Protective Immunity Afforded by Inactivated H5N1 (NIBRG-14) Vaccine Requires Antibodies against Both Hemagglutinin and Neuraminidase in Mice

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Background. Hemagglutination-inhibition (HI) antibody titers correlate with protective immunity to seasonal influenza viruses. However, inactivated H5N1 influenza vaccines from Vietnam 2004 strains afford protection without producing high or even detectable HI antibodies.

Methods. BALB/c mice were immunized twice (at a 3-week interval) with inactivated whole-virus influenza vaccine produced using reverse genetics, with the internal genes of A/PR/8/34 (a high-yield strain) and the hemagglutinin (HA) and neuraminidase (NA) genes of A/Vietnam/1194/04 (H5N1) virus (NIBRG-14) adjuvanted with alum (5 μg of HA). Either HA- or NA-binding antibodies were absorbed from the immune serum. The protective efficacy of these antibodies was determined by injecting the absorbed serum into severe combined immunodeficiency mice, which were then challenged with highly pathogenic H5N1 virus (A/Vietnam/Jp1203/2004; Japanese isolate of A/Vietnam/1203/2004).

Results. The NIBRG-14 vaccine elicited levels of anti-HA antibodies similar to levels elicited by the H1N1 vaccines, as well as levels of anti-NA antibodies higher than those elicited by the H1N1 vaccines. The absorption of either anti-HA or anti-NA antibody from immune serum samples obtained from NIBRG-14–vaccinated mice significantly reduced the protective efficacy of the serum.

Conclusions. For NIBRG-14 vaccines to confer protection to vaccinated hosts, both anti-HA and anti-NA antibodies are required. This finding implies that the measurement of both antibody levels may be required for accurate evaluation of vaccine efficacy.

To prepare for the emergence of pandemic viruses, H5N1 vaccine strains have been developed using reverse genetics [1]. However, in several clinical studies, it has been demonstrated that inactivated H5N1 vaccines induce lower levels of hemagglutination-inhibition (HI) and microneutralization (MN) antibody (Ab) titers than do seasonal influenza vaccines [2–6]. H5N1 viruses or vaccines derived from Vietnam 2004 strains poorly induced HI and MN Abs in immunologically naive ferrets and mice; this finding suggests that the immunogenicity of the vaccines, rather than the immunologic status of the vaccinated host, is responsible for the weak Ab response [7, 8]. Moreover, when influenza virus that bears a Ser223→Asn223 substitution in the hemagglutinin (HA) of Vietnam 2004 strains was used as detection antigen, the sensitivity of the HI test improved, demonstrating that the insufficient sensitivity of the HI test is also involved in the process [7].

The HI test predominantly detects anti-HA Abs, which are primarily responsible for virus neutralization in vivo. Therefore, the HI Ab titer is widely used as an immune correlate of vaccine efficacy [9]. However, H5N1 vaccines or viruses confer protection against Vietnam 2004 strains without there being any significant increase in HI titers [7, 8, 10]; this finding implies that the

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protective immunity provided by H5N1 vaccines requires immune components that cannot be detected by the HI test. The details of this protection and the immune correlates of vaccine efficacy remain to be determined.

In addition to the HA protein, the NA protein of influenza viruses can be the target of the protective Ab response. Although HA and NA are equivalently immunogenic on a molecular basis [11], the Ab response to NA is significantly suppressed when NA is presented in conjugation with HA. This phenomenon is known as intravirionic antigenic competition [12]. Thus, anti-NA Ab titers generated by whole inactivated vaccines are usually lower than those generated by immunization with equivalent amounts of purified NA, which may lead to less of a contribution of anti-NA Abs to protective immunity elicited by seasonal influenza vaccines.

Using a mouse model, we examined the relative contribution of anti-HA and anti-NA Abs elicited by whole inactivated H5N1 (NIBRG-14) vaccines adjuvanted with alum toward the induction of protective immunity. It was observed that the NIBRG-14 vaccine elicited anti-HA Abs with low HI and MN activity and higher levels of anti-NA Abs than did H1N1 vaccines. Of note, both anti-HA and anti-NA Abs are required for sufficient protection against infection with the highly pathogenic H5N1 virus. The data provide new insights into the protective immunity provided by H5N1 vaccines and prompt further investigation into the immune correlates of vaccine efficacy.

METHODS

Vaccine preparation. NIBRG-14 (H5N1) virus, which possesses modified HA and NA genes derived from the A/Vietnam/1194/2004 strain in the backbone of 6 internal genes of A/Puerto Rico/8/34 (PR8), was provided by the National Institute for Biological Standards and Controls. NIBRG-14, PR8 (H1N1), and A/New Caledonia/20/99 (NC20, H1N1) viruses were propagated in the allantoic cavity of the eggs of a 10-day-old embryonated hen and were purified through a 10%–50% sucrose gradient by means of ultracentrifugation. Viruses were resuspended in phosphate-buffered saline and were inactivated by treatment with 0.05% formalin at 4°C for 2 weeks. The protein and HA concentrations of the whole inactivated vaccines were determined as reported elsewhere [8]. The whole inactivated vaccines and alum solution (Pierce) were mixed in a 3:1 ratio and incubated at room temperature for 1 h before use.

