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A Synthetic Peptide Comprising Heterodimeric Epitopes of M Protein Elicits Mice Protective Immunity against H5N1 Influenza A Virus

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Background: The high degree of conservation of the M2 protein of influenza A virus and its cross-reactive immunity that decreased the severity of diseases caused by influenza virus in animal models make it a prime candidate for a universal influenza vaccine. Studies showed that recombinant HA, NA and M2 proteins were poorly immunogenic and required multiple doses or the inclusion of adjuvant for improved immunogenicity and efficacy. **Methods:** A synthetic peptide PEP-M2 comprising heterodimeric epitopes was employed to immunize mice in the presence or absence of a 'K' type CpG ODN adjuvant. **Results:** Mice vaccinated with PEP-M2 peptide elicited strong IgG ELISA antibody titers to this peptide and increased protection (with survival rates raised from 25% to 60%) for mice challenged by lethal influenza A virus (NIBRG-14, 1000$TCID_{50}$). The CpG ODN, used as an adjuvant for PEP-M2, elicited significantly higher antibody titers in mice. Further, PEP-M2 plus CpG ODN promoted in immunized mice higher survival rate (83.3%) and slower weight loss than PEP-M2 alone in lethal challenge assay. **Conclusions:** Results from this study revealed that peptide comprising heterodimeric epitopes of M Protein could elicit in mice protective immunity against influenza A virus in the presence of CpG ODN adjuvant.

Key words: H5N1, influenza, M2, epitope, CpG, adjuvant

INTRODUCTION

Interest in the development of pandemic influenza vaccines intensified with the outbreak of H5N1 influenza virus infection of humans in Hong Kong in 1997 and has increased further as H5N1 viruses have spread in birds and humans since 2003. The ongoing outbreak of HPAI H5N1 viruses in the bird population and the nearly 50% case-fatality rate among people infected with H5N1 viruses underscore the need for control strategies to prevent a potential influenza pandemic. Current influenza virus vaccines aim to induce strong antibody (Ab) responses to the ectodomains of hemagglutinin (HA) and neuraminidase (NA) molecules, since these antibodies (Abs) can provide potent protection against infection and/or disease. The main deficiency of this protection is that it targets highly variable viral determinants. Failure to anticipate the emergence of an epidemic strain with significant antigenic changes compared with the vaccine strain will greatly reduce vaccine-induced protection. M2e-specific immunity has been shown to decrease morbidity and mortality associated with influenza virus infection in several animal models and natural infection and current vaccines do not appear to induce a good M2e-specific antibody (Ab) response. Hence, M2e has been considered a potential vaccine for inducing cross-reactive protection against influenza type A viruses.[1]

M2 is a 97-amino-acid transmembrane protein of influenza type A virus.[2,3] The mature protein forms homotetramers[4,5] that have pH-inducible ion channel activity.[5,6] M2 tetramers are expressed at high density in the plasma membrane of infected cells and are well accessible to M2e-specific antibodies in this location, but only a few copies become incorporated into the envelope of mature infectious virus particles.[7,8] M2 has a small, nonglycosylated ectodomain (M2e) of 23 amino acids (aa), not counting the posttranslationally removed N-terminal Met. This region has shown only limited variation among human influenza A viruses. This remarkable degree of structural conservation of M2e is attributable mainly to its genetic relation with matrix protein 1 (M1),

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the most conserved protein of influenza A viruses with which it shares coding sequences. Thus, aa residues[1-9] of M2e and M1 are encoded by the same nucleotides in the same reading frame and aa[10-23] of M2e and 239-252 of M1 in a different reading frame.

Studies conducted on mice and ferrets have shown that although M2e-specific antibodies did not prevent infection, they restricted subsequent virus replication and reduced illness and proportion of deaths.[7,9-12] This antibody response was only poorly induced by infection, both in mice[12] and humans.[11,13] A possible reason for the poor M2e-specific antibody response is extensive antigenic competition with HA- and NA-specific responses.[14] Although replication of A/PR/8/34(H1N1) (PR8) virus for > 3 weeks in severe combined immunodeficient (SCID) mice that were chronically treated with M2e-specific monoclonal antibodies (MAbs) resulted in the emergence of M2e-escape mutants, only two distinct escape mutants emerged (P10L and P10H).[15] Thus, even though M2e is not totally invariant, it is remarkably stable, even under immune pressure.

