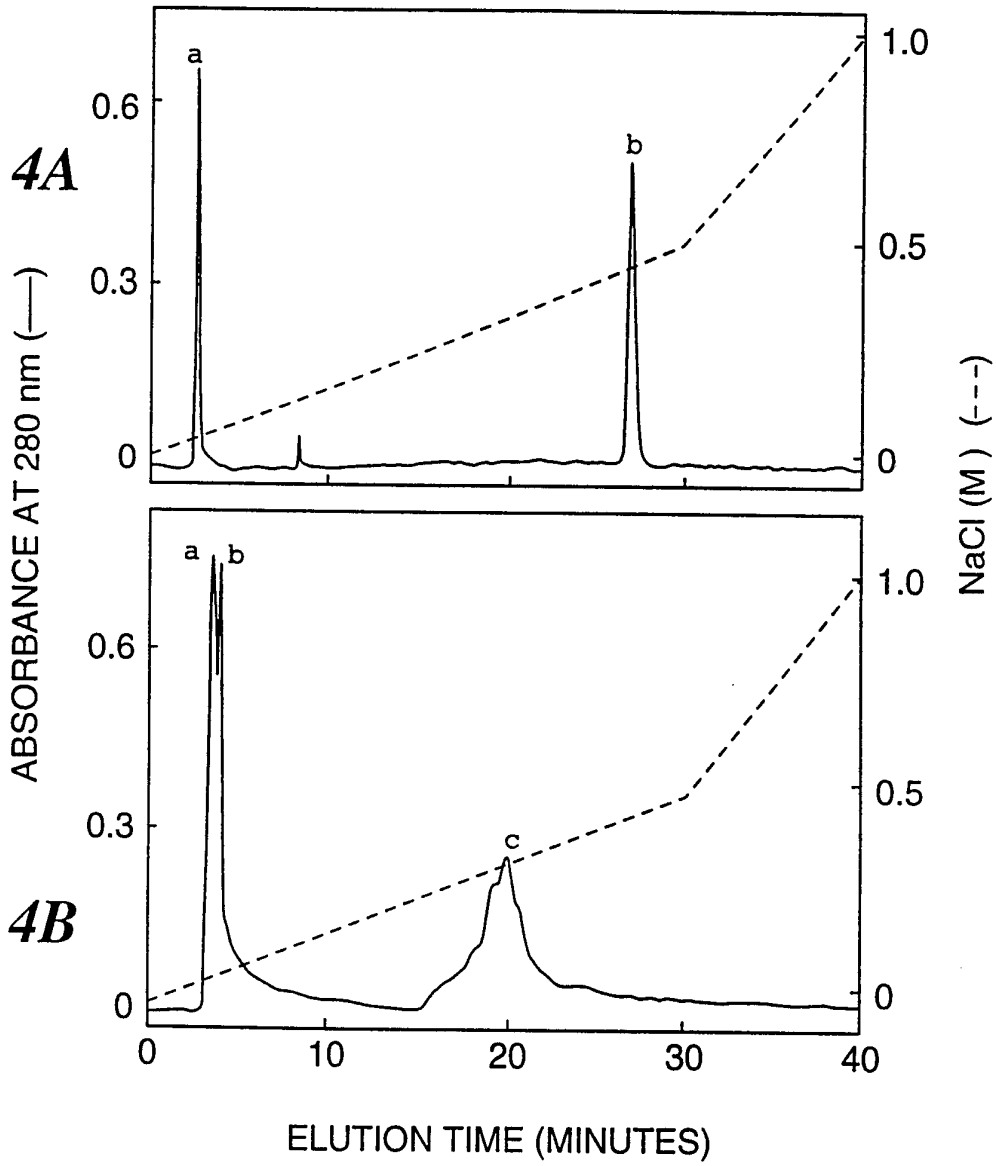


FIG. 3

FIG. 4A

FIG. 4B


a
b
c
0.6
0.3
0
1.0
0.5
0
10
20
30
40
ABSORBANCE AT 280 nm (——)
NaCl (M) (- - -)
ELUTION TIME (MINUTES)

