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(11)

EP 3 173 476 A1

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:

31.05.2017 Bulletin 2017/22

(51) Int Cl.:

C12N 7/00 (2006.01)

A61K 39/00 (2006.01)

(21) Application number: **16201293.4**

(22) Date of filing: **30.11.2016**

(84) Designated Contracting States:

AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

Designated Extension States:

BA ME

Designated Validation States:

MA MD

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(30) Priority: **30.11.2015 JP 2015234206**

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(54) **IMMUNITY INDUCER**

(57) Disclosed is an immunity inducer. The immunity inducer comprises virus like particles; the virus like particles comprise a virus-derived outer coat protein and an antigen-bound protein comprising an exogenous anti-

gen; the outer coat protein constitutes an outer coat of the virus like particles, and the antigen-bound protein is comprised in the outer coat; and the virus like particles induce an immune effect of a living body on the antigen.

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Description

TECHNICAL FIELD

5 **[0001]** The present invention relates to an immunity inducer. More specifically, the present invention relates to an immunity inducer comprising virus like particles.

BACKGROUND

10 **[0002]** It is known that a cytotoxic T lymphocyte (CTL) can be induced using virus like particles, and viral diseases and cancer can be treated. For example, US Patent Application Publication No. 2014-0286978 describes that a CTL epitope-specific CTL can be induced by immunizing with virus like particles obtained by introducing a CTL epitope into an SV40 VP1 loop region, and expressing it.

15 **[0003]** However, in US 2014-0286978, it is necessary to incorporate an epitope into a specific region of VP1, thus only an antigen of which the epitope is publicly known can be used.

SUMMARY OF THE INVENTION

20 **[0004]** As a result of intensive studies, the present inventors have found that an immune reaction of a living body can be induced even when using a virus like particle including an antigen in its outer coat, thereby completing the present invention.

25 **[0005]** The present invention provides an immunity inducer. The immunity inducer comprises virus like particles; the virus like particles comprise a virus-derived outer coat protein and an antigen-bound protein comprising an exogenous antigen; the outer coat protein constitutes an outer coat of the virus like particles, and the antigen-bound protein is also comprised in the outer coat; the virus like particles induce an immune effect in a living body towards the antigen.

[0006] The outer coat protein may be derived from a virus of the genus Polyomavirus. The virus of the genus Polyomavirus may be SV40, and the outer coat protein may be VP1.

30 **[0007]** The antigen-bound protein may be a fusion protein of the antigen and an inner peptide. The inner peptide may be derived from a virus in the genus Polyomavirus, and the inner protein may be VP2 and/or VP3. An amino acid sequence of the inner peptide may comprise an amino acid sequence represented by SEQ ID No. 9 or SEQ ID NO. 11.

[0008] The immunity inducer may be a solid preparation, a semisolid preparation, a liquid preparation, an injection, or a suppository.

[0009] The immunity inducer may be used in inducing an immunity effect towards the antigen in the living body by administering the immunity inducer to said living body.

35 **[0010]** The present invention also provides a method for producing the immunity inducer, comprising a step of contacting a virus-derived outer coat protein with an antigen-bound protein to prepare virus like particles.

[0011] In particular embodiments, the method may comprise a step of introducing a DNA encoding the outer coat protein and/or a DNA encoding the antigen-bound protein into a host cell to express the outer coat protein and/or the antigen-bound protein in the host cell, thereby obtaining the outer coat protein and/or the antigen-bound protein.

40 **[0012]** In these embodiments of the method, the host cell may be selected from a group consisting of an insect cell, Escherichia coli, a yeast and a plant cell.

[0013] In the method provided herein, the outer coat protein may be VP1 of SV40, and the antigen-bound protein may comprise VP2 and/or VP3 of SV40.

45 **[0014]** In the method provided herein, the immunity inducer may be a solid preparation, a semisolid preparation, a liquid preparation, an injection, or a suppository.

EFFECTS OF THE INVENTION

50 **[0015]** An immunity inducer capable of inducing an immune reaction of a living body on a specific antigen, irrespective of known or unknown epitope, is provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016]

55 Fig. 1 is a schematic view of an example of a virus like particle.
Fig. 2A is a SDS-PAGE photograph showing that VP2-M1 and wt VP1 are present in VLP purified from insect cells infected with a recombinant baculovirus.

Fig. 2B is an electron microscope photograph showing that VP2-M1/wt SV40 VP1 VLP is formed.

Fig. 2C is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse administered with VP2-M1/wt SV40 VP1 VLP purified from insect cells.

Fig. 3 is a vector map of pM01 vector.

5 Fig. 4A is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse intraperitoneally administered with a solution of ground silkworm pupa (phosphate buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

Fig. 4B is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse intraperitoneally administered with a solution of ground silkworm pupa (Tris buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

10 Fig. 4C is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse intraperitoneally administered with a solution of heat-treated ground silkworm pupa (phosphate buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

Fig. 4D is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse intraperitoneally administered with a solution of heat-treated ground silkworm pupa (Tris buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

15 Fig. 5 is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse nasally administered with a solution of ground silkworm pupa (phosphate buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

20 Fig. 6A is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse orally administered with a solution of ground silkworm pupa (phosphate buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

Fig. 6B is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse orally administered with a solution of ground silkworm pupa (Tris buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

25 Fig. 6C is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse orally administered with a solution of heat-treated ground silkworm pupa (Tris buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

Fig. 7 is figures showing a result of ICS analysis performed for lymphocytes obtained from the spleen of a mouse intestinally administered with a solution of ground silkworm pupa (phosphate buffer solution base) containing VP2-M1/wt SV40 VP1 VLP.

30 Fig. 8A is a figure showing a measurement result of anti-OVA antibody production in the serum of a mouse intraperitoneally administered with VP2-OVA/wt SV40 VP1 VLP.

Fig. 8B is a figure showing a measurement result of anti-OVA antibody production in the serum of a mouse nasally administered with VP2-OVA/wt SV40 VP1 VLP.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

40 **[0017]** An immunity inducer of a present embodiment contains virus like particles containing a virus-derived outer coat protein and an antigen-bound protein.

[0018] A virus like particle is usually a structure that has no viral genome, as is well known in the art. The outer coat protein and inner peptide described below can use a protein derived from virus.

45 **[0019]** A schematic view of an example of a virus like particle contained in the immunity inducer is shown in Fig. 1. The virus like particle represented by this schematic view has an outer coat constituted by outer coat protein VP1. The outer coat further includes VP2 fused with an antigen. Both VP1 and VP2 are derived from SV40.

[0020] The kind of virus that is an origin of an outer coat protein and inner peptide is not particularly limited as long as it is a virus having at least one outer coat protein and at least one inner peptide, which are different. Examples of the kind of virus include viruses in the genus Polyomavirus (including SV40 (Simian virus 40), JC virus, BK virus and the like), viruses belonging to the Papillomavirus family (including α , β , γ and μ viruses in the genus Papillomaviruses and the like), viruses belonging to the Siphoviridae family of the order Caudovirales (including HK97 virus and the like), viruses belonging to the Reoviridae family (including rice dwarf virus (RDV), blue tongue virus and the like), viruses belonging to the Tombusviridae family (including tomato bushy stunt virus (TBSV) and the like), and the like. The virus is preferably a virus in the genus Polyomavirus, more preferably SV40, JC virus, BK virus or the like, and particularly preferably SV40. The outer coat protein and inner peptide are preferably derived from the same virus.

55 **[0021]** The genus Polyomavirus (Genus: Polyomavirus) means a genus in the virus classification published by International Committee on Taxonomy of Viruses (ICTV) in 2014.

[0022] In the virus classification, reorganization of the classification and modification of generic name or the like are often made. Accordingly, even when the classification is reorganized, or the generic name or the like is modified in the

classification by ICTV or an equivalent academic authority in the future, viruses classified in the same genus as each virus belonging to the genus Polyomavirus in the ICTV classification in 2014 are defined to be included in the viruses in the genus Polyomavirus referred in this specification.

5 [0023] Since new kinds of viruses are often found in the art, new kinds of viruses that will belong to the current genus Polyomavirus or the classification group corresponding thereto are also defined to be included in the viruses in the genus Polyomavirus referred in this specification, in the virus classification by ICTV or an equivalent academic authority published in the future.

10 [0024] The orders, families, generic names and the like of the viruses referred above other than the genus Polyomavirus are also based on the ICTV classification published in 2014. The same as those stated for the genus Polyomavirus applies to the definition of these terms.

15 [0025] The outer coat protein is a protein that constitutes an outer coat of the virus like particles. The outer coat also can include an antigen-bound protein. The phrase "the outer coat protein constitutes an outer coat of the virus like particles" means that the outer coat substantially comprises the outer coat protein. More specifically, the outer coat may be constituted only by an outer coat protein. Alternatively, the outer coat may be constituted by an outer coat protein and a peptide or protein which binds to the outer coat protein in a manner whereby the structure of outer coat is maintained. For example, the outer coat of wild-type SV40 virus is constituted by assembling of 72 VP1 pentamer units, and VP2 and VP3 are bound to the inside of the constituted outer coat as lining. However, VP1 of SV40 can constitute an outer coat by itself even without VP2 and VP3. In the present embodiment, the outer coat protein means a protein capable of substantially constituting an outer coat by itself like SV40 VP1. The outer coat protein can be a peptide or protein containing at least an amino acid sequence minimally required for outer coat formation by virus-derived full-length amino acid sequences. The outer coat protein in the context of the virus particles provided herein is preferably a protein having a full-length amino acid sequence of a wild-type outer coat protein. Examples of suitable outer coat proteins include outer coat proteins of viruses belonging to the genus Polyomavirus, the Papillomavirus family, the Siphoviridae family of the order Caudovirales, the Reoviridae family and the Tombusviridae family, and more specific examples include VP1 of SV40 (Simian virus 40), JC viruses and the like that are viruses in the genus Polyomavirus, L1 of viruses belonging to the Papillomavirus family, P8 of rice dwarf virus (RDV), and the like. Examples of a peptide or protein at least containing an amino acid sequence minimally required to ensure outer coat formation include 24mer peptides forming a tomato bushy stunt virus (TBSV)-derived β -annulus structure, and the like. The outer coat protein is more preferably an outer coat protein of a virus in the genus Polyomavirus, further preferably an outer coat protein of SV40, JC virus, BK virus or the like, and particularly preferably SV40 VP1. The outer coat proteins provided in the outer coat of a particle as provided herein can be of one or several kinds.

25 [0026] A protein that is a part of the outer coat in a wild-type virus like VP2 and VP3 of SV40, but cannot constitute the outer coat by itself can be included as in "inner peptide" in the particles provided herein, as described below. However, the term "inner peptide" does not mean that these proteins or peptides like VP2 and VP3 of SV40 should not be considered as constituents of the outer coat. Such other substrates may be a non-essential member constituting the outer coat, or may involve in formation of the outer coat in some form.

30 [0027] The term "include as an inner protein" refers to the fact that at least the antigen in the antigen-bound protein should be present in the inside of the outer coat formed by the outer coat protein.