Several vaccination strategies have been evaluated in mouse and ferret models, including M2-expressing recombinant viruses, M2 recombinant proteins,[7,9] M2-encoding plasmid DNA[16], and synthetic M2e peptides that were chemically linked to carrier proteins or synthetically linked to defined helper T-cell determinants.[10,12,17] In most studies in which induction of an antibody response was confirmed, M2e-specific immunity reduced illness, but did not entirely prevent it. It has been suggested that M2e could be used as an adjunct to current vaccines and provide a protective safety net in the case of a major antigenic disparity between vaccine and circulating epidemic strains.[15]

Several studies have suggested that proper adjuvants might improve the immunity of influenza vaccine and reduce the dose of vaccine.[18-23] Aluminum hydroxide (alum) is currently the only human vaccine adjuvant approved for use in the United States, and although it is effective in boosting antibody responses, these responses require repeated administrations and tend to generate antiparasitic T helper 2 (T_H2), rather than antiviral and antibacterial T_H1, T cell immunity.[24] As a consequence, there is much effort devoted to developing prospective adjuvants that can establish protective immunity with fewer vaccinations and less injected material, through durable antibody and T_H1-dependent cytotoxic T cell activity. Other potential immune adjuvant might be considered and developed. Among these, CpG ODN is a potential adjuvant candidate. Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs mimic the activity of bacterial DNA motifs[25-27], that induce B cells, natural killer (NK) cells, and plasmacytoid dendritic cells to proliferate, mature, and secrete a variety of cytokines, chemokines, and/or Ig.[40] The ability of CpG-containing immunostimulatory ODNs (CpG ODNs) to induce both innate and adaptive cellular immune responses has made them a prospective prophylactic and therapeutic vaccine adjuvant for diseases requiring cellular immunity. CpG ODNs have been shown to stimulate macrophages and dendritic cells to synthesize several cytokines, including IL-12, IL-18, tumor necrosis factor alpha, alpha interferon (IFN-α), IFN-β, and IFN-γ, to upregulate costimulatory molecules such as CD40 and major histocompatibility complex class II and to enhance the ability of dendritic cells to present soluble protein to class I-restricted T cells.[28,29] Further, TLR9 activated by CpG DNA or synthetic oligodeoxynucleotides (ODNs) induces strong Th1-like immune activation, with the secretion of type-I IFN and activation of natural killer (NK) cells and strong $CD8^+$ T-cell responses.[30] The targeting of TLR9 has emerged as a powerful tool in the generation of Th1 adaptive immunity, and has shown promise for enhancing the efficacy of vaccination. Therefore, the use of CpG ODNs as a vaccine adjuvant in mice enables the antigen doses to be reduced by approximately two orders of magnitude, with antibody responses comparable to those induced by full-dose vaccine without CpG.[31] Furthermore, the addition of a CpG ODNs to a flu vaccine could enable the effective use of the vaccine with lower antigen doses.[32]

CpG ODNs have been suggested as an effective adjuvant that can promote or enhance systemic and mucosal immune responses to protein antigens.[33] In both mice and humans, CpG ODNs have been shown to stimulate multiple types of immune cell, leading to enhanced T_H1 and $CD8^+$ T-cell responses.[25] CpG DNA activates directly monocytes, macrophages, and dendritic cells to secrete Th1-like cytokines and express increased levels of cell surface costimulatory molecules.[34,35] NK cells are activated by CpG DNA to increase lytic activity and to secrete IFN-γ [31]. CpG DNA can also activate B cells and drive them to secrete IL-6, IL-10, and immunoglobulin[36,37] and to proliferate in a polyclonal T-cell independent manner.[38] These results indicate the likelihood of CpG DNA promoting the generation of antigen-specific immune responses. In this study, a synthetic peptide (PEP-M2), which contains heterodimeric epitope with H1N1 238-252aa, M1 $CD4^+$ T cell epitope (DQ249267) and H5N1 membrane ion channel 2 & M genes 10-25 aa (DQ094275), was employed to immunize

mice in the presence or absence of CpG ODNs as an adjuvant.