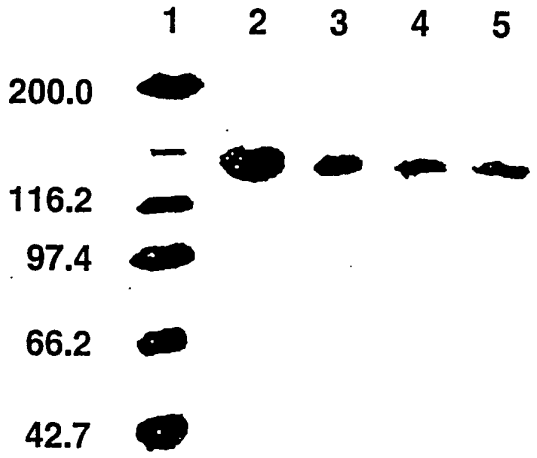


FIG. 5

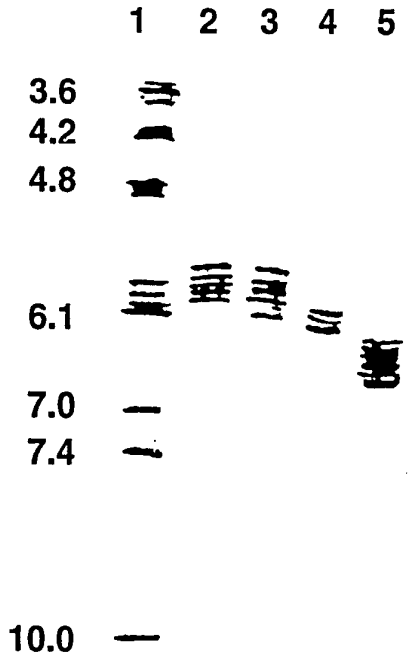


FIG. 6

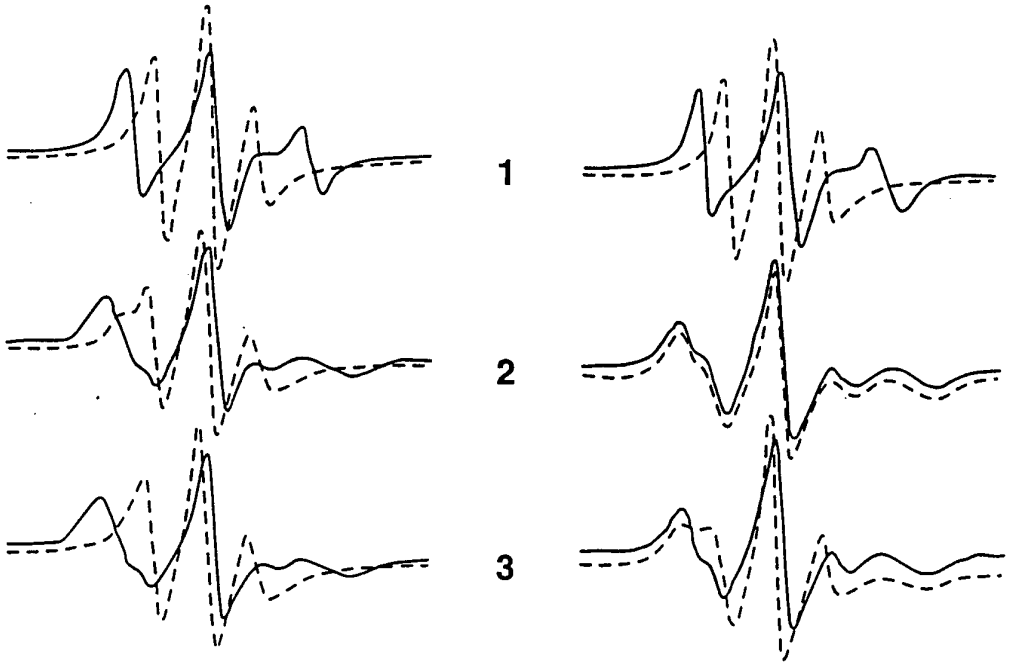


FIG. 7

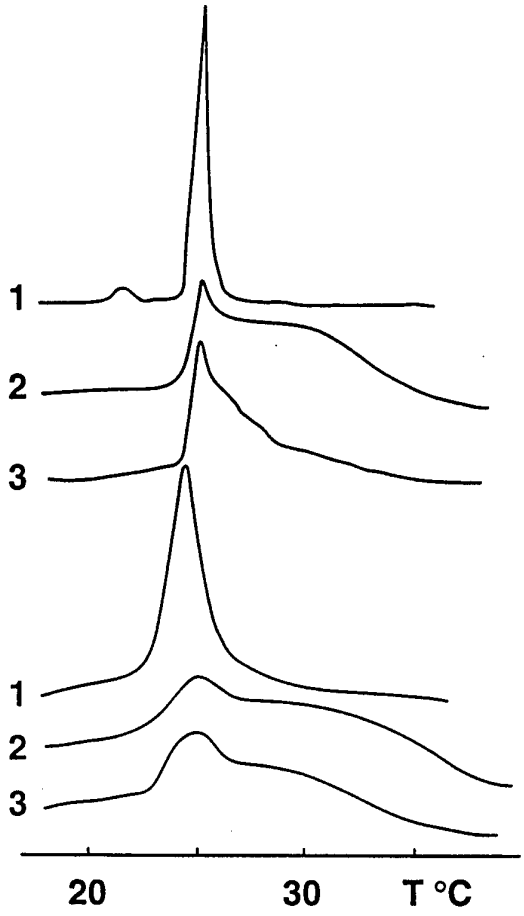


