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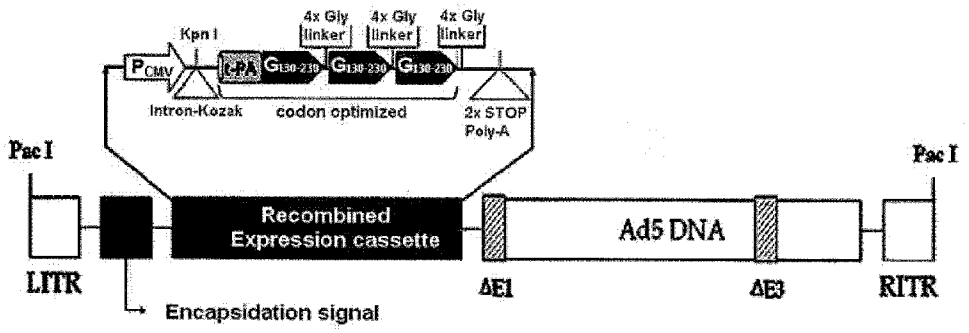
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(54) Title: RESPIRATORY SYNCYTIAL VIRUS VACCINE



Pac I-linearized recombinant adenoviral genome of rAd/3xG

(57) Abstract: The present invention relates to an adenovirus expression vector comprising a base sequence encoding amino acid residues 120 to 230 of RSV G protein, and to a vaccine composition for preventing and treating respiratory tract diseases, comprising the vector.

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【DESCRIPTION】

【Invention Title】

RESPIRATORY SYNCYTIAL VIRUS VACCINE

【Technical Field】

The present invention relates to an adenovirus expression vector comprising a base sequence encoding amino acid residues 120 to 230 of RSV G protein, and to a vaccine comprising the vector.

【Background Art】

Respiratory syncytial virus (RSV) is a non-segmented RNA virus, and belongs to the family paramyxoviridae. Respiratory syncytial virus can be divided into two major antigenic subgroups, A and B, and subgroup A is generally dominant. RSV synthesizes ten types of proteins such as NS1, NS2, P, N, M, SH, G, F, M2 and L proteins in an infected host cell. N (nucleocapsid), P (phosphoprotein), and L (large polymerase subunit) proteins of RSV form the nucleoprotein core, which is recognized as the minimum unit of infectivity (Brown et al., J.Virol, 1:368-373, 1967), and form the viral RNA dependent RNA transcriptase for transcription and replication of the RSV genome (Yu et al., J. Virol. 69:2412-2419, 1995; Grosfeld et al., J. Virol. 69:5677-5686, 1995). M2 protein is known to induce a cytotoxic T lymphocyte immune response, which is involved in the elimination of

virus-infected cells. Recent studies indicate that the M2 gene products (M2-1 and M 2-2) are involved in transcription (Collins et al., 1996, Proc. Natl. Acad. Sci. 93:81-85). F protein (fusion protein), G protein and SH protein are surface antigens. Among them, G protein and F protein having a relatively high molecular weight and being present in the outer membrane are highly glycosylated glycoproteins, and play a crucial role in protective antiviral immune responses against RSV infection and antigenicity. G protein is a major attachment protein, F protein is involved in giant cell formation and responsible for viral penetration and attachment, and F and G proteins induce neutralizing antibodies.

RSV is a major pathogen that causes lower respiratory tract infections in infancy and early childhood, and is the major cause of pneumonia and bronchitis. The high risk population includes infants and children with bronchopulmonary dysplasia, congenital heart disease, cystic fibrosis, cancer or various forms of immunodeficiency, as well as adult immunosuppressed prior to bone marrow transplantation. It was reported that RSV infection is common in the elderly, similar to influenza, and during RSV epidemic periods, the excess mortality is higher than influenza epidemic periods. In the United States, there is a relatively large population of about 100,000 to 200,000 at high risk of developing severe RSV illness, and RSV infection results in approximately

90,000 hospitalizations and 4,500 deaths annually. In Korea, RSV outbreaks occur each year, which is responsible for a large number of hospitalizations in infants. Among pathogens responsible for lower respiratory tract infections being isolated at the Seoul National University Children's Hospital, 60% was found to be caused by respiratory syncytial virus. Therefore, it can be seen that RSV also increases hospitalizations and deaths in Korea.

Despite such severity, there is still no currently available vaccine or specific therapeutic agent against RSV infection. In particular, a formalin-inactivated vaccine (FI-vaccine) was developed and its clinical trial was conducted, however, about 80% of infants administered with the FI-vaccine were hospitalized with RSV infection, some patients died from the infection, and severe lower respiratory tract complications were observed in the lungs. Ribavirin (Virazole) was approved for the treatment of lower respiratory tract infections caused by RSV. However, there are limitations in that its clinical efficacy is doubtful, toxicity is high, and given in aerosol form, its application is limited. In many countries including the United States, RSV-IGIV (Respigam) or an anti-RSV monoclonal antibody, palivizumab (Synagis) is used for the prevention of serious RSV infection in high-risk groups. However, it is a prophylactic, rather than therapeutic, treatment of RSV, and is expensive. Indeed, its expense means that it is unavailable for many people in need of

anti-RSV therapy. Accordingly, there is an urgent need for the development of effective therapeutic agents against RSV infection.

For the development of RSV vaccine, several factors need to be considered. First, vaccine-induced immunopathology should be excluded. Second, since the major target population for an RSV vaccine is infants, protective immunity should be induced by a single administration at birth. Third, the vaccine should not be neutralized by maternal antibodies.

As a RSV vaccine candidate, G protein lacks any MHC class I-restricted epitope and has not yet been demonstrated to elicit CTL (cytotoxic T lymphocyte) response. It has single immunodominant I-Ed epitope spanning RSV G amino acids 183 to 195, and largely induces a specific subset of CD4 T cells restricted to Vβ14 expression. Numerous studies have suggested that immunization of RSV G is associated with the induction of polarized Th2 type responses which leads to pulmonary eosinophilia upon RSV challenge of G-immunized mice (Hancock et al., J Virol 70:7783-91, 1996; J Virol et al., 72:2871-80, 1998; Openshaw, et al., Int Immunol 4:493-500, 1992; Tebbey, et al., J Exp Med 188:1967-72, 1998).

【Disclosure】

【Technical Problem】

It is an object of the present invention to provide an adenovirus expression vector comprising a base sequence encoding amino acid residues 120 to 230 of RSV G protein.

It is another object of the present invention to provide a vaccine composition comprising the adenovirus expression vector.

【Technical Solution】

Accordingly, the present inventors have made an effort to develop a more effective and safe RSV vaccine. They found that a partial sequence of RSV G protein is expressed using an adenoviral vector to elicit effective protective immunity against RSV replication without immunopathology such as eosinophilia, completing the present invention.

【Description of Drawings】

Fig. 1 is a diagram showing the structure of the adenovirus expression vector rAd/3xG according to the present invention;

Fig. 2 is a diagram showing the structure of the adenovirus expression vector rAd/6xG according to the present invention;

Fig. 3 is the result of Western blotting showing the G protein expression level by an adenovirus expression vector having three copies of RSV G fragment (rAd/3xG), an adenovirus expression vector having one copy of RSV G fragment (rAd/1xG), and an adenovirus expression vector having no RSV G fragment (rAd/control);

Fig. 4 is the result of Western blotting showing the G protein expression level by an adenovirus expression vector having three copies of RSV G fragment (rAd/3xG), an adenovirus expression vector having six copy of RSV G fragment (rAd/6xG), and an adenovirus expression vector having no RSV G fragment (rAd/control);

Fig. 5 is the result of measuring serum IgG titers after intranasal, intramuscular, and oral immunization with rAd/3xG (Preimmune: no administration, i.n.: intranasal administration, i.m.: intramuscular administration, oral: oral administration);

Fig. 6 is the result of measuring serum IgG titers after intranasal immunization with rAd/3xG and rAd/6xG;

Fig. 7 is the result of measuring IgA titers in the respiratory tract after intranasal, intramuscular, and oral immunization with rAd/3xG;

Fig. 8 is the result of ELISA, in which IgA titers in the respiratory tract were measured after immunization with rAd/control, rAd/3xG and live RSV, respectively;

Fig. 9 is the result of measuring IgA titers in the respiratory tract after intranasal immunization with rAd/3xG and rAd/6xG;

Fig. 10 is the results of measuring IgG titers and ELISA, in which IgG and IgA inductions were shown in the presence of pre-existing anti-adenovirus immunity;

Fig. 11 is the result of IFN-γ staining, in which G-specific T cell response was measured after immunization with rAd/control, rAd/3xG, and vaccinia virus expressing RSV G (vvG), respectively;

Fig. 12 is the result of counting eosinophils in bronchoalveolar lavage, after immunization with rAd/3xG and vvG, respectively;

Fig. 13 is the result of measuring RSV titers in the lung tissues from immunization group of rAd/control, intranasal and intramuscular immunization groups of rAd/3xG, and immunization group of live RSV;

Fig. 14 is the result of measuring weight loss by immunopathology in immunization groups of rAd/control, rAd/3xG, and live RSV; and

Fig. 15 is the result of measuring lung viral titers in immunization groups of rAd/control, rAd/3xG, adenovirus expression vector against RSV F protein (rAd/Fco), adenovirus expression vector against RSV M2 protein (rAd/M2), and mixed vaccine of rAd/3xG, rAd/Fco and rAd/M2 (rAd/Mix).

[Best Mode]

In one embodiment, the present invention provides an adenovirus expression vector comprising a base sequence encoding a RSV G protein.

In the present invention, the G protein of RSV, a major attachment protein, is a potentially important target for protective antiviral immune responses, and the wild type

nucleotide and amino acid sequences of the RSV G protein are disclosed in the art (Wertz et al., Proc. Natl. Acad. Sci. USA 92:4075-4079, 1985; Satake et al., Nucl. Acids Res. 13(21): 7795-7810, 1985). G protein lacks any MHC class I-restricted epitope and has not yet been demonstrated to elicit CTL (cytotoxic T lymphocyte). However, it has single immunodominant I-Ed epitope spanning RSV G amino acids 183 to 195 and largely induces a specific subset of CD4 T cells restricted to Vβ14 expression. The RSV G protein used in the present invention contains a fragment between amino acid residues 120 and 230, and more preferably a fragment between amino acid residues 130 and 230 from N-terminus.

In addition, the RSV G protein used in the present invention contains a protein having the wild type amino acid sequence, as well as a variant thereof. The term 'G protein variant' is intended to refer to G proteins, of which at least one amino acid is different from the wild-type due to the deletion, insertion, non-conservative substitution or conservative substitution of amino acid residues, or due to combinations thereof. Further, the G protein may be glycosylated or lipidated, and may also be derivatized to include molecules enhancing antigen presentation and targeting of antigens to antigen presenting cells.

The RSV G protein may be in the form of monomer containing a fragment of amino acid residues 120 to 230 or amino acid residues 130 to 230 from N-terminus, and for higher immunogenicity, may be also in the form of multimer by linking 2 or more, preferably

3 to 8, more preferably 3 to 6 of the fragment, and most preferably in the form of trimer by linking three of the fragment and hexomer by linking six of the fragment. In a specific embodiment of the present invention, three or six copies of the RSV G fragment are repeated tandem. In the present invention, to enhance the vaccine potential of an RSV G protein fragment (amino acid residues 130 to 230), the expression of the RSV G protein fragment is increased by codon optimization (SEQ ID NO. 1), and three or six copies of this RSV G fragment are repeated tandem for higher immunogenicity.