MATERIALS

Oligodeoxynucleotides

Owing to its high degree of conservation, M2 protein is a prime candidate for a universal influenza vaccine, especially with the nonglycosylated ectodomain (M2e) of 23 amino acids (aa) showing only limited variation among human influenza A viruses. There are aa residues 1-9 of M2e and M1 encoded by the same nucleotides in the same reading frame and aa 10-23 of M2e and 239-252 of M1 in a different reading frame. Therefore, a synthetic peptide (PEP-M2) containing heterodimeric epitopes of M protein was designed according to the alignment of H1N1 M1 protein and H5N1 M2 protein. ODNs used herein were: CG-ODN1, 5'-AGCTTTCGTCGTTTTGTCGTTTTGTCGTTGG-TAC-3'; CG-ODN2, 5'-CAACGAACAAAACGACGA-CAAAACGACGAA; (CpG dinucleotides underlined for phosphorothioate). All ODNs were synthesized with a nuclease-resistant phosphorothioate backbone by MDBio Inc., and the sodium salts of the ODN were ethanol precipitated and then resuspended in 10 mM Tris (pH 7.0) containing 1 mM EDTA for storage at -20°C. These two ODNs were complementary to each other and were annealed to form double-strand DNA by heating at 95°C for 2 min and then stayed at room temperature for 2 h. It was then stored at -20°C before dilution into saline for injection.

Ethics Statement

All animal experiments were reviewed by the Institutional Animal Care and Use Committee and approved by the regulatory authorities of Taiwan. The experiments were conducted in accordance with Taiwan's laws on animal experimentation and guidelines set out by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and the Office of Laboratory Animal Welfare (OLAW). The IACUC Certificate No. of this study was AN-96-01. Animals were housed according to OLAW and AAALAC guidelines in housing facilities accredited by the Center of Disease Center (CDC) of Taiwan.

Immunization of mice with synthetic peptide

Immunization with synthetic peptide was conducted on 6- to 8-wk-old female C3H/HeN mice (National Science Council, Executive Yuan, Taiwan). Each mouse received three doses (at days 0, 30 and 60) of subcutaneous injection of a solution containing 50 μg of PEP-M2 or STA-M2e in a total volume of 100 μl. Control groups (n = 5) received equal volume of phosphate buffer solution (PBS, pH 7.4) plus 50% (v/v) of Freund's adjuvant (GIBCO BRL, Cat. No. 15720-030). Experimental groups (n = 6) received PEP-M2 plus 50% (v/v) of Freund's adjuvant and PEP-M2 plus 50 μg of double-strand CpG ODNs. These experiments were performed with CpG ODNs in which the backbone was nuclease-resistant (phosphorothioate) to improve cell uptake and *in vivo* stability.[39] All component solutions were added at the same time, mixed with a vortex, and left on ice for about 30 min before injection. Sera were always assayed in triplicate. Each plate included an air blank, a negative control (pre-immunized serum in triplicate), as well as a row of different diluted positive controls for establishing a standard curve.

Evaluation of *in vivo* humoral response to synthetic peptide

Sera were recovered from mice at various times (days 0, 28, 56 and 84) after immunization. Abs specific to synthetic peptides (PEP-M2) were detected and quantified by ELISA assay (in triplicate) on samples from individual animals. In brief, the 96-well microtiter plates (Falcon #3912, BECTON DICKINSON) were coated with antigens (50 μl/well, 0.2 μg/ml in 0.05 M carbonate buffer, pH 9.6) at 4°C overnight. The contents of the plates were dumped, and the wells were filled with PBST containing 3% skimmed milk (150 μl/well). Plates were then incubated for 1 h at room temperature for blocking. After dumping the contents, the wells were rinsed once with PBST (PBS+0.05% Tween-20). Sample sera diluted in PBST were then added into wells (50 μl/well), and plates were then incubated at 37°C for 1 h. After dumping the contents, the wells were rinsed five times with PBST. This was followed by incubation with secondary antibodies (goat anti-mouse IgG or goat anti-mouse IgM conjugated with horseradish peroxidase, 1:3000 in PBST containing 3% skimmed milk, 50 μl/well) at room temperature for 1 h. After dumping the contents, the wells were rinsed five times with PBST again. The substrate solution [(TMB (3,3,5,5-tetramethylbenzidine), 50 μl/well with 100 μg/ml in phosphate-citrate buffer, pH 5.0 containing 1/1000 volume of 35% H_2O_2) was added and incubated at room temperature for 10 min. After the reaction was stopped by adding 1 M H_2SO_4 (50 μl/well), the optical densities (ODs) at 450 nm were measured. Sera were always assayed in triplicate. Each plate included an

air blank, a negative control (pre-immunized serum in triplicate), as well as a row of different diluted positive controls for establishing a standard curve.

