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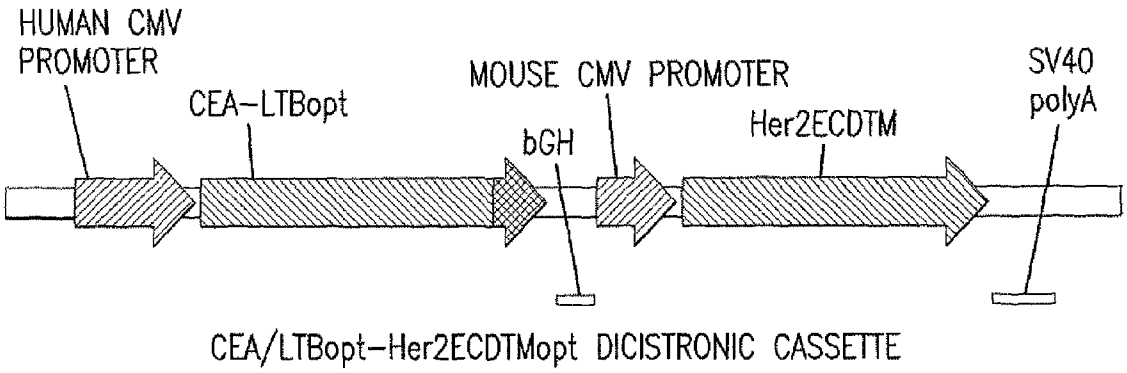
(54) **Title:** MULTI-ANTIGEN CONSTRUCT AND USES THEREOF



(57) **Abstract:** Dicistronic adenovirus and plasmid vectors expressing both the carcinoembryonic antigen (CEA) tumor-associated antigen, or portion thereof, and the human epidermal growth factor 2/neu antigen (HER2/neu), or portion thereof, are provided. The present invention also provides methods for inducing an immune response against CEA and/or HER2-associated tumors using the compositions and molecules disclosed herein. The CEA and HER2 genes encode antigens that are commonly associated with the development of human carcinomas. The present invention provides compositions and methods to elicit or enhance immunity to the protein products expressed by the CEA and/or HER2/neu tumor-associated antigens, wherein aberrant CEA and/or HER2 expression is associated with a carcinoma or its development. The dicistronic vectors of the present invention are useful in vaccines and pharmaceutical compositions for the prevention and/or treatment of cancer.

WO 2008/012237 A1

TITLE OF THE INVENTION
MULTI-ANTIGEN CONSTRUCT AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/832,980 filed July 24, 2006, the contents of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates generally to the therapy of cancer. More specifically, the present invention relates to dicistronic adenovirus and plasmid vectors expressing both the carcinoembryonic antigen (CEA) tumor-associated antigen, or portion thereof, and the human epidermal growth factor 2/neu antigen (HER2/neu), or portion thereof. The present invention also provides methods for inducing an immune response against CEA and/or HER2-associated tumors using the compositions and molecules disclosed herein.

BACKGROUND OF THE INVENTION

Vaccination has become a standard procedure for the prevention of numerous infectious diseases. The use of vaccines for the prevention and/or treatment of other types of diseases, such as cancer, is now an attractive possibility due to recent advances in molecular engineering and an increased understanding of tumor immunology.

Cancer is one of the leading causes of mortality worldwide. Despite an abundance of cancer-related research, conventional therapies that combine surgery, radiation, and chemotherapy, often fail to effectively treat established cancers. Reliable methods of prevention also remain unavailable.

Cancer typically involves the malfunction of genes that encode products that contribute to the regulation of the cell cycle or cell proliferation, such as growth factors and their receptors, oncogenes, and tumor suppressor genes. Many of these gene products are expressed on the surface of a variety of tumor cells; and, hence, are designated tumor-associated antigens (TAAs). The introduction of genes encoding TAAs directly into a subject has been shown to generate a protective immune response against the TAA in many experimental models, making these molecules a target for vaccine therapy. However, because many of these gene products are also expressed in normal cells, albeit at lower levels, many immunological therapies targeting TAAs have proven ineffective due to self-tolerance.

Genes coding for several TAAs have been isolated, characterized, and inserted into genetic vectors, such as plasmid DNA and viral vectors. Two tumor-associated antigens that have been implicated in the pathogenesis of cancer are epidermal growth factor 2, which is a transmembrane tumor associated antigen encoded by the HER2/neu proto-oncogene (also called c-erbB-2), and carcinoembryonic antigen (CEA).

Low levels of expression of the HER2/neu transcript and the encoded 185 kD protein are normally detected in adult epithelial cells of various tissues, including the skin and breast, and tissues of the gastrointestinal, reproductive, and urinary tracts (Press et al., *Oncogene* 5: 953-962 (1990)). Higher levels of HER2/neu expression are also detected in the corresponding fetal tissues during embryonic development (Press et al., *supra*).

Several observations make the HER2 antigen an attractive target for active specific immunotherapy. First, the HER2/neu gene is commonly overexpressed or amplified in various malignancies, such as carcinomas of the breast, ovary, uterus, colon, and prostate, and adenocarcinomas of the lung (reviewed in Disis and Cheever, *Adv. Cancer Research* 71: 343-371 (1997)). Overexpression of HER2/neu correlates with a poor prognosis and a higher relapse rate for cancer patients (Slamon et al., *Science* 244: 707-712 (1989)). Amplification of human HER2 leads to enhanced MAP kinase activity and cell proliferation, and contributes to the aggressive behavior of tumor cells (Ben-Levy et al. *Embo J* 13(14): 3302-11 (1994)). The high expression level of HER2 observed in tumors is in direct contrast with the low levels associated with normal adult tissues.

Additionally, many cancer patients suffering from malignancies associated with HER2/neu overexpression have had immune responses against the HER2 protein. Anti-hHER2 cytotoxic T lymphocytes (CTL) have been isolated from breast and ovarian cancer patients (Ioannides et al. *Cell Immunol* 151(1): 225-34 (1993); Peoples et al. *Proc Natl Acad Sci U S A* 92 (14): 6547-51 (1995)). Several HLA-A2.1-associated hHER2 peptides have been defined and peptide-specific T cells can be generated *in vitro* (Fisk et al. *Cancer Res* 57(1): 8-93 (1997); Yoshino et al. *Cancer Res* 54(13): 3387-90 (1994); Lustgarten et al. *Hum Immunol* 52(2): 109-18 (1997)).

The above findings demonstrate that anti-erbB-2 immune effector mechanisms are activated in cancer patients and highlight the potential benefit of enhancing such immune reactivity. An effective vaccine exploiting the immune response to HER2/neu must both enhance this immunity to a level that is protective and/or preventive and overcome self-tolerance.

Based on the above recitation, HER2/neu has been pursued as a target for the development of immunological treatments of malignancies. Anti-HER2 monoclonal antibodies have been investigated as therapies for breast cancer, with each antibody approach demonstrating various levels of success (for discussion, *see* Yarden, *Oncology* 61(suppl 2): 1-13 (2001)).

Additionally, DNA and peptide-based vaccines targeting HER2/neu have been reported. Amici et al. (U.S. Patent No. 6,127,344) disclose a method for inducing immunity against HER2/neu by administering an expression vector comprising the full-length human HER2/neu cDNA functionally linked to the human cytomegalovirus promoter. Morris et al. (WO 2004/041065) disclose a method of vaccination with dendritic cells modified by adenoviral vectors expressing a non-signaling HER2/neu gene. Cheever and Disis disclose methods for immunizing humans against HER2/neu-associated cancers by administration of HER2 peptides

(U.S. Patent No. 5,846,538). Additionally, HER2/neu peptide-based vaccines have been studied in rodent models (*for review, see* Disis and Cheever, *Adv. Cancer Res.* 71:343-71 (1997)).

Vectors encoding human HER2 and/or the extracellular and transmembrane domains of HER, and their use as vaccines have been disclosed (WO 05/012527), as have vectors encoding rhesus monkey HER2 (WO 04/061105) for use as vaccines.

In addition to the HER2 gene, the CEA gene is a TAA commonly associated with cancer. The CEA gene encodes a protein that acts as an intercellular adhesion molecule (Benchimol et al., *Cell* 57: 327-334 (1989)). CEA can also inhibit cell death resulting from detachment of cells from the extracellular matrix and can contribute to cellular transformation associated with certain proto-oncogenes such as *Bcl2* and *C-Myc* (*see* Berinstein, *J. Clin Oncol.* 20(8): 2197-2207 (2002)).

CEA is normally expressed during fetal development and in adult colonic mucosa. Aberrant CEA expression has long been correlated with many types of cancers, with the first report describing CEA overexpression in human colon tumors over thirty years ago (Gold and Freedman, *J. Exp. Med.* 121:439-462 (1965)). Overexpression of CEA has since been detected in nearly all colorectal tumors, as well as in a high percentage of adenocarcinomas of the pancreas, liver, breast, ovary, cervix, and lung. Moreover, it was demonstrated in transgenic mice immunized with a recombinant *vaccinia* vector expressing CEA that anti-CEA immune responses could be elicited without inducing autoimmunity, making CEA a particularly attractive target for active and passive cancer immunotherapy (Kass et al. *Cancer Res.* 59: 676-83 (1999)).

Therapeutic approaches targeting CEA include the use of anti-CEA antibodies (*see* Chester et al., *Cancer Chemother. Pharmacol.* 46 (Suppl): S8-S12 (2000)), as well as CEA-based vaccines (for review, *see* Berinstein, *supra*).

The development and commercialization of many vaccines have been hindered by an inability to generate an immune response against the target antigen of sufficient magnitude in treated individuals. Although DNA vaccines targeting various proteins have been developed, the resulting immune responses have been relatively weak compared with conventional vaccines. Also, vaccines targeting tumor-associated antigens are commonly ineffective or suffer limited efficacy due to immunotolerance. Thus, there is a need for vaccines that can elicit an immune response against TAAs that are efficacious and not hindered by self-tolerance.

SUMMARY OF THE INVENTION

As stated above, the carcinoembryonic antigen (CEA) and the HER2/neu genes are commonly associated with the development or presence of adenocarcinomas, including colorectal carcinomas. To this end, the present invention relates to compositions and methods to elicit or enhance immunity to the protein products expressed by the CEA and/or HER2/neu genes. Specifically, the present invention provides expression vectors comprising a first sequence of nucleotides and a second sequence of nucleotides; wherein the first sequence of nucleotides

encodes a human CEA protein, or variant thereof, and the second sequence of nucleotides encodes a human HER2/neu protein, or variant thereof. The expression vectors described herein are useful as therapeutic vaccines in individuals suffering from cancer, particularly those individuals in which CEA and/or HER2 are overexpressed at the tumor site. Said vaccines are useful as a monotherapy or as part of a therapeutic regime, said regime comprising administration of a second genetic vaccine or other vaccine such as a cell-based, protein, or peptide-based vaccine, or comprising radiotherapy or chemotherapy.

In preferred embodiments of the expression vector described herein, the first and/or second sequence of nucleotides comprises codons that have been optimized for high levels of expression in a human host cell.

The present invention further provides an adenoviral vector comprising a first sequence of nucleotides and a second sequence of nucleotides; wherein the first sequence of nucleotides encodes a human CEA protein, or variant thereof, and the second sequence of nucleotides encodes a human HER2/neu protein, or variant thereof. In further embodiments of the present invention, the use of the adenovirus vector in immunogenic compositions and vaccines for the prevention and/or treatment of CEA and or HER2-associated cancer is provided. In especially preferred embodiments of this portion of the invention, the adenovirus vector is an Ad6 vector.

In preferred embodiments of the present invention, the first sequence of nucleotides encodes a variant of a human CEA protein fused to the B subunit of the heat labile enterotoxin of *E.coli* (LTB), or substantial portion thereof, wherein the CEA portion of the encoded CEA fusion protein is deleted of its C-terminal anchoring domain.

In further preferred embodiments, the second sequence of nucleotides encodes a variant of a human HER2/neu protein, wherein the variant HER2 protein is a truncated form of human HER2 protein which comprises the extracellular and transmembrane domains of the HER2 protein, but not the intracellular domain.

The immunogenicity of an exemplary expression vector of the present invention comprising an Ad6 vector comprising nucleotide sequences encoding a CEA-LTB fusion as well as the extracellular and transmembrane domains of HER2 (HER2ECDTM) was confirmed in accordance with the present invention. The elicited immune response was measured in two different strains of mice against both the encoded antigens and in mice double-transgenic for CEA and HER2/neu, thus resulting in breakage of immune tolerance. Also determined in accordance with the present invention was the immunogenicity of an exemplary Ad6 vector encoding a variant of CEA and a variant of HER2 in immunodeficient mice that were engrafted with cells of the human immune system, thus contributing to the conclusion that the vectors described herein would be useful as a vaccine in human patients in need of treatment for HER2 and/or CEA-associated cancers.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

As used throughout the specification and appended claims, the following definitions and abbreviations apply:

The term "promoter" refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers".

The term "cassette" refers to a nucleotide or gene sequence that is to be expressed from a vector, for example, the nucleotide or gene sequence encoding the hCEA-LTB fusion or the nucleotide sequence encoding the HER2 extracellular and transmembrane domains (hereinafter HER2ECDTM). In general, a cassette comprises a gene sequence that can be inserted into a vector, which in some embodiments, provides regulatory sequences for expressing the nucleotide or gene sequence. In other embodiments, the nucleotide or gene sequence provides the regulatory sequences for its expression. In further embodiments, the vector provides some regulatory sequences and the nucleotide or gene sequence provides other regulatory sequences. For example, the vector can provide a promoter for transcribing the nucleotide or gene sequence and the nucleotide or gene sequence provides a transcription termination sequence. The regulatory sequences that can be provided by the vector include, but are not limited to, enhancers, transcription termination sequences, splice acceptor and donor sequences, introns, ribosome binding sequences, and poly(A) addition sequences.

The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, virus (including adenovirus), bacteriophages and cosmids.

The term "first generation," as used in reference to adenoviral vectors, describes adenoviral vectors that are replication-defective. First generation adenovirus vectors typically have a deleted or inactivated E1 gene region, and preferably have a deleted or inactivated E3 gene region.

The abbreviation "LT" refers generally to the heat labile enterotoxin of *E. coli*. "LT" may refer to the complete enterotoxin, comprising subunits A and B or a substantial portion of subunit A, or a substantial portion of subunit B. The abbreviation "LTB" refers to the B subunit of the heat labile enterotoxin of *E. coli*, or substantial portion thereof, including subunits which are truncated on the C-terminal or N-terminal end but maintain biological activity, as well as subunits that contain internal amino acid insertions, deletions, or substitutions but maintain biological activity.

The designation "Ad6 CEA-LTBopt.HER2ECDTM" refers to a construct, disclosed herein, which comprises an Ad6 adenoviral genome deleted of the E1 and E3 regions.

In the "Ad6 CEA-LTBopt.HER2ECDTM" construct, the E1 region is replaced by a codon-optimized human CEA-LTB gene in an E1 parallel orientation under the control of a human CMV promoter without intron A, followed by a bovine growth hormone polyadenylation (bGH) signal. Specifically, the codon-optimized human CEA sequence is devoid of the GPI anchor coding sequence and is fused at its C-terminus to the B subunit of *E. coli* heat labile enterotoxin. Proceeding in a 5' to 3' direction, following the bGH polyadenylation signal is a mouse CMV promoter, followed by a sequence of nucleotides that encodes the extracellular and transmembrane domains of HER2/neu (HER2ECDTM). Construction of the Ad6 dicistronic vector comprising CEA-LTB and HER2ECDTM nucleotide sequences is described in EXAMPLE 1.

As used herein, a "fusion protein" refers to a protein having at least two polypeptides covalently linked in which one polypeptide comes from one protein sequence or domain and the other polypeptide comes from a second protein sequence or domain. The fusion proteins of the present invention comprise a human CEA polypeptide or fragment or variant thereof, and a second polypeptide, which comprises the B subunit of *E. coli* heat labile enterotoxin (LT). CEA-LTB fusion proteins of the present invention are preferably linked N-terminus to C-terminus. The CEA polypeptide and the LTB polypeptide can be fused in any order. In preferred embodiments of this invention, the C-terminus of the CEA polypeptide is fused to the N-terminus of LTB, as exemplified in FIGURE 9. However, CEA-LTB fusion proteins in which the LTB polypeptide is fused to the N-terminus of the CEA polypeptide are also contemplated.

