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(54) Title: CYTOTOXIC T-LYMPHOCYTE-INDUCING IMMUNOGENS FOR PREVENTION, TREATMENT, AND DIAGNOSIS OF CANCER

(57) Abstract: The present invention relates to compositions and methods for the prevention, treatment, and diagnosis of cancer, especially carcinomas, such as ovarian carcinoma. The invention discloses peptides, polypeptides, and polynucleotides that can be used to stimulate a CTL response against cancer.

CYTOTOXIC T-LYMPHOCYTE-INDUCING IMMUNOGENS FOR PREVENTION, TREATMENT, AND DIAGNOSIS OF CANCER

This application claims priority of U.S. Provisional Applications 60/251,022, filed 4 December 2000, and 60/256,824, filed 20 December 2000, the disclosures of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to the field of immunogens whose structures incorporate polypeptides comprising epitopic peptides derived from proteins expressed by cancer cells and to uses of said immunogens in eliciting cytotoxic T lymphocyte (CTL) responses for the diagnosis, prevention and treatment of cancer, preferably carcinoma, most preferably ovarian carcinoma.

BACKGROUND OF THE INVENTION

The mammalian immune system has evolved a variety of mechanisms to protect the host from cancerous cells, an important component of this response being mediated by cells referred to as T cells. Cytotoxic T lymphocytes (CTLs) are specialized T cells that function primarily by recognizing and killing cancerous cells or infected cells, but also by secreting

soluble molecules referred to as cytokines that can mediate a variety of effects on the immune system.

Evidence suggests that immunotherapy designed to stimulate a tumor-specific CTL response would be effective in controlling cancer. For example, it has been shown that human CTLs recognize sarcomas (Slovin, S. F. et al., J.Immunol., 137:3042-3048, (1987)), renal cell carcinomas (Schendel, D. J. et al., J.Immunol., 151:4209-4220, (1993)), colorectal carcinomas (Jacob, L. et al., Int.J.Cancer, 71:325-332, (1997)), ovarian carcinomas (Ioannides, C. G. et al., J.Immunol., 146:1700-1707, (1991)) (Peoples, G. E. et al., Surgery, 114:227-234, (1993)), pancreatic carcinomas (Peiper, M. et al., Eur.J.Immunol., 27:1115-1123, (1997); Wolfel, T. et al., Int.J.Cancer, 54:636-644, (1993)), squamous tumors of the head and neck (Yasumura, S. et al., Cancer Res., 53:1461-1468, (1993)), and squamous carcinomas of the lung (Slingluff, C. L. Jr et al., Cancer Res., 54:2731-2737, (1994); Yoshino, I. et al., Cancer Res., 54:3387-3390, (1994)). The largest number of reports of human tumor-reactive CTLs have concerned cancers (Boon, T. et al., Ann.Rev.Immunol., 12:337-365, (1994)). The ability of tumor-specific CTLs to mediate tumor regression, in both human (Rosenberg, S. A. et al., N.Engl.J.Med., 319:1676-1680, (1988)) and animal models (Celluzzi, C. M. et al., J.Exp.Med., 183:283-287, (1996); Mayordomo, J. I. et al., Nat.Med., 1:1297-1302, (1995); Zitvogel, L. et al., J.Exp.Med., 183:87-97, (1996)), suggests that methods directed at increasing CTL activity would likely have a beneficial effect with respect to tumor treatment.

In order for CTLs to kill or secrete cytokines in response to a cancer cell, the CTL must first recognize that cell as being cancerous. This process involves the interaction of the T cell receptor, located on the surface of the CTL, with what is generically referred to as an MHC-peptide complex which is located on the surface of the cancerous cell. MHC (major histocompatibility-complex)-encoded molecules have been subdivided into two types, and are referred to as class I and class II MHC-encoded molecules.

In the human immune system, MHC molecules are referred to as human leukocyte antigens (HLA). Within the MHC, located on chromosome six, are three different genetic loci that encode for class I MHC molecules. MHC molecules encoded at these loci are referred to as HLA-A, HLA-B, and HLA-C. The genes that can be encoded at each of these loci are extremely polymorphic, and thus, different individuals within the population express different class I MHC molecules on the surface of their cells. HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8 are examples of different class I MHC molecules that can be expressed from these loci. The present disclosure involves peptides that are associated with the HLA-A1/A11 or HLA-A2 molecules, and with the gene and protein that gives rise to these peptides.

The peptides that associate with the MHC molecules can either be derived from proteins made within the cell, in which case they typically associate with class I MHC molecules (Rock, K. L. and Golde, U., Ann.Rev.Immunol., 17:739-779, (1999)) or they can be derived from proteins that are acquired from outside of the cell, in which case they typically associate with class II MHC molecules (Watts, C., Ann.Rev.Immunol., 15:821-850, (1997)). Peptides that evoke a cancer-specific CTL response most typically associate with class I MHC molecules. The peptides that associate with a class I MHC molecule are typically nine amino acids in length, but can vary from a minimum length of eight amino acids to a maximum of fourteen amino acids in length. A class I MHC molecule with its bound peptide, or a class II MHC molecule with its bound peptide, is referred to as an MHC-peptide complex.

The process by which intact proteins are degraded into peptides is referred to as antigen processing. Two major pathways of antigen processing occur within cells (Rock, K. L. and Golde, U., Ann.Rev.Immunol., 17:739-779, (1999); Watts, C., Ann.Rev.Immunol., 15:821-850, (1997)). One pathway, which is largely restricted to cells that are antigen presenting cells such as dendritic cells, macrophages, and B cells, degrades proteins that are typically

phagocytosed or endocytosed into the cell. Peptides derived in this pathway typically bind to class II MHC molecules. A second pathway of antigen processing is present in essentially all cells of the body. This second pathway primarily degrades proteins that are made within the cells, and the peptides derived from this pathway primarily bind to class I MHC molecules. It is the peptides from this second pathway of antigen processing that are referred to herein. Antigen processing by this latter pathway involves polypeptide synthesis and proteolysis in the cytoplasm. The peptides produced are then transported into the endoplasmic reticulum of the cell, associate with newly synthesized class I MHC molecules, and the resulting MHC-peptide complexes are then transported to the cell surface. Peptides derived from membrane and secreted proteins have also been identified. In some cases these peptides correspond to the signal sequence of the proteins that are cleaved from the protein by the signal peptidase. In other cases, it is thought that some fraction of the membrane and secreted proteins are transported from the endoplasmic reticulum into the cytoplasm where processing subsequently occurs.

Once bound to the class I MHC molecule and displayed on the surface of a cell, the peptides are recognized by antigen-specific receptors on CTLs. Mere expression of the class I MHC molecule itself is insufficient to trigger the CTL to kill the target cell if the antigenic peptide is not bound to the class I MHC molecule. Several methods have been developed to identify the peptides recognized by CTL, each method relying on the ability of a CTL to recognize and kill only those cells expressing the appropriate class I MHC molecule with the peptide bound to it (Rosenberg, S. A., Immunity, 10:281-287, (1999)). Such peptides can be derived from a non-self source, such as a pathogen (for example, following the infection of a cell by a bacterium or a virus) or from a self-derived protein within a cell, such as a cancerous cell. Examples of sources of self-derived proteins in cancerous cells have been reviewed (Gilboa, E., Immunity, 11:263-270, (1999); Rosenberg, S. A., Immunity, 10:281-287, (1999)) and include: (i) mutated genes; (ii) aberrantly expressed genes such as an alternative open reading frame or through an intron-exon boundary; (iii) normal

genes that are selectively expressed in only the tumor and the testis; and (iv) normal differentiation genes that are expressed in the tumor and the normal cellular counterpart.

Four different methodologies have typically been used for identifying the peptides that are recognized by CTLs. These are: (i) the genetic method; (2) motif analysis; (3) SErological analysis of REcombinant cDNA eXpression libraries (SEREX™); and (iv) the analytical chemistry approach or the Direct Identification of Relevant Epitopes for Clinical Therapeutics (DIRECT™).

The genetic method is an approach in which progressively smaller subsets of cDNA libraries from tumor cells are transfected into cells that express the appropriate MHC molecule but not the tumor-specific epitope. The molecular clones encoding T cell epitopes are identified by their ability to reconstitute tumor specific T cell recognition of transfected cells. The exact T cell epitope is then identified by a combination of molecular subcloning and the use of synthetic peptides based on the predicted amino acid sequence. Such methods, however, are susceptible to inadvertent identification of cross-reacting peptides, and are not capable of identifying important post-translational modifications.

Motif analysis involves scanning a protein for peptides containing known class I MHC binding motifs, followed by synthesis and assay of the predicted peptides for their ability to be recognized by tumor-specific CTL. This approach requires prior knowledge of the protein from which the peptides are derived. This approach is also greatly hampered by the fact that not all of the predicted peptide epitopes are presented on the surface of a cell (Yewdell, J. W. and Bennink, J. R., Ann.Rev.Immunol., 17:51-88, (1999)), thus additional experimentation is required to determine which of the predicted epitopes is useful.

The SEREX™ approach relies on using antibodies in the serum of cancer patients to screen cDNA expression libraries for a clone that expresses a protein

recognized by the antibody. This methodology presumes that an antibody response will necessarily have developed in the presence of a T cell response, and thus, the identified clone is a good candidate to encode a protein that can be recognized by T cells.

DIRECT™ involves a combination of cellular immunology and mass spectrometry. This approach involves the actual identification of CTL epitopes by sequencing the naturally occurring peptides associated with class I MHC molecules. In this approach, cells are first lysed in a detergent solution, the peptides associated with the class I MHC molecules are purified, and the peptides fractionated by high performance liquid chromatography (HPLC). The peptides are then used to reconstitute recognition by tumor-specific CTLs on a non-tumor cell expressing the appropriate MHC molecules. Sequencing is readily performed by tandem mass spectrometry (Henderson, R. A. et al., Proc.Natl.Acad.Sci.U.S.A, 90:10275-10279, (1993); Hogan, K. T. et al., Cancer Res., 58:5144-5150, (1998); Hunt, D. F. et al., Science, 255:1261-1263, (1992); Slingluff, C. L. Jr et al., J.Immunol., 150:2955-2963, (1993)).

