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(54) Promiscuous HER-2/NEU CD4 T cell epitopes

Promiskuitive HER-2/NEU-CD4-T-Zellepitope
Epitopes de lymphocytes T HER-2/NEU CD4 mêlés

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- **SOTIRIADOU R ET AL: "Peptide HER2(776-788) represents a naturally processed broad MHC class II-restricted T cell epitope", BRITISH JOURNAL OF CANCER, HARCOURT PUBLISHERS, vol. 85, no. 10, 16 November 2001 (2001-11-16), pages 1527-1534, XP002293865, ISSN: 0007-0920, DOI: 10.1054/BJOC.2001.2089**
- **KOBAYASHI H ET AL: "Defining promiscuous MHC class II Helper T-cell epitopes for the Her2/neu Tumor Antigen", CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 60, 15 September 2000 (2000-09-15), pages 5228-5236, XP002214629, ISSN: 0008-5472**

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Description

RELATED APPLICATIONS

5 BACKGROUND OF THE INVENTION

10 **[0001]** HLA class II-restricted CD4⁺ T cells play a critical role in cellular immunity and are a key component of anti-tumor immune responses. CD4⁺ T cells provide necessary help to tumor-specific CTLs (Topalian 1994. *Curr Opin Immunol* 6:741-745) and produce cytokines such as interferon gamma (IFN γ), which can activate antigen presenting cells and mediate other immunological effects (Corthay et al., 2005. *Immunity* 22:371-383). Experimental results in several systems have demonstrated that CD4⁺ T cells are necessary for an effective anti-tumor immune response. Given the importance of CD4⁺ T cells in generating a robust immune response, an optimally designed cancer immunotherapy or anti-tumor vaccine should induce both tumor-specific CD4⁺ and CD8⁺T cells for maximal efficacy.

15 **[0002]** The design of cancer immunotherapies and vaccines has benefited greatly from the identification of tumor-associated antigens. One such antigen, HER-2/Neu, is a prime target for such strategies due to its amplification in a variety of cancers, including breast and ovarian cancer. The HER-2/Neu oncogene encodes a transmembrane glycoprotein with homology to epidermal growth factor receptor (Cousens et al., 1985. *Science* 230:1132-1139). Overexpression of HER-2/Neu occurs in approximately 30% percent of breast adenocarcinomas and is associated with aggressive disease and a poor prognosis. As a result, several immunological approaches designed to increase T cell recognition of the HER-2/Neu protein have been tested in clinical trials. Characterizing the resulting HER-2/Neu-specific T cell responses in such studies has led to the identification of several HLA class I and class II-restricted T cell epitopes within the HER-2/Neu protein (Sotiriadou et al., 2001. *Br J Cancer* 85:1527-1534). The identification of these epitopes has, in turn, enhanced our ability to detect and quantitate HER-2/Neu-specific T cell response. Such information leads to improved designs for effective immunotherapies and provides a better understanding of the role of HER-2/Neu-specific T cell in eradicating HER-2/Neu-expressing tumors.

20 **[0003]** The usefulness of a defined T cell epitope is limited by its HLA-restriction. Peptide epitopes typically form productive peptide-MHC complexes with a small number of HLA alleles and stimulate T cell responses only in individuals expressing those alleles. This confines immunological studies and clinical trials to individuals of a specific HLA type, often 20% or less of the general population. So-called promiscuous T cell epitopes, which can be presented by a larger number of HLA alleles, have been described for several tumor antigens. Promiscuous T cell epitopes can bind to multiple HLA alleles to stimulate antigen-specific T cells, allowing for the induction and study of T cell responses in individuals of different HLA types. Additionally, promiscuous epitopes are valuable because the immunotherapies and vaccines based on these epitopes can be widely applicable to the general population for cancer treatment and prevention. Thus, there exists a clear need for new information relating to previously unknown promiscuous epitopes of tumor antigens.

25 **[0004]** EP1 568 373 A2 predicts a number of HER2/neu epitopes based on an analysis of motifs and supermotifs of epitope sequences.

30 **[0005]** The present inventors have identified a series of novel promiscuous T cell epitopes in the HER-2/Neu protein sequence. These epitopes, located within the region of 270-284 or 268-286 of the HER-2/Neu protein, are recognized by a CD4⁺ T cell clone generated from a patient treated with an autologous, active cellular immunotherapy for HER-2/Neu overexpressing carcinomas (Valone et al., 2001. *Cancer* 17 Suppl 2:S53-61). The T cell clone recognizes these peptide epitopes presented in the context of at least 25 different HLA- DRB1*alleles. Antibody blocking experiments confirm that the recognition is HLA-DR restricted. Furthermore, these epitopes are naturally processed and presented from exogenous protein antigen. The promiscuity of these epitopes for different HLA-DRB1* alleles makes these epitopes a valuable tool for evaluating HER-2/Neu-specific immune responses regardless of HLA type. Additionally, these epitopes can be used as a universal CD4 T helper cell epitope in peptide-based vaccines or immunotherapies for the treatment HER-2/Neu+ cancers.

BRIEF SUMMARY OF THE INVENTION

35 **[0006]** The present invention describes novel HER-2/Neu epitopes that can be presented by antigen presenting cells of a number of different HLA alleles to induce a HER-2/Neu specific T cell response. The present invention thus provides a composition for use in a method for inducing in a patient a T cell immune response specific to a HER-2/Neu protein, wherein said composition comprises a peptide consisting of the sequence TYNTDTFESMPN, and a physiologically acceptable excipient. The invention also provides a composition for use in a method for inducing in a patient a T cell immune response specific to a HER-2/Neu protein, wherein said composition comprises a peptide consisting of the sequence ALVTYNTDTFESMPN, VTYNTDTFESMP, TYNTDTFESMPN, LVTYNTDTFESMP, VTYNTDTFESMPN, ALVTYNTDTFESMP, LVTYNTDTFESMPN, PALVTYNTDTFESMPN, or CPALVTYNTDTFESMPN; and a physiologically acceptable excipient; wherein the route of administration of the composition is subcutaneous, intradermal, transder-

mal, intramuscular, intravenous, or intraperitoneal. The peptide may be in the form of a fusion product comprising one of the above HER-2/Neu derived peptides fused to a heterologous polypeptide. The invention is further defined by the claims. In some cases, the peptide is fused to the heterologous polypeptide by a peptide bond, such that the fusion product is in essence a recombinant fusion protein.

[0007] Preferably, the isolated peptide derived from the HER-2/Neu sequence or its fusion product is capable of inducing a T cell immune response specific to a HER-2/Neu protein when presented by an antigen-presenting cell of at least 10 different HLA-DR alleles, and more preferably, at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more different HLA-DR alleles.

[0008] In some embodiments, the HLA-DR alleles are selected from the group consisting of 0101, 0102, 0103, 1503, 160201, 0301, 0302, 0401, 0402, 040301, 040501, 1101, 1102, 1103, 1104, 110401, 1201, 1301, 1302, 1401, 1402, 0701, 080101, 080201, and 0901.

[0009] In some embodiments, the HER-2/Neu derived peptide has the amino acid sequence of SEQ ID NO:2. In other embodiments, the heterologous polypeptide is a granulocyte-macrophage colony-stimulating factor (GM-CSF).

[0010] In a second aspect, the present invention provides an isolated nucleic acid comprising a polynucleotide sequence encoding a HER-2/Neu derived peptide described above or a fusion protein joining a HER-2/Neu derived peptide and a heterologous polypeptide by a peptide bond, an expression cassette comprising the nucleic acid, and a host cell comprising the expression cassette.

[0011] In some cases, the polynucleotide sequence encodes the peptide having the amino acid sequence of SEQ ID NO:2. In other cases, the polynucleotide sequence encodes a fusion protein in which the heterologous polypeptide is GM-CSF.

[0012] In some embodiments, the expression cassette is a recombinant viral vector. In other embodiments, the expression cassette directs the expression of the peptide having the amino acid sequence of SEQ ID NO:2 or a recombinant fusion protein in which the heterologous polypeptide is GM-CSF.

[0013] In a third aspect, the present invention provides a composition comprising a HER-2/Neu derived peptide as described above or a fusion product of the peptide fused with a heterologous polypeptide, in addition to a physiologically acceptable excipient.

[0014] In some embodiments, the peptide has the amino acid sequence of SEQ ID NO:2. In other embodiments, the heterologous polypeptide is a granulocyte-macrophage colony-stimulating factor (GM-CSF). In yet other embodiments, the composition further comprises an antigen-presenting cell, which has the HER-2/Neu derived peptide forming a complex with a major histocompatibility complex (MHC) molecule on the surface of the cell.

[0015] In a fourth aspect, the present invention provides a method for inducing in a patient a T cell immune response specific to a HER-2/Neu protein. This method comprises the step of administering to the patient an effective amount of the composition comprising a HER-2/Neu derived peptide as described above or a fusion product of the peptide fused with a heterologous polypeptide, as well as a physiologically acceptable excipient.

[0016] In some embodiments, the peptide has the amino acid sequence of SEQ ID NO:2. In other embodiments, the heterologous polypeptide is a granulocyte-macrophage colony-stimulating factor (GM-CSF).

[0017] In another aspect, the invention provides an *in vitro* method for detecting a T cell immune response specific to a HER-2/Neu protein, the method comprising the steps of (a) contacting an antigen-presenting cell and a T cell, in a sample comprising blood, tumor infiltrate, lymph nodes or lymphatic fluids obtained from a patient, with a peptide consisting of the sequence: ALVTYNTDTFESMPN, VTYNTDTFESMP, TYNTDTFESMPN, LVTYNTDTFESMP, VTYNTDTFESMPN, ALVTYNTDTFESMP, LVTYNTDTFESMPN, PALVTYNTDTFESMPN, or CPALVTYNTDTFESMPN; and (b) detecting a T cell response, wherein the detection of a T cell response indicates the presence of a T cell immune response specific to a HER-2/Neu protein in the patient.

[0018] In some embodiments, step (c) is performed by ELISPOT, proliferation assay, or flow cytometry. In other embodiments, the HER-2/Neu derived peptide has the amino acid sequence of SEQ ID NO:2.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

Figure 1. T cell clone HER500.23c21 is HER-2/Neu specific. (A) HER500 (amino acids ■ and BA7072 (HER500/GM-CSF fusion protein) ♦ specific IFN γ production by HER500.23c21 was measured using ELISA after autologous PBMC presentation of the antigens; background was also tested A. Assay was set up in triplicate in 96 well round bottom plates in complete IMDM media with 10% PBS. Autologous PBMCs were used at 2×10^5 cells/well and T cell clone HER500.23c21 was added at 1×10^5 cells/well. Antigens were at the final concentrations shown. Assay was incubated for 48 hours at 37°C with 5% CO $_2$ and supernatant was removed from wells to test for cytokine production. (A) IFN γ production. Data points shown represent the calculated pg/mL for each antigen concentration. (B) IL-2 production for the same assay was measured using HT-2 cell proliferation. Data points represent the mean CPM

values for each antigen concentration.

Figure 2. T cell clone HER500.23.21 is specific for Peptide #63 (HERp270-284). The HER-2/neu epitope was mapped for clone HER500.23.21 and specificity was determined using autologous EBV-LcLs and each individual HER500 peptide #1-125 (15 amino acids in length). The assay was set up in 96 well round bottom plates with autologous EBV-LcLs at 2×10^5 cells/well and the T cell clone HER500.23.21 added at 1×10^5 cells/well. Each peptide was used at a final concentration of $1 \mu\text{g/mL}$. The assay was incubated at 37°C with 5% CO_2 for 48 hours at which time supernatant was removed to test for cytokine production. (A) IFN_γ production by clone HER500.23.21 was measured using ELISA, calculated pg/mL is shown for each HER500 peptide. (B) IL-2 production was measured using HT-2 cell proliferation.

Figure 3. HLA-DR Restriction. Autologous EBV-LcL at 2×10^5 cells/mL and HERp270-284 was used to stimulate the T cell clone HER500.23c21 (1×10^5 cells/well), with and without the addition of blocking anti-MHC class II antibodies. Anti-HLA-DR \blacklozenge and anti-HLA-DQ/anti-HLA-DP \blacksquare were titrated at the concentrations shown in cRPMI+10% FBS. Wells also contained EBV-LcLs, HER500.23c21T cell clone and peptide ($1 \mu\text{g/mL}$). Assay was incubated for 48 hours at 37°C with 5% CO_2 . Supernatants were harvested and frozen at -20°C and at a later date, analyzed for IFN_γ production using ELISA. Multiple experiments were performed, mean triplicate values for one representative experiment is shown.

Figure 4. HERp270-284 is a promiscuous MHC Class II HER-2/Neu epitope. (A) EBV-LcL lines homozygous for various HLA-DR alleles were tested for their ability to present HERp270-284 to clone HER500.23c21. Antigen-specific IFN_γ production by HER500.23c21 was above the upper detection limit of the IFN_γ ELISA (4000pg/mL) with all EBV-LcL lines. Negative controls included an irrelevant HER500 peptide, no peptide and each EBV-LcL line alone (all values not detectable, not shown). The assay was set up in 96 well round bottom plates in cRPMI+10% FBS with 2×10^5 EBV-LcL/well and 1×10^5 HER500.23c21 cells/well. Peptides were used at a final concentration of $1 \mu\text{g/mL}$. The assay was incubated for 48 hours at 37°C with 5% CO_2 . Supernatants were harvested, frozen at -20°C and then analyzed for IFN_γ by ELISA. Results are shown for one representative experiment. (B) HERp270-284 was titrated with each EBV LcL line to determine the sensitivity of HER500.23c21 to different alleles. The assay was set up as in (A) except HERp270-284 concentrations are: 10ng/mL , 33ng/mL , 66ng/mL , 100ng/mL and 250ng/mL . All HERp270-284 concentrations above 250ng/mL resulted in IFN_γ production by HER500.23c21 above the upper detection limit of the IFN_γ ELISA (4000pg/mL). Results are shown for one representative experiment.

Figure 5. Graphic depiction of IFN_γ production by HER500.23c21 following exposure to peptides presented by EBV LcL lines of diverse HLA-DRb1 alleles. The peptides were 9-mers within the HER-2/Neu peptide 270-284.

Figure 6. Graphic depiction of IFN_γ production by HER500.23c21 following exposure to peptides presented by EBV LcL lines of diverse HLA-DRb1 alleles. The peptides were 10- or 11-mers within the HER-2/Neu peptide 270-284.

Figure 7. Graphic depiction of IFN_γ production by HER500.23c21 (measured by absorbance at 492 nm) following exposure to peptides presented by EBV LcL lines of diverse HLA-DRb1 alleles. The peptides were 12-mers within the HER-2/Neu peptide 270-284.