Preparation of splenocyte cells for cytokines studies

Spleens were obtained from 6- to 8-wk-old female C3H/HeN mice that had been maintained under specific pathogen-free conditions in the Institute of Preventive Medicine Animal Care Facility. Splenocyte suspensions were collected, washed in phosphate buffer solution (PBS, pH 7.4), and suspended in complete medium consisting of RPMI 1640 with FCS (fetal calf serum, 10%), penicillin (100 unit/ml), streptomycin (100 unit/ml), L-glutamine (2 mM), and 2-ME (0.05 mM). Cultures were stimulated with LPS (Sigma, Cat. no. 0111:B4; 10 μg / ml), 5 μg /ml of PEP-M2, 5 μg /ml of PEP-M2 plus CpG ODNs (0.25 μM), or ConA (5 μg /ml). Culture supernatants were harvested after 24 h or 48 h and assayed for cytokine levels. Each sample was assayed in triplicate.

Isotype-specific Ig ELISA assays

Mouse Hybridoma IsoTyping Kit (Biomeda Corp., cat. no. 22-201) was used for the measurement of total IgA, IgM, IgG1, IgG2a, IgG2b, IgG3, κ light chain and λ chain from mice sera. For each ELISA, 50 μl of diluted serum (1/1000 in PBS, PH 7.4) was assayed and quantified according to a standard curve. All experimental procedures followed the instructions of the manual.

Cytokine ELISAs

The levels of IL-4, IL-12, and IFN-γ in culture supernatants were measured by sandwich ELISAs with paired cytokine-specific monoclonal antibodies according to the manufacturer's instructions (Quantikine M Mouse IL-12, IL-4, and IFN-γ Immunoassay ; R & D SYSTEM).

Cells and virus

MDCK (Madin-Darby canine kidney) cells were obtained from the American Type Culture Collection (ATCC), and maintained in Dulbecco's minimum essential medium (DMEM; GIBCO, Invitrogen, USA) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum (GIBCO, Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. Influenza A virus NIBRG-14 (NIBSC, VN/04 reverse genetic reassortant H5N1 virus; virus was obtained from the CDC of Taiwan) was amplificated in 10-day-old embryonic eggs at 35°C for 40 h. Virus was harvested from allantoic fluid. To determine the LD50 of each batch of virus, female BALB/c mice (6-7 weeks) (n = 10/group) were anesthetized subcutaneously with Zoletil 50 (Virbac Laboratories, France) (0.375 mg/mice) and inoculated intranasally with serial dilutions of the virus. LD_{50} was the dilution of the virus that produced lethality in 50% of the mice and LD_{50} titers were calculated using the method of Reed and Muench (Reed and Muench, 1938). LD_{50} equals 20 $TCID_{50}$.

Peptides, peptide conjugates and antibodies.

Synthetic peptides (PEP-M2) were commercially synthesized at MDBio, Inc. (Taipei, Taiwan). PEP-M2: H-MQAYQKRMGVQMQRFKSQPPTRNEWECRC-SDSSDP-MAP (Fig. 1A). For immunization, mice were injected subcutaneously with PBS or 50μg peptide per mouse in complete Freund's adjuvant (1:1 ratio) or in CpG ODN solution at a final volume of 200 μl. Boosters were given in incomplete Freund's adjuvant or CpG ODN solution on days 14 and 35. Sera were separated on days -1, 14, 28, 49 and 77. All separated sera were mixed with 50% glycerol and stored at -70°C.

Plaque-Assay

To measure virus titer, MDCK cells (5×10^6 /well) were inoculated into 6-well microplates and were incubated at 37°C in a humidified atmosphere with 5% CO_2 for overnight. On the second day, a serial of 10-fold dilutions of virus were prepared in PBS; MDCK cells were washed two times with PBS and 100 μl of viral dilutions were inoculated into 6-well microplates for adsorption. After 1 h of adsorption, virus suspensions were removed and cells were washed two times with PBS; then 1% Oxoid agars in DMEM/BSA medium were inoculated into microwells, and microplates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for three days. MDCK cells were fixed and stained with crystal violet solution for 1-2 hour, then agars were removed, and stained cells were washed with tap water. Microplates were air dried at room temperature for several hours, and plaques were calculated for virus concentration. LD_{50} titers of virus were calculated using the method of Reed and Muench.[40]

$TCID_{50}$

To calculate virus titer, MDCK cells ($1x10^4$ /well) in DMEM medium with 10%FBS were inoculated into 96-well microplates and were incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. On the second

