Suboptimal Humoral Immune Response against Influenza A(H7N9) Virus Is Related to Its Internal Genes

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Influenza A(H7N9) virus pneumonia is associated with a high case fatality rate in humans. Multiple viral factors have been postulated to account for the high virulence of the virus. It has been reported that patients with influenza A(H7N9) virus infection have relatively low titers of neutralizing antibodies compared to those with seasonal influenza virus infections. In this study, we compared serum hemagglutination inhibition (HI) and microneutralization (MN) antibody titers of mice challenged with wild-type A(H7N9) viruses [H7N9(Anhui) and H7N9(Zhejiang)], an A(H1N1)pdm09 virus [pH1N1(2009)], and a recombinant A(H7N9) virus with PR8/H1N1 internal genes (rg-PR8-H7-N9). All mice infected by H7N9(Anhui) and H7N9(Zhejiang) developed serum HI antibodies at 14 days postinfection (dpi) but no detectable MN antibodies, even at 28 dpi. A low level of neutralizing activity was detected in H7N9(Anhui)- and H7N9(Zhejiang)-infected mice using fluorescent focus MN assay, but convalescent-phase serum samples obtained from H7N9(Anhui)-infected mice did not reduce the mortality of naive mice after homologous virus challenge. Reinfection with homologous A(H7N9) virus induced higher HI and MN titers than first infection. In contrast, pH1N1(2009) virus infection induced robust HI and MN antibody responses, even during the first infection. Moreover, rg-PR8-H7-N9 induced significantly higher HI and MN antibody titers than H7N9(Zhejiang). In conclusion, the internal genes of A(H7N9) virus can affect the humoral immune response against homologous viral surface proteins, which may also contribute to the virulence of A(H7N9) virus.

The avian influenza A(H7N9) virus causes severe pneumonia in humans, which is often complicated by extrapulmonary complications (1–4). As of 23 June 2015, the laboratory-confirmed case-fatality rate of A(H7N9) virus infection was 41%, which was lower than that of A(H5N1) infection (53%) but much higher than that in the 2009 pandemic caused by the A(H1N1)pdm09 virus (~0.1 to 5%) (5, 6). In mice, the virulence of A(H7N9) virus is between that of the highly pathogenic A(H5N1) and A(H1N1)pdm09 viruses (7, 8). A transcriptomic study also showed that the perturbation of the host gene expression profile of A(H7N9) virus infection is intermediate to that of A(H5N1) and A(H1N1)pdm09 virus infections (7).

Previous studies have tried to identify viral determinants that contribute to A(H7N9) disease severity in humans. Genomic analysis of A(H7N9) virus showed that although many human isolates contain mutations that are associated with human adaptation, such as polymerase basic 2 (PB2) Glu627Lys and hemagglutinin (HA) Gln226Leu, they lack the important virulence determinant internal genes of A/H5N1 virus, such as the multibasic amino acid at the cleavage site of the HA protein (3). Although some studies showed that A(H7N9) virus can preferentially bind to α2,3-linked sialic acid, which is abundant in alveoli, this binding preference was not found in other studies (1). A study using reassortant viruses showed that the PB2, matrix (M), and nucleoprotein (NP) genes of A(H7N9) virus are critical for virulence (9).

An immunoinformatic study demonstrated that the HA gene of the A(H7N9) virus encodes 14 to 24% fewer T cell epitopes per full-length HA protein compared with those of other influenza viruses, such as A/California/07/2009 (H1N1) (10, 11). This suggests a possibility of lower immunogenicity during natural infection by A(H7N9) virus and perhaps also lower immunogenicity of the A(H7N9) influenza virus. In order to better understand the relevance of the immune response to A(H7N9) infection to the virulence of the virus, we studied the antibody responses to A(H7N9) virus using a mouse model. We found that the antibody response to A(H7N9) infection in mice was impaired and characterized by low titers of serum hemagglutination inhibition (HI) antibody, with no or very weak virus-neutralizing activity. In contrast, normal neutralizing-antibody production in mice was observed with a reverse-genetically engineered A(H7N9) virus containing internal genes derived from A/Puerto Rico/8/34 (H1N1) virus (PR8). This finding suggested that the internal genes of the A(H7N9) virus may play a more important role than the immunogenicity of the two surface proteins of A(H7N9) virus, the hemagglutinin and neuraminidase, in modulating the host immune response against the virus surface protein.