35 [0028] The outer coat protein is not required to have completely same amino acid sequence as that of a wild-type virus. The amino acid sequence may be varied as long as it does not hinder outer coat formation and an antigen-bound protein can be included in the formed outer coat. Variation of amino acid sequence means that one or more amino acid residues are substituted, deleted or added as compared to the wild-type sequence. The outer coat protein may form an outer coat by its self-assembling ability. The outer coat protein may form an outer coat by the action of factors inherent in a host. The outer coat protein may form an outer coat, as monomers. The outer coat protein may form outer coat-forming units (capsomere) constituted from multimers and form an outer coat by assembling of the units. The outer coat protein forms an outer coat by assembling of preferably dimers to decamers and more preferably trimers to pentamers of about 50 to 500 capsomeres. The outer coat protein may be an extracted and purified natural protein. The outer coat protein may be artificially synthesized by a genetic engineering technique or the like.

40 [0029] The shape of outer coat is not particularly limited. The shape may be spherical or tubular. The shape of outer coat is, for example, approximately spherical, regular octahedron to regular icosahedron.

45 [0030] When the outer coat is constituted from monomers of outer coat protein, the number of monomers constituting one outer coat is not particularly limited. The number of monomers constituting one outer coat is preferably 100 to 1000 and more preferably 150 to 500.

50 [0031] When the outer coat is constituted from capsomeres, the number of capsomeres constituting one outer coat is preferably 50 to 390 and more preferably 72 to 260.

55 [0032] The diameter of outer coat is not particularly limited. The diameter of outer coat is preferably 30 to 300 nm and more preferably 45 to 200 nm.

[0033] The antigen-bound protein is a fusion protein of an antigen and an inner peptide. The antigen-bound protein

contains an amino acid sequence of a desired antigen and an amino acid sequence of a virus-derived inner peptide. The inner peptide is not particularly limited as long as it can constitute the virus like particles of the present embodiment. In particular embodiments, the inner peptide contains, of a virus-derived full-length amino acid sequence, at least the amino acid sequence necessary for constituting the virus like particles as described herein. The inner peptide preferably has full-length amino acid sequences of the wild-type amino acid sequences or a sequence derived therefrom. Examples of the inner peptide include VP2 and VP3 of SV40 (Simian virus 40), JC viruses and the like, L2 of viruses belonging to the Papillomavirus family, P3 of RDV, and the like. Examples of peptides which contain, of the virus-derived full-length amino acid sequences, at least an amino acid sequence necessary for constituting the virus like particles provided herein,, include peptides comprising an amino acid sequence of SEQ ID No. 8 (specifically, peptides comprising a VP1 binding domain common to VP2 and VP3 of SV40), and the like. The inner peptide is more preferably a protein having an amino acid sequence of SEQ ID No. 9, and is further preferably a protein comprising an amino acid sequence of SEQ ID No. 9 or 11 (specifically, each of VP3 and VP2 of SV40; nucleic acid sequences are each shown in SEQ ID Nos. 10 and 12). The inner peptide can be one or more kinds.

[0034] The inner peptide may have completely the same amino acid sequence as that of a wild-type virus. The amino acid sequence may be varied as long as it constitutes the virus like particles of the present embodiment. The inner peptide may bind to the inside of the outer coat protein so as to form a lining of an outer coat. The inner peptide may be included in an outer coat without binding to the outer coat protein. The inner peptide may form an inner coat in the inside of the outer coat, for example, like P3 of RDV. Where the inner peptide forms an inner coat, the term "inner" is used, but in practice it is present in the outer coat. A part of the inner peptide may be exposed from the outer coat. To the inner peptide may be added, for example, a tag for confirming expression of the antigen-bound protein, for example, a FLAG tag or the like.

[0035] In a preferred embodiment, the virus is the genus Polyomavirus SV40. In this embodiment, the outer coat protein is VP1, and the antigen-bound protein is a fusion protein of VP2 and/or VP3 and an antigen. VP1 is also called a major capsid protein, and VP2 and VP3 are also called a minor capsid protein. When using VP1 and VP2 and/or VP3 of SV40, VP2 and VP3 bind to the inside of the outer coat protein VP1 so as to form a lining of the outer coat. Generally, an outer coat of a virus is often called capsid, thus a protein involved in outer coat formation like VP2 and VP3 of SV40 and the like could also be considered to be an outer coat protein in a broad sense. However, in the context of the present invention, only a protein which is capable of constituting an outer coat itself by itself, like VP1 of SV40, is called as "outer coat protein". Namely, even a protein that may be generally considered as an outer coat protein or capsid protein can be considered an "inner peptide" in the context of the present invention, like VP2 and VP3 of SV40. In these embodiments, an antigen can be included in the outer coat by binding the antigen to VP2 and/or VP3.

[0036] The kind of antigen is not particularly limited, and examples thereof include polypeptides, sugar chains, nucleic acids, lipids, and the like. The antigen is exogenous. The term "exogenous antigen" means the antigen is not derived from a virus from which the outer coat protein and inner peptide are derived and is not derived from the living body to which the immunity inducer is to be administered. The outer coat protein and the inner peptide are not included in the term "antigen" in this specification.

[0037] Among the suitable antigens, polypeptides are preferred. When using a polypeptide as an antigen, a fusion protein containing an antigen and an inner peptide can be easily produced by a genetic engineering method.

[0038] As an antigen, it is preferred to use a polypeptide derived from a pathogen. The polypeptide derived from a pathogen may be a full-length polypeptide. The polypeptide derived from a pathogen may be a polypeptide containing only a part of sequences. Examples include HA, NA, M1, M2, NP, NS1, NS2, PA, PB1, PB2, PB1-F2 and the like of influenza viruses, Gag, Pol, Env, Tat, Nef, Rev and the like of HIV, E1, E2, Core, NS2, NS3, NS4, NS5 and the like of hepatitis C viruses (HCV), E6, E7 and the like of viruses belonging to the Papillomavirus family, Melan-A/MART-1, gp100, MAGEA3, MAGE-A10, CEA, HER2/new, NY-E50-1, WT-1, hTERT and the like that are proteins specific to cancer cells, and the like. Among them, M1, NP, NS1, PA, PB1 and PB2 of influenza viruses, HER2/new, WT-1 and MAGE-A3 that are proteins specific to cancer cells and the like are preferred.

[0039] The antigen is bound to an inner peptide so as to be included in the outer coat of the virus like particles provided herein. Conventionally, in virus like particles, an antigen epitope is incorporated into an outer coat protein exposed to the outside of the outer coat, whereby the outer coat protein forms the outer coat (specifically, virus like particles in which an epitope is incorporated into a DE loop or an HI loop of SV40 VP1) are known (Patent Document 1). In these cases, when the amino acid length of the antigen epitope to be incorporated is too long, the outer coat structure can be destroyed, and it is necessary to incorporate the epitope into a specific region of the outer coat protein. Thus, only an antigen with a short epitope (about 5 to 15 amino acids) of which the sequence is publicly known can be practically used. In contrast, in immunity inducers provided herein, the antigen is included in a separate protein of the outer coat, thus it becomes possible to include an antigen having longer amino acid length, as compared to a conventional method of incorporating an epitope into the outer coat structure. Thus, a full-length antigen can be also used. Moreover, it is possible to induce immunity without adding an adjuvant to the antigen. The present inventors have unexpectedly found this point.

[0040] The size of the antigen is not particularly limited as long as it can be included in an outer coat as described

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herein. The size of the antigen is preferably 200 to 600 amino acid length.

[0041] The method of fusing an antigen and an inner peptide is not particularly limited, and any method known to a person skilled in the art, for example, a gene recombination technique and the like, can be used. An antigen and an inner peptide may be fused via one or more linkers known to a person skilled in the art, for example, a GGGGS linker.

[0042] An immunity inducer as provided herein induces an immune effect to an antigen included in an outer coat of virus like particles in a living body. The phrase "induces an immune effect" refers to activation of immunity of a living body by inducing an antibody or a cytotoxic T lymphocyte. For example, an action of producing an antibody by administration to a living body prior to onset of a disease (cancer, etc.) or infection of a pathogen (virus, etc.) and/or an action of inducing a memory CTL is enhanced to prevent a future disease (use as a preventive vaccine), and a cytotoxic T lymphocyte (CTL) of a living body is induced by administration to a living body having a disease (cancer, infection, etc.) to treat the disease (use for immunotherapy), and the like.

[0043] An immunity inducer as provided herein can be used as a vaccine for preventing or treating viral diseases or a vaccine for preventing or treating various cancers. Specifically, an immunity inducer can be used as a vaccine for preventing or treating diseases such as infections (influenza, immunodeficiency syndrome, hepatitis C, etc.), cancers (cervical cancer, pharyngeal papilloma, etc.), verrucas (verruca vulgaris, inclusion body of verruca vulgaris, verruca plana, etc.), HPV-associated epidermoid cyst, epidermodyplasia verruciformis, condyloma acuminatum and bowenoid papulosis.

[0044] In the immunity inducer as provided herein, virus like particles may be formulated with a pharmaceutical additive known to a person skilled in the art. The pharmaceutical additive as described above is not particularly limited, and examples thereof include excipients, lubricants, binders, disintegrants, coating agents, capsule base materials, plasticizers, colorants, solvents, stabilizers, preservatives, buffers, analgesics, bases, emulsifiers and suspending agents, other corrigents, sweeteners, absorbents, dissolution adjuvants, pH adjusting agents, thickeners, tonicity agents, dispersants, antiseptics, wetting agents, flavoring agents, antioxidants, and the like.

[0045] The excipient is not particularly limited, and examples thereof include mannitol, sucrose, glucose, corn starch, crystalline cellulose, calcium hydrogen phosphate, and the like.

[0046] The lubricant is not particularly limited, and examples thereof include magnesium stearate, talc, colloidal silica, and the like.

[0047] The binder is not particularly limited, and examples thereof include gum arabic, hydroxypropylcellulose (HPC), hydroxypropylmethylcellulose (HPMC), methylcellulose (MC), povidone (PVP), polyvinyl alcohol (PVA), and the like.

[0048] The disintegrant is not particularly limited, and examples thereof include cross-linked carmellose sodium, carmellose calcium, cross-linked povidone, sodium carboxymethyl starch, and the like.

[0049] The coating agent is not particularly limited, and examples thereof include coating agents for sugar coating such as sucrose and talc, enteric coating agents such as carboxymethylethyl cellulose, gastrosoluble coating agents such as polyvinyl acetal diethyl aminoacetate, and the like.

[0050] The capsule base material is not particularly limited, and examples thereof include gelatin and the like.

[0051] The plasticizer is not particularly limited, and examples thereof include triacetin, medium-chain triglyceride, and the like.

[0052] The colorant is not particularly limited, and examples thereof include edible tar colors, lake pigments, iron sesquioxide, and the like.

[0053] The solvent is not particularly limited, and examples thereof include aqueous solvents such as water for injection and sterile purified water, nonaqueous solvents such as vegetable oils (including olive oil, soybean oil and sesame oil), and the like.

[0054] The stabilizer is not particularly limited, and examples thereof include inert gas such as nitrogen and carbon dioxide, chelating agents such as EDTA, reduction substrates such as L-ascorbic acid, and the like.

[0055] The preservative is not particularly limited, and examples thereof include p-oxybenzoic ester, chlorobutanol, and the like.

[0056] The buffer is not particularly limited, and examples thereof include sodium salts of citric acid, acetic acid, phosphoric acid, and the like.