Mice, vaccination, and challenge. BALB/c mice (age, 6–8 weeks; body weight [± standard deviation {SD}], 20.0 ± 0.8 g; Japan SLC) were subcutaneously primed with whole inactivated vaccines that contained 5 μg of HA and were boosted with the same vaccines at a 3-week interval. Two weeks later, serum samples were collected from each mouse. For virus challenge, anesthetized CB17–severe combined immunodeficient (SCID) mice (age, 8 weeks; body weight [±SD], 20.3 ± 0.6 g; CLEA Japan) were challenged intranasally with a lethal dose (5 × 50% lethal dose, 5 LD_{50}) of PR8 or A/VN/Jp1203/04 (volume, 20 μL) and monitored daily for survival. All experiments with highly pathogenic viruses were conducted in a biosafety level 3 containment facility. All mice were treated in accordance with the guidelines of the institutional animal care and use committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Baculoviral expression of recombinant HA (rHA) and recombinant NA (rNA). The HA and NA coding genes of NIBRG-14, PR8, and NC20 strains were amplified by polymerase chain reaction (PCR) to attach a 6× His tag to the C-terminal of HA and the N-terminal of NA. The amplified DNAs were cloned into pBacPAK8 (Clontech) and transfected into Sf-21 *(Spodoptera frugiperda)* insect cells. Recombinant baculoviruses that contained rHA and rNA genes were isolated, and Sf-21 cells were infected with them. The recombinant proteins tagged with 6× His were purified using Talon columns (Clontech), in accordance with the manufacturer's protocol. The purity of the purified proteins was analyzed using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Detection of serum Abs. Serum Abs against vaccine viruses were detected using an HI test performed with red blood cells from chickens, MN assay, and enzyme-linked immunosorbent assay (ELISA). The HI and MN assays were performed as described elsewhere [8], and titers below the limit of detection were assigned a value that was one-half of that limit. The concentrations of HA- or NA-binding immunoglobulin G1 (IgG1) Abs were determined by ELISA performed using baculovirus-produced rHA and rNA proteins as the coating antigen. Ninety-six–well plates were coated with 10 μg/mL rHA or rNA in 0.1 mol/L carbonate buffer (pH 9.0) at 4°C and blocked with 1% bovine serum albumin. For the use of rNA, 0.3 mmol/L Ca^{2+} was included in carbonate buffer to increase stability. Serially diluted serum samples were then added to each well, along with standard serum samples obtained from hyperimmunized mice. Horseradish peroxidase–conjugated goat anti–mouse immunoglobulin G1 (Southern Biotechnology) was added, and horseradish peroxidase activity was visualized using o-phenylenediamine dihydrochloride (Sigma). The relative concentrations of anti-HA or anti-NA antibodies were estimated by comparison to standard curves on each plate.

Detection of virus attachment by flow cytometry. The inactivated viruses were labeled with AlexaFluor647 (Invitrogen), and HA activity was determined using red blood cells from chickens. We confirmed that the HA activities of the inactivated viruses were not affected by the labeling procedure. After preincubation of the AlexaFluor647-labeled inactivated viruses (equivalent to 4HA) with 1/100-diluted heat-inactivated immune serum, the mixtures were added to Madin-Darby canine kidney (MDCK) cells (1×10^5 cells) and were further incubated at 4°C for 30 min. The stained cells were analyzed using FACS Vantage (BD Immunocytometry Systems), and the mean fluorescence intensity was analyzed using FlowJo software, version 8.8.2 (TreeStar).

Absorption of HA- and NA-binding serum Abs. His-tagged rHA and rNA proteins (1 mg) were conjugated with 1 mg of

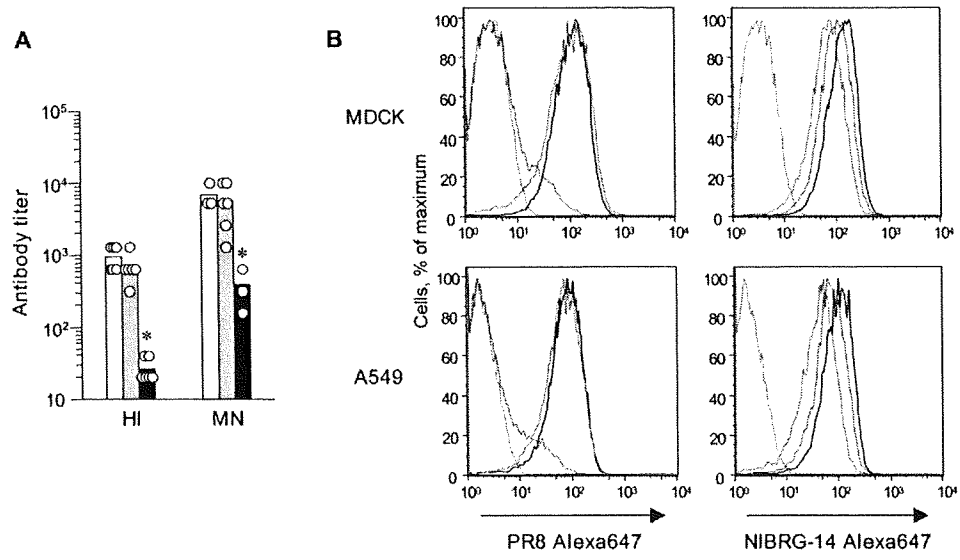

Figure 1. Poor inhibition of virus attachment in vitro by serum antibodies (Abs) elicited by the NIBRG-14 vaccine. *A,* Serum samples collected from PR8-, NC20-, or NIBRG-14–vaccinated mice (denoted by *white, gray,* and *black,* respectively) and determination of hemagglutination-inhibition (HI) and microneutralization (MN) activities of serum samples by use of homologous viruses. $*P < .05$, by Mann-Whitney nonparametric test (for HI) and Student's *t* test (for MN). *B,* Analysis of amounts of inactivated PR8 or NIBRG-14 viruses attached to Madin-Darby canine kidney (MDCK) or A549 cells *(black lines).* Inactivated viruses were preincubated with anti-PR8 serum *(blue lines)* or anti–NIBRG-14 serum *(red lines)* to estimate the inhibitory effects of each serum type. The data are representative of 3 independent experiments.

