Highly Pathogenic Avian Influenza A(H5N1) Mutants Transmissible by Air Are Susceptible to Human and Animal Neutralizing Antibodies

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A laboratory-generated reassortant H5 hemagglutinin (HA)/influenza A(H1N1) strain containing 4 mutations in influenza A(H5N1) HA has become transmissible by air among mammals. Here, we constructed 15 influenza A(H5N1) pseudoviruses containing a single mutation or a combination of mutations and showed that the pseudoviruses were susceptible to neutralizing antibodies from patients with influenza A(H5N1) infection and from mice immunized with a vaccine containing the conserved HA1 sequence of influenza A(H5N1). These results indicate that antibodies in patients currently infected by influenza A(H5N1) and antibodies induced by vaccines containing conserved sequences in HA1 of wild-type influenza A(H5N1) are highly effective in cross-neutralizing future influenza A(H5N1) mutants with airborne transmissibility, suggesting that human influenza pandemics caused by these influenza A(H5N1) variants can be prevented.

Keywords. Patient serum specimens; Neutralizing antibodies; H5N1 influenza virus; Air-transmissibility.

There is still a serious threat to global public health due to influenza A viruses, particularly the highly pathogenic avian influenza A(H5N1) strain, which has caused 633 infections, including 377 deaths, since 2003 [1]. Recently, Imai et al [2] showed that a laboratory-generated reassortant H5 hemagglutinin (HA)/influenza A(H1N1) strain containing the mutations N158D, N224K, Q226L, and T318I (hereafter, H3 numbering is used) in the HA of A/Vietnam/1203/2004(H5N1, clade 1) could be transmitted among ferrets via aerosol or respiratory droplets, suggesting that these 4 HA mutations contribute to the acquisition of airborne transmissibility by influenza A(H5N1) among mammals [2, 3].

This report and another by Herfst et al [4] have raised concerns about the potential influenza pandemic that may be caused by a highly pathogenic avian influenza A(H5N1) variant with natural mutations in HA or by laboratory-generated H5N1 mutants accidentally released from a laboratory if at-risk populations lack immunity to these emerging viruses [5–9]. Therefore, it is essential to determine the susceptibility of these mutant viruses to neutralizing antibodies from patients infected by the currently circulating influenza A(H5N1) strains and from animals immunized with vaccines based on the conserved sequences in the HA of influenza A(H5N1).

In this study, we generated a series of influenza A(H5N1) pseudoviruses containing single and combination forms of the above-noted mutations in influenza A(H5N1) HA, as reported by Imai et al [2], and assessed their susceptibility to neutralizing antibodies in serum specimens from influenza A(H5N1)–infected patients and a broadly cross-neutralizing monoclonal antibody (mAb) generated from mice immunized with a vaccine containing the conserved HA1 sequence of wild-type influenza A(H5N1) [10].

MATERIALS AND METHODS

The broadly neutralizing mAb HA-7 was generated previously from mice immunized with a recombinant protein expressing codon-optimized HA1 of A/Anhui/1/2005(H5N1) (AH/1, clade 2.3.4; GenBank accession no. ABD28180) fused with foldon (Fd) and Fc of human immunoglobulin G (IgG) 1 (HA1-Fdc) [10, 11]. Human serum specimens were collected from patients infected with A/Shenzhen/406H/2006(H5N1) (SZ/406H, clade 2.3.4; GenBank accession no. ABO36644), A/Fuyang/2006(H5N1) (FY/06; no reported accession number), and A/Anhui/1/2006(H5N1) (AH/06; GenBank accession no. AEO89065) in China during 2006–2007. The study of serum specimens from influenza A(H5N1)–infected patients was approved by the ethics review committee of the Beijing Institute of Microbiology and Epidemiology.
Single and multiple mutations of influenza A(H5N1) HA at positions N158D, N224K, Q226L, and T318I were constructed as follows. Briefly, a total of 15 mutant HAs containing single or combined mutations at positions N158D, N224K, Q226L, and T318I in the HA of A/ Qinghai/59/05(H5N1) (QH-HA, clade 2.2; GenBank accession no. ABE68921) were constructed using the QuikChange Site-Directed and Multi Site-Directed Mutagenesis Kits, according to the manufacturer’s protocols (Agilent Technologies, Santa Clara, CA).