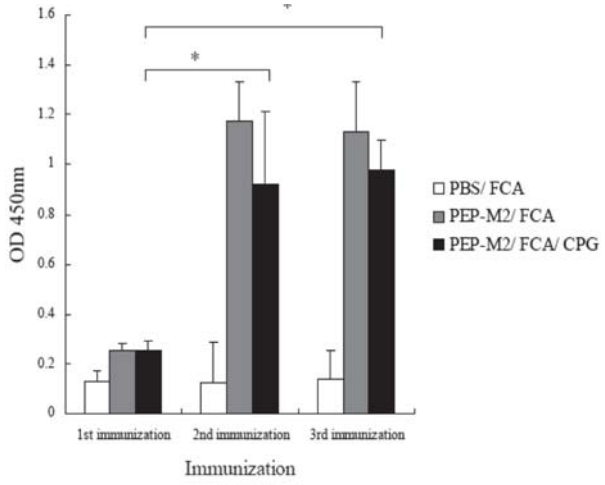

Fig. 1 Evaluation of mice specific humoral response to synthetic peptide PEP-M2 by ELISA. Mice (n = 4-8 per group) were immunized with PEP-M2 and bled retroorbitally. The titers of serum-specific antibodies were evaluated by ELISA as described in Materials and Methods. Sera from individual mice were used at 1:400 dilutions. Data are representative of two separate experiments. Kinetics of the anti-PEP-M2 humoral responses in mice immunized with PBS, 50 μg of PEP-M2 plus Freund's adjuvant, and 50 μg of PEP-M2 plus Freund's adjuvant and 50 μg of CpG ODNs. FCA denotes Freund's complete adjuvant. The data represent the mean titers±SD (error bars) of antibodies in each group of mice. Error bars indicate standard deviations. "*" denotes significant difference between two test groups.

day, a serial of 10-fold dilutions of virus were prepared in PBS; MDCK cells were washed two times with PBS and 100 μl of viral dilutions were inoculated into 96-well microplates in pent-plicate for adsorption. After 1 hr of adsorption, virus suspensions were removed and cells were washed two times with PBS; then DMEM/BSA medium were inoculated into microwells and microplates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for another five days. $TCID_{50}$ titers of virus were calculated using the method of Reed and Muench.[40]

Intranasal influenza challenge

One to two weeks after the final boost, immunized mice were lightly anaesthetized and challenged intranasally with 50 LD_{50} (1000 $TCID_{50}$) or 150 LD_{50} (3000 $TCID_{50}$) of influenza virus NIBRG14. Over the following 14-17 days, weights and survival rates of each group of mice were monitored daily.

Statistical analysis of the data

In all figures, vertical error bars denote the standard deviation (SD). Significances of differences in antibody responses and cellular responses were evaluated by one-way analysis of variance (ANOVA). T-test was used for the comparison of two specific groups in one-way ANOVA. To test the significance of difference in survival rates between each group of immunized mice, a Z' (alternative critical ratio) test was used.[41,42] A P value of < 0.05 was considered significant.

REESULTS

PEP-M2 elicited strong immune responses of mice after three doses of vaccination.

To evaluate the immune responses of synthetic peptide PEP-M2, mice (n = 4-8 per group) were immunized with PBS, 50 μg of PEP-M2 plus Freund's adjuvant, 50 μg of PEP-M2 plus Freund's adjuvant and 50 μg of CpG ODNs. After three doses of immunization, the specific IgG antibody titers of sera were evaluated by ELISA, as described in Materials and Methods. As shown in Fig. 1, Peptide PEP-M2 elicited strong IgG immune response in the presence of FCA or CpG ODNs as the adjuvant. Peptide PEP-M2 elicited immune response after second immunization both in the presence of adjuvant FCA or FCA plus CpG ODNs.

Synthetic peptide PEP-M2 induced protective immunity of mice against influenza A H5N1 virus.

To evaluate the immunization efficacy of synthetic peptide PEP-M2 against H5N1 influenza A virus. Four groups (n = 4-8) of mice were immunized with PEP-M2, followed by lethal challenge with 50 LD_{50} (1000 $TCID_{50}$) influenza A virus (NIBRG-14) intranasally, as described in Materials and Methods. After 17 days of observation, survival rates and weight changes in each group are compared. As shown in Fig. 2A, mice immunized with PEP-M2 had good survival rates; mice immunized with PEP-M2 plus FCA had 60% survival rate, while PEP-M2 plus CpG ODNs had 83.33% survival rate under the challenge of influenza A virus. Meanwhile, the loss of body weight was less significant for mice immunized with PEP-M2 (about 20%) than for mice immunized with PBS alone. Moreover, the recovery of mice body weight was sooner under PEP-M2 immunization, as compared with that under PBS immunization (about 30%) 8-9 days post virus challenge (Fig. 2B). Results indicated PEP-M2 could induce mice to produce protective immunity against H5N1 influenza A virus; while CpG ODNs strengthened