Immunization with cancer-derived, class I MHC-encoded molecule-associated peptides, or with a precursor polypeptide or protein that contains the peptide, or with a gene that encodes a polypeptide or protein containing the peptide, are forms of immunotherapy that can be employed in the treatment of cancer. These forms of immunotherapy require that immunogens be identified so that they can be formulated into an appropriate vaccine. Although a variety of cancer-derived antigens have been identified (Rosenberg, S. A., Immunity, 10:281-287, (1999)), not all of these are appropriate for broad-based immunotherapy as the expression of some peptides is limited to the tumor derived from a specific patient. Furthermore, the number of class I MHC molecules from which tumor-derived peptides have been discovered is largely restricted to HLA-A2. Thus, it would be useful to identify additional peptides that complex with class I MHC molecules other than HLA-A2. Such peptides would be particularly useful in the treatment of

cancer patients who do not express the HLA-A2 molecule. The HLA-A1/A11 antigens of the present invention (SEQ ID NO: 17-20) would be examples. It is also particularly useful to identify antigenic peptides that are derived from different parent proteins, even if the derived peptides associate with the same class I MHC molecule. Because an active immune response can result in the outgrowth of tumor cells that have lost the expression of a particular precursor protein for a given antigenic peptide, it is advantageous to stimulate an immune response against peptides derived from more than one parent protein, as the chances of the tumor cell losing the expression of both proteins is the multiple of the chances of losing each of the individual proteins.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to Immunogens comprising polypeptides with amino acid sequences comprising epitopic sequences selected from the sequences of SEQ ID NO: 1 - 20 and which immunogens facilitate a cytotoxic T lymphocyte (CTL)-mediated immune response against cancers. The present invention also relates to nucleic acid molecules that encode for the polypeptides and/or the full length proteins from which the polypeptides are derived, of such immunogens, and which can also be used to facilitate an immune response against cancer.

The present invention provides compositions comprising the immunogen described herein, and polynucleotides that direct the synthesis of such polypeptides, whereby the oligopeptides and polypeptides of such immunogens are capable of inducing a CTL response against cells expressing a protein comprising an epitopic sequence of SEQ ID NO: 1 – 16 presented in association with HLA-A2 and SEQ ID NO: 17 - 20 presented in association with HLA-AI/AII. The cells are usually cancer cells, preferably carcinoma cells, most preferably ovarian carcinomas expressing such proteins.

The present invention further relates to polynucleotides comprising the gene coding for a polypeptide of the immunogens disclosed herein.

The present invention also provides methods that comprise contacting a lymphocyte, especially a CTL, with an immunogen of the invention under conditions that induce a CTL response against a tumor cell, and more specifically against a cancer cell. The methods may involve contacting the CTL with the immunogenic peptide *in vivo*, in which case the peptides, polypeptides, and polynucleotides of the invention are used as vaccines, and will be delivered as a pharmaceutical composition comprising a pharmaceutically acceptable carrier and the immunogen, typically along with an adjuvant or one or more cytokines.

Alternatively, the immunogens of the present invention can be used to induce a CTL response *in vitro*. The generated CTL can then be introduced into a patient with cancer, more specifically cancer, colorectal carcinoma, ovarian carcinoma, lung carcinoma, or prostate carcinoma. Alternatively, the ability to generate CTL *in vitro* could serve as a diagnostic for cancer generally, including colorectal carcinoma, ovarian carcinoma, lung carcinoma, or prostate carcinoma.

DEFINITIONS

As used herein and except as noted otherwise, all terms are defined as given below.

The term "peptide" is used herein to designate a series of amino acid residues, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of the adjacent amino acids. The peptides are typically 9 amino acids in length, but can be as short as 8 amino acids in length, and as long as 14 amino acids in length.

The term "oligopeptide" is used herein to designate a series of amino acid residues, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of the adjacent amino acids. The length of the oligopeptide is not critical to the invention as long as the correct epitope or epitopes are maintained therein. The oligopeptides are typically less than about 30 amino acid residues in length, and greater than about 14 amino acids in length.

The term "polypeptide" designates a series of amino acid residues, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of the adjacent amino acids. The length of the polypeptide is not critical to the invention as long as the correct epitopes are maintained. In contrast to the terms peptide or oligopeptide, the term polypeptide is meant to refer to protein molecules of longer than about 30 residues in length.

A peptide, oligopeptide, protein, or polynucleotide coding for such a molecule is "immunogenic" (and thus an "immunogen" within the present invention) if it is capable of inducing an immune response. In the case of the present invention, immunogenicity is more specifically defined as the ability to induce a CTL-mediated response. Thus, an "immunogen" would be a molecule that is capable of inducing an immune response, and in the case of the present invention, a molecule capable of inducing a CTL response.

A T cell "epitope" is a short peptide molecule that binds to a class I or II MHC molecule and that is subsequently recognized by a T cell. T cell epitopes that bind to class I MHC molecules are typically 8-14 amino acids in length, and most typically 9 amino acids in length. T cell epitopes that bind to class II MHC molecules are typically 12-20 amino acids in length. In the case of epitopes that bind to class II MHC molecules, the same T cell epitope may share a common core segment, but differ in the length of the carboxy- and amino- terminal flanking sequences due to the fact that ends of the peptide

molecule are not buried in the structure of the class II MHC molecule peptide-binding cleft as they are in the class I MHC molecule peptide-binding cleft.

As used herein, reference to a DNA sequence includes both single stranded and double stranded DNA. Thus, the specific sequence, unless the context indicates otherwise, refers to the single strand DNA of such sequence, the duplex of such sequence with its complement (double stranded DNA) and the complement of such sequence.

The term "coding region" refers to that portion of a gene which either naturally or normally codes for the expression product of that gene in its natural genomic environment, i.e., the region coding *in vivo* for the native expression product of the gene. The coding region can be from a normal, mutated or altered gene, or can even be from a DNA sequence, or gene, wholly synthesized in the laboratory using methods well known to those of skill in the art of DNA synthesis.

The term "nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. The nucleotide sequence encoding for a particular peptide, oligopeptide, or polypeptide may be naturally occurring or they may be synthetically constructed. Generally, DNA segments encoding the peptides, polypeptides, and proteins of this invention are assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon.

The term "expression product" means that polypeptide or protein that is the natural translation product of the gene and any nucleic acid sequence coding equivalents resulting from genetic code degeneracy and thus coding for the same amino acid(s).

The term "fragment," when referring to a coding sequence, means a portion of DNA comprising less than the complete coding region whose expression product retains essentially the same biological function or activity as the expression product of the complete coding region.

The term "DNA segment" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the segment and its component nucleotide sequences by standard biochemical methods, for example, by using a cloning vector. Such segments are provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Sequences of non-translated DNA may be present downstream from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

The term "primer" means a short nucleic acid sequence that is paired with one strand of DNA and provides a free 3'OH end at which a DNA polymerase starts synthesis of a deoxyribonucleotide chain.

The term "promoter" means a region of DNA involved in binding of RNA polymerase to initiate transcription.

The term "open reading frame (ORF)" means a series of triplets coding for amino acids without any termination codons and is a sequence (potentially) translatable into protein.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated

from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polynucleotides, and recombinant or immunogenic polypeptides, disclosed in accordance with the present invention may also be in "purified" form. The term "purified" does not require absolute purity; rather, it is intended as a relative definition, and can include preparations that are highly purified or preparations that are only partially purified, as those terms are understood by those of skill in the relevant art. For example, individual clones isolated from a cDNA library have been conventionally purified to electrophoretic homogeneity. Purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. Furthermore, the claimed polypeptide which has a purity of preferably 0.001%, or at least 0.01% or 0.1%; and even desirably 1% by weight or greater is expressly contemplated.

The nucleic acids and polypeptide expression products disclosed according to the present invention, as well as expression vectors containing such nucleic acids and/or such polypeptides, may be in "enriched form." As used herein, the term "enriched" means that the concentration of the material is at least about 2, 5, 10, 100, or 1000 times its natural concentration (for example), advantageously 0.01%, by weight, preferably at least about 0.1% by weight. Enriched preparations of about 0.5%, 1%, 5%, 10%, and 20% by weight are also contemplated. The sequences, constructs, vectors, clones, and other materials comprising the present invention can advantageously be in enriched or isolated form.

The term "active fragment" means a fragment that generates an immune response (i.e., has immunogenic activity) when administered, alone or optionally with a suitable adjuvant, to an animal, such as a mammal, for example, a rabbit

or a mouse, and also including a human, such immune response taking the form of stimulating a CTL response within the recipient animal, such as a human. Alternatively, the "active fragment" may also be used to induce a CTL response *in vitro*.

As used herein, the terms "portion," "segment," and "fragment," when used in relation to polypeptides, refer to a continuous sequence of residues, such as amino acid residues, which sequence forms a subset of a larger sequence. For example, if a polypeptide were subjected to treatment with any of the common endopeptidases, such as trypsin or chymotrypsin, the oligopeptides resulting from such treatment would represent portions, segments or fragments of the starting polypeptide. This means that any such fragment will necessarily contain as part of its amino acid sequence a segment, fragment or portion, that is substantially identical, if not exactly identical, to a sequence of SEQ ID NOs: 1-4. When used in relation to polynucleotides, such terms refer to the products produced by treatment of said polynucleotides with any of the common endonucleases.

In accordance with the present invention, the term "percent identity" or "percent identical," when referring to a sequence, means that a sequence is compared to a claimed or described sequence after alignment of the sequence to be compared (the "Compared Sequence") with the described or claimed sequence (the "Reference Sequence"). The Percent Identity is then determined according to the following formula:

$$\text{Percent Identity} = 100\,[1-(C/R)]$$

wherein C is the number of differences between the Reference Sequence and the Compared Sequence over the length of alignment between the Reference Sequence and the Compared Sequence wherein (i) each base or amino acid in the Reference Sequence that does not have a corresponding aligned base or amino acid in the Compared Sequence and (ii) each gap in the Reference Sequence and (iii) each aligned base or amino acid in the Reference Sequence

that is different from an aligned base or amino acid in the Compared Sequence, constitutes a difference; and R is the number of bases or amino acids in the Reference Sequence over the length of the alignment with the Compared Sequence with any gap created in the Reference Sequence also being counted as a base or amino acid.

If an alignment exists between the Compared Sequence and the Reference Sequence for which the percent identity as calculated above is about equal to or greater than a specified minimum Percent Identity then the Compared Sequence has the specified minimum percent identity to the Reference Sequence even though alignments may exist in which the herein above calculated Percent Identity is less than the specified Percent Identity.

DETAILED SUMMARY OF THE INVENTION

The present invention relates generally to immunogens and immunogenic compositions, and methods of use therefor, for the prevention, treatment, and diagnosis of cancer, especially carcinomas, including ovarian carcinomas. Disclosed according to the invention are immunogens comprising proteins or polypeptides whose amino acid sequences comprises one or more epitopic oligopeptides with sequences selected from the group SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20. In addition, the invention further relates to polynucleotides that can be used to stimulate a CTL response against cancer, and more specifically carcinoma, especially ovarian carcinomas.