Generation of mutant influenza A(H5N1) pseudoviruses and detection of their susceptibility to serum specimens from influenza A(H5N1)-infected patients and HA-7 mAb were performed using a pseudovirus neutralization assay, as described by us elsewhere [12]. Briefly, 293T cells were cotransfected with pNL4-3.luc.RE plasmid and each of the plasmids encoding mutant HAs of QH-HA, and supernatants were harvested 72 hours later for single-cycle infection. Pseudoviruses were directly added to target cells or were incubated with either HA-7 mAb or human serum specimens at 37°C for 1 hour before they were added to cells. Infected cells were lysed 72 hours later and assayed for luciferase activity, using an Ultra 384 Luminometer (Tecan, San Jose, CA). The infection rate of pseudoviruses was expressed as relative luciferase units (RLU). The neutralization of mutant pseudoviruses against HA-7 mAb and human serum specimens was calculated as % neutralization [10, 13].

The human immunodeficiency virus type 1 (HIV-1) p24 content in influenza A(H5N1) pseudoviruses was quantified by enzyme-linked immunosorbent assay (ELISA) as described elsewhere [12]. Briefly, ELISA plates were precoated with HIV-1 immune globulin (5 µg/mL) overnight at 4°C and blocked at 37°C for 2 hours. Lysed pseudoviruses were added to the plates and incubated at 37°C for 1 hour. After washes, the plates were respectively incubated with anti-p24 mAb (183-H12-5C; 1:20) and then with horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG at 37°C for 1 hour. The substrate 3,3′,5,5′-tetramethylbenzidine (Zymed, Carlsbad, CA) was added, and the reaction was stopped by the addition of 1 N H2SO4. The absorbance at 450 nm was measured by an ELISA plate reader (Tecan).

The expression of HIV-1 p24 and HA in influenza A(H5N1) pseudoviruses was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by Western blot, according to our previously described protocols [12]. Briefly, lysed pseudoviruses were resolved by 10% Tris-glycine gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), and then blocked in 5% nonfat milk overnight at 4°C. The blots were incubated with anti-HIV-1 p24 mAb (183-H12-5C; 1:200) and then with anti-hA mAb (1C9; 1:100). After 3 washes, the blots were incubated with HRP-conjugated goat anti-mouse IgG (1:5000; Zymed) for 1 hour at room temperature. Signals were visualized with ECL Western blot substrate reagents and Amersham Hyperfilm (GE Healthcare, Piscataway, NJ).

RESULTS AND DISCUSSION

Previous studies have revealed some correlations between the above-noted mutations and the receptor-binding specificity and antigenicity of HA. For example, 3 of the 4 mutations, N158D, N224K, and Q226L, are located in the receptor-binding region, while the T318I mutation is located proximally to the fusion peptide region of HA, allowing efficient transmission of viruses with 4 mutations in HA among mammals. Thus, the mutation at these sites might affect the receptor-binding specificity and stability of HA [2]. Reports have also shown that glycosylation at N158 of HA and receptor-binding specificity synergistically affect the antigenicity and immunogenicity of a live attenuated influenza A(H5N1) vaccine virus in ferrets and that only in conjunction with the removal of N158 glycosylation will the introduction of the Q226L mutation result in better receptor binding and virus immunogenicity [14]. No other mutations at the above 4 sites have been reported to significantly affect the antigenicity of HA.

