Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein

Ze Chen,1 Tomoki Yoshikawa,1 Shin-etsu Kadowaki,1 Yukari Hagiwara,1 Kazutoshi Matsuo,1 Hideki Asanuma,1 Chikara Aizawa,2 Takeshi Kurata1 and Shin-ichi Tamura1

1 Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan
2 Research Center for Biologicals, Kitasato Institute, 6-111 Arai, Kitamoto-shi, Saitama 364-0026, Japan

Protection against influenza virus infection and antibody responses in mice vaccinated with plasmid DNAs encoding haemagglutinin (HA), neuraminidase (NA) and nucleoprotein (NP) were compared among BALB/c (H-2d), B10 (H-2b) and C3H (H-2k) mice. Mice were inoculated with each DNA construct twice, 3 weeks apart, at a dose of 1 µg per mouse by particle-mediated DNA transfer (gene gun) to the epidermis. They were challenged with a lethal dose of the homologous virus 7 days after the second vaccination. NA-DNA provided significant protection in all strains of mouse, whereas HA-DNA afforded significant protection only in BALB/c mice. The serum antibody titres against NA or HA molecules in BALB/c, C3H and B10 mice were high, intermediate and low, respectively. NP-DNA failed to provide protection in any strain of mouse, and elicited low titres of anti-NP antibodies. These results suggest that NA-DNA can be used as a vaccine component to provide effective protection against influenza virus infection in various strains of mouse.

Introduction

A series of global studies on the DNA vaccine has revealed that it is capable of eliciting persistent humoral and cell-mediated immune responses to a wide range of viral antigens, following delivery by various routes (Tighe et al., 1998; Fynan et al., 1993; Raz et al., 1994; Ramsay et al., 1997; Macgregor et al., 1998). Several studies have demonstrated that plasmid DNA encoding haemagglutinin (HA) or nucleoprotein (NP) of influenza viruses, given by gene gun or intramuscular injection, elicited specific immune responses and provided protection against influenza virus in mice, ferrets and chicken (Ulmer et al., 1993, 1994; Robinson et al., 1993; Webster et al., 1994; Kodihalli et al., 1997). NP-DNA vaccination by intramuscular injection has been reported to provide cross-subtype cytotoxic T lymphocyte-mediated protection (Ulmer et al., 1993; Fu et al., 1997), but conventional immunization with NP and matrix protein (M1) failed to provide protection. In addition, when DNA vaccines against NP and M1 are given to mice, together with HA, by intramuscular injection, they are more effective, particularly against different strains of virus, than inactivated viruses or subvirion vaccines (Donnelly et al., 1995). On the other hand, we have demonstrated that in mice immunized using the gene gun, in which only a low dose of DNA is used, plasmid DNAs encoding the viral surface glycoproteins, HA and neuraminidase (NA), were the most protective DNAs among those encoding HA, NA, M1, NP or nonstructural protein (NS1) (Chen et al., 1998). We have also shown that a mixture of plasmid DNAs encoding HA and NA provided almost complete protection against a lethal virus infection (Chen et al., 1999).

The murine immune responses to various antigens and the susceptibility of mice to some pathogens are known to be controlled by H-2 or non-H-2 genes (Shreffler et al., 1975). We have previously reported that anti-HA antibody (Ab) responses in mice immunized intranasally with inactivated influenza virus vaccines were dependent on mouse strain: H-2b, H-2d and H-2k strains were high, intermediate and low responders, respectively (Hirabayashi et al., 1991). This variation in response among mouse strains to HA induced by the inactivated vaccines may not always be the same as that induced by vaccination with HA-DNA because the immune stimulation by plasmid DNAs encoding HA is similar to a virus
infection but dissimilar to exogenous protein antigens (Tighe et al., 1998; Ramsay et al., 1997; Donnelly et al., 1997). In this regard, the variation in Ab response to other viral components, supplied by DNA vaccines, remains to be clarified.

In these experiments, we compared the ability of plasmid DNAs encoding HA, NA or NP to protect against a homologous influenza virus challenge, together with the ability to induce Ab responses, in three different strains of mice: BALB/c, B10 and C3H. The results showed that NA-encoding plasmid DNA could provide effective protection against lethal virus infection in any strain of mouse. In addition, HA-DNA afforded protection only in BALB/c mice and NP-DNA failed to provide protection in any strain of mouse.

### Methods

#### Plasmid DNAs
Plasmids pCAGGSP7/HA, pCAGGSP7/NA and pCAGGSP7/NP were constructed by cloning the PCR products of HA, NA and NP genes from the A/PR/8/34 (PR8, H1N1) influenza virus strain into the plasmid expression vector pCAGGSP7, as described previously (Niwa et al., 1991; Chen et al., 1998).