MATERIALS AND METHODS

Viruses, animals, and cell lines. The three wild-type influenza A viruses used in this study included 2 influenza A(H7N9) viruses, A/Anhui/1/2013 [H7N9(Anhui)] (12) and A/Zhejiang/DTID-ZJU01/2013 [H7N9(Zhejiang)] (4), and an A(H1N1)pdm09 virus, A/Hong Kong/415742/09 [pH1N1(2009)] (13). For a passive transfer study, mouse-adapted A/Hong Kong/415742/09...
[mouse-adapted pH1N1(2009)] was also used (13). A recombinant virus, rg-PR8-H7-N9, consists of HA and neuraminidase (NA) genes from H7N9(Zhejiang) and 6 internal genes from the PR8 virus, and the virus was generated by a reverse genetics approach, as we previously reported (14, 15). The viruses were propagated in 10-day-old specific-pathogen-free (SPF) chicken embryos, and the viral titers, expressed by PFU and 50% tissue culture infective dose (TCID50), were determined in Madin-Darby canine kidney (MDCK) cells. The mouse 50% lethal dose (LD50) was determined to be 10^3.1 PFU for H7N9(Anhui) and >10^6 PFU for H7N9(Zhejiang) (16).

Six- to 8-week-old female BALB/c mice were kept in an SPF animal facility with 12-h light/dark cycles and had free access to standard pellet food and water. The serum samples of all prechallenge mice were checked by HI and microneutralization (MN). All virus infection-related experiments were performed in biosafety level 3 facilities and were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong, Hong Kong.

**Virus infection of BALB/c mice and blood sample collection.** BALB/c mice were inoculated intranasally with 10^3 (n = 10 mice), 10^4 (n = 10 mice), or 10^5 (n = 20 mice) PFU of H7N9(Anhui) virus, and serum-virus mixtures were then added to MDCK cells. After 1 h of viral adsorption at 37°C, the cells were washed 3 times with PBS and further incubated for 6 h at 37°C. The serum-virus mixtures were then added to MDCK cells. After 1 h of viral adsorption at 37°C, the cells were washed and incubated with minimum essential medium (MEM) containing 1% penicillin-streptomycin and 2 μg/ml l-1-tosylamide-2-phenyl-ethyl chloromethyl ketone (TPCK)-treated trypsin at 37°C and 5% CO2. Cytopathic effects were evaluated 72 h after incubation.

**Fluorescent focus microneutralization (FFMN) assay.** As we previously described (18), MDCK cells were seeded in chamber slides and cultured overnight. The mouse convalescent-phase serum was treated and 2-fold serially diluted as in the HI assay. The diluted serum samples were incubated with H7N9(Anhui) virus (multiplicity of infection [MOI], 1, according to the number of MDCK cells) at room temperature for 1 h. The serum-virus mixtures were then added to MDCK cells. After 1 h of viral adsorption at 37°C, the cells were washed 3 times with PBS and further incubated for 6 h at 37°C. The cells were fixed in chilled acetone and methanol (1:1) at −20°C for 20 min and stained with mouse anti-influenza nucleoprotein (NP) antibody, followed by fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG. The percentage of positive cells was examined under a fluorescence microscope and analyzed quantitatively with the Image-Pro Plus 4.5 software.

**Statistical analysis.** Mouse survival rates in different groups were analyzed by the Kaplan-Meier method and log rank test using GraphPad Prism 6.0. HI and MN titers between groups were analyzed by the Student t test. All statistical calculations involving the geometric mean titers (GMTs) of antibodies were performed with log-transformed titers. A P value of <0.05 was considered statistically significant.