In the case where the RSV G protein takes the form of multimer, the amino acid sequence constituting the monomer may be covalently linked to each other, either directly or by a linker. In the case of using a linker, for example, one to five amino acids selected from glycine, alanine, leucine, isoleucine, proline, serine, threonine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, lysine, and arginine may be used, and preferred examples thereof may include valine, leucine, aspartic acid, glycine, alanine and proline. More preferably, to facilitate genetic manipulation, several amino acids selected from glycine, valine, leucine, and aspartic acid may be used by linking to each other. In a specific embodiment of the present invention, the region of RSV G protein is juxtaposed three times and six times with four glycine residues as a linker between each repeated region.

In a specific embodiment of the present invention, multiple

bands of approximately 20 kDa to 30 kDa are detected in the culture supernatant from HEp-2 cells infected with rAd/3xG (three copies of the RSV G fragment inserted into adenoviral DNA) and multiple bands of approximately 20 kDa to 30 kDa and approximately 40 kDa to 50 kDa are detected in the culture supernatant from HEp-2 cells infected with rAd/6xG (six copies of the RSV G fragment inserted into adenoviral DNA), but any specific bands are not detected in the supernatant from HEp-2 cells infected with rAd/1xG (one copy of the RSV G fragment inserted into adenoviral DNA) (Fig. 3 and Fig. 4), indicating the specific and enhanced expression of RSV G antigen by rAd/3xG and rAd/6xG. In addition, mice were inoculated once via intranasal and intramuscular routes with the same doses of rAd/1xG and rAd/3xG. As a result, the immunization of rAd/3xG induced strong serum total IgG responses even after single injection, and the immunization of the same dose of rAd/1xG failed to elicit any detectable IgG response above the control (Fig. 5). Notably, it can be seen that the rAd/3xG vaccine prepared by using the three repeated copies of the RSV G fragment can more effectively elicit immune response. Futher, it can be seen that IgG and IgA response are more effectively elicited after immunization with rAd/6xG than after immunization with rAd/3xG (Fig. 6 and Fig. 9).

As used herein, the term "expression vector" is a recombinant vector capable of expressing a target protein in a suitable host

cell, and referred to a gene construct containing essential regulatory factors operably linked to express a DNA insert.

As used herein, the term "operably linked" refers to a functional linkage between a nucleic acid expression control sequence and a nucleic acid sequence encoding the target protein to perform the general functions. For example, a promoter and the nucleic acid sequence encoding the target protein are operably linked to affect the expression of the nucleic acid sequence encoding the target protein. The operable linkage with recombinant vector can be prepared using the gene recombinant method known in the art, and the site-specific DNA linkage and cleavage are performed with the known enzymes in the art.

As used herein, "adenovirus expression vector" refers to any adenoviral vector comprising exogenous DNA, which is inserted into its own genome encoding polypeptides. The adenoviral vector desirably contains at least a portion of each terminal repeat required to support the replication of the viral DNA (preferably at least about 90% of the full ITR sequence), and the DNA required to encapsidate the genome into a viral capsid. Many suitable adenoviral vectors have been described in the art (see US Patent Nos. 6,440,944; 6,040,174 (replication-defective E1-deleted vector and differentiated packaging cell line)). The preferred adenovirus expression vector is a replication-defective vector, which does not replicate in normal cells.

To enhance an antigen-specific immune response, the vector of the present invention further include a nucleic acid sequence encoding a secretory signal sequence at N-terminus of the nucleic acid sequence encoding G protein. As used herein, the term "secretory signal sequence" (also designated as "signal sequence", "signal peptide", "leader sequence", or "leader peptide") refers to a short peptide sequence containing about 20 to 30 amino acids synthesized at the N-terminus of polypeptide (generally hydrophobic, herein), and to a peptide capable of directing the transport of a polypeptide linked thereto. It is preferable that a eukaryotic secretory signal sequence induces to secret the products of exogenous DNA of adenovirus expression vector. As the suitable sequence, various sequences are disclosed in the art, and include secretory signal sequences such as human growth hormone and immunoglobulin kappa chain. In a specific embodiment, the secretory signal sequence derived from human tissue plasminogen activator (t-PA) (5'-ATGGACGCCATGAAGAGGGGCCTGTGCTGCGTGCTGCTGCTGTGCGGCGCCGTGTT TGTGAGCCCCAGCGCT-3') is used. The sequence may be artificially synthesized or produced by genetic recombination, and is provided by operably linking to a vector capable of expressing the sequence.

In addition, to facilitate the detection of G protein, the vector of the present invention may further include a protein tag removed by using endopeptidase. As used herein, the term "tag"

refers to a molecule, which exhibits a quantifiable activity or characteristic, and examples thereof may include fluorescent molecules including chemical fluorescers such as fluorescein, and polypeptide fluorescers such as green fluorescent protein (GFP) and related proteins, and epitope tags such as a Myc tag, a Flag tag, a His tag, a leucine tag, an IgG tag, and a streptavidin tag. The preferred tag polypeptide generally has 6 or more amino acid residues, and typically about 8 to 50 amino acid residues. In the present invention, a six stretch of histidine is used as a tag, and the tag is preferably present at the C-terminus of G protein.

Further, the vector of the present invention may further include a stop codon at the C-terminus of G protein, and the preferred vector is shown in the diagram of Fig. 1 or Fig 2.

In addition, the expression vector of the present invention includes expression regulatory elements such as a promoter, an operator, an initiation codon, a polyadenylation signal and an enhancer. Both of the initiation codon and stop codon are generally regarded as a part of the nucleotide sequence coding for the antigenic target protein, and are necessary in order to be functional in an individual to whom a genetic construct has been administered, and must be in frame with the coding sequence. The promoter of the vector may be constitutive or inducible.

In a specific embodiment of the present invention, it was

observed that the immunization with the mixture (rAd/Mix) of rAd/3xG, RSV F protein (rAd/Fco) and RSV M2 protein (rAd/M2) adenovirus vaccines elicited the similar protective immunity to the immunization with the same dose of rAd/3xG vaccine (Fig. 11). However, the immunization with only the RSV F protein (rAd/Fco) or RSV M2 protein (rAd/M2) adenovirus vaccine was found to elicit lower protective immunity (Fig. 15), indicating that the adenoviral vector comprising the G protein has significant preventive and therapeutic effect against RSV, compared to other vectors comprising RSV proteins.

In another embodiment, the present invention relates to a vaccine composition for preventing and treating respiratory tract diseases caused by RSV, comprising the vector.

As used herein, the term "prevention" refers to all actions that inhibit or delay the diseases caused by RSV infection through the administration of composition. As used herein, the term "treatment" refers to all actions that restore or beneficially change the diseases caused by RSV infection through the administration of composition. Examples of the symptoms and diseases caused by RSV infection include cough, sneeze, high fever, stridor, bronchitis, bronchiolitis, pneumonia, asthma, tracheobronchitis, croup and respiratory failure.

As used herein, the adenoviral vector of the present invention

is administered as a vaccine to elicit immunity against respiratory tract diseases caused by RSV infection. The viral vector may be suitably formulated with a pharmaceutically acceptable carrier. For oral administration, the pharmaceutically acceptable carrier may include a binder, a lubricant, a disintegrator, an excipient, a solubilizer, a dispersing agent, a stabilizer, a suspending agent, a coloring agent, and a perfume. The injectable preparation may be formulated using an aqueous solution such as a saline solution and a Ringer's solution, and a non-aqueous solution such as vegetable oil, higher fatty acid ester (e.g., ethyl oleate), and alcohols (e.g., ethanol, benzylalcohol, propyleneglycol, or glycerine), and mixed with a buffering agent, a preserving agent, an analgesic, a solubilizer, an isotonic agent, and a stabilizer. For spray formulation, the viral vector of the present invention may be conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or nebulizer using a suitable propellant such as compressed air, nitrogen, carbon dioxide, and a hydrocarbon based low boiling solvent.

The pharmaceutical composition of the present invention may be formulated into various formulations by mixing with the above described pharmaceutically acceptable carriers. For oral administration, the pharmaceutical composition may be formulated into tablets, troches, capsules, elixirs, suspensions, syrups or wafers. For injectable preparations, the pharmaceutical composition may be formulated into an ampule as a single-dose

dosage form or a unit dosage form, such as a multidose container.

The composition of the present invention may be administered via any of the common routes, as long as it is able to reach a desired tissue.

As used herein, the term "administration" refers to the introduction of a predetermined material into a patient using any suitable method. The composition may be formulated for human or veterinary use, and administered via various routes. The viral vector may be administered via parenteral routes such as intravascular, intravenous, intraarterial, intramuscular and subcutaneous route. Further, the viral vector may be administered via oral, nasal, rectal, or transdermal route, or via the inhalation route using aerosol. The viral vector may be administered by either bolus injection or slow infusion. The vector is preferably administered by the subcutaneous route.

The vaccine composition of the present invention is administered in a pharmaceutically effective amount. As used herein, the phrase "pharmaceutically effective amount" refers to an amount enough to exert the vaccine effects, and further an amount not to cause an adverse reaction, or serious or excessive immunity response. An exact effective dose level may vary depending on a variety of factors including the disorder being treated, the severity of the disorder, activity of the compound employed, the route of administration, the sequestration rate of viral vector, the duration of the treatment, drugs used in combination or

coincidental with the viral vector, patient's age, weight, sex, diet, health condition, and the factors well known in the medicinal and medical field. In addition, various general considerations taken into account in determining the "therapeutically effective amount" are known to those of skill in the art, and are described in [Gilman et al., eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990] and [Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990]. The adenoviral vector is generally administered at a dose of about 1×10^{7} to 1×10^{11}, preferably 1×10^{8} to 5×10^{10}, more preferably 5×10^{8} to 2×10^{10} viral particles.

The composition of the present invention may be used alone or in combination with other therapeutic agents, and sequentially or simultaneously administered with conventional therapeutic agents once or several times. Considering all of the above factors, the minimum dose that produces the maximum effect, while avoiding side effects, must be used, which can be easily determined by those skilled in the art.

The vaccine composition of the present invention may further include an adenovirus expression vector containing all or a portion of RSV F protein. In addition, the vaccine composition of the present invention may further include an adenovirus expression vector containing all or a portion of RSV M2 protein. Moreover, the vaccine composition of the present invention may further

include all or a portion of RSV F protein and all or a portion of RSV M2 protein.

The RSV F protein is regarded as a one of antigens involved in virus neutralization. The RSV M2 protein is known as a protein which elicits cytotoxic T-cell response against virus-infected cells. In a specific embodiment of the present invention, it was observed that the immunization with the mixture (rAd/Mix) of rAd/3xG, RSV F protein (rAd/Fco) and RSV M2 protein (rAd/M2) adenovirus vaccines elicited the similar protective immunity to the immunization with the same dose of rAd/3xG vaccine (Fig. 15). However, a single immunization with the recombinant adenoviral vaccine expressing RSV F protein (rAd/Fco) or RSV M2 protein (rAd/M2) was found to induce lower protective immunity (Fig. 15). Accordingly, the immunization with the mixture of rAd/3xG, rAd/Fco, and rAd/M2 adenoviral vaccines can elicit protective immunity, which is the same as or better than the immunization with the same dose of rAd/3xG only.