The term "CEA-LTB fusion" refers to a nucleic acid sequence in which at least a portion of the CEA gene is fused to a substantial portion of the LTB subunit of *E. coli* heat labile enterotoxin. The term "CEA-LTB fusion protein" refers to a polypeptide encoded by a CEA-LT fusion as described. The terms "CEA-LTB fusion" and "CEA-LTB fusion protein" are also understood to refer to fragments thereof, homologs thereof, and functional equivalents thereof (collectively referred to as "variants"), such as those in which one or more amino acids is inserted, deleted or replaced by other amino acid(s). The CEA-LT fusions of the present invention, upon administration to a mammal such as a human being, can stimulate an immune response by helper T cells or cytotoxic T cells, or stimulate the production of antibodies at least as well as a "wild-type" CEA sequence. In preferred embodiments of the invention, the CEA-LTB fusions can enhance the immune response as compared to a wild-type CEA.

The abbreviation "AD" refers to the anchoring domain of a CEA gene or protein. The anchoring domain of the wild-type human CEA is located from about amino acid 679 to about amino acid 702 of SEQ ID NO:6 (FIGURE 10).

The term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with the molecules of the present invention, including the nucleic acid molecules described herein and the fusion proteins that are encoded by said nucleic acid molecules. Encompassed by the term "disorder" are chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. The molecules of the present invention are intended for use as treatments for disorders or conditions characterized by aberrant cell proliferation, including, but not limited to, breast cancer, colorectal cancer, and lung cancer.

The term "effective amount" means sufficient vaccine composition is introduced to produce the adequate levels of the polypeptide, so that a protective or therapeutic immune response results. One skilled in the art recognizes that this level may vary.

A "conservative amino acid substitution" refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (e.g., arginine for lysine; glutamic acid for aspartic acid).

"hCEA" and "hCEAopt" refer to a human carcinoembryonic antigen and a human codon-optimized carcinoembryonic antigen, respectively.

"hHER2.wt" and "hHER2.opt" refer to a human epidermal growth factor 2 antigen and a human codon-optimized epidermal growth factor 2 antigen, respectively.

"hHER2ECDTM.wt" and "hHER2ECDTM.opt" refer to a truncated human epidermal growth factor 2 antigen and a truncated human codon-optimized epidermal growth factor 2 antigen, respectively. The truncated forms of HER2, "hHER2ECDTM.wt" and "hHER2ECDTM.opt," comprise the extracellular and transmembrane domains of the human HER2 protein.

"Substantially similar" means that a given nucleic acid or amino acid sequence shares at least 75%, preferably 85%, more preferably 90%, and even more preferably 95% identity with a reference sequence. In the present invention, the reference sequence can be relevant portions of the wild-type human CEA nucleotide or amino acid sequence, the wild-type HER2 or HER2ECDTM nucleotide or amino acid sequence, or the wild-type nucleotide or amino acid sequence of the LTB subunit of the *E.coli* heat labile enterotoxin, as dictated by the context of the text. Thus, a CEA protein sequence that is "substantially similar" to the wild-type human CEA protein or fragment thereof will share at least 75% identity with the relevant fragment of the wild-type human CEA, along the length of the fragment, preferably 85% identity, more preferably 90% identity and even more preferably 95% identity. Whether a given CEA, HER2, or LTB protein or nucleotide sequence is "substantially similar" to a reference sequence can be determined for example, by comparing sequence information using sequence analysis software such as the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman

and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2:482, 1981).

A "substantial portion" of a gene, variant, fragment, or subunit thereof, means a portion of at least 50%, preferably 75%, more preferably 90%, and even more preferably 95% of a reference sequence.

A "gene" refers to a nucleic acid molecule whose nucleotide sequence codes for a polypeptide molecule. Genes may be uninterrupted sequences of nucleotides or they may include such intervening segments as introns, promoter regions, splicing sites and repetitive sequences. A gene can be either RNA or DNA. A preferred gene is one that encodes the invention peptide.

The term "nucleic acid" or "nucleic acid molecule" is intended for ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), probes, oligonucleotides, fragment or portions thereof, and primers. DNA can be either complementary DNA (cDNA) or genomic DNA, e.g. a gene encoding a CEA fusion protein.

"Wild-type protein" or "wt protein" refers to a protein comprising the major sequence of amino acids that occurs in nature, often designated as the reference sequence or "normal" allele. The amino acid sequence of wild-type human CEA is shown in FIGURE 10 (SEQ ID NO:6). "Wild-type CEA gene" refers to any gene comprising a sequence of nucleotides that encodes a naturally occurring CEA protein, including proteins of human origin or proteins obtained from another organism, including, but not limited to, other mammals such as rat, mouse and rhesus monkey. The amino acid sequence of wild-type human HER2 protein is shown in FIGURE 11 (SEQ ID NO:7) and has been reported (Coussens et al., *Science* 230: 1132-39 (1985); King et al., *Science* 229: 974-76 (1985)).

The term "mammalian" refers to any mammal, including a human being.

The abbreviation "Ag" refers to an antigen.

The term "TAA" refers to a tumor-associated antigen.

The abbreviation "ORF" refers to the open reading frame of a gene.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a schematic representation of an exemplary Ad6-CEA-HER2 dicistronic cassette of the present invention, as described in EXAMPLE 1. The two expression cassettes are oriented in tandem and hCMV and mCMV drive the expression of CEA-LTB and Her2 ECD.TM, respectively.

FIGURE 2 shows the results of an analysis of the genomic stability of the MRKAd6 CEA-LTB/HER2-ECDTM construct. Three plaque isolates were amplified and repeatedly passaged in PERC6 cells prior to analysis of the genomic structure, as described in EXAMPLE 1. Restriction digest of the plaque isolates was compared to that of the bulk vector preparation and of the original pre-adeno plasmid.

FIGURE 3 demonstrates the immunogenic potency of MRKAd6 CEA-LTB/HER2-ECDTM. Groups of BALB/c and C57BL/6 mice were immunized with two injections of MRKAd6 vectors encoding the human CEA-LTB and HER2-ECDTM (10^9 and 10^8 vp, see EXAMPLE 2). Immune response was measured by IFNγ ELIspot assay two weeks after the last injection.

FIGURE 4 shows a comparison of an exemplary Ad6 dicistronic (MRKAd6 CEA-LTB/HER2-ECDTM) vector to monocistronic vectors encoding HER2ECDTM and CEA. C57BL/6 mice (5-10/group) received two injections of 10^8 vp, two weeks apart. Two weeks later, mice were sacrificed and splenocytes were analyzed by intracellular staining upon stimulation with pools of peptides covering CEA, LTB and the ECD.TM portion of Her2/Neu. Panel A shows the CD8+ specific immune response; and panel B shows the CD4+ specific immune response. Black dots represent the immune response per each single mouse; white circles represent the geometric mean of the group.

FIGURE 5 demonstrates that MRKAd6 CEA-LTB/HER2-ECDTM can break tolerance to both target antigens. Groups of 5 CEA/HER2 double transgenic mice were immunized with 10^{10} vp of the MRKAd6 vector encoding both target antigens. Immune response was measured by IFNγ intracellular staining on mouse splenocytes 14 days after the last injection. Average values are also shown (black line).

FIGURE 6 shows the immune response elicited by MRKAd6 CEA-LTB/HER2-ECDM in engrafted NOD/scid–DR1 mice. Mice engrafted intrahepatically with CBMNC 5 days after birth received two injections of 10^{10} vp dicistronic Ad6, two weeks apart. Intracellular staining for IFNγ was performed three weeks after the last boost. CEA and Neu responses were measured with 15mer peptides covering the antigen sequence. PMA at 5 ng/ml was added as costimulus.

FIGURE 7 shows the immune response elicited by MRKAd6 CEA-LTB/HER2-ECDM in engrafted NOD/scid–DR1 mice. Mice engrafted under the kidney capsule with CBMNC, received two injections of 10^{10} vp dicistronic Ad6 two weeks apart. Intracellular staining for IFNγ was performed two weeks after last boost. CEA and Neu responses were measured with 15mer peptides covering the antigen sequence. PMA at 5 ng/ml was added as costimulus.

FIGURE 8, panel A, shows the nucleotide sequence of a codon-optimized polynucleotide (hHER2ECDTM.opt, SEQ ID NO:1) that encodes a truncated human HER2 protein, said protein comprising the extracellular and transmembrane domains of the HER2 protein. Panel B shows a second polynucleotide that encodes the extracellular and transmembrane domains of the HER2 protein, the second polynucleotide comprising "wild-type" nucleotide sequences, which have not been codon optimized (hHER2ECDTM.wt, SEQ ID NO:2).

FIGURE 9 shows the nucleotide sequence (SEQ ID NO:3) of an exemplary hCEA-LTB fusion (Panel A). Also shown is the nucleotide sequence of an exemplary hCEAopt-LTB fusion (SEQ ID NO:4, Panel B). Panel C shows the nucleotide sequence (SEQ ID NO:5) of an exemplary fully optimized hCEA-LTB fusion, designated herein hCEAopt-LTBopt. Nucleotide sequences encoding LTB are shown in bold. Junction sequences, created by the cloning strategy employed to fuse the CEA and LTB sequences are underlined.

FIGURE 10 shows the amino acid sequence of wild-type human CEA (SEQ ID NO:6), which was previously described (*see, e.g.,* U.S. Patent No. 5,274,087).

FIGURE 11 shows the amino acid sequence of the wild-type human HER2 protein (SEQ ID NO:7; *see, e.g.* Coussens et al., *Science* 230: 1132-39 (1985); King et al., *Science* 229: 974-76 (1985)).

FIGURE 12 shows the nucleotide sequence of a portion of the wild-type human CEA cDNA from nt 1 to nt 2037 (SEQ ID NO:8, Panel A), encoding a portion of the hCEA protein from aa 1 to aa 679 (SEQ ID NO:9, Panel B).

DETAILED DESCRIPTION OF THE INVENTION

Carcinoembryonic antigen (CEA) and HER2/neu are commonly associated with the development of adenocarcinomas. The present invention relates to compositions and methods to elicit or enhance immunity to the protein product expressed by the CEA and/or HER2/neu tumor-associated antigens, wherein aberrant CEA and/or HER2 expression is associated with the carcinoma or its development. Association of aberrant CEA and/or HER2 expression with a carcinoma does not require that the CEA and/or HER2 protein be expressed in tumor tissue at all timepoints of its development, as abnormal CEA and/or HER2 expression may be present at tumor initiation and not be detectable late into tumor progression or vice-versa.

To this end, the present invention provides compositions and methods to elicit or enhance immunity to the protein products expressed by the CEA and/or HER2/neu genes. Specifically, the present invention provides expression vectors comprising a first sequence of nucleotides and a second sequence of nucleotides; wherein the first sequence of nucleotides encodes a human CEA protein, or variant thereof, and the second sequence of nucleotides encodes a human HER2/neu protein, or variant thereof. It is believed that the dicistronic vectors of the present invention, which comprise nucleotide sequences encoding two tumor-associated antigens (specifically CEA and HER2/neu) will elicit a more efficacious immune response than a vaccine that targets only a single TAA. Because the gene expression pattern of a tumor may be modified as a result of immune selection, a tumor that initially expresses one TAA at tumor initiation or during early tumor development, may not express that same TAA as tumor development progresses. Likewise, a tumor that expresses numerous tumor markers at one time point may lose expression of one or more tumor antigens later in time. Thus, a vaccine that elicits an immune response against more than one TAA would be more likely to induce tumor regression

and/or halt tumor growth than a vaccine that targets only a single TAA because a tumor that expresses any one of the TAA's targeted by the vaccine would be susceptible to lysis by CTLs and/or antibodies. Additionally, the dicistronic vectors of the present invention are more cost-effective to develop as vaccines, requiring less production materials (e.g. controls) than the use of multiple monocistronic vectors.

The expression vectors described herein are useful as therapeutic vaccines in individuals suffering from cancer, particularly those individuals in which CEA and/or HER2 are overexpressed at the tumor site. Said vaccines are useful as a monotherapy or as part of a therapeutic regime, said regime comprising administration of a second genetic vaccine or other vaccine such as a cell-based, protein, or peptide-based vaccine, or comprising radiotherapy or chemotherapy.

The CEA and HER2 nucleotide sequences of the present invention encode the full-length human CEA and human HER2 proteins; or variants thereof, as stated above. Contemplated variants include, but are not necessarily limited to: sequences that are C- or N-terminally truncated, sequences with conservative substitutions, and sequences with internal deletions or insertions; relative to the wild-type CEA and HER2 protein sequences. The encoded CEA and HER2 protein variants of the present invention must be sufficient to elicit a CEA or HER2-specific immune response in a patient in need thereof. Reference sequences for the wt human CEA and wt human HER2 have been reported (*see, e.g.,* U.S. Patent No. 5,274,087; U.S. Patent No 5,571,710; and U.S. Patent No 5,843,761; Coussens et al., *supra*; King et al., *supra*).

In preferred embodiments of the present invention, the full length CEA gene, or variant thereof, is fused in-frame to nucleotide sequences encoding the B subunit of *E. coli* heat labile enterotoxin (LTB). Exemplary CEA-LTB sequences are shown in FIGURES 9A – 9C and set forth as SEQ ID NOs: 3-5. The LTB subunit, or substantial portion thereof, of the CEA-LTB fusion of may be fused to the amino terminus or the carboxy terminus of the CEA sequence. Further, the LTB sequence and the CEA sequence can be fused N-terminus to N-terminus, C-terminus to C-terminus, C-terminus to N-terminus or N-terminus to N-terminus. In preferred embodiments of the present invention, the C-terminus of the CEA polypeptide is fused to the N-terminus of LTB.

As stated above, contemplated for use in the dicistronic vectors of the present invention are nucleotide sequences encoding variants or mutants of the CEA, HER2 and/or LTB sequences described herein. Any such biologically active fragment and/or mutant will encode either a protein or protein fragment which at least substantially mimics the pharmacological properties of the hCEA protein and the hHER2 protein, including but not limited to the hCEAΔAD protein as set forth in SEQ ID NO:9 and the HER2ECDTM protein encoded by the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:2. The CEA and/or HER2 variants encoded by the nucleotide sequences in the dicistronic vectors of the present invention are sufficient to elicit an immune response against CEA and HER2. The dicistronic vectors of this

embodiment of the present invention are, therefore, useful as prophylactic and/or therapeutic vaccines against cancers that overexpress CEA and/or HER2.

In exemplary embodiments of this aspect of the present invention, an LTB variant is fused to the CEA sequence of the CEA fusion, wherein the LTB variant is truncated of its signal sequence (*see, e.g.* SEQ ID NO's: 3-5). While not being bound by theory, deletion of the toxin signal sequence, e.g. the LTB signal sequence, ensures that posttranslational processing of the CEA fusion is driven by the CEA signal sequence.

In preferred embodiments of the present invention, the CEA protein of the expression vector is human CEA (SEQ ID NO: 6) or a functional equivalent thereof, for example, a human CEA deleted of its C-terminal anchoring domain (CEAΔAD) (SEQ ID NO: 9), which is located from about amino acid 679 to about amino acid 702 of the full-length human CEA. While not being bound by theory, deletion of the anchoring domain increases secretion of the CEA fusion protein, thereby enhancing cross priming of the CEA-LTB immune response.

The dicistronic vectors of the present invention comprise nucleotide sequences expressing CEA, HER2 and/or variants thereof that are assembled into an expression cassette. The cassette preferably contains CEA and HER2 protein-encoding gene sequences, with related transcriptional and translational control sequences operatively linked to it, such as a promoter, and termination sequences.