Figure 8. Graphic depiction of IFN_γ production by HER500.23c21 (indicated by absorbance at 492 nm) following exposure to peptides presented by EBV LcL lines of diverse HLA-DRb1 alleles. The peptides were 13-mers within the HER-2/Neu peptide 270-284.

Figure 9. Graphic depiction of IFN_γ production by HER500.23c21 (indicated by absorbance at 492 nm) following exposure to peptides presented by EBV LcL lines of diverse HLA-DRb1 alleles. The peptides were 14-mers within the HER-2/Neu peptide 270-284.

Figure 10. Graphic depiction of IFN_γ production by HER500.23c21 (indicated by absorbance at 492 nm) following exposure to peptides presented by EBV LcL lines of diverse HLA-DRb1 alleles. The peptides were 16-mers within the HER-2/Neu peptide 268-286.

Figure 11. Graphic depiction of IFN_γ production by HER500.23c21 (indicated by absorbance at 492 nm) following exposure to peptides presented by EBV LcL lines of diverse HLA-DRb1 alleles. The peptides were 17-mers within the HER-2/Neu peptide 268-286.

DEFINITIONS

5 [0020] The term "**isolated**," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames that flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

15 [0021] In this application, the term "**amino acid**" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure different from the general chemical structure of an amino acid, but capable of functioning in a manner similar to a naturally occurring amino acid.

25 [0022] The term "**nucleic acid**" or "**polynucleotide**" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, or mRNA encoded by a gene.

30 [0023] When the relative locations of elements in a polynucleotide sequence are concerned, a "downstream" location is one at the 3' side of a reference point, and an "upstream" location is one at the 5' side of a reference point.

35 [0024] The terms "**polypeptide**," "**peptide**," and "**protein**" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins (*i.e.*, antigens), wherein the amino acid residues are linked by covalent peptide bonds. In this application, the amino acid sequence of a polypeptide is presented from the N-terminus to the C-terminus. In other words, when describing an amino acid sequence of a peptide, the first amino acid from the N-terminus is referred to as the "first amino acid."

45 [0025] When used in the context of describing partners of a fusion peptide, the term "**heterologous**" refers to the relationship of one peptide fusion partner to the another peptide fusion partner: the manner in which the fusion partners are present in the fusion peptide is not one that can be found a naturally occurring protein. For instance, a "heterologous polypeptide" fused with a HER-2/Neu epitope to form a fusion peptide may be one that is originated from a protein other than a HER-2/Neu protein, such as a granulocyte-macrophage colony-stimulating factor (GM-CSF). On the other hand, a "heterologous polypeptide" may be one derived from another portion of the HER-2/Neu protein that is not immediately contiguous to the HER-2/Neu epitope. A "heterologous polypeptide" may contain modifications of a naturally occurring protein sequence or a portion thereof, such as deletions, additions, or substitutions of one or more amino acid residues. Regardless of the origin of the "heterologous polypeptide" (*i.e.*, whether it is derived from the HER-2/Neu protein or another protein), the fusion peptide should not contain a subsequence of the human HER-2/Neu that encompasses the amino acid sequence of SEQ ID NO: 1 and have more than 18 amino acids in length. In some exemplary embodiments, a "heterologous polypeptide" for use in the present invention has no more than 15-20 amino acids in length; in other embodiments, a "heterologous polypeptide" has at least 100 amino acids in length.

55 [0026] The word "**fuse**" or "**fused**," as used in the context of describing a peptide that comprises a HER-2/Neu epitope joined with a heterologous polypeptide, refers to a connection between the epitope and the heterologous polypeptide by any covalent bond, including a peptide bond.

[0027] The phrase "**a nucleic acid sequence encoding**" refers to a nucleic acid which contains sequence information

for a structural RNA such as rRNA, a tRNA, or the primary amino acid sequence of a specific protein or peptide, or a binding site for a trans-acting regulatory agent. This phrase specifically encompasses degenerate codons (*i.e.*, different codons which encode a single amino acid) of the native sequence or sequences that may be introduced to conform to codon preference in a specific host cell.

5 [0028] An "**expression cassette**" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular polynucleotide sequence in a host cell. An expression cassette may be part of a plasmid, viral genome, or nucleic acid fragment. Typically, an expression cassette includes a polynucleotide to be transcribed, operably linked to a promoter.

10 [0029] The term "**recombinant**," when used with reference, *e.g.*, to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a nucleic acid or protein from an outside source or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under-expressed or not expressed at all.

15 [0030] The term "**administration**" or "**administering**" refers to various methods of contacting a substance with a mammal, especially a human. Modes of administration may include, but are not limited to, methods that involve contacting the substance intravenously, intraperitoneally, intranasally, transdermally, topically, subcutaneously, parentally, intramuscularly, orally, or systemically, and via injection, ingestion, inhalation, implantation, or adsorption by any other means. One exemplary means of administration of a HER-2/Neu peptide or a fusion peptide comprising a HER-2/Neu peptide and a heterologous polypeptide is via intravenous delivery, where the peptide or fusion peptide can be formulated as a pharmaceutical composition in the form suitable for intravenous injection, such as an aqueous solution, a suspension, or an emulsion, *etc.* Other means for delivering a HER-2/Neu peptide or a fusion peptide includes intradermal injection, subcutaneous injection, intramuscular injection, or transdermal application as with a patch.

20 [0031] An "**effective amount**" of a certain substance refers to an amount of the substance that is sufficient to effectuate a desired result. For instance, an effective amount of a composition comprising a peptide that is intended to induce an anti-Her-2/Neu immunity is an amount sufficient to achieve the goal of inducing the immunity when administered to a subject. The effect to be achieved may include the prevention, correction, or inhibition of progression of the symptoms of a disease/condition and related complications to any detectable extent. The exact quantity of an "effective amount" will depend on the purpose of the administration, and can be ascertainable by one skilled in the art using known techniques (*see, e.g.*, Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); and Pickar, *Dosage Calculations* (1999)).

25 [0032] A "**physiologically acceptable excipient**" is an inert ingredient used in the formulation of a composition, which contains the active ingredient(s) of a HER-2/Neu peptide or a fusion peptide comprising a HER-2/Neu peptide and a heterologous polypeptide and is suitable for use, *e.g.*, by injection into a patient in need thereof. This inert ingredient may be a substance that, when included in a composition, provides a desired pH, consistency, color, smell, or flavor of the composition.

30 [0033] As used herein, the term "**T cell immune response**" refers to activation of antigen specific T cells as measured by proliferation or expression of molecules on the cell surface or secretion of proteins such as cytokines.

DETAILED DESCRIPTION OF THE INVENTION

I. INTRODUCTION

35 [0034] The present inventors have identified a series of novel promiscuous T cell epitopes from the HER-2/Neu protein. These peptide epitopes demonstrate remarkable HLA promiscuity as they can be presented in the context of at least 25 different HLA-DRB1 alleles. The presentation of these epitopes by such a wide range of HLA-DRB1 alleles makes these epitopes extremely valuable as universal CD4 T helper cell epitopes in preparation of vaccines or immunotherapies for the treatment of cancers overexpressing HER-2/Neu in the general human population.

II. CHEMICAL SYNTHESIS OF PEPTIDES

40 [0035] The peptides of the present invention, particular those of relatively short length (*e.g.*, no more than 50-100 amino acids), may be synthesized chemically using conventional peptide synthesis or other protocols well known in the art.

45 [0036] Peptides may be synthesized by solid-phase peptide synthesis methods using procedures similar to those described by Merrifield *et al.*, *J. Am. Chem. Soc.*, 85:2149-2156 (1963); Barany and Merrifield, *Solid Phase Peptide Synthesis*, in *The Peptides: Analysis, Synthesis, Biology* Gross and Meienhofer (eds.), Academic Press, N.Y., vol. 2, pp. 3-284 (1980); and Stewart *et al.*, *Solid Phase Peptide Synthesis* 2nd ed., Pierce Chem. Co., Rockford, Ill. (1984). During synthesis, N- α -protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to a solid support, *i.e.*, polystyrene beads. The peptides are synthesized by linking an

amino group of an N- α -deprotected amino acid to an α -carboxy group of an N- α -protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N- α -protecting groups include Boc, which is acid labile, and Fmoc, which is base labile.

[0037] Materials suitable for use as the solid support are well known to those of skill in the art and include, but are not limited to, the following: halomethyl resins, such as chloromethyl resin or bromomethyl resin; hydroxymethyl resins; phenol resins, such as 4-(α -[2,4-dimethoxyphenyl]-Fmoc-aminomethyl)phenoxy resin; tert-alkyloxycarbonyl-hydrazidated resins, and the like. Such resins are commercially available and their methods of preparation are known by those of ordinary skill in the art.

[0038] Briefly, the C-terminal N- α -protected amino acid is first attached to the solid support. The N- α -protecting group is then removed. The deprotected α -amino group is coupled to the activated α -carboxylate group of the next N- α -protected amino acid. The process is repeated until the desired peptide is synthesized. The resulting peptides are then cleaved from the insoluble polymer support and the amino acid side chains deprotected. Longer peptides can be derived by condensation of protected peptide fragments. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are not discussed in detail herein (See, e.g., Atherton et al., *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press (1989), and Bodanszky, *Peptide Chemistry, A Practical Textbook*, 2nd Ed., Springer-Verlag (1993)).

III. RECOMBINANT PRODUCTION OF PEPTIDES

A. General Recombinant Technology

[0039] Basic texts disclosing general methods and techniques in the field of recombinant genetics include Sambrook and Russell, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and Ausubel et al., eds., *Current Protocols in Molecular Biology* (1994).

[0040] For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[0041] Oligonucleotides that are not commercially available can be chemically synthesized, e.g., according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Lett.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter et. al., *Nucleic Acids Res.* 12:6159-6168 (1984). Purification of oligonucleotides is performed using any art-recognized strategy, e.g., native acrylamide gel electrophoresis or anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983).

[0042] Recombinant production is an effective means to obtain peptides particularly those of relatively large molecular weight, for example, a fusion peptide of a HER-2/Neu epitope and a GM-CSF. The sequence of a polynucleotide encoding a peptide of this invention, and synthetic oligonucleotides can be verified after cloning or subcloning using, e.g., the chain termination method for sequencing double-stranded templates of Wallace et al., *Gene* 16: 21-26 (1981).

B. Construction of an Expression Cassette

Obtaining a Polynucleotide Sequence Encoding a Peptide for use in the Invention

[0043] A polynucleotide sequence encoding a peptide can be obtained by chemical synthesis, or can be purchased from a commercial supplier, which may then be further manipulated using standard techniques of molecular cloning.

Modification of Nucleic Acids for Preferred Codon Usage in a Host Organism

[0044] The polynucleotide sequence encoding a peptide can be optionally altered to coincide with the preferred codon usage of a particular host. For example, the preferred codon usage of one strain of bacterial cells can be used to derive a polynucleotide that encodes a peptide and includes the codons favored by this strain. The frequency of preferred codon usage exhibited by a host cell can be calculated by averaging frequency of preferred codon usage in a large number of genes expressed by the host cell (e.g., calculation service is available from web site of the Kazusa DNA Research Institute, Japan). This analysis is preferably limited to genes that are highly expressed by the host cell.

[0045] At the completion of modification, the coding sequences are verified by sequencing and are then subcloned into an appropriate expression vector for recombinant production of the peptides.

[0046] Following verification of the coding sequence, the peptide can be produced using routine techniques in the field of recombinant genetics.

C. Expression Systems

5 [0047] To obtain high level expression of a nucleic acid encoding a peptide, one typically subclones a polynucleotide encoding the peptide into an expression vector that contains a strong promoter to direct transcription, a transcription/trans-
lation terminator and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in
the art and described, e.g., in Sambrook and Russell, *supra*, and Ausubel *et al.*, *supra*. Bacterial expression systems
for expressing a peptide of this invention are available in, e.g., *E. coli*, *Bacillus sp.*, *Salmonella*, and *Caulobacter*. Kits
for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast,
10 and insect cells are well known in the art and are also commercially available. In one embodiment, the eukaryotic
expression vector is an adenoviral vector, an adeno-associated vector, or a retroviral vector.

[0048] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application.
The promoter is optionally positioned about the same distance from the heterologous transcription start site as it is from
the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be
accommodated without loss of promoter function.

15 [0049] In addition to the promoter, the expression vector typically includes a transcription unit or expression cassette
that contains all the additional elements required for the expression of a peptide in host cells. A typical expression
cassette thus contains a promoter operably linked to the polynucleotide sequence encoding the peptide and signals
required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic
acid sequence encoding the peptide is typically linked to a cleavable signal peptide sequence to promote secretion of
20 the peptide by the transformed cell. Such signal peptides include, among others, the signal peptides from tissue plas-
minogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional
elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene (e.g., encoding the
heterologous polypeptide), introns with functional splice donor and acceptor sites.

[0050] In addition to a promoter sequence, the expression cassette should also contain a transcription termination
25 region downstream of the structural gene to provide for efficient termination. The termination region may be obtained
from the same gene as the promoter sequence or may be obtained from different genes.

[0051] The particular expression vector used to transport the genetic information into the cell is not particularly critical.
Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial
expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems
30 such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of
isolation, e.g., c-myc.

[0052] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic
expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other
exemplary eukaryotic vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other
35 vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein
promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters
shown effective for expression in eukaryotic cells.

[0053] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin
40 B phosphotransferase, and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene
amplification are also suitable, such as a baculovirus vector in insect cells, with a polynucleotide sequence encoding
the peptide under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[0054] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*,
a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique
restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic
45 resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic
sequences are optionally chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if
necessary. Similar to antibiotic resistance selection markers, metabolic selection markers based on known metabolic
pathways may also be used as a means for selecting transformed host cells.

[0055] When periplasmic expression of a recombinant protein (e.g., a peptide for use in the present invention) is
50 desired, the expression vector further comprises a sequence encoding a secretion signal, such as the *E. coli* OppA
(Periplasmic Oligopeptide Binding Protein) secretion signal or a modified version thereof, which is directly connected to
5' of the coding sequence of the protein to be expressed. This signal sequence directs the recombinant protein produced
in cytoplasm through the cell membrane into the periplasmic space. The expression vector may further comprise a
coding sequence for signal peptidase 1, which is capable of enzymatically cleaving the signal sequence when the
55 recombinant protein is entering the periplasmic space. More detailed description for periplasmic production of a recom-
binant protein can be found in, e.g., Gray et al., Gene 39: 247-254 (1985), U.S. Patent Nos. 6,160,089 and 6,436,674.

C. Transfection Methods

[0056] Standard transfection methods are used to produce bacterial, mammalian, yeast, insect, or plant cell lines that express large quantities of a peptide, which are then purified using standard techniques (*see, e.g.,* Colley et al., J. Biol. Chem. 264:17619-17622 (1989); Guide to Protein Purification, in Methods in Enzymology, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.,* Morrison, J. Bact. 132:349-351 (1977); Clark-Curtiss & Curtiss, Methods in Enzymology 101:347-362 (Wu et al., eds, 1983)).