In accordance with the present invention there are disclosed specific oligopeptide sequences with amino acid sequences shown in SEQ ID NO: 1 - 20, which represent epitopic peptides (i.e. immunogenic oligopeptide sequences) of at least about 8 amino acids in length, preferably about 9

amino acids in length (i.e., nonapeptides), and no longer than about 10 amino acids in length and present as part of a larger structure, such as a polypeptide or full length protein. Proteins already known as immunogens in the art, such as MAGE 4 and MFG-E8, are specifically excluded from the present invention, especially where the epitopic peptide is one of SEQ ID NO: 17, 18, 19 and 20.

The polypeptides forming the immunogens of the present invention have amino acid sequences that comprise at least one stretch, possibly two, three, four, or more stretches of about 8 to 10 residues in length and which stretches differ in amino acid sequence from the sequences of SEQ ID NO: 1-20 by no more than about 1 amino acid residue, preferably a conservative amino acid residue, especially amino acids of the same general chemical character, such as where they are hydrophobic amino acids.

Said polypeptides can be of any desired length so long as they have immunogenic activity in that they are able, under a given set of desirable conditions, to elicit *in vitro* or *in vivo* the activation of cytotoxic T lymphocytes (CTLs) (i.e., a CTL response) against a presentation of a cancer specific protein, especially a carcinoma or sarcoma specific protein, most especially MAGE D, MAGE 4, MFG-E8 or human retinoblastoma-like protein, especially when such proteins are presented along with MHC-1 proteins, such as where said proteins are presented *in vitro* or *in vivo* by an antigen presenting cell (APC). The proteins and polypeptides forming the immunogens of the present invention can be naturally occurring or may be synthesized chemically.

The present invention is also directed to an isolated polypeptide, especially one having immunogenic activity, the sequence of which comprises within it one or more stretches comprising any 2 or more of the sequences of SEQ ID NO: 1-20 and in any relative quantities and wherein said sequences may differ by one amino acid residues from the sequences of SEQ ID NO: 1-20 in any given stretch of 8 to 10 amino acid residues. Thus, within the present invention, an by way of a non-limiting example only, such polypeptide

may contain as part of its amino acid sequence, nonapeptide fragments having up to 8 amino acids identical to a sequence of SEQ ID NO: 1-4 such that the polypeptide comprises, in a specific embodiment, 2 segments with at least 8 residues identical to SEQ ID NO: 1 and one segment with at least 8 residues identical to SEQ ID NO: 3. In other embodiments, other combinations and permutations of the epitopic sequences disclosed herein may be part of an immunogen of the present invention or of such a polypeptide so long as any such polypeptide comprises at least 2 such epitopes, whether such epitopes are different or the same. Thus, in a specific embodiment, a polypeptide of the present invention may comprise 2 copies of the sequence of SEQ ID NO: 2 at some point or points within its length. Of course, any combinations and permutations of the epitopes disclosed herein, as long as they are present at least two in number in such polypeptides, are expressly contemplated.

All of the epitopic peptides of SEQ ID NO: 1-20 are derived from proteins expressed by cancer cells and sequences and were identified through the method of Automated High Through-put Sequencing (HTPS).

Epitopic nonapeptide of SEQ ID NO: 1 (KIMDQVQQA) is derived from the human APC gene expressed by an ovarian carcinoma cell line, OVCAR3. While having been used in a vector for a vaccine [See: Chada et al, U.S. Patent No. 5,693,522], this protein has not been described as an immunogen but has been linked to other types of cancer [See: Spirio et al, *Nat. Genetics*, 20, 385-8 (1998)].

The epitopic decapeptide of SEQ ID NO: 2 (RLQEDPPAGV) is derived from the human ubiquitin conjugating enzyme (HHR6A) and was expressed by the OVCAR3 ovarian carcinoma cell line. The sequence was identified using HTPS. [See: Lal et al, U.S. Patent 6,013,702 for a description of this protein; Pati et al, *Mol. Cell Biol.*, 19:5001-13 (1999) for a description of the functions of the enzyme in targeting cyclic AMP-induced transcription].

The epitopic nonapeptide of SEQ ID NO: 3 (KLDVGNAEV) is derived from accessory protein BAP 31 and was expressed by the OVCAR3 ovarian carcinoma cell line. [See: Granville et al, *FEBS Lett.* 437:5-10 (1998); Annaert et al, *J. Cell Biol.*, 139:1397-1410 (1997); Li et al, *Eur. J. Biochem.*, 238:631-638 (1996); Ng et al, *J. Cell Biol.*, 139:327-338 (1997); Ng and Shore, *J. Biol. Chem.*, 273:3140-43 (1998)] The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 4 (FLYDDNQRV) is derived from Topoisomerase II and was expressed by the OVCAR3 ovarian carcinoma cell line. [See: Kim et al, *Anticancer Res.*, 19:5393-8 (1999); Fortune and Osheroff, *Prog. Nuc. Acid Res. Mol. Biol.*, 64:221-253 (2000); Willman and Holden, *Prostate*, 42:280-286 (2000); Cowell et al, *Exp. Cell Res.*, 255:86-94 (2000); Lage et al, *Br. J. Cancer*, 82:488-91 (2000)] The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 5 (ALMEQQHYV) is derived from Integrin beta-8 subunit precursor and was expressed by the OVCAR3 ovarian carcinoma cell line. [See:Nishimura et al, *J. Biol. Chem.*, 269:28708-15 (1994); Moyle et al, *J. Biol. Chem.*, 266:19650-8 (1991)] The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 6 (YLMDTSGKV) is derived from replication protein A and was expressed by the SKOV3 A2 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 7 (ILDDIGHGV) is derived from Abl binding protein 3 and was expressed by the SKOV3 A2 and OVCAR3 ovarian carcinoma cell lines. The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 8 (LLDRFLATV) is derived from Cyclin protein and was expressed by the SKOV3 A2 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic eicosapeptide of SEQ ID NO: 9 (LLIDDKGDIKL) is derived from protein encoded by human CDC2 gene involved in control of the celll cycle and was expressed by the SKOV3 A2 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic decapeptide of SEQ ID NO: 10 (RLYPWGVVEV) is derived from the protein encoded by human messenger RNA for the KIAA0158 gene and was expressed by the SKOV3 A2 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 11 (KLQELNYNL) is derived from a signal transducer and activator protein and was expressed by the SKOV3 A2 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 12 (ILIEHLYGL) is derived from LDL receptor related protein and was expressed by the SKOV3 A2 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 13 (YLIELIDRV) is derived from TNF-alpha converting enzyme and was expressed by the OVCAR3 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 14 (NLMEQPIKV) is derived from Plakoglobin protein and was expressed by the OVCAR3 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic decapeptide of SEQ ID NO: 15 (FLAEDALNTV) is derived from EDDR1 gene for receptor tyrosine kinase and was expressed by the OVCAR3 ovarian carcinoma cell line. The sequence was identified using HTPS.

The epitopic nonapeptide of SEQ ID NO: 16 (TLLNVIKSV) is derived from inositol triphosphate receptor type II and was expressed by the OVCAR3 and SKOV3 ovarian carcinoma cell lines. The sequence was identified using HTPS.

Epitopic nonapeptide of SEQ ID NO: 17 (MLKDIIKEY) is derived from the protein MAGE D, expressed by an ovarian carcinoma cell line. MAGE D is also known as hepatocellular carcinoma associated protein – JCL-1, as Snt-1 breast cancer associated protein, and as MAGE-like protein. [See: Lucas et al, *Cancer Res.*, **59**:4100-4103 (1999)].

The epitopic nonapeptide of SEQ ID NO: 18 (TSYVKVLEH) is derived from the protein MAGE 4 and was expressed by an ovarian carcinoma cell line different from that for the other epitopic peptides disclosed herein. The sequence was identified using HTPS. [See: Boon-Falleur et al, U.S. Patent 5,695,994 for a description of a MAGE 3 protein; Chen et al, *Liver*, **19**:110-114 (1999) for a description of the expression of MAGE genes in human carcinomas].

The epitopic nonapeptide of SEQ ID NO: 19 (HEYLKAFKV) is derived from the protein MFG-E8 protein and was expressed by an ovarian carcinoma cell line different from that for the other epitopic peptides disclosed herein. This protein is also known as the milk fat globule EGF (epidermal growth factor) Factor 8 protein and as BA46 and lactadherin. [See: Taylor et al, *DNA Cell Biol.*, **16**:861-869 (1997)]

The epitopic nonapeptide of SEQ ID NO: 20 (IYIKQIKTF) is derived from the human retinoblastoma-like protein (p130), also called E1A-associated 130k protein or E1A-associated cyclin-binding 130k protein, and was expressed by an ovarian carcinoma cell line different from that for the other epitopic peptides disclosed herein. [See: Claudio et al, *Cancer Res.*, **60**:372-382 (2000)]

Oligopeptides as disclosed herein may themselves be prepared by methods well known to those skilled in the art. (Grant, G. A., Synthetic Peptides: A User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York).

Besides the sequences of SEQ ID NOs:1 - 20, the proteins and polypeptides forming the immunogens of the present invention may also comprise one or more other immunogenic amino acid stretches known to be associated with cancer, and more specifically with carcinomas and melanomas, including colorectal carcinoma, ovarian carcinoma, lung carcinoma, or prostate carcinoma, and which may stimulate a CTL response whereby the immunogenic peptides associate with HLA-A1/A11, or HLA-A2, or another class I MHC (i.e., MHC-1) molecule.

The immunogens of the present invention can be in the form of a composition of one or more of the different immunogens and wherein each immunogen is present in any desired relative abundance. Such compositions can be homogeneous or heterogeneous with respect to the individual immunogenic peptide components present therein, having only one or more than one of such peptides.

The oligopeptides and polypeptides useful in practicing the present invention may be derived by fractionation of naturally occurring proteins by methods such as protease treatment, or they may be produced by recombinant or synthetic methodologies that are well known and clear to the skilled artisan (Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor). The polypeptide may comprise a recombinant or synthetic polypeptide that necessarily comprises at least one of SEQ ID NOs:1-20 which sequences may also be present in multiple copies.

