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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 $>10$ $\mu$g/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 $>1{,}000$ $\mu$g/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 $\mu$g/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 $\mu$g/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 $\mu$l of each plasmid (1 $\mu$g/$\mu$l) 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 $\mu$l of Luciferase Assay Substrate (Promega) was added to 10 $\mu$l 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 ($\mu$g) 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 $\mu$l 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-$\mu$l 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 $\mu$g/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

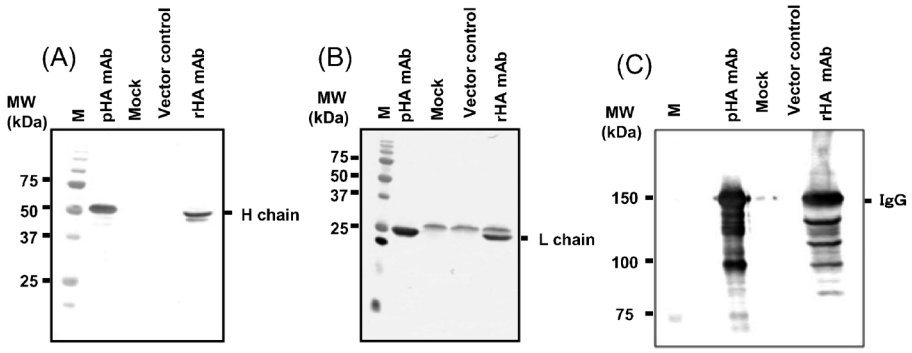


Fig. 1. In vitro expression of rHA mAb. Western blot analysis to determine the expression of the recombinant H chain (A), L chain (B), and IgG (C). Purified anti-HA mAb (pHA mAb) was compared with the supernatants of the 293T cells not transfected with any plasmid (Mock), transfected with pCADEST1-null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

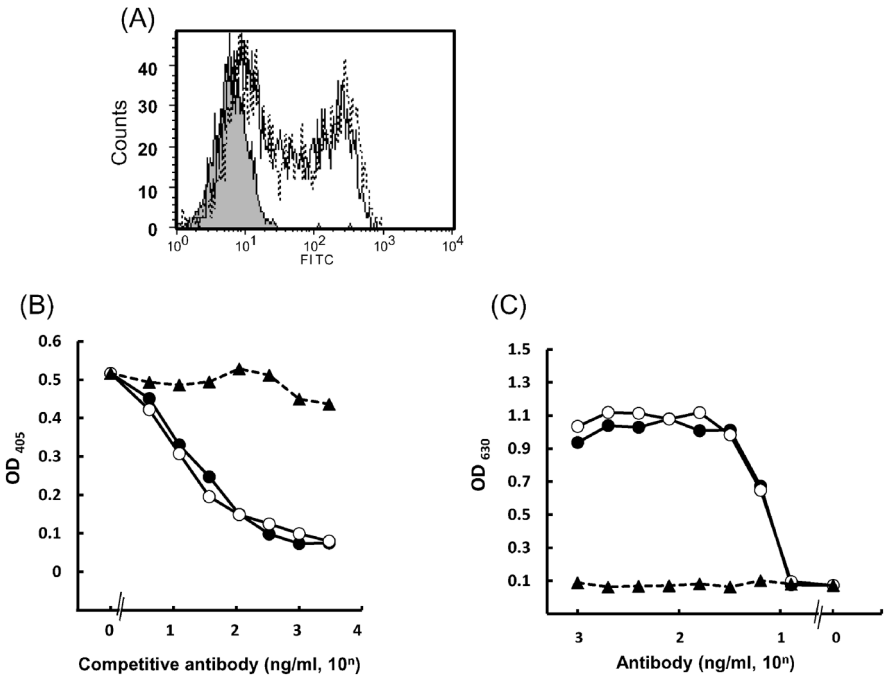


Fig. 2. Characterization of the rHA mAb in vitro. (A) The reactivity of rHA mAb with HA as a membrane protein was evaluated by flow-cytometric (FCM) analysis. 293T cells transiently transfected with pCAGGS-HA were stained with the pHA mAbs (solid line), the rHA mAbs (dotted line) and isotype control (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.

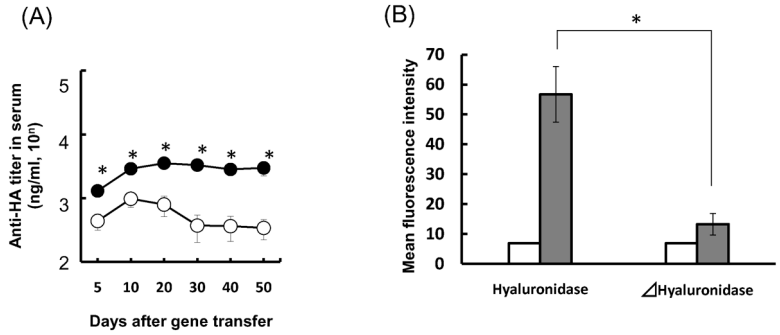


Fig. 3. In vivo expression of the rHA mAbs. (A) The time-course of the changes in the expression levels of the rHA mAbs in the sera of the mice that received the gene transfer into hyaluronidase-pretreated muscle (●, $n=5$) or into muscle without hyaluronidase pretreatment (○, $n=5$). The mAbs were evaluated 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). $^{*}P < 0.05$.