In a specific embodiment of the present invention, examined was the effect of the adenovirus expression vector according to the present invention on the prevention and treatment of respiratory tract diseases caused by RSV.

From the result of mouse in vivo test, it was found that a single immunization of replication-defective adenoviral vaccine (rAd/3xG) according to the present invention can elicit very

efficient serum IgG and respiratory IgA, and also even in the presence of pre-existing anti-adenovirus immunity (Figs. 5 to 10). Further, the intranasal immunization of rAd/3xG completely prevented any detectable RSV replication (Fig. 13).

In addition, each group of mice was immunized with live RSV, vaccinia virus expressing RSV G (vvG), and rAd/3xG, respectively and was challenged with RSV at three weeks after immunization. The bronchoalveolar lavage cells were collected to count eosinophils. As a result, eosinophilia was markedly enhanced in vvG-immunized groups (Fig. 12, 15 to 25% of total BAL cells), whereas a weak infiltrate of eosinophils, ranging from 1 to 3% of the total BAL cells, was observed in mice previously immunized with rAd/3xG vaccine. This influx was similar in magnitude to that observed in mice previously infected with live RSV virus. Accordingly, it can be seen that the immunization with the adenovirus expression vector according to the present invention hardly increase the risk of development of eosinophil-related lung pathology, unlike the vaccinia virus expression vector. Further, in keeping with potent lung protection, there was no significant weight loss by immunopathology upon RSV challenge in rAd/3xG-immune mice and disease score, when compared to vvG-scarified mice (Fig. 14).

Accordingly, a single immunization with the vaccine according to the present invention can effectively elicit

protective immunity in infants and young children against RSV infection without vaccine-enhanced immunopathology such as eosinophilia.

【Mode for Invention】

Hereinafter, the preferred Examples are provided for better understanding. However, these Examples are for illustrative purposes only, and the invention is not intended to be limited by these Examples.

Example 1: Preparation of RSV stock

A RSV A2 strain was propagated in HEp-2 cells (ATCC, Manassas, VA) in DMEM medium (Dulbecco's Modified Eagle Medium; Life Technologies, Gaithersburg, MD) supplemented with 3% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 20 mM 2-[4-(2-hydroxyethyl)-1- piperazinyl]ethanesulfonic acid (HEPES), and nonessential amino acids. Specifically, HEp-2 cells at about 70% confluency were infected with diluted RSV A2 virus at a multiplicity of infection of MOI=0.01, and the culture medium and cells were recovered after 72~96 hrs, when maximum cytopathic effect by viral replication was observed. The cells were ruptured by several freeze-thaw cycles, and then the supernatant was collected, followed by ultracentrifugation. The virus precipitate was suspended in a suitable amount of DMEM medium to prepare a stock.

Example 2: Construction of replication-defective adenovirus expressing RSV G fragment

A coding sequence of RSV G protein (RSV A2 strain) spanning from amino acid residues 130 to 230 (333 mer) was synthesized in which codon substitutions were made for minimized usage of rare codons (Bioneer Corp., Daejeon, Korea). PCR was performed using this DNA as a template and primers (5′: GCTAGC TACCCCTAC GAC GTG CCC GAC TAC GCC GTG AAG ACC AAA AAC ACC (SEQ ID NO. 2), 3′: GGATCC GCCGCCTCCGCC AGG CTT GGT GGT GGG CAC (SEQ ID NO. 3)), and then another PCR was performed using the synthesized oligonucleotide primer. A six stretch of histidine as a tag and two consecutive stop codons were attached at 3′ terminus. The region of RSV G protein was again amplified with various oligonucleotide primers containing appropriate restriction enzyme sites and juxtaposed three and six times with four glycine residues as a linker between each repeated region (Fig. 1 and Fig. 2). Then, a start codon and the signal sequence of human tissue plasminogen activator (t-PA) (5'-ATGGACGCCATGAAGAGGGGCCTGTGCTGCGTGCTGCTGCTGTGCGGCGCCGTGTT TGTGAGCCCCAGCGCT -3'; SEQ ID NO. 4) were inserted into the N-terminus of RSV G fragments. Finally, the entire open reading frame from initiation codon to stop codon was excised with Kpn I/Xho I digestion and inserted into the same sites of pShuttle-CMV vector. Briefly, the shuttle vector plasmid was electroporated into electrocompetent BJ5183 cells carrying the pAdEasy-1 adenoviral genomic DNA to obtain a recombinant adenoviral DNA.

The recombinant adenoviral DNA was isolated and transfected

into HEK293 cells to generate a replication-defective adenovirus expression vector rAd/3xG and rAd/6xG. Control adenovirus was generated by the same method using the empty pShuttle-CMV vector. The control virus and rAd/3xG virus were amplified in HEK293 cells, purified on $CsCl_2$ gradients, and the virus stock was titrated using HEK293 cells, and determined by $TCID_{50}$ (Tissue Culture Infectious Dose 50%). The correct expression of RSV G protein fragments by replication-defective adenovirus (rAd/3xG and rAd/6xG)-infected HEp-2 cells were verified by Western blotting using G-specific monoclonal antibody (clone No. 131-2G) and HRP (Horseradish peroxidase)-conjugated streptavidin (Zymed Laboratories, San Francisco, CA).

As a result, multiple bands of approximately 20 kDa to 30 kDa were detected in the culture supernatant from HEp-2 cells infected with rAd/3xG, but not either in rAd/control- or rAd/1xG-infected HEp-2 supernatant (Fig. 3). Futher, multiple bands of approximately 20 kDa to 30 kDa and 40 kDa to 50 kDa were detected in the culture supernatant from HEp-2 cells infected with rAd/6xG. These results indicate that rAd/3xG and rAd/6xG can induce the specific and enhanced expression of RSV G antigen.

Example 3: Immunization and RSV infection

For the immunization, 6 to 8-week-old female BALB/c mice (Charles River Laboratories Inc.Yokohama, Japan) were inoculated with varying doses of replication-defective adenovirus via intranasal, intramuscular or oral route to confirm whether the rAd/3xG vaccine induces antigen-specific immune response in vivo.

For intranasal immunizations, mice were lightly anesthetized by ether or chloroform inhalation, and 5×10^7 PFU of rAd/3xG or rAd/6xG vaccine in a volume of 50 μl was applied to the nostril. Intramuscular immunization was performed by injection of 5×10^7 PFU of rAd/3xG vaccine in 100 μl into mouse hind limbs. For oral immunization, mice were deprived of water and food at 2~3 hours before injection, and immunized with 5×10^7 PFU of rAd/3xG vaccine in 200 μl of PBS by proximal esophageal intubation with a mouse feeding needle. Vaccinia virus expressing RSV G (vvG, 5×10^7 PFU) were inoculated at the base of the tail by scarification using a 26-gauge needle. Four to twelve weeks later, the mice were lightly anesthetized by ether or chloroform inhalation, and then were challenged via intranasal route with 1×10^6 PFU of live RSV A2.

Example 4: Measurement of IgG response

From the retro-orbital plexus of mice immunized according to the method of Example 3, blood was obtained by a heparinized capillary tube, centrifuged, and sera were obtained. Anti-RSV IgG titers specific for RSV G protein were measured by ELISA. Briefly, 96-well plates were coated overnight at 4°C with 100 μl/well of 108 μg/ml RSV A2 Ag (US Biological, Swampscott, MA) or 2×10^4 PFU, and then blocked with PBS containing 2% BSA for 2 hrs. Sera or lavage fluids were then added in serial dilutions with 1% BSA PBST (Phosphate Buffered Saline Tween 20) and incubated at room temperature for 2 hrs. The plates were washed five times with 0.05% PBST, and incubated for 1 hr with a varying dilution

of a HRP-conjugated affinity-purified rabbit anti-mouse total IgG, IgG1, IgG2a, or IgA secondary antibody (Zymed Laboratories, San Francisco, CA). The plates were washed five times, developed with 3,3′,5,5′-tetramethylbenzidine, stopped with 1 M H_3PO_4, and analyzed at 450 nm.

As a result, the immunization of rAd/3xG via intranasal and intramuscular routes induced strong serum total IgG responses even after single injection. The immunization of rAd/3xG via oral route induced relatively low serum total IgG responses as compared to the immunization via intranasal and intramuscular routes. However, the immunization of the same dose of rAd/1xG via intranasal route failed to elicit any detectable IgG response above the control (Fig. 5). Notably, the level of serum IgG responses in rAd/3xG-immunized mice (via intranasal route) was comparable with that induced by live RSV inoculation, indicating potent immunogenicity of rAd/3xG vaccine in vivo.

Futher, the immunization of rAd/6xG via intranasal routes induced more stronger serum total IgG responses than the immunization of same doses of rAd/1xG, indicating superior immunogenicity of rAd/6xG vaccine (Fig. 6).

Example 5: Measurement of IgA response

A mucosal secretory IgA is the first line of host defense against aerial pathogens. Thus, an effective RSV vaccine should induce virus-specific secretory IgA preferably in the respiratory mucosal area. To examine whether rAd/3xG vaccination elicits IgA response in the respiratory tract, the experiment was performed

as follows.

At four days postchallenge, a subset of mice from each group was sacrificed under anesthesia, and tracheotomy was performed. The lung airways were washed with 0.8 ml of PBS three times through a tube connected to a 1 ml syringe. The BAL cells were collected by centrifugation, and the supernatant was subjected to ELISA to determine the titer of secretory IgA.

Consequently, the level of mucosal IgA was much higher in intranasally immunized group than the other groups, when BAL fluid was tested at 21 days after immunization, as shown in Fig. 7. Each group of mice immunized intramuscularly or orally with rAd/3xG exhibited basal levels of IgA response. The present inventors also checked the level of anti-RSV IgA in the lungs on day 4 after RSV challenge, and rAd/3xG-immune and live RSV-immune mice showed significantly higher levels of IgA, as compared to control mice ($p<0.01$; Fig. 8). The difference between rAd/3xG-immune and live RSV-immune mice was not statistically significant, although the level was slightly higher in the rAd/3xG group.

Futher, to compare mucosal IgA immunogenicity of rAd/3xG and rAd/6xG, mice of two group were each immunized with rAd/3xG and rAd/6xG and BAL fluid was obtained at 14 days after immunization. A mucosal secretory IgA level was measured by ELISA. As a result, IgA level of rAd/6xG group was more higher than that of rAd/3xG, and the result was consistently maintained within a certain range of dilution.

Example 6: Measurement of IgG and IgA responses in the presence of pre-existing immunity

To examine the effect of pre-existing immunity against adenovirus vector, the mice were preexposed with rAd/control, rested for three weeks, and then immunized with rAd/3xG vaccine. There was no significant difference in the anti-RSV IgG antibody response between the non-preexposed group and preexposed group with 1×10^7 PFU of adenoviral vector (Fig. 10). More interestingly, the levels of secreted anti-RSV IgA in BAL fluid were not significantly different among all groups of mice. All groups of mice showed complete protection, when RSV was challenged at four weeks after immunization. Consequently, the results suggest that a single immunization of rAd/3xG vaccine via intranasal route is sufficient and more efficient than other administration routes in inducing both serum IgG and respiratory IgA even in the presence of pre-existing anti-adenovirus immunity.