Thus, oligopeptides and polypeptides of the present invention may have one, two, three, or more such immunogenic peptides within the amino acid sequence of said oligopeptides and polypeptides, and said immunogenic peptides, or epitopes, may be the same or may be different, or may have any number of such sequences wherein some of them are identical to each other in amino acid sequence while others within the same polypeptide sequence are different from each other and said epitopic sequences may occur in any order within said immunogenic polypeptide sequence. The location of such sequences within the sequence of a polypeptide forming an immunogen of the invention may affect relative immunogenic activity. In addition, immunogens of the present invention may comprise more than one protein comprising the amino acid sequences disclosed herein. Such polypeptides may be part of a single composition or may themselves be covalently or non-covalently linked to each other.

The immunogenic peptides disclosed herein may also be linked directly to, or through a spacer or linker to: an immunogenic carrier such as serum albumin, tetanus toxoid, keyhole limpet hemocyanin, dextran, or a recombinant virus particle; an immunogenic peptide known to stimulate a T helper cell type immune response; a cytokine such as interferon gamma or GMCSF; a targeting agent such as an antibody or receptor ligand; a stabilizing agent such as a lipid; or a conjugate of a plurality of epitopes to a branched lysine core structure, such as the so-called "multiple antigenic peptide" described in (Posnett, D. N. et al., J.Biol.Chem., 263:1719-1725, (1988)); a compound such as polyethylene glycol to increase the half life of the peptide; or additional amino acids such as a leader or secretory sequence, or a sequence employed for the purification of the mature sequence. Spacers and linkers are typically comprised of relatively small, neutral molecules, such as amino acids and which are substantially uncharged under physiological conditions. Such spacers are typically selected from the group of nonpolar or neutral polar amino acids, such as glycine, alanine, serine and other similar amino acids. Such optional spacers or linkers

need not be comprised of the same residues and thus may be either homo- or hetero-oligomers. When present, such linkers will commonly be of length at least one or two, commonly 3, 4, 5, 6, and possibly as much as 10 or even up to 20 residues (in the case of amino acids). In addition, such linkers need not be composed of amino acids but any oligomeric structures will do as well so long as they provide the correct spacing so as to optimize the desired level of immunogenic activity of the immunogens of the present invention. The immunogen may therefore take any form that is capable of eliciting a CTL response.

In addition, the immunogenic peptides of the present invention may be part of an immunogenic structure via attachments other than conventional peptide bonds. Thus, any manner of attaching the peptides of the invention to an immunogen of the invention, such as an immunogenic polypeptide as disclosed herein, could provide an immunogenic structure as claimed herein. Thus, immunogens, such as proteins of the invention, are structures that contain the peptides disclosed according to the present invention but such immunogenic peptides may not necessarily be attached thereto by the conventional means of using ordinary peptide bounds. The immunogens of the present invention simply contain such peptides as part of their makeup, but how such peptides are to be combined to form the final immunogen is left to the talent and imagination of the user and is in no way restricted or limited by the disclosure contained herein.

The peptides that are naturally processed and bound to a class I MHC molecule, and which are recognized by a tumor-specific CTL, need not be the optimal peptides for stimulating a CTL response. See, for example, (Parkhurst, M. R. et al., J.Immunol., 157:2539-2548, (1996); Rosenberg, S. A. et al., Nat.Med., 4:321-327, (1998)). Thus, there can be utility in modifying a peptide, such that it more readily induces a CTL response. Generally, peptides may be modified at two types of positions. The peptides may be modified at amino acid residues that are predicted to interact with the class I MHC molecule, in which case the goal is to create a peptide that has a higher affinity for the class I MHC

molecule than does the parent peptide. The peptides can also be modified at amino acid residues that are predicted to interact with the T cell receptor on the CTL, in which case the goal is to create a peptide that has a higher affinity for the T cell receptor than does the parent peptide. Both of these types of modifications can result in a variant peptide that is related to a parent peptide, but which is better able to induce a CTL response than is the parent peptide. As used herein, the term "parent peptide" means an oligopeptide with the amino acid sequence of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20.

The parent peptides disclosed herein can be modified by the substitution of one or more residues at different, possibly selective, sites within the peptide chain. Such substitutions may be of a conservative nature, for example, where one amino acid is replaced by an amino acid of similar structure and characteristics, such as where a hydrophobic amino acid is replaced by another hydrophobic amino acid. Even more conservative would be replacement of amino acids of the same or similar size and chemical nature, such as where leucine is replaced by isoleucine. In studies of sequence variations in families of naturally occurring homologous proteins, certain amino acid substitutions are more often tolerated than others, and these are often show correlation with similarities in size, charge, polarity, and hydrophobicity between the original amino acid and its replacement, and such is the basis for defining "conservative substitutions."

Conservative substitutions are herein defined as exchanges within one of the following five groups: Group 1—small aliphatic, nonpolar or slightly polar residues (Ala, Ser, Thr, Pro, Gly); Group 2—polar, negatively charged residues and their amides (Asp, Asn, Glu, Gln); Group 3—polar, positively charged residues (His, Arg, Lys); Group 4—large, aliphatic, nonpolar residues (Met, Leu, Ile, Val, Cys); and Group 4—large , aromatic residues (Phe, Tyr, Trp).

Less conservative substitutions might involve the replacement of one amino acid by another that has similar characteristics but is somewhat different

in size, such as replacement of an alanine by an isoleucine residue. Highly nonconservative replacements might involve substituting an acidic amino acid for one that is polar, or even for one that is basic in character. Such radical substitutions cannot, however, be dismissed as potentially ineffective since chemical effects are not totally predictable and radical substitutions might well give rise to serendipitous effects not otherwise predictable from simple chemical principles.

Of course, such substitutions may involve structures other than the common L-amino acids. Thus, D-amino acids might be substituted for the L-amino acids commonly found in the antigenic peptides of the invention and yet still be encompassed by the disclosure herein. In addition, amino acids possessing non-standard R groups (i.e., R groups other than those found in the common 20 amino acids of natural proteins) may also be used for substitution purposes to produce immunogens and immunogenic polypeptides according to the present invention.

If substitutions at more than one position are found to result in a peptide with substantially equivalent or greater antigenic activity as defined below, then combinations of those substitutions will be tested to determine if the combined substitutions result in additive or syngeneic effects on the antigenicity of the peptide. At most, no more than 4 positions within the peptide would simultaneously be substituted.

Based on cytotoxicity assays, an epitope is considered substantially identical to the reference peptide if it has at least 10% of the antigenic activity of the reference peptide as defined by the ability of the substituted peptide to reconstitute the epitope recognized by a CTL in comparison to the reference peptide. Thus, when comparing the lytic activity in the linear portion of the effector:target curves with equimolar concentrations of the reference and substituted peptides, the observed percent specific killing of the target cells incubated with the substituted peptide should be equal to that of the reference

peptide at an effector:target ratio that is no greater than 10-fold above the reference peptide effector:target ratio at which the comparison is being made.

Preferably, when the CTLs specific for a peptide of SEQ ID NOs:1-20 are tested against the substituted peptides, the peptide concentration at which the substituted peptides achieve half the maximal increase in lysis relative to background is no more than about 1 mM, preferably no more than about 1 μM, more preferably no more than about 1 nM, and still more preferably no more than about 100 pM, and most preferably no more than about 10 pM. It is also preferred that the substituted peptide be recognized by CTLs from more than one individual, at least two, and more preferably three individuals.

Thus, the epitopes of the present invention may be identical to naturally occurring tumor-associated or tumor-specific epitopes or may include epitopes that differ by no more than 4 residues from the reference peptide, as long as they have substantially identical antigenic activity.

It should be appreciated that an immunogen consisting only of a peptide of SEQ ID NO:1, or of a polypeptide that does not correspond to a protein of SEQ ID NO:2, but nonetheless comprises a peptide of SEQ ID NO:1, or of a plurality of peptides of SEQ ID NOs:1-16 for HLA-a1/a11 or SEQ ID NO: 17-20 for HLA-A2, or of a polynucleotide coding for a plurality of peptides of SEQ ID NOs:1-20 is necessarily limited to stimulating a CTL response that is specific for those peptides found in the immunogen. The immunogens of the invention can also comprise a polypeptide that itself comprises one or more of the epitopic peptides of SEQ ID NOS: 1-20.

The immunogenic peptides and polypeptides of the invention can be prepared synthetically, by recombinant DNA technology, or they can be isolated from natural sources such as tumor cells expressing the parent protein product.

The polypeptides and oligopeptides disclosed herein can be synthesized in solution or on a solid support in accordance with conventional techniques.

Various automated peptide synthesizers are commercially available and can be used in accordance with known protocols. See, for example, (Grant, G. A., Synthetic Peptides: A User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York). Fragments of polypeptides of the invention can also be synthesized as intermediates in the synthesis of a larger polypeptide.

Recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide or polypeptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell, and cultivated under conditions suitable for expression. These procedures are well known in the art to the skilled artisan, as described in (Coligan, J. E. et al, Current Protocols in Immunology, 1999, John Wiley & Sons, Inc., New York; Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor). Thus, recombinantly produced peptides or polypeptides can be used as the immunogens of the invention.

The coding sequences for peptides of the length contemplated herein can be synthesized on commercially available automated DNA synthesizers using protocols that are well know in the art. See for example, (Grant, G. A., Synthetic Peptides: A User's Guide, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York). The coding sequences can also be modified such that a peptide or polypeptide will be produced that incorporates a desired amino acid substitution. The coding sequence can be provided with appropriate linkers, be ligated into suitable expression vectors that are commonly available in the art, and the resulting DNA or RNA molecule can be transformed or transfected into suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are available, and their selection is left to the skilled artisan. For expression of the fusion proteins, the coding sequence will be

provided with operably linked start and stop codons, promoter and terminator regions, and a replication system to provide an expression vector for expression in the desired host cell. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Of course, yeast, insect, and mammalian host cells may also be used, employing suitable vectors and control sequences.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium

phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor). Such cells can routinely be utilized for assaying CTL activity by having said genetically engineered, or recombinant, host cells express the immunogenic peptides of the present invention.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The polypeptide can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. High performance liquid chromatography (HPLC) can be employed for final purification steps.