**RESULTS**

A(H7N9)-infected mice had impaired neutralizing-antibody production. All prechallenge mice were checked by HI and MN assays and were shown to have undetectable antibody titers against the virus strains used. Mice infected with a high inoculum of H7N9(Anhui) (10^5 PFU) had severe body weight loss and a survival rate of 30% within 14 dpi (Fig. 1A and B and Table 1). Mice receiving lower inocula of H7N9(Anhui) (10^3 and 10^4 PFU) had <20% body weight loss, and all survived.

The HI and MN antibody titers were determined in blood samples collected from 5 mice in each group at 7 dpi and all the surviving mice at 14 and 28 dpi. Low HI GMTs of 26.4 and 45.9 against homologous virus H7N9(Anhui) were detected at 7 dpi in mice infected with 10^3 and 10^4 PFU, respectively. However, the surviving mice infected with 10^5 PFU had a GMT of only 11.5. At 14 dpi, significant increases in the HI titers were detected in all infected mice, irrespective of inoculum. The surviving mice had HI GMTs of 72.5 for the 10^5 PFU group (n = 6 mice), 52.7 for the 10^4 PFU group (n = 10 mice), and 40 for the 10^3 PFU group (n = 10 mice). At 28 dpi, the HI GMT further increased to 201.6 with the 10^5 PFU group and 171.5 with the 10^4 PFU group. The differences in HI GMTs between different challenge doses were not statistically significant (Fig. 1C). Using the MN antibody assay, no MN titer was detected at 7 and 14 dpi in all 3 groups. At 28 dpi, only 1 of 10 mice challenged with 10^6 PFU had an MN titer of 40 against H7N9(Anhui), and 4 mice had an MN titer of 20 (Fig. 1D). Since the MN GMT was low for H7N9(Anhui)-infected mice, we also determined the MN titers of mice infected with 10^5 PFU of H7N9(Zhejiang) or pH1N1(2009). At an infectious dose of 10^5 PFU, the 14-day survival rate was 100% for mice challenged with H7N9(Zhejiang) or pH1N1(2009). H7N9(Zhejiang) was also isolated from a patient with severe A(H7N9) influenza during the same period. The PB2 of H7N9(Zhejiang) has 627E/701N, while H7N9(Anhui) and pH1N1(2009) were infected with 10^5 PFU of H7N9(Zhejiang), the HI GMT was 91.9, while H7N9(Anhui) had an HI GMT of 100 at 14 dpi. None of the H7N9(Zhejiang)-infected mice had a detectable...
The MN titer of A(H7N9) virus/Anhui/01/2013 [H7N9(Anhui)] was 40 (GMT, 6.3), and only one of the 15 mice infected with H7N9(Anhui) had an MN titer of 40 (GMT, 7.2) (Fig. 2). There was no significant difference in the MN GMTs between mice infected with H7N9(Anhui) and those infected with H7N9(Zhejiang). On the contrary, mice challenged with 10^5 PFU of pH1N1(2009) had high HI and MN titers. At 14 dpi, the HI GMT was 367.6, and the MN GMT was 463.1. These antibody titers were significantly higher than those induced by the H7N9(Anhui) and H7N9(Zhejiang) viruses (P < 0.0001) (Fig. 2).

Mouse convalescent-phase serum infected with A(H7N9) had weak neutralizing activity in vitro. Twelve available serum

**TABLE 1** Survival rates of mice infected with influenza A viruses

| Inoculum titer (PFU) | Virus strain       | % survival rate at 14 dpi (no. survived/total no.)^a |
|----------------------|--------------------|-------------------------------------------------------|
| 10^3                 | H7N9(Anhui)        | 100 (10/10)                                           |
| 10^3                 | pH1N1(2009)        | 100 (10/10)^b                                         |
| 10^3                 | rg-PR8-H7-N9       | 100 (6/6)                                              |
| 10^5                 | H7N9(Anhui)        | 30 (6/20)                                              |
| 10^5                 | pH1N1(2009)        | 100 (16/16)                                            |
| 10^5                 | rg-PR8-H7-N9       | 100 (10/10)                                            |

^a dpi, days postinfection.
^b Data based on reference 14.
samples from mice challenged with $10^5$ PFU of H7N9(Anhui) taken at 14 dpi and with HI titers of 40 or 80 were tested by FFMN. All 12 tested serum samples at dilutions of 1:20 to 1:160 inhibited NP expression, and the inhibitory effect increased with increasing concentration of convalescent-phase serum (Fig. 3A). The test was considered valid, since an average of 60% reduction in the number of NP-positive cells was observed at the highest serum dilution (1:160) (Fig. 3B).