Talon resin (Clontech), and 3 mL of serum was applied onto the column that contained the rHA- or rNA-conjugated Talon resin. The flow-through was collected and applied again to repeat the absorption procedure twice. To estimate the protective efficacy of the serum Abs, 200 μL of serum that was deficient in HA- or NA-binding Abs was intravenously administered into the tail vein of CB17-SCID mice. The mice were challenged with 5 LD_{50} of PR8 or A/VN/Jp1203/04 (volume, 20 μL) viruses 6 h later.

Histopathologic examination and immunohistochemical (IHC) analysis. Mice were killed and dissected ventrally along the median line from the xiphoid process to the point of the chin. Excised lung, brain, and nasal tissue specimens were fixed with

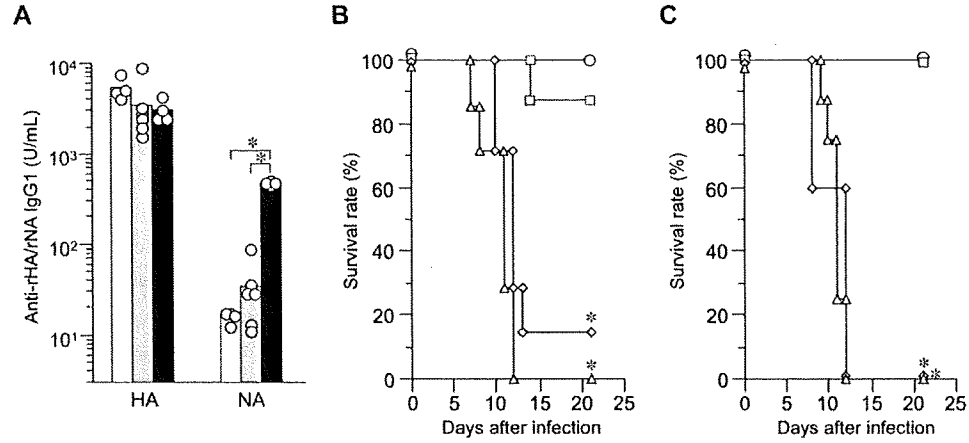

Figure 2. Levels of anti–hemagglutinin (HA)/neuraminidase (NA) serum antibodies (Abs) and the protection provided by immune serum. *A,* Anti–recombinant HA (rHA)/recombinant NA (rNA) immunoglobulin G1 (IgG1) Ab titers in the serum of PR8-, NC20-, or NIBRG-14–vaccinated mice (denoted by *white, gray,* and *black bars,* respectively) were determined by enzyme-linked immunosorbent assay. Each circle denotes the result obtained for an individual mouse. $*P < .05$, by Mann-Whitney nonparametric test (2-tailed test). *B* and *C,* CB17–severe combined immunodeficient (SCID) mice were administered 200 μL of serially diluted serum (*circles,* 1:1; *squares,* 1:2; *diamonds,* 1:4; and *triangles,* 1:20) obtained from mice vaccinated with NIBRG-14 *(B)* or PR8 *(C).* The mice were challenged with a lethal dose of A/VN/Jp1203/04 *(B)* or PR8 *(C),* and then survival curves were created using the Kaplan-Meier method. The generalized Wilcoxon test was used to calculate *P* values for the mice that were administered nondiluted and diluted serum. The data are representative of 2 independent experiments.