Example 7: Examination of eosinophilia level

7-1. G protein-specific T-cell response

It has been well demonstrated that sensitization of mice with vaccinia virus expressing RSV G (vvG) primes G-specific CD4 T-cell responses and results in pulmonary eosinophilia following RSV challenge. Since the G protein fragment expressed by rAd/3xG vaccine contains I-Ed-restricted CD4 T-cell epitope, the present inventors examined whether rAd/3xG immunization primes G-specific CD4 T cells or not. The mice immunized with rAd/3xG or vvG were infected with RSV. Lung mononuclear cells were prepared from the

lungs at four days after challenge, and stimulated with I-Ed-restricted G(183-195) epitope peptide. Then, cells were stained for IFN-γ.

The preparation of lymphocytes was performed as follows. The lungs were perfused with 5 ml of PBS containing 10 U/ml heparin (Sigma-Aldrich, St. Louis, MO) through the right ventricle using a syringe fitted with 25-gauge needle. The lungs were then removed and placed into RPMI Medium supplemented with 10% FBS (HyClone, Logan, UT). The tissue was then processed through a steel screen to obtain single cell suspension, and particulate matter was removed by passing through 70 μm cell strainer (BD Labware, Franklin Lakes, NJ). Then, lung cells were purified by centrifugation, and were stained in a buffer (PBS/3% FBS/0.09% NaN_3) using fluorochrome-conjugated antibodies. The used antibodies were anti-CD3e (clone 145-2C11), anti-CD4 (clone RM4-5), anti-CD44 (clone IM7), anti-CD49d (clone R1-2), and anti-Vβ14 TCR (clone 14-2). All antibodies were purchased from BD PharMingen (San Diego, CA). After staining, cells were fixed in PBS/2% (wt/vol) paraformaldehyde, and events acquired using a FACSCalibur® flow cytometer (BD Biosciences, San Diego, CA).

To analyze IFN-γ-producing cells, intracellular cytokine staining was performed as follows. In brief, 2×10^6 freshly explanted lung lymphocytes were cultured in culture tube. Cells were stimulated with 10 μM G(183-195) peptide (WAICKRIPNKKPG), and incubated for 5 hrs at 37°C in a 5% CO_2 incubator. Brefeldin A (5 μg/ml; Sigma-Aldrich) was added for the duration of the culture period to facilitate intracellular IFN-γ accumulation. Then,

cells were stained for surface markers, washed, fixed and permeabilized with a FACS buffer containing 0.5% saponin (Sigma-Aldrich, Seoul, Korea), and stained with anti-IFN-γ antibodies (clone XMG1.2). Dead cells were excluded on the basis of forward and side light scatter patterns. Data were collected using CELLQuest™ software (BD Biosciences) and analyzed with CELLQuest™ and WinMDI version 2.9 software (The Scripps Research Institute, La Jolla, CA).

Consequently, as shown in Fig. 11, G-specific T-cell response was barely detected in the lungs of rAd/3xG-immunized group (less than about 0.2% of lung CD4 T cells), while strong response (more than 10% of lung CD4 T cells) including Vβ14+IFN + cells was observed in vvG-immunized group by intracellular IFN-γ staining.

It was previously reported that CD4 T-cell response directed to the I-Ed-restricted immunodominant epitope spanning amino acid 183 to 195 of RSV G is sufficient to induce enhanced disease after RSV challenge. The results have indicated that rAd/3xG immunization barely primes G-specific CD4 T cells. Thus, it is quite unlikely that rAd/3xG immunization elicit vaccine-enhanced eosinophilia mediated by G-specific CD4 T-cell response after RSV challenge.

7-2. Count of eosinophil

To determine whether the immunization of rAd/3xG potentates vaccine-enhanced immunopathology, groups of immune mice were challenged with RSV at three weeks after immunization. After five days, mice were sacrificed under anesthesia and tracheotomy was

performed. The lung airways were washed with 0.8 ml of PBS three times through a tube connected to a 1 ml syringe. The bronchoalveolar lavage cells were collected by centrifugation, and then attached to a slide by a cytospin. H&E (hematoxylin-eosin) staining was performed to count eosinophils.

As a result, eosinophilia was markedly enhanced in vvG-immunized groups (Fig. 12, 15 to 25% of total BAL cells). However, a weak infiltrate of eosinophils, ranging from 1 to 3% of the total BAL cells, was observed in mice previously immunized with rAd/3xG vaccine. This influx was similar in magnitude to that observed in mice previously infected with live RSV virus, indicating that rAd/3xG hardly increase the risk of development of eosinophil-related lung pathology.

Example 8: Measurement of RSV titer in lung tissue

Having characterized the immune responses, immune protection was examined from pulmonary RSV challenge conferred by mucosal and parenteral rAd/3xG vaccination. To this end, immune mice were challenged with 5×10^6 PFU of RSV A2 at four weeks after immunization. At four days after RSV challenge, subsets of mice were euthanized and the lungs were removed into Eagle's modified essential medium. The tissues were then processed through a steel screen to obtain single cell suspension and particulate matter was removed by passing through 70 μm cell strainer (BD Labware, Franklin Lakes, NJ). The supernatants were collected and RSV titers in the supernatants were measured by plaque assay.

As a result, while there was active RSV replication in the

lungs of the control mice, intranasal immunization of rAd/3xG completely prevented any detectable RSV replication in the lungs during the course of infection (Fig. 13). A group of mice previously infected with live RSV A2 virus also exhibited protection from the challenge. However, intramuscular immunization of rAd/3xG resulted in partial protection at the peak of viral replication (day 4 after challenge). In keeping with potent lung protection, there was no significant weight loss by immunopathology upon RSV challenge in rAd/3xG-immune mice and disease score, when compared to vvG-scarified mice (Fig. 14). These results suggest that single mucosal rAd/3xG vaccination give rise to protective immunity in the absence of priming of pathologic CD4 T cells and subsequent vaccine-enhanced immunopathology accompanied by eosinophilia.

Example 9: Efficacy of mixed vaccine containing rAd/3xG

To examine whether upon immunization with a lower dose of rAd/3xG, protective immunity is maintained or not, intranasal immunization was performed at a dose of 1×10^7 PFU, and at 4 days after RSV challenge, RSV titers in the lung were measured. As a result, the protective immunity was found to slightly reduce (Fig. 15), as compared to the immunization at a dose of 1×10^8 PFU (Fig. 13).

In addition, the immunization with the mixture (rAd/Mix) of rAd/3xG, rAd/Fco, and rAd/M2 adenoviral vaccines at each dose of 1×10^7 PFU was found to enhance protective immunity, which is similar to the immunization with 1×10^8 PFU of rAd/3xG (Fig. 15). However, a single intranasal immunization with the

recombinant adenoviral vaccine expressing RSV F protein (rAd/Fco) or RSV M2 protein (rAd/M2) at a dose of 1×10^7 PFU was found to induce lower protective immunity at 4 days after RSV challenge (Fig. 15). This result indicates that the immunization with the mixture of rAd/3xG, rAd/Fco, and rAd/M2 adenoviral vaccines can elicit protective immunity, which is the same as or better than the immunization with 1×10^8 PFU of rAd/3xG only.

【Industrial Applicability】

A single immunization with the replication-defective adenoviral vaccine (rAd/3xG and rAd/6xG) according to the present invention can effectively elicit serum IgG and respiratory IgA, and exhibit effective protection against RSV infection without vaccine-enhanced immunopathology.

【CLAIMS】

1. An adenovirus expression vector comprising a base sequence encoding amino acid residues 120 to 230 of RSV G protein from the N-terminus.

2. The adenovirus expression vector according to claim 1, comprising a base sequence encoding amino acid residues 130 to 230 of RSV G protein.

3. The adenovirus expression vector according to claim 1, comprising three copies of the base sequence encoding amino acid residues 120 to 230 of RSV G protein.

4. The adenovirus expression vector according to claim 1, comprising six copies of the base sequence encoding amino acid residues 120 to 230 of RSV G protein.

5. The adenovirus expression vector according to claim 3 or claim 4, comprising a linker sequence between the base sequence encoding amino acid residues 120 to 230 of RSV G protein and the repeated base sequence.

6. The adenovirus expression vector according to claim 5, wherein the linker sequence is glycine.

7. The adenovirus expression vector according to claim 1, further comprising a secretory signal sequence at the N-terminus of RSV G protein.

8. The adenovirus expression vector according to claim 1, further comprising a protein tag and a stop codon at the C-terminus of RSV G protein.

9. The adenovirus expression vector according to claim 1, represented by the diagram in Fig. 1 or Fig. 2.

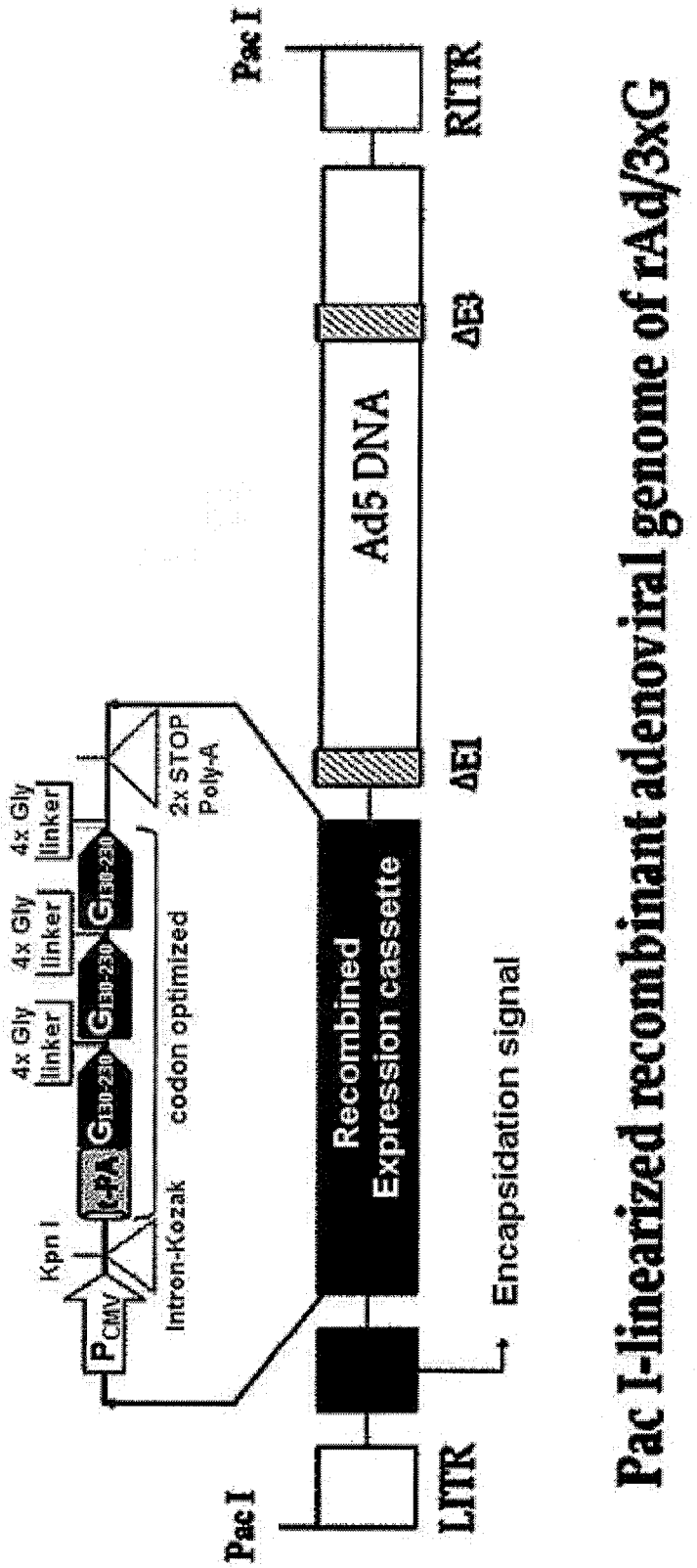
10. A vaccine composition for preventing and treating respiratory tract diseases caused by RSV, comprising the vector of claim 1.