FIG. 8

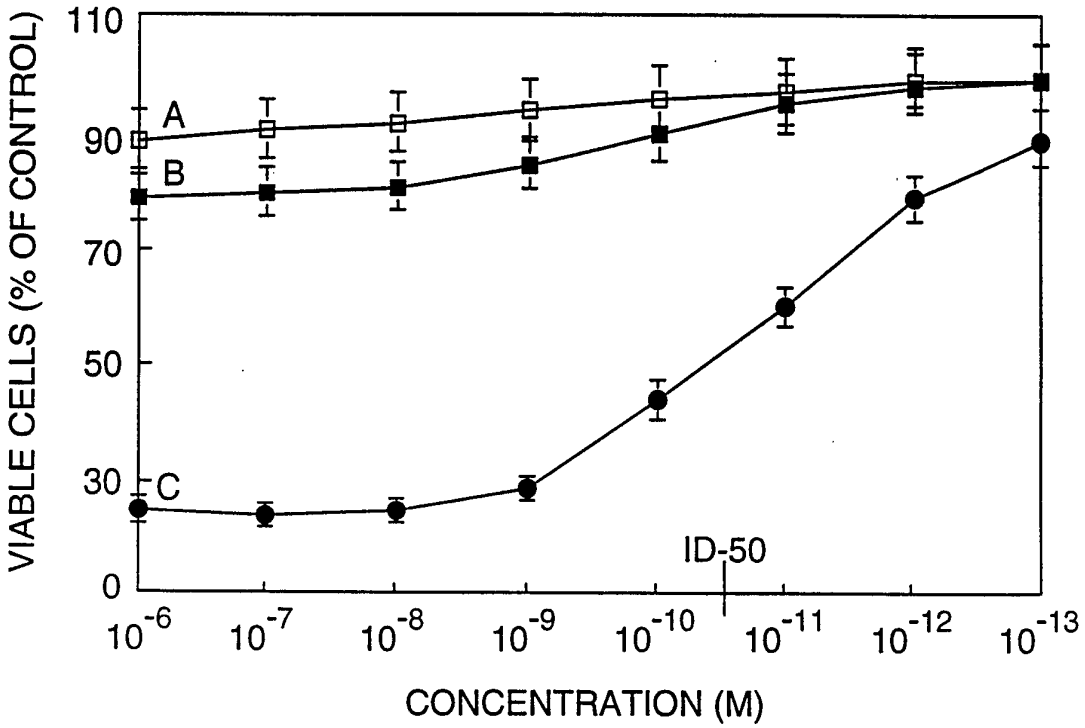

VIABLE CELLS (% OF CONTROL)
110
90
70
50
30
0
A
B
C
ID-50
10^{-6}
10^{-7}
10^{-8}
10^{-9}
10^{-10}
10^{-11}
10^{-12}
10^{-13}