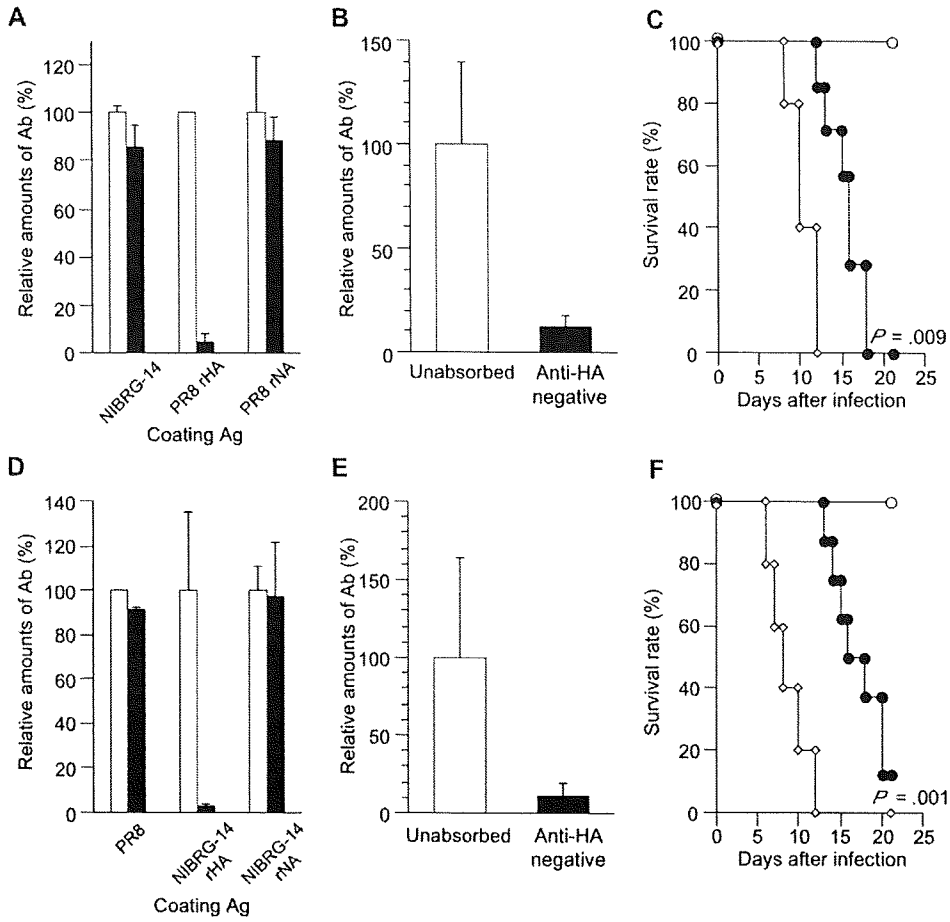

Figure 3. Protective role of hemagglutinin (HA)–binding antibodies (Abs) against homologous virus challenge. Immune serum samples obtained from mice that were vaccinated with PR8 *(A–C)* or NIBRG-14 *(D–F)* were applied to recombinant HA (rHA)–conjugated columns, and HA-binding Abs were absorbed. *A* and *D*, Amounts of immunoglobulin G1 Abs to rHA, recombinant neuraminidase (rNA), NIBRG-14 virus *(A)*, and PR8 virus *(D)* were determined by enzyme-linked immunosorbent assay and are expressed as the relative amounts against those of unabsorbed serum. *White bars* and *black bars* denote unabsorbed control and absorbed serum samples, respectively. Data are the mean ± standard deviation (SD) ($n = 3$). *B* and *E*, Amounts of neutralizing Abs were estimated by microneutralization (MN) assays performed using PR8 *(B)* and NIBRG-14 *(E)* as challenge viruses. Ab titers are expressed as relative amounts, compared with Ab titers of unabsorbed serum. Data are the mean ± SD ($n = 3$). *C* and *F*, CB17–severe combined immunodeficient (SCID) mice were administered 200 μL of serum, which had been passed through rHA-conjugated columns *(black circles)* or columns not conjugated with rHA HA *(white circles)*. As unprotected controls, serum samples from mice that were primed only with alum were also injected *(white diamonds)*. The mice were challenged with a lethal dose of PR8 *(C)* or A/VN/Jp1203/04 *(F)*. Survival curves were then created. The generalized Wilcoxon test was used to calculate *P* values for the mice that were administered absorbed serum or unabsorbed control serum. The data are representative of 2 independent experiments. Ag, antigen.

10% neutral-buffered formalin. The nasal tissue specimens were decalcified in ethylenediaminetetraacetic acid solution. After fixation, tissues were embedded in paraffin by use of conventional methods and were stained with hematoxylin-eosin or subjected to IHC staining using antiserum against the nucleoprotein of PR8 virus. IHC staining was performed using 3-3' diaminobenzidine as the substrate.

RESULTS

Poor inhibition of the virus attachment by serum Abs induced by NIBRG-14 vaccine. Serum Ab titers of vaccinated mice were evaluated by performing HI and MN assays. Consistent with reports published elsewhere [7, 8, 10], the levels of both HI and MN Abs were lower in mice vaccinated with NIBRG-14 than in mice vaccinated with PR8 and NC20 (figure 1*A*). HI and MN assays measure the level of anti-HA Abs, which block virus attachment to sialylated cellular receptors. In addition, the MN assay used in the present study detects anti-NA Abs at a lower sensitivity [13]. To confirm that the immune serum elicited by NIBRG-14 poorly inhibits virus attachment to cellular receptors, we quantitated the viruses attached to MDCK cells by means of flow cytometry. It was observed that both inactivated viruses exhibited similar levels of attachment to MDCK cells (figure 1*B*). Pretreatment of PR8 virus with anti-PR8 serum