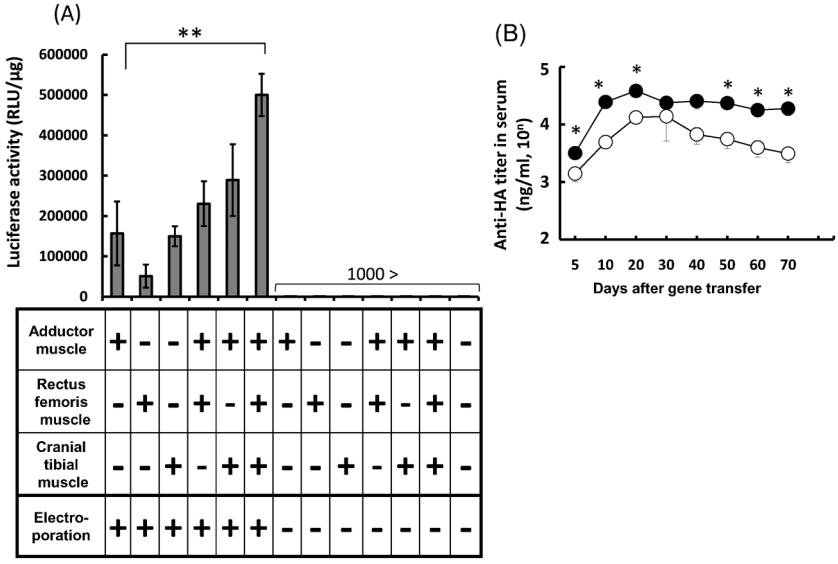


Fig. 4. (A) Effects of the number of injection muscles on the expression of the luciferase by the gene transfer. The luciferase gene transfer was carried out by a single intramuscular injection of 30 μg pCADEST1-Luc plasmid DNA into the each of muscle with or without electroporation. Two days after the 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,

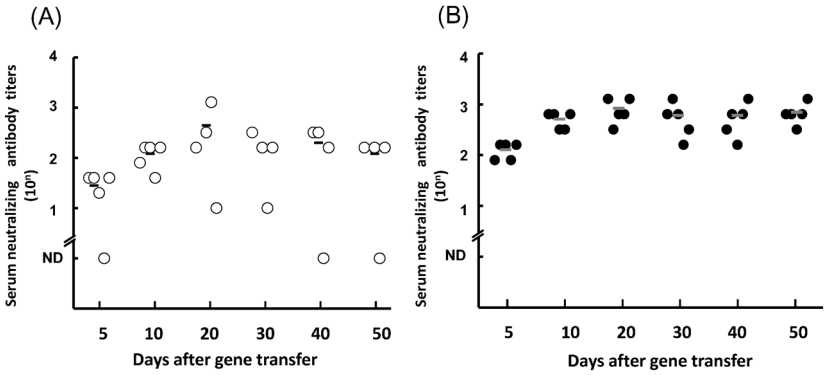


Fig. 5. Time-course of changes in the expression levels of the influenza virus (IFV)-neutralizing activity in the sera of mice (Fig. 4B) that received the gene transfer into adductor muscle (1 muscle, ○, $n=4$) and 3 muscles (●, $n=5$). Neutralizing antibody titer of the sera was measured by the use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

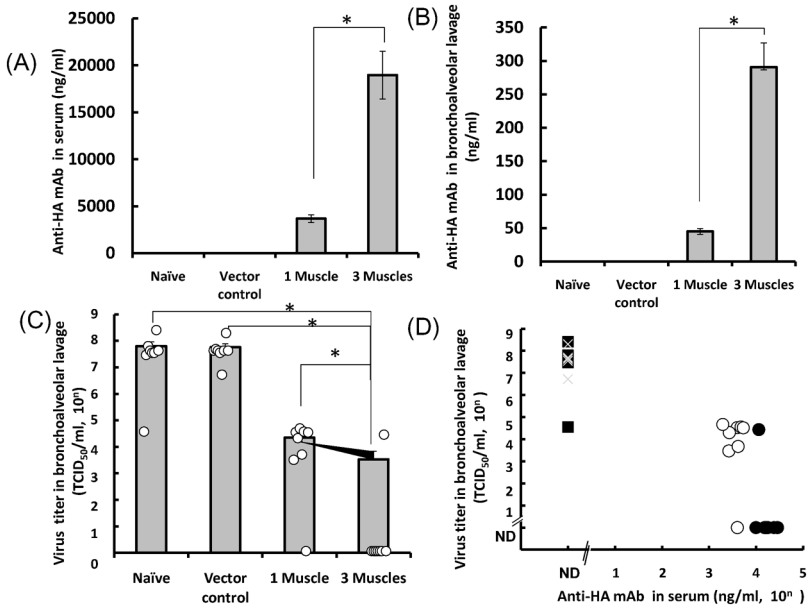


Fig. 6. Protection against IFV infection in ddY mice that received the mAb gene transfer. The mice were challenged with IFV 20 days after the mAb gene transfer. At 3 days after the lethal virus challenge, the expression levels of the rHA mAbs were measured in the serum samples (A) and bronchoalveolar 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