#### Immunization and challenge
Plasmid DNAs encoding viral proteins were inoculated twice, 3 weeks apart, into the abdominal epidermis of BALB/c, B10 and C3H female mice (6 to 8 weeks old) obtained from Japan SLrc (Hamamatsu-shi, Shizuoka) using the Helios gene gun system (Bio-Rad Laboratories). One day before inoculation, abdominal fur in the local area was removed with a depilatory agent. Each epidermal inoculation employing the gene gun (a helium pressure-setting of 400 p.s.i. (ca. 2760 kPa) contained 1 µg DNA and 1 mg of 1 µm-sized gold powder. Seven days after the last inoculation, mice were challenged with the mouse-adapted strain of influenza virus, A/PR/8/34 (PR8, H1N1) (40 LD₅₀, 10⁻³⁶ EID₅₀ per mouse for BALB/c, 10⁻¹⁰ EID₅₀ per mouse for C3H and 10⁻¹¹ EID₅₀ per mouse for B10) by intranasal administration of 20 µl virus suspension. This infection caused rapid, widespread virus replication in the lungs and death in 6 to 8 days in unimmunized mice (Yetter et al., 1980; Tamura et al., 1992).

#### Specimens
Mice were anaesthetized with chloroform and then bled from the heart with a syringe. Serum was collected from the blood and used for IgG Ab assays. After bleeding, a ventral incision was made along the median line from the xiphoid process to the point of the chin. The trachea and lungs were excised and washed twice by injecting a total of 2 ml PBS containing 0·1% BSA. The bronchoalveolar lavage was used for virus titration after removing cellular debris by centrifugation (Tamura et al., 1992, 1996).

#### Purified NA molecules
The PR8 viruses, grown in 10-day-old embryonated eggs and purified by centrifugation across a continuous 30% to 60% sucrose gradient, were stored at −80 °C. They were disrupted by adding Triton X-100 to a final concentration of 4% (v/v) to freshly thawed virus concentrate, stirred and warmed at 37 °C for 1 h. The disrupted virus preparation was centrifuged at 15 000 r.p.m. at 4 °C for 30 min, to separate core proteins from surface proteins. PBS was added to the supernatant to make a final concentration of 1% Triton X-100. The diluted supernatant was applied to the Sepharose column-immobilized anti-HA monoclonal Abs and then to the Sepharose column-immobilized rabbit anti-NP polyclonal Abs. The effluent containing NA molecules was concentrated and applied to a Superdex G-200 column (Pharmacia Biotech). NA-rich fractions were eluted by PBS containing 0·1% Triton X-100 and identified by NA activity (Potier et al., 1979). They were pooled and used in ELISA as NA molecules.

#### Antibody (Ab) assays
The amount of IgG Ab to HA or NP molecules purified from PR8 virus was measured by ELISA. ELISA was performed sequentially from the solid phase (EIA plate, Coster) with a ladder of reagents consisting of: first HA or NP molecules purified from the PR8 virus according to the procedure of Phelan et al. (1980); second, serial twofold dilutions of sera from each group of immunized or preimmune mice; third, goat anti-mouse IgG Ab (r-chain specific) (Amersham) conjugated with biotin; fourth, streptavidin conjugated with alkaline phosphatase (GIBCO BRL); and finally, p-nitrophenyl phosphate. The chromogen produced was measured for absorbance at 410 nm in an Sj EIA Autoreader (model er-8000, Sanko Junyaku). Ab-positive cut-off values were set as mean ± 2 SD of preimmune sera. ELISA Ab titre was expressed as the highest serum dilution giving a positive reaction.

The amount of IgG Ab to PR8 NA molecules was also measured by ELISA, according to the method of Khan et al. (1982). The inhibition assay for neuraminidase activity by Ab (NI assay) was performed with the substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylmuramic acid according to the method described by Potier et al. (1979) and Deroo et al. (1996).

#### Virus titrations
Serial tenfold dilutions of the bronchoalveolar wash were prepared, and each dilution was injected into five embryonated eggs. The presence of virus in each egg was determined by the haemagglutination capacity of the allantoic fluid 2 days after injection. The virus titre of each specimen, expressed as the 50% egg-infecting dose (EID₅₀), was calculated from the lowest dilution of eggs with virus. The virus titre of each experimental group was represented by the mean ± SD of virus titre/ml of each specimen from all mice in each group (Tamura et al., 1992).