The immunogenic peptides of the present invention may be used to elicit CTLs *ex vivo* from either healthy individuals or from cancer patients with cancer, such as colorectal carcinoma, lung carcinoma, ovarian carcinoma, or prostate carcinoma. Such responses are induced by incubating in tissue

culture the individual's CTL precursor lymphocytes together with a source of antigen presenting cells and the appropriate immunogenic peptide. Examples of suitable antigen presenting cells include dendritic cells, macrophages, and activated B cells. Typically, the peptide at concentrations between 10 and 40 μg/ml, would be pre-incubated with the antigen presenting cells for periods ranging from 1 to 18 hrs. β_2-microglobulin (4 μg/ml) can be added during this time period to enhance binding. The antigen presenting cells may also be held at room temperature during the incubation period (Ljunggren, H.-G. et al., Nature, 346:476-480, (1990)) or pretreated with acid (Zeh, H. J., III et al., Hum.Immunol., 39:79-86, (1994)) to promote the generation of denatured class I MHC molecules which can then bind the peptide. The precursor CTLs (responders) are then added to the antigen presenting cells to which the immunogenic peptide has bound (stimulators) at responder to stimulator ratios of between 5:1 and 50:1, and most typically between 10:1 and 20:1. The co-cultivation of the cells is carried out at 37°C in RPMI 1640, 10% fetal bovine serum, 2 mM L-glutamine, and IL-2 (5-20 Units/ml). Other cytokines, such as IL-1, IL-7, and IL-12 may also be added to the culture. Fresh IL-2-containing media is added to the cultures every 2-4 days, typically by removing one-half the old media and replenishing it with an equal volume of fresh media. After 7-10 days, and every 7-10 days thereafter, the CTL are re-stimulated with antigen presenting cells to which immunogenic peptide has been bound as described above. Fresh IL-2-containing media is added to the cells throughout their culture as described above. Three to four rounds of stimulation, and sometimes as many five to eight rounds of stimulation, are required to generate a CTL response that can then be measured *in vitro*. The above described protocol is illustrative only and should not be considered limiting. Many *in vitro* CTL stimulation protocols have been described and the choice of which one to use is well within the knowledge of the skilled artisan. The peptide-specific CTL can be further expanded to large numbers by treatment with anti-CD3 antibody. For example, see (Riddell, S. R. and Greenberg, P. D., J.Immunol.Methods, 128:189-201, (1990); Walter, E. A. et al., N.Engl.J.Med., 333:1038-1044, (1995)).

Antigen presenting cells that are to be used to stimulate a CTL response are typically incubated with peptide of an optimal length, most commonly a nonapeptide, that allows for direct binding of the peptide to the class I MHC molecule without additional processing. Larger oligopeptides and polypeptides are generally ineffective in binding to class I MHC molecules as they are not efficiently processed into an appropriately sized peptide in the extracellular milieu. There a variety of approaches that are known in the art, however, that allow oligopeptides and polypeptides to be exogenously acquired by a cell, which then allows for their subsequent processing and presentation by a class I MHC molecule. Representative, but non-limiting examples of such approaches include electroporation of the molecules into the cell (Harding, C. H. III, Eur.J.Immunol., 22:1865-1869, (1992)), encapsulation of the molecules in liposomes which are fused to the cells of interest (Reddy, R. et al., J.Immunol.Methods, 141:157-163, (1991)), or osmotic shock in which the molecules are taken up via pinocytosis (Moore, M. W. et al., Cell, 54:777-785, (1988)). Thus, oligopeptides and polypeptides that comprise one or more of the peptides of the invention can be provided to antigen presenting cells in such a fashion that they are delivered to the cytoplasm of the cell, and are subsequently processed to allow presentation of the peptides.

Antigen presenting cells suitable for stimulating an *in vitro* CTL response that is specific for one or more of the peptides of the invention can also be prepared by introducing polynucleotide vectors encoding the sequences into the cells. These polynucleotides can be designed such that they express only a single peptide of the invention, multiple peptides of the invention, or even a plurality of peptides of the invention. There are a variety of approaches that are known in the art, that allow polynucleotides to be introduced and expressed in a cell, thus providing one or more peptides of the invention to the class I MHC molecule binding pathway. Representative, but non-limiting examples of such approaches include the introduction of plasmid DNA through particle-mediated gene transfer or electroporation (Tuting, T. et

al., J.Immunol., 160:1139-1147, (1998)), or the transduction of cells with an adenovirus expressing the polynucleotide of interest (Perez-Diez, A. et al., Cancer Res., 58:5305-5309, (1998)). Thus, oligonucleotides that code for one or more of the peptides of the invention can be provided to antigen presenting cells in such a fashion that the peptides associate with class I MHC molecules and are presented on the surface of the antigen presenting cell, and consequently are available to stimulate a CTL response.

By preparing the stimulator cells used to generate an *in vitro* CTL response in different ways, it is possible to control the peptide specificity of CTL response. For example, the CTLs generated with a particular peptide will necessarily be specific for that peptide. Likewise, CTLs that are generated with a polypeptide or polynucleotide expressing or coding for particular peptides will be limited to specificities that recognize those peptides. More broadly, stimulator cells, and more specifically dendritic cells, can be incubated in the presence of the whole protein. As a further alternative, stimulator cells, and more specifically dendritic cells, can be transduced or transfected with RNA or DNA comprising the polynucleotide sequence encoding the protein. Under these alternative conditions, peptide epitopes that are naturally cleaved out of the protein, and which are generated in addition to peptide epitopes of SEQ ID NOs:1-20 can associate with an appropriate class I MHC molecule, which may or may not include HLA-A1, -A2, or -A3. The selection of antigen presenting cells and the type of antigen with which to stimulate the CTL, is left to the ordinary skilled artisan.

In specific embodiments, the methods of the present invention include a method for inducing a CTL response *in vitro* that is specific for a tumor cell expressing HLA-A1/A11 or HLA-A2, whereby the method comprises contacting a CTL precursor lymphocyte with an antigen presenting cell that has bound an immunogen comprising one or more of the peptides disclosed according to the invention.

In specific embodiments, the methods of the present invention include a method for inducing a CTL response *in vitro* that is specific for a tumor cell expressing HLA-A1/A11 or HLA-A2, whereby the method comprises contacting a CTL precursor lymphocyte with an antigen presenting cell that has exogenously acquired an immunogenic oligopeptide or polypeptide that comprises one or more of the peptides disclosed according to the invention.

A yet additional embodiment of the present invention is directed to a process for inducing a CTL response *in vitro* that is specific for a tumor cell expressing HLA-A1/A11 or HLA-A2, comprising contacting a CTL precursor lymphocyte with an antigen presenting cell that is expressing a polynucleotide coding for a polypeptide of the invention and wherein said polynucleotide is operably linked to a promoter.

A variety of techniques exist for assaying the activity of CTL. These techniques include the labeling of target cells with radionuclides such as $Na_2{}^{51}Cr0_4$ or ^{3}H-thymidine, and measuring the release or retention of the radionuclides from the target cells as an index of cell death. Such assays are well-known in the art and their selection is left to the skilled artisan. Alternatively, CTL are known to release a variety of cytokines when they are stimulated by an appropriate target cell, such as a tumor cell expressing the relevant class I MHC molecule and the corresponding peptide. Non-limiting examples of such cytokines include IFN-γ, TNFα, and GM-CSF. Assays for these cytokines are well known in the art, and their selection is left to the skilled artisan. Methodology for measuring both target cell death and cytokine release as a measure of CTL reactivity are given in (Coligan, J. E. et al, Current Protocols in Immunology, 1999, John Wiley & Sons, Inc., New York).

After expansion of the antigen-specific CTLs, the latter are then adoptively transferred back into the patient, where they will destroy their specific target cell. The utility of such adoptive transfer is demonstrated in (North, R. J. et al., Infect.Immun., 67:2010-2012, (1999); Riddell, S. R. et al.,

Science, 257:238-241, (1992)). In determining the amount of cells to reinfuse, the skilled physician will be guided by the total number of cells available, the activity of the CTL as measured *in vitro*, and the condition of the patient. Preferably, however, about 1 x 10^6 to about 1 x 10^{12}, more preferably about 1 x 10^8 to about 1 x 10^{11}, and even more preferably, about 1 x 10^9 to about 1 x 10^{10} peptide-specific CTL are infused. Methodology for reinfusing the T cells into a patient are well known and exemplified in U.S. Patent No. 4,844,893 to Honski, et al., and U.S. Patent No. 4,690,915 to Rosenberg.

The peptide-specific CTL can be purified from the stimulator cells prior to infusion into the patient. For example, monoclonal antibodies directed towards the cell surface protein CD8, present on CTL, can be used in conjunction with a variety of isolation techniques such as antibody panning, flow cytometric sorting, and magnetic bead separation to purify the peptide-specific CTL away from any remaining non-peptide specific lymphocytes or from the stimulator cells. These methods are well known in the art, and are their selection is left to the skilled artisan. It should be appreciated that generation of peptide-specific CTL in this manner, obviates the need for stimulating the CTL in the presence of tumor. Thus, there is no chance of inadvertently reintroducing tumor cells into the patient.

Thus, one embodiment of the present invention relates to a process for treating a subject with cancer characterized by tumor cells expressing complexes of HLA-A1/A11 or HLA-A2, whereby CTLs produced *in vitro* according to the present invention are administered in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

Another embodiment of the present invention is directed to a process for treating a subject with cancer characterized by tumor cells expressing any class I MHC molecule and an epitope of SEQ ID NO: 1-20, whereby the CTLs are produced *in vitro* and are specific for the epitope or parent protein and are administered in an amount sufficient to destroy the tumor cells through direct

lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

In the foregoing embodiments the cancer to be treated may include a colorectal carcinoma, an ovarian carcinoma, a lung carcinoma, and prostate carcinoma, but especially ovarian carcinoma.

The *ex vivo* generated CTL can be used to identify and isolate the T cell receptor molecules specific for the peptide. The genes encoding the alpha and beta chains of the T cell receptor can be cloned into an expression vector system and transferred and expressed in naïve T cells from peripheral blood, T cells from lymph nodes, or T lymphocyte progenitor cells from bone marrow. These T cells, which would then be expressing a peptide-specific T cell receptor, would then have anti-tumor reactivity and could be used in adoptive therapy of cancer, and more specifically cancer, colorectal carcinoma, ovarian carcinoma, lung carcinoma, and prostate carcinoma.

In addition to their use for therapeutic or prophylactic purposes, the immunogenic peptides of the present invention are useful as screening and diagnostic agents. Thus, the immunogenic peptides of the present invention, together with modern techniques of gene screening, make it possible to screen patients for the presence of genes encoding such peptides on cells obtained by biopsy of tumors detected in such patients. The results of such screening may help determine the efficacy of proceeding with the regimen of treatment disclosed herein using the immunogens of the present invention.

