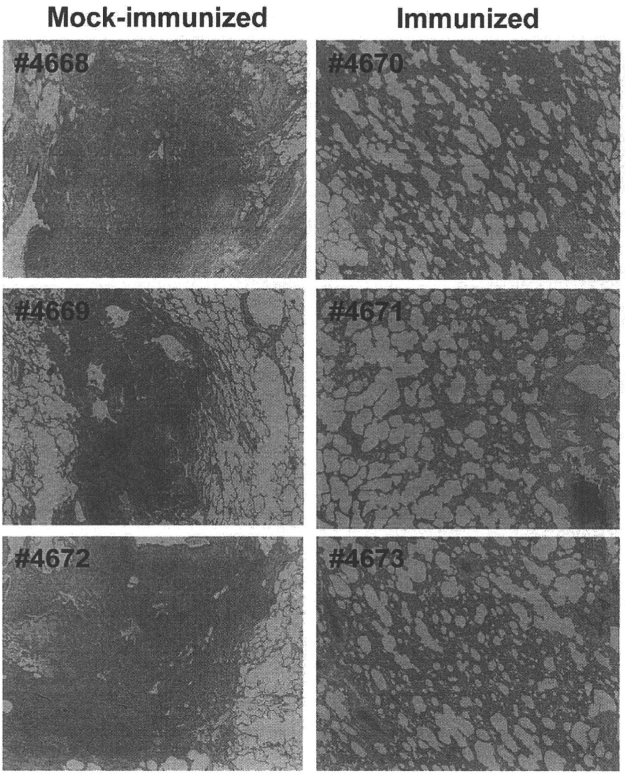

Fig. 3. Histopathological changes in the lungs from monkeys immunized intranasally with the Ampligen-combined NIBRG14 vaccine (#4670, #4671, and #4673) or from mock-immunized control monkeys (#4668, #4669, and #4672) 14 days after challenge with A/Vietnam/1194/04 virus (40×, H&E).

DISCUSSION

In this study, the effectiveness of the influenza A H5N1 vaccine (NIBRG14) in cynomolgus macaques was examined when it was delivered intranasally together with Ampligen. Intranasal vaccinations are known to induce mucosal immune responses by respiratory tract mucosa (which is the initial site of virus infection) and thus could be the most effective immunization strategy to deliver protection from influenza virus infection [Tamura et al., 2005]. However, such a vaccine is more likely to induce effective mucosal antibody responses if it is combined with a potent mucosal adjuvant. While cholera toxin and *Escherichia coli* heat-labile toxin are potent adjuvants that can enhance mucosal immune responses [Tamura et al., 2005], they have several undesirable side-effects in humans, including VIIth cranial nerve dysfunction [Mutsch et al., 2004]. Therefore, for intranasal influenza vaccines in humans, other adjuvants that are both clinically safe and effective should be developed. In this study, Ampligen®, which is a synthetic double-stranded RNA polyI/polyC$_{12}$U that has both a good safety profile as shown by clinical trials [Unknown, 2004] and good mucosal adjuvant activity in mice when co-administered intranasally with NIBRG14 [Ichinohe et al., 2007a,b] was used.

When administered to the monkeys, the adjuvant-combined vaccine elicited salivary IgA and serum IgG

antibody responses, as detected by ELISA (Fig. 1). Significantly, these vaccine-induced serum antibodies have neutralizing activity against both homologous and heterologous influenza A H5N1 viruses (Table II). Previously, it has been demonstrated in the influenza model mouse that this intranasal vaccination induces both cross-reactive mucosal antibodies and less cross-reactive serum antibodies [Ichinohe et al., 2005, 2007b], and that the ability of the mucosal antibodies to cross-react with various strains of influenza virus could be attributed to the secretory IgA antibodies [Ichinohe et al., 2007a]. These observations, together with the fact that cross-reactive neutralizing antibodies were detected in the serum of the vaccinated monkeys (Table II), suggest that more cross-reactive mucosal antibodies may also have been induced in the vaccinated monkeys. The cross-reactive neutralization activity was not detected in the salivary IgA antibodies, but this may have been due to the low concentration of the IgA antibodies (data not shown). Somewhat surprisingly, IgA antibody in saliva of both vaccinated and mock-immunized monkeys decreased quickly after 9 and 2 days post infection, respectively (Fig. 1B). The decrease of IgA antibody in mock-immunized monkey might be a background level (below broken line). However, the immunized monkeys sustained significant levels of salivary IgA antibody responses after the infection. Since saliva is so sticky and impure, it is required to optimize collection of saliva samples for IgA-ELISA to reduce background levels. Further work will be required to determine whether the vaccine also induces mucosal antibodies in monkeys that have greater cross-reactive neutralization activity than the serum antibodies.

Concomitant with these antibody responses, the vaccinated monkeys were protected completely from a challenge infection with the homologous virus, as shown by the inability to isolate the A/Vietnam/1194/04 virus from the vaccinated monkeys. In contrast, this virus was isolated readily from the nasal and throat swabs of the mock-immunized monkeys (Fig. 2 and Table III). Notably, it has been shown that intranasal administration of mice with NIBRG14 combined with Ampligen elicited protective immunity against both the homologous virus (A/Vietnam/1194/2004) and heterologous viruses, namely, A/Hong Kong/483/97 and the recent A/Indonesia/6/2005 virus [Ichinohe et al., 2007a]. These observations suggest that the monkeys that were immunized intranasally with the Ampligen-combined influenza A H5N1 vaccine may also have developed cross-protective immunity against influenza A H5N1 virus challenge.

Cynomologous macaques have been used as nonhuman primate models for studying influenza virus infection [Rimmelzwaan et al., 2001]. They demonstrated that when cynomologus monkeys are infected intratracheally with the A/Hong Kong/156/97 (H5N1) virus, they develop acute respiratory distress syndrome along with fever, and the virus can be isolated 4 days after infection from tissue samples of the trachea, lung, tracheobronchial lymph nodes, and heart [Rimmelzwaan et al., 2001]. In the present experiments, the results showed that when the mock-immunized monkeys were infected by the A/Vietnam/1194/2004 (H5N1) virus delivered intranasally and intratracheally, they developed pneumonia (Fig. 3), the virus-associated symptoms of tachypnea, diarrhea, nasal discharge, cough, and intention tremor (Table I), and lost their appetite, although their body weights and body temperatures did not change significantly (data not shown). Furthermore, the viruses were isolated from the nasal, throat or rectal swabs of the mock-immunized monkeys at 5 and/or 2 days post infection (Table III and Fig. 2), although the virus could not be isolated from the 14 dpi tissue samples of the frontal lobe, vertex, cerebellum, brain stem, trigeminal nerve, lung, and ileum (data not shown). However, although Rimmelzwaan et al. [2001] could also detect viral antigen (influenza virus nucleoprotein) in the lung on 4 and 7 days post-infection by immunohistochemistry, the viral antigen was not detected by immunohistochemical staining of the lungs of either mock-immunized or immunized monkeys at 14 days post infection (data not shown). The differences between the study of Rimmelzwaan et al. and present study in terms of the detection of influenza virus in tissue samples and the clinical signs may be due to the time point of virus collection (4 and 14 days after challenge, respectively), the virus strains (A/Hong Kong/156/97 and A/Vietnam/1194/04, respectively), and the infecting influenza virus dose (2.5×10^4 50% tissue culture infective dose and 3×10^5 PFU, respectively).

Itoh et al. [2008] demonstrated clearly that intranasal vaccination of cynomologus monkeys with a formalin-inactivated vaccine prepared from a non-pathogenic influenza A H5N1 virus conferred protective immunity against highly pathogenic influenza A H5N1 virus infection. However, in their experiments, the monkeys were given a high dose of whole virus vaccine (1 mg/dose). In the present experiments, the monkeys were immunized three times intranasally with 90 μg/dose (less than 10% of the whole virus vaccine dose) of NIBRG14, which is a medicinal product for human use, together with Ampligen as an adjuvant, and this regimen protected the monkeys from highly pathogenic influenza A H5N1 influenza. This suggests that this adjuvant-combined intranasal vaccine may overcome the problem of a limited supply of influenza A H5N1 virus vaccine.

In summary, nonhuman primates immunized intranasally with an Ampligen-combined NIBRG14 vaccine derived from a highly pathogenic influenza virus clinical isolate developed mucosal and systemic immunity that protected them from homologous A/Vietnam/1194/04 influenza virus infection. The intranasal administration of NIBRG14 and Ampligen was well tolerated. The vaccinated monkeys did not exhibit any clinical signs after challenge. Although the safety of Ampligen when administered intranasally with an influenza vaccine should be examined further, previous results using an intravenous protocol suggest that Ampligen may also be

useful in nasal vaccines destined for humans [2004]. Further clinical studies are needed to clarify these issues.

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Original Article

Passive Immune-Prophylaxis against Influenza Virus Infection by the Expression of Neutralizing Anti-Hemagglutinin Monoclonal Antibodies from Plasmids

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SUMMARY: The genetic delivery of therapeutic monoclonal antibodies (mAbs) by in vivo production may offer a new solution to the current problems in the mAb therapy for microbial diseases. Herein, plasmids encoding the neutralizing mAb against hemagglutinin (HA) of A/PR/8/34 influenza virus (IFV) were electro-transferred into mouse muscle and the relationship between serum recombinant anti-HA mAb (rHA mAb) levels and the prophylactic efficacy against lethal IFV infection were analyzed. Pretreatment of the muscle with hyaluronidase before electroporation and gene transfer into 3 muscles resulted in a marked enhancement of the mAb expression. After single gene transfer, peak serum concentrations were reached around 20 days after the gene transfer following sustained expression of >10 μg/ml of rHA mAbs. This level of rHA mAb expression was sufficient to protect all mice against a lethal IFV infection. Furthermore, a significant rHA mAb expression level sufficient to protect the host against lethal IFV infection was maintained for at least 130 days. Passive immune-prophylaxis with gene transfer using the plasmid encoding neutralizing mAbs may therefore provide effective protection against viral infections, including IFV.

