(12)特許協力条約に基づいて公開された国際出願

(19) 世界知的所有権機関
国際事務局

(43) 国際公開日
2004年7月15日 (15.07.2004)

PCT

(10) 国際公開番号
WO 2004/058299 A1

(51) 国際特許分類[7]: A61K 39/00, 48/00, A61P 35/00

(21) 国際出願番号: PCT/JP2003/016548

(22) 国際出願日: 2003年12月24日 (24.12.2003)

(25) 国際出願の言語: 日本語

(26) 国際公開の言語: 日本語

(30) 優先権データ:
特願2002-372103
2002年12月24日 (24.12.2002) JP

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(81) 指定国 (国内): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) 指定国 (広域): ARIPO特許 (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), ユーラシア特許 (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), ヨーロッパ特許 (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI特許 (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

添付公開書類:
— 国際調査報告書

２文字コード及び他の略語については、定期発行される各PCTガゼットの巻頭に掲載されている「コードと略語のガイダンスノート」を参照。

(54) Title: VACCINES COMPRISING POLYNUCLEOTIDES

(54) 発明の名称: ポリヌクレオチドからなるワクチン

(57) Abstract: A composition containing an expression vector which encodes an antigen recognized by CD4+ helper T lymphocyte and an antigen recognized by CD8+ cytotoxic T lymphocyte (CTL) (in particular, a tumor-specific antigen, a tumor-associated antigen or a cell-associated antigen), preferably containing an expression vector encoding interferon-gamma. Specific examples of the antigen recognized by CD4+ helper T lymphocyte include cancer/testis antigens such as NY-ESO-1, while specific examples of the antigen recognized by CD8+ cytotoxic T lymphocyte (CTL) (in particular, a tumor-specific antigen, a tumor-associated antigen or a cell-associated antigen) include HER-2/neu and cancer/testis antigens such as NY-ESO-1, MAGE and SAGE.

(57) 要約: CD4+ヘルパーT細胞により認識される抗原、およびCD8+細胞障害性T細胞(CTL)により認識される抗原、特に腫瘍特異性抗原、腫瘍関連抗原または細胞関連抗原をコードする発現ベクターを含む組成物であり、インターフェロンガンマをコードする発現ベクターを含むことが好ましい。具体的には、例えばCD4+ヘルパーT細胞により認識される抗原としてNY-ESO-1等の癌/精巣抗原を、CD8+細胞障害性T細胞(CTL)により認識される抗原、特に腫瘍特異性抗原、腫瘍関連抗原または細胞関連抗原としてHER-2／neu等あるいはNY-ESO-1,MAGE,SAGE等の癌/精巣抗原等を挙げることができる。

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DESCRIPTION

VACCINES COMPRISING POLYNUCLEOTIDES

Technical Field

The present invention relates to vaccines for developing tumor-specific immunity *in vivo*. More specifically, the invention relates to polynucleotide vaccines comprising one or more expression vectors that encode an antigen recognized by CD4+ helper T cells, and a tumor-specific, tumor-associated, or cell-related antigen. The antigens recognized by CD4+ helper T cells are preferably cancer/testis antigens identified by the SEREX (serological identification of antigen by recombinant cDNA expression cloning) method. To reliably achieve activation of CD4+ helper T cells for developing tumor immunity, an expression vector encoding interferon gamma is preferably administered at the same time. The tumor-specific, tumor-associated, or cell-related antigens are preferably antigens showing high rates of expression in one or more cancer types, and comprising an antigenic site that exhibits suitable compatibility with, and thus binds to, major histocompatibility complex (MHC) class I molecules of mammals including humans. Furthermore, so that they can be expressed and/or presented on the same cell, the expression vectors encoding the antigen recognized by CD4+ helper T cells and the tumor-specific, tumor-associated, or cell-related antigen, as well as expression vectors encoding interferon gamma are preferably immobilized on the same carrier particle or the like and administered to a cell; however, this invention is not restricted to this.

Background Art

For more than a decade, cancer has been the leading cause of death in Japan. Immunotherapy is expected to be a fourth method of cancer treatment, along with surgery, radiotherapy and chemotherapy. Immunotherapy is a treatment method utilizing the *in vivo* immune response system. An immune response is elicited and controlled by interactions between B lymphocytes, T lymphocytes, antibodies, and antigen presenting cells (APC). First, an exogenous antigen is processed by an APC, bound to a major histocompatibility complex (MHC) class I or class II molecule, and presented to helper T cells. Helper T cell recognition of this MHC-bound exogenous antigen leads to T cell activation and cytokine secretion. The secreted cytokines help differentiate antigen-stimulated B cells into antibody-forming cells and also promote the differentiation of killer T cells. Cells expressing the antigens are eliminated by secreted antibodies and activated killer T cells, and thus cellular and humoral responses progress to eliminate exogenous antigens.