To that end, in further preferred embodiments of the present invention, the expression of the CEA nucleotide sequence, or CEA variant thereof, is driven by a first promoter while the expression of the HER2 nucleotide sequence, or HER2 variant thereof, is driven by a second promoter. In particularly preferred embodiments of the invention, the first promoter and the second promoter are not the same. While not wishing to be bound by theory, the use of two different promoters reduces the possibility of internal recombination events and avoids the reduction of gene expression due to squelching of transcription factors. In exemplary embodiments of the invention described herein, expression of a CEA-LTB nucleotide sequence, for example CEA-LTBopt (SEQ ID NO:5), is controlled by a human cytomegalovirus (CMV) promoter and expression of a HER2ECDTM nucleotide sequence, for example HER2ECDTMopt (SEQ ID NO:1), is controlled by a mouse CMV promoter. In a specific exemplary embodiment of the invention described herein, CEA-LTBopt expression is driven by the CMV immediate-early (IE) promoter and expression of HER2ECDTM is driven by the murine CMV promoter. One of skill in the art will recognize that any of a number of other known promoters may be chosen for purposes of driving expression of the CEA and HER2 nucleotide sequences of the present invention. Additional examples of promoters include naturally occurring promoters such as the EF1 alpha promoter, Rous sarcoma virus promoter, and SV40 early/late promoters and the p-actin promoter; and artificial promoters such as a synthetic muscle specific promoter and a chimeric muscle- specific/CMV promoter (Li et al., *Nat. Biotechnol.* 17:241-245 (1999); Hagstrom et al., *Blood* 95:2536-2542 (2000)).

In addition to CEA and HER2 encoding sequences and promoter sequences, the expression cassette of the dicistronic vector of the present invention comprises a termination sequence(s)/polyadenylation signal following the gene-encoding nucleotide sequences. The polyadenylation signal is responsible for cleaving the transcribed RNA and the addition of a poly (A) tail to the RNA. The poly (A) tail is important for the mRNA processing.

Polyadenylation signals that can be used as part of a gene expression cassette include the minimal rabbit -globin polyadenylation signal and the bovine growth hormone polyadenylation (BGH). (Xu et al., *Gene* 272: 149-156 (2001), Post et al., U.S. Patent U. S. 5,122,458.) Additional examples include the Synthetic Polyadenylation Signal (SPA) and SV40 polyadenylation signal. In preferred embodiments of the present invention, nucleotide sequences coding for the CEA and HER2 variants are followed by the BGH and SV40 polyadenylation signals.

Also included within the scope of the present invention are dicistronic vectors comprising variant nucleotide sequences that encode CEA and HER2, or variants thereof, the variant nucleotide sequences including but not necessarily limited to: nucleotide substitutions, deletions, additions, amino-terminal truncations and carboxy-terminal truncations. In some cases, it may be advantageous to add specific point mutations to the nucleotide sequences encoding the TAA or variant thereof to reduce or eliminate toxicity of the encoded protein. Also included within the scope of this invention are mutations in the DNA sequence that do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in the functionality of the polypeptide. The mutations of the present invention encode mRNA molecules that express a CEA, CEA fusion protein, HER2, or variants thereof that are sufficient to elicit an immune response against CEA and/or HER2.

In some embodiments of this aspect of the present invention, the dicistronic vectors comprise nucleotide sequences that are codon-optimized for high level expression in a human host cell. In these embodiments, at least a portion of the codons of the CEA and/or HER2 nucleotide sequence within the dicistronic vector are designed so as to use the codons preferred by the projected host cell, which in preferred embodiments is a human cell. The dicistronic vectors comprising codon-optimized sequences may be used for the development of recombinant adenovirus or plasmid-based DNA vaccines and immunogenic compositions, which provide effective immunoprophylaxis against CEA and/or HER2-associated cancer through neutralizing antibody and cell-mediated immunity.

To this end, in accordance with this embodiment of the present invention, the dicistronic vectors comprise nucleic acid sequences that encode a desired CEA, HER2, or variant thereof, and that are converted to a polynucleotide sequence having an identical translated sequence but with alternative codon usage as described by Lathe, "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data: Theoretical and Practical Considerations" *J.*

Molec. Biol. 183:1-12 (1985), which is hereby incorporated by reference. The methodology generally consists of identifying codons in the wild-type sequence that are not commonly associated with highly expressed human genes and replacing them with optimal codons for high expression in human cells. The new gene sequence is then inspected for undesired sequences generated by these codon replacements (e.g., "ATTTA" sequences, inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, etc.). Undesirable sequences are eliminated by substitution of the existing codons with different codons coding for the same amino acid. The synthetic gene segments are then tested for improved expression.

The methods described above were used to create synthetic gene sequences, described herein, which encode CEA and/or HER2 and form a portion of the expression cassette of the dicistronic vectors of the present invention, resulting in an expression vector comprising a gene or genes comprising codons optimized for high level expression. While the above procedure provides a summary of our methodology for designing codon-optimized genes for use in cancer vaccines, it is understood by one skilled in the art that similar vaccine efficacy or increased expression of genes may be achieved by minor variations in the procedure or by minor variations in the sequence.

In preferred embodiments of the present invention, the sequence of nucleotides that encodes the CEA protein, or variant or fusion thereof, is codon-optimized for high-level expression in human cell (*see, e.g.* FIGURE 9). In other preferred embodiments, the nucleotide sequence encoding the HER2 protein or HER2 variant, such as HER2ECDTM is codon-optimized for high-level expression in human cells (*see, e.g.* FIGURE 8A). In still further preferred embodiments, both the first sequence of nucleotides and the second sequence of nucleotides, encoding the CEA and HER2 proteins or variants are codon-optimized for high-level expression in human cells.

The present invention also relates to host cells transformed or transfected with the dicistronic vectors of the present invention. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of bovine, porcine, monkey and rodent origin; and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Such recombinant host cells can be cultured under suitable conditions to produce a CEA and HER2 protein, or biologically equivalent variants. In a preferred embodiment of the present invention, the host cell is human. As defined herein, the term "host cell" is not intended to include a host cell in the body of a transgenic human being, human fetus, or human embryo.

The dicistronic vectors of the present invention are preferably adenoviral or plasmid vectors, although linear DNA linked to a promoter, or other vectors, such as adeno-associated virus or a modified vaccinia virus, retroviral or lentiviral vector may also be used.

In preferred embodiment of the invention, the vector is an adenovirus vector (used interchangeably herein with "adenovector"). Adenovectors can be based on different adenovirus

serotypes such as those found in humans or animals. Examples of animal adenoviruses include bovine, porcine, chimp, murine, canine, and avian (CELO). Preferred adenovectors are based on human serotypes, more preferably Group B. C, or D serotypes. Examples of human adenovirus Group B. C, D, or E serotypes include types 2 ("Ad2"), 4 ("Ad4"), 5 ("Ad5"), 6 ("Ad6"), 24 ("Ad24"), 26 ("Ad26"), 34 ("Ad34") and 35 ("Ad35"). In particularly preferred embodiments of the present invention, the expression vector is an adenovirus type 6 (Ad6) vector.

If the vector chosen is an adenovirus, it is preferred that the vector be a so-called first-generation adenoviral vector. These adenoviral vectors are characterized by having a non-functional E1 gene region, and preferably a deleted adenoviral E1 gene region. Adenovectors do not need to have their E1 and E3 regions completely removed. Rather, a sufficient amount the E1 region is removed to render the vector replication incompetent in the absence of the E1 proteins being supplied in bans; and the E1 deletion or the combination of the E1 and E3 deletions are sufficiently large enough to accommodate a gene expression cassette.

In some embodiments, the expression cassette is inserted in the position where the adenoviral E1 gene is normally located. In addition, these vectors optionally have a non-functional or deleted E3 region. It is preferred that the adenovirus genome used be deleted of both the E1 and E3 regions (ΔE1ΔE3). The adenoviruses can be multiplied in known cell lines which express the viral E1 gene, such as 293 cells, or PERC.6 cells, or in cell lines derived from 293 or PERC.6 cell which are transiently or stably transformed to express an extra protein. For examples, when using constructs that have a controlled gene expression, such as a tetracycline regulatable promoter system, the cell line may express components involved in the regulatory system. One example of such a cell line is T-Rex-293; others are known in the art.

For convenience in manipulating the adenoviral vector, the adenovirus may be in a shuttle plasmid form. This invention is also directed to a shuttle plasmid vector which comprises a plasmid portion and an adenovirus portion, the adenovirus portion comprising an adenoviral genome which has a deleted E1 and optional E3 deletion, and has an inserted expression cassette comprising a first sequence of nucleotides that encodes a CEA protein, or variant thereof, and a second sequence of nucleotides that encodes a HER2 protein, or variant thereof. In preferred embodiments, there is a restriction site flanking the adenoviral portion of the plasmid so that the adenoviral vector can easily be removed. The shuttle plasmid may be replicated in prokaryotic cells or eukaryotic cells.

In a preferred embodiment of the invention, the expression cassette is inserted into the pMRKAd6-HV0 adenovirus plasmid (*See* Emini et al., WO2003031588A2), which is hereby incorporated by reference). This plasmid comprises an Ad6 adenoviral genome deleted of the E1 and E3 regions. Advantageously, this enhanced adenoviral vector is capable of maintaining genetic stability following high passage propagation.

Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the adenoviruses, shuttle plasmids, and DNA immunogens of this invention.

The dicistronic CEA-HER2 vectors of the present invention allow for the development of a therapeutic or prophylactic cancer vaccine by providing a dicistronic expression cassette that encodes both a CEA and a HER2 protein, or variants thereof, which can elicit an immune response against both the CEA and HER2 TAAs when administered to a mammal such as a human being. To this end, one aspect of the instant invention is a method of preventing or treating CEA and/or HER2-associated cancer comprising administering to a mammal a vaccine vector comprising a first sequence of nucleotides and a second sequence of nucleotides; wherein the first sequence of nucleotides encodes a human CEA protein, or variant thereof, and the second sequence of nucleotides encodes a human HER2/neu protein, or variant thereof.

In preferred embodiments of this aspect of the invention, the method comprises administering an expression vector comprising a first sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a B subunit of *E. coli* heat labile enterotoxin (LTB); and a second sequence of nucleotides that encodes a truncated human HER2/neu protein, wherein the truncated HER2 protein comprises the extracellular and transmembrane domains of HER2/neu.

In accordance with the method described above, the dicistronic vaccine vector may be administered for the treatment or prevention of a cancer in any mammal, including but not limited to: lung cancer, breast cancer, and colorectal cancer. In a preferred embodiment of the invention, the mammal is a human.

Further, one of skill in the art may choose any type of vector suitable for therapeutic administration for use in the treatment and prevention method described. Suitable vectors can deliver nucleic acid into a target cell without causing an unacceptable side effect.

Preferably, the vector is an adenovirus vector or a plasmid vector. In a preferred embodiment of the invention, the vector is an adenoviral vector comprising an adenoviral genome with a deletion in the adenovirus E1 region, and an insert in the adenovirus E1 region, wherein the insert comprises an expression cassette comprising: a first sequence of nucleotides and a second sequence of nucleotides; wherein the first sequence of nucleotides encodes a human CEA protein, or variant thereof, and the second sequence of nucleotides encodes a human HER2/neu protein, or variant thereof.

The instant invention further relates to an adenovirus vaccine vector comprising an adenoviral genome with a deletion in the E1 region, and an insert in the E1 region, wherein the insert comprises an expression cassette comprising: (a) a first sequence of nucleotides that encodes a human CEA protein, or variant thereof; (b) a second sequence of nucleotides that encodes a human HER2/neu protein, or variant thereof; (c) a first promoter operably linked to the first sequence of nucleotides and (d) a second promoter operably linked to the second sequence of

nucleotides. In preferred embodiments of this aspect of the invention, the first promoter and the second promoter are not the same. In further preferred embodiments of this aspect of the invention, the adenovirus vector is an Ad 6 vector.

In some embodiments of this invention, the recombinant adenovirus and plasmid-based polynucleotide vaccines disclosed herein are used in various prime/boost combinations in order to induce an enhanced immune response. In this case, the two vectors are administered in a "prime and boost" regimen. For example the first type of vector is administered one or more times, then after a predetermined amount of time, for example, 2 weeks, 1 month, 2 months, six months, or other appropriate interval, a second type of vector is administered one or more times. Preferably the vectors carry expression cassettes encoding the same TAAs or combination of TAAs, i.e. CEA and HER2. In the embodiment where a plasmid DNA is also used, it is preferred that the vector contain one or more promoters recognized by mammalian or insect cells. In a preferred embodiment, the plasmid would contain a strong promoter such as, but not limited to, the CMV promoter. The synthetic CEA fusion gene or other gene to be expressed would be linked to such a promoter. An example of such a plasmid would be the mammalian expression plasmid V1Jns as described (J. Shiver *et. al.* in *DNA Vaccines*, M. Liu et al. eds., N.Y. Acad. Sci., N.Y., 772:198-208 (1996), which is herein incorporated by reference).

Also contemplated within the scope of this invention are administration of the dicistronic vectors described herein as part of a therapeutic regime, said therapeutic regime including administration of a second dicistronic vector as described herein or, alternatively, administration of a second genetic vaccine or other vaccine such as a cell-based, protein, or peptide-based vaccine, or comprising radiotherapy or chemotherapy.

As stated above, an adenoviral vector vaccine and a plasmid vaccine may be administered to a vertebrate as part of a therapeutic regime to induce an immune response. To this end, the present invention relates to a method of treating a mammal with a CEA and/or HER2-associated cancer comprising: (a) introducing into the mammal a first vector comprising: i) a first sequence of nucleotides and a second sequence of nucleotides; wherein the first sequence of nucleotides encodes a human carcinoembryonic antigen (CEA) protein, or variant thereof, and the second sequence of nucleotides encodes a human HER2/neu protein, or variant thereof.; and ii) a promoter operably linked to the polynucleotide; (b) allowing a predetermined amount of time to pass; and (c) introducing into the mammal a second vector comprising: i) a first sequence of nucleotides and a second sequence of nucleotides; wherein the first sequence of nucleotides encodes a human carcinoembryonic antigen (CEA) protein, or variant thereof, and the second sequence of nucleotides encodes a human HER2/neu protein, or variant thereof and ii) a promoter operably linked to the polynucleotide.

In a preferred embodiment of the method of treatment described above, the first vector is a plasmid and the second vector is an adenovirus vector. In an alternative embodiment, the first vector is an adenovirus vector and the second vector is a plasmid.

In the method described above, the first type of vector may be administered more than once, with each administration of the vector separated by a predetermined amount of time. Such a series of administration of the first type of vector may be followed by administration of a second type of vector one or more times, after a predetermined amount of time has passed. Similar to treatment with the first type of vector, the second type of vector may also be given one time or more than once, following predetermined intervals of time.

The amount of expressible DNA or transcribed RNA to be introduced into a vaccine recipient will depend partially on the strength of the promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 10 μg to 300 μg of a plasmid vaccine vector is administered directly into muscle tissue. An effective dose for recombinant adenovirus is approximately $10^6 - 10^{12}$ particles and preferably about 10^7—10^{11} particles. Subcutaneous injection, intradermal introduction, impression though the skin, and other modes of administration such as intraperitoneal, intravenous, intramuscular or inhalation delivery are also contemplated.

In preferred embodiments of the present invention, the vaccine vectors are introduced to the recipient through intramuscular injection.

The vaccine vectors of this invention may be naked, i.e., unassociated with any proteins, or other agents which impact on the recipient's immune system. In this case, it is desirable for the vaccine vectors to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, it may be advantageous to administer an agent which assists in the cellular uptake of DNA, such as, but not limited to calcium ion. These agents are generally referred to as transfection facilitating reagents and pharmaceutically acceptable carriers. Those of skill in the art will be able to determine the particular reagent or pharmaceutically acceptable carrier as well as the appropriate time and mode of administration.

It is a common goal of vaccine development to augment the immune response to the desired antigen to induce long lasting protective and therapeutic immunity. Co-administration of vaccines with compounds that can enhance the immune response against the antigen of interest, known as adjuvants, has been extensively studied. In addition to increasing the immune response against the antigen of interest, some adjuvants may be used to decrease the amount of antigen necessary to provoke the desired immune response or decrease the number of injections needed in a clinical regimen to induce a durable immune response and to provide protection from disease and/or induce regression of disease.