[0057] Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA, or other foreign genetic material into a host cell (*see, e.g.,* Sambrook and Russell, *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the peptide

D. Detection of Recombinant Expression of a Peptide in Host Cells

[0058] After the expression vector is introduced into appropriate host cells, the transfected cells are cultured under conditions favoring expression of the peptide. The cells are then screened for the expression of the recombinant peptide, which is subsequently recovered from the culture using standard techniques (*see, e.g.,* Scopes, Protein Purification: Principles and Practice (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra*; and Sambrook and Russell, *supra*).

[0059] Several general methods for screening gene expression are well known among those skilled in the art. First, gene expression can be detected at the nucleic acid level. A variety of methods of specific DNA and RNA measurement using nucleic acid hybridization techniques are commonly used (*e.g.,* Sambrook and Russell, *supra*). Some methods involve an electrophoretic separation (*e.g.,* Southern blot for detecting DNA and Northern blot for detecting RNA), but detection of DNA or RNA can be carried out without electrophoresis as well (such as by dot blot). The presence of nucleic acid encoding a peptide of this invention in transfected cells can also be detected by PCR or RT-PCR using sequence-specific primers.

[0060] Second, gene expression can be detected at the polypeptide level. Various immunological assays are routinely used by those skilled in the art to measure the level of a gene product, particularly using polyclonal or monoclonal antibodies that react specifically with a peptide, particularly one containing a sufficiently large heterologous polypeptide (*e.g.,* Harlow and Lane, Antibodies, A Laboratory Manual, Chapter 14, Cold Spring Harbor, 1988; Kohler and Milstein, Nature, 256: 495-497 (1975)). Such techniques require antibody preparation by selecting antibodies with high specificity against the peptide or an antigenic portion thereof. The methods of raising polyclonal and monoclonal antibodies are well established and their descriptions can be found in the literature, *see, e.g.,* Harlow and Lane, *supra*; Kohler and Milstein, Eur. J. Immunol., 6: 511-519 (1976).

IV. PURIFICATION OF PEPTIDES

A. Purification of Chemically Synthesized Peptides

[0061] Purification of synthetic peptides is accomplished using various methods of chromatography, such as reverse phase HPLC, gel permeation, ion exchange, size exclusion, affinity, partition, or countercurrent distribution. The choices of appropriate matrices and buffers are well known in the art.

B. Purification of Recombinantly Produced Peptides

1. **Purification of Peptides from Bacterial Inclusion Bodies**

[0062] When a peptide is produced recombinantly by transformed bacteria in large amounts, typically after promoter induction, although expression can be constitutive, the peptides may form insoluble aggregates. There are several protocols that are suitable for purification of protein inclusion bodies. For example, purification of aggregate proteins (hereinafter referred to as inclusion bodies) typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, *e.g.,* by incubation in a buffer of about 100-150 $\mu\text{g/ml}$ lysozyme and 0.1% Nonidet P40, a non-ionic detergent. The cell suspension can be ground using a Polytron grinder (Brinkman Instruments, Westbury, NY). Alternatively, the cells can be sonicated on ice. Alternate methods of lysing bacteria are described in Ausubel *et al.* and Sambrook and Russell, both *supra*, and will be apparent to those of skill in the art.

[0063] The cell suspension is generally centrifuged and the pellet containing the inclusion bodies resuspended in

buffer which does not dissolve but washes the inclusion bodies, *e.g.*, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 150 mM NaCl and 2% Triton-X 100, a non-ionic detergent. It may be necessary to repeat the wash step to remove as much cellular debris as possible. The remaining pellet of inclusion bodies may be resuspended in an appropriate buffer (*e.g.*, 20 mM sodium phosphate, pH 6.8, 150 mM NaCl). Other appropriate buffers will be apparent to those of skill in the art.

5 [0064] Following the washing step, the inclusion bodies are solubilized by the addition of a solvent that is both a strong hydrogen acceptor and a strong hydrogen donor (or a combination of solvents each having one of these properties). The proteins that formed the inclusion bodies may then be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to, urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents that are capable of solubilizing aggregate-forming proteins, such as SDS (sodium dodecyl sulfate) and 70% formic acid, may be inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of the immunologically and/or biologically active protein of interest. After solubilization, the protein can be separated from other bacterial proteins by standard separation techniques. For further description of purifying recombinant polypeptides from bacterial inclusion body, *see, e.g.*, Patra et al., Protein Expression and Purification 18:182-190 (2000).

10 [0065] Alternatively, it is possible to purify recombinant polypeptides, *e.g.*, a peptide for use in this invention, from bacterial periplasm. Where the recombinant polypeptide is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to those of skill in the art (*see e.g.*, Ausubel *et al.*, *supra*). To isolate recombinant peptides from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO₄ and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant peptides present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

2. Standard Protein Separation Techniques for Purification

30 [0066] When a recombinant polypeptide, *e.g.*, a peptide for use in the present invention, is expressed in host cells in a soluble form, its purification can follow the standard protein purification procedure described below. This standard purification procedure is also suitable for purifying peptides obtained from chemical synthesis.

i. Solubility Fractionation

35 [0067] Often as an initial step, and if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest, *e.g.*, a peptide for use in the present invention. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol is to add saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This will precipitate the most hydrophobic proteins. The precipitate is discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, through either dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

ii. Size Differential Filtration

50 [0068] Based on a calculated molecular weight, a protein of greater and lesser size can be isolated using ultrafiltration through membranes of different pore sizes (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of a protein of interest, *e.g.*, a peptide for use in the present invention. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the peptide of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

iii. Column Chromatography

[0069] A protein of interest (such as a peptide for use in the present invention) can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, or affinity for ligands. In addition, antibodies raised against a peptide of this invention can be conjugated to column matrices and the peptide immunopurified. All of these methods are well known in the art.

[0070] It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

C. Confirmation of Peptide Sequence

[0071] The amino acid sequence of a peptide can be confirmed by a number of well established methods. For example, the conventional method of Edman degradation can be used to determine the amino acid sequence of a peptide. Several variations of sequencing methods based on Edman degradation, including microsequencing, and methods based on mass spectrometry are also frequently used for this purpose.

D. Modification of Peptides

[0072] The peptides can be modified to achieve more desirable properties. The design of chemically modified peptides and peptide mimics that are resistant to degradation by proteolytic enzymes or have improved solubility or binding ability is well known.

[0073] Modified amino acids or chemical derivatives of the HER-2/Neu peptides or fusion peptides may contain additional chemical moieties of modified amino acids not normally a part of the HER-2/Neu protein. Covalent modifications of the peptides are within the scope of the present disclosure. Such modifications may be introduced into a peptide by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following examples of chemical derivatives are provided by way of illustration and not by way of limitation.

[0074] The design of peptide mimics which are resistant to degradation by proteolytic enzymes is known to those skilled in the art. See e.g., Sawyer, Structure-Based Drug Design, P. Verapandia, Ed., N.Y. (1997); U.S. Patent Nos. 5,552,534 and 5,550,251. Both peptide backbone and side chain modifications may be used in designing secondary structure mimicry. Possible modifications include substitution of D-amino acids, N^α-Me-amino acids, C_α-Me-amino acids, and dehydroamino acids. To this date, a variety of secondary structure mimetics have been designed and incorporated in peptides or peptidomimetics.

[0075] Other modifications include substitution of a natural amino acid with an unnatural hydroxylated amino acid, substitution of the carboxy groups in acidic amino acids with nitrile derivatives, substitution of the hydroxyl groups in basic amino acids with alkyl groups, or substitution of methionine with methionine sulfoxide. In addition, an amino acid of a HER-2/Neu peptide or a fusion peptide can be replaced by the same amino acid but of the opposite chirality, i.e., a naturally-occurring L-amino acid may be replaced by its D-configuration.

V. FUSING A HER-2/NEU EPITOPE WITH A HETEROLOGOUS POLYPEPTIDE

[0076] In one aspect of this disclosure, a peptide corresponding to a HER-2/Neu promiscuous T cell epitope is attached to a heterologous polypeptide via a covalent bond to form a fusion peptide, such that the ability of the HER-2/Neu epitope to induce a T cell response is enhanced. Frequently, this covalent bond is a peptide bond and the HER-2/Neu epitope and the heterologous polypeptide form a new polypeptide. This peptide bond may be a direct peptide bond between the HER-2/Neu epitope and the heterologous polypeptide, or it may be an indirect peptide bond provided by way of a peptide linker between the HER-2/Neu epitope and the heterologous polypeptide.

[0077] Other covalent bonds are also suitable for the purpose of fusing the HER-2/Neu peptide with the heterologous polypeptide. For instance, a functional group (such as a non-terminal amine group, a non-terminal carboxylic acid group, a hydroxyl group, and a sulfhydryl group) of one peptide may easily react with a functional group of the other peptide and establish a covalent bond, other than a peptide bond, that conjugates the two peptides. A covalent connection between a peptide of a HER-2/Neu epitope and a heterologous polypeptide can also be provided by way of a linker molecule with suitable functional group(s). Such a linker molecule can be a peptide linker or a non-peptide linker. A linker may be derivatized to expose or to attach additional reactive functional groups prior to conjugation. The derivatization may involve attachment of any of a number of molecules such as those available from Pierce Chemical Company, Rockford, Illinois.

VI. FUNCTIONAL ASSAYS

[0078] A HER-2/Neu epitope of this disclosure (or a fusion peptide comprising a HER-2/Neu peptide and a heterologous polypeptide) is useful for its capability to induce a T cell immune response specific to a HER-2/Neu protein, when the epitope is presented by an antigen-presenting cell that may have one of at least 10 different HLA-DR alleles, more preferably at least 15, 20, or 25 different HLA-DR alleles. Various functional assays can be used to confirm the ability of a HER-2/Neu epitope to induce such a HER-2/Neu specific T cell immune response in a promiscuous manner with regard to antigen presenting cells of different HLA-DR alleles, including proliferation assay and flow cytometry assays detecting the binding between a T cell receptor and a peptide epitope or the production of cytokines by T cells.

[0079] The functional assay system used in the Examples of this application is particularly suitable for this purpose. Briefly, a panel of at least 10, preferably at least 15, 20 or 25, antigen presenting cell lines, each homozygous for a different HLA-DR allele, is employed to present HER-2/Neu-derived peptides to a clone of CD4⁺ T cells (e.g., clone HER500.23c21) that is specifically responsive to HER-2/Neu protein (e.g., by production of cytokines such as IFN γ or IL-2). Epitope 270-284 having the amino acid sequence of SEQ ID NO:2 is used as a positive control, whereas an irrelevant HER-2/Neu derived peptide, no peptide, and each antigen presenting cell line alone are used as negative controls for the assays. The assays are set up in multi-welled cell culture plates in appropriate medium with antigen presenting cells and CD4⁺ T cells in each well. Peptides are diluted to a suitable concentration and added to each well. Following incubation of an appropriate time period, supernatants are collected from the wells and analyzed for cytokine production, which can be measured by ELISA based on absorbance at 492 nm. Typically, the effect of a HER-2/Neu class II promiscuous epitope described herein in inducing a HER-2/Neu specific CD4⁺ T cell response is at least 25% of the effect of HER-2/Neu epitope 270-284 (which has the amino acid sequence set forth in SEQ ID NO:2) under the same assay conditions, e.g., at the same molar concentration and presented by antigen-presenting cells of the same, individual HLA-DR allele. More preferably such effect is at least 30%, 40%, 50%, 60%, 70%, 80% or higher of that shown by HER-2/Neu epitope 270-284 under the same conditions.

VII. COMPOSITIONS AND ADMINISTRATION

[0080] The present disclosure also provides compositions comprising an effective amount of (1) a HER-2/Neu peptide; or (2) a fusion peptide comprising a HER-2/Neu peptide and a heterologous polypeptide; or (3) an antigen presenting cell (APC) with the peptide of (1) or (2) forming a complex with an MHC molecule on the cell surface for inducing a T cell immune response specific against a HER-2/Neu protein in both prophylactic and therapeutic applications. Pharmaceutical compositions are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, Science 249: 1527-1533 (1990).

[0081] Antigen presenting cells (APCs) can be generated for peptide-loading by a variety of methods. The starting raw material is peripheral blood or a leukapheresis with or without mobilization. APCs can be isolated by multiple methods, e.g., boyant density centrifugation, elutriation, magnetic beads and plastic adherence used alone or in combination. After isolation, APCs are cultured for 1-14 days with or without the presence of cytokines, growth factors, activation agents, and maturation agents. APCs are loaded with a peptide by addition of peptide to the culture in concentrations from 1 μ g to 1 mg/mL for 6-48 hrs. APCs are harvested, washed, and resuspended in a suitable formulation for infusion. APCs can be delivered fresh or can be kept in frozen storage for delivery at a later time.

[0082] The pharmaceutical compositions can be administered by various routes, e.g., subcutaneous, intradermal, transdermal, intramuscular, intravenous, or intraperitoneal. The preferred routes of administering the pharmaceutical compositions are subcutaneous or intradermal at biweekly doses of about 1 μ g - 10 mg, preferably 50 μ g-1 mg, of a peptide for a 70 kg adult human. The appropriate dose may be administered in weekly, biweekly, or monthly intervals.

[0083] Peptide pulsed APCs can be administered by various routes, e.g., subcutaneous, intradermal, intravenous or intraperitoneal. The peptide pulsed APCs are delivered in weekly, biweekly, or monthly intervals at doses of 1 million to 10 billion cells.

[0084] For preparing pharmaceutical compositions containing a peptide, inert and pharmaceutically acceptable excipients or carriers are used. Liquid pharmaceutical compositions include, for example, solutions, suspensions, and emulsions suitable for intradermal, subcutaneous, parenteral, or intravenous administration. Sterile water solutions of the active component (e.g., a HER-2/Neu peptide or fusion peptide) or sterile solutions of the active component in solvents comprising water, buffered water, saline, PBS, ethanol, or propylene glycol are examples of liquid compositions suitable for parenteral administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents, and the like.

[0085] Sterile solutions can be prepared by dissolving the active component (e.g., a HER-2/Neu peptide or fusion peptide) in the desired solvent system, and then passing the resulting solution through a membrane filter to sterilize it

or, alternatively, by dissolving the sterile compound in a previously sterilized solvent under sterile conditions. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9, and most preferably from 7 to 8.