Elimination of antigen-expressing cells by T cells can classified into three main groups:

1) humoral immunity (activated helper T cells stimulate proliferation/differentiation of specific B cell clones to produce antibodies that function in the recognition and elimination of antigens), 2) specific cellular immunity (activated helper T cells induce cytotoxic T cells (CTL) that respond in an antigen-specific manner and act directly on the target), and 3) non-specific cellular immunity (activated helper T cells induce non-specific natural killer cells, activated macrophages, and such, and these cells function to eliminate antigens). As described above, T cells play a central role in recognizing target antigens to elicit an immune response.

Regarding tumor rejection, appropriate immunization using congenital or self-derived tumor cells or their fractions has been known to induce antitumor immune responses in host cells (L. Gross, Cancer Res. 3: 326-333 (1943); E.J. Foley, Cancer Res. 13: 835-837 (1953); R.T. Prehn and J.M. Maln, J. Natl. Cancer Inst. 18: 769-778 (1957); G. Klein *et al.*, Cancer Res. 20: 1561-1572 (1980); L. Old *et al.*, Ann. N.Y. Acad. Sci. 101: 80-106 (1962); A. Globerson and M. Feldman, J. Natl. Cancer Inst. 32: 1229-1243 (1964)). The role of CD8+ and CD4+ T cells in these antitumor immune systems has been of enormous interest (R.J. North, Adv. Immunol. 35: 89-155 (1984); P.D. Greenberg, Adv. Immunol. 49: 281-355 (1991); D.M. Pardoll and S.L. Topalian, Curr. Opin. Immunol. 10: 588-594 (1998)). CD8+ T cells derived from specifically immunized mice are reported to be capable of destroying tumor target cells *in vitro* (H. Wagner *et al.*, Adv. Cancer Res. 31: 77-124 (1980)). It has also been reported that adoptive transfer of CD8+ T cells from an immunized donor confers resistance to tumor transplants to susceptible mice (R.J. North, Adv. Immunol. 35: 89-155 (1984); P.D. Greenberg, Adv. Immunol. 49: 281-355 (1991); C. J.M. Melief, Adv. Cancer Res. 58: 143-175 (1992)). In addition, anti-CD8+ antibodies are known to abolish resistance to tumor transplantation in preimmunized mice (E. Nakayama and A. Uenaka, J. Exp. Med. 161: 345-355 (1985); X.G. Gu *et al.*, Cancer Res., 58: 3385-3390 (1998); Y. Noguchi *et al.*, Proc. Natl. Acad. Sci. USA 92: 2219-2223 (1994)). Over the past decade, MHC class I binding peptides derived from the tumor cells of mice and humans and recognized by CD8+ T cells have been discovered (T. Boon *et al.*, Annu. Rev. Immunol. 12: 337-368 (1994); S.A. Rosenberg, Immunity 10: 281-287 (1999)).

There are two forms of tumor antigen (i.e., target molecules on tumor cells): tumor peptides presented by MHC class I molecules and targeted by CD8+ CTL, which plays a major part in cellular immunity (tumor rejection antigens); and molecules called tumor-associated antigens, which are targeted by humoral immunity (antibodies) and expressed on tumor cell membranes. Following the genetic level identification of human tumor antigens recognized by T lymphocytes, various human tumor rejection antigens have been discovered. In melanomas, a clear antitumor effect has been observed due to vaccination therapy, which is a specific immunotherapy using tumor rejection antigens. Furthermore, attempts are being made to enhance the immunotherapeutic effect by the combined use of cytokines, and to apply dendritic

cells pulsed with antigenic peptides, or dendritic cells to which an antigen gene has been introduced. Recently, DNA vaccines are also being tested.

A number of approaches have been attempted to augment the helper action of CD4+ T cells (D. Pardoll and S.L. Topalian, Curr. Opin. Immunol. 10: 588-594 (1998); R.F. Wang, Trends Immunol. 5: 269-276 (2001)). Earlier methods can be divided into three categories. The first method is modification of the immunizing antigens themselves by, for example, haptenizing an antigen (Y. Mizushima *et al.*, J. Natl. Cancer Inst. 74: 1269-1273 (1985)) or directly linking a heterologous immunogenic peptide to an antigen (R.W. Chesnut *et al.*, Vaccine Design, eds. M.F. Powell and M.J. Newman (Plenum, New York) 847-874 (1995); J. Rice *et al.*, J. Immunol. 167: 1558-1565 (2001)). The second method is co-immunization with tumor antigens and molecules having a strong helper determinant (R. Romieu *et al.*, J. Immunol. 161: 5133-5137 (1998); N. Casares *et al.*, Eur. J. Immunol. 31: 1780-1789 (2001)), such as viral vectors encoding tumor antigens (M. Wang *et al.*, J. Immunol. 154: 4685-4692 (1995)). The last method is based on the discovery of molecular signals such as CD40 ligands (J.P. Ridge *et al.*, Nature (London) 393: 474-478 (1998); S.R.M. Bennett *et al.*, Nature (London) 393: 478-480 (1998); S.P. Schoenberg *et al.*, Nature (London) 393: 480-483 (1998)) and other stimulatory or co-stimulatory signals regulating the helper function of CD4+ T cells and the interaction of APC with CD4+ T cells (A. Porgador *et al.*, J. Exp. Med. 188: 1075-1082 (1998)). The discovery of such signals is thought to provide methods for augmenting CD8+ T cell response.