CONCENTRATION (M)

FIG. 9

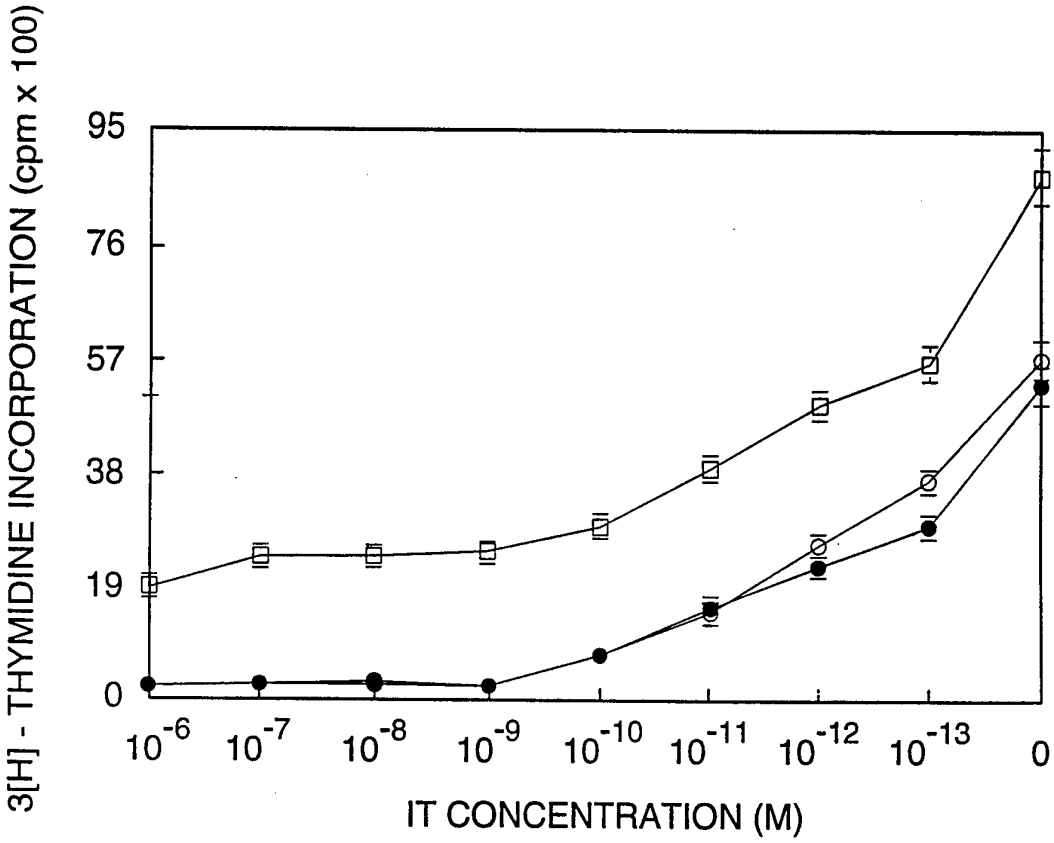

3[H] - THYMIDINE INCORPORATION (cpm x 100)
95
76
57
38
19
0
10-6
10-7
10-8
10-9
10-10
10-11
10-12
10-13
0
IT CONCENTRATION (M)