11. The vaccine composition according to claim 10, further comprising one or two selected from an RSV F expressing adenoviral vector and an RSV M2 expressing adenoviral vector.

12. A method for preventing or treating respiratory tract diseases caused by RSV, comprising administering to a subject the vaccine composition of claim 10 or claim 11.

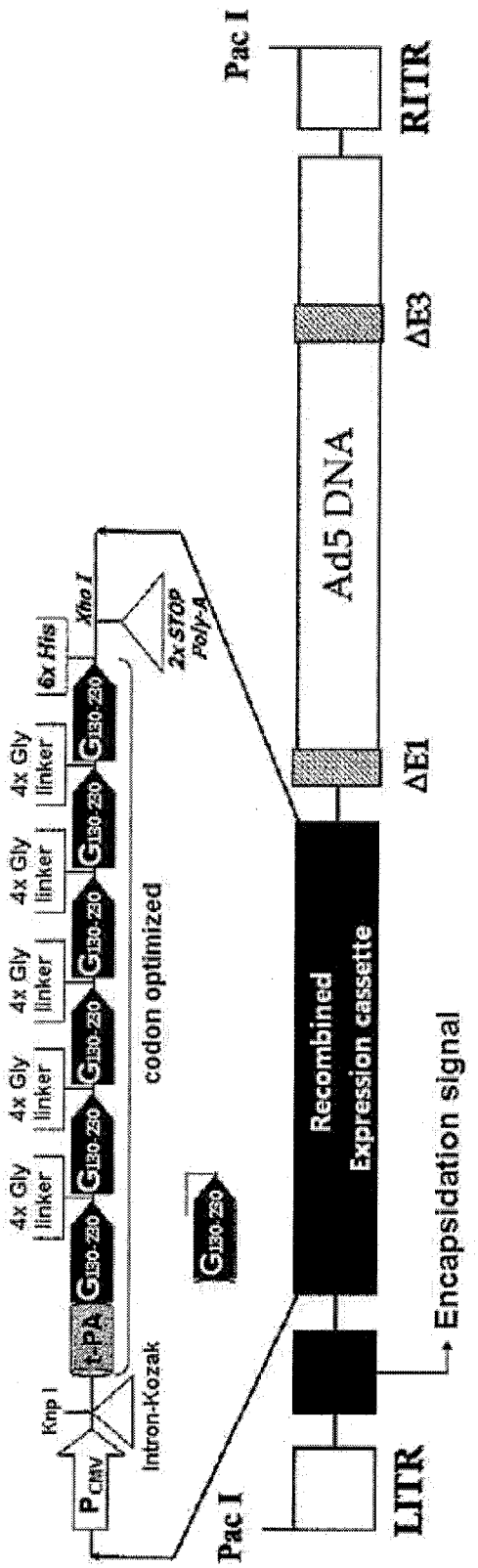
【DRAWINGS】

[Fig. 1]

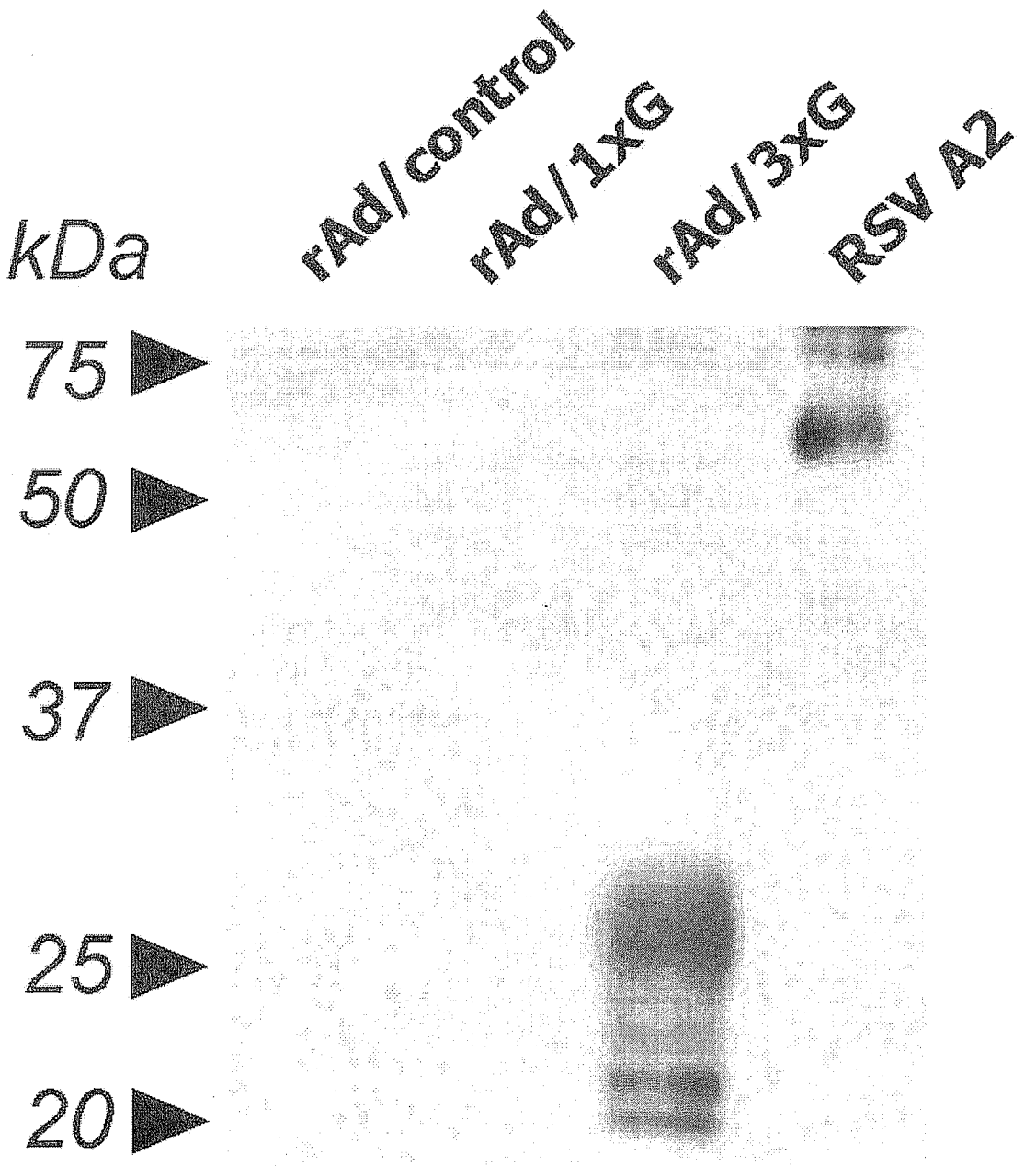


Pac I-linearized recombinant adenoviral genome of rAd/3xG

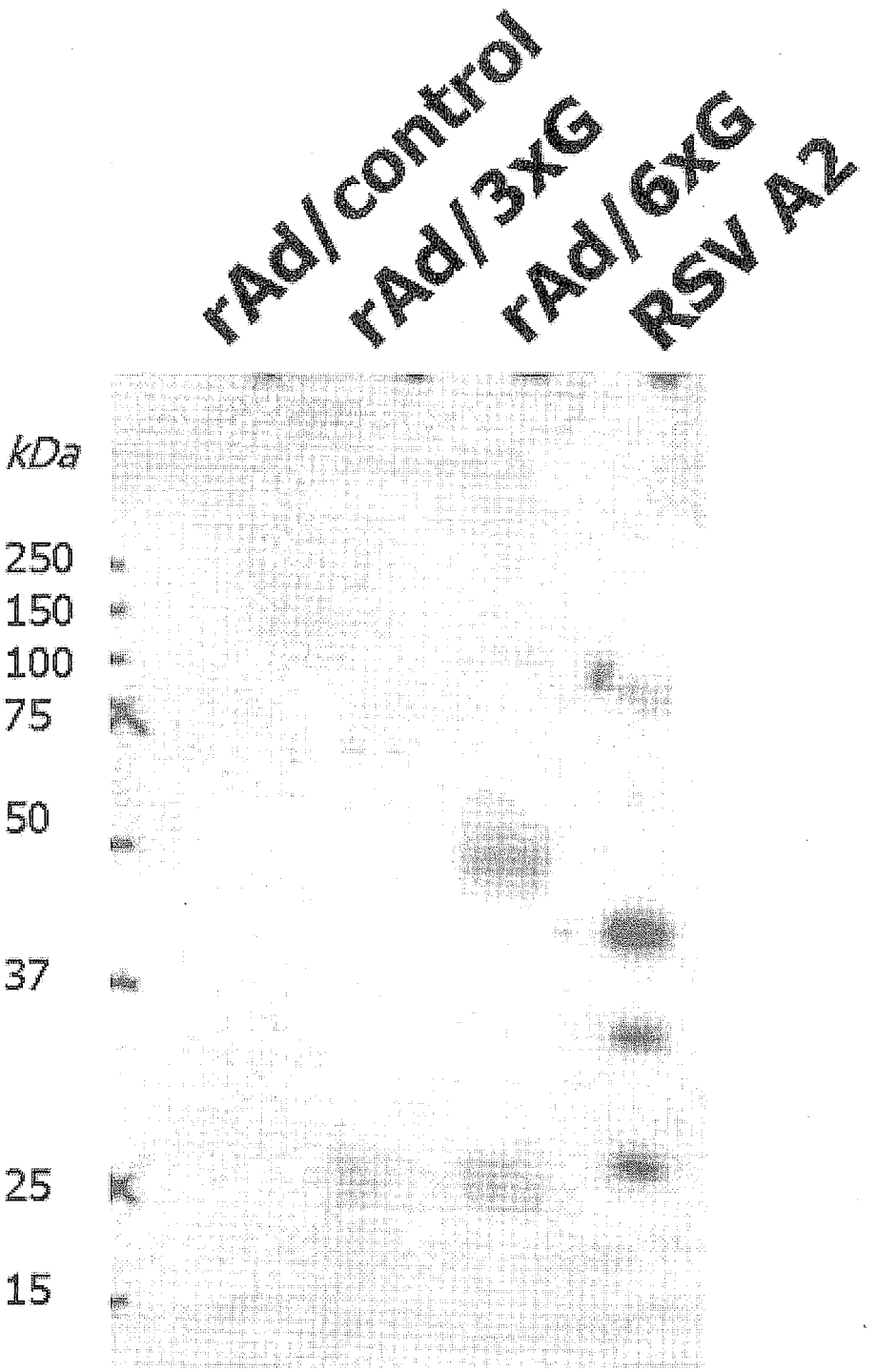
[Fig. 2]