[0086] The pharmaceutical compositions containing a HER-2/Neu peptide or fusion peptide can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a condition that may be exacerbated by the proliferation of tumor cells overexpression the HER-2/Neu protein in an amount sufficient to prevent, cure, reverse, or at least partially slow or arrest the symptoms of the condition and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease or condition and the weight and general state of the patient, but generally range from about 1 μ g to about 10 mg of the HER-2/Neu peptide or fusion peptide biweekly for a 70 kg patient, with dosages of from about 50 μ g to about 1 mg of the peptide biweekly for a 70 kg patient being more commonly used. The appropriate dose may be administered in weekly, biweekly, or monthly intervals.

[0087] Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of a HER-2/Neu peptide or fusion peptide sufficient to effectively inhibit HER-2/Neu overexpressing tumor cell proliferation in the patient for therapeutic purposes.

VIII. METHOD FOR DETECTING T CELL RESPONSE SPECIFIC TO HER-2/NEU PROTEIN

[0088] The present invention further provides an *in vitro* method for detecting whether a T cell immune response specific to a HER-2/Neu protein is present in a patient as defined in the claims. This method includes the following steps: first, lymphocytes including at least a T cell and an antigen-presenting cell are obtained from a patient. Suitable samples that yield such lymphocytes include blood, tumor infiltrate, and lymph nodes or lymphatic fluids. Second, the T cell and antigen-presenting cells are exposed to a HER-2/Neu peptide (or a fusion peptide comprising the HER-2/Neu peptide and a heterologous peptide) of this invention under conditions that would allow proper presentation of a T cell epitope by the antigen-presenting cell to the T cell. Third, signs of a T cell response is measured *in vitro* by means well known in the art such as ELISPOT, proliferation assay, or flow cytometry. When a T cell response is detected by any of these methods, it can be concluded that there exists a T cell immune response specific to a HER-2/Neu protein in the patient.

EXAMPLES

[0089] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially similar results.

EXAMPLE 1

Materials and Methods

[0090] Recombinant Proteins and Synthetic Peptides. BA7072 is a proprietary recombinant fusion protein manufactured by Dendreon Corporation (Seattle, WA) for use in the investigational vaccine APC8024, for the treatment of HER-2/neu+ cancers. BA7072 contains protein sequences from both the extracellular domain (ECD) and intracellular domain (ICD) of HER-2/neu. HER500 is a recombinant protein also produced by Dendreon Corporation containing sequences from both the ECD and ICD of HER-2/neu and is the HER-2/neu portion of the antigen, BA7072. For defining HER500 specific immune responses *in vitro*, 125 peptides were generated from the HER500 protein sequence. These peptides were 15 amino acids in length, overlapping by 11-mers (Genemed Synthesis, South San Francisco, CA). HER500 peptide #63 (HERp270-284, ALVYNTDTFESMPN) of the 125 HER500 peptides, corresponds to amino acids 270-284 of the natural HER-2/neu sequence. In addition to the HER500 15-mer peptides generated, 9-, 10-, 11-, 12-, 13-, 14-, 16-, and 17-mer peptides were synthesized from residues 268-286 of the HER-2/Neu sequence. These peptides were derived after NH₂- and COOH- terminal truncations or the addition of 1-2 amino acids to HERp270-284 to give the peptides described. All HER500 peptides were sequenced and determined to be at >95% purity by analytical HPLC and mass spectroscopy (Genemed Synthesis).

[0091] Subject and Healthy Donor Sample Collection. All subject and healthy donor specimens were collected according to investigator sponsored protocols approved by the appropriate Investigational Review Board. After receiving informed consent, whole blood samples were collected by venipuncture into heparinized vacutainer tubes or syringes and prepared for transport and/or processing. After receipt of blood samples by our laboratory, peripheral blood mononuclear cells (PBMC) were collected under sterile conditions by density gradient centrifugation and prepared for use in

specified assays.

[0092] In Vitro Generation of HER-2/neu specific T cell clones. PBMC from a subject receiving APC8024, an investigational treatment for HER-2/neu positive cancer, were stimulated in a T-25 tissue culture flask with 10 μ g/mL of BA7072 overnight in RPMI 1640 with 2mM L-glutamine, 50U/mL Penicillin, 50 μ g/mL Streptomycin and 20mM HEPES buffer with 10% Human AB serum (Gemini BioProducts, Calabasas, CA) (cRPMI+10%HS). The following day IFN γ secreting cells were isolated from the PBMC culture using the IFN γ Secretion Assay Cell Enrichment and Detection kit (Miltenyi Biotech, Auburn, CA). The IFN γ enriched population was plated by limiting dilution in 96 well round bottom plates with 10U/mL recombinant human IL-2 (Invitrogen). Non-IFN γ secreting cells were irradiated (3000 rads) and added at 50 μ L per well to give a final volume in all wells of 150 μ L. Plates were incubated for seven days at 37°C with 5% CO $_2$. EBV-transformed lymphoblastoid cells (EBV-LcL) were also generated from this subject using autologous PBMC and supernatant from the B95-8 cell line (ATCC, Manassas, VA) for the expansion and testing of autologous T cell clones. On day 7 of the cloning, IFN γ secreting cells were non-specifically expanded in 96 well plates as previously described (Yee et al., 2002. Proc Natl Acad Sci USA 99:16168-16173). Briefly, to each well, 100 μ L of cRPMI+10%HS media with 25U/mL recombinant human IL-2 and 10ng/mL anti-human CD3 antibody (BD Pharmingen, San Diego, CA) was added with 1x10 4 /well irradiated autologous EBV-LcLs and 1x10 5 /well irradiated allogeneic PBMC. Plates were incubated for 14 days at 37°C and then wells were visually inspected for positive growth. Growth positive clones, clone HER.23c21 and others, were transferred into 24 well plates and expanded using rIL-2, anti-CD3 and accessory cells as above. Final volume in each well was 2.4mL and accessory cells were increased in number to give 2x10 6 /well irradiated allogeneic PBMC and 1x10 5 /well irradiated autologous EBV-LcL per well.

[0093] Identification and Characterization of HER-2/neu specific T cell clones. Clones were screened for antigen specificity using autologous PBMC and antigen or autologous EBV-LcL and Her500 peptides. Stimulations were set up in 96 well round bottom plates in cRPMI+10% FBS media and incubated for 48 hours at 37°C with 5% CO $_2$. Additionally, clones were stained for CD4 and CD8 surface expression by flow cytometry.

[0094] Cytokine Production. To determine cytokine production in the antigen specific stimulation assays, after 48 hours, 200 μ L of supernatant was pulled from cultures and tested for both IL-2 and IFN γ production. IL-2 production was measured using the IL-2 dependent line HT-2 (ATCC). HT-2 cells were grown in IMDM with 10% FBS and 100uM NEAA, 1 mM Sodium Pyruvate, 2mM L-glutamine, 50 mM Penicillin, 50 U/mL Streptomycin, 20 mM HEPES and 20 uM 2-Mercaptoethanol (cIMDM+10%FBS) and fed twice a week with 20ng/mL rIL-2. For the assay, 4 days after HT-2 cells were split, cells were washed with IMDM+10% FBS to remove all rIL-2, and cells were added to 96 well round bottom plates at 1x10 4 cells/well in cIMDM + 10%FBS. Supernatants from antigen specific stimulation were added to wells and plates were incubated 24-30 hours at 37°C. The following day 1 μ Ci of 3 [H] TdR was added for the final 6 hours of the assay and plates were harvested to glass fiber filter mats and DNA incorporation of the radioisotope, or proliferative response, was determined by counts per minute using a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Inc. Boston, MA). IFN γ production was measured using anti-human IFN γ antibody pairs for ELISA (BD Pharmingen, San Diego, CA). Briefly, Immulon 4 plates (Thermo Labsystems/ VWR, Brisbane, CA) were coated overnight with purified anti-human IFN γ antibody (NIB42) at 3 μ g/mL. The next day coating antibody was discarded and 4% Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO) in PBS (Invitrogen) was added to wells and plates were incubated for 2 hours at 37°C. Plates were washed with PBS+ 0.05%Tween 20 and 100 μ L of supernatant samples from the antigen specific stimulation was added to wells and incubated at room temperature for 1.25-2 hours. Plates were washed and biotinylated anti-human IFN γ antibody (4S.B3) was diluted in 1% BSA in PBS (1 μ g/mL) and added to plates for 1 hour at room temperature. After washing plates, Strep-Avidin HRP (BD Pharmingen) was diluted 1:1000 in PBST and added to wells for 30 minutes at room temperature. Finally, plates were washed and Sigma $^{\circledR}$ Fast OPD was added for 15 minutes in the dark. 2M HCl was added to stop the reaction and plates were read for absorbance at 492nm on a spectrophotometer.

[0095] HLA-DR Restriction and Promiscuity. To determine HLA-DR restriction of the T cell epitope HERp270-284, anti-HLA-DR mAb L243, HLA-DQ mAb 1a3 or HLA-DP mAb B7/21 (20-1.25 μ g/mL) were cultured with T cell clone HER.23c21, peptide HERp270-284 and autologous EBV-LcL in cRPMI+10%FBS media. Supernatants were harvested after 48 hours and tested for IL-2 and IFN γ production. HERp270-284 was also tested for HLA-DR promiscuity using EBV-LcL lines purchased from the European Collection of Cell Cultures originating from the 12 $^{\text{th}}$ International Histocompatibility Workshop (IHW) held in Strasbourg, France. The IHW Lines listed in Table I are homozygous for various HLA-DR β 1 alleles and were propagated in RPMI 1640 with 10% Fetal Bovine Serum, 20mM HEPES, 2mM L-glutamine, 50mM Penicillin and 50U/mL Streptomycin (cRPMI+10%FBS) (Invitrogen, Carlsbad, CA). To test for MHC class II promiscuity, HERp270-284 was added at 1 μ g/mL with each separate EBV-LcL line (2x10 5 cells/well) and the T cell clone HER.23c21 (1x10 5 cells/well) in a 96 well round bottom plate in cRPMI+10%FBS media at 37°C. Supernatants were harvested after 48 hours and tested for IFN γ production.

Results

[0096] APC8024 is an investigational autologous cell immunotherapy for Her2/neu-expressing breast cancer. To characterize the immune response induced by this immunotherapy, we isolated T cell clones from a clinical trial subject treated with APC8024. One of the CD4+ T cell clones generated, HER500.23c21, showed a specific response to exogenous HER-2/Neu protein sequences presented by autologous PBMCs (Figure 1). In this experiment, HER500.23c21 was stimulated with increasing doses of HER500, a recombinant protein containing the intracellular and extracellular domains of HER-2/Neu, and BA7072, which consists of HER500 expressed as a fusion protein with hGM-CSF. HER500.23c21 specifically produced IFN γ and IL-2 in response to both antigens but no cytokine production was observed in the absence of antigen. HER500.23c21 responded to each of these proteins in a dose dependent manner, indicating that HER500.23c21 recognizes a HER-2/Neu epitope that is naturally processed and presented from exogenous protein antigen.

[0097] Because of the clear response of HER500.23c21 to HER-2/Neu protein sequences, the specific epitope recognized by HER500.23c21 was mapped using peptides. HER500.23c21 was tested against a panel of 125 individual overlapping 15-mer peptides covering the HER-2/Neu protein sequences within HER500. Each peptide was used at 1ug/ml with autologous EBV LCL cells as antigen presenting cells. In these experiments, HER500.23c21 responded strongly to the pool of all 125 individual peptides, as measured by either IFN γ or IL2 production (Figure 2 A and B, top bar). In addition, Her500.23c21 responded to only one individual peptide, peptide 63, indicating that this peptide contains the epitope recognized by HER500.23c21 (Figure 2). Peptide 63 corresponds to amino acids 270-284 of the HER-2/Neu protein sequence and overlaps the adjacent peptides by 11 amino acids. Thus, the lack of response to peptide 62 or 64 suggests that the HER500.23c21 T cell epitope is contained entirely in the HERp270-284 sequence and the amino acids common to the flanking peptides do not contain the complete epitope for this T cell clone. To define the minimal epitope recognized by HER500.23c21, we designed 9-mer, 10-mer or 11-mer peptides with NH₂- and COOH- terminal truncations of HERp270-284. Stimulation of HER500.23c21 with these peptides was successful with only 11-mer sequences VTYN-TDTFESM and TYNTDTFESMP when presented by EBV LCL lines representing HLA-DRB1 alleles *0301, *0302, *1301 and *1402.

[0098] In order to determine which HLA molecules were responsible for presenting HERp270-284 to HER500.23c21, HLA-blocking antibodies were used to inhibit presentation and T cell activation. HER500.23c21 was stimulated with 1ug/ml HERp270-284 and autologous EBV-LcL in the absence or presence of increasing concentrations of antibodies specific for HLA-DR or HLA-DP/-DQ. T cell stimulation was determined by IFN γ production. The antibody against HLA-DR inhibited the stimulation of HER500.23c21 by HERp270-284 in a dose-dependent manner whereas the HLA-DP/-DQ blocking antibody had no effect on T cell activation, even at high concentrations of 20ug/ml (Figure 3). These results demonstrate that HER500.23c21 recognition of HERp270-284 is HLA-DR restricted.

[0099] To further define the HLA restriction, we tested the ability of HERp270-284 to activate HER500.23c21 using a panel of 25 EBV LcL lines homozygous for different HLA-DRB1* alleles, representing 13 DR serological families (Table 1). Every EBV-LcL line tested was able to efficiently stimulate HER500.23c21 in an antigen-specific manner, indicating that the HERp270-284 epitope is promiscuous for at least 25 HLA-DRB1* alleles (Figure 4A). In these experiments, 1ug/ml of HERp270-284 peptide induced a maximal T cell response, regardless of the EBV-LCL line used. However, differences in the ability of the various alleles to stimulate Her500.23c21 were evident at lower concentrations of peptide (Figure 4B), with some alleles, such as the DRB1*0401, able to stimulate as low as 10ng/ml, and other alleles were unable to stimulate below 250ng/ml (DRB1*1103). These differences likely reflect a range of binding affinities of HERp270-284 for different DRB1* alleles. However, the high degree of promiscuity observed in these experiments suggests that HERp270-284 may contain a universal helper T cell epitope for HER-2/Neu.

Discussion

[0100] The study of anti-tumor immune responses is often restricted to a small number of antigens presented by specific HLA types due to reagent limitations. The identification of promiscuous T cell epitopes can help alleviate those limitations by permitting the analysis of anti-tumor immune responses in individuals of diverse HLA types. Because of their value, both as a research tool and potential therapeutic, much effort has been focused on the identification and characterization of promiscuous CD4 and CD8 T cell epitopes. Peptide epitopes with varying degrees of promiscuity in their HLA-binding have been identified in infectious disease antigens- HIV(van der Burg et al., 1999. J Immunol 162:152-160), mycobacteria (Valle et al., 2001. Clin Exp Immunol 123:226-232), and *p.falciparum* (Contreras et al., 1998. Infect Immun 66:3579-3590), as well as tumor antigens such as NY-ESO (Zarour et al., 2002. Cancer Res 62:213-218), MAGE (Consogno et al., 2003. Blood 101:1038-1044), Tert (Schroers et al., 2003. Clin Cancer Res 9:4743-4755), and Her2/neu (Kobayashi et al., 2000. Cancer Res 60:5228-5236). Computer programs such as TEPITOPE (Bian and Hammer. 2004. Methods 34:468-475) use known common binding motifs to predict promiscuous epitopes based on protein sequence and have identified many potential new T cell epitopes from a variety of sources.