#### Statistics
Comparisons of experimental groups were evaluated by Student’s t-test; P < 0·05 was considered significant. For survival, probability was calculated by using Fisher’s exact test, comparing the frequency of survival in mice with viral protein expressing DNA versus control groups.

### Results

The ability of different strains of mouse immunized with HA-, NA- or NP-DNA to mount immune responses against influenza virus

The effects of DNA vaccination on protection against lethal influenza virus infection were investigated in BALB/c, B10 and C3H mice. The mice were immunized twice, 3 weeks apart, with 1 µg of plasmid DNA encoding PR8 virus HA, NA or NP using a gene gun to deliver DNA-coated gold beads into the skin of the abdomen. Negative control mice were immunized with a vector lacking the inserted protein gene. One week after the second DNA immunization, mice were challenged with a lethal dose of PR8 virus. Lung virus titres 3 days after, body weights 1 week after, and survival 3 weeks
Table 1. Protection against a lethal PR8 virus challenge in mice immunized with plasmid DNA encoding the HA-, NA- or NP gene

Different strains of mouse, BALB/c, B10 and C3H, were immunized twice, 3 weeks apart, with 1 µg plasmid DNA encoding various viral proteins from PR8 and challenged with a lethal dose of PR8 virus. Lung virus titres 3 days after infection were measured to evaluate the ability of each plasmid DNA to protect mice from acute lung infection (Table 1).

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>Mouse strain</th>
<th>H-2</th>
<th>Lung virus titre (log₁₀ EID₅₀/ml)</th>
<th>Weight loss† (%) of pre-challenge weight</th>
<th>No. of survivors/ no. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>BALB/c</td>
<td>d</td>
<td>4.2 ± 0.4*</td>
<td>5.2 ± 0.4*</td>
<td>36/40*</td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td>k</td>
<td>0.0 ± 0.5</td>
<td>19.1 ± 0.6*</td>
<td>6/20</td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td>b</td>
<td>0.2 ± 0.2</td>
<td>18.3 ± 0.7*</td>
<td>6/20</td>
</tr>
<tr>
<td>NA</td>
<td>BALB/c</td>
<td>d</td>
<td>4.4 ± 0.7*</td>
<td>5.3 ± 0.2*</td>
<td>33/40*</td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td>k</td>
<td>5.0 ± 0.3*</td>
<td>1.9 ± 0.5*</td>
<td>13/20*</td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td>b</td>
<td>4.8 ± 0.4*</td>
<td>11.3 ± 0.1*</td>
<td>13/20*</td>
</tr>
<tr>
<td>NP</td>
<td>BALB/c</td>
<td>d</td>
<td>0.1 ± 0.2</td>
<td>26.7 ± 0.4*</td>
<td>4/40</td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td>k</td>
<td>0.0 ± 0.1</td>
<td>20.4 ± 0.4*</td>
<td>0/20</td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td>b</td>
<td>0.1 ± 0.2</td>
<td>26.2 ± 0.2*</td>
<td>3/20</td>
</tr>
<tr>
<td>Vector</td>
<td>BALB/c</td>
<td>d</td>
<td>0.2 ± 0.3</td>
<td>25.9 ± 0.3*</td>
<td>4/40</td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td>k</td>
<td>0.9 ± 0.3</td>
<td>24.8 ± 0.3*</td>
<td>3/20</td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td>b</td>
<td>0.4 ± 0.3</td>
<td>26.7 ± 0.4*</td>
<td>3/20</td>
</tr>
</tbody>
</table>

* Significant difference (P < 0.05).
† Values represent mean ± SD of each group.

Table 2. Antibody titres in mice immunized by gene gun with HA-, NA- or NP-DNA

Mice were immunized twice, 3 weeks apart, with 1 µg of HA-, NA- or NP-DNA. Serum samples from the immunized mice were obtained 10 days after the second immunization. Values represent mean ± SD of each group of mice.

<table>
<thead>
<tr>
<th>Plasmid DNA</th>
<th>Mouse strain</th>
<th>Anti-HA Ab (2⁰)</th>
<th>Anti-NA Ab (2⁰)</th>
<th>NI assay (2⁰)</th>
<th>Anti-NP Ab (2⁰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>BALB/c</td>
<td>15.2 ± 3.2</td>
<td>10.4 ± 1.8</td>
<td>4.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>BALB/c</td>
<td>9.6 ± 0.6</td>
<td>7.7 ± 0.6</td>
<td>5.8 ± 2.6</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td></td>
<td></td>
<td></td>
<td>3.8 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td></td>
<td></td>
<td></td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>NP</td>
<td>BALB/c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

after infection were measured to evaluate the ability of each plasmid DNA to protect mice from acute lung infection (Table 1).