INTRODUCTION

Classical passive immunity has been used for over a century in the prevention of infectious diseases (1). However, this field has largely missed out on the recent benefits provided by therapeutic monoclonal antibodies (mAbs) (2). The underdevelopment and underutilization of mAb therapy for microbial diseases has various complex explanations, including the current availability of antimicrobial drugs, small markets, high costs, and microbial antigenic variation (3). In addition, in cases where an effective vaccine is already available, it is not necessary, in principle, to consider the use of passive immunity. However, there are signs that the climate for mAb therapeutics in infectious diseases is changing because of recent increases in antibiotic drug resistance, the emergence of new pathogenic microbes for which no therapy is available, and mAb cocktail formulations (3).

The genetic delivery of therapeutic mAbs by in vivo production offers a new potential solution to these problems and promises to eliminate the inconvenience of weekly infusions of mAbs over a long period of time (4). The genetic delivery of mAb genes can be achieved using both viral and non-viral gene-transfer methods, and significant efforts have been devoted to the expression of full-length antibodies in vivo after gene transfer (5). Recently, Fang et al. (2005) provided convincing evidence that in vivo therapeutic antibody gene transfer is indeed possible, at least at the preclinical level (6). Thus, a single dose of a recombinant adeno-associated virus vector encoding the VEGF2-neutralizing mAb resulted in long-term expression of $>1{,}000$ μg/ml of the mAb in mice and significant anti-tumor efficacy. On the other hand, non-viral mAb gene transfer is still in its immature stage; the highest mAb serum level (1.5 μg/ml) achieved to date was obtained by electroporation of mAb plasmid into mouse muscle (7). Furthermore, no analysis of the functional activity of in vivo expressed mAb has been reported thus far. To the best of our knowledge, no other studies have been conducted on non-viral mAb gene transfer, therefore development of this technology remains a challenge.

We have demonstrated recently that in vivo therapeutic mAb gene transfer is possible by the improved electroporation of mAb plasmid in muscle using nude mouse and tetanus toxin (TT) neutralizing human anti-TT mAbs (8). The goal of the studies presented herein was to determine whether this in vivo therapeutic mAb gene transfer method can also be applied to influenza prophylaxis. Influenza virus (IFV) infection, one of the most serious infectious diseases, continues to be a major public health problem. Indeed, annual influenza epidemics cause severe illness in some 5 million people around the world. Parenteral inactivated vaccine and live-attenuated vaccine are currently used to control influenza (9). Hemagglutinin (HA), one of the major viral surface glycoproteins, is the most effective component

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in the conventional vaccine for providing protection against IFV infection by inducing anti-HA antibodies (10–12). Neutralizing anti HA-specific antibodies are known to play a critical role in protecting against IFV infection (13) as IFVs invade the host epithelial cells by binding to receptors (sialic acid) on the cell surface via HA (12). It has also been reported that passive immunization by neutralizing anti-HA mAbs could afford effective protection against H1N1 and H5N1 viral infections in mice (13–15).

In the present experiments, in vivo therapeutic mAb gene transfer was carried out by the improved electroporation of mAb plasmids encoding IFV neutralizing anti-HA IgG mAbs in muscle. The levels and duration of serum mAb expression were examined and the ability of mAb to be transudated into the respiratory tract (16,17) to prevent viral infection after lethal challenge infection with IFV determined. A single dose of an mAb-encoding plasmid vector with the enhancer injection resulted in long-term expression of over 10 μg/ml of the functional mAbs in mouse serum and complete prophylactic efficacy. In addition, effective prophylaxis for IFV infection was possible 130 days after mAb gene transfer. The gene transfer-based passive immune-prophylaxis with plasmid encoding neutralizing mAbs described here may provide effective protection against a wide range of acute-severe viral infections and an opportunity to reconsider primary immunization with current vaccines for immunocompromised patients and infants in developing countries.

MATERIALS AND METHODS

Plasmid construction: Total RNA from hybridoma cells secreting mAbs against the HA molecules of A/Puerto Rico/8/34 (A/PR8) virus (18) was purified using the RNeasy kit (Qiagen, Valencia, Calif., USA). cDNA from the variable regions of the heavy chain (V_H), including their signal peptide sequences, was amplified using Ig-primer sets (Novagen, Madison, Wis., USA), which were added with a CACC sequence at the 5′ end for subsequent subcloning into the pENTR/D-TOPO vector (Invitrogen, Tokyo, Japan). The heavy-chain variable region (V_H) and constant region 1 (C_H1) were cloned into the pGEM-T vector using the TA cloning kit (Promega, Madison, Wis., USA) and then sequenced. The gene of the heavy-chain constant region (C_H1, C_H2, and C_H3) was also cloned into the pGEM-T vector in the same manner. V_H and C_H were then joined by overlap PCR via C_H1 and the fragment obtained was subcloned into the pENTR/D-TOPO vector using the pENTR Directional TOPO Cloning Kit (Invitrogen). The cDNA of the light chains was amplified using the same primers and subcloned directly into the pENTR/D-TOPO vector. Finally, the genes were then subcloned separately into the pCADEST1 vector, which was constructed on the basis of pCA5, the CAG promoter-driven plasmid, and pDEST12.2 (Invitrogen) (19,20), using an LR-recombination reaction, in accordance with the manufacturer's protocol (Invitrogen).

Cell culture and transfection: 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). For the expression of recombinant anti-HA antibodies (rHA mAbs) in vitro, 293T cells were co-transfected with pCADEST1-anti-HA IgG1 and pCADEST1-anti-HA kappa chains using the FuGENE HD Transfection Reagent (Roche, Tokyo, Japan).

To purify rHA mAbs, free-style 293-F cells (Invitrogen) were co-transfected with the plasmid constructs described above using the Freestyle 293 Cell Transfection Kit (Invitrogen) in accordance with the manufacturer's protocol. The recombinant antibodies were purified from the culture supernatants by precipitation with 50% ammonium sulfate, followed by affinity chromatography using a Protein G Sepharose Fast Flow system (GE Healthcare, Tokyo, Japan).

Western blot analysis: The rHA mAbs expressed in the culture supernatant of the transiently transfected 293T cells were separated by SDS-PAGE under reducing conditions or non-reducing conditions, and transferred to PDVF membranes (Millipore, Bedford, Mass., USA) blocked with Block-Ace solution (Snow Brand Milk Products, Tokyo, Japan). Thereafter, to detect the heavy-chain or full-length antibodies, the membrane was incubated with alkaline phosphatase-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, Ala., USA). To detect the light chains, the membrane was incubated with biotin-conjugated goat anti-mouse kappa chain (Southern Biotechnology Associates), followed by incubation with alkaline phosphatase-streptavidin conjugate (Invitrogen, Carlsbad, Calif., USA). Both chains were visualized using an NBT/BCIP substrate solution.

Flow-cytometric (FCM) analysis: 293T cells were transfected with the pCAGGS-HA encoding HA gene of A/PR8 then, 48 h after transfection, the 293T cells were collected, followed by incubation with pHA mAb, rHA mAb, mouse anti-OVA IgG1 mAb (isotype control), and the diluted sera of mice that were to receive the gene transfer. After washing, the cells were incubated with FITC-conjugated goat anti mouse IgG (H + L) (Jackson ImmunoResearch, West Grove, Pa., USA). After further washing, the stained cells were measured using FACSCalibur and data analysis was performed using the CellQuest software (Becton Dickinson, San Jose, Calif., USA). The serum titer was assigned a mean fluorescence intensity (MFI).

Mice: ddY mice (5–7 weeks old) were purchased from Sankyo Labo Service Co. (Tokyo, Japan). All animal experiments were performed according to the guidelines of the Tokyo University of Science.

Antibody expression in vivo by electro-gene transfer: The expression plasmids were electro-transferred into mouse muscle using a previously described method (20–22) with minor modifications. Briefly, 30 μl of each plasmid (1 μg/μl) was injected into the adductor and/or rectus femoris and/or cranial tibial muscle using a syringe fitted with a two-stage needle (26-gauge), and needle electrodes (26-gauge) with a spacing of 5 mm were placed in each of the muscles. Six pulses (100 V, 50 ms, polarity reversal every 3 pulses) were then delivered into the injection site. All muscles were pretreated by injection of bovine hyaluronidase about 10 min before the gene transfer to decrease the viscosity of the extracellular matrix and facilitate DNA diffusion (23). Mice in the control group were not treated (naïve group) and

received the gene transfer with pCADEST1-null (vector control group).

Luciferase assay: Two days after gene transfer with pCADEST1-Luc-expressing firefly luciferase gene, the muscles were removed. The samples were cut into pieces and homogenized in 10 ml of 50 mM Tris-HCl (pH 8.0). The same volume of lysis buffer (50 mM Tris/phosphate buffer, 16 mM $MgCl_2$, 2 mM DTT, 2% TritonX-100, 30% Glycerol) was then added and the specimens were incubated for 1 h at 4°C. After centrifugation at 11,300 × *g* for 30 min at 4°C, 50 μl of Luciferase Assay Substrate (Promega) was added to 10 μl of each supernatant. The luciferase activity was assessed using a Wallace EnVision system (Perkin-Elmer Life, Boston, Mass., USA) and the results were expressed in relative light units (RLU) per protein content (μg) in the supernatant. The protein was measured using a Bradford assay with the Bradford assay reagent (Bio-Rad, Tokyo, Japan).