Therefore, the vaccines and immunogenic compositions described herein may be formulated with an adjuvant in order to primarily increase the immune response elicited by administration of the expression vectors described herein. Adjuvants which may be used in conjunction with the expression vectors of the present invention, include, but are not limited to,

adjuvants containing CpG oligonucleotides, or other molecules acting on toll-like receptors such as TLR9 (for review, *see*, Daubenberger, C.A., *Curr. Opin. Mol. Ther.* 9(1):45-52 (2007)), T-helper epitopes, lipid-A and derivatives or variants thereof, liposomes, cytokines, (e.g. granulocyte macrophage-colony stimulating factor (GMCSF)), CD40, CD28, CD70, IL-2, heat-shock protein (HSP) 90, CD134 (OX40), CD137, non ionic block copolymers, incomplete Freund's adjuvant, and chemokines. Additional adjuvants for use with the compositions described herein are adjuvants containing saponins (*e.g.* QS21), either alone or combined with cholesterol and phospholipid in the characteristic form of an ISCOM ("immune stimulating complex," *for review, see* Barr and Mitchell, *Immunology and Cell Biology* 74: 8-25 (1996); and Skene and Sutton, *Methods* 40: 53-59 (2006)). Additionally, aluminum-based compounds, such as aluminum hydroxide ($Al(OH)_3$), aluminum hydroxyphosphate ($AlPO_4$), amorphous aluminum hydroxyphosphate sulfate (AAHS) or so-called "alum" ($KAl(SO_4)\cdot 12H_2O$), many of which have been approved for administration into humans by regulatory agencies worldwide, may be combined with the compositions provided herein.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

EXAMPLE 1

Construction, rescue and genomic stability of Ad6- CEA-LTB/HER2-ECDM dicistronic vector.

A CEA-LTB fusion was engineered by joining the cDNA of the CEA protein deleted of the anchoring sequence (nucleotide 1 to 2037) to the LTB subunit of the *E. coli* heat labile enterotoxin to which the signal peptide coding sequence had been removed (nucleotide 64 to 375), as previously described (WO 2005/077977). In addition, a C-terminal deletion mutant of p185 (HER2/neu protein) retaining the extra-cellular and the transmembrane domain (hereinafter HER2ECDTM) was constructed as previously described (WO 2005/012527). Nucleotide sequences encoding both the CEA-LTB fusion protein and the HER2ECDTM were codon-optimized for optimal expression in human cells.

To construct an adenovirus vector expressing both CEA-LTB and HER2ECD.TM, a dicistronic cassette was assembled in tandem (FIGURE 1). The specific design

of the cassette included both a human cytomegalovirus (CMV) promoter and a mouse CMV promoter, which enable the expression of the CEA-LTB fusion protein and the HER2ECD.TM protein, respectively. Two different promoters were chosen to reduce the possibility of internal recombination events and to avoid the reduction of gene expression due to squelching of transcription factors. Additionally, the nucleotide sequences coding for the CEA and HER2 variants were followed by the BGH and SV40 polyadenylation signals, respectively.

Shuttle plasmid pNEBAd6-CEA-LTB/HER2ECDTM was constructed by removing the dicistronic expression cassette from polyMRK-CEA-LTB/HER2ECDTM-SV40 by *SpeI* and *AflII* and inserting it in the same restriction sites of pNEBAd6-2HCMVnefMCMVgagpol. The genetic structure of pNEBAd6-CEA-LTB/HER2ECDTM was verified by restriction enzyme analysis. To construct pre-adenovirus pMRKAd6CEA-LTB-HER2ECDTM, the transgene containing fragment was liberated from shuttle plasmid pNEBAd6-CEA-LTB/HER2ECDTM by digestion with restriction enzymes *Pac*1 and *PmeI* and gel purified. The purified transgene fragment was then co-transformed into *E. coli* strain BJ5183 with linearized (*Cla*I-digested) adenoviral backbone plasmid, pAd6MRKDE1DE3. Plasmid DNA isolated from BJ5183 transformants was then transformed into competent *E. coli* DH5α for screening by restriction analysis. The desired plasmid pMRKAd6CEA-LTB-HER2ECDTM was verified by restriction enzyme digestion and DNA sequence analysis. This plasmid was cut with *Pac*I to release the Ad ITRs and subsequently transfected in PerC-6 cells, which were serially passaged until a cytopathic effect was observed.

To determine whether the MRKAd6 CEA-LTB/HER2-ECDM dicistronic vector was stable upon repeated passages in PERC6 cells, the genomic structure of three different isolates at passage 10 was compared to the genomic structure of both the bulk preparation of the MRKAd6 CEA-LTB/HER2-ECDM vector and the original pre-adeno plasmid. To do so, DNA was isolated from each of the three single plaque isolates, the bulk vector sample, and of the original plasmids, and digested with the restriction enzyme *Bgl*II. The resulting *Bgl*II-fragments were visualized by radioactive fill-in. No differences in the *Bgl*II fragment sizes were revealed by comparison of each of the five samples (see FIGURE 2) Thus, these results suggest that the MRKAd6 CEA-LTB/HER2-ECDM vector does not undergo rearrangement upon serial passage in PERC6 cells.

EXAMPLE 2

IFN-γ ELISPOT Assay

Ninety-six wells MAIP plates (Millipore Corp., Billerica, MA) were coated with 100 µl/ well of purified rat anti-mouse IFN- γ (IgG1, clone R4-6A2, Pharmingen) diluted to 2.5 µg/ml in sterile PBS. After washing with PBS, blocking of plates was carried out with 200µl/well of R10 medium for 2 hrs at 37°C

Splenocytes were obtained by removing the spleen from euthanized mice in a sterile manner and by spleen disruption by grating on a metal grid. Red blood cells were removed by osmotic lysis by adding 1 ml of 0.1X PBS to the cell pellet and vortexing for approximately 15s. One ml of 2x PBS was then added and the volume was brought to 4ml with 1x PBS. Cells were pelleted by centrifugation at 1200 rpm for 10 min at RT, and the pellet was resuspended in 1 ml R10 medium. Viable cells were counted using Türks staining.

Splenocytes were plated at $5x10^5$ and $2.5x10^5$ ells/well in duplicate and incubated for 20h at 37°C with 1μg/ml suspension of each peptide. Concanavalin A (ConA) was used as positive internal control for each mouse at 5μg/ml. After washing with PBS, 0.05% Tween 20, plates were incubated O/N at 4°C with 50μl/well of biotin-conjugated rat anti-mouse IFN γ (RatIgG1, clone XMG 1.2, PharMingen) diluted to 1:2500 in assay buffer. After extensive washing, plates were developed by adding 50 μl/well NBT/B-CIP (Pierce) until development of spots was clearly visible. The reaction was stopped by washing plates thoroughly with distilled water. Plates were air dried and spots were then counted using an automated ELISPOT reader.

EXAMPLE 3

Immunogenic potency of Ad6 dicistronic vector in wild type mice.

To verify the ability of the MRKAd6 vector to elicit immune responses to both the CEA and HER2 antigens, groups of BALB/c and C57BL/6 mice were immunized with different doses of MRKAd6 CEA-LTB/HER2-ECDTM at day 0 and 14. Two weeks later, the immune response to CEA and HER2 elicited by the MRKAd6 vector was analyzed by IFN γ ELISPOT assay, as described in EXAMPLE 2. Significant cellular immune responses against CEA, LTB, and HER2 were detected in both mouse strains (see FIGURE 3).

The immune response elicited by vaccination of BALB/c mice with the Ad6 dicistronic vector was primarily biased towards the N-terminal region of both the CEA and HER2 proteins, as evidenced by the immunoreactivity of the Neu-1 and CEA-1 peptide pools (N-terminal peptide pools). By contrast, the immune response in C57BL/6 mice was mainly targeted to the C-terminal region of CEA (CEA-2). Also, both the C-terminal and N-terminal peptide pools of HER2 showed some immunoreactivity in C57BL/6 mice, albeit to a lower extent than that which was observed in BALB/c mice with the N-terminal peptide pool of Neu-1. Thus, these data demonstrate that the dicistronic MRKAd6 vector can elicit a cell-mediated immune response to both the CEA and HER2 antigens in different mouse strains.

EXAMPLE 4

Comparison of immunogenicity of Ad6 dicistronic vector encoding CEA-LTB and HER2-ECDTM with Ad6 monocistronic vectors.

To further evaluate the efficiency and immunogenic potency of the Ad6 dicistronic vector, two Ad6 monocistronic vectors were constructed: one encoding CEA-LTB under the

transcriptional control of the human CMV promoter, and one encoding the ECD.TM portion of HER2/Neu controlled by the mouse CMV promoter. The vectors were rescued and amplified. Subsequently, the genomic structures of the vectors were compared by restriction analysis, revealing no major rearrangements.

Expression of each of the antigens was verified by ELISA and by FACS staining upon infection of HeLa cells (not shown). The expression levels of each of the monocistronic Ad6 vectors encoding the single TAAs was comparable to that of the dicistronic Ad6 vector encoding both the CEA and HER2 variants.

Groups of C57BL/6 mice were immunized with: 1) Ad6-CEA-LTB; 2) Ad6-ECD.TM; 3) Ad6-CEA-LTB+Ad6-ECD.TM; 4) Ad6-dicistronic. Each mouse received IM injections of 10^8 viral particles, two weeks apart and immune response was measured by intracellular staining for IFN γ 14 days later. Both a $CD8^+$ (FIGURE 4A) and a $CD4^+$ (FIGURE 4B) immune response was elicited in immunized mice. Importantly, single vector and co-administration of Ad6 monocistronic vectors resulted in comparable immunogenicity for each relative antigen, thus showing that efficacy of Ad6 dicistronic is not affected by mechanisms such as transcription factors squelching between human and mouse CMV promoter within the same antigen presenting cell.

EXAMPLE 5

Immunization of CEA-HER2 Transgenic Mice with MRKAd6 Dicistronic Vector.

To further elucidate the immunogenic potency of the MRKAd6 vector, human HER2 and human CEA (CEA-HER2.Tg) double transgenic mice were generated and used as animal model for immunization studies. CEA-HER2.Tg mice were derived by crossing human CEA transgenic mice with a line of human HER2 transgenic mice developed by Wei-Zen Wei (Wayne State University, Detroit). CEA.tg mice ($H\text{-}2^b$) were provided by J. Primus (Vanderbilt University) and kept in standard conditions (Clarke et al. *Cancer Res.* 58:1469-77 (1998)). The HER2 transgenic mice carry the full length wild type cDNA of human HER2 under the control of the whey acidic protein promoter (WAP) (Piechocki et al. Human ErbB-2 (Her-2) transgenic mice: a model system for testing Her-2 based vaccines. *J Immunol.* 171(11):5787-94 (2003)). These mice express HER2 protein in the secretory mammary epithelia during pregnancy and lactation, and constitutively express HER2 in the Bergman glial cells within the molecular layer of the cerebellum (Piechocki et al., *supra*). No neoplastic transformation was detected in any tissue of the HER2 transgenic mice (Piechocki et al.).

A group of CEA-HER2.Tg mice were immunized with two intramuscular (IM) injections of $1x10^{10}$ vp of the MRKAd6 vector two weeks apart. Fifteen days later, mice were euthanized and splenocytes were analyzed by intracellular staining for HER2 and CEA specific IFN γ production. A significant immune response was measured against CEA and HER2 peptide pools (see FIGURE 5), particularly against the C-terminal region of CEA (CEA-2) and of HER2-

ECDTM (Neu-2). The immune response against LTB was similarly high. These data demonstrate that dicistronic MRKAd6 is indeed able to break tolerance to both TAA.

EXAMPLE 6

Ad6 dicistronic vector induces immune response in mice engrafted with human immune system.

A mouse model of the human immune system, with functional circulating human T and B cells, has recently been described (Camacho et al. *Cell. Immunol.* 232(1-2): 86-95 (2004)). This model was produced by grafting thymus and spleen fragments of HLA-DR1 transgenic mice (NOD/scid-DR1) and subsequently injecting human CBMNC into transplanted tissues (Camacho et al. *supra*). It was demonstrated that human cells from spleen and engrafted thymus/spleen tissues of these mice can proliferate with anti-human CD3 antibody (Camacho et al. *supra*). Moreover, humoral and cellular immune responses to allogeneic human cells were detected in these NOD/scid-DR1 chimeric mice, making them a suitable mouse model for studying vaccine efficacy.

To assess the immunogenic potency of MRKAd6 CEA-LTB/HER2-ECDM in this model, NOD/scid–DR1 mice were engrafted with HLA-A2 human cord blood MNC (CBMNC), either at 5 days old or 8-12 weeks old. 2 (adult) or 6-8 (neonates) weeks after engraftment, mice were immunized twice with MRKAd6 CEA-LTB/HER2-ECDM IM (10^{10} viral particles), 2 weeks apart. Two-three weeks after the boost, immune response was analyzed by intracellular staining for IFN γ using 15mer peptide pools encompassing CEA, LTB and HER2ECD.TM. The immune response of two mice engrafted intrahepatically at 5 days old is shown in FIGURE 6. A strong cellular immune response was measured both against CEA and HER2/Neu, and reactivity was both for CD4+ and CD8+ cells. Consistently, strong immune response was also measured in adult mice engrafted in the kidney capsule. The immune response measured from one mouse, which was mainly CD8+ specific, is shown in FIGURE 7.

These data show that dicistronic Ad6 is strongly immunogenic in this mouse model, consistent with the evidence obtained in CEA/HER2 double transgenic mice.

WHAT IS CLAIMED IS:

1. An expression vector comprising a first sequence of nucleotides and a second sequence of nucleotides; wherein the first sequence of nucleotides encodes a human carcinoembryonic antigen (CEA) protein, or variant thereof, and the second sequence of nucleotides encodes a human HER2/neu protein, or variant thereof.

2. An expression vector comprising a first sequence of nucleotides that encodes a CEA fusion protein, wherein the CEA fusion protein comprises a CEA protein or variant thereof, fused to a B subunit of *E. coli* heat labile enterotoxin (LTB); and a second sequence of nucleotides that encodes a truncated human HER2/neu protein, wherein the truncated HER2 protein comprises the extracellular and transmembrane domains of HER2/neu.

3. The expression vector of claim 2, wherein the first sequence of nucleotides is operably linked to a first promoter and the second sequence of nucleotides is operably linked to a second promoter; wherein the first promoter and the second promoter are not the same.

4. The expression vector of any of the preceding claims, wherein the CEA protein is C-terminally truncated.

5. The expression vector of claim 4, wherein the CEA protein is truncated at amino acid 679 and comprises a sequence of amino acids as set forth in SEQ ID NO:9.

6. The expression vector of any of the preceding claims, wherein the first sequence of nucleotides or the second sequence of nucleotides is codon-optimized for optimal expression in human cells.

7. The expression vector of claim 2, wherein the LTB subunit is truncated of its signal sequence.

8. The expression vector of any of the preceding claims, wherein the vector is an adenovirus vector.

9. The expression vector of claim 8, wherein the vector is an Ad 6 vector.

10. An immunogenic composition comprising an expression vector of any of the preceding claims, a pharmaceutically acceptable carrier, and an adjuvant.

11. A host cell comprising the vector of any of the preceding claims.

12. A method of treating cancer comprising administering to a patient in need thereof the vector of claim 1.

13. An adenoviral vector comprising an adenoviral genome with a deletion in the adenovirus E1 region, and an insert in the adenovirus E1 region, wherein the insert comprises an expression cassette comprising:

(a) a first polynucleotide comprising a sequence of nucleotides that encodes a CEA protein or variant thereof fused to a sequence of nucleotides that encodes a B subunit of *E. coli* heat labile enterotoxin and,

(b) a second polynucleotide comprising a sequence of nucleotides that encodes a truncated HER2/neu protein, the truncated HER2/neu protein comprising an extracellular domain and a transmembrane domain;

(c) a first promoter operably linked to the first polynucleotide, and

(d) a second promoter operably linked to the second polynucleotide;

wherein the first promoter and the second promoter are not the same.

14. The adenoviral vector of claim 13 which is an Ad6 vector.

15. The Ad6 vector of claim 14 wherein the first polynucleotide comprises a sequence of nucleotides as set forth in SEQ ID NO:5 and the second polynucleotide comprises a sequence of nucleotides as set forth in SEQ ID NO:1.

16. The Ad6 vector of claim 14 wherein the first polynucleotide comprises a sequence of nucleotides as set forth in SEQ ID NO:5 and the second polynucleotide comprises a sequence of nucleotides as set forth in SEQ ID NO:2.