In mice immunized with HA-DNA, significantly lower lung virus titres were detected in BALB/c mice, but not in B10 or C3H mice. BALB/c mice also showed a significantly higher survival rate (90%) and a significantly lower weight loss (5.2%) compared with those of the negative control mice (10%, 25-9%). On the other hand, B10 and C3H mice did not show a significantly higher survival rate, although weight loss was low. Thus, HA-DNA provided effective protection against the lethal virus challenge in BALB/c mice, but it failed to afford effective protection in B10 and C3H mice. In mice immunized with NA-DNA, significantly lower lung virus titres were
detected in all three strains of mouse. A significantly higher survival rate and significantly lower weight loss were also observed in all strains of mouse. Thus, NA-DNA provided protection against the virus challenge in all three strains of mouse. In mice immunized with NP-DNA, a high lung virus titre, high weight loss and low survival rate were seen in all strains of mouse. Thus, NP-DNA failed to provide protection in any of the three strains of mouse.

The ability of different strains of mouse immunized with HA-, NA- or NP-DNA to produce Abs

Sera from the three strains of mouse taken 10 days after the second DNA immunization (3 days after challenge) were analysed for Ab titres to HA, NP or NA proteins by ELISA or NI assays as described in Methods. As shown in Table 2, Ab responses to each protein were different in each strain of mouse. In mice immunized with HA-DNA, the levels of Ab titres against PR8 HA molecules in BALB/c, C3H and B10 mice were high, intermediate and low, respectively. Similar results were obtained for mice immunized with NA-DNA; BALB/c, C3H and B10 mice were high, intermediate and low responders to NA molecules, respectively. However, the difference in anti-NA IgG Ab titre, estimated by both ELISA and NI assay, among the three strains was less than that of the anti-HA IgG Ab titre. On the other hand, NP-DNA immunization generated relatively low Ab responses in all strains of mouse.

Next, anti-HA IgG Ab subclass titres, for IgG1, IgG2a, IgG2b and IgG3, of the sera from three strains of mouse taken 10 days after the second HA-DNA immunization were determined by ELISA (Table 3). In BALB/c mice, IgG1 Ab responses which were about three times higher than those of IgG2a or IgG2b Ab were seen. In B10 mice, a low level of IgG1 Ab was generated, but IgG2a and IgG2b Abs were not detected. In C3H mice, high levels of IgG1, IgG2a and IgG2b Abs were produced. The IgG3 Ab could not be detected in any of the three mouse strains. These data suggest that the levels of IgG subclass Ab generated by DNA immunization using the gene gun are different in the three strains of mouse.

Table 3. Immunoglobulin G subclass induced by HA-DNA vaccination via gene gun

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Serum IgG subclass</th>
<th>ELISA (2)(^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>IgG1</td>
<td>9.0 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>IgG2a</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>IgG2b</td>
<td>2.5 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>IgG3</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>C3H</td>
<td>IgG1</td>
<td>7.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>IgG2a</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>IgG2b</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td></td>
<td>IgG3</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>B10</td>
<td>IgG1</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>IgG2a</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td></td>
<td>IgG2b</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td></td>
<td>IgG3</td>
<td>&lt; 1.0</td>
</tr>
</tbody>
</table>

* Values represent mean ± SD of each group.

Discussion

In this experiment, the ability of HA-, NA- and NP-expressing DNAs to provide protection against influenza virus was tested in three different strains of mouse immunized with 1 µg of plasmid DNA by the gene gun method (Table 1). HA-DNA provided effective protection in BALB/c mice, but only slight protection in C3H and B10 mice. NA-DNA provided protection in all strains of mouse tested, while NP-DNA failed to provide protection in any of the tested strains. In our previous experiments, we have shown that plasmid DNAs encoding HA and NA, but not M1, NP and NS1, protect BALB/c mice from lethal influenza virus challenge (Chen et al., 1998). These results suggest that both HA- and NA-DNAs (or both HA and NA molecules) are protective against influenza virus, and that NA-DNAs (or NA molecules) are the most protective in various strains of mouse.