Quantification of A/PR8 HA-specific antibodies and competitive ELISA: The amount of rHA mAbs in the mice sera was measured by ELISA, as described previously (24). Briefly, 96-well flat-bottomed micro titer plates (Costar EIA/RIA plate No. 3690; Corning, Corning, N.Y., USA) were coated with HA molecules purified from A/PR8 viruses and blocked with PBS (−) containing 25% Block Ace® (Snow Brand Milk Products). After washing the plates with PBS (−) containing 0.5% Tween20 (PBS-Tween), serially diluted mouse serum was added to each well, in duplicate, and incubated. HA-specific antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates) diluted 1:10,000 in PBS-Tween. The plates were then developed with 1 mg/ml *p*-nitrophenyl phosphate (pNPP) in 1.0 M diethanolamine buffer containing 0.5 mM $MgCl_2$ (pH 9.8) and the absorbance of the plates was read at an optical density of 405 nm using an AUTO READER III (Sanko-junyaku, Tokyo, Japan). Parental anti-HA antibodies (pHA mAbs) were used as the standard (24). The concentration of the antibodies in the specimens was determined from the standard regression curve constructed for each assay with the programmed AUTO READER III.

For the competitive ELISA, serially diluted rHA mAbs were added to each well pre-coated with the purified A/PR8 HA molecules. After removing the solution, the biotin-conjugated pHA mAbs were added and the plate incubated for 1 h at room temperature. Alkaline phosphatase-streptavidin conjugate (Invitrogen) was then added, and incubation continued for 1 h at room temperature. After this time, pNPP solution was added and the absorbance measured as described above.

Virus challenge and virus titration: Virus challenge was carried out as described previously (25) with modifications for the ddY mouse. Thus, the A/PR8 virus was grown in the allantoic cavities of 10- to 11-day-old fertile chicken eggs, and stored at −80°C until used. Around 20 and 130 days after the gene transfer, the ddY mice were anesthetized and intranasally challenged with 10^5 times the 50% tissue culture infective dose ($TCID_{50}$) of A/PR8 viruses in 20 μl PBS (−), which resulted in lethal pneumonia. Three days after the virus challenge, the mice were sacrificed under anesthesia and serum samples collected. Bronchoalveolar lavage and nasal wash specimens were obtained by washing the isolated lungs and the nasal cavity of the isolated upper head with 2 ml and 1 ml of PBS (−) containing 0.1% bovine serum albumin, respectively.

The sera were assayed for A/PR8 HA-specific antibody responses as described above. The virus titers in the bronchoalveolar lavage specimens and nasal washings were estimated as $TCID_{50}$ according to a previously described method (26). Briefly, 100-μl aliquots of serial 10-fold dilutions of the bronchoalveolar lavage specimens or nasal washings were inoculated into Madin-Darby canine kidney (MDCK) cells in 96-well plates and incubated for 3 days at 37°C in a humidified 5% CO_2 atmosphere. After incubation, the MDCK cells were fixed with 10% formaldehyde-PBS (−) for 10 min, and the plates were then stained with 1.62 mM naphthol blue black (Sigma, Tokyo, Japan) in 1.57 M acetic acid buffer containing 12.2 mM sodium acetate. The plates were then washed and 0.1 M NaOH added to each well. The levels of the cytopathic effect (CPE) resulting from the IFV infection were evaluated by reading the absorbance of the plates at an optical density of 630 nm using the AUTO READER III system. The virus titer was calculated as $TCID_{50}$ by the Reed-Muench method.

Independently, the other groups of ddY mice, which underwent gene transfer in the same manner, were prepared for the estimation of survival rate. Survival and weight change were monitored for 2 weeks after virus challenge.

Measurement of the neutralization titer of the viruses: The neutralizing antibody titer of either purified antibodies or antisera was measured using a micro-neutralization assay, as described previously (27,28). Briefly, 100 $TCID_{50}$ of A/PR8 viruses were mixed with an equal volume of serial twofold dilutions of either purified antibodies (from 1 μg/ml) or antisera (from 1:10) treated with Receptor Destroying Enzyme (Denka Seiken, Tokyo, Japan) and then incubated for 30 min at 37°C. The mixtures were then inoculated into MDCK cells in duplicate, and incubated for 3 days. CPE for the IFV infection was evaluated by reading the absorbance of the plates at an optical density of 630 nm using the AUTO READER III. The neutralization titer was defined as either the antibody concentration or the reciprocal of the highest serum dilution that showed no CPE.

Statistics: Comparisons between experimental groups were performed using Students' *t* test. Probability values < 0.05 were considered to indicate significance.

RESULTS

Characterization of the rHA mAb in vitro: The ORFs of the full-length heavy and light chains were cloned from the murine hybridoma previously found to produce mouse anti-A/PR8 HA antibodies, which have been characterized with respect to their binding potential for intact A/PR8 HA and their neutralizing activities against A/PR8 IFV infection (18). They were then subcloned separately into the expression plasmid, pCADEST1 driven by a CAG promoter (20,22).

Plasmids encoding the heavy and light chains were co-transfected into 293T cells, and rHA mAbs expression

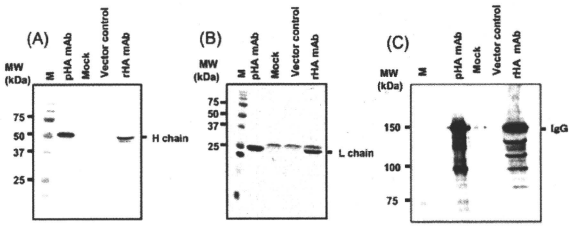

Fig. 1. In vitro expression of rHA mAb. Western blot analysis to determine the expression of the recombinant H chain (A), L chain (B), and IgG (C). Purified anti-HA mAb (pHA mAb) was compared with the supernatants of the 293T cells not transfected with any plasmid (Mock), transfected with pCADEST1-null (vector control), and co-transfected with pCADEST1-anti-HA heavy chain and pCADEST1-anti-HA kappa chain. Heavy chain (A) and kappa chain (B) separated by reduced SDS-PAGE were proved with anti-mouse γ1 and anti-mouse κ antibodies, respectively. IgG (C) was separated by non-reduced SDS-PAGE and proved with anti-mouse γ1 antibodies.

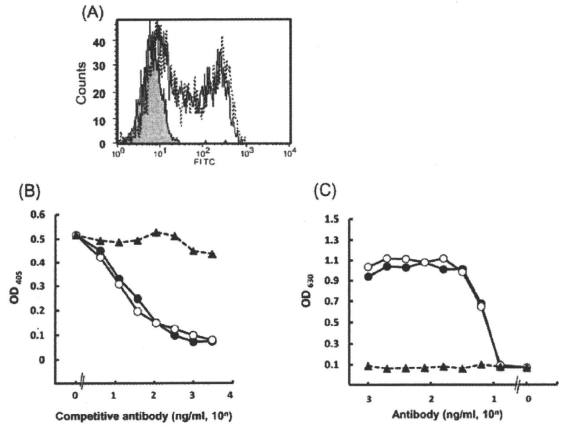

Fig. 2. Characterization of the rHA mAb in vitro. (A) The reactivity of rHA mAb with HA as a membrane protein was evaluated by flow-cytometric (FCM) analysis. 293T cells transiently transfected with pCAGGS-HA were stained with the pHA mAbs (solid line), the rHA mAbs (dotted line) and isotype control (shaded area). (B) Dose dependent inhibition of binding of the biotin-conjugated pHA mAb by autochtonous pHA mAb (●), rHA mAb (○), and isotype control (▲) by competitive ELISA. (C) Dose dependent neutralizing activity against A/PR8 influenza virus by incubating with the pHA mAb (●), the rHA mAb (○) and isotype control (▲) by $TCID_{50}$ assay as described in Materials and Methods.

in the culture supernatants were determined by Western blotting (Fig. 1A-1C). Protein bands corresponding to molecular weights of approximately 50 kDa (Fig. 1A) and 25 kDa (Fig. 1B), which correspond to the heavy and light chains of the IgG1 protein of rHA mAbs, were detected under reducing conditions. The size of each of these bands was similar to that reported for the pHA mAbs from hybridoma cells. Several bands were detected in the culture supernatants of both the transfected 293T cells and the pHA mAbs under non-reducing conditions (Fig. 1C). The largest single band corresponded to a molecular weight of approximately 150 kDa, thereby suggesting a full-length antibody containing two heavy and two light chains.

FCM analysis showed that the rHA mAbs had reacted with the A/PR8-HA on the cell membrane, as is the case for pHA mAbs (Fig. 2A). Indeed, preincubation of an ELISA plate coated with A/PR8-HA with rHA mAbs inhibited the binding of pHA mAbs (Fig. 2B), thereby suggesting that the two antibodies recognize the same epitope. Finally, the neutralizing effect of the rHA mAbs was determined by evaluating the levels of the CPE under a microscope. The results indicated that rHA mAbs exhibit a similar neutralizing activity against the A/PR8-IFV to the pHA mAbs (Fig. 2C). These findings indicate that the rHA mAbs expressed from the plasmid construct exhibit the full range of biological activity of the parental antibodies.