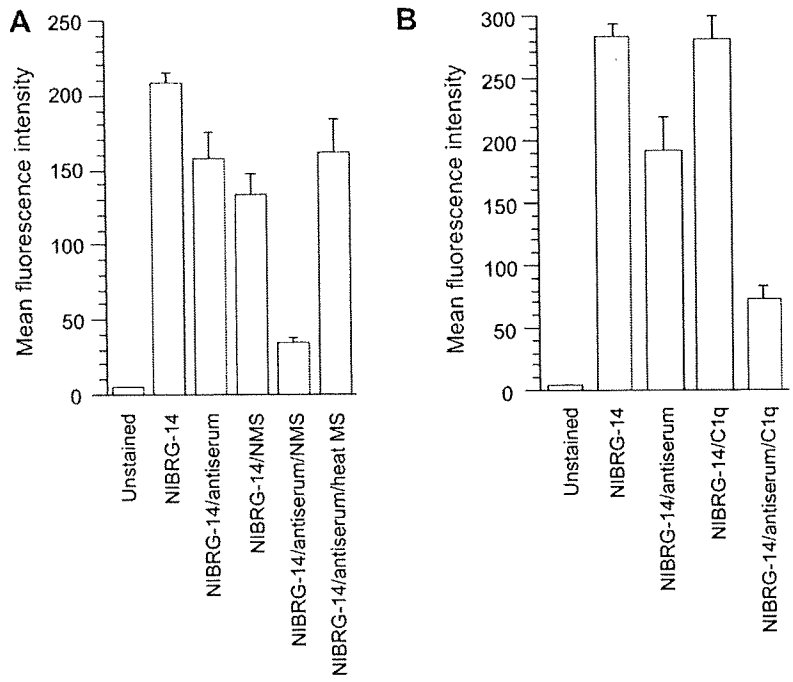

Figure 4. Complement enhanced the inhibition of virus attachment by immune serum. The mean fluorescence intensity of the viruses attached to Madin-Darby canine kidney (MDCK) cells was determined by flow cytometry, as shown in figure 2*B*. *A*, Inactivated NIBRG-14 viruses were preincubated with anti–NIBRG-14 serum in the presence or absence of naive mouse serum (NMS) or heat-inactivated naive mouse serum (heat MS) (final 10%). *B*, Inactivated NIBRG-14 viruses were preincubated in the presence or absence of purified human C1q (10 μg/mL). Data are the mean ± standard deviation ($n = 3$). Data are representative of 2 independent experiments.

completely inhibited virus attachment, whereas pretreatment of NIBRG-14 virus with anti-NIBRG-14 serum did not inhibit the attachment of NIBRG-14 virus. A similar result was obtained using type II alveolar epithelial cell line A549, which showed that anti–NIBRG-14 serum cannot efficiently inhibit virus attachment, irrespective of the cell type used.

Efficient elicitation of protective serum Abs by NIBRG-14 vaccine in vivo. The poor HI and MN activity of serum from NIBRG-14–vaccinated mice could be attributed to the small quantity or compromised activity of HA-binding Abs. To examine this possibility, the levels of serum Abs specific to HA and NA were determined by ELISA performed using rHA and rNA as antigens. When alum was used as an adjuvant, it was observed that the IgG1 isotype dominated the specific Ab response [14]; therefore, we examined the levels of HA- and NA-binding IgG1 titers in the serum of vaccinated mice (figure 2*A*). Inactivated NIBRG-14, PR8, and NC20 vaccines elicited similar amounts of HA-binding IgG1 Abs, suggesting that the H5 protein of NIBRG-14 possesses sufficient immunogenicity in this immunization protocol. In contrast, NIBRG-14 vaccines induced significantly higher levels of NA-binding IgG1 Ab than did PR8 and NC20 vaccines. This increase in Ab levels was not the result of a lack of sensitivity in the detection of Abs specific for PR8 and NC20 rNAs, because PR8 rNA captured anti-NA Abs induced by NIBRG-14 vaccine at levels >14-fold higher than those induced by H1N1 vaccines. These results suggest that the N1 protein of NIBRG-14 might be more immunogenic and/or stable than those of PR8 and NC20, which easily undergo degradation without Ca^{2+} [15].

The protective immunity induced by subcutaneous injection of alum-adjuvanted inactivated influenza vaccines largely depends on the generation of neutralizing Abs in the serum samples, and the transfer of 200 μL of anti–NIBRG-14 serum from vaccinated mice into immunocompromised SCID mice conferred protection against lethal challenge with H5N1 virus (figure 2*B*). Serial dilution of anti–NIBRG-14 serum and anti-PR8 serum comparably reduced their protective efficacy, and the 4-fold dilution of both types of serum diminished their protective effect. These results suggest that inactivated NIBRG-14 vaccines have sufficient immunogenicity and induce the generation of protective virus-binding Abs, compared with inactivated H1N1 vaccines, although their HI and MN activities are low.

Contribution of non–HI, HA-binding Abs to protection. The protective role of anti-HA Abs in vivo was investigated. After the absorption of HA-binding Abs, levels of residual HA-binding Abs were determined by ELISA and expressed as the percentage of unabsorbed controls. Results showed that the amount of HA-binding Abs in the postabsorbed serum from both NIBRG-14–vaccinated and PR8-vaccinated mice was reduced to <5% of the amount in the unabsorbed control serum (figure 3*A* and 3*D*). MN Ab titers in the absorbed serum from both NIBRG-14–vaccinated and PR8-vaccinated mice were also reduced to approximately one-