Alternatively, the immunogenic peptides disclosed herein, as well as functionally similar homologs thereof, may be used to screen a sample for the presence of CTLs that specifically recognize the corresponding epitopes. The lymphocytes to be screened in this assay will normally be obtained from the peripheral blood, but lymphocytes can be obtained from other sources, including lymph nodes, spleen, tumors, and pleural fluid. The peptides of the present invention may then be used as a diagnostic tool to evaluate the

efficacy of the immunotherapeutic treatments disclosed herein. Thus, the *in vitro* generation of CTL as described above would be used to determine if patients are likely to respond to the peptide *in vivo*. Similarly, the *in vitro* generation of CTL could be done with samples of lymphocytes obtained from the patient before and after treatment with the peptides. Successful generation of CTL *in vivo* should then be recognized by a correspondingly easier ability to generate peptide-specific CTL *in vitro* from lymphocytes obtained following treatment in comparison to those obtained before treatment.

The oligopeptides of the invention, such as SEQ ID NO: 1 - 20, can also be used to prepare class I MHC tetramers which can be used in conjunction with flow cytometry to quantitate the frequency of peptide-specific CTL that are present in a sample of lymphocytes from an individual. Specifically, for example, class I MHC molecules comprising HLA-A2 and peptides of SEQ ID NO: 1-16, or HLA-A1/A11 and peptides of SEQ ID NO:17 - 20, would be combined to form tetramers as exemplified in U.S. Patent 5,635,363. Said tetramers would find use in monitoring the frequency of CTLs specific for the combination of HLA-A1/A11 and, for example, a peptide of SEQ ID NO:17 in the peripheral blood, lymph nodes, or tumor mass of an individual undergoing immunotherapy with the peptides, proteins, or polynucleotides of the invention, and it would be expected that successful immunization would lead to an increase in the frequency of the peptide-specific CTL. Said tetramers could also be developed for peptides of SEQ ID NOs:18-20 in combination with HLA-A1/A11. The same would hold true for uses of HLA-A2 and SEQ ID NO: 1-16.

As stated above, a vaccine in accordance with the present invention may include one or more of the hereinabove described polypeptides or active fragments thereof, or a composition, or pool, of immunogenic peptides disclosed herein. When employing more than one polypeptide or active fragment, such as two or more polypeptides and/or active fragments may be

used as a physical mixture or as a fusion of two or more polypeptides or active fragments. The fusion fragment or fusion polypeptide may be produced, for example, by recombinant techniques or by the use of appropriate linkers for fusing previously prepared polypeptides or active fragments.

The immunogenic molecules of the invention, including vaccine compositions, may be utilized according to the present invention for purposes of preventing, suppressing or treating diseases causing the expression of the immunogenic peptides disclosed herein, such as where the antigen is being expressed by tumor cells. As used in accordance with the present invention, the term "prevention" relates to a process of prophylaxis in which an animal, especially a mammal, and most especially a human, is exposed to an immunogen of the present invention prior to the induction or onset of the disease process. This could be done where an individual has a genetic pedigree indicating a predisposition toward occurrence of the disease condition to be prevented. For example, this might be true of an individual whose ancestors show a predisposition toward certain types of cancer. Alternatively, the immunogen could be administered to the general population as is frequently done for infectious diseases. Alternatively, the term "suppression" is often used to describe a condition wherein the disease process has already begun but obvious symptoms of said condition have yet to be realized. Thus, the cells of an individual may have become cancerous but no outside signs of the disease have yet been clinically recognized. In either case, the term prophylaxis can be applied to encompass both prevention and suppression. Conversely, the term "treatment" is often utilized to mean the clinical application of agents to combat an already existing condition whose clinical presentation has already been realized in a patient. This would occur where an individual has already been diagnosed as having a tumor.

It is understood that the suitable dosage of an immunogen of the present invention will depend upon the age, sex, health, and weight of the

recipient, the kind of concurrent treatment, if any, the frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can be tailored to the individual subject, as determined by the researcher or clinician. The total dose required for any given treatment will commonly be determined with respect to a standard reference dose as set by a manufacturer, such as is commonly done with vaccines, such dose being administered either in a single treatment or in a series of doses, the success of which will depend on the production of a desired immunological result (i.e., successful production of a CTL-mediated response to the antigen, which response gives rise to the prevention and/or treatment desired). Thus, the overall administration schedule must be considered in determining the success of a course of treatment and not whether a single dose, given in isolation, would or would not produce the desired immunologically therapeutic result or effect.

The therapeutically effective amount of a composition containing one or more of the immunogens of this invention, is an amount sufficient to induce an effective CTL response to the antigen and to cure or arrest disease progression. Thus, this dose will depend, among other things, on the identity of the immunogens used, the nature of the disease condition, the severity of the disease condition, the extent of any need to prevent such a condition where it has not already been detected, the manner of administration dictated by the situation requiring such administration, the weight and state of health of the individual receiving such administration, and the sound judgment of the clinician or researcher. Thus, for purposes of prophylactic or therapeutic administration, effective amounts would generally lie within the range of from 1.0 µg to about 5,000 µg of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 µg to about 1,000 µg of peptide pursuant to a boosting regimen over days, weeks or even months, depending on the recipient's response and as necessitated by subsequent monitoring of CTL-mediated activity within the bloodstream. Of course, such dosages are to be considered only a general guide and, in a given situation, may greatly exceed

such suggested dosage regimens where the clinician believes that the recipient's condition warrants more a aggressive administration schedule. Needless to say, the efficacy of administering additional doses, and of increasing or decreasing the interval, may be re-evaluated on a continuing basis, in view of the recipient's immunocompetence (for example, the level of CTL activity with respect to tumor-associated or tumor-specific antigens).

For such purposes, the immunogenic compositions according to the present invention may be used against a disease condition such as cancer by administration to an individual by a variety of routes. The composition may be administered parenterally or orally, and, if parenterally, either systemically or topically. Parenteral routes include subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. One or more such routes may be employed. Parenteral administration can be, for example, by bolus injection or by gradual perfusion over time.

Generally, vaccines are prepared as injectables, in the form of aqueous solutions or suspensions. Vaccines in an oil base are also well known such as for inhaling. Solid forms which are dissolved or suspended prior to use may also be formulated. Pharmaceutical carriers, diluents and excipients are generally added that are compatible with the active ingredients and acceptable for pharmaceutical use. Examples of such carriers include, but are not limited to, water, saline solutions, dextrose, or glycerol. Combinations of carriers may also be used. These compositions may be sterilized by conventional, well known sterilization techniques including sterile filtration. The resulting solutions may be packaged for use as is, or the aqueous solutions may be lyophilized, the lyophilized preparation being combined with sterile water before administration. Vaccine compositions may further incorporate additional substances to stabilize pH, or to function as adjuvants, wetting agents, or emulsifying agents, which can serve to improve the effectiveness of the vaccine.

The concentration of the CTL stimulatory peptides of the invention in pharmaceutical formulations are subject to wide variation, including anywhere

from less than 0.01% by weight to as much as 50% or more. Factors such as volume and viscosity of the resulting composition must also be considered. The solvents, or diluents, used for such compositions include water, possibly PBS (phosphate buffered saline), or saline itself, or other possible carriers or excipients.

The immunogens of the present invention may also be contained in artificially created structures such as liposomes, ISCOMS, slow-releasing particles, and other vehicles which increase the immunogenicity and/or half-life of the peptides or polypeptides in serum. Liposomes include emulsions, foams, micelies, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. Liposomes for use in the invention are formed from standard vesicle-forming lipids which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally determined by considerations such as liposome size and stability in the blood. A variety of methods are available for preparing liposomes as reviewed, for example, by (Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York)and see also U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

Liposomes containing the peptides or polypeptides of the invention can be directed to the site of lymphoid cells where the liposomes then deliver the selected immunogens directly to antigen presenting cells. Targeting can be achieved by incorporating additional molecules such as proteins or polysaccharides into the outer membranes of said structures, thus resulting in the delivery of the structures to particular areas of the body, or to particular cells within a given organ or tissue. Such targeting molecules may a molecule that binds to receptor on antigen presenting cells. For example an antibody that binds to CD80 could be used to direct liposomes to dendritic cells.

The immunogens of the present invention may also be administered as solid compositions. Conventional nontoxic solid carriers including

pharmaceutical grades of mannitol, lactose, starch, magnesium, cellulose, glucose, sucrose, sodium saccharin, and the like. Such solid compositions will often be administered orally, whereby a pharmaceutically acceptable nontoxic composition is formed by incorporating the peptides and polypeptides of the invention with any of the carriers listed above. Generally, such compositions will contain 10 - 95% active ingredient, and more preferably 25 - 75% active ingredient.

Aerosol administration is also an alternative, requiring only that the immunogens be properly dispersed within the aerosol propellant. Typical percentages of the peptides or polypeptides of the invention are 0.01% - 20% by weight, preferably 1% - 10%. The use of a surfactant to properly disperse the immunogen may be required. Representative surfactants include the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1 - 20% by weight of the composition, preferably 0.25 – 5%. Typical propellants for such administration may include esters and similar chemicals but are by no means limited to these. A carrier, such as lecithin for intranasal delivery, may also be included.

The peptides and polypeptides of the invention may also be delivered with an adjuvant. Adjuvants include, but are not limited to complete or incomplete Freund's adjuvant, Montanide ISA-51, aluminum phosphate, aluminum hydroxide, alum, and saponin. Adjuvant effects can also be obtained by injecting a variety of cytokines along with the immunogens of the invention. These cytokines include, but are not limited to IL-1, IL-2, IL-7, IL-12, and GM-CSF.

The peptides and polypeptides of the invention can also be added to professional antigen presenting cells such as dendritic cells that have been prepared ex vivo. For example, the dendritic cells could be prepared from

CD34 positive stem cells from the bone marrow, or they could be prepared from CD14 positive monocytes obtained from the peripheral blood. The dendritic cells are generated ex vivo using cytokines such as GM-CSF, IL-3, IL-4, TNF, and SCF. The cultured DC are then pulsed with peptides at various concentrations using standard methods that are well known in the art. The peptide-pulsed dendritic cells can then be administered either intraveneously, subcutaneously, or intradermally , and the immunization may also include cytokines such as IL-2 or IL-12.