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The biological relevance of the epitopes identified *in silico* is being addressed in a number of systems (Ruiz et al., 2004. Clin Cancer Res 10:2860-2867; and Al-Attayah and Mustafa. 2004. Scand J Immunol 59:16-24).

[0101] In this study, a novel promiscuous T cell epitope was discovered from the tumor associated antigen, HER-2/Neu. This T cell epitope was identified with a CD4+ T cell clone isolated from a patient treated with an autologous cell immunotherapy for HER-2/Neu+ cancer. The epitope is contained within amino acids 270-284 of the HER-2/Neu sequence and is naturally processed and presented from exogenous protein antigen. The fact that the T cell clone specific for this epitope was isolated from an individual treated with a HER-2/Neu-specific immunotherapy suggests that this epitope may play a role *in vivo* as part of a clinically relevant anti-tumor immune response. In addition to being identified in a biologically relevant context, this peptide epitope is interesting because it has a very broad HLA-DR promiscuity and it can be presented to the T cell clone HER500.23c21 by at least 25 different HLA-DRB1* alleles representing 13 serological DR families. Most other peptides identified as promiscuous T cell epitopes are presented by just a few different HLA alleles but we have yet to identify an HLA-DRB1* allele incapable of presenting HERp270-284 to T cell clone HER500.23c21. The relative lack of MHC restriction for presentation of this epitope makes it an ideal candidate for a universal HER-2/Neu CD4 T cell epitope.

[0102] A major goal of tumor immunology is to develop effective cancer immunotherapies and vaccines against tumor-associated antigens. Such treatments are designed to stimulate an anti-tumor immune response to eradicate the tumor. Focusing on specific tumor associated antigens has been successful in generating anti-tumor immune responses and led to the identification of specific T cell epitopes within some tumor antigens. While most early work focused solely on generating tumor-specific CD8 T cell responses, there has been a growing appreciation of the importance of CD4 T cells in generating an effective anti-tumor immunity. Because of this, the study of anti-tumor CD4 T cell responses and identification of class II-restricted T cell epitopes from tumor associated antigens has expanded. The utility of such epitopes is greatly increased if such epitopes can be presented by more than one HLA type. Peptide-based cancer vaccine strategies are hampered by the HLA restriction of the peptide epitope within the vaccine. The inclusion of promiscuous T cell epitopes such as the one describe herein broadens the usefulness of such vaccines within the general population. Inducing an immune response against a tumor antigen by a promiscuous epitope is an efficient way to reach a larger percentage of the general population. Such a promiscuous epitope also provides useful means to analyze the response.

Table I. HLA-defined EBV-LCL cell lines

HLA-DRB1* allele	DR serological family	Cell line name
0101	DR1	KAS116
0102	DR1	PMG075
0103	DR103	TER-ND
1503	DR15	AMAI
160201	DR16	RML
0301	DR17	VAVY
0302	DR18	RSH
0401	DR4	BM14
0402	DR4	YAR
040301	DR4	SSTO
040501	DR4	LKT3
1101	DR11	BM21
1102	DR11	BM15
1103	DR11	TISI
1104	DR11	BOB
110401	DR11	FPAF
1201	DR12	BM16
1301	DR13	OMW
1302	DR13	EMJ

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(continued)

HLA-DRB1* allele	DR serological family	Cell line name
1401	DR14	EK
1402	DR14	AMALA
0701	DR7	BER
080101	DR8	BM9
080201	DR8	SPL
0901	DR9	T7526

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[0103] Cell lines were obtained from the ECACC European Collection of Cell Cultures and are listed in the IMGT/HLA cell directory (website ebi.ac.uk/imgt/hla/cell_query.html).

EXAMPLE 2

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[0104] In this series of experiments, additional peptides derived from the HER-2/Neu protein were tested for their potential as promiscuous MHC Class II HER-2/neu epitopes. Epitope HERp270-284 (SEQ ID NO:2) was used as a positive control in the experiments. A panel of EBV-LcL lines, homozygous for various HLA-DR alleles, was used to present HERp270-284, as well as various lengths of peptides within this 15-amino acid sequence or longer length peptides within the 19-amino acid sequence of HER-2/Neu 268-286 (SEQ ID NO:1), to clone HER500.23c21. Negative controls for the assays included an irrelevant HER500 peptide, no peptide, and each EBV-LcL line alone. Autologous EBV-LcLs were also run with each condition as a control for clone HER500.23c21 specificity. The assays were set up in 96 well round bottom plates in cRPMI+10% FBS with 2×10^5 EBV-LcL/well and 1×10^5 HER500.23c21 cells/well. Peptides were diluted at a final concentration of $1 \mu\text{g/mL}$ added at $100 \mu\text{L/well}$. The assay was incubated for 48 hours at 37°C with 5% CO_2 . Supernatants were harvested and analyzed for $\text{IFN}\gamma$ production using ELISA. Results are shown for one representative experiment for each peptide series (Figures 5-11). Results of the $\text{IFN}\gamma$ produced by clone HER500.23c21 in response to peptide and each HLA-DR allele are reported in pg/mL of $\text{IFN}\gamma$ or as absorbance at 492nm.

SEQUENCE LISTING

35

[0105]

SEQ ID NO:1 (268-286 of HER-2/Neu protein)
CPALVTYNTDTFESMPNPE

40

SEQ ID NO:2 (270-284 of HER-2/Neu protein, or residues 3-17 of SEQ ID NO:1)
ALVTYNTDTFESMPN

SEQUENCE LISTING

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[0106]

<110> Dendreon Corporation

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<130> AHB/FP6830145

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EP 2 570 422 B1

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EP 2 570 422 B1

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 1 5 10 15

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4. The composition for use according to claim 2 wherein the peptide consists of the amino acid sequence LVITYNTDTFESMP or VTYNTDTFESMPN.
5. The composition for use according to claim 2 wherein the peptide consists of the amino acid sequence ALVITYNTDTFESMP, LVITYNTDTFESMPN, PALVITYNTDTFESMPN, or CPALVITYNTDTFESMPN.
6. The composition for use according to claim 1 or 2 wherein said peptide is fused to a heterologous polypeptide.
7. The composition for use according to claim 6, wherein the peptide is fused to a heterologous polypeptide via a peptide bond.
8. The composition for use according to claim 6, wherein the heterologous polypeptide is a granulocyte-macrophage colony-stimulating factor (GM-CSF).
9. The composition for use according to any of the preceding claims, wherein the composition further comprises an antigen presenting cell, the peptide forming a complex with a major histocompatibility complex (MHC) molecule on the surface of the cell.
10. The composition for use according to claim 9 wherein said MHC molecule is an HLA-DR allele that is any one of the alleles 0101, 0102, 0103, 1503,160201, 0301, 0302, 0401, 0402, 040301, 040501, 1101, 1102, 1103,1104,110401,1201,1301,1302, 1401, 1402, 0701, 080101, 080201, and 0901.
11. The composition for use according to any of claims 1-10, wherein the route of administration is subcutaneous or intradermal at biweekly doses of about 1 µg-10 mg for a 70 kg adult human.
12. An *in vitro* method for detecting a T cell immune response specific to a HER-2/Neu protein, the method comprising the steps of (a) contacting an antigen-presenting cell and a T cell, in a sample obtained from a patient, with a peptide consisting of the sequence:
- TYNTDTFESMPN; and
- (b) detecting a T cell response, wherein the detection of a T cell response indicates the presence of a T cell immune response specific to a HER-2/Neu protein in the patient.
13. An *in vitro* method for detecting a T cell immune response specific to a HER-2/Neu protein, the method comprising the steps of (a) contacting an antigen-presenting cell and a T cell, in a sample comprising blood, tumor infiltrate, lymph nodes or lymphatic fluids obtained from a patient, with a peptide consisting of the sequence:
- ALVITYNTDTFESMPN, VTYNTDTFESMP, TYNTDTFESMPN, LVITYNTDTFESMP, VTYNTDTFESMPN, ALVITYNTDTFESMP, LVITYNTDTFESMPN, PALVITYNTDTFESMPN, or CPALVITYNTDTFESMPN; and
- (b) detecting a T cell response, wherein the detection of a T cell response indicates the presence of a T cell immune response specific to a HER-2/Neu protein in the patient.
14. The method of claim 12 or 13, wherein step (b) is performed by ELISPOT, proliferation assay, or flow cytometry.
15. The method of claim 12 or 13, wherein the peptide is fused to a heterologous polypeptide via a peptide bond.
16. The method of claim 15, wherein the heterologous polypeptide is a granulocyte-macrophage colony-stimulating factor (GM-CSF).

Patentansprüche

1. Zusammensetzung zur Verwendung in einem Verfahren zur Auslösung einer für ein HER-2/Neu-Protein spezifischen T-Zell-Immunantwort bei einem Patienten, worin die Zusammensetzung ein Peptid, das aus der Sequenz TYNTDTFESMPN besteht, und einen physiologisch annehmbaren Exzipienten umfasst.

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2. Zusammensetzung zur Verwendung in einem Verfahren zur Auslösung einer für ein HER-2/Neu-Protein spezifischen T-Zell-Immunantwort bei einem Patienten, worin die Zusammensetzung ein Peptid, das aus der Sequenz ALV-TYNTDTFESMPN, VTYNTDTFESMP, TYNTDTFESMPN, LVTYNTDTFESMP, VTYNTDTFESMPN, ALVTYNTDT-FESMP, LVTYNTDTFESMPN, PALVTYNTDTFESMPN oder CPALVTYNTDTFESMPN besteht; und einen physiologisch annehmbaren Exzipienten umfasst; worin der Verabreichungsweg der Zusammensetzung subkutan, intradermal, transdermal, intramuskulär oder intraperitoneal ist.
3. Zusammensetzung zur Verwendung nach Anspruch 2, worin das Peptid aus der Aminosäuresequenz VTYNTDT-FESMP besteht.
4. Zusammensetzung zur Verwendung nach Anspruch 2, worin das Peptid aus der Aminosäuresequenz LVTYNTDT-FESMP oder VTYNTDTFESMPN besteht.
5. Zusammensetzung zur Verwendung nach Anspruch 2, worin das Peptid aus der Aminosäuresequenz ALVTYNTDT-FESMP, LVTYNTDTFESMPN, PALVTYNTDTFESMPN oder CPALVTYNTDTFESMPN besteht.
6. Zusammensetzung zur Verwendung nach Anspruch 1 oder 2, worin das Peptid an ein heterologes Polypeptid fusioniert ist.
7. Zusammensetzung zur Verwendung nach Anspruch 6, worin das Peptid über eine Peptidbindung an ein heterologes Polypeptid fusioniert ist.
8. Zusammensetzung zur Verwendung nach Anspruch 6, worin das heterologe Polypeptid ein Granulozyten-Makrophagen-Kolonie-stimulierender Faktor (GM-CSF) ist.
9. Zusammensetzung zur Verwendung nach einem der vorangegangenen Ansprüche, worin die Zusammensetzung des Weiteren eine Antigen präsentierende Zelle umfasst, wobei das Peptid einen Komplex mit einem Haupthistokompatibilitätskomplex- (MHC-) Molekül an der Oberfläche der Zelle bildet.
10. Zusammensetzung zur Verwendung nach Anspruch 9, worin das MHC-Molekül ein HLA-DR-Allel ist, das eines der Allele 0101, 0102, 0103, 1503, 160201, 0301, 0302, 0401, 0402, 040301, 040501, 1101, 1102, 1103, 1104, 110401, 1201, 1301, 1302, 1401, 1402, 0701, 080101, 080201 und 0901 ist.
11. Zusammensetzung zur Verwendung nach einem der Ansprüche 1 bis 10, worin der Verabreichungsweg für einen 70 kg schweren erwachsenen Menschen subkutan oder intradermal in Dosen von etwa 1 µg bis 10 mg alle zwei Wochen ist.
12. *In-vitro*-Verfahren zur Detektion einer für ein HER-2/Neu-Protein spezifischen T-Zell-Immunantwort, wobei das Verfahren die folgenden Schritte umfasst: (a) das Kontaktieren einer Antigen präsentierenden Zellen und einer T-Zelle in einer dem Patienten entnommenen Probe mit einem Peptid, das aus der folgenden Sequenz besteht:
- TYNTDTFESMPN; und
- (b) das Detektieren einer T-Zell-Antwort, worin die Detektion einer T-Zell-Antwort ein Indikator für die Gegenwart einer für ein HER-2/Neu-Protein spezifischen T-Zell-Immunantwort bei dem Patienten ist.
13. *In-vitro*-Verfahren zur Detektion einer für ein HER-2/Neu-Protein spezifischen T-Zell-Immunantwort, wobei das Verfahren die folgenden Schritte umfasst: (a) das Kontaktieren einer Antigen präsentierenden Zellen und einer T-Zelle in einer dem Patienten entnommenen Probe, die Blut, Tumoringfiltrat, Lymphknoten- oder lymphatische Flüssigkeiten umfasst, mit einem Peptid, das aus der folgenden Sequenz besteht:
- ALVTYNTDTFESMPN, VTYNTDTFESMP, TYNTDTFESMPN, LVTYNTDTFESMP, VTYNTDTFESMPN, ALV-TYNTDTFESMP, LVTYNTDTFESMPN, PALVTYNTDTFESMPN oder CPALVTYNTDTFESMPN; und
- (b) das Detektieren einer T-Zell-Antwort, worin die Detektion einer T-Zell-Antwort ein Indikator für die Gegenwart einer für ein HER-2/Neu-Protein spezifischen T-Zell-Immunantwort bei dem Patienten ist.

14. Verfahren nach Anspruch 12 oder 13, worin Schritt (b) mittels ELISPOT, Proliferationstest oder Durchflusszytometrie durchgeführt wird.

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15. Verfahren nach Anspruch 12 oder 13, worin das Peptid über eine Peptidbindung an ein heterologes Polypeptid fusioniert ist.

16. Verfahren nach Anspruch 15, worin das heterologe Polypeptid ein Granulozyten-Makrophagen-Kolonie-stimulierender Faktor (GM-CSF) ist.

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Revendications

1. Composition destinée à être utilisée dans un procédé pour induire chez un patient une réponse immunitaire de cellules T spécifique à une protéine HER-2/Neu, où ladite composition comprend un peptide consistant en la séquence TYNTDTFESMPN, et un excipient physiologiquement acceptable.