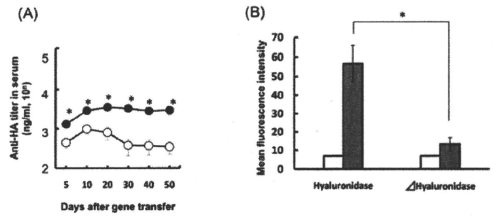

Fig. 3. In vivo expression of the rHA mAbs. (A) The time-course of the changes in the expression levels of the rHA mAbs in the sera of the mice that received the gene transfer into hyaluronidase-pretreated muscle (●, $n=5$) or into muscle without hyaluronidase pretreatment (○, $n=5$). The mAbs were evaluated by quantitative ELISA. (B) FCM analysis of the reactivity of the rHA mAbs in the serum for A/PR8-HA on the cell membrane. 293T cells transiently transfected with pCAGGS-HA were stained with the pre-sera (open column) and the sera collected 20 days after the gene transfer with or without (⊿) hyaluronidase (filled column). Data were expressed as mean fluorescence intensity (MFI) ± standard error of the mean (S.E.M). $^{*}P < 0.05$.

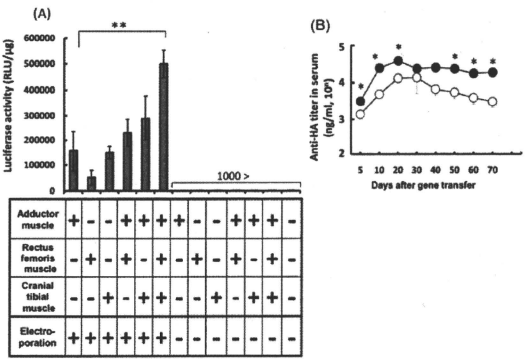

Fig. 4. (A) Effects of the number of injection muscles on the expression of the luciferase by the gene transfer. The luciferase gene transfer was carried out by a single intramuscular injection of 30 μg pCADEST1-Luc plasmid DNA into the each of muscle with or without electroporation. Two days after the gene transfer, the luciferase activity was determined in the muscle homogenate. Each column represents the mean activity of luciferase ± S.E.M. The gene was transduced into each or combinations of adductor, rectous femous, and cranial muscles of a mouse (electroporation (+), $n=6$; electroporation (−), $n=3$). $^{**}P < 0.005$. (B) Time-course of changes in the expression levels of the rHA mAbs in the sera of mice that received the gene transfer into adductor muscle (1 muscle, ○, $n=4$) and 3 muscles (●, $n=5$). The serum mAbs were determined by quantitative ELISA. Data were expressed as mean ± S.E.M. $^{*}P < 0.05$.

Analysis of the expression level of rHA mAbs in mice that underwent gene transfer: The plasmids encoding the heavy and light chains of anti-HA mAbs were electro-transferred into the adductor muscle of ddY mice and the time-course of the change in expression levels of the rHA mAbs in sera monitored for 50 days. The effects of hyaluronidase pretreatment of the muscle prior to electro-gene transfer on gene expression were also evaluated. Thus, the serum mAb concentration peaked at approximately 1,000 ng/ml 10 days after the gene transfer in hyaluronidase-untreated mice. In contrast, pretreatment with the enhancer resulted in a significant increase of serum rHA mAbs, with the peak mAb concentration of approximately 3,500 ng/ml, which is 3.5-fold higher than that obtained without the pretreatment, being reached around 20 days after gene transfer (Fig. 3A). The serum mAb concentrations obtained following gene transfer into hyaluronidase-pretreated muscle remained close to 3,000 ng/ml for 50 days (Fig. 3A). Furthermore, the binding activity of the rHA mAbs to recombinant A/PR8-HA molecules on the cell membrane was higher in the sera from the hyaluronidase-pretreated mice than in the sera from untreated mice (Fig. 3B).

Effect of the number of injection muscles on gene expression: In order to further improve the serum rHA mAb expression levels, the ability of gene transfer into additional muscles to increase the mAb expression levels was also tested.

Before comparing the levels of mAb expression,

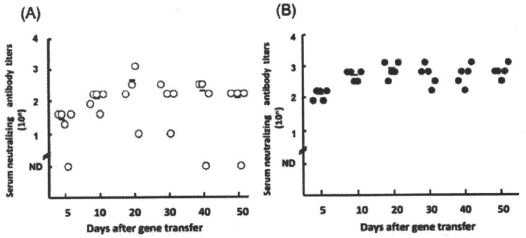

Fig. 5. Time-course of changes in the expression levels of the influenza virus (IFV)-neutralizing activity in the sera of mice (Fig. 4B) that received the gene transfer into adductor muscle (1 muscle, ○, $n=4$) and 3 muscles (●, $n=5$). Neutralizing antibody titer of the sera was measured by the use of micro-neutralization assay. Horizontal bars represent the average for each group at the day. ND, not detected.

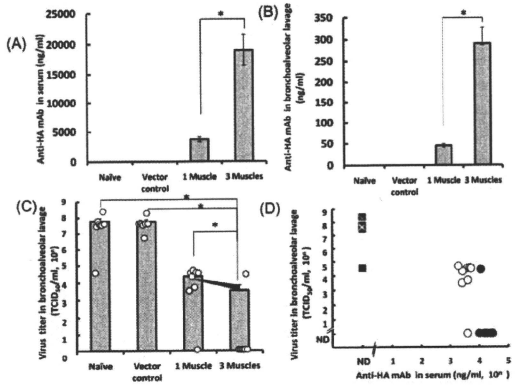

Fig. 6. Protection against IFV infection in ddY mice that received the mAb gene transfer. The mice were challenged with IFV 20 days after the mAb gene transfer. At 3 days after the lethal virus challenge, the expression levels of the rHA mAbs were measured in the serum samples (A) and bronchoalveolar lavage specimens (B) by quantitative ELISA. (C) The viral titers in the bronchoalveolar lavage specimens were determined by the $TCID_{50}$ assay, as an index of protection against infection. Each of the error bars represents the S.E.M. $^*P < 0.05$. (D) The viral titers in the bronchoalveolar lavage specimens in (C) were expressed relative to the expression levels of the rHA mAbs in the respective serum samples in (A). Each plot indicates the values for individual mice. Naïve (■, $n=8$); vector control (×, $n=8$); gene transfer into 1 muscle (○, $n=8$); gene transfer into 3 muscles (●, $n=8$); ND, not detected.

firefly luciferase-encoding plasmids were injected into several muscles and the expression level of the gene ascertained by luciferase expression assay. Electro-transfer was then performed on the adductor muscle, rectus femoris muscle, and cranial tibial muscle, or combinations of the muscles of the mice. Electro-transfer of the plasmid construct into 3 muscles together (3 muscles) induced the highest luciferase-expression level, which was an approximately 3-fold higher than that obtained following plasmid injection into only 1 site of the adductor muscle (1 muscle) (Fig. 4A).

In a similar manner, but with the antibody-encoding plasmid, the serum titers for the rHA mAb were determined by HA-ELISA following gene transfer into 1 or 3 muscles. mAb gene transfer into 3 muscles was found to induce higher serum rHA mAb concentrations than gene transfer into 1 muscle, and the resulting high concentrations remained above 10,000 ng/ml (Fig. 4B) for 70 days. Likewise, the expression levels were approximately 3-fold higher than that obtained with gene transfer into only 1 muscle (Fig. 4B). Finally, the neutralizing activity of serum rHA mAbs against A/PR8 virus following gene transfer into 1 or 3 muscles was examined in vitro. The results showed that the neutralizing antibody titers correlated well with serum rHA mAb concentrations (Fig. 5A, 5B). Furthermore, the neutralizing titer following gene transfer into 3 muscles was slightly higher than that following gene transfer into 1 muscle. These results demonstrate that gene transfer into 3 muscles could induce higher serum rHA mAb

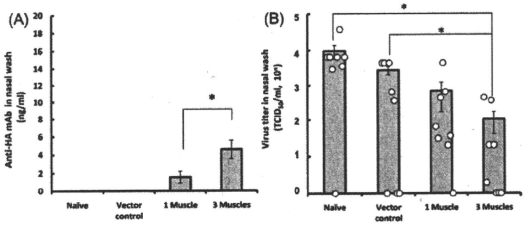

Fig. 7. The expression levels of the rHA mAbs and virus titer in the nasal washings obtained from the same ddY mice in Fig. 6. (A) The expression levels of the rHA mAbs were measured in the nasal washing by quantitative ELISA. (B) The viral titers in the nasal washings were determined by the $TCID_{50}$ assay. Each of the error bars represents S.E.M. $^*P < 0.05$. NS, not significant ($P > 0.05$).

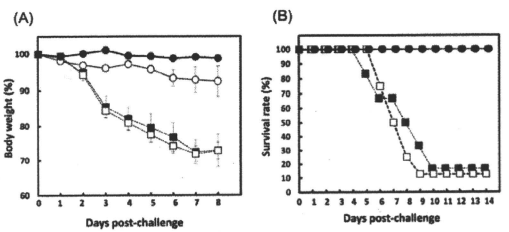

Fig. 8. Protection of the mice that received the mAb gene transfer from lethal A/PR8 virus challenge. The mice were challenged with IFV 20 days after the gene transfer and the body weight (A) and the survival rates (B) were monitored for 14 days. The body weight was expressed relative to the initial mean body weight of each group. Naïve (■, $n=6$); vector control (□, $n=8$); gene transfer into 1 muscle (○, $n=8$); gene transfer into 3 muscles (●, $n=8$). Each of the error bars represents the S.E.M.

concentrations with neutralizing activity than that into 1 muscle, thereby suggesting that rHA mAb concentration is a functional indicator of the protective efficacy against IFV infection.