[0057] The analgesic is not particularly limited, and examples thereof include benzyl alcohol, procaine hydrochloride, glucose, and the like.

[0058] The base is not particularly limited, and examples thereof include bases for suppositories such as cacao butter and gelatin, bases for ointments such as liquid paraffin and carnauba wax, and the like.

[0059] The emulsifier is not particularly limited, and examples thereof include gum arabic, polysorbate, sodium lauryl sulfate, and the like.

[0060] The suspending agent is not particularly limited, and examples thereof include gum arabic, sodium alginate, tragacanth, aluminum monostearate, and the like.

[0061] An immunity inducer as provided herein has a sufficient immunity inducing effect by itself. Thus, an immunity inducer need not contain an adjuvant, but may contain an adjuvant. When an immunity inducer contains an adjuvant,

examples of the adjuvant to be used include aluminum hydroxide gel, complete Freund's adjuvant, incomplete Freund's adjuvant, pertussis adjuvant, poly(I,C), CpG-DNA, and the like. In particular embodiments, the immunity inducer does not contain an adjuvant.

5 **[0062]** An immunity inducer may be any of a solid preparation, a semisolid preparation, a liquid preparation, an injection, a suppository, or any other preparation form known to a person skilled in the art. Specific dosage form is not particularly limited, and examples thereof include tablets, pills, granules, powders, capsules, troches, injections, liquid agents, elixirs, syrups, limonades, suppositories, ointments, suspension agents, emulsions, liniments, lotions, percutaneous absorption preparations, patches, cataplasms, aerosols, and the like.

10 **[0063]** In particular embodiments, the immunity inducer is a liquid preparation. The liquid preparation is prepared, for example, by extracting virus like particles from a host cell expressing the virus like particles and diluting the virus like particles with an appropriate solvent as necessary.

15 **[0064]** In particular embodiments, the immunity inducer is a suspension agent. The suspension agent is prepared, for example, by producing a homogenate obtained by homogenizing a host cell expressing the virus like particles, and extracting, purifying, and diluting the homogenate with a solvent as necessary. Specifically, when a lepidopterous insect individual such as a silkworm is used as a host, a suspension agent can be prepared by grinding an individual expressing the virus like particles, and roughly purifying the ground individual.

20 **[0065]** The administration route of an immunity inducer as provided herein is not particularly limited, and examples thereof include oral administration, transmucosal administration (for example, transnasal administration, intranasal administration, buccal administration, enema administration, and the like), parenteral administration (for example, intraperitoneal injection, subcutaneous injection, intravenous injection, intramuscular injection, injection into a space between tissues, and the like), transdermal administration, and the like. More specifically, an immunity inducer of the present embodiment can be used by not only a high burden administration by injection or the like, but also a low burden administration by oral ingestion, administration by collarium, enema or the like. For example, an immunity inducer can be used as a vaccine for animals by mixing into animal feed.

25 **[0066]** The dose and number of doses of an immunity inducer as provided herein can be properly set by a person skilled in the art according to the kind of an antigen, animal species of an administration target, and symptom, age, body weight, administration form and the like, of an administration target. The dose is usually 0.01 μg to 100 mg, preferably 0.1 μg to 50 mg, and more preferably 1.0 μg to 10 mg, and it is preferred to administer an immunity inducer once per few days to few months.

30 **[0067]** The administration target of an immunity inducer as provided herein can be biological bodies, more specifically, human or animals other than human (mammals other than human, birds, reptiles, and the like). Examples of animals other than human include bovine, equine, porcine, chicken, canine, feline, mouse, rat, lagomorph, simian, and the like.

35 **[0068]** In particular embodiments, the immunity inducer provided herein is a pharmaceutical composition containing a pharmacologically effective amount of virus like particles from the viewpoint of immunity induction. Virus like particles may cause a pharmaceutically acceptable side effect when administered, but it is preferred for the animal of an administration target that there is no pathogenicity, and a side effect is not caused.

[0069] An immunity inducer as provided herein can be produced by preparing virus like particles according to a method known to a person skilled in the art, and formulating the virus like particles by mixing with a pharmaceutically acceptable excipient or the like as necessary.

40 **[0070]** Virus like particles can be prepared, for example, by mixing a virus-derived outer coat protein with an antigen-bound protein. Mixing conditions can be properly set by a person skilled in the art. An outer coat protein is mixed with an antigen-bound protein, whereby the antigen-bound protein is included in the outer coat formed by the outer coat protein.

45 **[0071]** In particular embodiments, before preparing the virus like particles, the virus-derived outer coat protein and the antigen-bound protein are produced. In particular embodiments, a DNA encoding an outer coat protein and/or a DNA encoding an antigen-bound protein are incorporated into a host cell, and the outer coat protein and/or the antigen-bound protein are expressed in the host cell, whereby the outer coat protein and/or the antigen-bound protein may be obtained. These proteins can be obtained by methods known to a person skilled in the art, for example, gene recombination and the like. In particular embodiments, where they are expressed together, the outer coat protein and the antigen-bound protein may come into contact with each other directly upon expression by the host. In further embodiments, the outer coat protein and/or the antigen-bound protein are expressed and then mixed.

50 **[0072]** The host cell for production of the proteins is not particularly limited as long as it does not hinder the formation of virus like particles. The host cell is selected, for example, from a group consisting of insect cells (including insect individuals such as silkworm), *Escherichia coli*, yeasts and plants. The host cell is preferably an insect cell, more preferably a lepidopterous insect individual, and further preferably a silkworm.

55 **[0073]** When an outer coat protein and an antigen-bound protein are expressed in a host cell, virus like particles may be prepared by direct contact of the outer coat protein with the antigen-bound protein as expressed within the host cell. Alternatively, virus like particles may be formed in a production process such as homogenization, purification and extraction from the host cell.

[0074] The virus like particles formed in the host cell may be collected as necessary. Collection method is not particularly limited, but a person skilled in the art can properly select, mainly depending on the kind of the host cell. For example, when the host cell is an insect cell, an Escherichia coli cell or the like, cytolysis and the like by ultrasonication or the like can be used. When the host cell is a pupa of a lepidopterous insect, a method of eluting virus like particles by grinding or the like, and collecting a supernatant after centrifugation can be used.

[0075] In a preferred embodiment, first, an insect cell or insect individual is infected with a baculovirus into which a DNA encoding an outer coat protein and a DNA encoding an antigen-bound protein are incorporated. Next, the insect cell or insect individual is subjected to ultrasonic treatment or ground, then centrifuged or filtered, and the supernatant is collected, whereby virus like particles can be obtained. Alternatively, a host cell may be infected with a first baculovirus into which a DNA encoding an outer coat protein and a second baculovirus into which a DNA encoding an antigen-bound protein are incorporated, in place of the baculovirus into which a DNA encoding an outer coat protein and a DNA encoding an antigen-bound protein are incorporated.

[0076] The virus like particles may be purified as necessary. The purification method is not particularly limited, and examples thereof include methods known to a person skilled in the art such as density gradient centrifugation and chromatography and the like.

[0077] The virus like particles may be heat treated as necessary. The heat treatment method is not particularly limited, and examples thereof include methods of incubating silkworm pupae as a host cell in boiling water and the like.

[0078] Formulation can be performed, for example, by mixing virus like particles with an appropriate pharmaceutical additive, molding into a desired dosage form, and coating the dosage form as necessary.

[0079] Specifically, when a dosage form is formed into a solid preparation, for example, a tablet, it can be formulated, for example, by mixing virus like particles with an appropriate excipient, binder and/or disintegrant, adding an appropriate lubricant, further mixing the ingredients, tableting the mixture, and coating the dosage form as necessary.

[0080] When a dosage form is formed into an injection or a liquid preparation, it can be formulated, for example, by dispersing virus like particles in an appropriate solvent, filtering or sterilizing the dispersion as necessary, and filling the dispersion in a predetermined container.

[0081] When a dosage form is formed into an ointment, it can be formulated, for example, by melting an appropriate ointment in a mixer equipped with a warming device, stopping warming, mixing at a low speed until it coagulates in the form of an ointment, adding virus like particles immediately before coagulation, and filling the mixture in a predetermined container.

[0082] When a dosage form is formed into a suppository, it can be formulated, for example, by mixing virus like particles with an appropriate base for suppositories previously melted at a low temperature, pouring the mixture into a mold, and cooling it to harden.

[0083] Another aspect of the invention relates to virus like particles for inducing an immune effect in a living body. More specifically, the invention provides virus like particles containing a virus-derived outer coat protein and an antigen-bound protein, for inducing an immune effect in a living body on the antigen, in which the outer coat protein constitutes an outer coat of the virus like particles, and the antigen-bound protein is included in the outer coat.

[0084] The virus like particles, virus, outer coat protein, outer coat, antigen-bound protein, antigen and the like are as described above.

[0085] The virus like particles as provided herein are used as a vaccine for preventing or treating viral diseases or a vaccine for preventing or treating various cancers, by being administered to a living body. In this way, an immune effect is induced in the living body against an antigen included in the outer coat. Therefore, the virus like particles described above can be used as a vaccine for preventing or treating diseases such as infections (influenza, HIV, hepatitis C, etc.), cancers (cervical cancer, pharyngeal papilloma, etc.), verrucas (verruca vulgaris, inclusion body of verruca vulgaris, verruca plana, etc.), HPV-associated epidermoid cyst, epidermodysplasia verruciformis, condyloma acuminatum and bowenoid papulosis.

[0086] Accordingly, it can be also said that another aspect of the invention relates to virus like particles for use in the treatment of a disease. More specifically, the present embodiment relates to virus like particles containing a virus-derived outer coat protein and an antigen-bound protein, for use in the treatment of a disease, in which the outer coat protein constitutes an outer coat of the virus like particles, and the antigen-bound protein is included in the outer coat.

[0087] The virus like particles, virus, outer coat protein, outer coat, antigen-bound protein, antigen, disease and the like are as described above.

[0088] Another aspect of the invention relates to a method for inducing an immune effect in a living body including administering an immunity inducer containing virus like particles to the living body. More specifically, this aspect provides a method for inducing an immune effect in a living body including administering an immunity inducer containing virus like particles to the living body, in which the virus like particles contain a virus-derived outer coat protein and an antigen-bound protein, the outer coat protein constitutes an outer coat of the virus like particles, and the antigen-bound protein is included in the outer coat, and induces an immune effect of the living body on the antigen.

[0089] The virus like particles, virus, outer coat protein, outer coat, antigen-bound protein, antigen, immunity inducer,

administration method thereof, living body and the like are as described above.

[0090] It can be also said that further aspect of the invention relates to a method for preventing or treating diseases including administering an immunity inducer containing virus like particles to a living body. More specifically, the present invention relates to a method for preventing or treating diseases including administering an immunity inducer containing virus like particles to a living body, in which the virus like particles contain a virus-derived outer coat protein and an antigen-bound protein, the outer coat protein constitutes an outer coat of the virus like particles, and the antigen-bound protein is included in the outer coat, and induces an immune effect of a living body on the antigen.

[0091] The virus like particles, virus, outer coat protein, outer coat, antigen-bound protein, antigen, immunity inducer, administration method thereof, living body, disease and the like are as described above.

[0092] Another aspect of the invention relates to use of virus like particles in the production of an immunity inducer.