Conventionally, antibodies have not been thought to play a large part in antitumor effector functions. However, it is now clear that tumor antigens elicit a strong and complete immune response involving both cellular and humoral immunity (for example, Y.-T. Chen *et al.*, Proc. Natl. Acad. Sci. USA 94: 1914-1918 (1997); E. Jager *et al.*, J. Exp. Med. 187: 625-630 (2000); E. Jager *et al.*, Proc. Natl. Acad. Sci. USA 97: 12198-12203 (2000)). Recently, M. Pfreundschuh and his colleagues developed a method called SEREX (Y.-T. Chen *et al.*, Proc. Natl. Acad. Sci. USA 94: 1914-1918 (1997)) to identify tumor-associated antigens that may serve as vaccines against tumors. In this method, human sera are used to screen human tumor cDNA expression libraries. More than 1800 kinds of genes identified by SEREX are registered on the Internet as the SEREX database (www.licr.org/SEREX.html).

However, many problems are left unsolved, such as what kind of adjuvant or APC to use to effectively induce tumor-specific immunity using the identified antigenic peptides/DNAs to completely treat tumors; or how to deal with the immune system evasion of tumors.

Helper T cells are often reported to be necessary for quantitative/qualitative amplification of CTL. However, little is known about the characteristics of antigen molecules recognized by these T cells and the functional impact of T cells on antitumor immune responses (P.D. Greenberg, Adv. Immunol. 49: 281-355 (1991); D.M. Pardoll and S.L. Topalian, Curr. Opin.

Immunol. 10: 588-594 (1998); S.R. Bennett *et al.*, J. Exp. Med. 186: 65-70 (1997); R.F. Wang, Trends Immunol. 5: 269-276 (2001); C. Fayolle *et al.*, J. Immunol. 147: 4069-4073 (1991); M. Shiral *et al.*, J. Immunol. 152: 1549-1556 (1994); K. Hung *et al.*, J. Exp. Med. 188: 2357-2368 (1998); F. Ossendorp *et al.*, J. Exp. Med. 187: 693-702 (1998); Y. Shen and S. Fujimoto, Cancer Res. 56: 5005-5011 (1996); T. Nishimura *et al.*, J. Exp. Med. 190: 617-627 (1999); D.R. Surman *et al.*, J. Immunol. 164: 562-565 (2000); A. Franco *et al.*, Nat. Immunol. 1: 145-150 (2000); C.N. Baxevanis *et al.*, J. Immunol. 164: 3902-3912 (2000); F. Fallarino *et al.*, J. Immunol. 165: 5495-5501 (2000); A.L. Marzo *et al.*, Cancer Res. 59: 1071-3390 (1999); A.L. Marzo *et al.*, J. Immunol. 165: 6047-6055 (2000)). The current hypothesis for serial intercellular interaction amongst helper T cells, CTL and APC points to the possibility that helper T cells related to antitumor immune response can recognize a diverse and wide range of antigens (J.P. Ridge *et al.*, Nature 393: 474-478 (1998); S.R.M. Bennett *et al.*, Nature 393: 478-480 (1998); S.P. Schoenberger *et al.*, Nature 393: 480-483 (1998); Z. Lu *et al.*, J. Exp. Med. 191: 541-550 (2000)).

Analyses of humoral immune responses in human and murine tumors are greatly advancing due to the above-described SEREX method (Y.-T. Chen *et al.*, Proc. Natl. Acad. Sci. USA 94: 1914-1918 (1997); E. Jager *et al.*, J. Exp. Med. 187: 625-630 (2000); E. Jager *et al.*, Proc. Natl. Acad. Sci. USA 97: 12198-12203 (2000); U. Sahin *et al.*, Proc. Natl. Acad. Sci. USA 92: 11810-11813 (1995); L.J. Old and Y.-T. Chen, J. Exp. Med. 187: 1163-1167 (1998); Y.-T. Chen, "Principle and Practice of the Biologic Therapy of Cancer", ed. S.A. Rosenberg (Lippincott Williams & Wilkins, Philadelphia) 557-570 (2000); T. Ono *et al.*, Int. J. Cancer 88: 845-851 (2000)). It is reported that when many of the genes identified by SEREX are completely sequenced, their sequence is the same as the wild-type sequence. In other words, they do not comprise amino acid substitutions or such (L.J. Old and Y.-T. Chen, J. Exp. Med. 187: 1163-1167 (1998); Y.-T. Chen, "Principle and Practice of the Biologic Therapy of Cancer", ed. S.A. Rosenberg (Lippincott Williams & Wilkins, Philadelphia) 557-570 (2000)). Therefore, the immunogenicity of these molecules is not the result of mutations. Furthermore, although some SEREX antigens show tumor-restricted expression in normal tissues (e.g., cancer/testis antigens, melanocyte differentiation antigens, etc.), most of the SEREX-identified antigens are ubiquitously expressed. However, the serum samples of patients with associated or non-associated cancer show more antibodies with a high titer against these wild-type molecules than normal healthy subjects. At present, the immunogenic stimulus for eliciting humoral immunity is thought to be the enhanced expression of these tumor products. Since all of these molecules are detected by IgG class antibodies, it is possible that CD4+ helper T cells recognize these wild-type molecules. In view of the above, the present inventors examined and discovered that wild-type immunogenic molecules of tumor cells can amplify tumor-specific

CD8+ CTL via CD4+ helper T cell activation. Namely, they discovered the involvement of such molecules in antitumor immune response (H. Nishikawa *et al.*, Pro. Natl. Acad. Sci. U.S.A. 98(25):14571-14576 (2001)).