FIG. 10

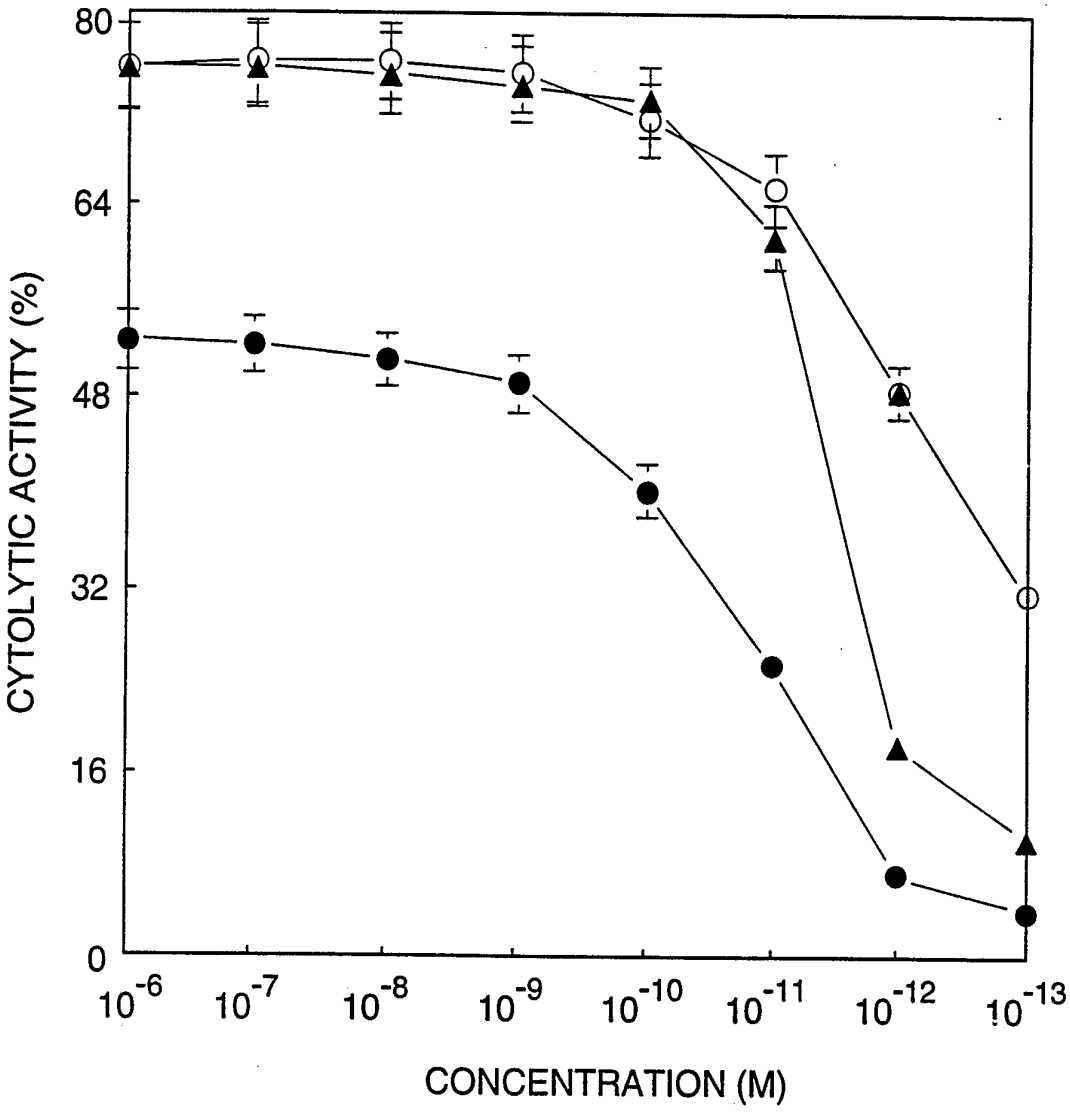

80
64
48
32
16
0
CYTOLYTIC ACTIVITY (%)
10-6
10-7
10-8
10-9
10-10
10-11
10-12
10-13
CONCENTRATION (M)

FIG. 11