Published in final edited form as:

Immunol Lett. 2011 March 30; 135(1-2): 59–63. doi:10.1016/j.imlet.2010.09.012.

Serologic cross-reactivity among humans and birds infected with highly pathogenic avian influenza A subtype H5N1 viruses in China

Zheng Li^a, Chi Ma^a, Zhonghua Liu^b, and Wei He^{a,*}

^a The Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & School of Peking Union Medical College

^b Center for AIDS, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & School of Peking Union Medical College

Abstract

To study immunogenicity and serologic cross-reactivity of hemagglutinins (HAs) among humans and birds infected with highly pathogenic avian influenza (HPAI) H5N1, four representative H5N1 HA genes from humans and birds infected with distinct genetic clusters of H5N1 viruses in China were cloned, and several H5N1 infected human serum and H5N1 positive bird serum samples were used. Recombinant HA proteins were generated for ELISA assays and pseudotype viruses containing HAs were produced for neutralization assays and hemagglutination inhibition (HI) tests. We found significant differences among clades compared to species in binding, neutralization and HI activity of H5N1 strains isolated from birds. While significant differences were observed among species in H5N1 isolated from humans, investigation of H5N1 infected human and avian sera provided evidence that the pressure from nAb may be a driving force for positive selection. Therefore, improved anti-viral nAb therapies could block avian influenza transmission in humans.

Keywords

Highly pathogenic avian influenza H5N1 virus; Hemagglutinin; Serologic cross-reactivity

1. Introduction

Sustained and widespread highly pathogenic avian influenza (HPAI) H5N1 epidemics represented a significant public health hazard, not only because they caused substantial mortality in poultry, but also because they increased the risk of a human influenza pandemic [1]. Given the two human-to-human transmission cases of H5N1 reported in China [2,3] and the recent H1N1 pandemic threat in the world [4,5], controlling the interspecies transmission of avian influenza is of critical importance. Therefore, the study of the immunogenicity and serologic cross-reactivity among H5N1 strains is of great importance. A number of studies had focused on the differences among avian influenza on a genetic level, but little was

* Author correspondence: Mailing address: Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & School of Basic Medicine, Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China, Phone: 86-10-65136981; Fax: 86-10-65105909; for Wei He: heweiimu@public.bta.net.cn.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

reported on the protein and sera level. Such studies may help us to understand the fundamentals of interspecies transmission of avian influenza.

Hemagglutinin (HA), which binds to sialic acid-containing receptors on host cells, is a protein that can induce the host to produce neutralizing antibodies (nAbs). Frequent mutation of HA is one of the mechanisms for viral escape. In this study, we used four HA genes isolated from humans and birds, several H5N1 infected human sera and H5N1 positive bird sera to demonstrate the cross-reactivity among the species and clades. H5N1 viruses are classified into ten phylogenetic clades based on their hemagglutinin genetic sequence. At least 4 major antigenic groups of H5N1 viruses are currently in circulation, and have caused infection in humans [6]. Clade 2.2 viruses were the most virulent, and caused outbreaks in more than 60 countries ranging from Azerbaijan to Nigeria and Pakistan [7]. In this study, we selected four H5N1 strains that belonged to clade 2.3.4, clade 2.2 and clade 1 and produced their HA proteins and pseudotype viruses. The H5N1 infected human sera and H5N1 positive bird sera were used to react with these HAs through HI tests, ELISA binding and pseudotype neutralization assays. We found significant differences among clades compared to species in binding, neutralization and HI activity of H5N1 strain isolated from birds. Significant differences among species were observed in H5N1 isolated human strains. Our results suggest that humans are more susceptible to avian influenza and immunoprophylaxis is a suitable way to prevent human infection.