With regards to DNA vaccines, intramuscular administration of naked DNA has been proven to induce both humoral and cellular immune responses. Though the precise mechanism by which DNA vaccines induce an immune response is currently unknown (see Pardoll *et al.*, Immunity 3: 165-169 (1995)), their effectiveness is shown by the induction of humoral and cellular immunities. These results indicate that naked DNA is expressed after administering a DNA vaccine, and peptide products of the naked DNA are presented as antigens with both the MHC class I and class II proteins.

Exogenous peptides bound to MHC class I and/or II molecules and derived from viruses, bacteria, and the like are recognized as antigens by T cell receptors on CTL. This promotes reactions such as the production of various lymphokines and proliferation of cells, killing cells infected with the virus, bacteria, or such from which the exogenous peptide was derived. Irrespective of the location or function in the pathogen, these antigenic peptides are fragments incorporated and processed by APC or other cells. To artificially generate CTL responses, replication vectors that produce protein antigens in cells can be used (J.R. Bennink and J.W. Yewdell, Curr. Top. Microbiol. Immunol. 163: 153 (1990); C.K. Stover *et al.*, Nature 351: 456 (1991); A. Aldovini and R.A. Young, Nature 351: 479 (1991); R. Schfer *et al.*, J. Immunol. 149: 53 (1992); C.S. Hahn *et al.*, Proc. Natl. Acad. Sci. USA 89: 2679 (1992)); as well as methods for introducing peptides to the cytosol (F.R. Carbone and M.J. Bevan, J. Exp. Med. 169: 603 (1989); K. Deres *et al.*, Nature 342: 561 (1989); H. Takahashi *et al.*, Nature 344: 873 (1990); D.S. Collins *et al.*, J. Immunol. 148: 3336 (1992); M.J. Newman *et al.*, J. Immunol. 148: 2357 (1992)).

Furthermore, a method for using naked polynucleotides as a vaccine to inoculate a vertebrate has been examined (WO90/11092 (October 4, 1990)). Expression of DNA precipitated with calcium chloride and administered into the abdominal cavity, veins, or muscles is possible (N. Benvenisty and L. Reshef, Proc. Natl. Acad. Sci. USA 83: 9551-9555 (1986)). In mice, a DNA expression vector was shown to be incorporated into myocytes and expressed in cells upon intramuscular injection (J.A. Wolff *et al.*, Science 247: 1465 (1990); G. Ascadi *et al.*, Nature 352: 815 (1991)). In this case, the vector was maintained as an episome and did not replicate. However, persistent expression of the vector was observed following injection into the skeletal muscle of rats, fish, and primates, as well as the cardiac muscle of rats (H. Lin *et al.*, Circulation 82: 2217 (1990); R.N. Kitsis *et al.*, Proc. Natl. Acad. Sci. USA 88: 4138 (1991); E. Hansen *et al.*, FEBS Lett. 290: 73 (1991); S. Jiao *et al.*, Hum. Gene Therapy 3: 21 (1992); J.A. Wolff *et al.*, Human Mol. Genet. 1: 363 (1992)). It was further reported that B7 on the surface

of APC and MHC presentation of epitopes play comparable roles in the activation of CTL during tumor elimination (Edington, Biotechnology 11: 1117-1119 (1993)). When an MHC molecule on the surface of APC presents an epitope to a T cell receptor, B7 expressed on the surface of the same APC binds to CTLA-4 or CD28 and functions as the second signal. As a result, CD4+ helper T cells, which emit signals to increase APC-destroying CD8+ T cells, rapidly proliferate.

To immunize with DNAs, the DNAs do not necessarily have to be administered intramuscularly. For example, Tang *et al.* demonstrates that mice produce anti-bovine growth hormone (BGH) antibodies following administration to the skin of gold particles coated with DNA coding for BGH (Tang *et al.*, Nature 356: 152-154 (1992)). In addition to skin, it is reported that a jet injector can be used to transfect DNA to living animal tissues, such as muscular, adipose, mammary gland tissues and such (Furth *et al.*, Analytical Biochemistry 205: 365-368 (1992)). Various methods for introducing nucleic acids have also been published as reviews (T. Friedman, Science 244: 1275-1281 (1989)). WO93/17706 describes a method for inoculating an animal with a vaccine against viruses, by coating a carrier particle with a gene construct, and administering the coated particle to a cell of the animal. Further, DNA immunization against herpes virus has been reported (Cox *et al.*, J. Virol. 67: 5664-5667 (1993)). In addition, DNA vaccines and their production and/or administration methods are also described in U.S. Patent No. 4,945,050, U.S. Patent No. 5,036,006, U.S. Patent No. 5,589,466, WO94/16737, and such.