Relationship between serum and respiratory tract mAbs and protection: To evaluate the prophylactic efficacy of rHA mAbs, mAb gene transfer was performed in mice which had received gene transfer into 1 or 3 muscles. Thus, the mice were challenged with a lethal dose (10^5 $TCID_{50}$ in 20 μl PBS (−)) of the A/PR8 virus at around 20 days after gene transfer. Three days after viral challenge, the virus titers in the bronchoalveolar lavage and nasal wash specimens from the infected mice were measured and the serum A/PR8 HA-specific antibody concentrations were determined by HA-ELISA. A serum rHA mAb concentration of approximately 20,000 ng/ml, almost 5-fold higher than that obtained following gene transfer into 1 muscle, was detected following gene transfer into 3 muscles (Fig. 6A). A concentration of approximately 300 ng/ml of rHA mAbs was detected in the bronchoalveolar lavage specimens of mice which underwent gene transfer into 3 muscles (Fig. 6B). This concentration was about 7-fold higher than that observed for mice which received gene transfer into 1 muscle, which was equivalent to that observed in the serum (Fig. 6A), thus indicating the diffusion of rHA mAbs from the plasma into the serous fluid of the alveolar epithelia. Gene transfer into 3 muscles resulted in a reduction of the viral titer in bronchoalveolar lavage to undetectable levels for almost all mice, whereas gene transfer into 1 muscle reduced it to 1/3,000 of that for control mice (Fig. 6C). The viral titers were inversely correlated with the serum antibody levels (Fig. 6D), thus meaning that the ability to mount a protective response to viral infection correlated well with the serum antibody titers.

The rHA mAb titers in the nasal wash specimens obtained from the mice were also determined and the influence of gene transfer on the viral titers in these specimens was examined. This study showed that rHA mAb titers, which were not detected in the nasal washings of untreated mice, were detected in the specimens obtained from those mice which had undergone mAb gene transfer (Fig. 7A). A significant reduction (1/10 ~ 1/100) in the viral titers for the nasal washings obtained from mice which had undergone gene transfer into 3 muscles was observed compared to those for untreated mice (Fig. 7B).

The changes in body weight and survival rates of those animals which underwent gene transfer were monitored after challenge with the lethal dose of virus. All mice that underwent gene transfer survived for more than 14 days, with almost no loss in body weight (Fig. 8A, B), whereas the untreated mice (naïve or vector control group) showed a very rapid decline in body weight, which resulted in a mortality of 80% within 14

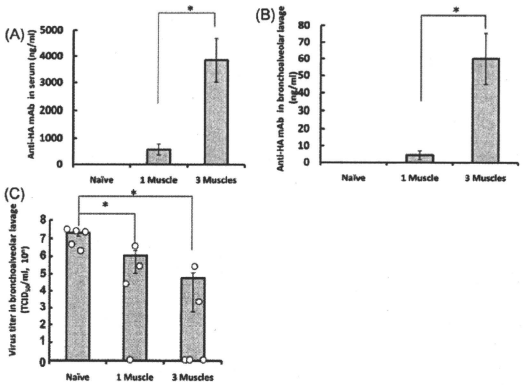

Fig. 9. Long-term expression of IFV-neutralizing rHA mAbs and the prophylactic efficacy against IFV infection. The mAb gene transfer into mice was executed as described above; naïve ($n=5$), gene transfer into 1 muscle ($n=4$), gene transfer into 3 muscles ($n=5$). Around 130 days after the gene transfer, the mice were received the lethal A/PR8 virus challenge. At 3 days after the virus challenge, expression levels of the rHA mAbs were measured in the serum (A) and bronchoalveolar lavage (B) specimens by quantitative ELISA. The viral titers in the bronchoalveolar lavage specimens were determined by the $TCID_{50}$ assay (C). Each error bar represents the S.E.M. $^*P < 0.05$.

days of viral challenge (Fig. 8A, B).

Long-term expression of IFV-neutralizing rHA mAbs and their prophylactic efficacy against IFV infection: To determine the length of time during which the prophylactic efficacy of the rHA mAb was sustained following mAb gene transfer, the mice were challenged with a lethal dose of the virus around 130 days after gene transfer. The serum and bronchoalveolar lavage specimens were collected as described for the experiment shown in Fig. 6 and rHA mAb concentration and virus titer were determined. Although the serum rHA mAb concentration obtained following gene transfer into 3 muscles decreased to approximately 20% of the peak level, it remained high (about 4,000 ng/ml) even 130 days after gene transfer (Fig. 9A); the rHA mAb concentration in bronchoalveolar lavage specimens also remained at a significant level (about 60 ng/ml). The viral titers in the bronchoalveolar lavage specimens for those mice which underwent gene transfer into 3 muscles were also much lower (1/300) than those for specimens from the untreated group (Fig. 9C). Likewise, a lower but nevertheless significant reduction in virus titer (1/20) was observed (Fig. 9C) in the bronchoalveolar lavage specimens of those mice which underwent gene transfer into 1 muscle (serum concentration, 600 ng/ml rHA mAbs; bronchoalveolar lavage, 4 ng/ml). Consequently, these data indicate that passive immunotherapy using the mAb gene-transfer technique induces a protective response against IFV infection that is maintained for at least 130 days after the procedure.

DISCUSSION

The results of this study show that protection againt IFV can be obtained with neutralizing mAbs induced by passive immunization using an in vivo mAb gene- transfer technique involving plasmids. Thus, most of the viruses were eliminated from the bronchoalveolar lavage specimens around the time of the peak expression of mAbs following mAb gene transfer into 1 or more mouse muscles. Furthermore, the virus-elimination potency was correlated with the serum rHA mAb concentrations. Indeed, whereas difference in serum rHA mAb titers was >3-4-fold as regards gene transfer into 3 muscles and into 1 muscle, the difference in viral titers in the bronchoalveolar lavage specimens was much higher (1,000-10,000-fold). These results therefore suggest that if the antibody expression levels can be induced still further, the protective effect against IFV infection would also be much higher. In this context, it is interesting to note that the rHA mAb expression induced upon the mAb gene transfer remained high for at least 130 days, thus meaning that the hosts were protected against lethal IFV infection for at least this period of time. In our previous study (8) using other mAbs, the long-term expression of neutralizing mAbs in vivo was demonstrated to last for at least 260 days after mAb gene transfer. Future studies should therefore be aimed at determining the minimum serum mAb concentration required for prophylaxis against each type of viral infection.

The present study also showed that the viral titers in the nasal washings of those mice which underwent mAb gene transfer into 3 muscles were also reduced even though this passive immunotherapy could induce an IgG antibody production. We and others (16,29) have previously reported that serum IgG antibodies are secreted on the mucosal tissues and partially prevent virus infection. These facts suggest that the high serum rHA mAbs titers detected in this study are secreted on

the nasal mucosa, where they neutralize the A/PR8-IFV. Passive immunotherapy may therefore inhibit both infection by IFVs in the upper respiratory tract and the pneumonia which often arises upon IFV infection.

Furthermore, the antibody titers were still sufficient to exert a protective effect against IFV infection in those mice infected with a lethal dose of the virus over 130 days after gene transfer. Thus, these results suggest that this single-dose passive immunotherapy provides effective prophylaxis against influenza infection for a prolonged period of time.

The ability of the IFV to alter the antigenic properties of its surface HA so as to evade host immune defenses and cause annual influenza epidemics is well known (30,31). To solve this issue, genes from other neutralizing anti-HA antibodies will need to be obtained. Neutralizing mAbs which are broadly cross-reactive to variable influenza A virus were isolated recently (13,32). It should therefore be possible to protect against multiple IFVs infection, such as new strains or emerging pandemic strains, by the electro-transfer with the plasmid encoding the gene for such mAbs.

In the field of cancer therapeutics, recombinant adeno-associated viral vectors have been developed for the long-term delivery of therapeutic antibodies to neutralize cancer (33). Furthermore, some researchers have used viral vectors, such as adenoviral vectors, to express therapeutic antibodies (33–35). However, if a viral vector were used for gene therapy, an immune response against the viral backbone should also be induced in vivo. The majority of humans carry preexisting humoral and/or cellular immunity to such as Ad5-based vectors (36), thereby severely limiting the use of viral vectors for gene-therapy applications as they would inhibit transfer of the viral vector in vivo. However, in this study expression plasmid DNA, which is a non-viral vector, was transferred into mice by electroporation. It is expected that the gene therapy described however could be used iteratively for most individuals. Likewise, a similar hydrodynamics-based passive immunotherapy procedure, which induces maximal transgene expression approximately 8 h after gene transfer (37,38), could be used to treat IFV infection and could therefore lead to the commercial development of anti-viral mAbs.

In conclusion, a new gene therapy which can reliably and sustainably elicit protective immunity against IFV infection after single-dose administration has been developed. Furthermore, in a "proof of principle," it has been shown that passive immune-prophylaxis with a plasmid expressing anti-HA mAb can effectively protect mice from a lethal dose of influenza A virus. This technique may be applicable to the entire human population in the event of pandemic influenza.

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Conflict of interest None to declare.

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REVIEW

Development of a cytotoxic T-lymphocyte-based, broadly protective influenza vaccine

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ABSTRACT

The current vaccination strategy against influenza is to induce production of antibodies directed against the surface antigens of these viruses. However, frequent changes in the surface antigens of influenza viruses allow them to avoid antibody-mediated immunity. On the other hand, it is known that cytotoxic T-lymphocyte (CTL) populations directed against internal antigens of influenza A virus are broadly cross-reactive to influenza virus subtypes. The present authors have previously demonstrated that antigens chemically coupled to the surface of liposomes made using unsaturated fatty acids are cross-presented by APCs via MHC class I to $CD8^+$ T cells and induce antigen-specific CTLs. Based on this finding, a liposome vaccine that is capable of inducing CTL response against internal antigens of influenza viruses and removing virus-infected cells in the host has been developed. The CTL-based liposomal technique might be applicable for developing vaccines against influenza and other viruses, such as hepatitis C, HIV, and severe acute respiratory syndrome corona virus, which frequently change their surface antigenic molecules.