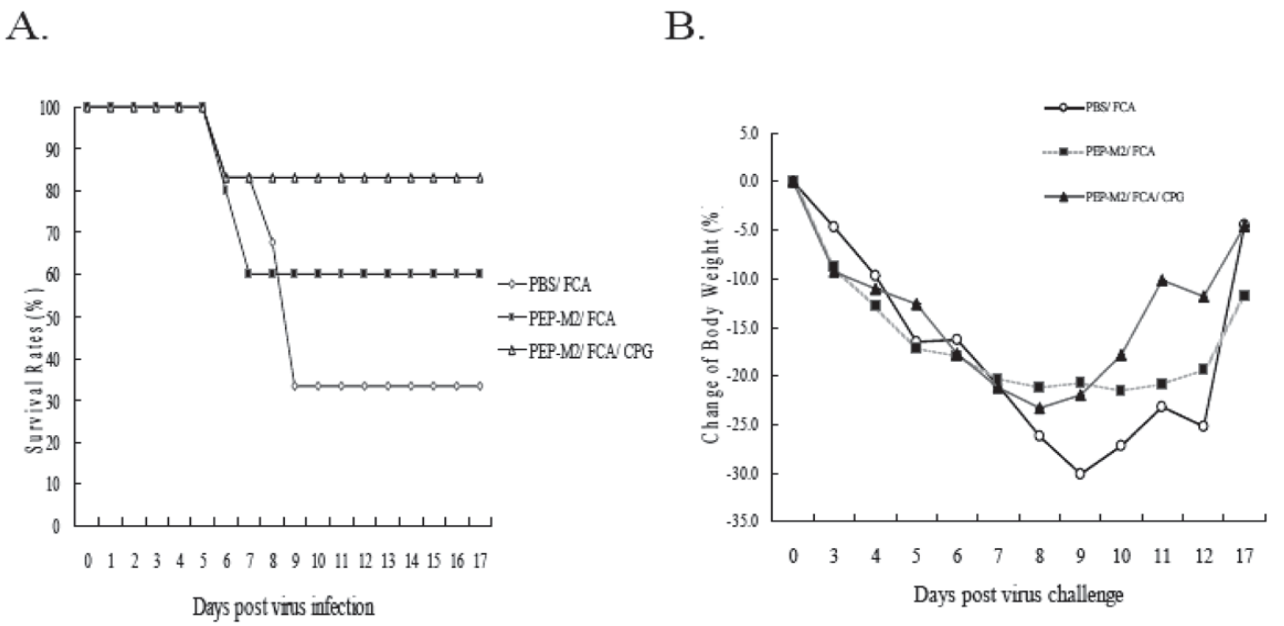

Fig. 2 Lethal challenge assays of mice post immunization with synthetic peptide PEP-M2. Four groups (n = 4-8) of mice were immunized with peptide PEP-M2, followed by lethal challenge with 50 LD_{50} (1000 $TCID_{50}$) influenza A virus (NIBRG-14) intranasally as described in Materials and Methods. After 17 days of observation, survival rates (A) and weight change (B) in each group are shown. As seen in (A), the survival rate of mice immunized with PEP-M2 plus FCA plus CpG ODN was significantly higher than that of mice immunized with PBS plus FCA. Meanwhile, the body weights of mice immunized with PEP-M2 plus FCA and PEP-M2 plus FCA plus CpG ODN recovered more quickly 7-8 days post challenge, as compared with those of mice immunized with PBS plus FCA (B).

this protective immunity, thus increasing the survival rate from 60% (for PEP-M2 plus FCA group) to 83.33% (for PEP-M2 plus FCA plus CpG group). Results from this study indicate that synthetic peptide PEP-M2 could induce protective immunity against lethal influenza infection, especially in the presence of CpG ODNs as adjuvant.

PEP-M2 elicited Th1-type immune responses of mice with significant IgG2a, IgG2b isotype antibodies, and IFN-γ in the presence of CpG ODNs.

To analyze IgG isotype of mouse antibodies specific to PEP-M2, antibody isotype profiles of mice immunized with PBS plus Freund's adjuvant, PEP-M2 plus Freund's adjuvant, PEP-M2 plus Freund's adjuvant and CpG ODNs; were assayed by Mouse Hybridoma IsoTyping Kit (Biomeda Corp., cat. no. 22-201) for the measurement of total IgG1, IgG2a, and IgG2b. As shown in Fig. 3A, PEP-M2 induced strong IgG1 response, and also significant IgG2a and IgG2b antibody responses. Further, both IgG2a and IgG2b immune responses were even stronger using CpG ODNs as adjuvant.