Convalescent-phase sera did not confer any protection upon mice challenged by H7N9(Anhui). We next determined whether this low level of neutralizing activity in mouse convalescent-phase serum observed in the FFMN assay could provide in vivo protection when passively transferred to infected mice. Five serum samples that had HI titers of 40 to 80 and FFMN titers of >160 were pooled, diluted with PBS at 1:10, and injected intraperitoneally into groups of 5 mice. The results showed that when the convalescent-phase serum was given to mice immediately after or at 24 h after a lethal-dose challenge with H7N9(Anhui) ($10^5$ PFU), no statistically significant improvement in body weight or survival

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**FIG 2** HI and MN titers against homologous virus in A(H7N9) [H7N9(Anhui) and H7N9(Zhejiang)] and A(H1N1)pdm09 [pH1N1(2009)] virus-infected mouse sera. Same doses ($10^5$ PFU) of the three different viruses were intranasally inoculated in groups of BALB/c mice, and serum samples were collected at 14 dpi for HI and MN antibody detection against each individual virus. The data presented are titers on a log2 scale. The horizontal dashed lines indicate a titer of 40 for HI and MN. The horizontal solid lines indicate the GMTs. In total, $n = 15$ for each group. $\dagger\dagger\dagger$, $P < 0.0001$ compared with pH1N1(2009) group.

**FIG 3** Fluorescent focus microneutralization (FFMN) assay. Mouse convalescent-phase serum samples taken at 14 days after $10^5$ PFU of H7N9(Anhui) infections were serially diluted 2-fold starting at 1:10. The diluted serum samples were mixed with H7N9(Anhui) (MOI, 1), incubated at room temperature for 1 h, then added to MDCK cells. The cells were fixed at 6 h after infection and stained with mouse anti-influenza nucleoprotein (NP) antibody and FITC-conjugated donkey anti-mouse IgG. (A) Representative images of stained MDCK cells. Top panel, DAPI (4',6-diamidino-2-phenylindole); lower panel, NP protein was labeled green (original magnification, $\times$200). (B) Percentage of reduction in NP-positive cells in FFMN assay. NP positivity in MDCK cells infected with H7N9(Anhui) without serum treatment was taken as 100%. $n = 12$ for H7N9(Anhui) convalescent mouse sera, and $n = 3$ for uninfected control mouse sera. The error bars indicate the standard deviations.
was achieved, but there was a trend toward better survival in mice receiving convalescent-phase serum, in which 60% of mice receiving convalescent-phase serum 24 hpi survived (Fig. 4A). Similarly, passive transfer of pH1N1(2009) convalescent-phase serum that was diluted to an HI titer of 40 did not protect mice from lethal pH1N1(2009) challenge (Fig. 4B).

Mice are protected from reinfection with neutralizing-antibody production. To study the long-term immune protection from reinfection with the homologous virus, mice surviving after H7N9(Anhui) challenge were rechallenged with 5 or 10^6 PFU (n = 5) at 4 weeks or 12 weeks after the first infection. At 12 weeks after the first infection, mouse serum samples were collected before rechallenge and tested to determine the HI titer. The HI GMT was 45.9 (data not shown). Mice reinfected 4 weeks after the first infection showed no signs of disease, and mice reinfected 12 weeks after the first infection showed ruffled fur and labored breathing at 3 and 4 dpi, but all fully recovered. No mortality was observed in any group of the rechallenged mice (Fig. 5A and B). Therefore, these mice were protected during reinfection from homologous virus despite low HI and MN titers. The protection may be conferred by antibodies against non-HA proteins of influenza virus, such as NP and NA, or cell-mediated immune responses that were induced during the first infection.