The ability of HA-, NA- and NP-expressing plasmids to induce Ab responses against HA, NA and NP molecules was also examined in the three mouse strains (Table 2). It has already been shown that Abs against HA generally neutralize virus infectivity, thereby preventing infection. In addition, Abs against NA reduce virus replication to below the pathogenic threshold so that infection occurs without frank disease, but Abs against NP antigens cannot provide protection (Couch et al., 1974; Ada & Jones, 1986). In our experiments, the Ab titres against HA in BALB/c, C3H and B10 mice, immunized with HA-DNA were high, intermediate and low, respectively (Table 2). Protection against influenza virus provided by HA-DNA in BALB/c mice and the lack of protection in B10 mice seem to be dependent on the ability of the mouse to produce anti-HA Abs (Tables 1 and 2). The lack of protection in C3H mice with a relatively high level of anti-HA Ab production may be explained by assuming that the highest level of anti-HA Abs is required for providing the protection in C3H mice. Anti-NA Ab titres in BALB/c, C3H and B10 mice, immunized with NA-DNAs, were high, intermediate and low, respectively, although the difference of anti-NA Ab titres among the three strains was not so great as that of the anti-HA IgG Ab (Table 2). This suggests that even a relatively low level of anti-NA Ab is enough to prevent virus replication in any strain of mouse. In addition, NP-DNA immunization generated a relatively low Ab response in any strain of mouse, and failed to provide...
Influenza DNA vaccines in different mouse strains

Donnelly et al., 1997; Tighe et al., 1998.)

The ability of HA-DNAs to induce anti-HA IgG subclass Ab was also examined in different strains of mouse; IgG1 responses were most prominent in BALB/c and B10 mice, while IgG2a and IgG2b, as well as IgG1, were detected equally in C3H mice (Table 3). Thus, the appearance of the IgG subclass seemed to be different in each mouse strain, although it has been reported that gene gun immunization elicits Th2 cells that stimulate production of IgG1 isotype Ab (Pertmer et al., 1996; Feltquate et al., 1997).

In these experiments it was shown that mouse strains with H-2^a (BALB/c), H-2^b (C3H) and H-2^k (B10) haplotypes were high, intermediate and low responders to HA-DNA, respectively. On the other hand, mouse strains with H-2^b (and H-2^a), H-2^a and H-2^b (and H-2^a) haplotypes were high, intermediate and low responders to inactivated influenza virus vaccines (Hirabayashi et al., 1991). The discrepancy between a high response to HA-DNA and an intermediate response to HA molecules in inactivated vaccines in BALB/c (H-2^a) mice may be explained by different antigen processing and presentation pathways (Tighe et al., 1998; Ramsay et al., 1997; Donnelly et al., 1997). However, we cannot exclude the possibility that the discrepancy is due to different immunization routes: immunization with HA-DNA by gene gun versus intranasal immunization of inactivated vaccines. It is well known that foreign antigens processed by antigen-presenting cells must be recognized in association with surface H-2 (I-E) molecules by class II-restricted helper T cells prior to Ab production by B cells (Brodsky et al., 1991). The low responsiveness of B10 (H-2^b) mice to both HA-DNA and inactivated vaccines may be explained by an undetectable level of I-E antigen expression on the antigen-presenting cells owing to a failure to synthesize a normal Eα chain (Ozato et al., 1980; Jones et al., 1981). In addition, we cannot exclude the possibility that the low responsiveness of B10 mice to both HA-DNA and inactivated vaccine is due to the shortage of influenza virus-specific T cell clones.

DNA vaccines could not only serve as potentially safer alternatives to immunization with certain live virus vaccines, but also provide a promising approach to the development of effective subunit vaccines (Donnelly et al., 1997). The suggestions, based on our experiments, that both HA- and NA-DNAs (or both HA and NA molecules) are highly protective against influenza virus and that NA molecules could provide protection against influenza virus in various mouse strains, are useful for the development of more efficient subunit vaccines against influenza virus. Thus, although the inactivated vaccines are standardized only according to the content of HA antigen, the use of NA as one of the major components is another approach towards prevention of influenza. The relatively slower antigenic evolution of NA, compared with HA, should also be considered in vaccine development (Kilbourne et al., 1990). In this regard, Johansson et al. (1987) have shown that HA and NA molecules are competitive, with dominant anti-HA Ab responses over anti-NA Ab responses when they are present together on an intact live influenza virus as well as commercially inactivated vaccine. They have also shown that immunization with a mixture of purified HA and NA eliminates antigenic competition between HA and NA (Johansson & Kilbourne, 1994, 1996) and that supplementation of the conventional influenza A vaccine with purified viral NA results in a balanced and broadened immune response (Johansson et al., 1998). Furthermore, the finding that NA is protective in the different strains of mouse shown in this paper (Table 1) suggests that NA together with HA could provide effective protection against influenza virus in genetically heterogeneous humans.

References


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