To evaluate cellular immune responses of mice immunized with synthetic peptides mixed with different adjuvants, spleen cells isolated from some immunized mice challenged by 1000 $TCID_{50}$ NIBRG-14 influenza virus and survived were cultured in RPMI medium and then stimulated with LPS, CpG, PEP-M2, and PEP-M2 plus CpG. After 24 h, IFN-γ in culture supernatants were measured by sandwich ELISAs using paired cytokine-specific monoclonal antibodies according to the manufacturer's instructions. As shown in Fig. 3B, significant IFN-γ secretion of spleen cells was shown for mice immunized either with PEP-M2 or PEP-M2 plus CpG than with control (PBS) after stimulation with LPS, PEP-M2, or PEP-M2 plus CpG. Further, mice immunized with PEP-M2 plus CpG had higher IFN-γ response to the stimulation of PEP-M2 plus CpG than those immunized with PEP-M2 alone. Results implicated that synthetic peptide PEP-M2 could elicit both humoral and cellular immune responses. Further, CpG ODNs as the adjuvant could induce Th1-like immunity activation, which has previously shown greater efficiency in clearing influenza virus.

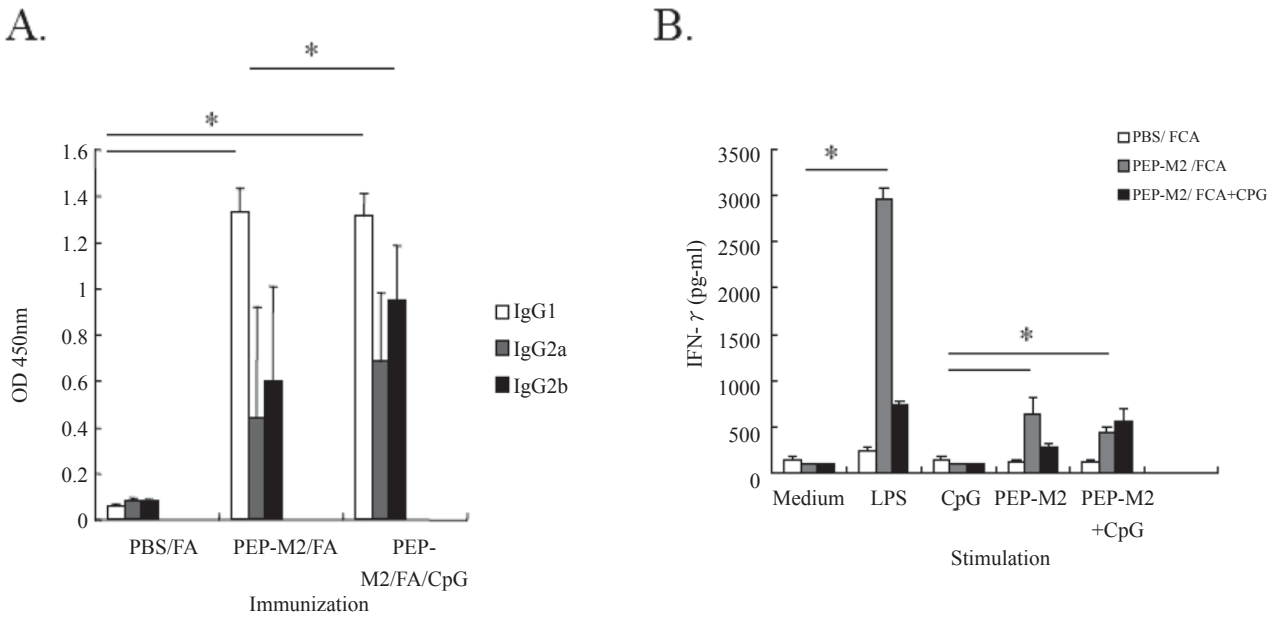

Fig. 3 Evaluation of immune responses for mice immunized with PEP-M2. (A) IgG isotype analysis of mice antibodies specific to PEP-M2. Antibody isotype profiles of mice immunized with PBS plus Freund's adjuvant, and PEP-M2 plus Freund's adjuvant, PEP-M2 plus Freund's adjuvant and CpG ODNs were assayed by Mouse Hybridoma IsoTyping Kit (Biomeda Corp., cat. no. 22-201) for the measurement of total IgG1, IgG2a, and IgG2b. For each ELISA, 50 μl of diluted serum was assayed and quantified according to a standard curve as described in Materials and Methods. Sera from individual mice were used at 1:400 dilutions. (B) IFN-γ responses of immunized mice spleen cells stimulated with antigens. To evaluate cellular immune responses of mice immunized with PEP-M2, and PEP-M2 plus CpG; spleen cells from immunized mice were cultured in RPMI medium and then stimulated with LPS, CpG, PEP-M2, and PEP-M2 plus CpG. After 24 h, IFN-γ in culture supernatants were measured by sandwich ELISAs with paired cytokine-specific monoclonal antibodies according to the manufacturer's instructions (Quantikine M Mouse IFN-γ Immunoassay; R & D SYSTEM). Error bars indicate standard deviations of triplicate tests.