The present invention is also directed to a vaccine in which an immunogen of the present invention is delivered or administered in the form of a polynucleotide encoding the a polypeptide or active fragment as disclosed herein, whereby the peptide or polypeptide or active fragment is produced *in vivo*. The polynucleotide may be included in a suitable expression vector and combined with a pharmaceutically acceptable carrier. For example, the peptides or polypeptides could be expressed in plasmid DNA and nonreplicative viral vectors such as vaccinia, fowlpox, Venezuelan equine encephalitis virus, adenovirus, or other RNA or DNA viruses. These examples are meant to be illustrative only and should not be viewed as self-limiting A wide variety of other vectors are available and are apparent to those skilled in the art from the description given herein. In this approach, a portion of the nucleotide sequence of the viral vector is engineered to express the peptides or polypeptides of the invention. Vaccinia vectors and methods useful in immunization protocols are described in U.S. Patent No. 4,722,848, the disclosure of which is incorporated herein by reference in its entirety.

Regardless of the nature of the composition given, additional therapeutic agents may also accompany the immunogens of the present invention. Thus, for purposes of treating tumors, compositions containing the immunogens disclosed herein may, in addition, contain other antitumor pharmaceuticals. The use of such compositions with multiple active ingredients is left to the discretion of the clinician.

In addition, the immunogens of the present invention can be used to stimulate the production of antibodies for use in passive immunotherapy, for use as diagnostic reagents, and for use as reagents in other processes such as affinity chromatography.

The present invention also relates to antibodies that react with immunogens, such as a polypeptide comprising one or more of the epitopic peptides of SEQ ID NO: 1-20 as disclosed herein. Active fragments of such antibodies are also specifically contemplated. Such antibodies, and active fragments of such antibodies, for example, and Fab structure, may react with, including where it is highly selective or specific for, an immunogenic structure comprising 2, 3, 4 or more of the epitopic peptides of the invention. Thus, the present invention also relates to a process of using antibodies to elicit an immune response, including the production of protective antibodies, following administration of immunogens of the invention to an immunocompetent animal (i.e., an animal whose immune system has not been compromised, either naturally or experimentally, so as to not exhibit such a reaction, such as a knock-out animal or the like).

With the advent of methods of molecular biology and recombinant technology, it is now possible to produce antibody molecules by recombinant means and thereby generate gene sequences that code for specific amino acid sequences found in the polypeptide structure of the antibodies. Such antibodies can be produced by either cloning the gene sequences encoding the polypeptide chains of said antibodies or by direct synthesis of said polypeptide chains, with *in vitro* assembly of the synthesized chains to form active tetrameric (H_2L_2) structures with affinity for specific epitopes and antigenic determinants. This has permitted the ready production of antibodies having sequences characteristic of neutralizing antibodies from different species and sources.

Regardless of the source of the antibodies, or how they are recombinantly constructed, or how they are synthesized, *in vitro* or *in vivo*, using transgenic animals, such as cows, goats and sheep, using large cell cultures of laboratory or commercial size, in bioreactors or by direct chemical synthesis employing no living organisms at any stage of the process, all antibodies have a similar overall 3 dimensional structure. This structure is often given as H_2L_2 and refers to the fact that antibodies commonly comprise 2 light (L) amino acid chains and 2 heavy (H) amino acid chains. Both chains have regions capable of interacting with a structurally complementary antigenic target. The regions interacting with the target are referred to as "variable" or "V" regions and are characterized by differences in amino acid sequence from antibodies of different antigenic specificity.

The variable regions of either H or L chains contains the amino acid sequences capable of specifically binding to antigenic targets. Within these sequences are smaller sequences dubbed "hypervariable" because of their extreme variability between antibodies of differing specificity. Such hypervariable regions are also referred to as "complementarity determining regions" or "CDR" regions. These CDR regions account for the basic specificity of the antibody for a particular antigenic determinant structure.

The CDRs represent non-contiguous stretches of amino acids within the variable regions but, regardless of species, the positional locations of these critical amino acid sequences within the variable heavy and light chain regions have been found to have similar locations within the amino acid sequences of the variable chains. The variable heavy and light chains of all antibodies each have 3 CDR regions, each non-contiguous with the others (termed L1, L2, L3, H1, H2, H3) for the respective light (L) and heavy (H) chains. The accepted CDR regions have been described by Kabat et al, *J. Biol. Chem.* **252**:6609-6616 (1977). The numbering scheme is shown in the figures, where the CDRs are underlined and the numbers follow the Kabat scheme.

In all mammalian species, antibody polypeptides contain constant (i.e., highly conserved) and variable regions, and, within the latter, there are the CDRs and the so-called "framework regions" made up of amino acid sequences within the variable region of the heavy or light chain but outside the CDRs.

The antibodies disclosed according to the invention may also be wholly synthetic, wherein the polypeptide chains of the antibodies are synthesized and, possibly, optimized for binding to the polypeptides disclosed herein as being receptors. Such antibodies may be chimeric or humanized antibodies and may be fully tetrameric in structure, or may be dimeric and comprise only a single heavy and a single light chain. Such antibodies may also include fragments, such as Fab and $F(ab_2)'$ fragments, capable of reacting with and binding to any of the polypeptides disclosed herein as being receptors.

A further embodiment of the present invention relates to a method for inducing a CTL response in a subject comprising administering to subjects that express HLA-A1 antigens an effective (i.e., CTL-stimulating amount) of an immunogen of the invention that does not comprise the entire protein expressing the epitopic peptides disclosed herein (i.e., one that comprises less than the entire protein where the protein is a naturally occurring polypeptide) in an amount sufficient to induce a CTL response to tumor cells expressing at least HLA-A1 or HLA-A2, as the case may be, thereby eliciting a cellular response against said tumor cells.

A still further embodiment of the present invention relates to a method for inducing a CTL response in a subject, wherein the immunogen is in the form of a polynucleotide. In one non-limiting example, the method comprises administering to subjects that express HLA-A1 at least one CTL epitope, wherein said epitope or epitopes are selected from a group comprising the peptides disclosed according to the invention, and are coded within a

polynucleotide sequence that does not comprise the entire protein coding region, in an amount sufficient to induce a CTL response to tumor cells expressing HLA-A1. A similar example could be drawn for HLA-A2.

While the below examples are provided to illustrate the invention, it is to be understood that these methods and examples in no way limit the invention to the embodiments described herein and that other embodiments and uses will no doubt suggest themselves to those skilled in the art. All publications, patents, and patent applications cited herein are hereby incorporated by reference, as are the references cited therein. It is also to be understood that throughout this disclosure where the singular is used, the plural may be inferred and vice versa and use of either is not to be considered limiting.

Example 1 - Cell Lines

For HLA-A1/A11 studies, ARGOV57, a HLA-A1/11 positive ovarian cell line, was established by culturing tumor cells from an ascitic fluid from an ovarian patient.

For HLA-A2 studies, OVCAR3, a HLA-A2 positive ovarian carcinoma cell line, was established by culturing tumor cells from an ascitic fluid from an ovarian patient.

SKOV3-A2, a HLA-A2 stably expressing ovarian carcinoma cell line, was established by culturing tumor cells from an ascitic fluid from an ovarian patient and transduced with HLA-A2 gene.

Example 2 - Immunoaffinity Purification

ARGOV57 cells were grown in 10-chamber Nunc cell factories (Fisher, Pittsburgh, PA). The cells were harvested by treatment with 0.45 % trypsin and 0.32 mM EDTA, washed two times in phosphate-buffered saline solution (pH 7.4), and stored as cell pellets at -80°C. Aliquots of 6-8 x 10^{10} cells were solubilized at 5-10 x 10^{6} cells/ml in 20 mM Tris, pH 8.0, 150 mM NaCl, 1% CHAPS, 18.5 μg/ml iodoacetamide, 5 μg/ml aprotonin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 5 mM EDTA, 0.2% sodium azide, and 17.4 μg/ml phenylmethylsulfonyl fluoride for 1 h. This and all subsequent steps were performed with ice-cold solutions and at 4°C. The lysates were then centrifuged at 100,000 x g, the pellets discarded, and the supernatants passed through a 0.22 μm filter. The supernatants were then passed over a series of columns with the first containing Sepharose, and the second containing the HLA-A1-specific monoclonal antibody, GAP-A1, bound to a protein A-Sepharose matrix. The second column was then sequentially washed with 20 column volumes of 20 mM Tris, pH 8.0, 150 mM NaCl, 20 column volumes of 20 mM Tris, pH 8.0, 1.0 M NaCl, and 20 column volumes of 20 mM Tris, pH 8.0. The peptides were eluted from the column with 5 column volumes of 10% acetic acid. The isolated HLA-A1 molecules were then boiled for 5 min to further dissociate any bound peptide from the heavy chains. The peptides were then separated from the co-purifying class I heavy chain and β_2-microglobulin by centrifugation on a Ultrafree-CL membrane with a nominal molecular weight cut-off of 5,000 Daltons (Millipore, Beford, MA).

For a separate study, OVCAR3 or SKOV3 cells were successfully prepared using the same procedure as just described but A2 molecules were prepared and using A2 specific antibodies.

Example 3 – Peptide Fractionation

The peptide extracts were fractionated by RP-HPLC (Reversed Phase – High Performance Liquid Chromatography) using an Applied Biosystems (ABI) model 140B system. The extracts were concentrated by vacuum centrifugation from about 20 ml down to 250 µl and injected into either a Brownlee (Norwalk, CT) C_{18} Aquapore column (2.1 mm x 3 cm; 300 Å; 7 µm) or a Higgins (Mountain View, CA) C18 Haisil column (2.1 mm x 4 cm; 300 Å; 5 µm). The peptides were eluted by first using a gradient of acetonitrile/0.085% TFA (trifluoroacetic acid) in 0.1% TFA/water, with the concentration of acetonitrile increasing from 0 – 9% (0 – 5 minutes), 9 – 36% (5 – 55 minutes), and 36 – 60% (55 – 62 minutes). A second dimension fractionation of combined fractions 17 and 18 from the first dimension (TFA) fraction was accomplished using the same gradient but with the substitution of HFBA (heptafluorobutyric acid) for TFA. The flow rate was 200 µl/min, and fractions were collected at 1 min (Brownlee column) or 40 second (Higgins column) intervals. A third dimension of RP-HPLC was achieved using an Eldex (Napa, CA) MicroPro Pump, a homemade C_{18} microcapillary column, and an ABI model 785A UV absorbance detector. The column was prepared by packing a 27 cm bed of 10 µm C_{18} particles in a section of 285 µm o.d./75 µm i.d. fused silica (Polymicro Technologies, Phoenix, AZ). Peptides in combined fractions 26 and 27 of the second dimension fraction were loaded onto this column and eluted with a gradient of acetonitrile/0.67% triethylamine acetate/water in 0.1% triethylamine acetate/water, with the concentration of acetonitrile increasing from 0 – 60% in 40 minutes. The flow rate was about 300 nl/min, and fractions were collected into 25 µl of water every 30 s. In all RP-HPLC experiments, peptides were detected by monitoring UV absorbance at 214 nm.