Key words cytoxic T lymphocyte, influenza, liposome, vaccine.

Adjuvants are indispensable for viral vaccines, such as inactivated, subunit, or component vaccines, in which the antigens possess weak immunogenicity. However, the currently used aluminum adjuvants are known to stimulate only humoral responses (1) and are also known to induce IgE antibody production, which elicits an allergic response in some individuals following vaccination (2). Therefore, there is a need for improved adjuvants that are suitable for clinical use. Because they are known to act as powerful adjuvants when physically associated with a protein antigen, liposomes as antigen carriers (vehicles) have been particularly attracting attention among the candidates for adjuvants for novel vaccines (3–6). Most of the liposomal vaccines which have been proposed have been prepared by antigen entrapment within the aqueous lumen of liposomes (7). However, it is known that encapsulated and surface-linked liposomal antigens induce differential immune responses in humoral- (8) and cellular (9) immunity.

We have previously reported that surface-linked liposomal antigens induce IgE-selective unresponsiveness (10). Our results were consistent even when different procedures for coupling antigens with liposomes (11), or for producing liposomes with different lipid components (12), were employed. During the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-coupled liposomal antigens, we discovered an alternative approach to regulating the production of IgE, one that is independent of the activity of T cells (13). The IgE-selective unresponsiveness

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List of Abbreviations: APCs, antigen presenting cells; CD, cluster of differentiation; CpG, cytosine-phosphate-guanine; CTL, cytotoxic T lymphocyte; DC, dendritic cell; IFN, interferon; HA, hemagglutinin; HLA, human leukocyte antigen; ICS, intracellular cytokine staining; IL, interleukin; MHC, major histocompatibility complex; NP, nucleoprotein; OVA, ovalbumin; S-OIV, swine-origin influenza (H1N1) virus; TLR, Toll-like receptor.

induced by the liposomal antigen involved direct effects on IgE, but not IgG switching *in vivo*. Thus, we expected that surface-linked liposomal antigen would be suitable for the development of a novel vaccine which would induce minimal IgE synthesis. Moreover, given the relatively low allergic response to, and increased antigenicity of, this allergen, this form of antigen preparation would be suitable for allergen immunotherapy (14, 15). In addition, we recently found that when lipid components are chosen for liposomes, surface-coupled liposomal antigens are cross-presented to $CD8^+$ T cells via MHC class I (16). Therefore, surface-linked liposomal antigens might be suitable for the development of tumor vaccines that would present tumor antigens to APCs and induce antitumor responses, and for the development of virus vaccines that would induce CTLs to eliminate virus-infected host cells.

A novel S-OIV which was first detected in Mexico in March 2009 spread globally via human-to-human contact. Although S-OIV belongs to the same HA serotype as seasonal human H1N1 influenza virus, vaccination with seasonal influenza vaccine does not result in cross-reactive antibodies (17), possibly because there is a wide protein sequence divergence of around 20–24% between S-OIV H1 and human seasonal influenza H1 (18). The current vaccination strategy is to elicit neutralizing antibody responses against HA, a surface glycoprotein of influenza viruses. Although antibody-mediated immunity to the surfaces of viruses reduces the probability of infection and morbidity, an antibody against one influenza virus type or subtype provides limited or no protection against another. Moreover, as a consequence of the frequent development of antigenic variants through antigenic drift, an antibody to one antigenic variant of an influenza virus might not protect against a new antigenic variant of the same type or subtype. Therefore, although vaccines designed to induce antibodies against HA provide reasonable protection against homologous viruses, it is feared that, due to the high rate of viral diversification, the vaccines currently being produced may have HA sequences so different from those of any pandemic strain that they would have little or no efficacy (19).

In natural infection, it is known that the host responds by inducing humoral and cellular immunity against the pathogen. Humoral immune responses consist of production of antibodies that bind to the surfaces of bacteria and viruses, whereas cellular immune responses mediate immunity to intracellular pathogens. Since Effros *et al.* reported in 1977 that influenza A virus-specific CTLs are broadly cross-reactive for cells of the same MHC class I type infected with serologically distinct H1N1 and H3N2 influenza viruses (20), it has been known that CTLs specific to internal proteins show high cross-reactivity between strains and between subtypes, reflecting more than 90% conservation of the internal proteins (21). Lee *et al.* recently reported that memory T-cells established by seasonal human influenza A infection cross-react with H5N1 in healthy individuals who have not been exposed to H5N1 viruses (22). The $CD8^+$ T-cells of the participants recognized multiple synthesized influenza peptides, including peptides from the H5N1 strain. Thus, vaccine formulas that include heterosubtypic T-cell-mediated immunity might confer broad protection against avian and human influenza A viruses.

In general, extracellular antigens are presented via MHC class II molecules to $CD4^+$ T-cells, whereas intracellular antigens are presented via MHC class I molecules to $CD8^+$ T-cells. To induce influenza-specific CTLs, vaccine antigens must be loaded onto the class I MHC processing pathway in the APCs via cross-presentation. In the process of cross-presentation, exogenous proteins cross over to the endogenous pathway, thus gaining access to MHC class I molecules (23). However, the currently approved alum adjuvant, which was first described by Glenny *et al.* in 1926 (24), is known to be effective only for induction of humoral immunity, and not for induction of cell-mediated immunity (25). Consequently, development of a novel vaccine adjuvant is essential for production of a $CD8^+$ T-cell vaccine.

Since liposomal conjugates induce CTLs efficiently when CTL epitope peptides are coupled to the surfaces of liposomes (16), they were expected to be suitable for development of peptide vaccines (26) . We recently demonstrated that liposome-coupled peptide $NP_{366–374}$, derived from NP of H3N2 influenza virus induces antigen-specific CTLs and successfully suppresses replication of H3N2 influenza virus in the lung in C57BL/6 mice (27). We further evaluated a possible application of liposome-coupled peptides to the development of an influenza vaccine using HLA-A*0201-restricted CTL epitope peptides and HLA-A*0201-transgenic mice.

In this review, we summarize data indicating that a correlation exists between the lipid component of liposomes and the immune response induced by surface-linked liposomal antigens, and discuss the potential of surface-linked liposomal antigens for the development of CTL-based viral vaccines.

Liposomes with different lipid components exert different adjuvanticity in antigen-liposome conjugates via differential recognition by antigen presenting cells

Based on our previous finding that liposomes with different lipid components display different adjuvant effects when antigens are coupled with liposomes via glutaraldehyde (12), we investigated adding antigen-liposome

conjugates prepared using liposomes with different lipid components to a macrophage culture, and phagocytosis and digestion of liposome-coupled antigen by the macrophages (28). When mice were immunized with OVA-liposome conjugates containing liposomes consisting of either saturated- or unsaturated fatty acids, the degree of anti-OVA IgG antibody production induced by these two OVA-liposome conjugates was significantly different; OVA-liposome conjugates made using the "unsaturated" liposomes induced more than ten-fold greater anti-OVA IgG production than that induced by OVA-liposome conjugates made using "saturated" liposomes, whereas the same dose of plain OVA solution induced far less anti-OVA IgG antibody production than that induced by OVA-liposome conjugates.

In order to examine whether the different adjuvant effects of these two liposome preparations were due to differential recognition of liposomal antigens by APCs, we investigated phagocytosis of OVA-liposome conjugates by macrophages by adding fluorescence-labeled OVA coupled with "saturated" or "unsaturated" liposomes to the macrophage culture. We found that more OVA were incorporated by macrophages when OVA were coupled to "unsaturated" liposomes than when OVA were coupled to "saturated" liposomes. To compare the processing of OVA coupled either with "saturated" or "unsaturated" liposomes by macrophages, we further investigated the fluorescence intensity of the macrophages by adding DQ-OVA-coupled liposomes to the macrophage culture. DQ-OVA exhibits green fluorescence upon proteolytic degradation. It appeared that the amount of OVA processed by macrophages in the same time period was greater when OVA was coupled to the "unsaturated" liposomes than when it was coupled to the "saturated" liposomes.

We further investigated antigen presentation by macrophages to an antigen-specific T-cell clone using the same set of conjugates. When we cultured macrophages in the presence of OVA-liposome conjugates prior to co-culture with the OVA-specific T-cell clone, 42–6A, significantly greater IL-2 production was observed when the macrophages were pre-cultured with OVA-liposomes made using "unsaturated" liposomes. Although a substantial amount of IL-2 was produced when the macrophages were pre-cultured with OVA-liposome conjugates made using "saturated" liposomes, the amount of IL-2 was still more than ten-fold less than that in the "unsaturated" liposome group.

Thus, these results clearly demonstrate that the adjuvant activity of liposomes observed primarily *in vivo* is closely correlated with recognition of antigen-liposome conjugates and the presentation of liposome-coupled antigen by macrophages, suggesting that the adjuvant effects of liposomes are exerted at the beginning of the immune response, that is, upon recognition of the antigen by APCs.