At 14 days after rechallenge, mouse sera had HI titers comparable to those from the first H7N9(Anhui) infection (GMT, 119.86 for 4-week group; GMT, 80 for 12-week group). However, significantly higher MN titers were detected in 10/11 and 9/11 mice rechallenged at 4 weeks and 12 weeks, respectively. The respective GMTs were 47.56 and 54.81, which were significantly higher than those after the first H7N9(Anhui) infection (P < 0.01) (Fig. 5C and D).

Roles of the internal genes of the A(H7N9) virus in impairing antibody response during first infection. To study whether the impaired MN antibody production is solely related to the proposed low immunogenicity of A(H7N9) virus, the experiment was repeated with a recombinant influenza rg-PR8-H7-N9 virus. The rg-PR8-H7-N9 virus contains 6 internal genes from A/PR/8/34 H1N1 virus, while the HA and NA genes were from the A(H7N9) virus H7N9(Zhejiang). Inoculation with 10^5 PFU of rg-PR8-H7-N9 virus caused about 10% body weight loss at 3 and 4 dpi. All infected mice gradually regained their initial body weight at 10 dpi, and all survived (data not shown). Mice infected with rg-PR8-H7-N9 had significantly lower pulmonary viral titers than those infected with H7N9(Zhejiang) on 6 dpi (P = 0.009), but there was no significant difference at 2 dpi and 4 dpi (Fig. 6A). Mice infected with rg-PR8-H7-N9 had significantly higher HI and MN titers than those challenged with the wild-type A(H7N9) virus H7N9(Zhejiang) (Fig. 6B). A temporal profile of serum antibody production showed that as early as 7 dpi, all rg-PR8-H7-N9-infected mice (n = 10) had a serum HI titer of 40 to 160 (GMT, 56.56), whereas only 1 of the 10 mice challenged with H7N9(Zhejiang) had a titer of 40 at 7 dpi. At 14 dpi, the HI GMT...
of rg-PR8-H7-N9-infected mice was significantly higher than that of H7N9(Zhejiang)-infected mice (GMT, 139.3 versus 74.6, respectively; \(P < 0.00079\)). At 28 dpi, the HI GMT further increased in both the rg-PR8-H7-N9- and H7N9(Zhejiang)-infected groups to 226 and 183.8, respectively, but the difference between the 2 groups was not statistically significant. Most importantly, a significant increase in MN antibody production was induced by rg-PR8-H7-N9 infection. Even at 7 dpi, the MN GMT was 18.8, which was significantly higher than the GMT of 5 in the H7N9(Zhejiang) group (\(P < 0.0001\)). The MN titers were 40 to 320 (GMT, 121.3) at 14 dpi and 160 to 640 (GMT, 278.5) at 28 dpi, and both were significantly higher than those induced by H7N9(Zhejiang). Among the 10 H7N9(Zhejiang)-infected mice, the MN titer was 40 for 2 mice and 20 for 7 mice (GMT, 20), even at day 28 dpi.

**DISCUSSION**

We describe here a systematic study on the antibody response induced by A(H7N9) virus in a mouse infection model. Similar to the situation in humans, we showed that surviving A(H7N9)-infected mice had lower titers of HI and MN antibodies during the first infection than those of surviving A(H1N1)pdm09-infected mice. Higher HI and MN titers were elicited only during reinfection by the same virus. The low antibody titers in convalescent A(H7N9)-infected mice correlated with the poor protection of convalescent-phase serum treatment of immunologically naive mice challenged by homologous virus. Mice infected with the recombinant A(H7N9) virus with the PR8 internal genes (rg-PR8-H7-N9) had lower pulmonary viral titers on 6 dpi and higher HI and MN titers from 7 dpi than those of mice infected with the wild-type A(H7N9) viruses. Collectively, the results suggest that the poor humoral immune response induced by A(H7N9) virus may be a contributing factor to the impairment of the host antiviral response, which may lead to the high mortality of A(H7N9) virus infection in humans. Furthermore, the impaired antibody response is attributable to internal genes.