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2. Composition destinée à être utilisée dans un procédé pour induire chez un patient une réponse immunitaire de cellules T spécifique à une protéine HER-2/Neu, où ladite composition comprend un peptide consistant en la séquence ALVTYNTDTFESMPN, VTYNTDTFESMP, TYNTDTFESMPN, LVTYNTDTFESMP, VTYNTDTFESMPN, ALVTYNTDTFESMP, LVTYNTDTFESMPN, PALVTYNTDTFESMPN, ou CPALVTYNTDTFESMPN ; et un excipient physiologiquement acceptable ; où la voie d'administration de la composition est la voie sous-cutanée, intradermique, transdermique, intramusculaire, intraveineuse, ou intrapéritonéale.

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3. Composition destinée à être utilisée selon la revendication 2, dans laquelle le peptide consiste en la séquence d'acides aminés VTYNTDTFESMP.

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4. Composition destinée à être utilisée selon la revendication 2, dans laquelle le peptide consiste en la séquence d'acides aminés LVTYNTDTFESMP ou VTYNTDTFESMPN.

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5. Composition destinée à être utilisée selon la revendication 2, dans laquelle le peptide consiste en la séquence d'acides aminés ALVTYNTDTFESMP, LVTYNTDTFESMPN, PALVTYNTDTFESMPN, ou CPALVTYNTDTFESMPN.

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6. Composition destinée à être utilisée selon la revendication 1 ou 2, dans laquelle ledit peptide est fusionné à un polypeptide hétérologue.

7. Composition destinée à être utilisée selon la revendication 6, dans laquelle le peptide est fusionné à un polypeptide hétérologue via une liaison peptidique.

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8. Composition destinée à être utilisée selon la revendication 6, dans laquelle le polypeptide hétérologue est un facteur de stimulation des colonies de granulocytes-macrophages (GM-CSF).

9. Composition destinée à être utilisée selon l'une quelconque des revendications précédentes, où la composition comprend en outre une cellule présentatrice d'antigène, le peptide formant un complexe avec une molécule du complexe majeur d'histocompatibilité (CMH) sur la surface de la cellule.

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10. Composition destinée à être utilisée selon la revendication 9, où ladite molécule du CMH est un allèle HLA-DR qui est l'un quelconque des allèles 0101, 0102, 0103, 1503, 160201, 0301, 0302, 0401, 0402, 040301, 040501, 1101, 1102, 1103, 1104, 110401, 1201, 1301, 1302, 1401, 1402, 0701, 080101, 080201, et 0901.

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11. Composition destinée à être utilisée selon l'une quelconque des revendications 1 à 10, où la voie d'administration est la voie sous-cutanée ou intradermique à des doses bihebdomadaires d'environ 1 µg-10 mg pour un humain adulte de 70 kg.

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12. Procédé *in vitro* pour détecter une réponse immunitaire de cellules T spécifique à une protéine HER-2/Neu, le procédé comprenant les étapes consistant à (a) mettre en contact une cellule présentatrice d'antigène et une cellule T, dans un échantillon obtenu chez un patient, avec un peptide consistant en la séquence :

TYNTDTFESMPN ; et

(b) détecter une réponse de cellules T, où la détection d'une réponse de cellules T indique la présence d'une réponse immunitaire de cellules T spécifique à une protéine HER-2/Neu chez le patient.

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13. Procédé *in vitro* pour détecter une réponse immunitaire de cellules T spécifique à une protéine HER-2/Neu, le procédé comprenant les étapes consistant à (a) mettre en contact une cellule présentatrice d'antigène et une cellule T, dans un échantillon comprenant du sang, un infiltrat tumoral, des ganglions lymphatiques ou des liquides lymphatiques obtenus chez un patient, avec un peptide consistant en la séquence :

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ALVTYNTDTFESMPN, VTYNTDTFESMP, TYNTDTFESMPN, LVTYNTDTFESMP, VTYNTDTFESMPN, ALVTYNTDTFESMP, LVTYNTDTFESMPN, PALVTYNTDTFESMPN, ou CPALVTYNTDTFESMPN ; et

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(b) détecter une réponse de cellules T, où la détection d'une réponse de cellules T indique la présence d'une réponse immunitaire de cellules T spécifique à une protéine HER-2/Neu chez le patient.

14. Procédé selon la revendication 12 ou 13, dans lequel l'étape (b) est réalisée par un ELISPOT, un essai de prolifération, ou par cytométrie en flux.

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15. Procédé selon la revendication 12 ou 13, dans lequel le peptide est fusionné à un polypeptide hétérologue via une liaison peptidique.

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16. Procédé selon la revendication 15, dans lequel le polypeptide hétérologue est un facteur de stimulation des colonies de granulocytes-macrophages (GM-CSF).

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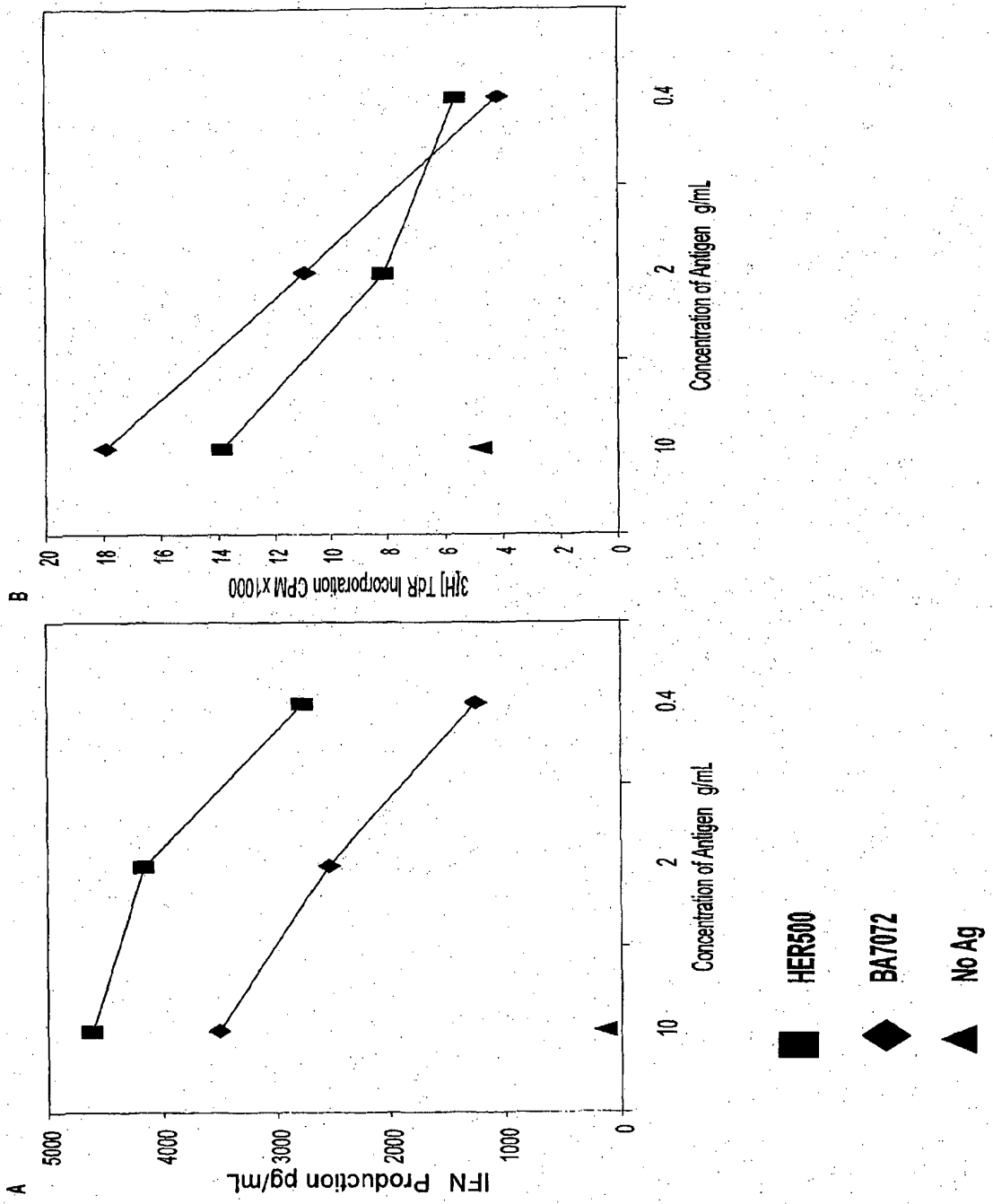