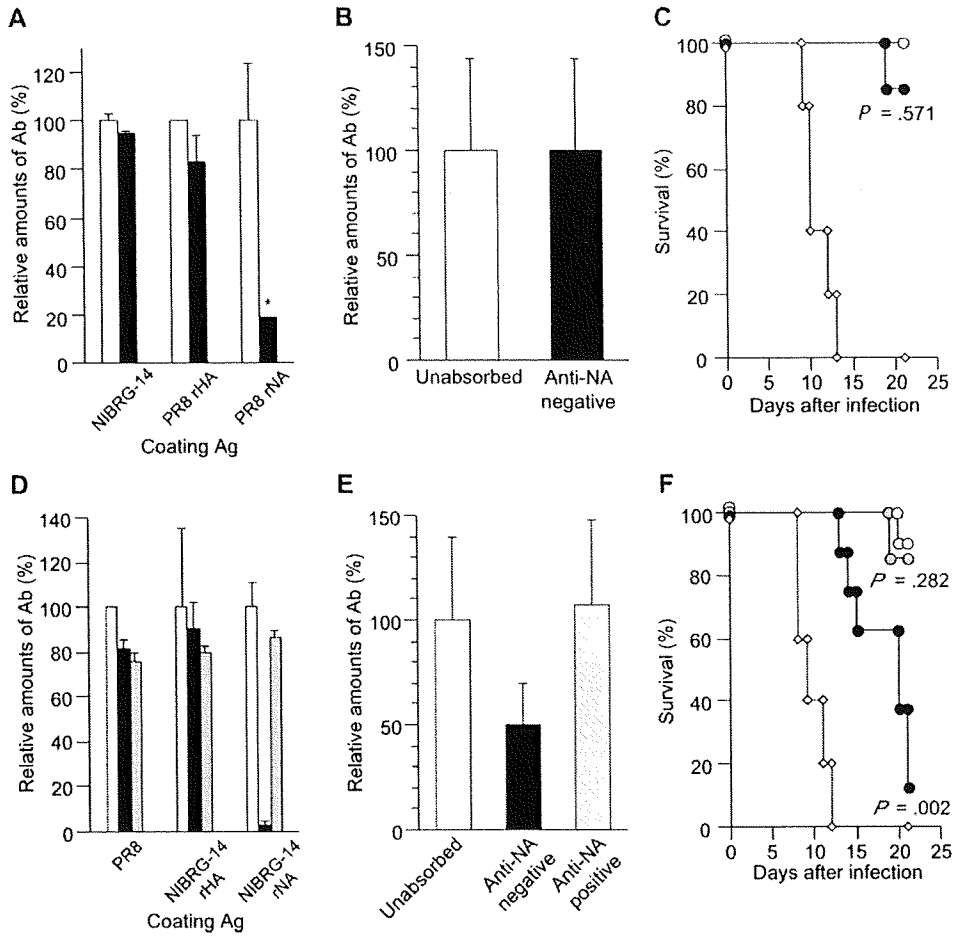

Figure 5. Protective role of neuraminidase (NA)–binding antibodies (Abs) against homologous virus challenge. Anti-NA Abs were absorbed from the serum of mice that were vaccinated with PR8 *(A–C)* or NIBRG-14 *(D–F)*, and the absorption efficiency was estimated by enzyme-linked immunosorbent assay, as shown in figure 3 *(A, D)*. For NIBRG-14, the serum from recombinant NA (rNA)–primed mice was added into NA Ab–depleted serum to restore the levels of anti-NA Abs to the levels in the unabsorbed control serum *(gray bars)*. Data are the mean ± standard deviation ($n = 3$). *Ab titers are below the detection limit. Amounts of neutralizing Abs were estimated using MN assays *(B, E)*, and the protective efficacy of each serum type in vivo *(C, F)* was estimated as shown in figure 3. Gray circles denote mice that were administered the serum mixture (anti–rNA Ab-depleted serum and anti-rNA Ab-containing serum). Data are representative of 2 independent experiments. Ag, antigen; rHA, recombinant hemagglutinin. *Black bars*, absorbed serum samples; *white bars*, unabsorbed control samples.

tenth of the MN Ab titers in the unabsorbed control serum (figure 3*B* and 3*E*). This result shows that the remaining Abs specific for the native form of HA are below the minimal threshold (one-fourth of unabsorbed serum) for the protection of infected mice (figure 1*B* and 1*C*). To compare the protective efficacy of immune serum with or without anti-HA Abs, CB17-SCID mice were intravenously administered each type of serum and then intranasally challenged with 5 LD_{50} of PR8 or a highly pathogenic H5N1 virus. Mice that were administered serum without anti-HA Abs were more susceptible to lethal infection with PR8 virus than were those that were administered unabsorbed serum (figure 3*C*) ($P = .009$, by the generalized Wilcoxon test); this finding confirms that anti-HA Abs play a pivotal role in virus neutralization in vivo. Similarly, the absorption of anti-HA Abs from the serum samples of mice that were administered NIBRG-14 vaccine significantly reduced the protective efficacy against a lethal challenge with the highly pathogenic A/Vietnam/Jp1203/2004 virus (figure 3*F*) ($P = .001$). Thus, these results support the possibility that HA-binding Abs with low HI and MN activity in anti–NIBRG-14 serum contribute to in vivo protection against a homologous virus challenge.

Association of complement with enhancement of the inhibitory effect of virus attachment by anti–NIBRG-14 serum. Feng et al. [16] have reported that low HI and MN activity of several H1-specific monoclonal Abs can be enhanced by the addition of the complement factor C1q, implying that the in vivo protective efficacy of anti–NIBRG-14 serum with compromised HI and MN activity may be improved by the addition of this complement factor to the serum. To investigate this hypothesis, we monitored the inhibitory effect of anti–NIBRG-14 serum against virus attachment to MDCK cells in the presence of C1q. Preincubation of inactivated NIBRG-14 virus with anti–NIBRG-14 serum in the presence of naive mouse serum or pu-