[0093] The virus like particles, virus, outer coat protein, outer coat, antigen-bound protein, antigen, immunity inducer, administration method thereof, living body, disease and the like are as described above.

[0094] Hereinbelow, the present invention will be described in detail by way of examples, but the present invention is not limited to these examples.

EXAMPLES

Example 1: Preparation of VP2-M1-including SV40 VP1 VLP and Induction of FMP:58-66 epitope specific-cytotoxic T lymphocyte by immunization therewith

Preparation of baculovirus expressing wild-type (wt) simian virus 40 (SV40) VP1

[0095] A wt SV40 VP1 gene (SEQ ID No. 1; the amino acid sequence is shown in SEQ ID No. 2) was inserted into Sal I site and Kpn I site of pFastBac1 plasmid (invitrogen). Escherichia coli DH10bac (invitrogen) holding a baculovirus genome was transformed with the obtained plasmid to prepare a recombinant baculovirus genome with VP1 1 incorporated therein. The recombinant baculovirus genome was transfected to Sf-9 cells. After three days, the supernatant thereof was collected to obtain a solution containing recombinant baculovirus. A part of this solution was again infected with Sf-9 cells (invitrogen), thereby increasing a recombinant baculovirus titer. The resulting solution was referred to as a stock solution of recombinant baculovirus.

Preparation of baculovirus expressing VP2 fused M1 protein

[0096] A coding sequence of FLAG tag (SEQ ID No. 13) was added to the upstream of a codon encoding an amino terminus (N-terminus) of wt SV40 VP2, and a BamHI site was introduced into the further upstream thereof. The stop codon of wt SV40 VP2 was eliminated, and an EcoRI site was introduced. The obtained polynucleotide was inserted via a BamHI site and an EcoRI site of pFastBac1 plasmid to prepare a plasmid containing a wt SV40 VP2 gene. A coding sequence of a GGGGSGGGGSGGGGS linker (SEQ ID No. 3; the nucleic acid sequence is shown in SEQ ID No. 14) was introduced into the upstream of a codon encoding an N-terminus of M1 protein, and an EcoRI site was introduced into the further upstream thereof. The stop codon was added to the downstream of M1 protein coding sequence, and a Sal I site was introduced into the further downstream thereof. The obtained polynucleotide was introduced via the EcoRI site and the Sal I site of the plasmid containing a wt SV40 VP2 gene to prepare a plasmid holding a gene fused with the M1 coding sequence in the downstream of the VP2 coding sequence.

[0097] Escherichia coli DH10bac (invitrogen) holding a baculovirus genome was transformed with this plasmid to prepare a recombinant baculovirus genome expressing protein VP2-M1 in which M1 was fused with wt SV40 VP2 (SEQ ID No. 4; the nucleic acid sequence is shown in SEQ ID No. 5). These recombinant baculovirus genomes were transfected to Sf-9 cells. After three days, the supernatant thereof was collected to obtain a solution containing recombinant baculovirus.

[0098] A part of the solution obtained above was again infected with Sf-9 cells, thereby increasing a recombinant baculovirus titer. The resulting solution was referred to as a stock solution of recombinant baculovirus.

Preparation of wt SV40 VP1 VLP containing VP2-M1

[0099] A recombinant baculovirus with wt SV40 VP1 incorporated therein (M.O.I. (multiplicity of infection) = 0.05 to 0.2) and a baculovirus with VP2-M1 incorporated therein (M.O.I. = 0.015 to 0.06) were coinfecting in a 15 cm culture dish in which 3×10^7 Sf-9 cells were inoculated (infection ratio of wt SV40 VP1 : VP2-M1 = 1 : 0.3 (M.O.I. base)). A total of 10 dishes was prepared. After three days of infection, a total of 3×10^8 Sf-9 cells inoculated on these 10 dishes were collected. After washing with PBS (-), the cells were resuspended in 10 ml of a buffer for VP1 ultrasonic treatment (20 mM Tris-HCl (pH 7.9), 1% (w/vol) deoxycholic acid). Thereafter, in order to suppress endogenous protease activity, 2

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mM phenylmethylsulfonyl fluoride (final concentration of 2 μ M), chymostatin (final concentration of 1 μ g/ml), aprotinin (final concentration of 1 μ g/ml), leupeptin (final concentration of 1 μ g/ml), antipain (final concentration of 1 μ g/ml) and pepstatin (final concentration of 1 μ g/ml) were added thereto, and the mixture was ultrasonically crushed.

[0100] Thereafter, the crushed substance was centrifuged at 15,000 rpm, 4°C for 5 minutes to separate into supernatant and pellet, and the supernatant was referred to as a lysate solution.

[0101] Each 1.5 ml of a 20% CsCl solution (20 mM Tris-HCl (pH 7.9), 20% (w/vol) cesium chloride), a 30% CsCl solution (20 mM Tris-HCl (pH 7.9), 30% (w/vol) cesium chloride), a 40% CsCl solution (20 mM Tris-HCl (pH 7.9), 40% (w/vol) cesium chloride), and a 50% CsCl solution (20 mM Tris-HCl (pH 7.9), 50% (w/vol) cesium chloride) were superposed in an Ultra-Clear centrifugation tube (14 x 89 mm, Beckman coulter) in descending order of density, for density gradient centrifugation of cesium chloride. Then, 5 ml of the lysate solution containing wt SV40 VP1 containing VP2-M1 was further superposed. Thereafter, the centrifugation tube was ultracentrifuged at 35,000 rpm, 4°C for 3 hours (SW41Ti rotor, Beckman).

[0102] After ultracentrifugation, a white band appeared at a center was collected with 23 G, 1 ml of a Terumo syringe (0.60 x 32 mm, TERUMO). This fraction was mixed with a 37% CsCl solution (20 mM Tris-HCl (pH 7.9), 37% (w/vol) cesium chloride), and the mixture was transferred to an Ultra-Clear centrifugation tube (11 x 60 mm, Beckman coulter). Thereafter, the centrifugation tube was ultracentrifuged at 50,000 rpm, 4°C for 20 hours (SW60Ti rotor, Beckman). After ultracentrifugation, a white band appeared at a center was collected with 23 G, 1 ml of a Terumo syringe (0.60 x 32 mm, TERUMO). This fraction was dialyzed (Slide-A-Lyzer MINI Dialysis Units, 3500 MWCO, Thermo SCIENTIFIC) against a PBS (-) solvent, and the fraction was centrifuged at 15,000 rpm, 4°C for 5 minutes. The supernatant was collected and referred to as a wt SV40 VP1 VLP fraction containing purified VP2-M1. This fraction was developed by SDS-PAGE and then stained with CBB. As a result, it became certainly clear that two bands, a VP2-M1-derived band and a wt SV40 VP1 VLP-derived band, were purified (Fig. 2A). A VP2-M1-including wt SV40 VP1 VLP (VP2-M1/wt SV40 VP1 VLP) sample contained in this fraction was observed under an electron microscope photograph. As a result, VLP formed by wt SV40 VP1 could be confirmed (Fig. 2B). It was suggested that VP2-M1 was included in the inside of VLP.

Immunization of VP2-M1/wt SV40 VP1 VLP

[0103] The following experiment used a transgenic mouse using C57BL/6 as a background and expressing a chimera of HLA-A*0201 and H-2Db further fused with human β 2m (hereinafter, HHD mouse). This mouse is a β 2m and H-2Db knockout mouse, thus it is considered that mouse-derived MHC class I is not exposed to the cell surface.

[0104] An 8-week old transgenic mouse was immunized by 100 μ l of the VP2-M1/wt SV40 VP1 VLP (500 μ g/ml) via the intraperitoneal route. For immunization, a 1 ml syringe with a 27-gauge needle inserted (Myjector, syringe with an injection needle, for insulin, TERUMO, SS-10M2713) was used.

[0105] Alternatively, 40 μ l of the VP2-M1/wt SV40 VP1 VLP (500 μ g/ml) was nasally administered to an 8-week old transgenic mouse under general anesthesia, by the transnasal route.

[0106] One week after administration, the spleen of the immunized mouse was collected. Lymphocytes were prepared by the following method, and Intra-cellular staining (ICS) analysis described below was performed.

Preparation of lymphocytes from spleen of mouse

[0107] The spleen was removed from the immunized mouse. The spleen was put in a ϕ 6 cm dish with 5 ml of RPMI-1640 medium. The spleen was well loosened using tweezers in the medium, and a solution containing lymphocytes eluted in the medium was transferred to a 15 ml tube. The ϕ 6 cm dish was again washed with 5 ml of RPMI-1640 medium. The supernatant was added to the 15 ml tube so that the total amount is 10 ml. The supernatant was again transferred to a new 15 ml tube, leaving tissue sections deposited at the bottom of the 15 ml tube. Thereafter, the 15 ml tube was centrifuged at 1,200 rpm at room temperature for 5 minutes to obtain a pellet containing lymphocytes. The supernatant was removed, and the pellet was loosened. Thereafter, in order to remove erythrocytes, 250 μ l of a NH_4Cl -tris solution was added thereto, and the mixture was stirred. Thereafter, 10 ml of RPMI-1640 medium was quickly added thereto, and the 15 ml tube was centrifuged at 1,200 rpm at room temperature for 5 minutes to obtain a pellet containing lymphocytes. The supernatant was removed, and the pellet was loosened. Thereafter, 10 ml of RPMI-1640 medium was again added thereto. The medium containing lymphocytes was transferred to a new 15 ml tube with a pipette so as not to suck modified erythrocytes as much as possible. Thereafter, the 15 ml tube was again centrifuged at 1,200 rpm at room temperature for 5 minutes. The supernatant was removed, and then the pellet was loosened. The pellet was again suspended in 10 ml of RPMI-1640 medium, and centrifuged at 1,200 rpm at room temperature for 5 minutes. The supernatant was removed, and the pellet was finally suspended in 2 ml of 10% FCS mixing RPMI-1640 medium. In order to count lymphocytes, 10 μ l of the above suspension was added to 490 μ l of a 2% acetic acid solution. The number of cells was counted with a Burkert-Turk hemocytometer. The resulting mixture was diluted with 10% FCS mixing RPMI-1640 medium so as to be 1×10^7 cells/ml.

Intra-cellular staining (ICS) analysis

[0108] After immunizing the mouse, ICS analysis was performed, in order to investigate that a CTL induced by reacting to M1 CTL epitope sequence (GILGFVFTL) is present in the lymphocytes collected from the spleen. BD GolgiPlug (trademark) (BD) diluted 25-fold with 10% FCS mixing RPMI-1640 medium was added to a 96-well round-bottom plate, at 5 μ l per well. Thereto was further added 100 μ l of a peptide of 20 μ M of M1 CTL epitope (GILGFVFTL, Operon) diluted with 10% FCS mixing RPMI-1640 medium. As a negative control, 100 μ l of a 10% FCS mixing RPMI-1640 medium not containing a peptide was added. To this well was added 100 μ l of the lymphocytes prepared above. Thereafter, the mixture was incubated at 37°C, 5% CO₂, for 5 hours.

[0109] After incubation, the mixture was spun down at 4°C, 1,400 rpm to remove the supernatant, and the cells were loosened with a Vortex mixer. Thereafter, FACS buffer (2% FCS, 0.1% sodium azide, 1 x PBS (-)) was added at 200 μ l per well. The mixture was again spun down at 4°C, 1,400 rpm to remove the supernatant, and the cells were loosened. Thereafter, 100 μ l of Mouse BD Fc Block (trademark) (BD Pharmingen) diluted to 5 μ g/ml with FACS buffer was added thereto, and the mixture was incubated at 4°C for 10 minutes.