Pac I
RITR
ΔE3
Ad5 DNA
ΔE1
Recombined
Expression cassette
Encapsidation signal
LITR
Pac I
Knp I
Intron-Kozak
t-PA
codon optimized
Xho I
6x His
2x STOP
Poly-A
4x Gly
linker
G130-230

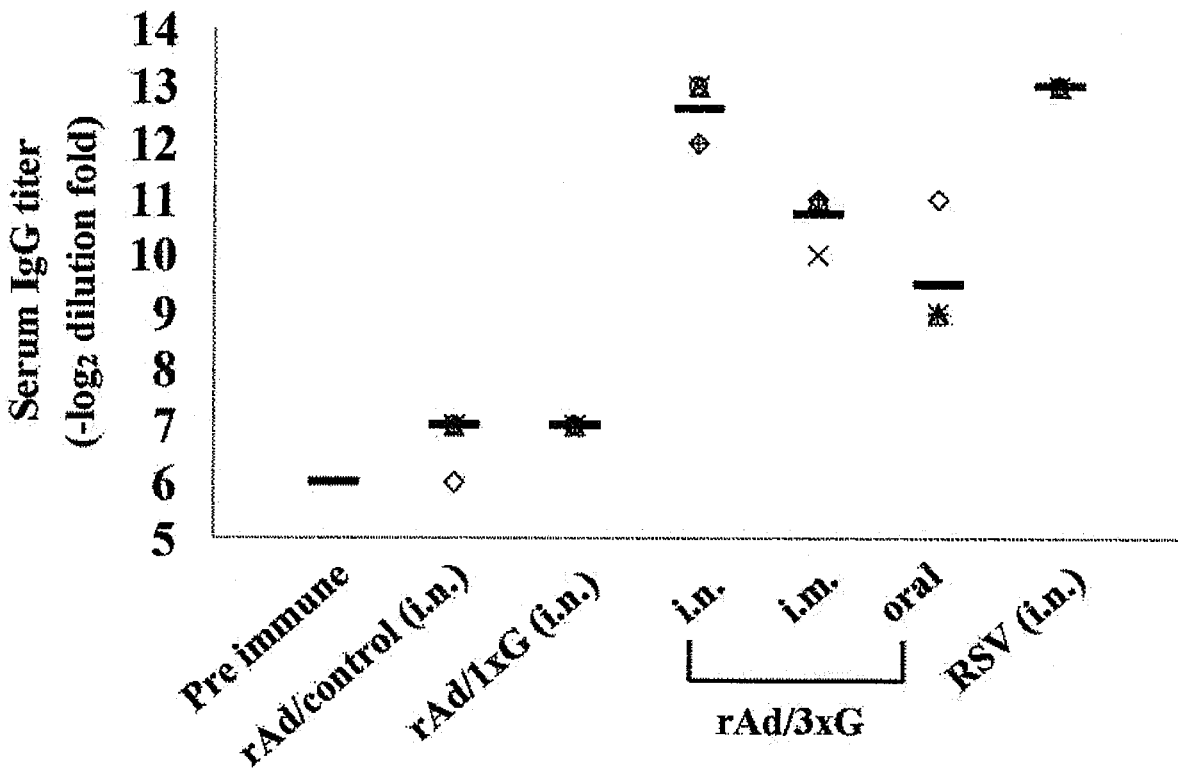
[Fig. 3]



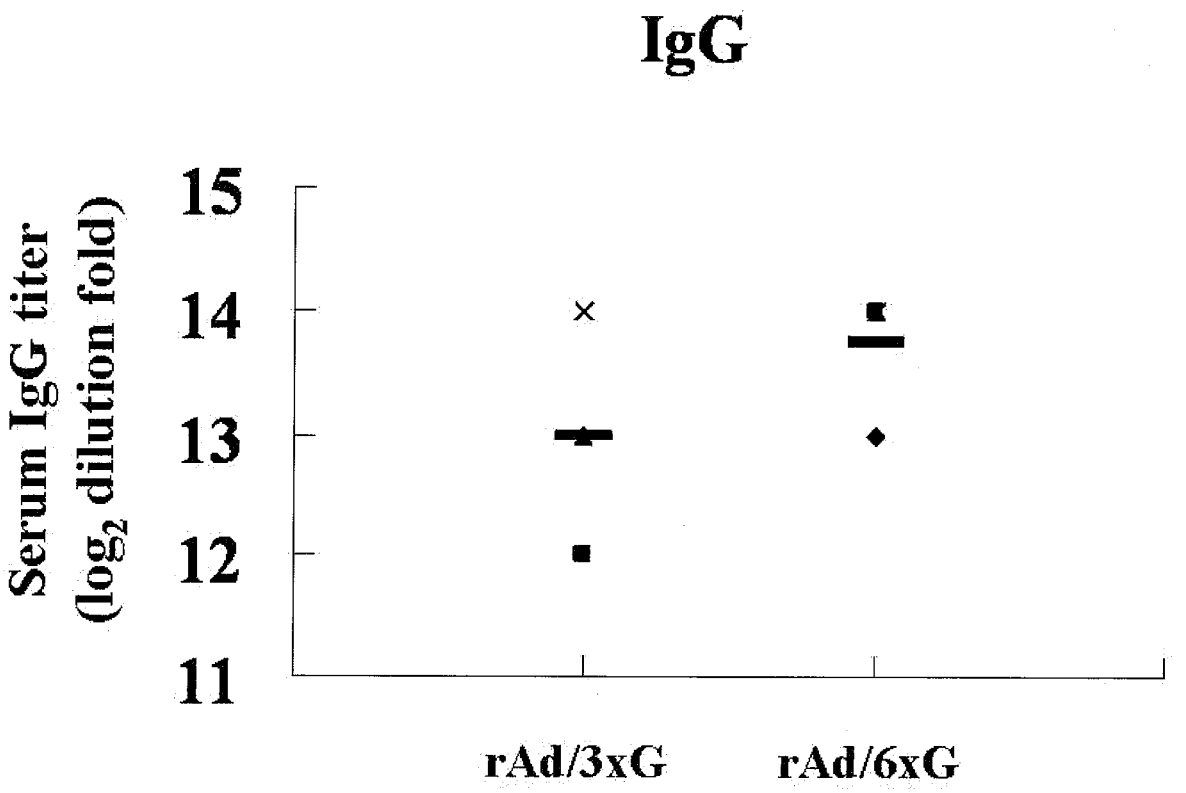
[Fig. 4]



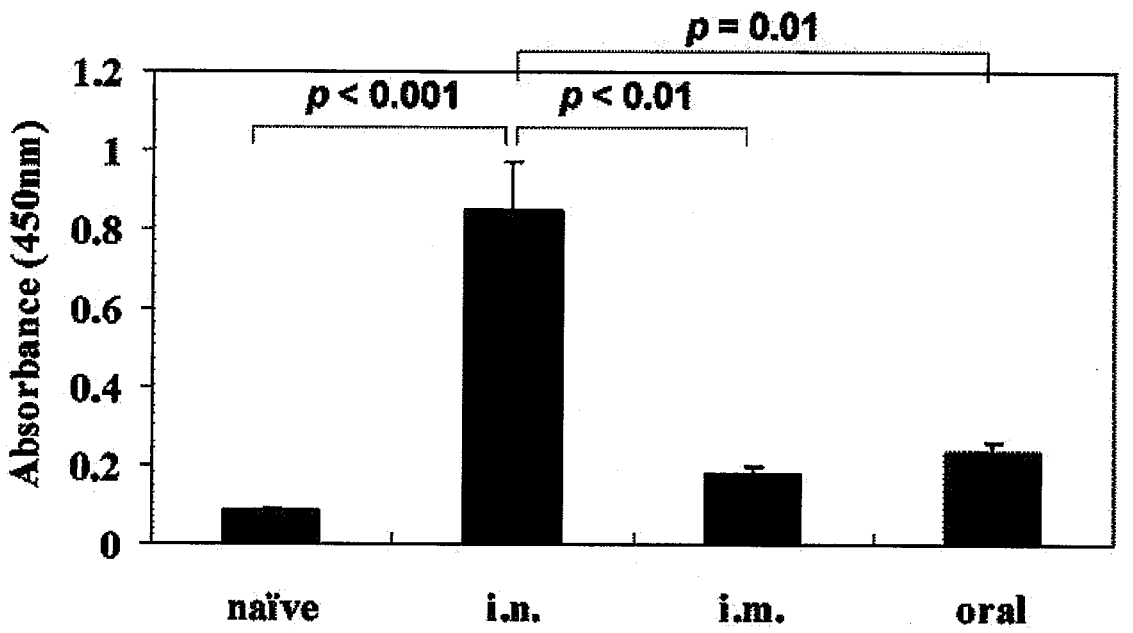
[Fig. 5]


Serum IgG titer
(-log2 dilution fold)
14
13
12
11
10
9
8
7
6
5
Pre immune
rAd/control (i.n.)
rAd/1xG (i.n.)
i.n.
i.m.
oral
rAd/3xG
RSV (i.n.)

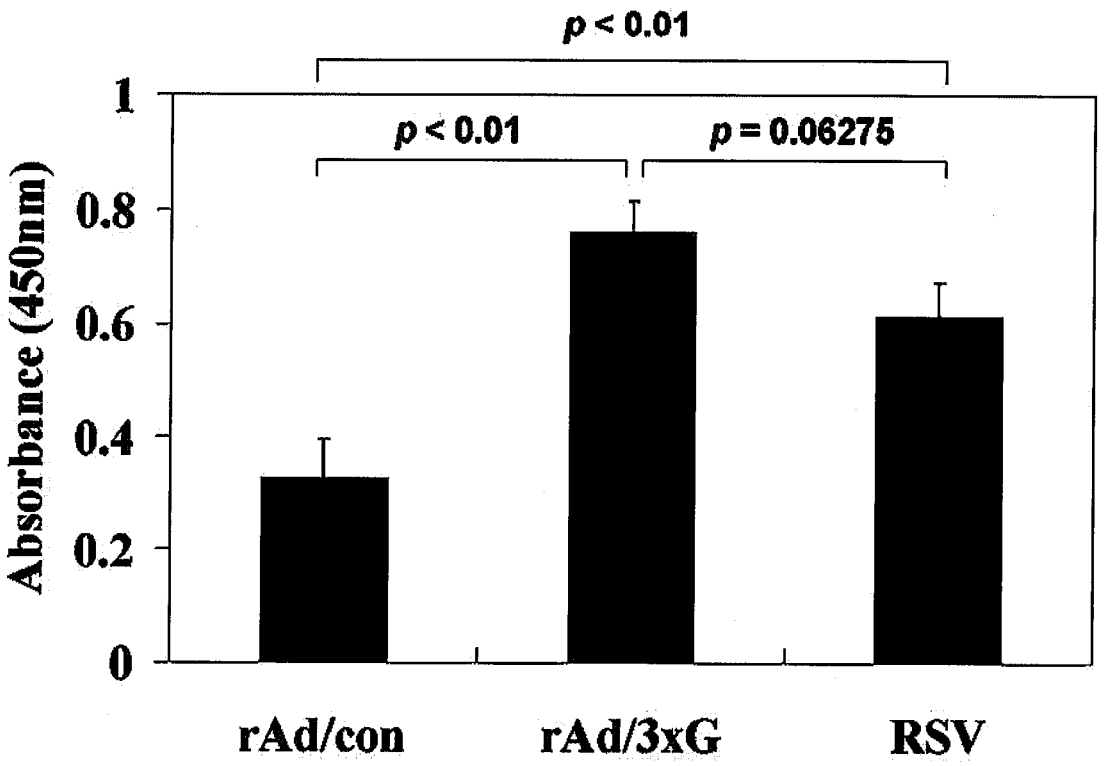
[Fig. 6]