17. The Ad6 vector of claim 15 wherein the first promoter is a human CMV promoter and the second promoter is a murine CMV promoter.

18. The Ad6 vector of claim 16 wherein the first promoter is a human CMV promoter and the second promoter is a murine CMV promoter.

19. A vaccine plasmid comprising a plasmid portion and an expression cassette portion, the expression cassette portion comprising:

(a) a first polynucleotide comprising a first sequence of nucleotides as set forth in SEQ ID NO:1;

(b) a second polynucleotide comprising a sequence of nucleotides as set forth in SEQ ID NO:5;

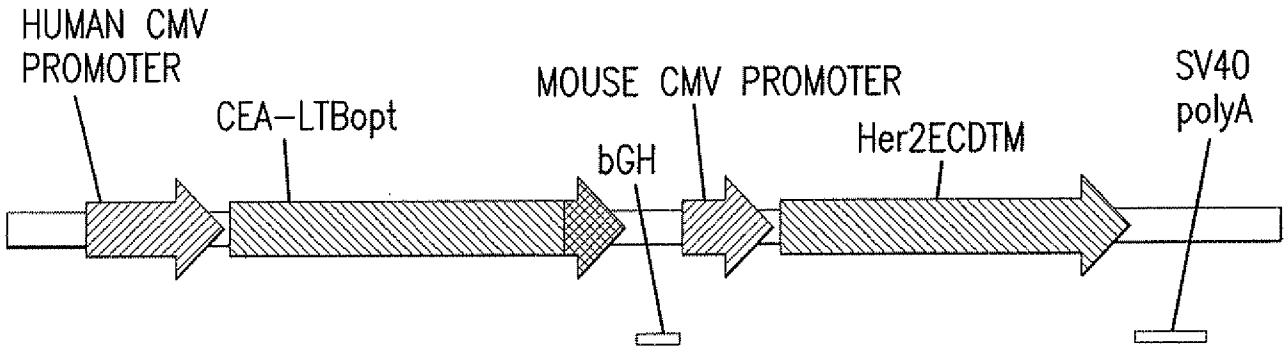
(c) a first promoter operably linked to the first polynucleotide; and

(d) a second promoter operably linked to the second polynucleotide;

wherein the first promoter and the second promoter are not the same.

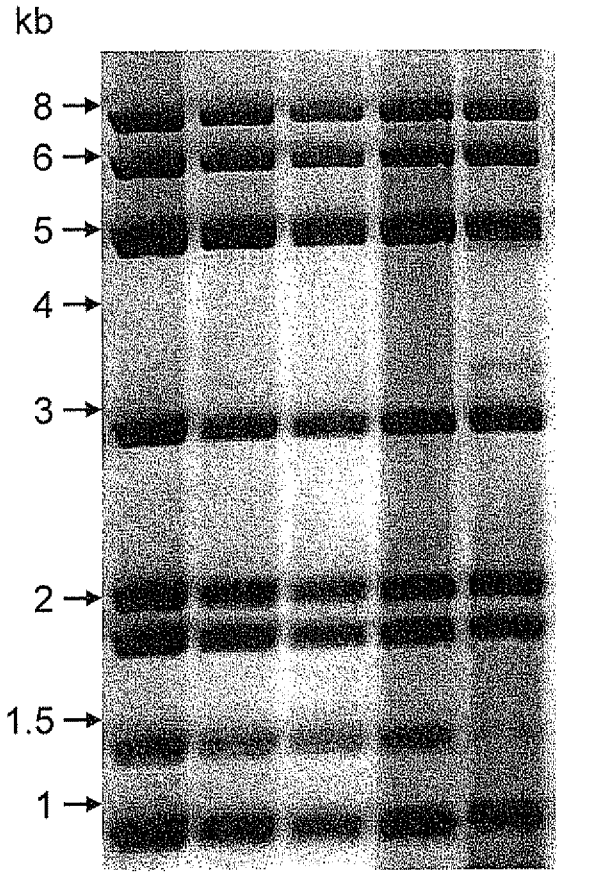
20. A method of treating CEA and/or HER2 associated cancer comprising administering the expression vector of claim 2 to a patient in need thereof.

21. A method of treating CEA and/or HER2 associated cancer comprising administering the expression vector of claim 12 to a patient in need thereof.



CEA/LTBopt–Her2ECDTMopt DICISTRONIC CASSETTE

FIG.1



Bgl II restriction digest

FIG.2

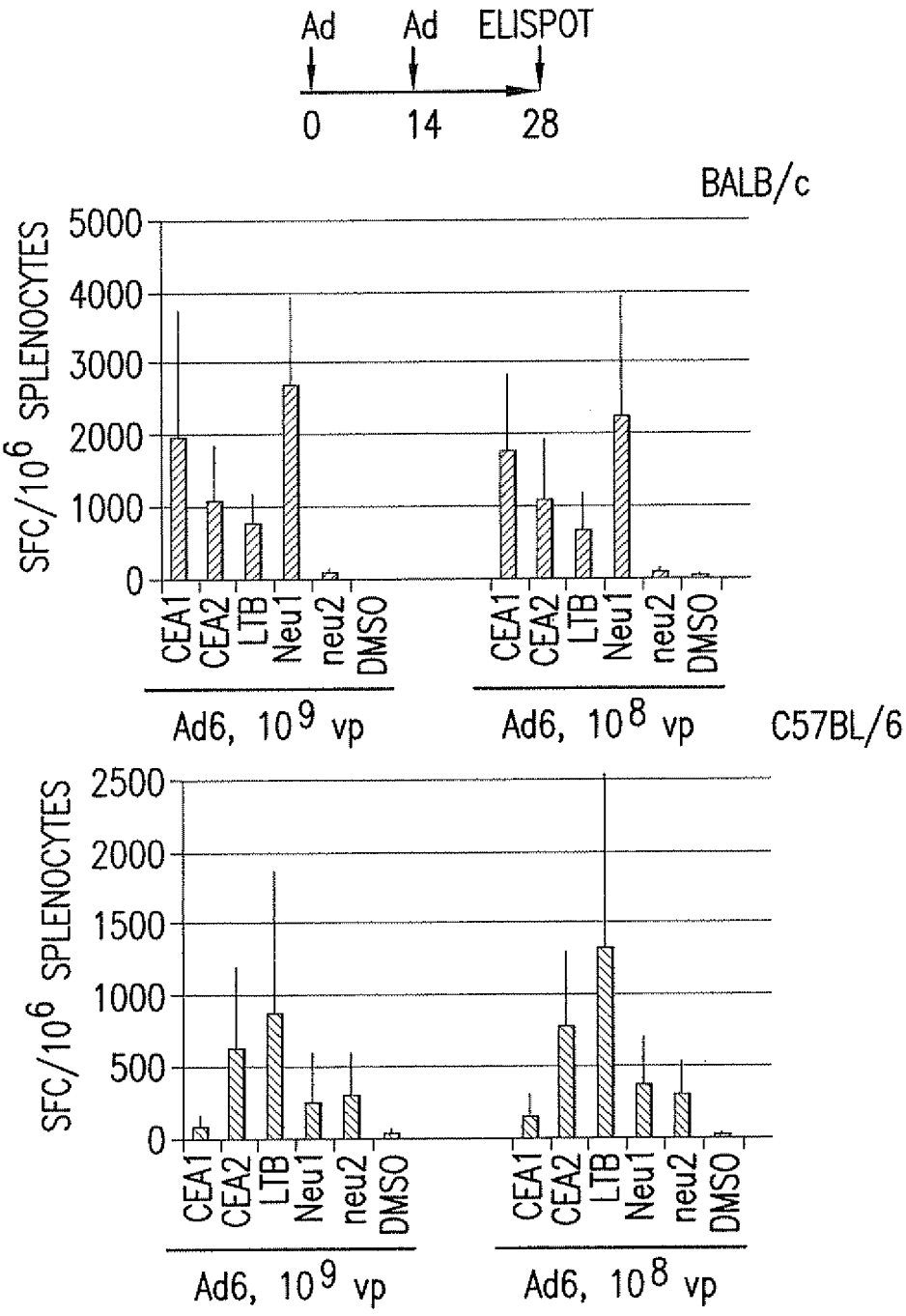

Ad
Ad
ELISPOT
0
14
28
BALB/c
SFC/10^6 SPLENOCYTES
5000
4000
3000
2000
1000
0
CEA1
CEA2
LTB
Neu1
neu2
DMSO
Ad6, 10^9 vp
Ad6, 10^8 vp
C57BL/6
2500
2000
1500
1000
500

FIG.3

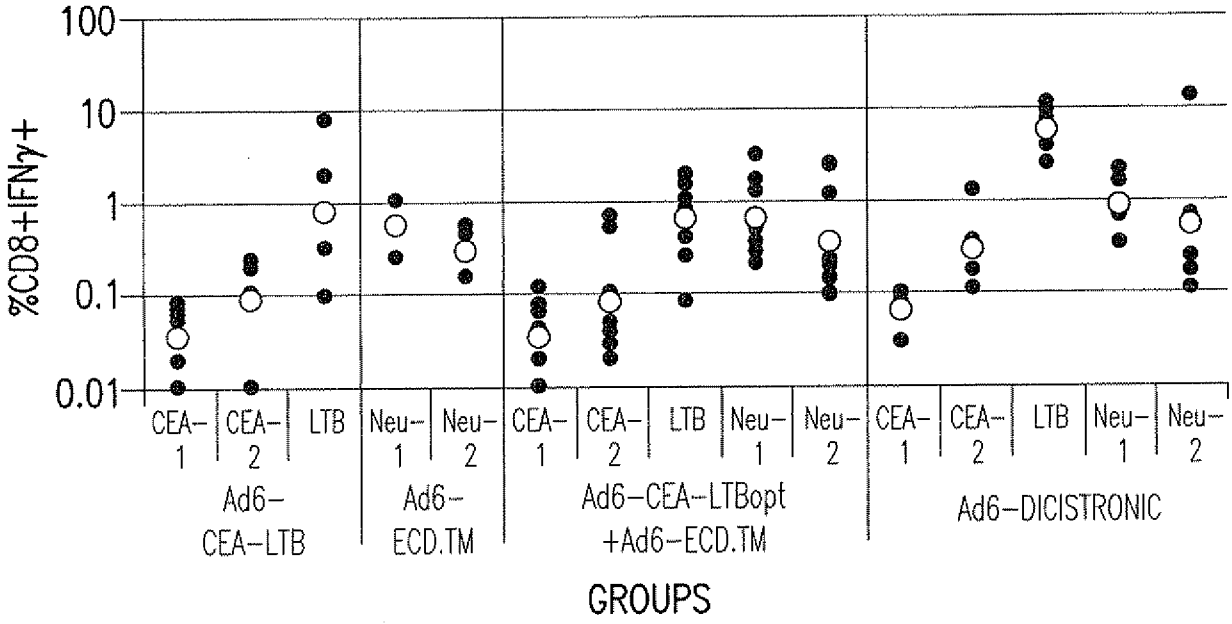

100
10
1
0.1
0.01
%CD8+IFNγ+
CEA-1
CEA-2
LTB
Neu-1
Neu-2
Ad6-CEA-LTB
Ad6-ECD.TM
Ad6-CEA-LTBopt +Ad6-ECD.TM
Ad6-DICISTRONIC
GROUPS

FIG.4A

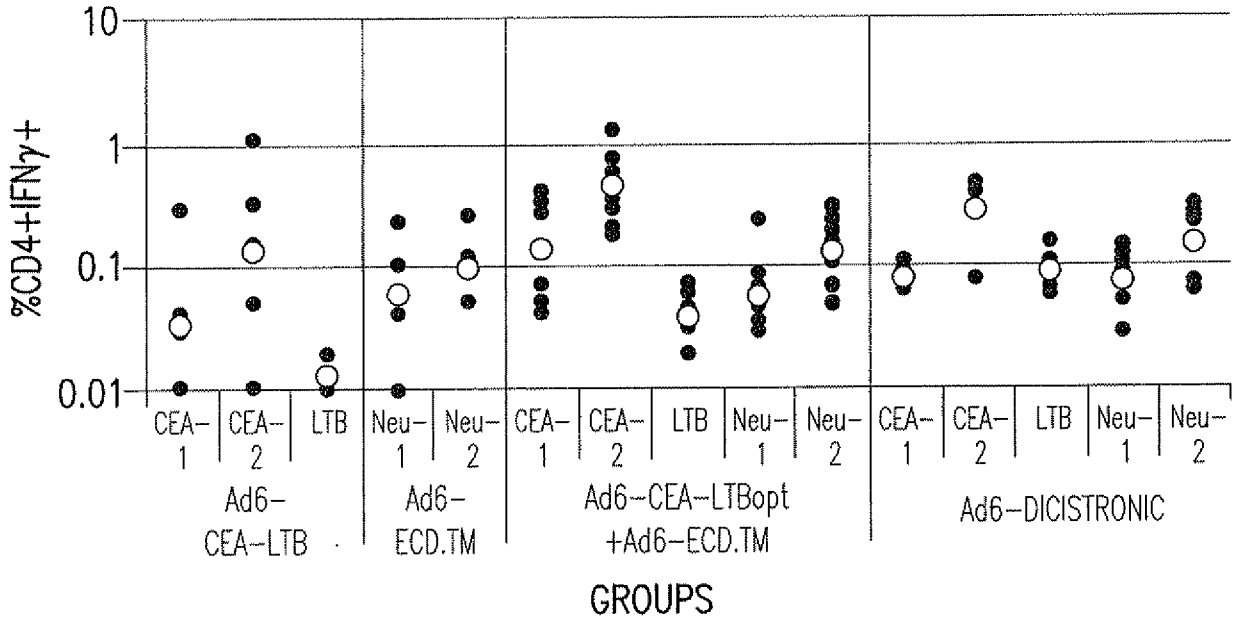

10
1
0.1
0.01
%CD4+IFNγ+
CEA-1
CEA-2
LTB
Neu-1
Neu-2
Ad6-CEA-LTB
Ad6-ECD.TM
Ad6-CEA-LTBopt +Ad6-ECD.TM
Ad6-DICISTRONIC
GROUPS