In this study, using a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE), we constructed 15 influenza A(H5N1) pseudoviruses expressing HAs of wild-type QH-HA with mutations, including N158D, N224K, Q226L, and T318I single mutations and N158D-N224K, N158D-Q226L, N158D-T318I, N224K-Q226L, N224K-T318I, Q226L-T318I, N158D-N224K-Q226L, N158D-N224K-T318I, N158D-Q226L-T318I, N224K-Q226L-T318I, and N158D-N224K-Q226L-T318I combination mutations. The reactivity of these pseudoviruses with HIV-1 p24–specific mAb and influenza A (H5N1) HA–specific mAb was assessed by Western blot. We found that all influenza A(H5N1) pseudoviruses with or without mutations in HA reacted strongly with anti-p24 mAb and anti-HA mAb (Figure 1A), indicating that the mutations in HAs of these influenza A(H5N1) pseudoviruses do not significantly affect their antigenicity.

We next tested the infectivity of the influenza A(H5N1) pseudoviruses bearing the wild-type and mutant HA in different target cells. HEK 293T (human kidney), MDCK (canine kidney), Vero (African green monkey kidney), and A549 (human lung) cells were selected because we previously showed that these cells could be effectively infected by pseudovirus with wild-type QH-HA [12]. We observed that all mutant pseudoviruses containing 50 pg of HIV-1 p24 were able to efficiently infect 293T and MDCK cells, with a luciferase activity of >2.6 × 103 RLU (Figure 1B). These mutant influenza A(H5N1) pseudoviruses could also infect Vero and A549 cells, although infection rates were relatively lower than those observed for 293T and MDCK cells (Figure 1C). The positive control VSV-G could effectively infect all tested target cells, while the
negative control Env-HIV-1 showed no infection in these cells (Figure 1). Since these influenza A(H5N1) pseudoviruses with or without mutations are single-cycle and replication-deficient viruses, they can be handled in a biosafety level 2 laboratory without a biosafety concern [12]. Accordingly, the use of pseudoviruses in this study cannot pose potential dual-use risks [15].

Subsequently, we tested the susceptibility of influenza A (H5N1) pseudoviruses bearing 1–4 of the mutations described above to neutralization by serum specimens from influenza A (H5N1)–infected patients. Like the wild-type QH-HA influenza A(H5N1) pseudovirus, all 15 mutant pseudoviruses were almost completely neutralized (>99%) by serum specimens (1:600) collected from patients on day 32 (Figure 2A) and day

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**Figure 1.** Analysis of protein expression of influenza A(H5N1) pseudoviruses with or without mutations in H5 hemagglutinin (HA) and their infectivity in different target cells. **A**, Pseudoviruses were detected by Western blot using anti–human immunodeficiency virus type 1 (HIV-1) p24 (183-H12-5C; 1:200) and anti-influenza A(H5N1) HA (1C8; 1:100) monoclonal antibodies (mAb; 75 and 12.5 pg of p24/well, respectively). Detection of influenza A(H5N1) pseudovirus infectivity (50 pg of p24/well) in 293T and MDCK cells (B) and in Vero and A549 cells (C). VSV-G and Env-HIV-1 were included as positive and negative pseudovirus controls, respectively. Data were presented as mean relative luciferase units (RLU) ± SD of 4 parallel wells in 96-well culture plates. QH-HA, influenza A(H5N1) pseudovirus with wild-type HA.
Figure 2. Neutralization of influenza A(H5N1) pseudoviruses by serum specimens from influenza A(H5N1)-infected patients and HA-7 monoclonal antibodies (mAb). Serum specimens were collected from patients on day 32 (A) and day 45 (B) after SZ/406H(H5N1) infection, 6 months after FY/06(H5N1) infection (C), and 11 months after AH/06(H5N1) infection (D). E: Neutralization of the N158D-N224K-Q226L-T318I mutant and influenza A(H5N1) pseudoviruses with wild-type HA (QH-HA) by serum specimens from 4 healthy individuals (donors 1–4) as the controls. F: Neutralization of influenza A(H5N1) pseudoviruses by HA-7 mAb. All data were presented as the mean percentage of neutralization ± SD of duplicate wells.
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Potential conflicts of interest. All authors: No reported conflicts.

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References


Notes

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