Example 4 – Mass Spectrometric Analysis

The second dimension HPLC fraction was analyzed using an affluent splitter on the microcapillary HPLC column. In this experiment, the column (360 µm o.d. x 100 µm i.d. with a 25 cm C_{18} bed) was butt connected with a zero dead volume tee (Valco, Houston, TX) to two pieces of fused silica of different lengths (25 µm and 40 µm i.d.). Peptides were eluted with a 34 min gradient of 0 – 60% acetonitrile. The 25 µm capillary deposited one-fifth of the HPLC effluent into the wells of a microtitre plate for use in CTL epitope reconstitution assays, whereas the remaining four-fifths of the effluent was directed into the mass spectrometer. Ions were formed by electrospray ionization, and mass spectra were recorded by scanning between mass to charge ratios (*m/z*) 300 and 1400 every 1.5 seconds. Peptide sequences were determined by CAD (collision-activated dissociation) tandem mass spectrometry as described in the literature (Hunt, D. F. et al., Proc.Natl.Acad.Sci.U.S.A, 83:6233-6237, (1986)).

Example 5 – Peptide Synthesis

Peptides were synthesized using a Gilson (Madison, WI) AMS 422 multiple peptide synthesizer. Ten µMol quantities were synthesized using conventional FMOC amino acids, resins, and chemical techniques. Peptides were purified by RP-HPLC using a 4.6 mm x 100 mm POROS (Perseptive Biosystems, Cambridge, MA) column and a 10 min, 0-60% acetonitrile in 0.1% TFA gradient.

Example 6 – Identification of peptides that associates with HLA-A1/A11.

To identify the antigens present on the surface of the ARGOV57 ovarian carcinoma tumor cell line, HLA-A1/A11 molecules were purified by

immunoaffinity chromatography from 8 x 10^{10} ARGOV57 tumor cells. After acid elution of the affinity column and dissociation of the class I/peptide complexes by boiling, the peptides were separated from the HLA-A1/A11 heavy chains and β2-microglobulin by membrane filtration, concentrated, and then fractionated by RP-HPLC using TFA as the organic modifier. Analysis of the fragmented masses obtained from the CAD allowed the determination of the peptide sequence as MLKDIIKEY (SEQ ID NO: 17). CAD of a synthetic peptide corresponding to SEQ ID NO:17 unequivocally identified the unknown as having the sequence of SEQ ID NO:17. A Genbank search revealed that a peptide of SEQ ID NO:17 corresponds exactly to amino acid residues 305-313 of the hepatocellular carcinoma associated protein JCL-1.

Other sequences (SEQ ID NOs: 18-20) were determined in the same way. Thus, analysis of the fragmented masses obtained from the CAD allowed the determination of the peptide sequence as TSYVKVLEH (SEQ ID NO: 18). CAD of a synthetic peptide corresponding to SEQ ID NO: 18 unequivocally identified the unknown as having the sequence of SEQ ID NO: 18. A Genbank search revealed that a peptide of SEQ ID NO: 18 corresponds exactly to amino acid residues 282-290 of the MAGE-4 protein. In the same way, HEYLKAFKV (SEQ ID NO: 19) was shown to correspond exactly to residues 152-160 of the milk fat globule-EGF factor 8 protein and IYIKQIKTF (SEQ ID NO: 20) corresponds exactly to a nonapeptide from human retinoblastoma-associated protein.

Example 7 – Identification of peptides that associates with HLA-A2.

To identify the antigens present on the surface of the OVCAR3 or SKOV3 ovarian carcinoma tumor cell line, HLA-A2 molecules were purified by immunoaffinity chromatography from 8 x 10^{10} OVCAR3 or SKOV3 tumor cells. After acid elution of the affinity column and dissociation of the class I/peptide complexes by boiling, the peptides were separated from the HLA-A2 heavy

chains and β2-microglobulin by membrane filtration, concentrated, and then fractionated by RP-HPLC using TFA as the organic modifier. Analysis of the fragmented masses obtained from the CAD allowed the determination of the peptide sequence as KIMDQVQQA (SEQ ID NO: 1). CAD of a synthetic peptide corresponding to SEQ ID NO:1 unequivocally identified the unknown as having the sequence of SEQ ID NO:1. A Genbank search revealed that a peptide of SEQ ID NO:1 corresponds exactly to amino acid residues 1745-1753 of the human APC gene protein.

Other sequences (SEQ ID NOs: 2 - 16) were determined in the same way using the appropriate protein source and GenBank sequences. Thus, for example, analysis of the fragmented masses obtained from the CAD allowed the determination of the peptide sequence as RLQEDPPAGV (SEQ ID NO: 2). CAD of a synthetic peptide corresponding to SEQ ID NO: 2 unequivocally identified the unknown as having the sequence of SEQ ID NO: 2. A Genbank search revealed that a peptide of SEQ ID NO: 2 corresponds exactly to amino acid residues of the human ubiquitin-conjugating enzyme.

Table 1. CTL-inducing peptide epitopes.

SEQ ID NO:	SEQUENCE	SEQ ID NO:	SEQUENCE
1	KIMDQVQQA	11	KLQELNYNL
2	RLQEDPPAGV	12	ILIEHLYGL
3	KLDVGNAEV	13	YLIELIDRV
4	FLYDDNQRV	14	NLMEQPIKV
5	ALMEQQHYV	15	FLAEDALNTV
6	YLMDTSGKV	16	TLLNVIKSV
7	ILDDIGHGV	17	MLKDIIKEY
8	LLDRFLATV	18	TSYVKVLEH
9	LLIDDKGTIKL	19	HEYLKAFKV
10	RLYPWGVVEV	20	IYIKQIKTF

WHAT IS CLAIMED IS:

1. An immunogen comprising an isolated polypeptide whose amino acid sequence comprises at least one epitopic peptide selected from the group consisting of SEQ ID NOS: 1 - 20 and wherein when the epitopic peptide is one of SEQ ID NO: 17-20 said polypeptide does not include MAGE 4 or MFG-E8 proteins.

2. The immunogen of claim 1 wherein said polypeptide comprises at least two of said epitopic peptides.

3. The immunogen of claim 1 wherein said polypeptide comprises at least three of said epitopic peptides.

4. The immunogen of claim 1 wherein said polypeptide comprises at least four of said epitopic peptides.

5. The immunogen of claim 1 wherein said epitopic peptide differs from SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 but wherein said difference is no more than one amino acid unit.

6. The immunogen of claim 5 wherein said one amino acid difference is the result of a conservative amino acid substitution.

7. The immunogen of claim 6 wherein said substitution is the substitution of one hydrophobic amino acid unit by another hydrophobic amino acid.

8. The immunogen of claim 5 wherein said amino acid difference is the addition or deletion of one amino acid to or from said oligopeptide.

9. An immunogen comprising a member selected from the group consisting of Mage D protein and an immunologically active fragment of Mage D protein.

10. A polynucleotide comprising a polynucleotide selected from the group consisting of:

(a) a polynucleotide that encodes a polypeptide selected from the group consisting of the polypeptides of claims 1, 2, 3, 4, 5, 6, 7, 8, and 9, and

(b) the full complement of (a).

11. The polynucleotide of claim 10 wherein the polynucleotide of (a) is a DNA.

12. The polynucleotide of claim 10 wherein the polynucleotide of (a) is an RNA.

13. A vector comprising a polynucleotide of claim 10.

14. A mammalian cell comprising the vector of claim 13 and expressing said polynucleotide.

15. A vaccine composition comprising an immunogen of claim 1, 2, 3, 4, 5, 6, 7, 8, or 9 present in a pharmaceutically acceptable carrier and in an amount sufficient to elicit production of antibodies or cells that react with said immunogen when said immunogen is administered to an immunologically competent animal.

16. An antibody specific for an immunogen of claim 1, 2, 3, 4, 5, 6, 7, 8 or 9.

17. A process for inducing a cytotoxic T lymphocyte (CTL) *in vitro* that is specific for a tumor cell expressing HLA-A1 comprising contacting a precursor CTL with an immunogen of claim 1 under conditions that generate a CTL response to the tumor cell.

18. A process for inducing a CTL response *in vitro* that is specific for a tumor cell expressing HLA-A1, said process comprising contacting a precursor CTL with a mammalian cell of claim 14.

19. A process for treating a subject with cancer characterized by tumor cells expressing HLA-A1, said process comprising administering CTLs induced by the processes of claims 17 or 18 in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

20. A process for treating a cancer-afflicted subject characterized by tumor cells expressing any class I MHC molecule and a gene coding for an epitopic sequence of at least one of SEQ ID NO: 17 - 20, whereby the CTLs of claim 17 are administered in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

21. A process for inducing a cytotoxic T lymphocyte (CTL) *in vitro* that is specific for a tumor cell expressing HLA-A2 comprising contacting a precursor CTL with an immunogen of claim 1 under conditions that generate a CTL response to the tumor cell.

22. A process for inducing a CTL response *in vitro* that is specific for a tumor cell expressing HLA-A2, said process comprising contacting a precursor CTL with a mammalian cell of claim 14.

23. A process for treating a subject with cancer characterized by tumor cells expressing HLA-A2, said process comprising administering CTLs

induced by the processes of claims 21 or 22 in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

24. A process for treating a cancer-afflicted subject characterized by tumor cells expressing any class I MHC molecule and a gene coding for an epitopic sequence of at least one of SEQ ID NO: 1-16, whereby the CTLs of claim 21 are administered in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

25. The process for claims 19, 20, 23 or 24 wherein said cancer is carcinoma.

26. The process for claims 19, 20, 23 or 24 wherein said cancer is ovarian carcinoma.

27. A process for inducing a CTL response in a subject, said process comprising administering at least one immunogen of claim 1, 2, 3, 4, 5, 6, 7, 8 or 9, including combinations thereof, to an HLA-A1 positive subject and in an amount sufficient to induce a CTL response to tumor cells expressing HLA-A1.

28. A process for inducing a CTL response in a subject, said process comprising administering at least one immunogen of claim 1, 2, 3, 4, 5, 6, 7, 8 or 9, including combinations thereof, to an HLA-A2 positive subject and in an amount sufficient to induce a CTL response to tumor cells expressing HLA-A2.

SEQUENCE LISTING

<110> Ramakrishna, Venky
Ross, Mark
Philip, Ramila

<120> Cytotoxic T-Lymphocyte-Inducing Immunogens for Prevention, Treatment and Diagnosis of Cancer

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