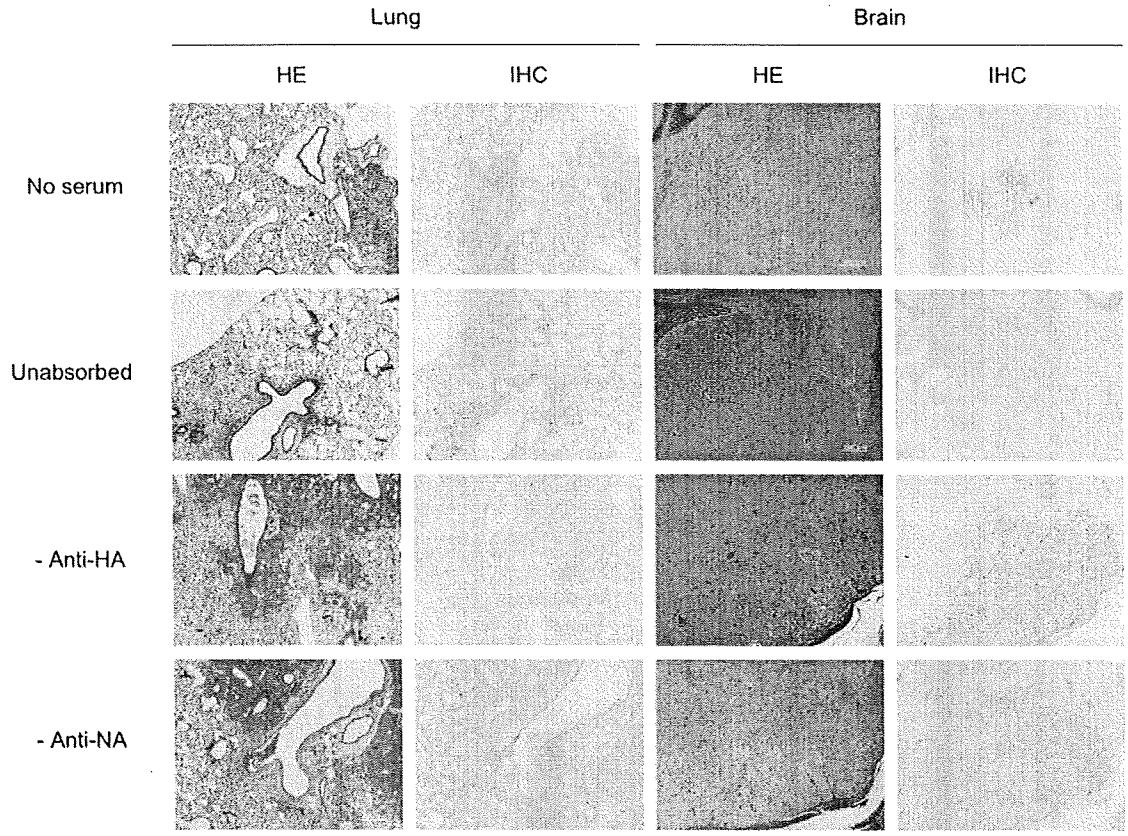

Figure 6. Histopathologic findings in mice infected with a highly pathogenic H5N1 virus. CB17–severe combined immunodeficient (SCID) mice were reconstituted with unabsorbed immune serum (unabsorbed serum) or with serum absorbed by a recombinant hemagglutinin (rHA) or an recombinant neuraminidase (rNA) column. Naive SCID mice that were not reconstituted with serum were used as control mice. At 8–10 days after infection, the lungs and brain of each mouse were collected, fixed with formalin, and analyzed by hematoxylin-eosin staining or antinucleoprotein immunohistochemical analysis (original magnification, ×50).

rified C1q partially enhanced the inhibition of virus attachment by the serum (figure 4). These data suggest that the inhibitory effect of anti–NIBRG-14 serum against virus attachment may depend on the presence of complement factors.

Contribution of NA-binding Abs to protection. The protective efficacy of anti-NA Abs was examined by an adoptive transfer of the absorbed serum. The amount of anti-NA Abs in the absorbed serum from PR8-vaccinated mice could not be detected, even by use of the highly sensitive ELISA. Therefore, the amount of NA-binding Abs remaining in this serum (<18.8% of the amount noted in control serum) could not be precisely determined (figure 5*A*). Both MN assay and the serum transfer experiments revealed that the neutralization ability of the absorbed serum was not significantly different from that of the unabsorbed control serum (figure 5*B* [$P = .500$] and 5*C* [$P = .571$]). In contrast, the absorption of anti-NA Abs from the immune serum of NIBRG-14–vaccinated mice significantly reduced the protective efficacy of the serum, and the mice that were administered this absorbed serum died more rapidly than did those that were administered the unabsorbed control serum (figure 5*F*) ($P = .002$). However, the MN assay did not detect a significant difference between the neutralization ability of the absorbed and unabsorbed serum (figure 5*E*) ($P = .056$).

To confirm whether the reduction in the protective efficacy of the serum is solely the result of the absorption of anti-NA Abs, we added the immune serum of rNA-immunized mice to the absorbed serum to restore the level of anti-NA Abs. This addition of immune serum from rNA-immunized mice restored the amount of anti-NA Abs in the absorbed serum to the level of that in the unabsorbed control serum (figure 5*D*). Of note, the adoptive transfer of serum supplemented with anti-NA Abs prolonged the survival of infected mice to the length of survival of control mice (figure 5*F*) ($P = .282$). This finding confirmed that the anti-NA Abs contained in the serum of NIBRG-14–vaccinated mice were responsible for providing immune protection. Thus, these data indicate that, even in the presence of anti-HA Abs, anti-NA Abs elicited by NIBRG-14 vaccines can afford protection against a lethal infection with homologous H5N1 virus.