[0110] After incubation, the mixture was again spun down at 4°C, 1,400 rpm to remove the supernatant, and the cells were loosened.

[0111] Thereafter, FACS buffer was added at 200 μ l per well, and the mixture was again spun down at 4°C, 1,400 rpm to remove the supernatant. Washing operation with FACS buffer was again carried out. FITC Rat Anti-Mouse CD8a Clone: 53-6.7 (BD Pharmingen) diluted to 10 μ g/ml with FACS buffer was added to the loosened cells, at 50 μ l per well, and the mixture was incubated in a dark place at 4°C for 30 minutes.

[0112] After incubation, washing operation with 200 μ l of FACS buffer was carried out twice. Thereafter, 100 μ l of BD Cytotfix/Cytoperm (trademark) (BD Biosciences) was added to the loosened cells, at 100 μ l per well, and the mixture was incubated in a dark place at 4°C for 20 minutes. After incubation, the washing operation as same as described above was carried out twice, using 200 μ l of 1 x BD Perm/Wash (trademark) (BD biosciences) in place of the FACS buffer. Thereafter, 50 μ l of PE anti-mouse IFN- γ Clone: XMG1.2 (BioLegend) diluted to 10 μ g/ml with 1 x BD Perm/Wash (trademark) was added to the loosened cells, and the mixture was incubated in a dark place at 4°C for 30 minutes.

[0113] After incubation, the washing operation as same as described above was carried out twice, using 200 μ l of 1 x BD Perm/Wash (trademark). Thereafter, FACS fixation buffer (1% formaldehyde, 1 x FACS buffer) was added to the loosened cells, at 100 μ l per well, and the mixture was incubated in a dark place at 4°C overnight.

[0114] After incubation, 400 μ l of FACS buffer was added to 5 ml of a polystyrene tube (BD Falcon, 5 ml polystyrene round-bottom tube 12 x 75 mm style). Thereto was added the sample fixed with 100 μ l of FACS fixation buffer. Thereafter, dot plot analysis was performed with FACScan (BD). Cell Quest (BD) software was used for the analysis. The result of ICS two-dimensional analysis is shown in Fig. 2C.

[0115] As a result of ICS analysis, CD8 + IFN- γ + T cells appeared in an FMP:58-66 peptide addition dependent manner in immunization by the intraperitoneal (i.p.) route and immunization by the transnasal route. Based on the above, it became certainly clear that FMP:58-66 epitope specific-cytotoxic T lymphocyte (CTL) contained in M1 protein was induced by VP2-M1/wt SV40 VP1 VLP immunization (Fig. 2C).

[0116] Based on these results, it was shown that VP2-M1/wt SV40 VP1 VLP purified from insect cells was intraperitoneally or nasally administered to a HHD mouse, and M1 CTL epitope specific CTL could be induced.

Example 2: Induction of FMP:58-66 epitope specific-cytotoxic T lymphocyte by immunization (intraperitoneal, transnasal, oral, enema administration) of solution of ground silkworm pupa in which VP2-M1 and SV40 wild-type VP1 are co-expressed

Preparation of VP2-M1 and wt SV40 VP1 co-expressing solution of ground silkworm pupa

[0117] A wt SV40 VP1 gene fragment was incorporated into a multicloning site (restriction enzyme SmaI) of pM01 vector (SYSMEX CORPORATION; the vector map is shown in Fig. 3). The resulting plasmid construct was called wt SV40 VP1_pM01.

[0118] A VP2-M1 gene was incorporated into a multicloning site (restriction enzyme SmaI) of pM01 vector (SYSMEX CORPORATION). The resulting plasmid construct was called VP2-M1_pM01.

[0119] A recombinant baculovirus was prepared by modifying a method of Maeda, et al. (Invertebrate Cell system and Applications, Vol.1, p.167-181, CRC Press, Boca Raton (1989)). Specifically, DNA (20 ng) of CPd baculovirus (cysteine protease deficient virus strain, SYSMEX) linearly linked to the above plasmid construct wt SV40 VP1_pM01 or VP2-M1_pM01 (each 50 ng) was co-transfected to BmN cells (Maeda, 1989), using a lipofection reagent (X-tremeGENE 9 DNA Transfection Reagent: Roche Diagnostics K.K.). The infection symptoms were confirmed, and then the culture supernatant was collected. Whereby, a recombinant baculovirus incorporating a wt SV40 VP1 gene or VP2-M1 was obtained.

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[0120] The recombinant baculovirus was inoculated to a silkworm pupa (variety: Kinsyu-showa, silkworm seeds were purchased from Ueda-sanshu and artificially bred to pupas in SYSMEX CORPORATION). After six days from virus inoculation, the pupas were collected, and frozen at -80°C.

[0121] To the frozen five pupas or five pupas incubated in boiled water for 10 minutes from a frozen state was added 25 mL of a Tris buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% (w/v) glycerol, 1 mM DTT, protease inhibitor cocktail (Roche Diagnostics K.K., Complete-EDTA-free)), or a phosphate buffer solution (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄ (pH 7.4)). The pupas were crushed using a homogenizer (ELMEX LIMITED, Model SH-IIM, paddle crushing) to obtain a suspension. This suspension was filtered with a mesh to remove the residue of the epidermis of the pupa and the like, and this filtrate was referred to as a solution of ground silkworm pupa.

(Intraperitoneal administration)

Immunization of VP2-M1/wt SV40 VP1 VLP

[0122] A 8-week HHD mouse was immunized by 100 µl of the homogenate prepared from silkworm expressing VP2-M1/wt SV40 VP1 VLP (a solution of ground silkworm pupa prepared with a phosphate buffer solution (Fig. 4A), a solution of ground silkworm pupa prepared with a Tris buffer solution (Fig. 4B), a solution of heat-treated ground silkworm pupa prepared with a phosphate buffer solution (Fig. 4C), and a solution of heat-treated ground silkworm pupa prepared with a Tris buffer solution (Fig. 4D)). For immunization, a 1 ml syringe with a 27-gauge needle inserted (Myjector, syringe with an injection needle, for insulin, TERUMO, SS-10M2713) was used.

[0123] One week after administration, the spleen of the immunized mouse was collected. Lymphocytes were prepared by the method described in Example 1, and Intra-cellular staining (ICS) analysis was performed.

[0124] As a result of ICS analysis, in i.p. immunization, appearance of CD8 + IFN-γ + T cells was confirmed in an FMP:58-66 peptide addition dependent manner (Figs. 4A to 4D). Based on these results, it became clear that FMP:58-66 epitope specific-cytotoxic T lymphocyte (CTL) contained in M1 protein was induced by i.p. immunization of the homogenate prepared from silkworm pupa expressing VP2-M1/wt SV40 VP1 VLP. This CTL induction was detected irrespective of the presence or absence of heat treatment of pupa, and irrespective of the kind of the buffer solution (Tris buffer solution or phosphate buffer solution) used in the preparation.

(Transnasal administration)

Immunization of VP2-M1/wt SV40 VP1 VLP

[0125] 20 µl of the homogenate prepared from silkworm expressing VP2-M1/wt SV40 VP1 VLP was nasally administered to an 8-week old HHD mouse under general anesthesia, by the transnasal route. After one week from administration, the spleen of the immunized mouse was collected. Lymphocytes were prepared by the method described in Example 1, and Intra-cellular staining (ICS) analysis was performed.

[0126] As a result of ICS analysis, in nasal immunization, CD8 + IFN-γ + T cells was appeared in an FMP:58-66 peptide addition dependent manner (Fig. 5). Based on the above, it became certainly clear that FMP:58-66 epitope specific-cytotoxic T lymphocyte (CTL) contained in M1 protein was induced by nasal immunization of VP2-M1/wt SV40 VP1 VLP.

(Oral administration)

Immunization of VP2-M1/wt SV40 VP1 VLP

[0127] The silkworm homogenate expressing VP2-M1/wt SV40 VP1 VLP (100 µl of a solution of non-heated ground silkworm pupa prepared with a phosphate buffer solution (Fig. 6A), 100 µl of a solution of non-heated ground silkworm pupa prepared with a Tris buffer solution (Fig. 6B), and 100 µl of a solution of heat-treated ground silkworm pupa prepared with a Tris buffer solution (Fig. 6C)) was administered to a 8-week HHD mouse under general anesthesia by the oral route. For administration, a 1 ml syringe with an oral sonde (oral sonde needle for mouse, φ0.9 x L50 mm (A), Natsume Seisakusho Co., Ltd., KN-348) inserted (TERUMO) was used. After one week from administration, the spleen of the immunized mouse was collected. Lymphocytes were prepared by the method described in Example 1, and Intra-cellular staining (ICS) analysis was performed.

[0128] Based on the result of ICS analysis, CD8 + IFN-γ + T cells were appeared in an FMP:58-66 peptide addition dependent manner in oral immunization (Figs. 6A to 6C). Based on the above, it became certainly clear that FMP:58-66 epitope specific-cytotoxic T lymphocyte (CTL) contained in M1 protein was induced by oral immunization of the silkworm homogenate containing non-heated or heated VP2-M1/wt SV40 VP1 VLP.

(Enema administration)

Immunization of VP2-M1/wt SV40 VP1 VLP

5 **[0129]** 100 μ l or 200 μ l of the silkworm homogenate expressing VP2-M1/wt SV40 VP1 VLP was administered to an 8-week old HHD mouse under general anesthesia, by the enema route. For administration, a 1 ml syringe with an oral sonde (sterilized DISPOSABLE oral sonde for mouse, 5200S, FUCHIGAMI) inserted (TERUMO) was used.

[0130] After one week from administration, the spleen of the immunized mouse was collected. Lymphocytes were prepared by the method described in Example 1, and Intra-cellular staining (ICS) analysis was performed.

10 **[0131]** As a result of ICS analysis, CD8 + IFN- γ + T cells were appeared in an FMP:58-66 peptide addition dependent manner in intestinal immunization (Fig. 7). Based on the above, it became certainly clear that FMP:58-66 epitope specific-cytotoxic T lymphocyte (CTL) contained in M1 protein was induced by VP2-M1/wt SV40 VP1 VLP immunization.

15 Example 3: Induction of anti-OVA antibody production by immunization with VP2-egg albumin (Ovalbumin, OVA)-including wt SV40 VP1 VLP.

Preparation of baculovirus expressing wild-type (wt) simian virus 40 (SV40) VP1

20 **[0132]** A baculovirus expressing wt SV40 VP1 was prepared as described in Example 1.

Preparation of baculovirus expressing VP2 fused OVA protein

25 **[0133]** First, a coding sequence of FLAG tag (SEQ ID No. 13) was added to the upstream of a codon encoding an amino terminus (N-terminus) of wt SV40 VP2, and a BamHI site was introduced into the further upstream thereof. The stop codon of wt SV40 VP2 was eliminated, and an EcoRI site was introduced. The obtained polynucleotide was inserted via a BamHI site and an EcoRI site of pFastBac1 plasmid to prepare a plasmid containing a wt SV40 VP2 gene. A coding sequence of a GGGGSGGGGSGGGGS linker (SEQ ID No. 3; the nucleic acid sequence is shown in SEQ ID No. 14) was introduced into the upstream of a codon encoding an N-terminus of OVA protein, and an EcoRI site was introduced into the further upstream thereof. The stop codon was added to the downstream of OVA protein coding sequence, and a Sal I site was introduced into the further downstream thereof.