FIG.4B

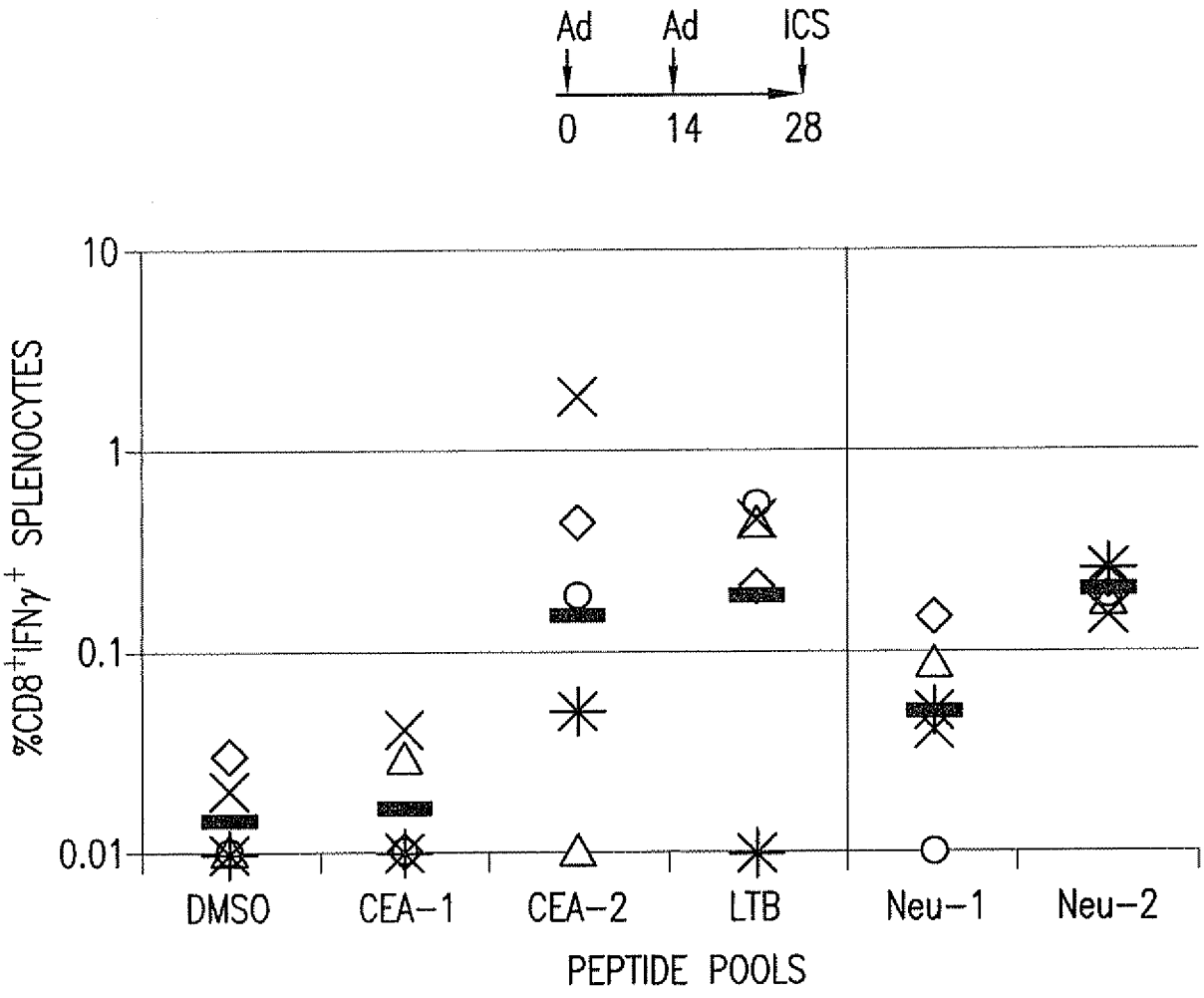

Ad
Ad
ICS
0
14
28
10
1
0.1
0.01
%CD8+IFNγ+ SPLENOCYTES
DMSO
CEA-1
CEA-2
LTB
Neu-1
Neu-2
PEPTIDE POOLS

FIG.5

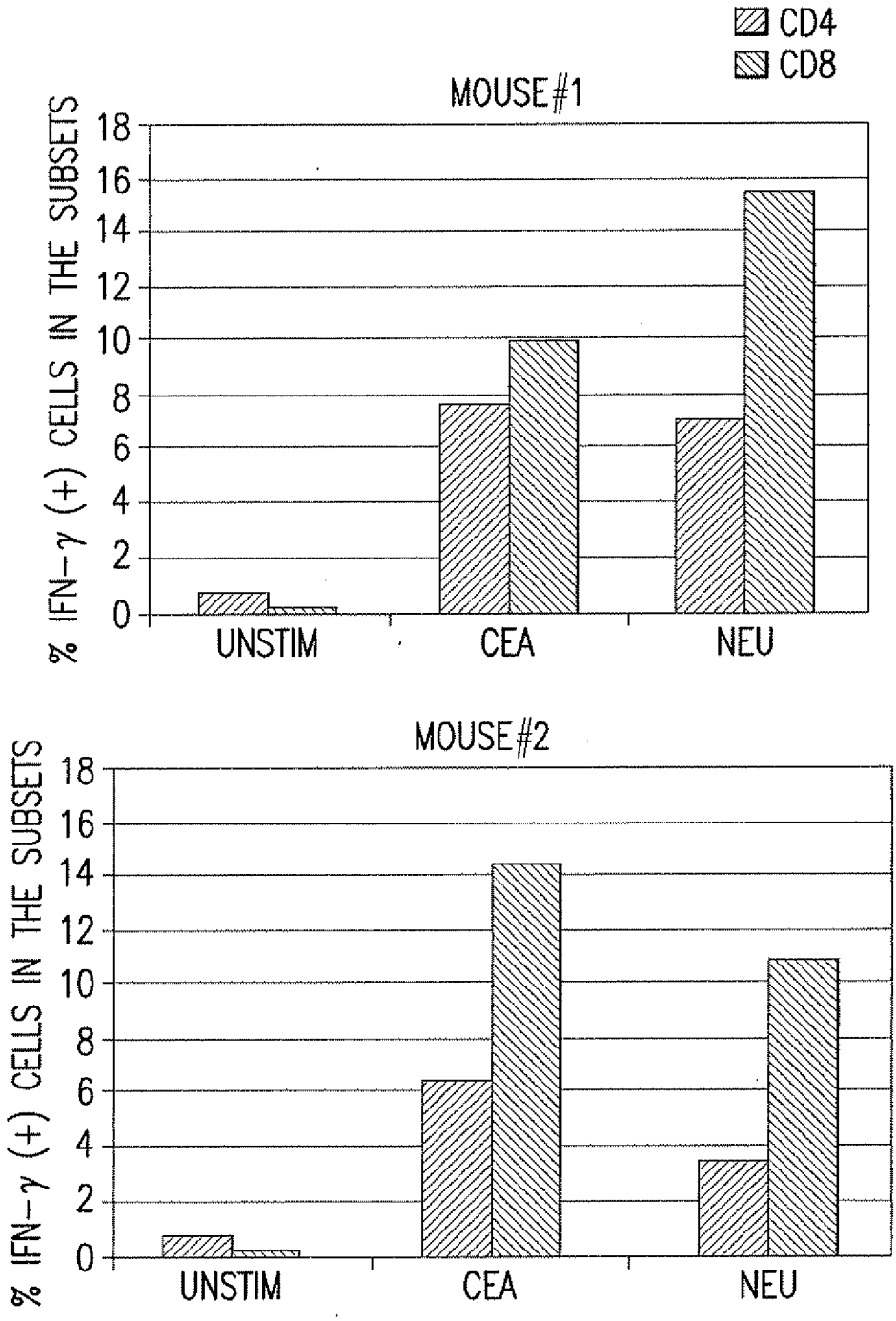

CD4
CD8
MOUSE#1
% IFN-γ (+) CELLS IN THE SUBSETS
0
2
4
6
8
10
12
14
16
18
UNSTIM
CEA
NEU
MOUSE#2
% IFN-γ (+) CELLS IN THE SUBSETS
0
2
4
6
8
10
12
14
16
18
UNSTIM
CEA
NEU

FIG.6

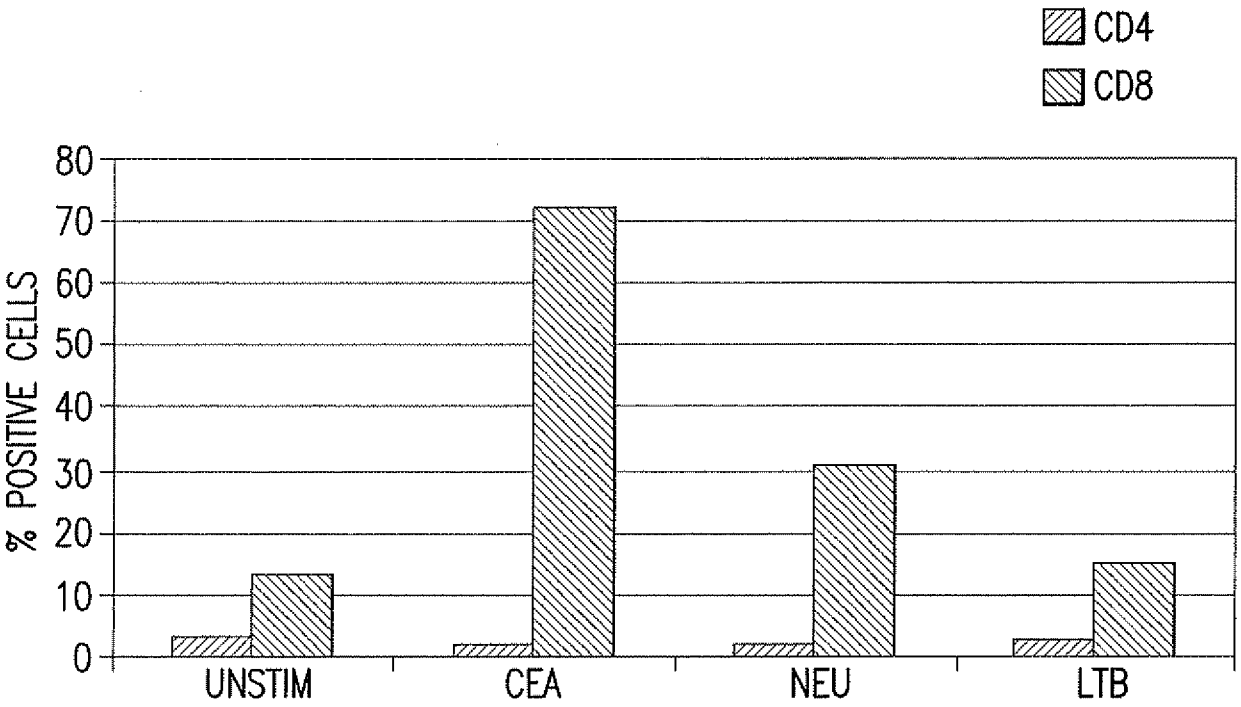

CD4
CD8
% POSITIVE CELLS
80
70
60
50
40
30
20
10
0
UNSTIM
CEA
NEU
LTB

FIG.7

Human Codon-Optimized HER2ECDTM Nucleotide Sequence

ATGGAGCTGG CCGCCCTGTG CCGCTGGGGC CTGCTGCTGG CCCTGCTGCC CCCCGGCGCC
GCCAGCACCC AGGTGTGCAC CGGCACCGAC ATGAAGCTGC GCCTGCCCGC CAGCCCCGAG
ACCCACCTGG ACATGCTGCG CCACCTGTAC CAGGGCTGCC AGGTGGTGCA GGGCAACCTG
GAGCTGACCT ACCTGCCCAC CAACGCCAGC CTGAGCTTCC TGCAGGACAT CCAGGAGGTG
CAGGGCTACG TGCTGATCGC CCACAACCAG GTGCGCCAGG TGCCCCTGCA GCGCCTGCGC
ATCGTGCGCG GCACCCAGCT GTTCGAGGAC AACTACGCCC TGGCCGTGCT GGACAACGGC
GACCCCCTGA ACAACACCAC CCCCGTGACC GGCGCCAGCC CCGGCGGCCT GCGCGAGCTG
CAGCTGCGCA GCCTGACCGA GATCCTGAAG GGCGGCGTGC TGATCCAGCG CAACCCCCAG
CTGTGCTACC AGGACACCAT CCTGTGGAAG GACATCTTCC ACAAGAACAA CCAGCTGGCC
CTGACCCTGA TCGACACCAA CCGCAGCCGC GCCTGCCACC CCTGCAGCCC CATGTGCAAG
GGCAGCCGCT GCTGGGGCGA GAGCAGCGAG GACTGCCAGA GCCTGACCCG CACCGTGTGC
GCCGGCGGCT GCGCCCGCTG CAAGGGCCCC CTGCCCACCG ACTGCTGCCA CGAGCAGTGC
GCCGCCGGCT GCACCGGCCC CAAGCACAGC GACTGCCTGG CCTGCCTGCA CTTCAACCAC
AGCGGCATCT GCGAGCTGCA CTGCCCCGCC CTGGTGACCT ACAACACCGA CACCTTCGAG
AGCATGCCCA ACCCCGAGGG CCGCTACACC TTCGGCGCCA GCTGCGTGAC CGCCTGCCCC
TACAACTACC TGAGCACCGA CGTGGGCAGC TGCACCCTGG TGTGCCCCCT GCACAACCAG
GAGGTGACCG CCGAGGACGG CACCCAGCGC TGCGAGAAGT GCAGCAAGCC CTGCGCCCGC
GTGTGCTACG GCCTGGGCAT GGAGCACCTG CGCGAGGTGC GCGCCGTGAC CAGCGCCAAC
ATCCAGGAGT TCGCCGGCTG CAAGAAGATC TTCGGCAGCC TGGCCTTCCT GCCCGAGAGC
TTCGACGGCG ACCCCGCCAG CAACACCGCC CCCCTGCAGC CCGAGCAGCT GCAGGTGTTC
GAGACCCTGG AGGAGATCAC CGGCTACCTG TACATCAGCG CCTGGCCCGA CAGCCTGCCC
GACCTGAGCG TGTTCCAGAA CCTGCAGGTG ATCCGCGGCC GCATCCTGCA CAACGGCGCC
TACAGCCTGA CCCTGCAGGG CCTGGGCATC AGCTGGCTGG GCCTGCGCAG CCTGCGCGAG
CTGGGCAGCG GCCTGGCCCT GATCCACCAC AACACCCACC TGTGCTTCGT GCACACCGTG
CCCTGGGACC AGCTGTTCCG CAACCCCCAC CAGGCCCTGC TGCACACCGC CAACCGCCCC
GAGGACGAGT GCGTGGGCGA GGGCCTGGCC TGCCACCAGC TGTGCGCCCG CGGCCACTGC
TGGGGCCCCG GCCCCACCCA GTGCGTGAAC TGCAGCCAGT TCCTGCGCGG CCAGGAGTGC
GTGGAGGAGT GCCGCGTGCT GCAGGGCCTG CCCCGCGAGT ACGTGAACGC CCGCCACTGC
CTGCCCTGCC ACCCCGAGTG CCAGCCCCAG AACGGCAGCG TGACCTGCTT CGGCCCCGAG
GCCGACCAGT GCGTGGCCTG CGCCCACTAC AAGGACCCCC CCTTCTGCGT GGCCCGCTGC
CCCAGCGGCG TGAAGCCCGA CCTGAGCTAC ATGCCCATCT GGAAGTTCCC CGACGAGGAG
GGCGCCTGCC AGCCCTGCCC CATCAACTGC ACCCACAGCT GCGTGGACCT GGACGACAAG
GGCTGCCCCG CCGAGCAGCG CGCCAGCCCC CTGACCAGCA TCATCAGCGC CGTGGTGGGC
ATCCTGCTGG TGGTGGTGCT GGGCGTGGTG TTCGGCATCC TGATCTGA (SEQ ID NO:1)