Disclosure of the Invention

Example embodiments may provide effective immunotherapy against tumors. The example embodiments may also provide permanent treatments for early stage cancers, therapies for suppressing postoperative recurrence or metastasis of cancers, and treatment methods for patients whose tumor has been detected but is inoperable, and for whom radiation and chemical treatments were ineffective.

Disclosed are vaccines for developing tumor-specific immunity, and is based on the finding that tumor-specific immunity can be induced by using, as a polynucleotide vaccine, expression vectors encoding an antigen recognized by CD4+ helper T cells, and a tumor-specific, tumor-associated, or cell-related antigen, and preferably an expression vector that encodes interferon gamma. In one aspect, there is provided a composition comprising one or more expression vectors encoding an antigen recognised by CD4+ helper T cells, and a tumor-specific, tumor-associated, or cell-related antigen. More preferably, the composition further comprises an expression vector encoding interferon gamma. The antigens recognized by CD4+ helper T cells are preferably molecules identified by the SEREX method, and more preferably SEREX antigens showing exclusive expression in tumors in ordinary tissue. In another aspect, there is provided a vaccine comprising the composition as defined above. In yet another aspect, there is provided the use of a composition as defined above in the manufacture of a medicament for inducing tumor-specific immunity in a mammal.

Examples of such antigens include NY-ESO-1, MAGE-A4, and such, categorized as cancer/testis antigens. The present inventors have shown that simultaneous presentation of a SEREX antigen with a tumor-specific, tumor-associated, or cell-related antigen strongly induces tumor-specific immunity (H. Nishikawa *et al.*, Pro. Nat. Acad. Sci. USA. 98(25):14571 (2001)). The inventors have also found that independent expression of only a SEREX antigen suppresses CD4+ helper T cell activity, thus suppressing tumor-specific immunity, whereas co-expression with interferon gamma blocks such suppression (Japanese Patent Application No. 2002-254967; H. Nishikawa *et al.*, Proc. Nat. Acad. Sci. USA. 100(19):10902 (2003)).

Thus, expression vectors encoding an antigen recognized by CD4+ helper T cells and a tumor-specific, tumor-associated, or cell-related antigen, and, more preferably an additional expression vector encoding interferon gamma, are immobilized onto the same carrier particles and administered to cells so as to be expressed and presented on the same cell. However, this invention is not limited to this.

As described herein, an "antigen recognized by CD4+ helper T cells" refers to a polypeptide that comprises a portion that serves as an epitope of a CD4+ glycoprotein expressed on mature helper T cells or precursor cells thereof. When a T cell receptor recognizes an antigen presented by an MHC class II, CD4 binds to the β2 domain of the MHC class II and thus enhances the ability of the T cell to recognize antigens. CD4 further transmits signals into the cell, and promotes proliferation and secretion of cytokines. In the present invention, molecules identified by SEREX are particularly preferred among the molecules recognized by CD4+ helper T cells. Such molecules include, but are not restricted to, cancer/testis antigens such as NY-ESO-1 and MAGE-A1, heat shock proteins such as DnaJ-like 2, DNA ligase 1, galactin 1, as well as poly (A) binding protein, *Homo sapiens* hexamethylene-bis-acetamide-inducible protein, human retinoic acid-responsive protein, *H. sapiens* hepatitis delta antigen interacting protein A (DIPA), *H. sapiens* cDNA FLJ20644 fis clone KATO02588 (FLJ20644fis), etc. Most of the molecules identified by the SEREX method are wild-type molecules that are also generally expressed in healthy people. However, to avoid unnecessary immune responses in healthy tissues, molecules specifically expressed in tumors are desirable among those identified by the SEREX method. Thus, cancer/testis antigens such as NY-ESO-1 and MAGE-A4 are particularly preferred.

Herein, a "tumor-specific, tumor-associated, or cell-related antigen" in the present invention refers to an antigen that is associated with tumors or expressed specifically in specific tumor cells. Antigens expressed only in cancers and testis include MAGE, BAGE, GAGE, SAGE, NY-ESO-1, etc. Cancer-specific mutant antigens, which are derived from mutant gene products produced during carcinogenesis, include CDK4, MUM-1, CASP-8, ras, bcr-abl, etc. Tissue-specific antigens, which are locally expressed in specific tissues, include MART-1, TRP,

tyrosinase, gp100, PSA, proteinase 3, etc. Protein antigens expressed in higher levels in cancers include HER2/neu, CEA, SART1, etc. Viral antigens include EBV, HPV, HTLV-1, etc. Herein, a tumor-specific, tumor-associated, or cell-related antigen may be a full-length protein or a fragment of such with immunogenicity.