30 **[0134]** The obtained polynucleotide was introduced via the EcoRI site and the Sal I site of the plasmid containing a wt SV40 VP2 gene to prepare a plasmid holding a gene fused with the OVA coding sequence in the downstream of the VP2 coding sequence.

35 **[0135]** Escherichia coli DH10bac (invitrogen) holding a baculovirus genome was transformed with this plasmid to prepare a recombinant baculovirus genome expressing protein VP2-OVA in which OVA was fused with wt SV40 VP2 (SEQ ID No. 6; the nucleic acid sequence is shown in SEQ ID No. 7). The recombinant baculovirus genome was transfected to Sf-9 cells. After three days, the supernatant thereof was collected to obtain a solution containing recombinant baculovirus. A part of this solution was again infected with Sf-9 cells, thereby increasing a recombinant baculovirus titer. The resulting solution was referred to as a stock solution of recombinant baculovirus.

40 Preparation of wt SV40 VP1 VLP containing VP2-OVA

[0136] A wt SV40 VP1 VLP containing VP2-OVA was prepared in the same manner as in "preparation of wt SV40 VP1 VLP containing VP2-M1" in Example 1.

45 Immunization of VP2-OVA/wt SV40 VP1 VLP (intraperitoneal administration and transnasal administration)

[0137] An 8-week old HHD mouse was immunized by 100 μ l of VP2-OVA/wt SV40 VP1 VLP (500 μ g/ml) via the intraperitoneal route. For immunization, a 1 ml syringe with a 27-gauge needle inserted (Myjector, syringe with an injection needle, for insulin, TERUMO, SS-10M2713) was used. 100 μ L of an OVA solution (1 mg/mL) was subcutaneously inoculated to a mouse as an object group of VP2-OVA/wt SV40 VP1 VLP immunization.

[0138] Alternatively, 40 μ l of VP2-OVA/wt SV40 VP1 VLP (500 μ g/ml) was nasally administered to an 8-week old HHD mouse under general anesthesia, by the transnasal route.

55 **[0139]** After one week from immunization, additional immunization was performed. After two weeks from initial immunization, the mouse was given general anesthesia, then the blood was collected from the heart of the mouse. Blood clotting was induced in the obtained blood, and the mouse serum in the supernatant was collected.

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Detection of antibody induced by immunization (ELISA method)

5 **[0140]** In order to confirm that an anti-OVA antibody induced in the serum by reaction to OVA is present, antibody detection was performed as follows, using the mouse serum collected as described above. OVA was dissolved in TBS (20 mM Tris-HCl (pH 8.0), 150 mM NaCl) so as to be 1 $\mu\text{g}/100 \mu\text{L}$. 100 μL of this solution was added to each well of a 96-well plate (Nunc MaxiSorp flat-bottom 96-well plate). The plate was allowed to stand at room temperature for 2 hours or at 4°C overnight to bond OVA to the bottom surface of the plate. After OVA immobilization, the plate was washed with TBS-T (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.005% (w/v) Tween 20). Thereafter, 300 μL of a 5% (w/v) skim milk solution was added to each well, and the plate was allowed to stand at room temperature for 2 hours or at 4°C overnight. The skim milk solution was removed, then each well was washed with TBS-T.

10 **[0141]** 50 μL of a solution obtained by diluting the mouse serum obtained above to a desired concentration with TBS-T was added to each well, and the plate was allowed to stand at room temperature for 1 hour. The mouse serum solution was removed, and each well was washed with TBS-T. Thereafter, 100 μL of HRP-labeled anti-mouse IgG (H+L chain) (MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.) diluted 2,000-fold with TBS-T, and the plate was allowed to stand at room temperature for 1 hour. After allowing the plate to stand, each well was washed with TBS-T. TMB peroxidase EIA complex substrate kit (Bio-Rad Laboratories, Inc.) was added to each well according to the accompanying instructions, and HRP-labeled anti-mouse IgG (H+L chain) present in each well was measured. The absorbance value at 655 nm was measured using iMark microplate Absorbance Reader (Bio Rad Laboratories, Inc.).

15 **[0142]** The results are shown in Fig. 8A and Fig. 8B. As shown in Fig. 8A, production of anti-OVA antibody was induced in a mouse intraperitoneally administered with VP2-OVA/SV40 wt VP1 VLP, and an antibody titer higher than a mouse singly administered with OVA was shown. Induction of anti-OVA antibody production was also seen in a mouse nasally administered with VP2-OVA/SV40 wt VP1 VLP (Fig. 8B). Based on these results, it was shown that production of an antibody specific to OVA can be induced by intraperitoneal administration or transnasal administration of VP2-M1/SV40 wt VP1 VLP.

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Saitama Medical University
Hiroshi HANDA

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 Gln Ala Tyr Ala Val Ile Ser Gly Ala Pro Ala Ala Ile Ala Gly Phe
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 Gly Tyr Arg Phe Phe Ser Asp Trp Asp His Lys Val Ser Thr Val Gly
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 Leu Tyr Gln Gln Pro Gly Met Ala Val Asp Leu Tyr Arg Pro Asp Asp
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 Tyr Tyr Asp Ile Leu Phe Pro Gly Val Gln Thr Phe Val His Ser Val
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 Gln Tyr Leu Asp Pro Arg His Trp Gly Pro Thr Leu Phe Asn Ala Ile
 145 150 155 160

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 Ser Gln Ala Phe Trp Arg Val Ile Gln Asn Asp Ile Pro Arg Leu Thr
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70
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45 **Claims**

1. An immunity inducer, wherein
 the immunity inducer comprises virus like particles;
 50 the virus like particles comprise a virus-derived outer coat protein and an antigen-bound protein comprising an
 exogenous antigen;
 the outer coat protein constitutes an outer coat of the virus like particles, and the antigen-bound protein is comprised
 in the outer coat.
- 55 2. The immunity inducer according to claim 1, wherein the outer coat protein is derived from a virus in the genus
 Polyomavirus.
3. The immunity inducer according to claim 2, wherein the virus in the genus Polyomavirus is SV40, and the outer coat

protein is VP1.

4. The immunity inducer according to any of claims 1 to 3, wherein the antigen-bound protein is a fusion protein of the antigen and an inner peptide.
5. The immunity inducer according to claim 4, wherein the inner peptide is derived from a virus in the genus Polyomavirus, and the inner protein is VP2 and/or VP3.
6. The immunity inducer according to claim 4 or 5, wherein an amino acid sequence of the inner peptide comprises an amino acid sequence represented by SEQ ID No. 9 or SEQ ID NO. 11.
7. The immunity inducer according to any of claims 1 to 6, wherein the immunity inducer is a solid preparation, a semisolid preparation, a liquid preparation, an injection, or a suppository.
8. The immunity inducer according to any of claims 1 to 7 for use in inducing immunity effect of the living body on the antigen by administering the immunity inducer.
9. A method for producing the immunity inducer according to any of claims 1 to 8, comprising a step of contacting a virus-derived outer coat protein with an antigen-bound protein to prepare virus like particles.
10. The method according to claim 9, comprising a step of introducing a DNA encoding the outer coat protein and a DNA encoding the antigen-bound protein into a host cell to express the outer coat protein and the antigen-bound protein in the host cell, thereby obtaining the outer coat protein and the antigen-bound protein.
11. The method according to claim 9 or 10, wherein the host cell is selected from a group consisting of an insect cell, Escherichia coli, a yeast and a plant cell.
12. The method according to any of claims 9 to 11, wherein the outer coat protein is VP1 of SV40, and the antigen-bound protein comprises VP2 and/or VP3 of SV40.
13. The method according to any of claims 9 to 12, wherein the immunity inducer is a solid preparation, a semisolid preparation, a liquid preparation, an injection, or a suppository.

FIG. 1

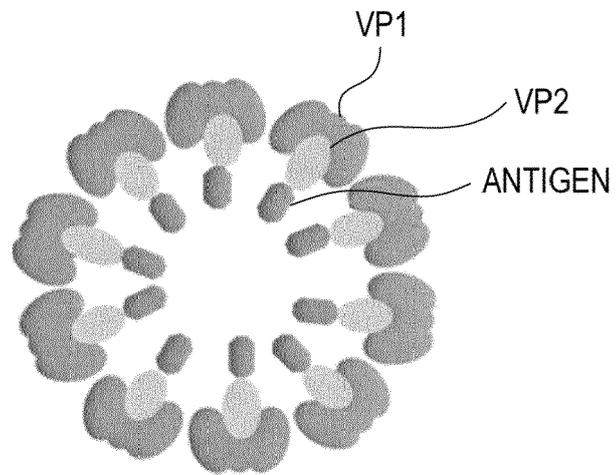


FIG. 2A

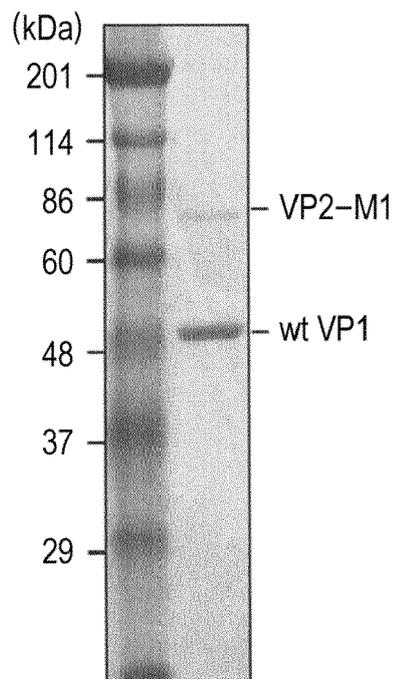
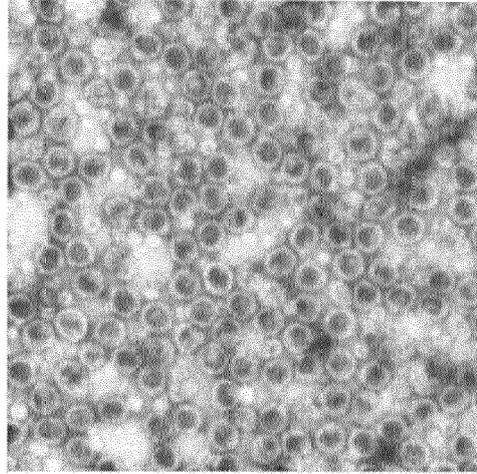