IgG
Serum IgG titer
(log_2 dilution fold)
15
14
13
12
11
rAd/3xG
rAd/6xG

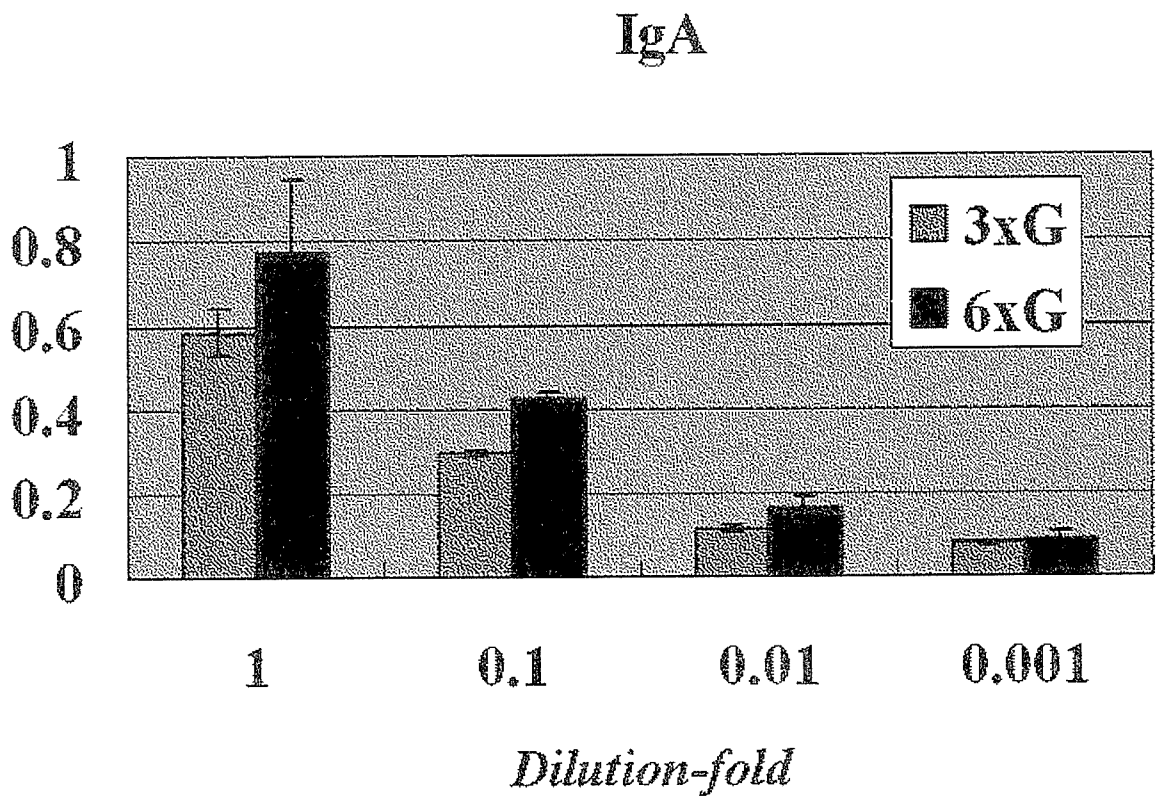
[Fig. 7]


p = 0.01
p < 0.001
p < 0.01
Absorbance (450nm)
1.2
1
0.8
0.6
0.4
0.2
0
naïve
i.n.
i.m.
oral

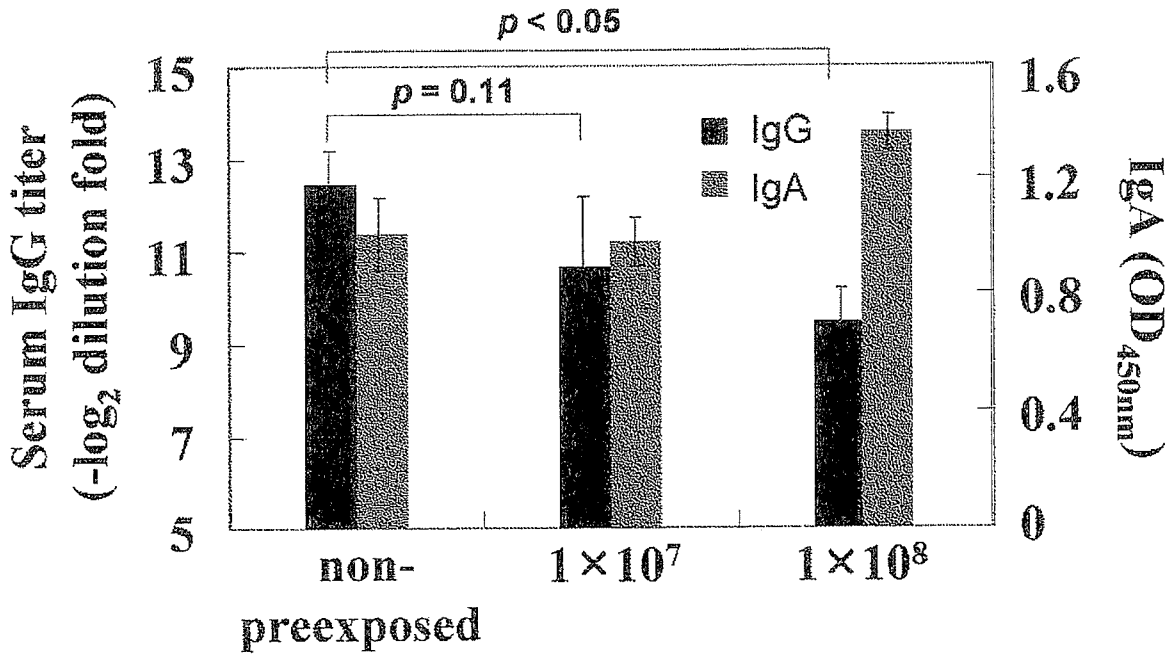
[Fig. 8]


p < 0.01
p < 0.01
p = 0.06275
Absorbance (450nm)
1
0.8
0.6
0.4
0.2
0
rAd/con
rAd/3xG
RSV

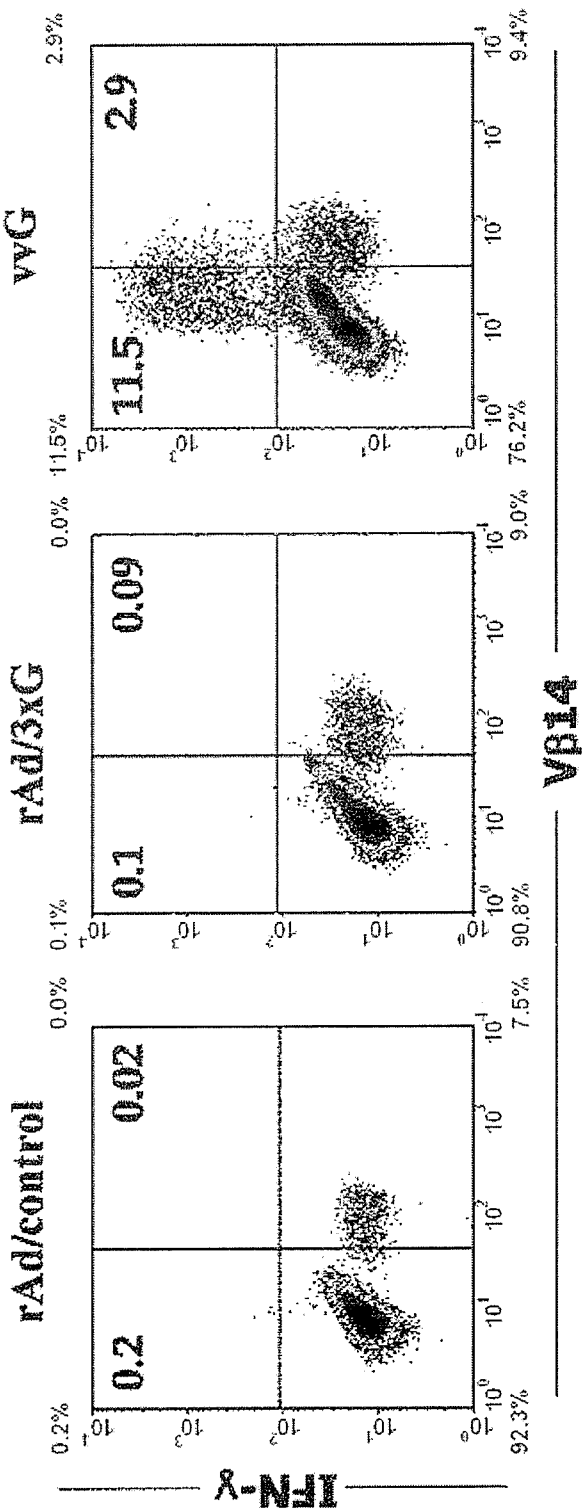
[Fig. 9]



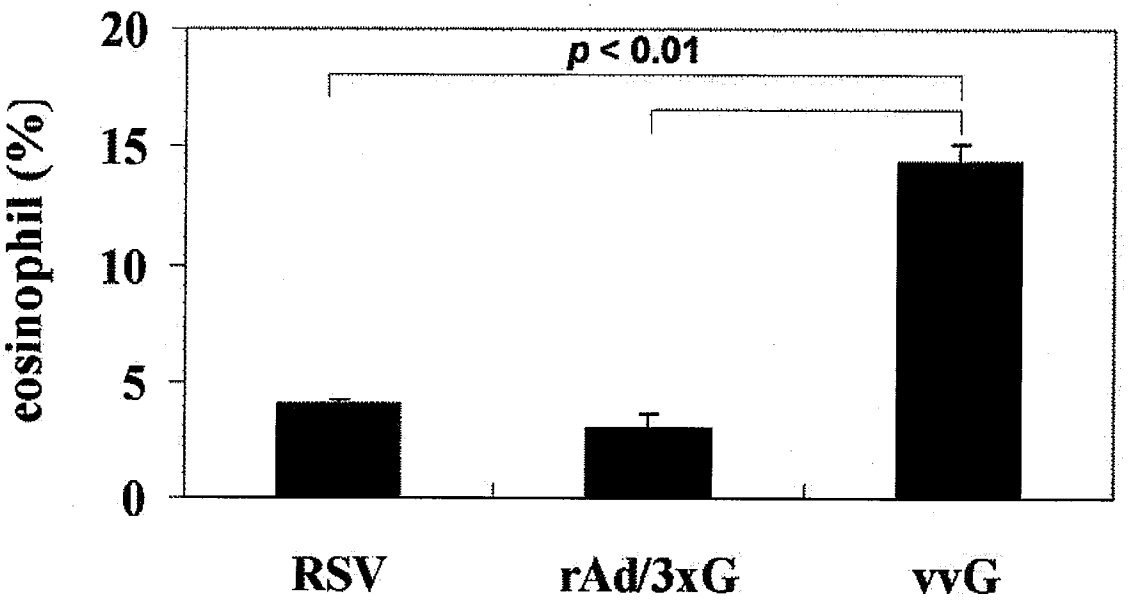
[Fig. 10]


p < 0.05
p = 0.11
IgG
IgA
Serum IgG titer (-log2 dilution fold)
IgA (OD450nm)
15
13
11
9
7
5
1.6
1.2
0.8
0.4
0
non-preexposed
1×10^7
1×10^8