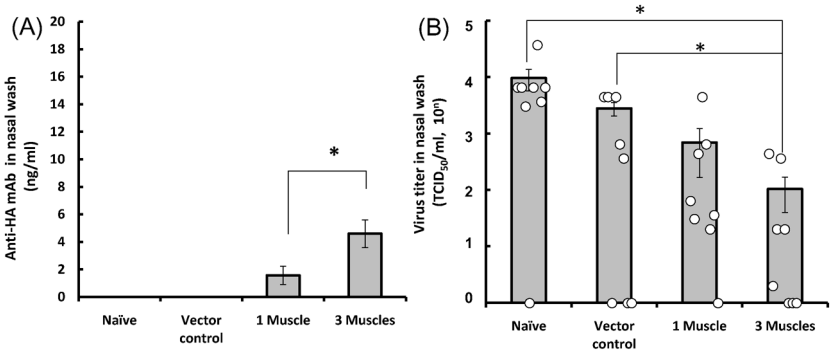


Fig. 7. The expression levels of the rHA mAbs and virus titer in the nasal washings obtained from the same ddY mice in Fig. 6. (A) The expression levels of the rHA mAbs were measured in the nasal washing by quantitative ELISA. (B) The viral titers in the nasal washings were determined by the $TCID_{50}$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

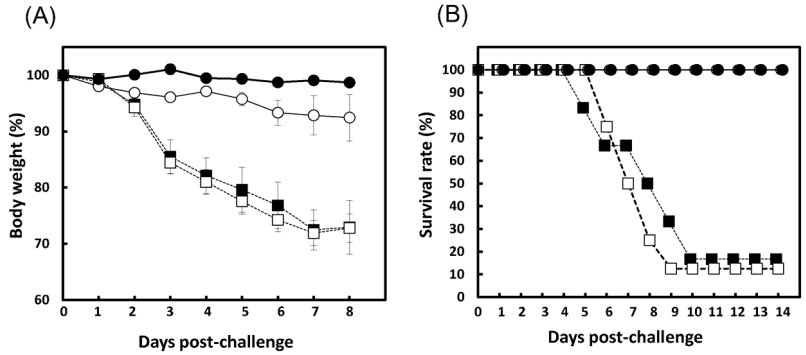


Fig. 8. Protection of the mice that received the mAb gene transfer from lethal A/PR8 virus challenge. The mice were challenged with IFV 20 days after the gene transfer and the body weight (A) and the survival rates (B) were monitored for 14 days. The body weight was expressed relative to the initial 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 $\mu$l 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

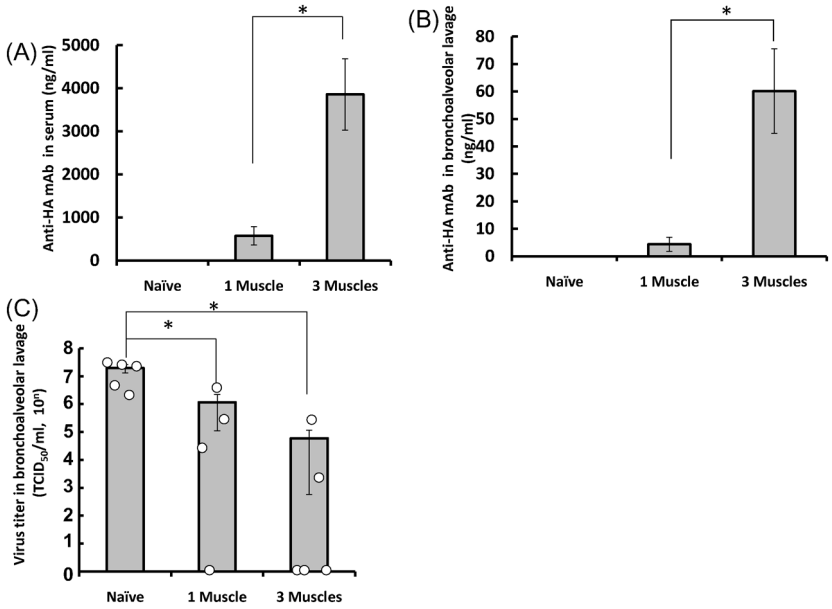


Fig. 9. Long-term expression of IFV-neutralizing rHA mAbs and the prophylactic efficacy against IFV infection. The mAb gene transfer into mice was executed as described above; naïve ($n = 5$), gene transfer into 1 muscle ($n = 4$), gene transfer into 3 muscles ($n = 5$). Around 130 days after the gene 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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