FIG. 2B

wt VP1-VLP/VP2-M1



100 nm

FIG. 2C

wt VP1-VLP/VP2-M1

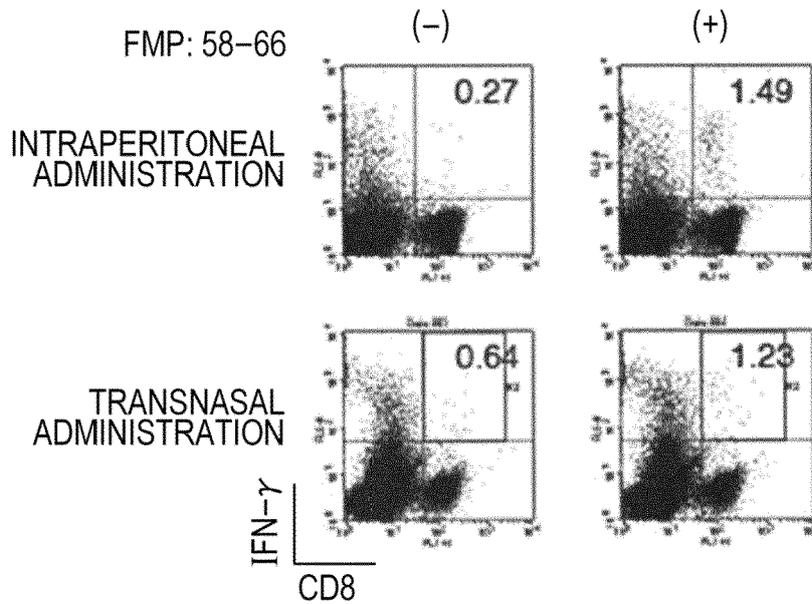
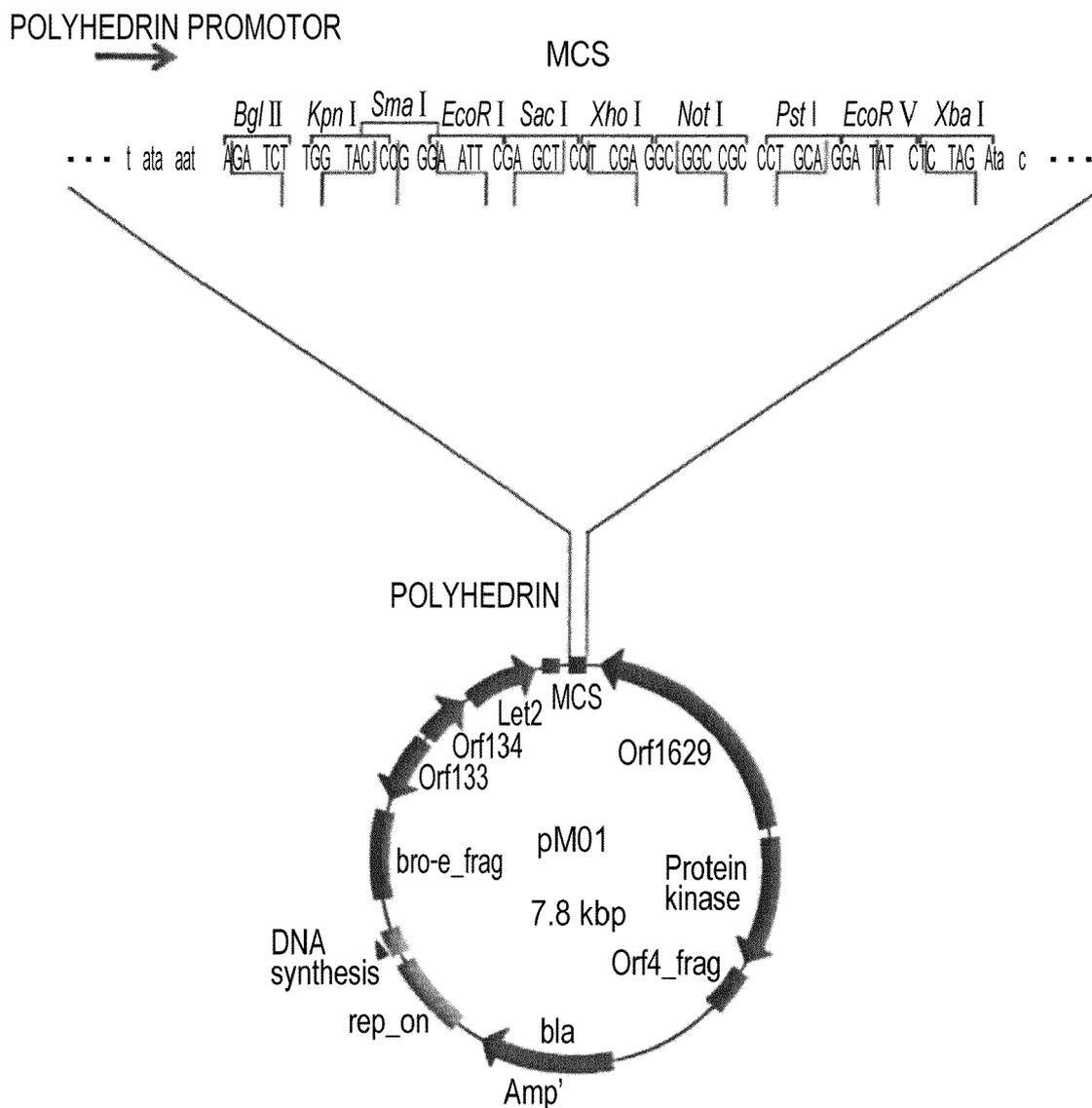


FIG. 3



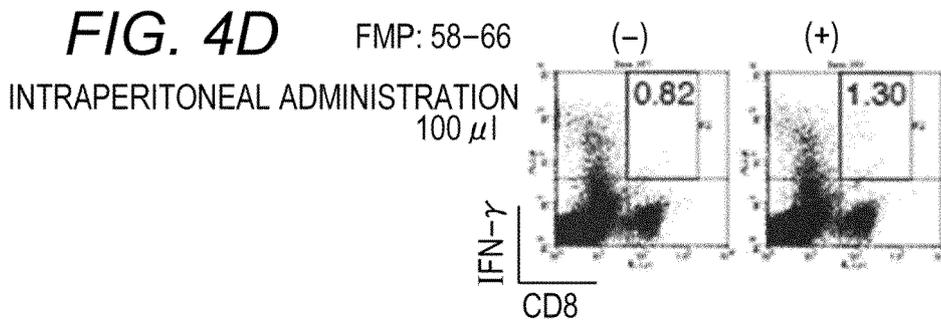
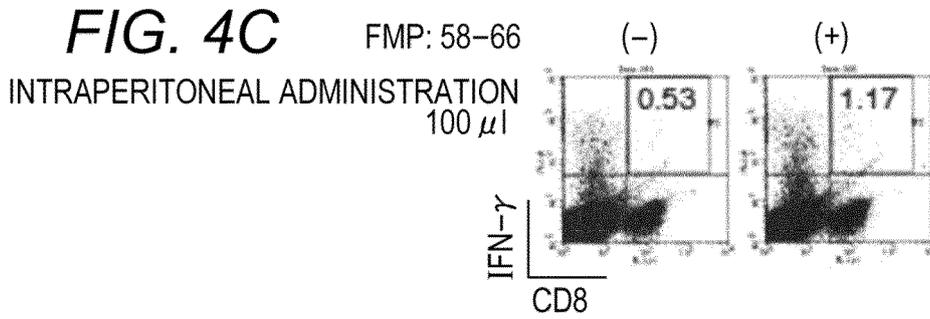
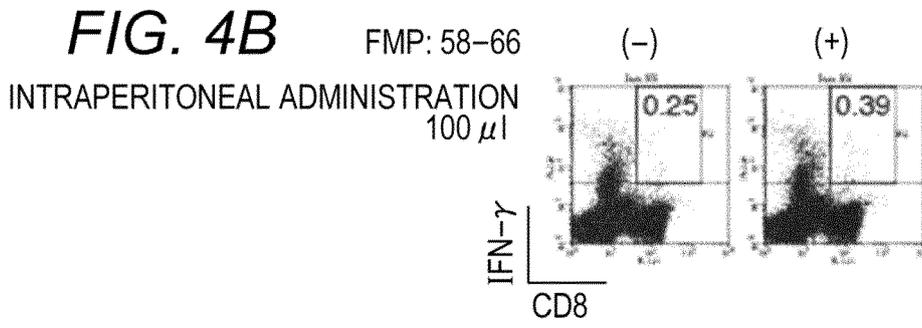
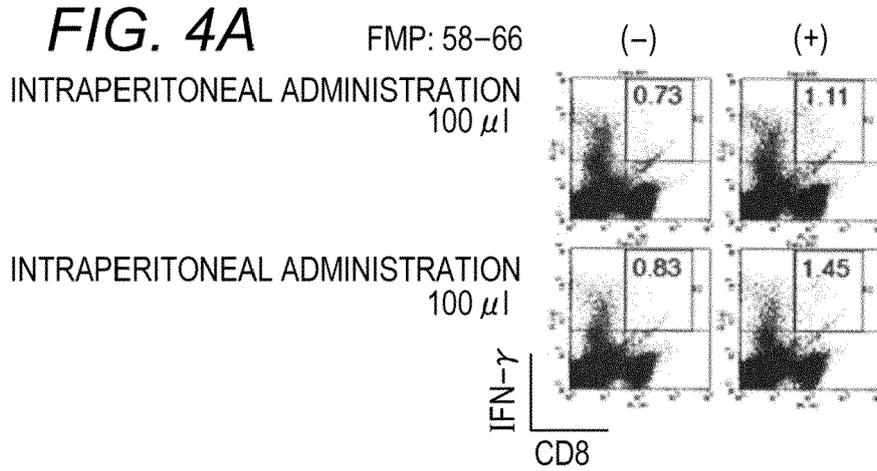