Tumor-specific and/or tumor-associated antigens show characteristic expression patterns in various cancer types. For example, Her2/neu is expressed in 20-30% of breast cancers, ovarian cancers, gastric cancers, lung cancers, and such. NY-ESO-1 is expressed in 20-30% of breast cancers, ovarian cancers, and esophageal cancers; MAGE-A4 in 50-60% of head and neck cancers and esophageal cancers, and in 20% of lung cancers; and SAGE in 20% of lung cancers, esophageal cancers, and head and neck cancers. Therefore, the above-described highly expressed antigens are particularly preferred as tumor-specific, tumor-associated, or cell-related antigens to be used in anti-cancer vaccines for treating these cancers. Furthermore, some of the cancer/testis antigens such as NY-ESO-1 and MAGE-A4 have been known to comprise in a single molecule 1) an antigenic site to bind an MHC class I molecule, thereby stimulating CD8+ CTL, and 2) an antigenic site to bind an MHC class II molecule, thus allowing identification as a SEREX antigen. Such antigens are particularly preferred.

The tumor-specific, tumor-associated, or cell-related antigens activating CD8+ CTL cells exert their function upon binding to an MHC class I molecule of a host animal to which a vaccine of the present invention is administered. When the host animal is a human, the antigen that activates CD8+ CTL cells needs to bind to a class I molecule of the HLA-A, -B or -C locus of each person. Incidentally, there are 23 known types of HLA-A locus, which is thought to be important in antigen presentation. Types A24 and A2 are common in Mongoloids including Japanese, and account for 70% or more. In Caucasians, including North American Caucasians and such, A1, A2, A3 and A29 are major types, and in Africans, A30 and A2 are common. Within its molecule, Her2/neu has been known to comprise epitopes that bind to HLA-A2 and/or HLA-A24. Tissue-specific antigens such as gp100, MART-1, and tyrosinase, as well as cancer/testis antigens such as MAGE-3 (A3), MAGE-A4, and NY-ESO-1, have also been known to comprise epitopes that bind to A2. Furthermore, tyrosinase and cancer/testis antigens such as MAGE-A4, NY-ESO-1, and SAGE, have been known to comprise an epitope that binds to A24. Accordingly, when a vaccine of the present invention is given to a host animal, it is preferable to examine the expression of these antigens in tumors, as well as the MHC type of the individual host animal, to select an antigen compatible to that type.

More specifically, the Genbank accession numbers for each antigen are shown below:

NY-ESO-1: AJ003149

BAGE1: AF499647

GAGE1:NM_001468

GAGE2: NM_001472
GAGE8: NM_012196
SAGE: NM_018666
HER-2/neu: M11730, NM_004448, NP_004439
MAGE-A3: NM_005362
MAGE-A4: NM_002362
MAGE-A12: NM_005367
DnaJ-like 2: NM_001539, NP_001530
PRAME: NM_006115
Homo sapiens hexamethylene-bis-acetamide-induced protein: XM_008348
Human retinoic acid-responsive protein: U50383
DIPA: XM_006503
FLJ20644fis: AK000651

In the present invention, the polynucleotides that encode the antigens are not restricted, and may be DNAs, RNAs, or such, as long as they can elicit a desired immune response when administered to a host animal by a method of the present invention. The polynucleotides that encode the antigens of the present invention may be those whose nucleotide sequence is artificially modified by one or more amino acid deletions, substitutions, insertions and/or additions, by known methods such as site-directed mutagenesis (see Current Protocols in Molecular Biology, John Wiley & Sons, Ausubel *et al.*, Section 8.1-8.5 (1987)), as long as the polypeptide encoded by the polynucleotide can generate a desired immune response in the host. Furthermore, as long as they can elicit a desired immune response in the host, the polynucleotides may encode naturally occurring polypeptides with mutations. Such mutants existing in nature may be isolated by known hybridization techniques (see Current Protocols in Molecular Biology, John Wiley & Sons, Ausubel *et al.*, Section 6.3-6.4 (1987)) and gene amplification techniques (PCR) (see Current Protocols in Molecular Biology, John Wiley & Sons, Ausubel *et al.*, Section 6.1-6.4 (1987)).

Further, if a gene encoding an antigenic protein is known, one skilled in the art can readily analyze the hydrophobic and/or hydrophilic regions within the amino acid sequence of the protein (Kyte and Doolittle, J. Mol. Biol. 157: 105-122 (1982)) and its secondary structure (Chou and Fasman, Ann. Rev. Biochem. 47: 251-276 (1978)) to predict antigenic regions in the amino acid sequence (for example, see Anal. Biochem. 151:540-546 (1985)), and can synthesize peptides having the predicted amino acid sequence to determine their antigenicity using the PEPSCAN method (Nature 314 (1985); Published Japanese Translation of International Publication No. Sho 60-500684), etc. Thus, a polynucleotide encoding a peptide fragment that comprises an epitope site that was determined based on an above-described method may be

produced by techniques such as chemical synthesis, and used in an expression vector for antigens of the present invention.