Figure 1

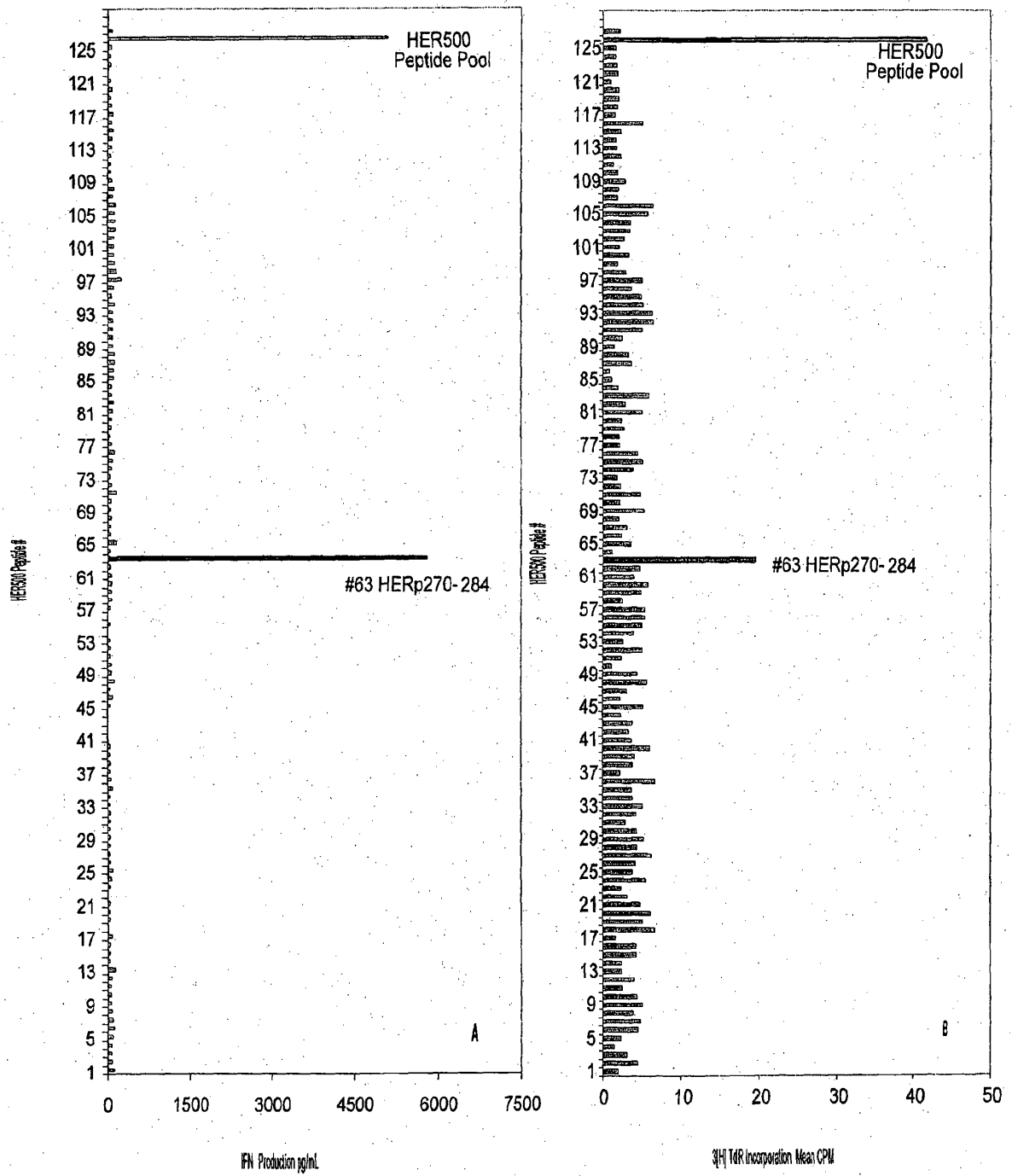


Figure 2

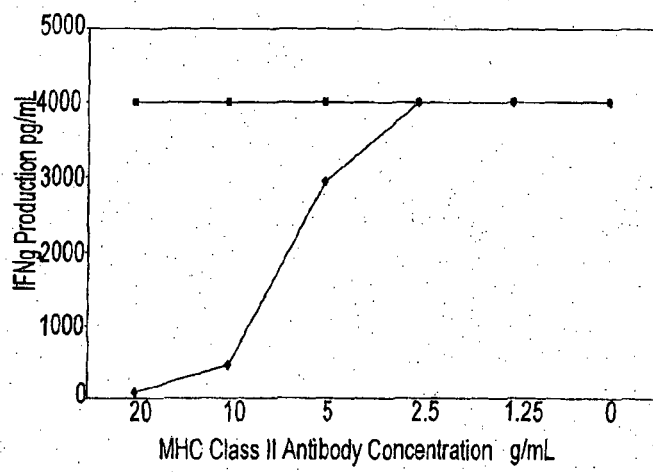


Figure 3

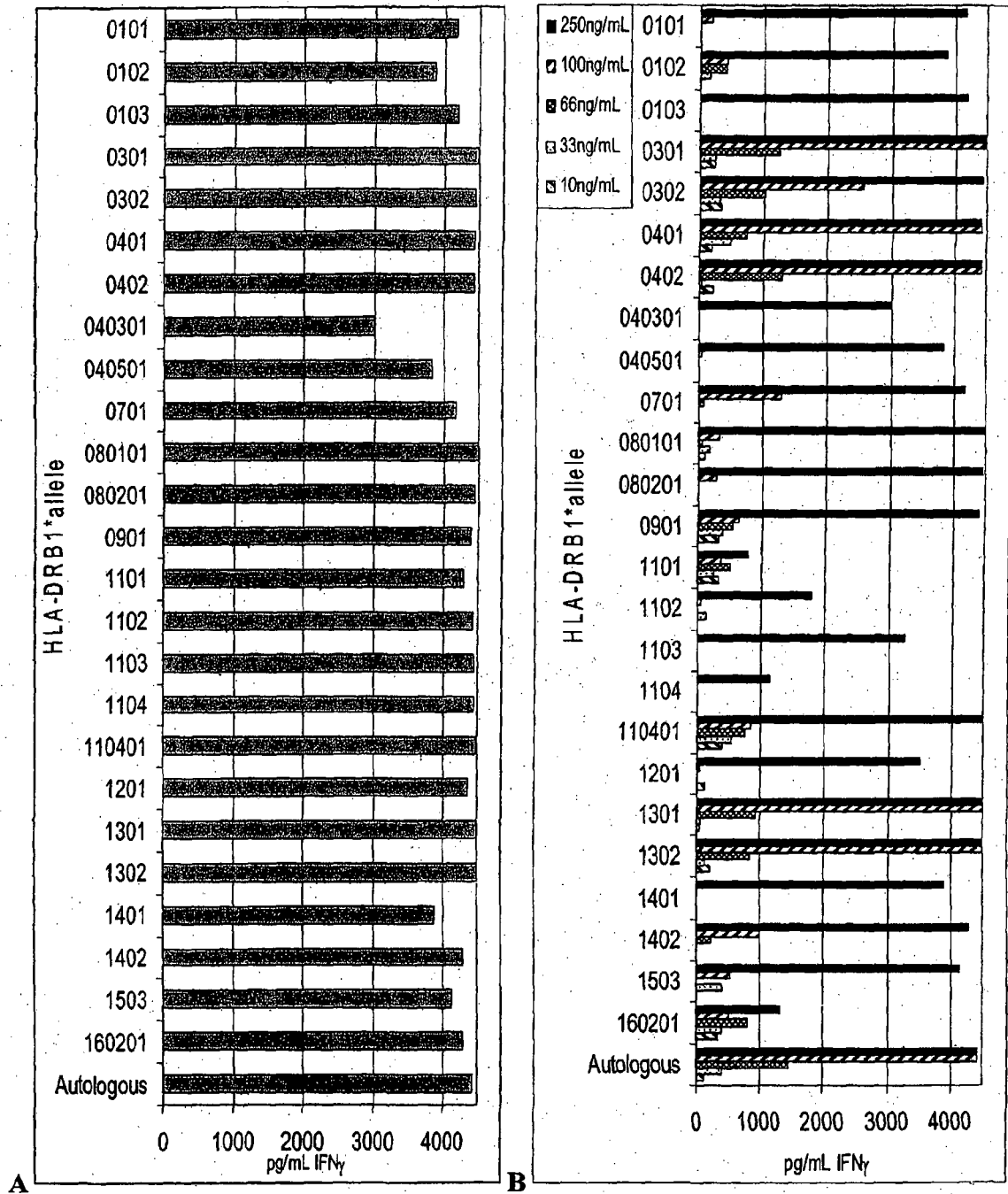


Figure 4

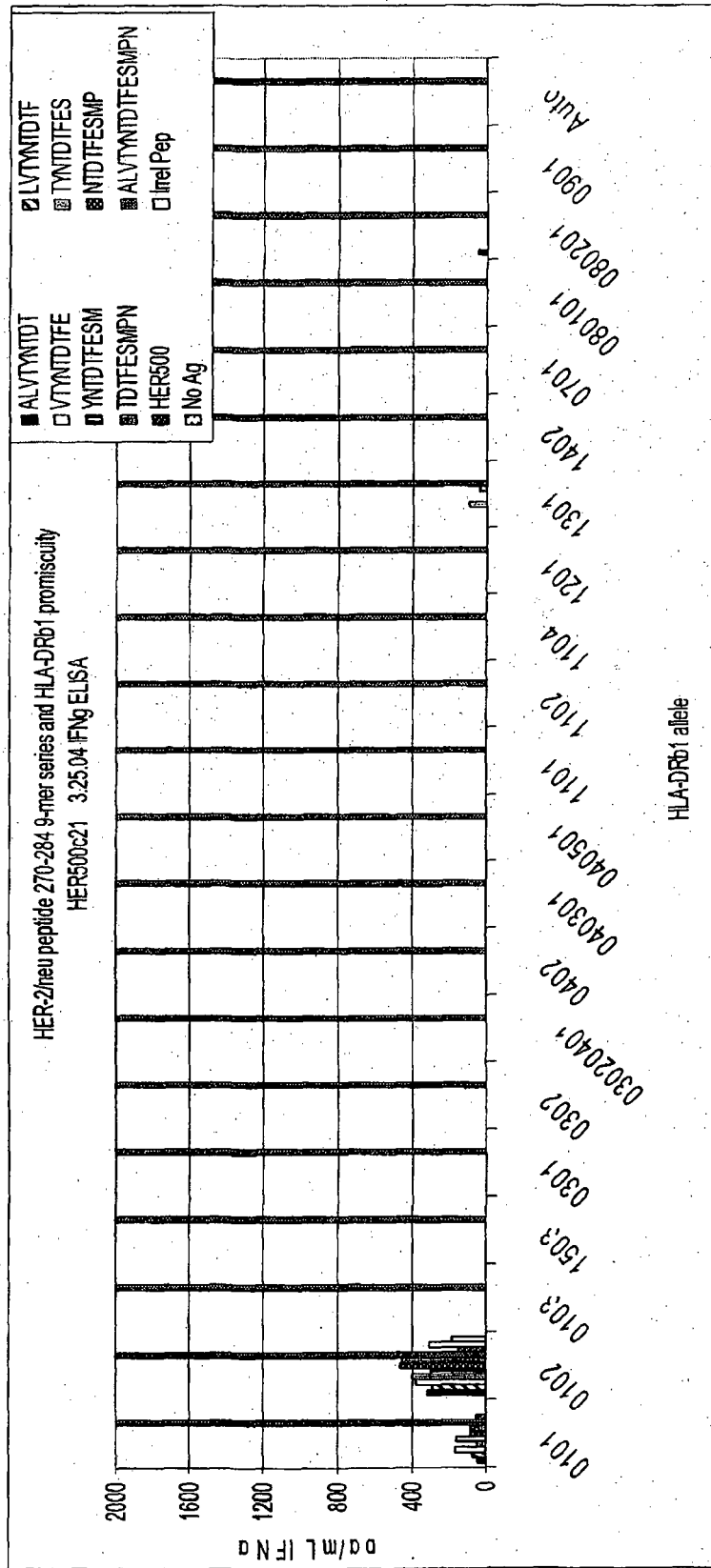


Figure 5

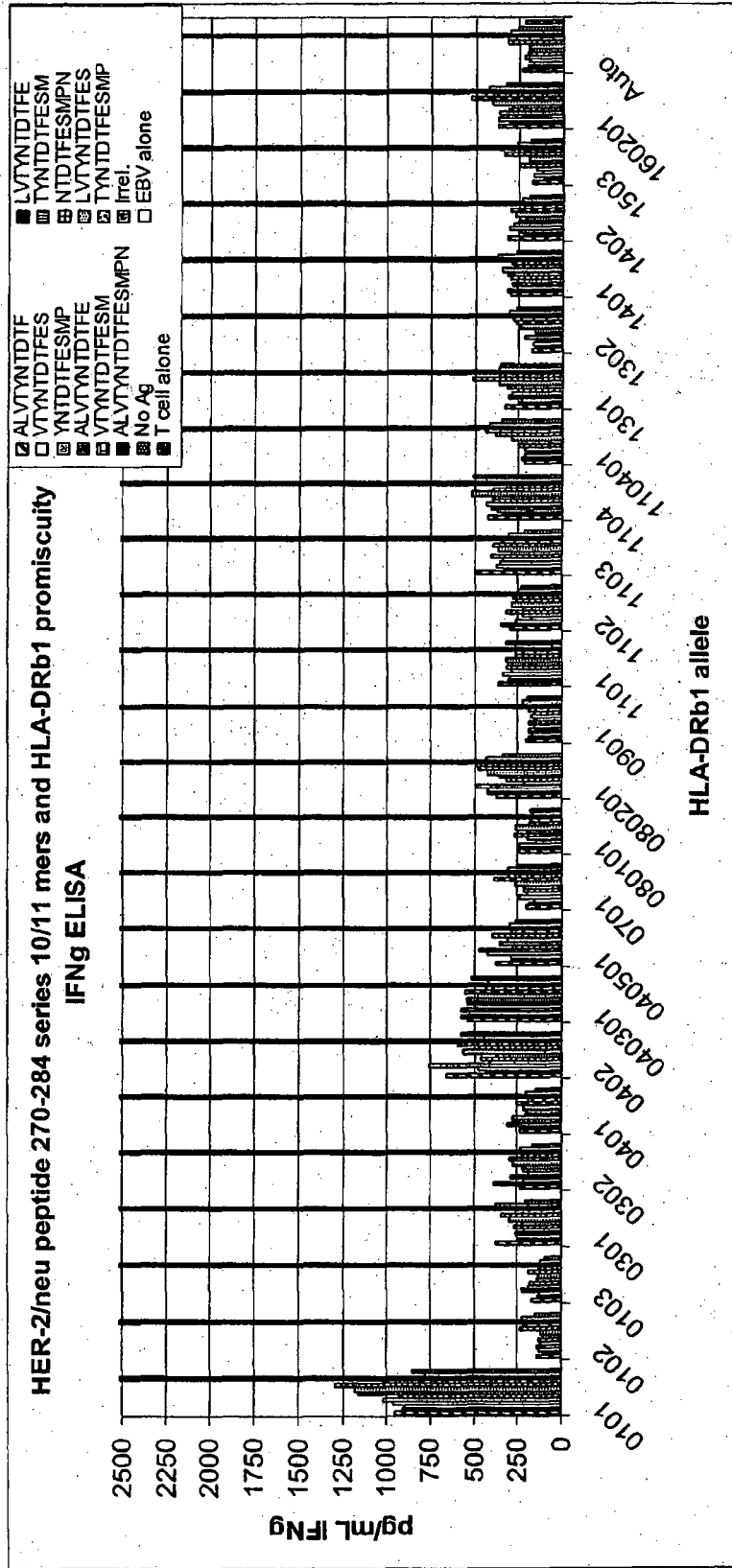


Figure 6

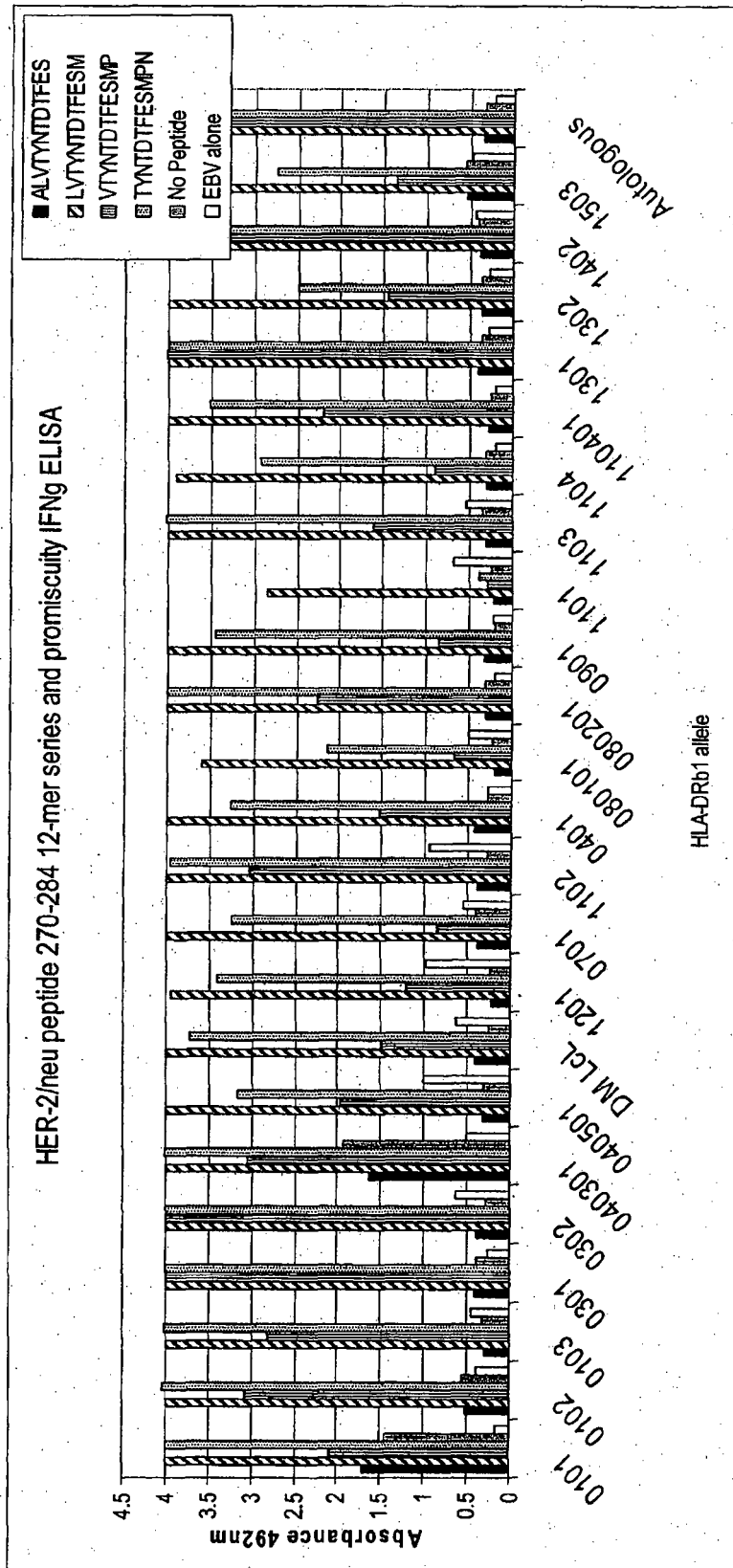


Figure 7

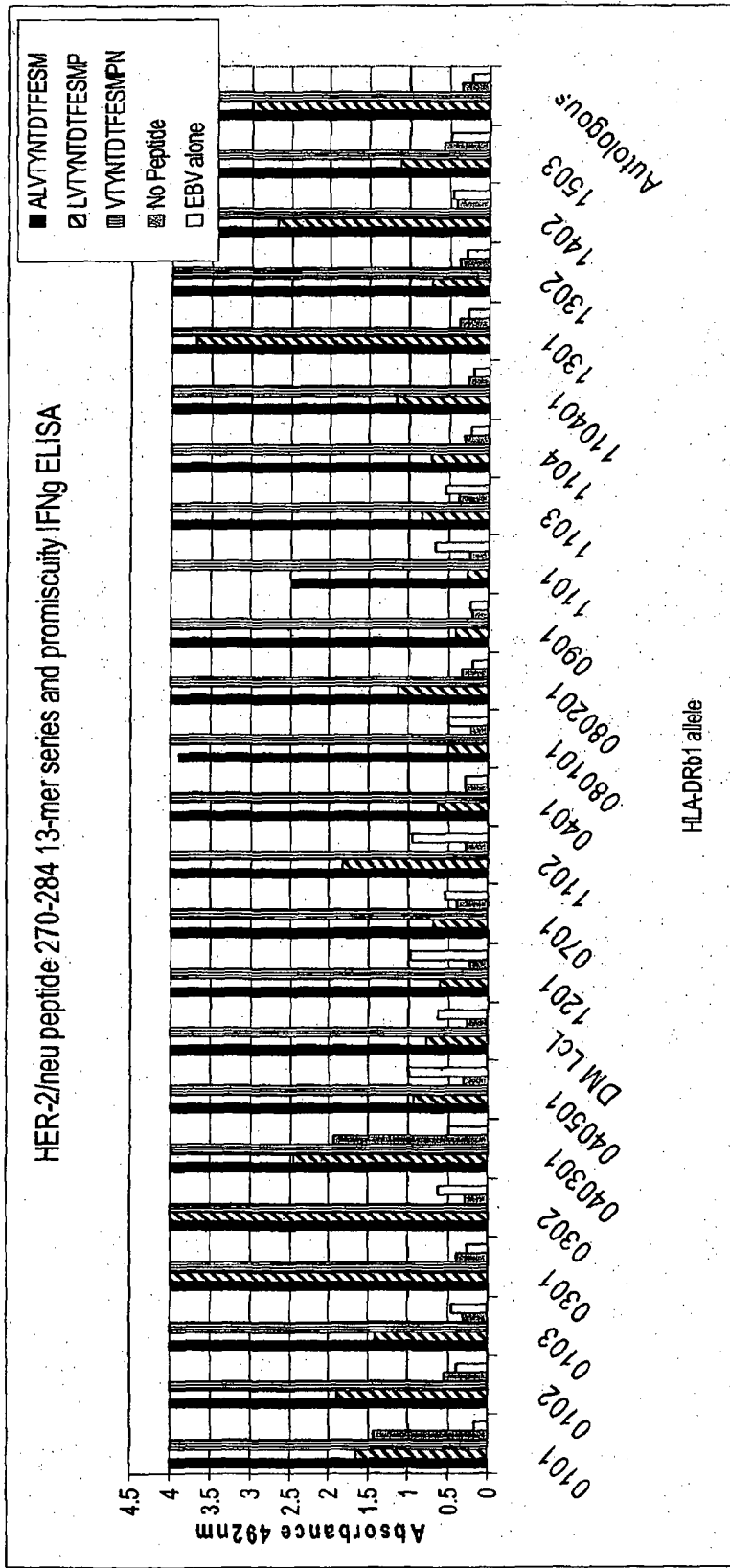


Figure 8

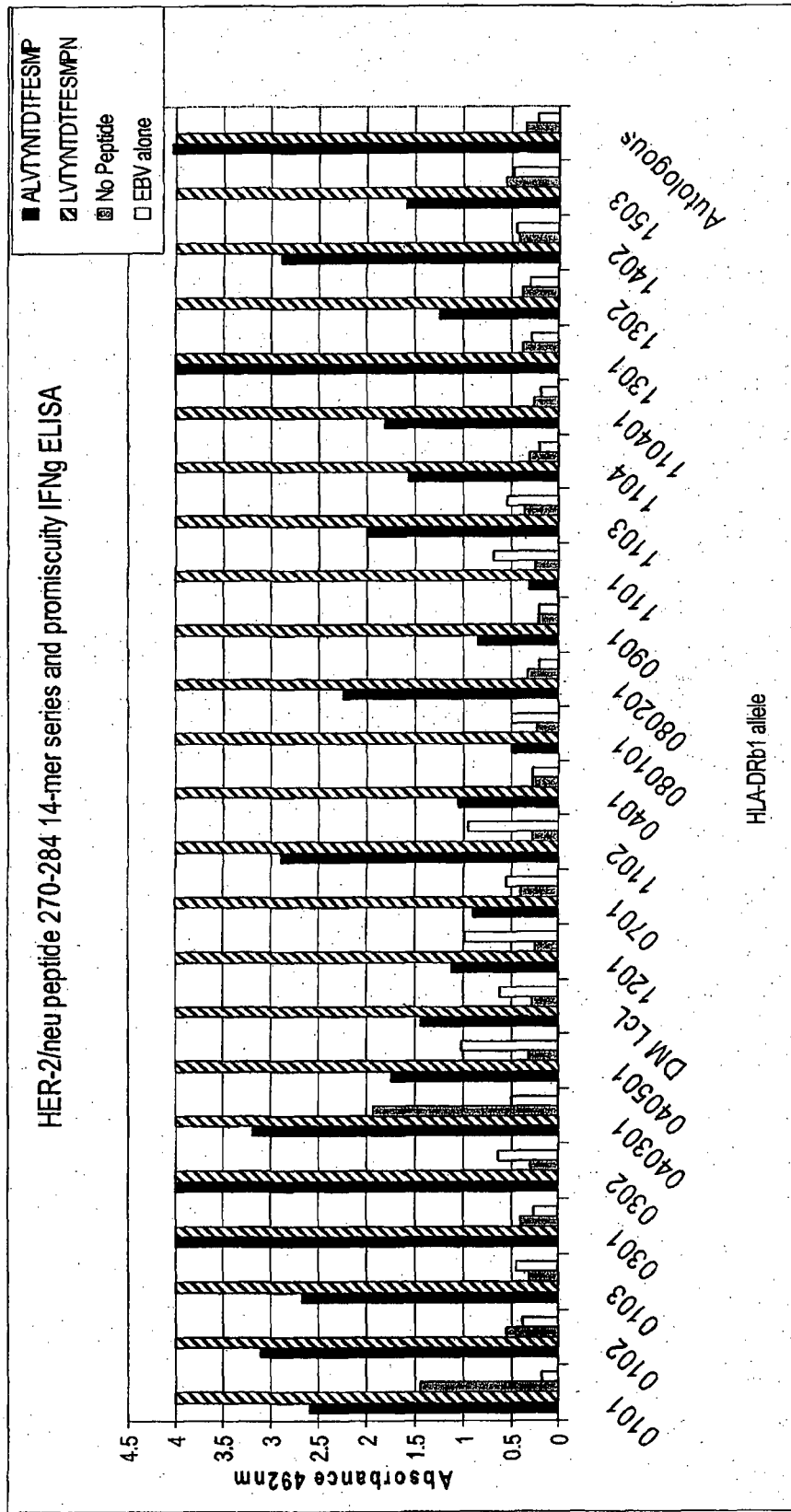


Figure 9

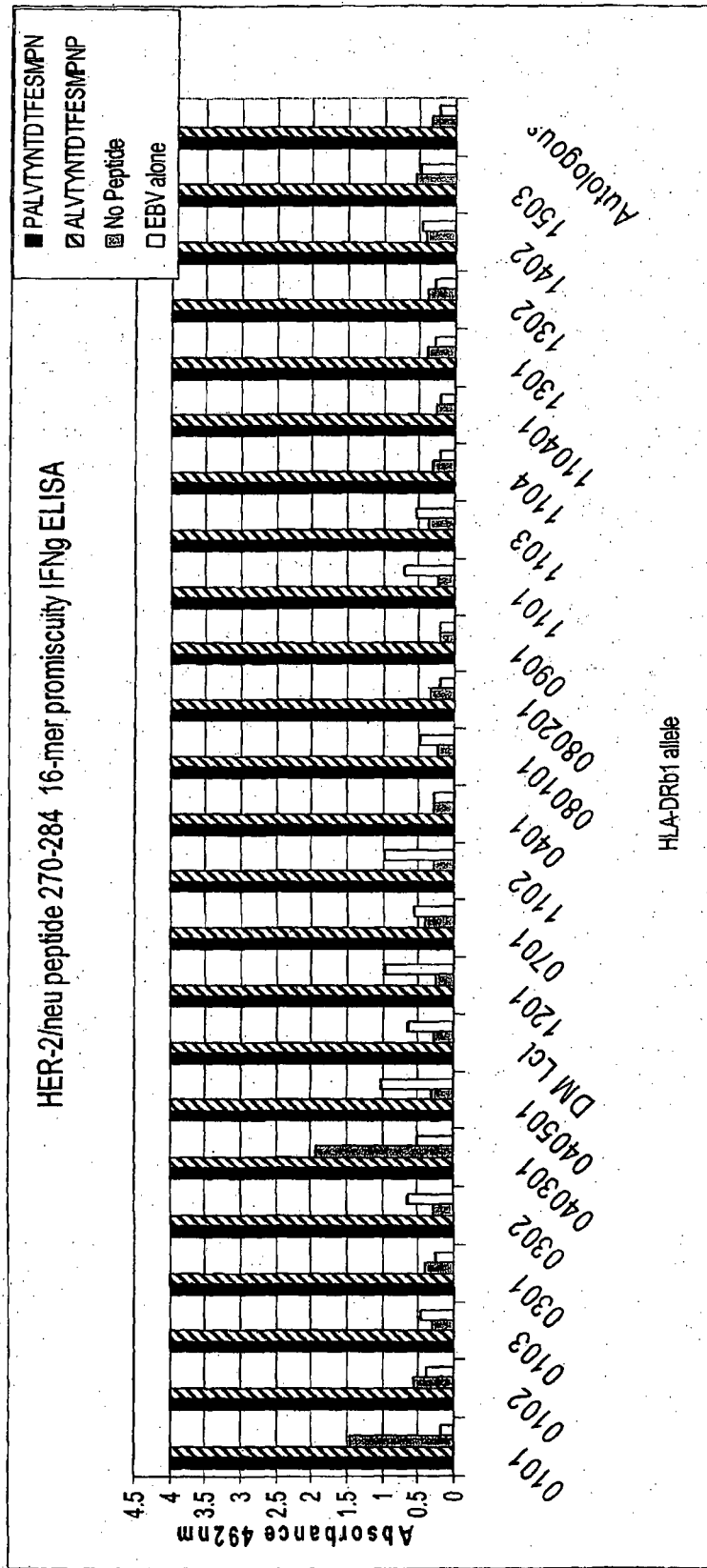


Figure 10

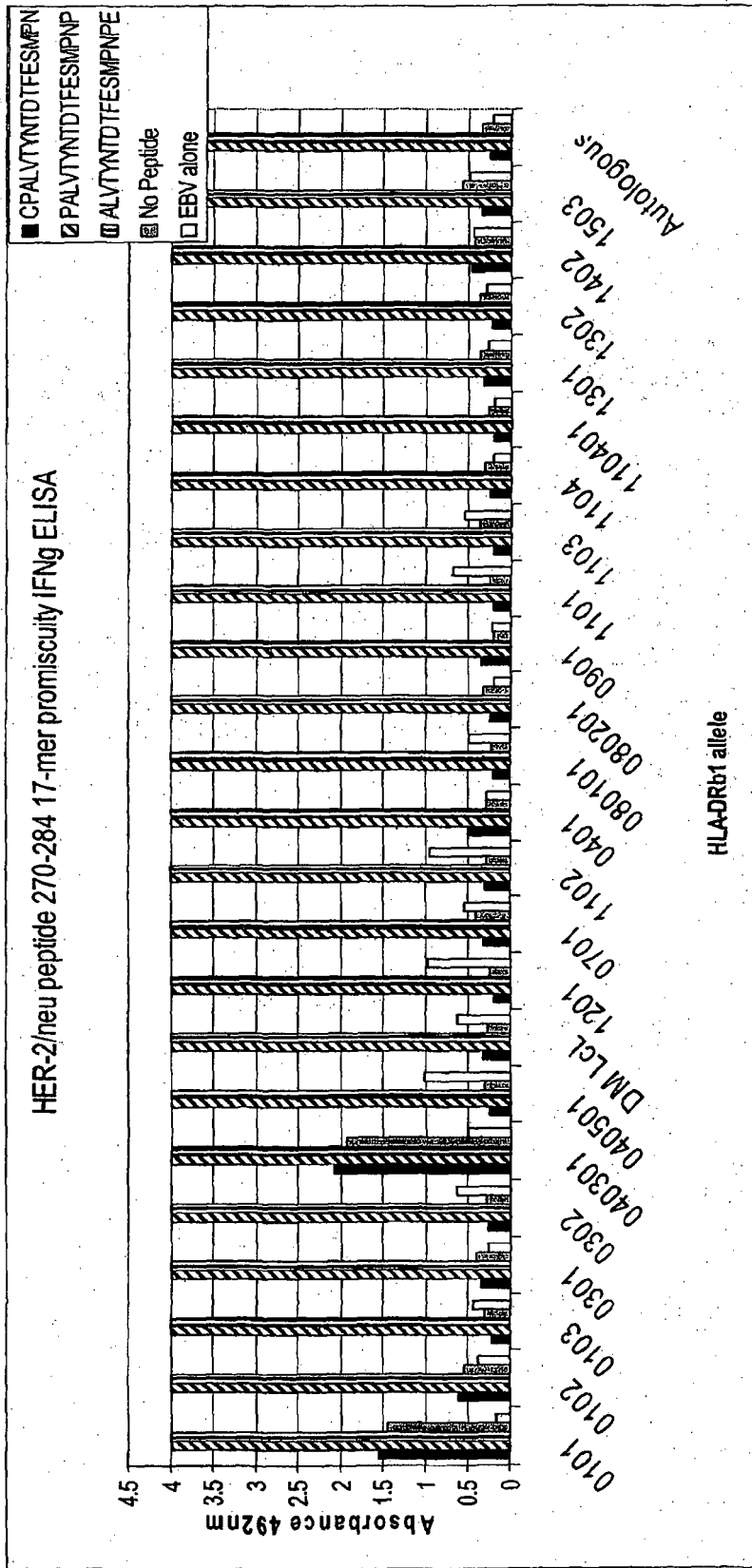


Figure 11

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专利名称(译)	混杂的HER-2 / NEU CD4 T细胞表位		
公开(公告)号	EP2570422B1	公开(公告)日	2015-12-16
申请号	EP2012168585	申请日	2007-08-10
[标]申请(专利权)人(译)	丹佐昂公司		
申请(专利权)人(译)	Dendreon公司CORPORATION		
当前申请(专利权)人(译)	Dendreon公司CORPORATION		
[标]发明人	WOLLAN JAMI B JONES LORI A		
发明人	WOLLAN, JAMI B. JONES, LORI A.		
IPC分类号	C07K14/00 A61K38/00 C12Q1/00 G01N33/53 C07H21/00		
CPC分类号	A61K38/00 A61P35/00 A61P37/00 A61P37/04 A61P43/00 C07K14/71		
优先权	60/837209 2006-08-11 US 11/836645 2007-08-09 US		
其他公开文献	EP2570422A3 EP2570422A2		
外部链接	Espacenet		

摘要(译)

本发明涉及人HER-2 / Neu蛋白的新T细胞表位的发现，其对于至少25种不同的HLA-DR等位基因是杂乱的。本发明还涉及含有一种新表位或这种表位的融合肽和异源多肽的组合物。本文进一步公开了表位或其融合肽的用途，以及含有表位或其融合肽的组合物。