FIG. 5

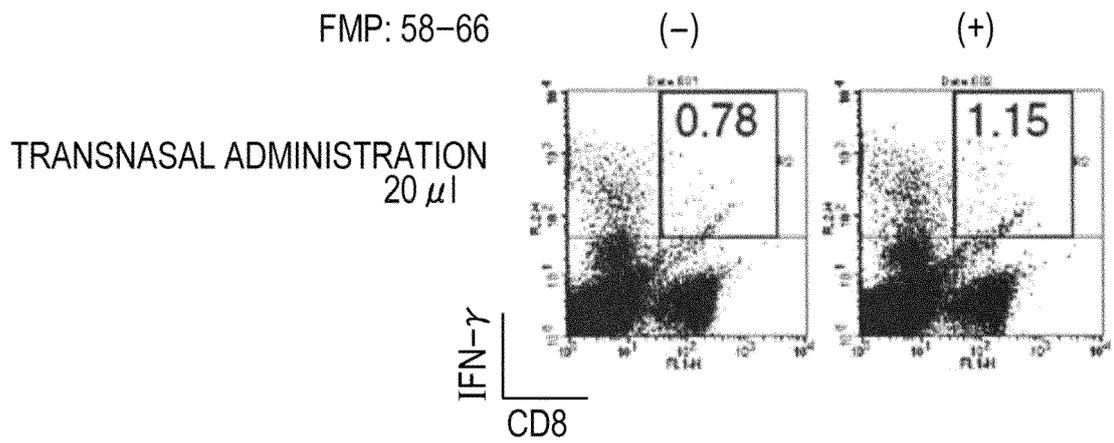


FIG. 6A

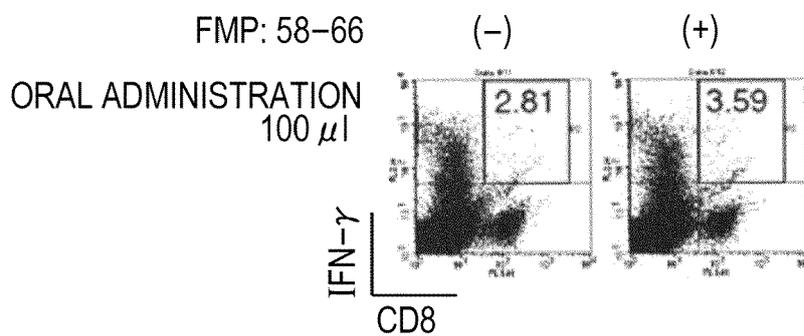


FIG. 6B

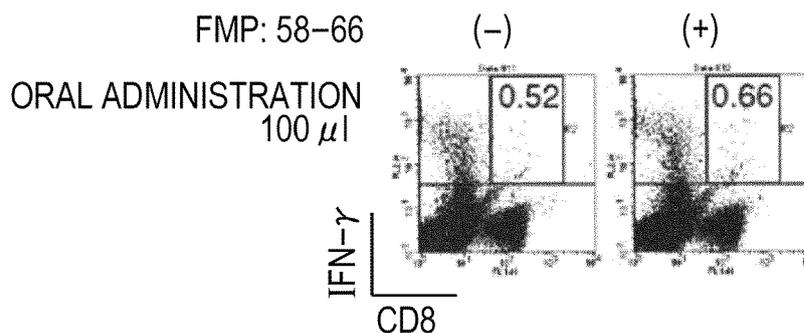


FIG. 6C

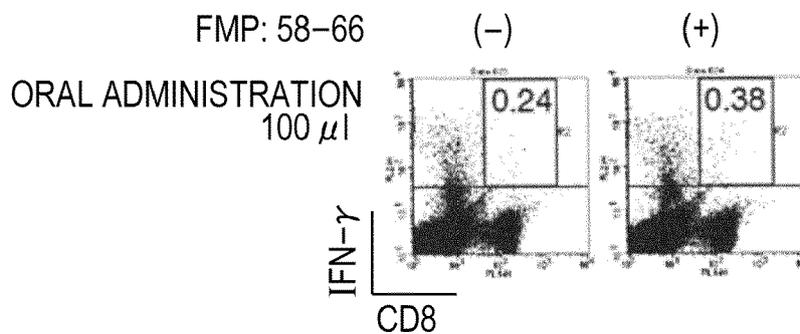


FIG. 7

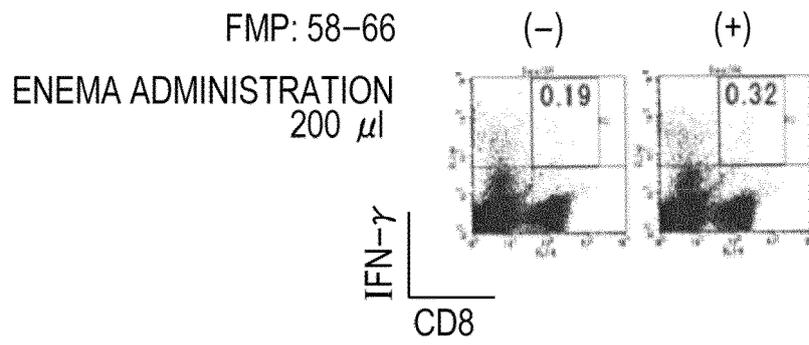


FIG. 8A

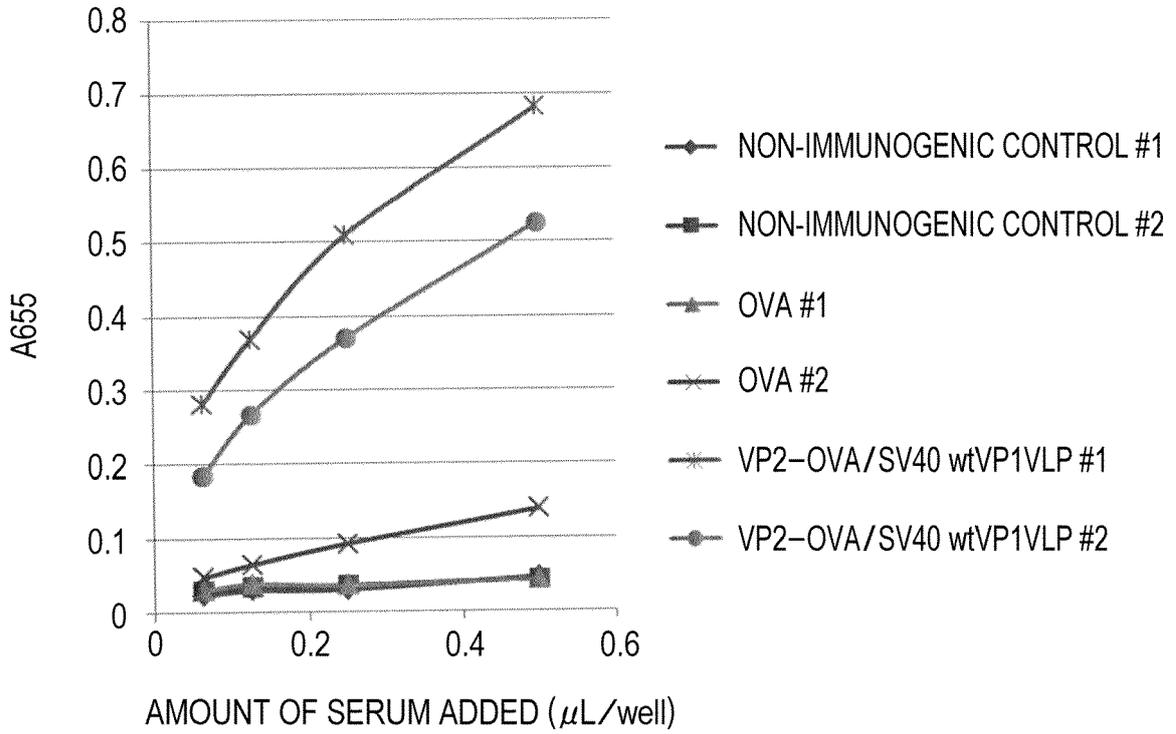
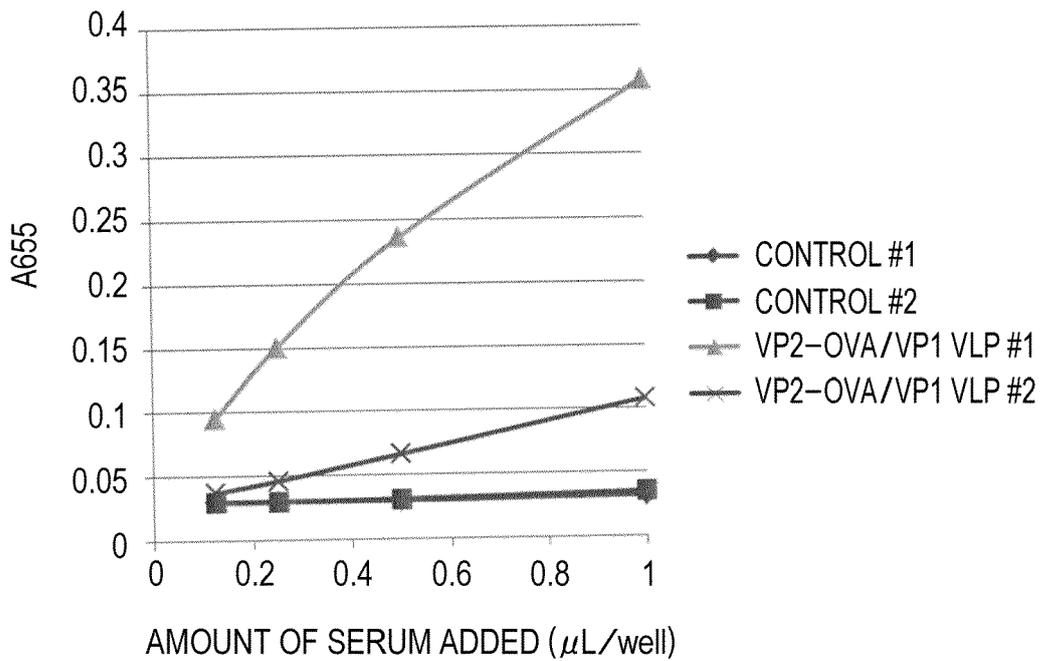


FIG. 8B





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DOCUMENTS CONSIDERED TO BE RELEVANT			
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X	INOUE ET AL: "Engineering of SV40-based nano-capsules for delivery of heterologous proteins as fusions with the minor capsid proteins VP2/3", JOURNAL OF BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 134, no. 1-2, 3 January 2008 (2008-01-03), pages 181-192, XP022500142, ISSN: 0168-1656, DOI: 10.1016/J.JBIOTEC.2007.12.006 * abstract * * Paragraphs 2.2., 2.3 and 2.6 * -----	1-13	INV. C12N7/00 A61K39/00
X	TEGERSTEDT KARIN ET AL: "A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors", CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 65, no. 13, 1 July 2005 (2005-07-01), pages 5953-5957, XP002387780, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-05-0335 * abstract * * See "Materials and methods" and "Vaccination and Tumor Challenge" * ----- -/--	1-13	TECHNICAL FIELDS SEARCHED (IPC) A61K C12N C07K
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 16 March 2017	Examiner Turri, Matteo
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	MASAAKI KAWANO ET AL: "SV40 virus-like particles as an effective delivery system and its application to a vaccine carrier", EXPERT REVIEW OF VACCINES, vol. 12, no. 2, 9 February 2013 (2013-02-09), pages 199-210, XP055348348, GB ISSN: 1476-0584, DOI: 10.1586/erv.12.149 * Page 203: "Delivery of foreign proteins into cells by SV40-VLPs"; figure 1 *	1-13	TECHNICAL FIELDS SEARCHED (IPC)
A	ABING A ET AL: "Efficient intracellular delivery of a protein and a low molecular weight substance via recombinant polyomavirus-like particles", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, US, vol. 279, no. 26, 25 June 2004 (2004-06-25), pages 27410-27421, XP002354397, ISSN: 0021-9258, DOI: 10.1074/JBC.M313612200	1-13	
A	TEUNISSEN ERIK A ET AL: "Production and biomedical applications of virus-like particles derived from polyomaviruses", JOURNAL OF CONTROLLED RELEASE, vol. 172, no. 1, 31 August 2013 (2013-08-31), pages 305-321, XP028772934, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2013.08.026 ----- -/--	1-13	
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 16 March 2017	Examiner Turri, Matteo
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A	TEGERSTEDT K ET AL: "MURINE POLYOMAVIRUS VIRUS-LIKE PARTICLES (VLPs) AS VECTORS FOR GENE AND IMMUNE THERAPY AND VACCINES AGAINST VIRAL INFECTIONS AND CANCER", ANTICANCER RESEARCH - INTERNATIONAL JOURNAL OF CANCER RESEARCH AND TREATMENT, INTERNATIONAL INSTITUTE OF ANTICANCER RESEARCH, GR, vol. 25, no. 4, 1 July 2005 (2005-07-01), pages 2601-2608, XP009068573, ISSN: 0250-7005 -----	1-13	
			TECHNICAL FIELDS SEARCHED (IPC)
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 16 March 2017	Examiner Turri, Matteo
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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