Expression vectors used in the present invention are recombinant vectors into which an antigen gene of the present invention, and preferably an interferon gamma gene, are inserted. The vectors to insert these genes include plasmids, phages, cosmids, viruses, and other conventional vectors in the technical field of the present invention. Those skilled in the art can construct various plasmids and vectors based on techniques described in, for example, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. (1989), edited by Sambrook *et al.*, and the above-described literature, edited by Ausubel *et al.*

Those skilled in the art can appropriately select factors such as promoters and terminators - used herein to regulate expression in the host - from known regulatory sequences, depending upon the host type and the purpose, and can also arrange them upstream and/or downstream of the antigen gene. Therefore, regulatory sequences derived from an antigen, or heterogeneous regulatory sequences, may be used in the present invention. Furthermore, if needed, markers such as antibiotic resistance markers may be used in the expression vectors of the present invention. Though many commercially available vectors can be used, nonessential polynucleotide sequences are preferably deleted from the vector in the present invention. The same holds for expression vectors for the interferon gamma gene. In addition, the polynucleotides encoding an antigen recognized by CD4+ helper T cells, those encoding a tumor-specific, tumor-associated, or cell-related antigen, and those encoding interferon gamma used in the present invention may be included in different expression vectors, or constructed to be expressed from a single vector, as long as they are arranged such that they can be expressed in the same cell.

Upon introduction into animal tissues, a vaccine of the present invention induces *in vivo* expression of a present antigen and elicits a desired immune response. Various methods are known for introducing nucleic acids into living bodies (T. Friedman, Science 244: 1275-1281 (1989)). Any method may be used as long as it induces *in vivo* expression of the present invention's antigens, and elicits a desired immune response.

The compositions of the present invention are useful as vaccines and can be used as naked plasmids. They may be packaged into liposomes, formed as various virus vectors including retrovirus vectors, adenovirus vectors, vaccinia virus vectors, poxvirus vectors, adeno-associated virus vectors, and HVJ (hemagglutinating virus of Japan) vectors (see K. Adolph "Virus genomic methods", CRC Press, Florida (1996), for example,), or coated on beads (carriers) such as colloidal gold particles. Preferably, vectors expressing an antigen recognized by CD4+ helper T cells, a tumor-specific, tumor-associated, or cell-related antigen, and preferably interferon gamma are adhered to a carrier particle, such as a gold particle, for

introduction into the body by gene gun or such; however, the present invention is not limited to this. Methods for coating carrier particles with polynucleotides are known (see, for example, WO93/17706). Finally, the polynucleotides can be prepared in solutions suitable for *in vivo* administration, such as physiological saline. To enhance immune reactions, the compositions of the present invention may be prepared as vaccines in combination with adjuvants such as known proteins or other carriers. Moreover, agents such as calcium ions, which help the uptake of plasmids in a cell, may be used in combination. In addition, pharmaceutically acceptable agents that make transfection easy may also be used as required.

The polynucleotide vaccines of the present invention may be administered by any method, so long as the method allows the vaccine to generate an immune response within a host animal. Preferably, the compositions of the present invention are administered at a dose sufficient to induce an immune response in the host animal, by methods including injections or infusions via appropriate parenteral routes such as intravenous, intraperitoneal, subcutaneous, intradermal, adipose tissues, mammary gland tissues, inhalation, or intramuscular; gas induced particle bombardment methods (with gene guns or such); or application methods through mucosal routes using nasal drops or such forms. Furthermore, a host animal may be immunized by administering the present composition into a blood cell, bone marrow-derived cell (APC, etc.), and such by using *ex vivo* methods such as liposome transfection, particle bombardment methods, and viral infection, and then reintroducing the cell into the animal. Of the above-mentioned administration methods, gene transformation methods using accelerated particles are described in U.S. Patent No. 4,945,050m, and devices based on modified methods thereof are commercially available (BioRad Laboratories). In particular, a preferred result can be obtained by transfecting dendritic cells *ex vivo,* and then returning the cells to the body.

The types of host animals of the present invention are not limited, as long as the animal's tumor immune response is enhanced by a composition of the present invention. Specific examples include mammals such as mice, rats, bovines, pigs, and primates like monkeys and humans, etc. Preferable host animals of the present invention include primates, and particularly humans.

The doses of the compositions of the present invention depend on the immunogenicity of the ingredients to the host. However, those skilled in the art can determine appropriate doses required for immunization by administering a given dose of the composition to test animals, and measuring the antibody titer using assay methods such as ELISA, detecting CTL response by chromium release assay or such, or observing immune response by detecting Th response using cytokine release measurement. Those skilled in the art will recognize that the immunogenicity of an ingredient in a composition also depends on the strength of regulatory sequences, such as transcription and translation promoters, used in expression vectors of the present invention.

Furthermore, those skilled in the art can readily adjust the dose of a composition of the present invention based on the types of expression vector to be used.

Thus, the present invention provides clinically useful vaccines for developing tumor-specific immunity *in vivo*. More specifically, the invention provides polynucleotide vaccines comprising expression vectors encoding an antigen recognized by CD4+ helper T cells and a tumor-specific, tumor-associated, or cell-related antigen, and preferably an expression vector encoding interferon gamma, as well as methods of using the same.

Brief Description of the Drawings

Fig. 1 is a graph showing the results of ELISPOT assays for the compositions of the present invention.

Best Mode for Carrying Out the Invention

Herein below, the present invention will be specifically described using an example, however, it is not to be construed as being limited thereto.