Virus spread and pathologic findings in mice reconstituted with either HA or NA Ab–absorbed serum. Mice that were administered immune serum lacking either anti-HA or anti-NA Abs were subjected to histopathologic analysis. In naive mice, an

abundance of viral nucleoprotein antigens was detected in both the lungs and brain, and interstitial infiltration of inflammatory cells was also observed (figure 6). In contrast, viral nucleoprotein antigen in the lungs of mice reconstituted with unabsorbed control serum was scarcely present in the lungs and was absent in the brain 10 days after infection. Absorption of either HA or NA Abs was associated with a similar phenotype, and viral antigens were abundantly detected in both the lungs and the brain. Thus, absorption of either type of Ab results in a distinct pathologic difference from SCID mice reconstituted with unabsorbed serum; however, the pathologic characteristics were comparable among mice given either HA Ab– or NA Ab–deficient serum.

DISCUSSION

The data presented in this study suggest that NIBRG-14 vaccines possess sufficient immunogenicity and induce comparable amounts of anti-HA Abs. Although the anti-HA Abs have weak HI and MN activity, they contributed to the neutralization of homologous H5N1 virus, partially with the help of complement factors in vivo. Of note, the higher level of anti-NA Abs elicited by NIBRG-14 vaccines also participated in protection, even in the presence of anti-HA Abs. These results provide new insights into the protective immunity elicited by currently stockpiled H5N1 vaccines and indicate that methods other than the conventional HI assay are required for the estimation of vaccine efficacy.

Induction of anti-HA Abs with low HI activity has been observed in mouse and ferret models of H5N1 and H2N2 avian influenza virus infection, suggesting that this phenomenon is not limited to a particular host species or to a subtype of avian influenza virus [7, 8, 17]. When whole influenza virus that bore a Ser223→Asn223 substitution in the HA of Vietnam 2004 strains was used as detection antigen, the sensitivity of the HI assay improved [7, 17]; this finding supports the insensitive detection of anti-HA Abs by conventional HI assay rather than the poor induction of anti-HA Abs (figures 1 and 2). Ser223 does not appear to be included in the antigen recognition sites of several mAbs against the HA of Vietnam 2004 strain, and the Ser223→Asn223 substitution may alter the conformation or receptor specificity of the H5 protein, which eventually increases HI sensitivity [7, 18]. Moreover, the sensitivity of the HI assay for antiavian H2 Abs was improved by using only the isolated H2 HA antigen instead of the whole virus [17]. These results suggest that the unique antigenic structure of HA or the interaction between HA and other viral antigens on the surface of avian influenza virus may significantly affect the sensitivity of the HI assay.

The addition of complement to the anti–NIBRG-14 serum partially restored the neutralization activity of the serum, similar to that of mAbs against H1 protein [16]. The large complex formed by the complement and the HA-bound Abs may cause steric interference between HA and sialic acid, as has been postulated elsewhere [19]. To our knowledge, steric interference on virus attachment has been reported only in monoclonal Abs against HA of PR8 virus. In the present study, we found that it can also occur in polyclonal Abs against H5N1 virus. Additional studies are required to clarify whether the phenomenon can be observed for all influenza viruses and whether the balance of HA and NA activities is involved in this process.

It has been shown that large amounts of anti-NA Abs contribute to providing protection to the host [11, 20]. However, when NA is conjugated with the HA antigen in the form of a whole virion, the anti-NA Ab response is inhibited by intravirionic antigenic competition [12]. Thus, the level of anti-NA Abs elicited by inactivated PR8 and NC20 vaccines may be insufficient for providing immune protection to the host (figure 5). In this context, the significant contribution of anti-NA Abs elicited by the NIBRG-14 vaccine to provision of immune protection is remarkable, and it may partially explain the protection that is independent of HI and MN Ab titers. The anti-NA Ab titer elicited by the NIBRG-14 vaccine reached a level that was approximately one-sixth of the anti-HA Ab titer (figure 2*A*), which is close to the ratio of HA to NA proteins noted on the surface of virion (5:1). This finding suggests that intravirionic competition is reduced in NIBRG-14 vaccine.

At present, we do not know the exact mechanisms underlying the enhanced induction of anti-NA Abs by NIBRG-14 vaccine. Given the labile nature of N1 protein, the differential induction of anti-NA Ab between NIBRG-14 and H1N1 vaccines may reflect that N1 protein from NIBRG-14 is more stable and immunogenic than N1 proteins from PR8 and NC20 after the process of vaccine preparation. In addition, the extent to which HA and NA antigens are cross-linked by formalin treatment may be weak on the surface of NIBRG-14, leading to less intravirionic competition.

Recent clinical studies have used clade 1 vaccines from A/Vietnam/04 strains, but most avian H5N1 viruses prevalent in the past year belong to clade 2. Thus, rgA/Indonesia/5/2005 (clade 2.1) and rgA/Anhui/1/2005 (clade 2.3) were selected as vaccine seed viruses in Japan, but the H5 proteins of both viruses possess the Ser223 residue, indicating that the HI and MN activity of the anti-HA Abs elicited by each vaccine may be compromised. Recently, it has been shown that anti-HA Abs elicited by rHA of the rgA/Indonesia/5/2005 strain showed a level of homologous HI activity that was 4.6-fold higher than that of Vietnam 2004 strain [21]. Thus, the interference of HI activity in clade 2 strains may be modest. We are currently characterizing the anti-HA and anti-NA Abs elicited by clade 2 vaccines, to clarify whether the data obtained for the NIBRG-14 vaccines can be generalized to other clades of H5N1 vaccines.

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