FIG.8A

Human HER2ECDTM wt Nucleotide Sequence

ATGGAGCTG GCGGCCTTG TGCCGCTGG GGGCTCCTC CTCGCCCTC TTGCCCCCC GGAGCCGCG
AGCACCCAA GTGTGCACC GGCACAGAC ATGAAGCTG CGGCTCCCT GCCAGTCCC GAGACCCAC
CTGGACATG CTCCGCCAC CTCTACCAG GGCTGCCAG GTGGTGCAG GGAAACCTG GAACTCACC
TACCTGCCC ACCAATGCC AGCCTGTCC TTCCTGCAG GATATCCAG GAGGTGCAG GGCTACGTG
CTCATCGCT CACAACCAA GTGAGGCAG GTCCCACTG CAGAGGCTG CGGATTGTG CGAGGCACC
CAGCTCTTT GAGGACAAC TATGCCCTG GCCGTGCTA GACAATGGA GACCCGCTG AACAATACC
ACCCCTGTC ACAGGGGCC TCCCCAGGA GGCCTGCGG GAGCTGCAG CTTCGAAGC CTCACAGAG
ATCTTGAAA GGAGGGGTC TTGATCCAG CGGAACCCC CAGCTCTGC TACCAGGAC ACGATTTTG
TGGAAGGAC ATCTTCCAC AAGAACAAC CAGCTGGCT CTCACACTG ATAGACACC AACCGCTCT
CGGGCCTGC CACCCCTGT TCTCCGATG TGTAAGGGC TCCCGCTGC TGGGGAGAG AGTTCTGAG
GATTGTCAG AGCCTGACG CGCACTGTC TGTGCCGGT GGCTGTGCC CGCTGCAAG GGGCCACTG
CCCACTGAC TGCTGCCAT GAGCAGTGT GCTGCCGGC TGCACGGGC CCCAAGCAC TCTGACTGC
CTGGCCTGC CTCCACTTC AACCACAGT GGCATCTGT GAGCTGCAC TGCCCAGCC CTGGTCACC
TACAACACA GACACGTTT GAGTCCATG CCCAATCCC GAGGGCCGG TATACATTC GGCGCCAGC
TGTGTGACT GCCTGTCCC TACAACTAC CTTTCTACG GACGTGGGA TCCTGCACC CTCGTCTGC
CCCCTGCAC AACCAAGAG GTGACAGCA GAGGATGGA ACACAGCGG TGTGAGAAG TGCAGCAAG
CCCTGTGCC CGAGTGTGC TATGGTCTG GGCATGGAG CACTTGCGA GAGGTGAGG GCAGTTACC
AGTGCCAAT ATCCAGGAG TTTGCTGGC TGCAAGAAG ATCTTTGGG AGCCTGGCA TTTCTGCCG
GAGAGCTTT GATGGGGAC CCAGCCTCC AACACTGCC CCGCTCCAG CCAGAGCAG CTCCAAGTG
TTTGAGACT CTGGAAGAG ATCACAGGT TACCTATAC ATCTCAGCA TGGCCGGAC AGCCTGCCT
GACCTCAGC GTCTTCCAG AACCTGCAA GTAATCCGG GGACGAATT CTGCACAAT GGCGCCTAC
TCGCTGACC CTGCAAGGG CTGGGCATC AGCTGGCTG GGGCTGCGC TCACTGAGG GAACTGGGC
AGTGGACTG GCCCTCATC CACCATAAC ACCCACCTC TGCTTCGTG CACACGGTG CCCTGGGAC
CAGCTCTTT CGGAACCCG CACCAAGCT CTGCTCCAC ACTGCCAAC CGGCCAGAG GACGAGTGT
GTGGGCGAG GGCCTGGCC TGCCACCAG CTGTGCGCC CGAGGGCAC TGCTGGGGT CCAGGGCCC
ACCCAGTGT GTCAACTGC AGCCAGTTC CTTCGGGGC CAGGAGTGC GTGGAGGAA TGCCGAGTA
CTGCAGGGG CTCCCCAGG GAGTATGTG AATGCCAGG CACTGTTTG CCGTGCCAC CCTGAGTGT
CAGCCCCAG AATGGCTCA GTGACCTGT TTTGGACCG GAGGCTGAC CAGTGTGTG GCCTGTGCC
CACTATAAG GACCCTCCC TTCTGCGTG GCCCGCTGC CCCAGCGGT GTGAAACCT GACCTCTCC
TACATGCCC ATCTGGAAG TTTCCAGAT GAGGAGGGC GCATGCCAG CCTTGCCCC ATCAACTGC
ACCCACTCC TGTGTGGAC CTGGATGAC AAGGGCTGC CCCGCCGAG CAGAGAGCC AGCCCTCTG
ACGTCCATC ATCTCTGCG GTGGTTGGC ATTCTGCTG GTCGTGGTC TTGGGGGTG GTCTTTGGG
ATCCTCATC TGA (SEQ ID NO:2)

FIG.8B

hCEA-LTB Sequence

```
   1  ATGGAGTCTC CCTCGGCCCC TCCCCACAGA TGGTGCATCC CCTGGCAGAG
  51  GCTCCTGCTC ACAGCCTCAC TTCTAACCTT CTGGAACCCG CCCACCACTG
 101  CCAAGCTCAC TATTGAATCC ACGCCGTTCA ATGTCGCAGA GGGGAAGGAG
 151  GTGCTTCTAC TTGTCCACAA TCTGCCCCAG CATCTTTTTG GCTACAGCTG
 201  GTACAAAGGT GAAAGAGTGG ATGGCAACCG TCAAATTATA GGATATGTAA
 251  TAGGAACTCA ACAAGCTACC CCAGGGCCCG CATACAGTGG TCGAGAGATA
 301  ATATACCCCA ATGCATCCCT GCTGATCCAG AACATCATCC AGAATGACAC
 351  AGGATTCTAC ACCCTACACG TCATAAAGTC AGATCTTGTG AATGAAGAAG
 401  CAACTGGCCA GTTCCGGGTA TACCCGGAGC TGCCCAAGCC CTCCATCTCC
 451  AGCAACAACT CCAAACCCGT GGAGGACAAG GATGCTGTGG CCTTCACCTG
 501  TGAACCTGAG ACTCAGGACG CAACCTACCT GTGGTGGGTA AACAATCAGA
 551  GCCTCCCGGT CAGTCCCAGG CTGCAGCTGT CCAATGGCAA CAGGACCCTC
 601  ACTCTATTCA ATGTCACAAG AAATGACACA GCAAGCTACA AATGTGAAAC
 651  CCAGAACCCA GTGAGTGCCA GGCGCAGTGA TTCAGTCATC CTGAATGTCC
 701  TCTATGGCCC GGATGCCCCC ACCATTTCCC CTCTAAACAC ATCTTACAGA
 751  TCAGGGGAAA ATCTGAACCT CTCCTGCCAC GCAGCCTCTA ACCCACCTGC
 801  ACAGTACTCT TGGTTTGTCA ATGGGACTTT CCAGCAATCC ACCCAAGAGC
 851  TCTTTATCCC CAACATCACT GTGAATAATA GTGGATCCTA TACGTGCCAA
 901  GCCCATAACT CAGACACTGG CCTCAATAGG ACCACAGTCA CGACGATCAC
 951  AGTCTATGCA GAGCCACCCA AACCCTTCAT CACCAGCAAC AACTCCAACC
1001  CCGTGGAGGA TGAGGATGCT GTAGCCTTAA CCTGTGAACC TGAGATTCAG
1151  AACACAACCT ACCTGTGGTG GGTAAATAAT CAGAGCCTCC CGGTCAGTCC
1101  CAGGCTGCAG CTGTCCAATG ACAACAGGAC CCTCACTCTA CTCAGTGTCA
1151  CAAGGAATGA TGTAGGACCC TATGAGTGTG GAATCCAGAA CGAATTAAGT
1201  GTTGACCACA GCGACCCAGT CATCCTGAAT GTCCTCTATG GCCCAGACGA
1251  CCCCACCATT TCCCCCTCAT ACACCTATTA CCGTCCAGGG GTGAACCTCA
1301  GCCTCTCCTG CCATGCAGCC TCTAACCCAC CTGCACAGTA TTCTTGGCTG
1351  ATTGATGGGA ACATCCAGCA ACACACACAA GAGCTCTTTA TCTCCAACAT
1401  CACTGAGAAG AACAGCGGAC TCTATACCTG CCAGGCCAAT AACTCAGCCA
1451  GTGGCCACAG CAGGACTACA GTCAAGACAA TCACAGTCTC TGCGGAGCTG
1501  CCCAAGCCCT CCATCTCCAG CAACAACTCC AAACCCGTGG AGGACAAGGA
1551  TGCTGTGGCC TTCACCTGTG AACCTGAGGC TCAGAACACA ACCTACCTGT
1601  GGTGGGTAAA TGGTCAGAGC CTCCCAGTCA GTCCCAGGCT GCAGCTGTCC
1651  AATGGCAACA GGACCCTCAC TCTATTCAAT GTCACAAGAA ATGACGCAAG
1701  AGCCTATGTA TGTGGAATCC AGAACTCAGT GAGTGCAAAC CGCAGTGACC
1751  CAGTCACCCT GGATGTCCTC TATGGGCCGG ACACCCCCAT CATTTCCCCC
1801  CCAGACTCGT CTTACCTTTC GGGAGCGAAC CTCAACCTCT CCTGCCACTC
1851  GGCCTCTAAC CCATCCCCGC AGTATTCTTG GCGTATCAAT GGGATACCGC
1901  AGCAACACAC ACAAGTTCTC TTTATCGCCA AAATCACGCC AAATAATAAC
1951  GGGACCTATG CCTGTTTTGT CTCTAACTTG GCTACTGGCC GCAATAATTC
2001  CATAGTCAAG AGCATCACAG TCTCTGCATC TGGAACTCTA GATGCTCCCC
2051  AGTCTATTAC AGAACTATGT TCGGAATATC GCAACACACA AATATATACG
2101  ATAAATGACA AGATACTATC ATATACGGAA TCGATGGCAG GTAAAAGAGA
2151  AATGGTTATC ATTACATTTA AGAGCGGCGC AACATTTCAG GTCGAAGTCC
2201  CGGGCAGTCA ACATATAGAC TCCCAAAAAA AAGCCATTGA AAGGATGAAG
2251  GACACATTAA GAATCACATA TCTGACCGAG ACCAAAATTG ATAAATTATG
2301  TGTATGGAAT AATAAAACCC CCAATTCAAT TGCGGCAATC AGTATGGAAA
      ACTAG (SEQ ID NO:3)
```

FIG.9A

CEAopt-LTB Nucleotide Sequence

```
   1  ATGGAGAGCC CCAGCGCCCC CCCCCACCGC TGGTGCATCC CCTGGCAGCG
      CCTGCTGCTG ACCGCCAGCC TGCTGACCTT CTGGAACCCC CCCACCACCG
 101  CCAAGCTGAC CATCGAGAGC ACCCCCTTCA ACGTGGCCGA GGGCAAGGAG
      GTGCTGCTGC TGGTGCACAA CCTGCCCCAG CACCTGTTCG GCTACAGCTG
 201  GTACAAGGGC GAGCGCGTGG ACGGCAACCG CCAGATCATC GGCTACGTGA
      TCGGCACCCA GCAGGCCACC CCCGGCCCCG CCTACAGCGG CCGCGAGATC
 301  ATCTACCCCA ACGCCAGCCT GCTGATCCAG AACATCATCC AGAACGACAC
      CGGCTTCTAC ACCCTGCACG TGATCAAGAG CGACCTGGTG AACGAGGAGG
 401  CCACCGGCCA GTTCCGCGTG TACCCCGAGC TGCCCAAGCC CAGCATCAGC
      AGCAACAACA GCAAGCCCGT GGAGGACAAG GACGCCGTGG CCTTCACCTG
 501  CGAGCCCGAG ACCCAGGACG CCACCTACCT GTGGTGGGTG AACAACCAGA
      GCCTGCCCGT GAGCCCCCGC CTGCAGCTGA GCAACGGCAA CCGCACCCTG
 601  ACCCTGTTCA ACGTGACCCG CAACGACACC GCCAGCTACA AGTGCGAGAC
      CCAGAACCCC GTGAGCGCCC GCCGCAGCGA CAGCGTGATC CTGAACGTGC
 701  TGTACGGCCC CGACGCCCCC ACCATCAGCC CCCTGAACAC CAGCTACCGC
      AGCGGCGAGA ACCTGAACCT GAGCTGCCAC GCCGCCAGCA ACCCCCCCGC
 801  CCAGTACAGC TGGTTCGTGA ACGGCACCTT CCAGCAGAGC ACCCAGGAGC
      TGTTCATCCC CAACATCACC GTGAACAACA GCGGCAGCTA CACCTGCCAG
 901  GCCCACAACA GCGACACCGG CCTGAACCGC ACCACCGTGA CCACCATCAC
      CGTGTACGCC GAGCCCCCCA AGCCCTTCAT CACCAGCAAC AACAGCAACC
1001  CCGTGGAGGA CGAGGACGCC GTGGCCCTGA CCTGCGAGCC CGAGATCCAG
      AACACCACCT ACCTGTGGTG GGTGAACAAC CAGAGCCTGC CCGTGAGCCC
1101  CCGCCTGCAG CTGAGCAACG ACAACCGCAC CCTGACCCTG CTGAGCGTGA
      CCCGCAACGA CGTGGGCCCC TACGAGTGCG GCATCCAGAA CGAGCTGAGC
1201  GTGGACCACA GCGACCCCGT GATCCTGAAC GTGCTGTACG GCCCCGACGA
      CCCCACCATC AGCCCCAGCT ACACCTACTA CCGCCCCGGC GTGAACCTGA
1301  GCCTGAGCTG CCACGCCGCC AGCAACCCCC CCGCCCAGTA CAGCTGGCTG
      ATCGACGGCA ACATCCAGCA GCACACCCAG GAGCTGTTCA TCAGCAACAT
1401  CACCGAGAAG AACAGCGGCC TGTACACCTG CCAGGCCAAC AACAGCGCCA
      GCGGCCACAG CCGCACCACC GTGAAGACCA TCACCGTGAG CGCCGAGCTG
1501  CCCAAGCCCA GCATCAGCAG CAACAACAGC AAGCCCGTGG AGGACAAGGA
      CGCCGTGGCC TTCACCTGCG AGCCCGAGGC CCAGAACACC ACCTACCTGT
1601  GGTGGGTGAA CGGCCAGAGC CTGCCCGTGA GCCCCCGCCT GCAGCTGAGC
      AACGGCAACC GCACCCTGAC CCTGTTCAAC GTGACCCGCA ACGACGCCCG
1701  CGCCTACGTG TGCGGCATCC AGAACAGCGT GAGCGCCAAC CGCAGCGACC
      CCGTGACCCT GGACGTGCTG TACGGCCCCG ACACCCCCAT CATCAGCCCC
1801  CCCGACAGCA GCTACCTGAG CGGCGCCAAC CTGAACCTGA GCTGCCACAG
      CGCCAGCAAC CCCAGCCCCC AGTACAGCTG GCGCATCAAC GGCATCCCCC
1901  AGCAGCACAC CCAGGTGCTG TTCATCGCCA AGATCACCCC CAACAACAAC
      GGCACCTACG CCTGCTTCGT GAGCAACCTG GCCACCGGCC GCAACAACAG
2001  CATCGTGAAG AGCATCACCG TGAGCGCCAG CGGCACCTCT AGAGCTCCCC
      AGACTATTAC AGAACTATGT TCGGAATATC GCAACACACA AATATATACG
2101  ATAAATGACA AGATACTATC ATATACGGAA TCGATGGCAG GCAAAAGAGA
      AATGGTTATC ATTACATTTA AGAGCGGCGA AACATTTCAG GTCGAAGTCC
2201  CGGGCAGTCA ACATATAGAC TCCCAGAAAA AAGCCATTGA AAGGATGAAG
      GACACATTAA GAATCACATA TCTGACCGAG ACCAAAATTG ATAAATTATG
2301  TGTATGGAAT AATAAAACCC CCAATTCAAT TGCGGCAATC AGTATGGAAA
      ACTAG (SEQ ID NO:4)
```