Antigens coupled to the surface of liposomes made using unsaturated fatty acid are cross-presented to CD8$^+$ T cells and induce potent antitumor immunity

Liposomes with different lipid components have been demonstrated to display different adjuvant effects when antigens are chemically coupled to their surfaces (12, 28). Here, we investigated the antigen presentation of liposome-coupled OVA *in vitro*, and found that OVA coupled to "unsaturated" liposomes are presented to both CD4$^+$ and CD8$^+$ T-cells while OVA coupled to "saturated" liposomes are presented only to CD4$^+$ T cells (16). When we co-cultured APCs derived from BALB/c mice with OVA-liposome conjugates made using liposomes with the above two different lipid components, and subsequently cultured them with splenic CD4$^+$ or CD8$^+$ T cells of OVA-immune BALB/c mice, OVA-liposome conjugates with the two types of liposome induced production of comparable amounts of IFN-γ by CD4$^+$ T cells. However, OVA-liposome conjugates made using "saturated" liposomes did not induce IFN-γ production by CD8$^+$ T cells, while OVA-liposome conjugates made using "unsaturated" liposomes induced significant production of IFN-γ.

In most APCs, exogenous antigens cannot be presented via the MHC class I pathway because exogenous antigens are unable to gain access to the cytosolic compartment. This segregation of exogenous antigens from the class I pathway is important in preventing CTLs from killing healthy cells that have been exposed to foreign antigens but have not become infected (29). Consequently, in general, exogenous antigens do not prime CTL responses *in vivo*. However, there are several exceptions to this rule, reflecting an ability of the antigens to be delivered into the cytosolic compartments in some cases (30–34). We have shown that antigens coupled to "unsaturated" liposomes are presented to both CD4$^+$ and CD8$^+$ T cells. Confocal laser scanning microscopic analysis demonstrated that a portion of the OVA coupled to liposomes received processing beyond the MHC class II compartment, suggesting that degradation of OVA occurs in the cytosol, and that peptides generated in this manner are presented to CD8$^+$ T cells via MHC class I. We further confirmed cross-presentation induced by OVA coupled to liposomes consisting of unsaturated fatty acids in *in vivo* CTL induction experiments. CTLs were successfully induced *in vivo* only when OVA or $OVA_{257–264}$ chemically coupled to "unsaturated" liposomes was inoculated into mice.

We further investigated the ability of antigen-liposome conjugates to induce antitumor immunity. The aim of cancer vaccination is to generate an immune-mediated anti-tumor associated antigen response that results in elimination of the tumor. The antigen of choice may be the whole protein alone or one with immune stimulatory components, or defined epitopes (e.g., peptides), of the target antigen (35). Recent preclinical studies have demonstrated that combined therapies involving the use of vaccines with cytokines, activators of DCs such as TLR ligands or mAb to CD40, or recombinant vectors that provide a stimulus to the innate immune system, result in enhanced antitumor responses (36). In this study, antigenic peptides were chemically coupled to the surface of liposomes and inoculated into tumor-bearing mice in combination with CpG and anti-IL-10 monoclonal antibodies. This treatment successfully induced eradication of the tumor mass, whereas inoculation of mice with CpG and anti-IL-10 monoclonal antibodies with peptide solution containing the same amount of $OVA_{257-264}$ as liposome-coupled $OVA_{257-264}$ did not affect E.G7 tumor growth. It has been reported that CpG and the anti-IL-10 receptor antibody reverse tumor-induced DC paralysis, resulting in tumor rejection by CTL activated by DC (37). These results suggest that, by choosing appropriate lipid components for liposomes, tumor vaccines that use surface-coupled liposomal antigens to present tumor antigens to APCs and induce antitumor responses might be developed.

Thus, in contrast to previous investigations of liposome-based drug-delivery systems which have focused on encapsulation of antigens into liposomes, the potential of surface-linked liposomal antigens for application to vaccine development has been investigated (7, 38). During the course of this investigation, several advantages of liposome-coupled antigens over liposome-encapsulated antigens have become apparent: (i) A predominant coupling efficiency of antigens to liposomes; we have previously reported a procedure for coupling antigens to liposomes whereby approximately 50% of the antigens become bound to the surface of liposomes (11), whereas with antigen-encapsulation, a 60-fold greater volume of antigens is required to obtain the same amount of conjugates (unpublished observation). (ii) Antigen-specific and IgE-selective unresponsiveness induced by surface-linked liposomal antigens; antigens chemically coupled to the surface of liposomes induce antigen-specific IgG but not IgE antibody production in mice (10) and also in monkeys (39), suggesting that it might be possible to develop vaccines with minimal allergic side-effects by utilizing surface-linked liposomal antigens. In addition, during the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-linked liposomal antigen, we identified an alternative mechanism, not involving T cells, in the regulation of IgE synthesis (13). (iii) Enhanced recognition of liposomal antigens by APCs; since liposomes basically consist of immunologically inert fatty acid, they are barely recognized by APCs. Therefore, some contrivance, such as the introduction of mannose on their surfaces (40), is required in antigen-encapsulated liposomes to enhance the recognition of liposomes by APCs. On the other hand, with surface-linked liposomal antigens, antigens expressed on the surface of liposomes might be recognized more efficiently by APCs, which could result in enhanced presentation to T cells. In fact, surface-linked liposomal antigens induce significantly greater antigen-specific IgG production than do liposome-encapsulated antigens in mice (unpublished observation). In addition, a significant difference has been observed between liposomes with different lipid components in the recognition of liposomal antigens by APCs, this difference correlating closely with the adjuvant activity of liposomes. More antigens coupled to "unsaturated" liposomes are engulfed by macrophages *in vitro*, and greater antigen-specific antibody production is induced *in vivo*, than when "saturated" liposomes are used, suggesting that the adjuvant effects of liposomes are exerted at the beginning of the immune response, that is, upon recognition of antigens by APCs (28). In addition to this quantitative difference between liposomes with different lipid components, a qualitative difference (i.e., a difference in ability to induce cross-presentation) has also been observed between "saturated" and "unsaturated" liposomes. Although the precise mechanism underlying this difference is currently unclear, the significant difference in membrane mobility observed between these liposomes (12) might affect their ability to induce cross-presentation. These data suggest that differences in lipid components in liposomes lead to differential processing and presentation of liposomal antigens in APCs.

Induction of antigen-specific $CD8^+$ T-cells and cytotoxic T lymphocytes by liposome-coupled cytotoxic T lymphocyte epitopes derived from internal proteins of influenza viruses

Based on the results described above, we evaluated liposomal conjugates with CTL epitope peptides derived from highly conserved internal antigens of influenza viruses for their ability to protect against infection by influenza viruses (41). HLA-A*0201 (A2)-binding epitopes were predicted among the amino-acid sequences of six coding regions—M1, NP, PA, PB1, PB2, and NS—in the H1N1 influenza virus (PR8 strain) using programs available on the Internet. The predicted epitopes were then

Table 1. Amino acid sequences of CTL epitopes derived from internal antigens of influenza viruses

Virus strain	M1 58–66	NS1 128–136	PB1 410–418
H1N1 (A/PR/8/34)	GILGFVFTL	IILKANFSV	GMFNMLSTV
H3N2 (A/Aichi/2/68)	GILGFVFTL	VMLKANFSV	GMFNMLSTV
H1N1 (A/New York/4290/2009)	GILGFVFTL	IVLKANFSV	GMFNMLSTV
H5N1 (A/Hong Kong/483/97)	GILGFVFTL	IILKANFSV	GMFNMLSTV

*Disagreements are underlined.

synthesized and chemically coupled to liposomes to evaluate their abilities to induce antigen-specific CD8$^+$ T-cells and CTLs by means of ICS and an *in vivo* cytotoxicity assay, respectively. Immunization of the liposome-coupled, HLA A2-restricted epitope peptides, M1 58–66, NS1 128–136, and PB1 410–418 (Table 1) induced significant numbers of antigen-specific CD8$^+$ T-cells in HLA-A2-transgenic (A2Tg) mice, as evaluated by ICS (Fig. 1a). Moreover, as shown in Figure 1b, all the peptide-liposome conjugates in Figure 1a induced significant *in vivo* CTL responses, indicating that all the predicted CTL epitopes were loaded onto MHC class I and recognized by CTLs. On the other hand, even in the presence of CpG solutions of the predicted peptides did not induce antigen-specific CD8$^+$ T-cells in A2Tg immunized mice (Fig. 1a). As shown in Table 1 all three peptides were preserved well, not only in H1N1 (A/PR/8/34) and H3N2 (A/Aichi/2/68) seasonal influenza viruses, but also in the 2009 pandemic H1N1 (A/New York/4290/2009) influenza virus and in a highly pathogenic H5N1 (A/Hong Kong/483/97) influenza virus In contrast, NS1 128–136 had partially changed in H3N2 (A/Aichi/2/68) and H1N1 (A/New York/4290/2009) influenza viruses. Among the CTL epitopes determined in the present study, peptide M1 58–66 had already been reported by Gotch *et al.* in 1987 (42).

Virus challenge experiment

We performed virus challenge experiments using peptide M1 58–66 on the above-described HLA-A2-restricted CTL epitopes. A2Tg mice were immunized subcutaneously with liposome-coupled, HLA-A2-restricted peptide M1 58–66. One week after the immunization, we infected them intranasally with H1N1 (A/PR/8/34) or H3N2 (A/Aichi/2/68) influenza virus. As shown in Figure 2, viral growth in the lung was suppressed significantly in the immunized mice 2 to 6 days after infection by either H1N1 (A) or H3N2 (B) influenza viruses. Thus, in A2Tg mice, immunization with liposome-coupled peptides successfully induced protection against influenza viruses regardless of the influenza virus subtypes.