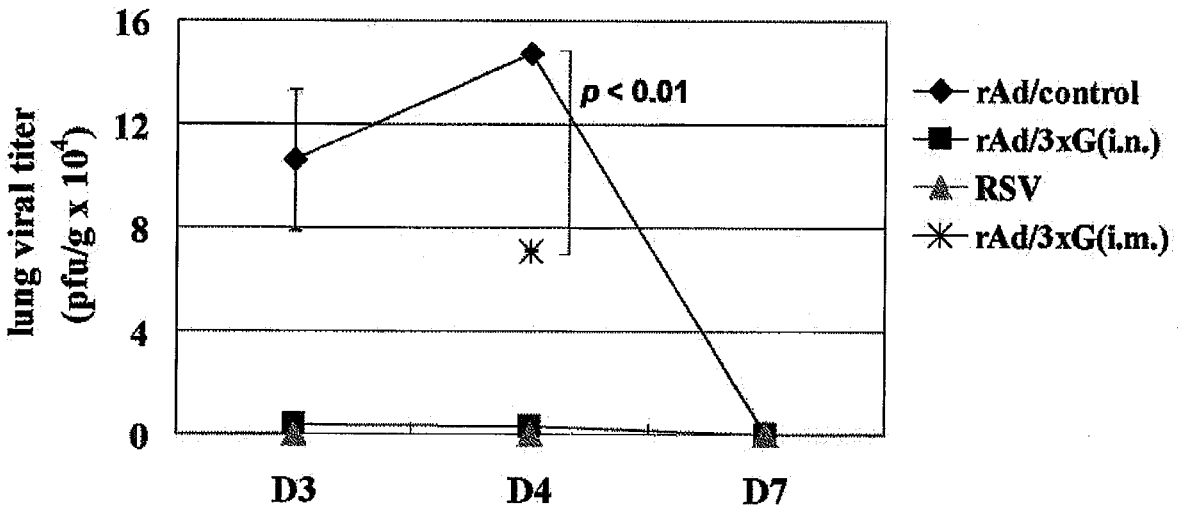
[Fig. 11]


rAd/control
rAd/3xG
VVG
0.2
0.02
0.1
0.09
11.5
2.9
0.2%
0.0%
0.1%
0.0%
11.5%
2.9%
92.3%
7.5%
90.8%
9.0%
76.2%
9.4%
IFN-γ
VR14

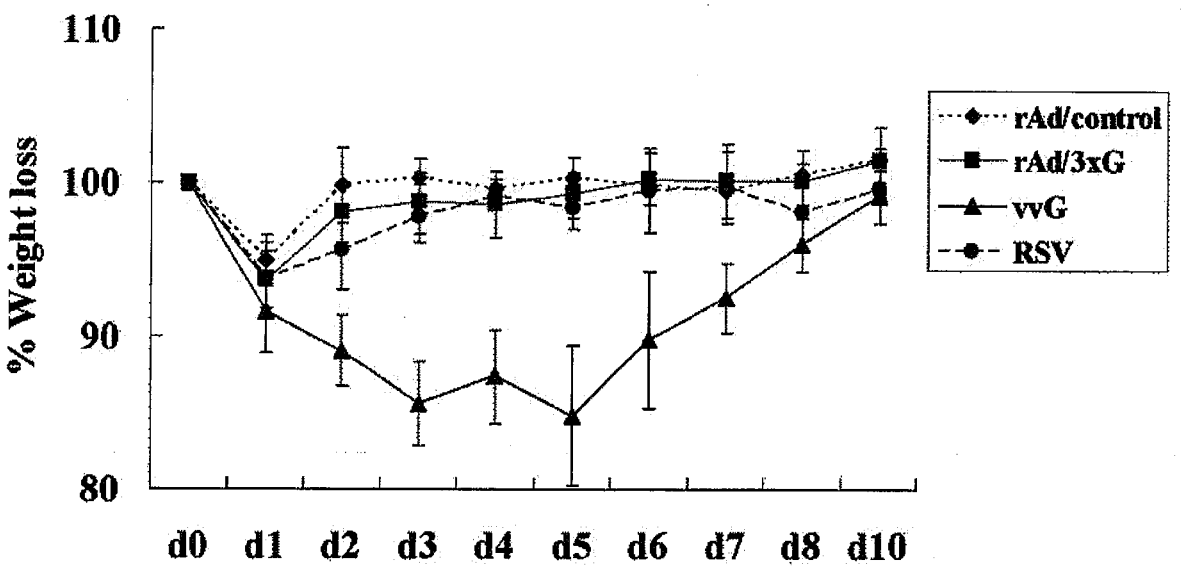
[Fig. 12]



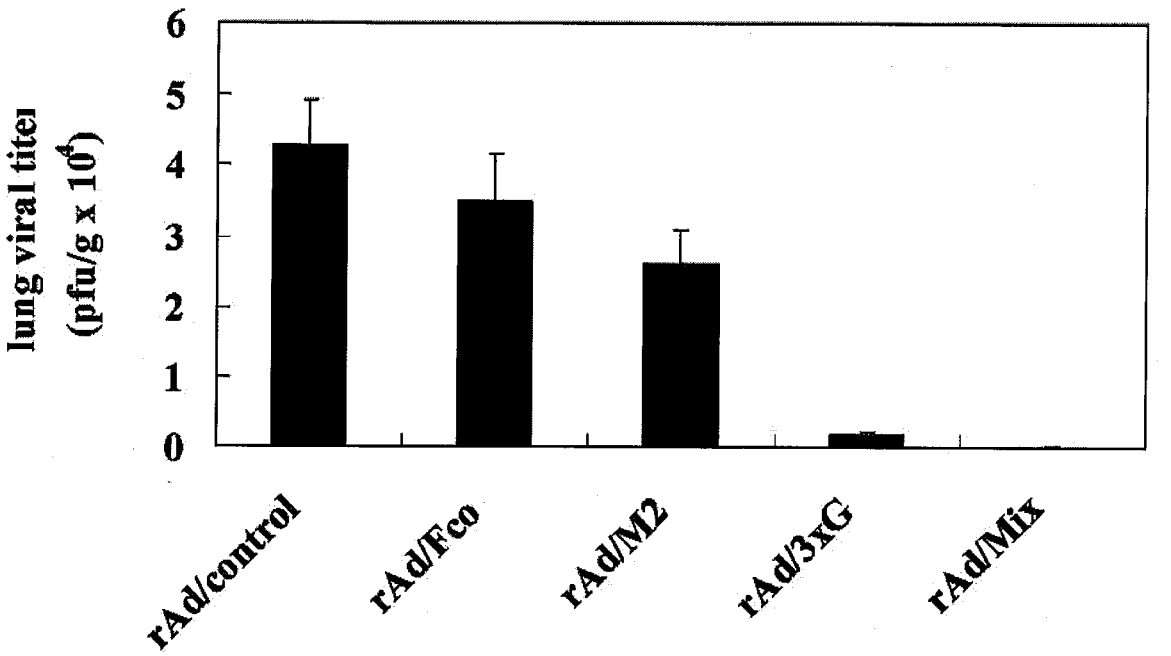
[Fig. 13]


16
12
8
4
0
lung viral titer
(pfu/g x 10^4)
p < 0.01
D3
D4
D7
rAd/control
rAd/3xG(i.n.)
RSV
rAd/3xG(i.m.)

[Fig. 14]


110
100
90
80
% Weight loss
d0
d1
d2
d3
d4
d5
d6
d7
d8
d10
rAd/control
rAd/3xG
vvG
RSV

[Fig. 15]


lung viral titer
(pfu/g x 10⁶)
6
5
4
3
2
1
0
rAd/control
rAd/Fco
rAd/M2
rAd/3xG
rAd/Mix

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/002762

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/861(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 C12N 15/86, C12N 15/80, A61K 39/21

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

N. A.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS, WPI, USPTO, PAJ, NCBI, PubMed : INSPECT "recombinant adenovirus vector, Respiratory Syncytial Virus, RSV, glycoprotein, etc."

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99/04010 A1 (Connaught Lab. Ltd., US) 28 Jan 1999 - see abstract; page 4, line 28 ~ page 7, line 21; page 14, lines 15 ~ 19; and claims	1, 2, 7, 8, 10, 11
Y	US 6964762 B2 (Wang, D., et al., US) 15 Nov 2005 - see abstract; col.3, lines 56 ~ 60; and col.13, lines 30 ~ 35	1, 2, 7, 8, 10, 11
Y	J Infect Dis. Vol.166(4):769-775. (Hsu, K.H., et al., US) Oct 1992 "Immunogenicity of recombinant adenovirus-respiratory syncytial virus vaccines with adenovirus types 4, 5, and 7 vectors in dogs and a chimpanzee." - see the whole document	1, 2, 7, 8, 10, 11
Y	US 6616930 B2 (Binz, H., et al., FR) 9 Sep 2003 - see abstract; col.1, line 62 ~ col.4, line 17; and claims	1, 2, 7, 8, 10, 11
A	US 20030021808 A1 (Tripp, R.A., et al., US) 30 Jan 2003 - see abstract; and claims	1-11

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 OCTOBER 2008 (23.10.2008)	**23 OCTOBER 2008 (23.10.2008)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
SHIN, Weon Hye
Telephone No. 82-42-481-5591

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/002762

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6180398 B1 (Klein, M.L., et al., US) 30 Jan 2001 - see the whole document	1-11
A	FEMS Immunol Med Microbiol. Vol.48(1):116-122 (Falcone, V., et al., DE) Oct 2006 "Systemic and mucosal immunity to respiratory syncytial virus induced by recombinant Streptococcus gordonii surface-displaying a domain of viral glycoprotein G." - see the whole document	1-11
A	US 5149650 A (Wertz, G.W., et al., US) 22 Sep 1992 - see the whole document	1-11
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/002762

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [x] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [x] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/002762

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

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

1. ☒ Claims Nos.: 12
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 12 is directed to a method for preventing or treating a disease, which falls into the category of methods for treatment of the human body or animal body by surgery or therapy as well as diagnostic methods. [Article 17(2)(a)(i), Rule 39.1(iv) PCT]

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

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

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

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

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2008/002762

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