We have shown that the convalescent MN titers of H7N9(Anhui)- and H7N9(Zhejiang)-infected mice, and to a less extent the HI titers, were lower than those induced by the pH1N1(2009) virus. MN titers were also lower than HI titers in patients infected with A(H7N9) virus. Guo et al. (19) showed that the MN titers of A(H7N9) patients were only 20 to 80 in serum samples collected 102 to 125 days after symptom onset, compared to HI titers of 80 to 640 for the same samples (19). The MN titers after A(H7N9) infection were also much lower than those induced by A(H5N1) virus infection, in which the MN GMT in convalescent patients...
was 540 at 1 to 2 months after symptom onset (20). In a mouse study, a low HI titer after A(H7N9) infection was reported, in which inoculation of $10^6$ to $10^8$ PFU of H7N9(Anhui) virus in mice induced HI titers of only 80 to 160 at 14 dpi (21). We have shown that the internal genes play an important role in the generation of neutralizing antibodies. By replacing the internal genes of wild-type A(H7N9) viruses with the internal genes of PR8, both MN and HI titers increased significantly. In particular, the MN GMT increased by almost 25-fold. Several internal genes have been shown to affect HI and MN antibody responses. Influenza vaccines with a deletion or truncation of the nonstructural 1 (NS1) gene have been demonstrated to confer higher immunogenicity (22–24). NS1 inhibits the type I interferon-mediated antiviral response (25). Since the antibody response was enhanced without changing the HA or NA, it is unlikely that HA or NA contributes to the poor immunogenicity of the wild-type A(H7N9) viruses.

Our previous studies showed that convalescent blood products with high MN titers can improve the outcome of patients with severe A(H1N1)pdm09 infection (26, 27). In the current study, the passive transfer of convalescent-phase sera with neutralizing activity detectable only by FFMN assay, but not by the standard MN assay, did not exhibit a significant in vivo protective effect for mice challenged with the A(H7N9) virus. These results indicated that the low level of neutralizing anti-A(H7N9) antibody in convalescent-phase serum is not sufficient to provide protection.

Currently, the development of A(H7N9) vaccines has encountered the problems of a low seroconversion rate and low titer of antibody response in animal models and several human clinical trials (28–31). Accumulated evidence has demonstrated that A(H7N9) vaccines have poor immunogenicity without an adjuvant. Even at a higher dosage of 45 μg of HA, only minimal antibody responses were induced (32), while the incorporation of an adjuvant, such as MF-59, and Iscomatrix in the A(H7N9) vaccine formulation enhanced the immunogenicity significantly (30, 31). Thus, the conventional vaccination regimen may not be adequate to elicit the optimal neutralization antibody response unless a higher antigen dose or adjuvant or immune potentiation measures are considered (33–35).

This study has several limitations. First, we did not assess the antibody response against other influenza virus proteins, such as NA and NP, or the cell-mediated immune responses after A(H7N9) virus infection in mice. Information on the roles of the other immune mechanisms in the protection of hosts against subsequent infection may provide some insight on how surviving H7N9(Anhui)-infected mice were protected from...
H7N9(Anhui) reinfection. Second, we could not compare the antibody response of rg-PR8-H7-N9 with that of the original PR8 virus. This is because all mice challenged with the original PR8 virus died, even at a low infectious dose of 10^2 PFU; therefore, the convalescent antibody titer could not be measured. Third, we tested only the antibody response of a small number of virus strains from each group. Fourth, the higher antibody response might be related to the increased number of HA or NA per virion of rg-PR8-H7-N9 virus compared to that of H7N9(Anhui).

The humoral immune response plays a pivotal role in the protection against influenza virus infection in humans. This study showed that the avian-origin (A(H7N9) virus triggers a much weaker antibody response than that of the human pandemic influenza A(H1N1) virus in mice. A better understanding of the factors affecting antibody response will greatly enhance our understanding of the pathogenesis of A(H7N9) virus infection and may allow the development of novel strategies in the design of A(H7N9) vaccines with better protective efficacy.

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