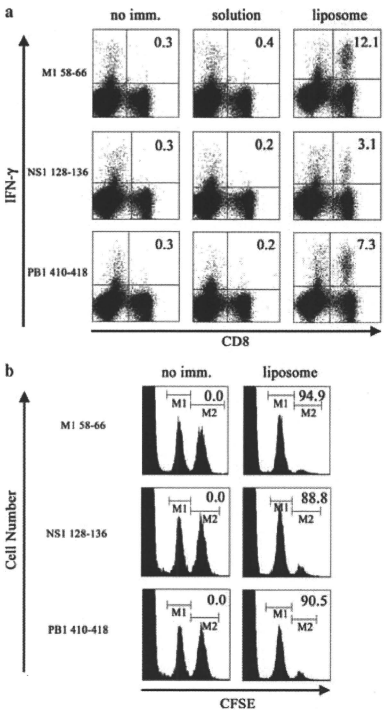

Fig. 1. Induction of antigen-specific CD8$^+$ T-cell and CTL responses. Mice received immunization with the liposome-coupled peptides M1 58–66, NS1 128–136, and PB1 410–418 (liposome) or liquefied peptides in the same amounts as liposome conjugates (solution) in the presence of CpG. Control mice received no immunization. One week after immunization ICS and *in vivo* CTL assay were performed. (a) ICS of antigen-specific CD8$^+$ T-cells among spleen cells of A2Tg mice. Cells were stained for their surface expression of CD8 (x axis) and their intracellular expression of IFN-γ (y axis). The numbers shown indicate the percentage of CD8$^+$ cells that were positive for intracellular IFN-γ. (b) Induction of CTLs by liposome-coupled peptides. The numbers shown indicate the percentage of total cells killed. The data shown are representative of three independent experiments. Imm., immunization.

Induction of memory CD8$^+$ T-cells

After confirming induction of protective ability by liposomal peptide in the effector phase, we investigated whether or not this immunization induced memory CD8$^+$ T-cells. As shown in Figure 3, significant numbers of CD8$^+$ T-cells

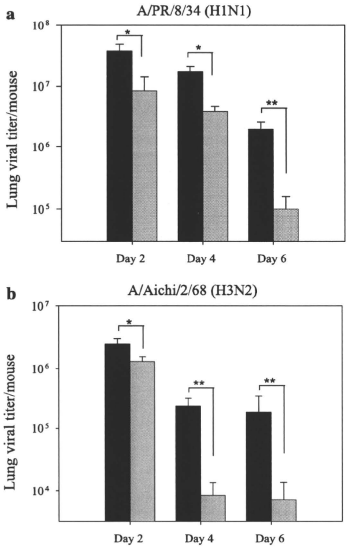

Fig. 2. Viral titers in lungs of mice following challenge with H1N1 or H3N2 influenza virus. Mice received immunization with either liposome-coupled peptide M1 58–66 liposome conjugates in the presence of CpG (gray bars) or none (black bars). One week later, mice were challenged intranasally with either (a) the H1N1 or (b) the H3N2 influenza virus. On days 2, 4, or 6 post-infection, viral titers in the lungs were determined by calculating the 50% tissue culture infective dose using MDCK cells. Data represent mean and standard error of five mice per group. *, $P < 0.05$; **, $P < 0.01$.

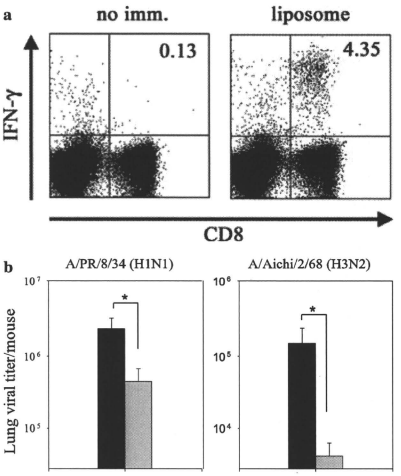

Fig. 3. Induction of long-lasting immunity by peptide-liposome conjugates. Mice were immunized with liposome-coupled peptide M1 58–66 either in the presence or absence of CpG. 90 days after immunization, ICS and viral challenge experiments were performed. (a) ICS of $CD8^+$ T-cells specific for peptide M1 58–66 among spleen cells of A2Tg mice immunized with liposome-coupled peptide (liposome) or not (no imm.). (b) Viral replication in the lungs of mice immunized with liposome-coupled peptide (gray bars) or not (black bars) 6 days after nasal infection with influenza viruses. Data represent the mean and standard error of five mice per group. imm., immunization; *, $P < 0.05$.

specific to peptides M1 58–66 were detected in the immunized mice at 90 days after immunization (Fig. 3a). In addition, viral growth in the lung was significantly suppressed after nasal challenge with either the H1N1 or H3N2 influenza virus (Fig. 3b). Thus, we demonstrated that immunization readily induces memory $CD8^+$ T-cells.

Induction of long-lasting protection against lethal doses of influenza viruses

We next investigated whether or not the long-lasting immunity demonstrated above would help protect mice against infection with lethal doses of influenza viruses. Six months after immunization with liposome-coupled M1 58–66 peptides, mice were challenged with a lethal dose of influenza virus H1N1 PR8 strain. As shown in Figure 4, although the immunized mice lost body weight up to 8 days after infection, at 4 to 7 days the rate of loss was significantly less than that observed in a non-immunized control group, and the immunized mice recovered body weight thereafter (Fig. 4a). Finally, five out of six immunized mice survived, whereas all of the non-immunized control mice died within 7 days of infection with significant loss of body weight and morbidity (Fig. 4b).

The data cited above indicate that liposome-coupled CTL epitope peptides derived from internal antigens of influenza virus protect against infection with heterosubtypic influenza viruses. Since the early finding that influenza A virus-specific CTLs are broadly cross-reactive for cells of the same MHC class I type infected with serologically distinct H1N1 and H3N2 viruses (20), numerous investigators have reported on immunodominant and cross-reactive CTL epitopes derived from conserved internal antigens, such as NP (43, 44), M1 (42, 44, 45), and NS1 (21) influenza viruses. It was hoped that CTL epitopes could be used to develop a broadly protective

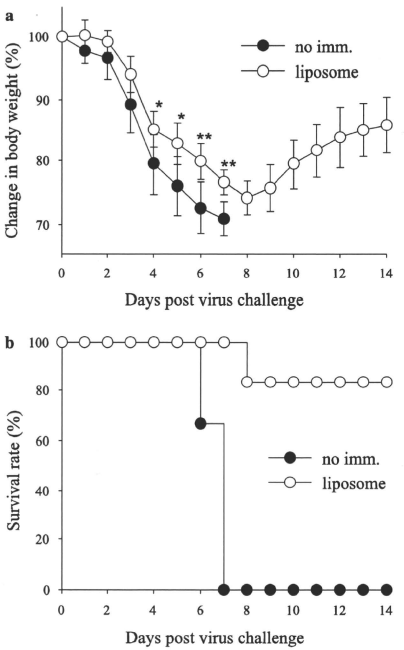

Fig. 4. Maintenance of protective ability against a lethal dose of H1N1 virus in mice immunized with liposome-coupled peptide. Mice were immunized with either liposome-coupled peptide M1 58–66 in the presence (open symbols) or absence (solid symbols) of CpG. Six months after immunization, mice were challenged intranasally with 5 × median lethal dose of H1N1 influenza virus (PR8 strain). The mice were weighed and monitored daily for mortality up to 14 days post-challenge. (a) Changes in body weight of mice calculated as a percentage of the mean weight per group in comparison with starting body weight. Data represent mean body weight and standard error of six mice per group. *, $P < 0.05$: **, $P < 0.01$ as compared with non-immunized control mice. (b) Survival percentage. The mice were killed once they had lost 30% of their initial body weight.

influenza vaccine (46). To date, candidate $CD8^+$ T-cell vaccines have been reported to induce partial protection (47), suggesting that $CD8^+$ T-cells certainly help to protect against influenza. Therefore, the $CD8^+$ T-cell vaccine strategy against influenza was expected to support antibody-focused vaccine strategies by "reducing" (46) or "dampening" (21) the impact of the next pandemic.

Liposome-coupled CTL epitope peptides efficiently induced antigen-specific $CD8^+$ T-cells and CTLs (Fig. 1), and suppressed viral replication in the lungs of mice infected with either H1N1 or H3N2 influenza virus (Fig. 2). In addition, since a single immunization just one week before infection successfully reduced viral replication in the lung, this vaccination protocol would be expected to counter the rapid spread of an influenza pandemic. The efficacy of liposome-coupled peptides in inducing CTL-based protective immunity is likely due to the characteristics of liposome-coupled antigens, which are very readily recognized by APCs (28) and effectively induce cross-presentation via MHC class I in APCs (16) In addition to their safety, they are less likely to induce allergic responses (13). Moreover, vaccines consisting of liposome-coupled CTL epitope peptides without $CD4^+$ T-cell epitopes are capable of inducing long-lived $CD8^+$ memory T-cells (Fig. 3). Mice immunized with liposome-coupled M1 58–66 peptides remained protected for at least 6 months after immunization (Fig. 4).

The CTL epitopes employed in this study are contained not only in the seasonal influenza viruses, but also in the currently emerging S-OIV and in the extremely virulent avian H5N1 influenza viruses (Table 1), suggesting that this liposomal peptide vaccine might be effective in protecting against infection by both seasonal and pandemic influenza viruses. In addition, the liposome employed in this study was originally developed as an antigenic carrier that effectively induces humoral immunity (i.e., antibody production) against liposome-coupled antigens (14). Therefore, antigen-liposome conjugates are capable of inducing both humoral and cellular immunity against influenza viruses, by combined coupling of antibody- and CTL epitopes to the surfaces of liposomes.

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