[Example 1]

The HER2-specific CD8 positive T cell enhancing effect of helper epitopes including NY-ESO-1

[Method]

1) Plasmids

The following genes were incorporated into the expression vector pCAGGS to construct plasmids. Each of the purified plasmids were adjusted to 1 μg/μl, coated on to gold particles, and used for immunization using the Helios Gene Gun System.

- 147HER2: a gene encoding the 147 N-terminal amino acid residues of c-erbB-2/HER2/neu (HER2);
- p63(T) mini gene: a gene encoding the CTL epitope of HER2 ; namely, HER2p63(T), TYLPTNASL ;
- NY-ESO-1: a gene encoding NY-ESO-1, which is a cancer/testis antigen; and
- mIFN-g: a gene encoding mouse interferon gamma.

2) Experimental Protocol

Six to eight week old BALB/c mice from the groups below (four animals per group) were immunized twice, at two weeks intervals. One week after immunization, two animals per

group were sacrificed and CD8 positive T cells were prepared from their spleens using MACS system. Using P1.HTR (DBA/2 mice derived mastocytoma) pulsed with HER2p63(T) peptides as the target cell, ELISPOT assays were performed to detect interferon gamma producing cells. P1.HTR pulsed with the HER2p780 (PYVSRLLGI) peptide were used as a negative control. Each experiment was conducted in duplicate.

(Experiment 1)

- 147HER2
- 147HER2 + mIFN-g
- 147HER2 + NY-ESO-1
- 147HER2 + mIFN-g + NY-ESO-1

(Experiment 2)

- p63(T) mini gene
- p63(T) mini gene + mIFN-g
- p63(T) mini gene + NY-ESO-1
- p63(T) mini gene + mIFN-g + NY-ESO-1

The results are shown in the following table and in Fig. 1.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
p63(T) pulsed 200000 cells	40.3	46	109.3	100.6
p63(T) pulsed 100000 cells	16.6	17.3	44	38
p63(T) pulsed 50000 cells	7	9	17.6	17
p780 pulsed 200000 cells	0.3	2	1	2.6
p780 pulsed 100000 cells	1	0.6	1	0.6
p780 pulsed 50000 cells	0.3	0.6	0.3	0.3

Ex.1: 147HER2 GG 2x day 7

Ex.2: 147HER2 + mIFN-g GG 2x day 7

Ex.3: 147HER2 + NY-ESO-1 GG 2x day 7

Ex.4: 147HER2 + mIFN-g + NY-ESO-1 GG 2x day 7

("GG" means administration using a gene gun.)

Industrial Applicability

As described above, it was clearly shown that a combination of an antigen recognized by CD4+ helper T cells and a tumor-specific, tumor-associated, or cell-related antigen, and, more preferably, co-expression of interferon gamma with such a combination causes an increase in

interferon gamma producing cells, and induces tumor-specific immunity. Examples of such antigen combinations include, for example, the combination of HER-2/neu and NY-ESO-1.

CLAIMS

1. A composition comprising one or more expression vectors encoding an antigen recognized by CD4+ helper T cells, and a tumor-specific, tumor-associated, or cell-related antigen.

2. The composition of claim 1, wherein the antigen recognized by CD4+ helper T cells is a molecule identified by the SEREX method.

3. The composition of claim 1 or 2, wherein the antigen recognized by CD4+ helper T cells is a cancer/testis antigen identified by the SEREX method.

4. The composition of claim 1 or 2, wherein the antigen recognized by CD4+ helper T cells is an antigen selected from the group consisting of NY-ESO-1, MAGE, BAGE, GAGE, and SAGE.

5. The composition of any one of claims 1 to 4, wherein the tumor-specific, tumor-associated, or cell-related antigen is HER-2/neu.

6. The composition of any one of claims 1 to 4, wherein the tumor-specific, tumor-associated, or cell-related antigen is a cancer/testis antigen.

7. The composition of any one of claims 1 to 5, wherein the antigen recognized by CD4+ helper T cells is a cancer/testis antigen, and the tumor-specific, tumor-associated, or cell-related antigen is HER-2/neu antigen.

8. The composition of any one of claims 1 to 4 or claim 6, wherein both the antigen recognized by CD4+ helper T cells and the tumor-specific, tumor-associated, or cell-related antigen are cancer/testis antigens.

9. The composition of any one of claims 1 to 8, wherein the antigen recognized by CD4+ helper T cells is NY-ESO-1, and the tumor-specific, tumor-associated, or cell-related antigen is HER-2/neu.

10. The composition of any one of claims 1 to 9, wherein polynucleotides encoding each antigen are comprised in separate expression vectors.

11. The composition of claim 10, wherein the expression vectors encoding each antigen have been immobilized on the same carrier particle.

12. The composition of claim 11, wherein the expression vectors encoding each antigen and an expression vector encoding interferon gamma have been immobilized on the same carrier particle.

13. A vaccine comprising the composition of any one of claims 1 to 12.

14. The vaccine of claim 13, wherein the vaccine is for administration with a gene gun.

15. The vaccine of claim 13 or 14, wherein the vaccine is for prevention and/or treatment of cancers.

16. Use of a composition of any one of claims 1 to 12 in the manufacture of a medicament for inducing tumor-specific immunity in a mammal.