FIG.9B

hCEA-LTBopt Coding Sequence

```
   1  ATGGAGAGCC CCAGCGCCCC CCCCCACCGC TGGTGCATCC CCTGGCAGCG
      CCTGCTGCTG ACCGCCAGCC TGCTGACCTT CTGGAACCCC CCCACCACCG
 101  CCAAGCTGAC CATCGAGAGC ACCCCCTTCA ACGTGGCCGA GGGCAAGGAG
      GTGCTGCTGC TGGTGCACAA CCTGCCCCAG CACCTGTTCG GCTACAGCTG
 201  GTACAAGGGC GAGCGCGTGG ACGGCAACCG CCAGATCATC GGCTACGTGA
      TCGGCACCCA GCAGGCCACC CCCGGCCCCG CCTACAGCGG CCGCGAGATC
 301  ATCTACCCCA ACGCCAGCCT GCTGATCCAG AACATCATCC AGAACGACAC
      CGGCTTCTAC ACCCTGCACG TGATCAAGAG CGACCTGGTG AACGAGGAGG
 401  CCACCGGCCA GTTCCGCGTG TACCCCGAGC TGCCCAAGCC CAGCATCAGC
      AGCAACAACA GCAAGCCCGT GGAGGACAAG GACGCCGTGG CCTTCACCTG
 501  CGAGCCCGAG ACCCAGGACG CCACCTACCT GTGGTGGGTG AACAACCAGA
      GCCTGCCCGT GAGCCCCCGC CTGCAGCTGA GCAACGGCAA CCGCACCCTG
 601  ACCCTGTTCA ACGTGACCCG CAACGACACC GCCAGCTACA AGTGCGAGAC
      CCAGAACCCC GTGAGCGCCC GCCGCAGCGA CAGCGTGATC CTGAACGTGC
 701  TGTACGGCCC CGACGCCCCC ACCATCAGCC CCCTGAACAC CAGCTACCGC
      AGCGGCGAGA ACCTGAACCT GAGCTGCCAC GCCGCCAGCA ACCCCCCCGC
 801  CCAGTACAGC TGGTTCGTGA ACGGCACCTT CCAGCAGAGC ACCCAGGAGC
      TGTTCATCCC CAACATCACC GTGAACAACA GCGGCAGCTA CACCTGCCAG
 901  GCCCACAACA GCGACACCGG CCTGAACCGC ACCACCGTGA CCACCATCAC
      CGTGTACGCC GAGCCCCCCA AGCCCTTCAT CACCAGCAAC AACAGCAACC
1001  CCGTGGAGGA CGAGGACGCC GTGGCCCTGA CCTGCGAGCC CGAGATCCAG
      AACACCACCT ACCTGTGGTG GGTGAACAAC CAGAGCCTGC CCGTGAGCCC
1101  CCGCCTGCAG CTGAGCAACG ACAACCGCAC CCTGACCCTG CTGAGCGTGA
      CCCGCAACGA CGTGGGCCCC TACGAGTGCG GCATCCAGAA CGAGCTGAGC
1201  GTGGACCACA GCGACCCCGT GATCCTGAAC GTGCTGTACG GCCCCGACGA
      CCCCACCATC AGCCCCAGCT ACACCTACTA CCGCCCCGGC GTGAACCTGA
1301  GCCTGAGCTG CCACGCCGCC AGCAACCCCC CCGCCCAGTA CAGCTGGCTG
      ATCGACGGCA ACATCCAGCA GCACACCCAG GAGCTGTTCA TCAGCAACAT
1401  CACCGAGAAG AACAGCGGCC TGTACACCTG CCAGGCCAAC AACAGCGCCA
      GCGGCCACAG CCGCACCACC GTGAAGACCA TCACCGTGAG CGCCGAGCTG
1501  CCCAAGCCCA GCATCAGCAG CAACAACAGC AAGCCCGTGG AGGACAAGGA
      CGCCGTGGCC TTCACCTGCG AGCCCGAGGC CCAGAACACC ACCTACCTGT
1601  GGTGGGTGAA CGGCCAGAGC CTGCCCGTGA GCCCCCGCCT GCAGCTGAGC
      AACGGCAACC GCACCCTGAC CCTGTTCAAC GTGACCCGCA ACGACGCCCG
1701  CGCCTACGTG TGCGGCATCC AGAACAGCGT GAGCGCCAAC CGCAGCGACC
      CCGTGACCCT GGACGTGCTG TACGGCCCCG ACACCCCCAT CATCAGCCCC
1801  CCCGACAGCA GCTACCTGAG CGGCGCCAAC CTGAACCTGA GCTGCCACAG
      CGCCAGCAAC CCCAGCCCCC AGTACAGCTG GCGCATCAAC GGCATCCCCC
1901  AGCAGCACAC CCAGGTGCTG TTCATCGCCA AGATCACCCC CAACAACAAC
      GGCACCTACG CCTGCTTCGT GAGCAACCTG GCCACCGGCC GCAACAACAG
2001  CATCGTGAAG AGCATCACCG TGAGCGCCAG CGGCACCTCT AGAGCCCCCC
      AGAGCATCAC CGAGCTGTGC AGCGAGTACC GGAACACCCA GATCTACACC
2101  ATCAACGACA AGATCCTGAG CTACACCGAG AGCATGGCCG GCAAGAGGGA
      GATGGTGATC ATCACCTTCA AGAGCGGCGC CACCTTCCAG GTGGAGGTGC
2201  CCGGCAGCCA GCACATCGAC AGCCAGAAGA AGGCCATCGA GCGGATGAAG
      GACACCCTGC GGATCACCTA CCTCACCGAG ACCAAGATCG ACAAGCTGTG
2301  CGTGTGGAAC AACAAGACCC CCAACAGCAT CGCCGCCATC AGCATGGAGA
      ATTGATAA (SEQ ID NO:5)
```

FIG.9C

```
  1  MESPSAPPHR WCIPWQRLLL TASLLTFWNP PTTAKLTIES TPFNVAEGKE
 51  VLLLVHNLPQ HLFGYSWYKG ERVDGNRQII GYVIGTQQAT PGPAYSGREI
101  IYPNASLLIQ NIIQNDTGFY TLHVIKSDLV NEEATGQFRV YPELPKPSIS
151  SNNSKPVEDK DAVAFTCEPE TQDATYLWWV NNQSLPVSPR LQLSNGNRTL
201  TLFNVTRNDT ASYKCETQNP VSARRSDSVI LNVLYGPDAP TISPLNTSYR
251  SGENLNLSCH AASNPPAQYS WFVNGTFQQS TQELFIPNIT VNNSGSYTCQ
301  AHNSDTGLNR TTVTTITVYA EPPKPFITSN NSNPVEDEDA VALTCEPEIQ
351  NTTYLWWVNN QSLPVSPRLQ LSNDNRTLTL LSVTRNDVGP YECGIQNELS
401  VDHSDPVILN VLYGPDDPTI SPSYTYYRPG VNLSLSCHAA SNPPAQYSWL
451  IDGNIQQHTQ ELFISNITEK NSGLYTCQAN NSASGHSRTT VKTITVSAEL
501  PKPSISSNNS KPVEDKDAVA FTCEPEAQNT TYLWWVNGQS LPVSPRLQLS
551  NGNRTLTLFN VTRNDARAYV CGIQNSVSAN RSDPVTLDVL YGPDTPIISP
601  PDSSYLSGAN LNLSCHSASN PSPQYSWRIN GIPQQHTQVL FIAKITPNNN
651  GTYACFVSNL ATGRNNSIVK SITVSASGTS PGLSAGATVG IMIGVLVGVA
701  LI (SEQ ID NO:6)
```

FIG.10

```
   1 MELAALCRWG LLLALLPPGA ASTQVCTGTD MKLRLPASPE THLDMLRHLY QGCQVVQGNL
  61 ELTYLPTNAS LSFLQDIQEV QGYVLIAHNQ VRQVPLQRLR IVRGTQLFED NYALAVLDNG
 121 DPLNNTTPVT GASPGGLREL QLRSLTEILK GGVLIQRNPQ LCYQDTILWK DIFHKNNQLA
 181 LTLIDTNRSR ACHPCSPMCK GSRCWGESSE DCQSLTRTVC AGGCARCKGP LPTDCCHEQC
 241 AAGCTGPKHS DCLACLHFNH SGICELHCPA LVTYNTDTFE SMPNPEGRYT FGASCVTACP
 301 YNYLSTDVGS CTLVCPLHNQ EVTAEDGTQR CEKCSKPCAR VCYGLGMEHL REVRAVTSAN
 361 IQEFAGCKKI FGSLAFLPES FDGDPASNTA PLQPEQLQVF ETLEEITGYL YISAWPDSLP
 421 DLSVFQNLQV IRGRILHNGA YSLTLQGLGI SWLGLRSLRE LGSGLALIHH NTHLCFVHTV
 481 PWDQLFRNPH QALLHTANRP EDECVGEGLA CHQLCARGHC WGPGPTQCVN CSQFLRGQEC
 541 VEECRVLQGL PREYVNARHC LPCHPECQPQ NGSVTCFGPE ADQCVACAHY KDPPFCVARC
 601 PSGVKPDLSY MPIWKFPDEE GACQPCPINC THSCVDLDDK GCPAEQRASP LTSIISAVVG
 661 ILLVVVLGVV FGILIKRRQQ KIRKYTMRRL LQETELVEPL TPSGAMPNQA QMRILKETEL
 721 RKVKVLGSGA FGTVYKGIWI PDGENVKIPV AIAVLRENTS PKANKEILDE AYVMAGVGSP
 781 YVSRLLGICL TSTVQLVTQL MPYGCLLDHV RENRGRLGSQ DLLNWCMQIA KGMSYLEDVR
 841 LVHRDLAARN VLVKSPNHVK ITDFGLARLL DIDETEYHAD GGKVPIKWMA LESILRRRFT
 901 HQSDVWSYGV TVWELMTFGA KPYDGIPARE IPDLLEKGER LPQPPICTID VYMIMVKCWM
 961 IDSECRPRFR ELVSEFSRMA RDPQRFVVIQ NEDLGPASPL DSTFYRSLLE DDDMGDLVDA
1021 EEYLVPQQGF FCPDPAPGAG GMVHHRHRSS STRSGGGDLT LGLEPSEEEA PRSPLAPSEG
1081 AGSDVFDGDL GMGAAKGLQS LPTHDPSPLQ RYSEDPTVPL PSETDGYVAP LTCSPQPEYV
1141 NQPDVRPQPP SPREGPLPAA RPAGATLERP KTLSPGKNGV VKDVFAFGGA VENPEYLTPQ
1201 GGAAPQPHPP PAFSPAFDNL YYWDQDPPER GAPPSTFKGT PTAENPEYLG LDVPV*
(SEQ ID NO:7)
```

FIG.11

atggagtctc cctcggcccc tccccacaga tggtgcatcc cctggcagag gctcctgctc 60
acagcctcac ttctaacctt ctggaacccg cccaccactg ccaagctcac tattgaatcc 120
acgccgttca atgtcgcaga ggggaaggag gtgcttctac ttgtccacaa tctgccccag 180
catcttttg gctacagctg gtacaaaggt gaaagagtgg atggcaaccg tcaaattata 240
ggatatgtaa taggaactca acaagctacc ccagggcccg catacagtgg tcgagagata 300
atatacccca atgcatccct gctgatccag aacatcatcc agaatgacac aggattctac 360
accctacacg tcataaagtc agatcttgtg aatgaagaag caactggcca gttccgggta 420
tacccggagc tgcccaagcc ctccatctcc agcaacaact ccaaacccgt ggaggacaag 480
gatgctgtgg ccttcacctg tgaacctgag actcaggacg caacctacct gtggtgggta 540
aacaatcaga gcctcccggt cagtcccagg ctgcagctgt ccaatggcaa caggaccctc 600
actctattca atgtcacaag aaatgacaca gcaagctaca aatgtgaaac ccagaaccca 660
gtgagtgcca ggcgcagtga ttcagtcatc ctgaatgtcc tctatggccc ggatgccccc 720
accatttccc ctctaaacac atcttacaga tcaggggaaa atctgaacct ctcctgccac 780
gcagcctcta acccacctgc acagtactct tggtttgtca atgggacttt ccagcaatcc 840
acccaagagc tctttatccc caacatcact gtgaataata gtggatccta tacgtgccaa 900
gcccataact cagacactgg cctcaatagg accacagtca cgacgatcac agtctatgca 960
gagccaccca aacccttcat caccagcaac aactccaacc ccgtggagga tgaggatgct 1020
gtagccttaa cctgtgaacc tgagattcag aacacaacct acctgtggtg ggtaaataat 1080
cagagcctcc cggtcagtcc caggctgcag ctgtccaatg acaacaggac cctcactcta 1140
ctcagtgtca caaggaatga tgtaggaccc tatgagtgtg gaatccagaa cgaattaagt 1200
gttgaccaca gcgacccagt catcctgaat gtcctctatg gcccagacga ccccaccatt 1260
tccccctcat acacctatta ccgtccaggg gtgaacctca gcctctcctg ccatgcagcc 1320
tctaacccac ctgcacagta ttcttggctg attgatggga acatccagca acacacacaa 1380
gagctcttta tctccaacat cactgagaag aacagcggac tctatacctg ccaggccaat 1440
aactcagcca gtggccacag caggactaca gtcaagacaa tcacagtctc tgcggagctg 1500
cccaagccct ccatctccag caacaactcc aaacccgtgg aggacaagga tgctgtggcc 1560
ttcacctgtg aacctgaggc tcagaacaca acctacctgt ggtgggtaaa tggtcagagc 1620
ctcccagtca gtcccaggct gcagctgtcc aatggcaaca ggaccctcac tctattcaat 1680
gtcacaagaa atgacgcaag agcctatgta tgtggaatcc agaactcagt gagtgcaaac 1740
cgcagtgacc cagtcaccct ggatgtcctc tatgggccgg acacccccat catttccccc 1800
ccagactcgt cttacctttc gggagcgaac ctcaacctct cctgccactc ggcctctaac 1860
ccatccccgc agtattcttg gcgtatcaat gggataccgc agcaacacac acaagttctc 1920
tttatcgcca aaatcacgcc aaataataac gggacctatg cctgttttgt ctctaacttg 1980
gctactggcc gcaataattc catagtcaag agcatcacag tctctgcatc tggaact
(SEQ ID NO:8)

FIG.12A

```
  1  MESPSAPPHR WCIPWQRLLL TASLLTFWNP PTTAKLTIES TPFNVAEGKE
 51  VLLLVHNLPQ HLFGYSWYKG ERVDGNRQII GYVIGTQQAT PGPAYSGREI
101  IYPNASLLIQ NIIQNDTGFY TLHVIKSDLV NEEATGQFRV YPELPKPSIS
151  SNNSKPVEDK DAVAFTCEPE TQDATYLWWV NNQSLPVSPR LQLSNGNRTL
201  TLFNVTRNDT ASYKCETQNP VSARRSDSVI LNVLYGPDAP TISPLNTSYR
251  SGENLNLSCH AASNPPAQYS WFVNGTFQQS TQELFIPNIT VNNSGSYTCQ
301  AHNSDTGLNR TTVTTITVYA EPPKPFITSN NSNPVEDEDA VALTCEPEIQ
351  NTTYLWWVNN QSLPVSPRLQ LSNDNRTLTL LSVTRNDVGP YECGIQNELS
401  VDHSDPVILN VLYGPDDPTI SPSYTYYRPG VNLSLSCHAA SNPPAQYSWL
451  IDGNIQQHTQ ELFISNITEK NSGLYTCQAN NSASGHSRTT VKTITVSAEL
501  PKPSISSNNS KPVEDKDAVA FTCEPEAQNT TYLWWVNGQS LPVSPRLQLS
551  NGNRTLTLFN VTRNDARAYV CGIQNSVSAN RSDPVTLDVL YGPDTPIISP
601  PDSSYLSGAN LNLSCHSASN PSPQYSWRIN GIPQQHTQVL FIAKITPNNN
651  GTYACFVSNL ATGRNNSIVK SITVSASGT (SEQ ID NO:9)
```

FIG.12B

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/057358

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/09

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/052917 A (EPIMMUNE INC [US]; KEOGH ELISSA A [US]; SOUTHWOOD SCOTT [US]; FIKES JO) 24 June 2004 (2004-06-24) page 6, paragraph 17 page 23, paragraph 78 page 27, paragraph 86	1,3,4, 10-12
A	FACCIABENE ANDREA ET AL: "DNA and adenoviral vectors encoding carcinoembryonic antigen fused to immunoenhancing sequences augment antigen-specific immune response and confer tumor protection" January 2006 (2006-01), HUMAN GENE THERAPY, VOL. 17, NR. 1, PAGE(S) 81-92 , XP002458937 ISSN: 1043-0342 the whole document	1-21

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 November 2007	30/11/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Hoff, Céline

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/057358

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KASS ERIK ET AL: "Induction of protective host immunity to carcinoembryonic antigen (CEA), a self-antigen in CEA transgenic mice, by immunizing with a recombinant vaccinia-CEA virus" 1 February 1999 (1999-02-01), CANCER RESEARCH, VOL. 59, NR. 3, PAGE(S) 676-683 , XP002458938 ISSN: 0008-5472 the whole document	1-21
A	WO 2006/008154 A (CUREVAC GMBH [DE]; HOERR INGMAR [DE]; PASCOLO STEVE [DE]) 26 January 2006 (2006-01-26) claims 1-32	1-21
A	BERNHARD H ET AL: "Vaccination against the HER-2/neu oncogenic protein" ENDOCRINE-RELATED CANCER, JOURNAL OF ENDOCRINOLOGY LTD., BRISTOL, GB, vol. 9, no. 1, March 2002 (2002-03), pages 33-44, XP003016518 ISSN: 1351-0088 the whole document	1-21
A	WO 2005/019455 A (ANGELETTI P IST RICHERCHE BIO [IT]; AURISICCHIO LUIGI [IT]; CILIBERTO) 3 March 2005 (2005-03-03) the whole document	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/057358

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 12, 20-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2007/057358

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004052917 A	24-06-2004	AU 2003296330 A1	30-06-2004
		CA 2511775 A1	24-06-2004
		EP 1583548 A2	12-10-2005
		US 2006094649 A1	04-05-2006
WO 2006008154 A	26-01-2006	DE 102004035227 A1	16-02-2006
		EP 1768703 A1	04-04-2007
WO 2005019455 A	03-03-2005	CA 2534547 A1	03-03-2005
		EP 1658370 A1	24-05-2006