DISCUSSION

An ideal influenza vaccine would be effective against a range of virus subtypes and could be useful during pandemic and inter-pandemic periods. That is to say, a universal influenza virus vaccine should be the ideal vaccine. The high degree of conservation of the M2 protein makes it a prime candidate for a universal influenza vaccine. In this study, a synthetic peptide (PEP-M2) comprising heterodimeric epitopes of M protein was designed according to the alignment of H1N1 M1 protein and H5N1 M2 protein. Previous research indicated that high density of M2e epitopes could efficiently induce protective immunity for mice against the challenge of influenza virus.[17,43] In this study, all mice produced high immune responses (IgG) to H5N1 influenza A virus after immunization with 2-3 doses of synthetic peptide PEP-M2. PEP-M2 induced both humoral (IgG1) and cellular (IgG2a, IgG2b, IFN-γ) immune responses. To evaluate the cellular immune responses of PEP-M2, a quantitative RT-PCR assay was employed to quantify expressions of different cytokines. In this experiment, splenocytes from PEP-M2-immunized mice were stimulated with different reagents (including PBS, ConA, CpG, PEP-M2, PEP-M2 plus CpG, and LPS); as compared with those from the control (PBS). Both humoral and cellular cytokines (including IL-2, IL-4, IL-10, and IFN-γ; as compared with the internal control β-actin) were significantly increased under the stimulation of PEP-M2 (an average increase of 3.83 folds for IL-2, 20.71 folds for IL-4, 13.83 folds for IL-10, and 8.06 folds for IFN-γ relative to PBS) (data not shown). Further, after survival rate evaluation in mice, peptide PEP-M2 could induce protective immunity against lethal influenza infection, especially in the presence of CpG as adjuvant.

In most studies in which induction of an antibody response was confirmed, M2e-specific immunity reduced illness, but did not entirely prevent it. Thus, M2e will not be a substitute for the currently licensed vaccines that can induce much stronger protection if a reasonably good match exists between the vaccine and epidemic strain. Rather, M2e could be used as an adjuvant to current vaccines and provide a protective safety net in the case of a major antigenic disparity between vaccine and circulating epidemic strains. Results from this study implicated that a synthetic peptide comprising heterodimeric epitopes (PEP-M2) could elicit sufficient, although not total, protective immunity of mice (with 83.3% survival rate) against the H5N1 influenza virus in the presence of a proper adjuvant.

According to previous studies, CpG ODNs have been recognized as a powerful vaccine adjuvant, inducing faster and stronger humoral and cellular immune responses. To date, the safety of TLR9 activation with CpG ODNs appears good[44], the usage and its effect of CpG ODNs as an adjuvant for influenza virus might be considered after larger clinical trials and a longer duration of follow-up. In this study, CpG ODNs were evaluated for their immunostimulatory activities in mice vaccinated with a synthetic peptide comprising heterodimeric epitopes of M protein of influenza virus. Further, mixture of Freund's adjuvant and CpG ODNs promoted stronger cellular immune responses (both IgG2a and IgG2b) to synthetic peptide PEP-M2 in mice than Freund's adjuvant; it also induced stronger immune responses than did aluminum hydroxide and CpG ODNs alone (data not shown). Hence, CpG ODNs might be used with other adjuvants to enhance the immune responses of mice to influenza virus.

In short, results from this study revealed that synthetic peptide PEP-M2, comprising heterodimeric epitopes of M protein, could elicit sufficient protective immunity of mice against influenza A virus in the presence of CpG ODNs as adjuvant. Further, with immunizations of synthetic peptide PEP-M2, CpG ODNs could promote immune responses and increase survival rates of mice under lethal challenge of H5N1 influenza A virus. The synergistic effects of CpG ODNs and alum adjuvants for the promotion of protective immunity against H5N1 influenza A virus merit further investigation.

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DISCLOSURE

All authors declare that this study has no conflict of interest.

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