2. Materials and methods

2.1. Serum samples and HA genes

Three hemagglutination inhibition (HI)-positive bird serum samples and one HI-negative bird serum sample against A/Bar-headed Goose/Qinghai/1/05 (QH) H5N1 strain were provided by Chinese Academy of Sciences (CAS), which were collected from the Qing Hai Lake in 2005, and were named as b1, b2, b3 and b4 respectively. All of the human serum samples were obtained from the China Center for Disease Control (CCDC), among which two samples were infected. One infected serum sample was obtained from a H5N1 convalescent patient in Anhui province in the south of China, named as Patient 1. The other was isolated from a patient who died of H5N1 infection in Xinjiang province in the north of China, named as Patient 2. Eight human serum samples were collected from healthy volunteers vaccinated with H5N1 vaccine (A/Vietnam/1203/2004), named as Vaccinee 1–8. Six healthy control human sera also were collected, named as Health 1–6. All sera were heat-inactivated at 56°C for 30 min prior to the following experiments.

Four representative H5N1 HA genes were obtained from Hongkong University (HKU), China Center for Disease Control (China CDC) and Chinese Academy of Sciences (CAS). See Table 1 for more information regarding the HA genes.

2.2. Production of recombinant HAs

The baculovirus/insect cell system is used widely for recombinant protein production [8,9]. Briefly, the *Spodoptera frugiperda* (Sf9) cells was propagated and maintained in Sf-900 II SFM (Gibco BRL). The HA ectodomain genes were cloned into the transfer vector PacGP67b (BD Biosciences), respectively. The transfer vector was then co-transfected into the Sf9 cells with linearized baculovirus DNA (Invitrogen) to produce a recombinant baculoviruses. Viral supernatant was collected at 72 hours post-infection (PI). rHA proteins were expressed after three times of infections. 6-His tags were added to the HA protein C-terminals, and this tag was used to purify the supernatant of infected Sf-9 (Ni-NTA by GE healthy care). Western blotting using anti-His or anti-HA polyclone antibodies was performed to identify the rHA proteins.

2.3. Neutralization assay

Full-length HA and neuraminidase (NA) genes were used to produce pseudotype H5N1 viruses. Briefly, all HA and NA genes were cloned into pcDNA3.1 V5His TOPO expression vectors after sequencing. Western blotting was used to identify the expression of HA and NA in 293T cells. Three plasmids, pcDNA3.1-HA, pcDNA3.1-NA, and trunk bone plasmid pNL4-3 encoding HIV Gag-pol and a firefly luciferase reporter gene were co-transfected into 293T cells to produce a pseudotype virus. At 48 hours post-transfection, viral supernatants were harvested for neutralization assays. Briefly, various dilutions of serum were incubated with adequate pseudotype H5N1 viruses for 30 minutes at room temperature (RT). The mixture was then added into MDCK cells in 96-well plates. Infection efficiency was quantified by measuring the luciferase activity in the target cells with an EG&G Berthold Microplate Luminometer LB 96V. All of the experiments with pseudovirus were performed in a P2 laboratory.

The neutralization activity of sera was calculated according to the following equation: $(A-B)/A \times 100\%$. “A” represents the positive wells that contained only pseudotype viruses, and “B” represents the testing wells that contained the mixture of testing serum samples and pseudotype viruses.

2.4. ELISA assay

Briefly, rHAs were coated on the polystyrene plate at 4°C overnight, and the plate was blocked with 5% bovine serum albumin (BSA) (Sigma) at 37°C for 2h. The human and wild bird sera (at a dilution of 1:5000) were incubated in wells at 37°C for 1h. HRP-labeled secondary anti-human IgG (1:5000) (Sigma) and HRP-anti-avian IgY (1:5000) (Sigma) were added at 37°C for 1h, then OPD/H₂O₂ was added and color development was stopped by the addition of H₂SO₄. Plates were read at 450/630nm.

2.5. Hemagglutination inhibition (HI) assay

The HI assay was carried out according to a standard hemagglutination-inhibition protocol [10]. Sera were treated overnight with Vibrio cholerae receptor-destroying enzyme (Denka-Seiken, Tokyo), and were inactivated for 30 min at 56°C to destroy non-specific inhibitors. Serum samples were diluted twice, and were mixed with pseudotype H5N1 virus (4 HA unit). After a 30 minute incubation, 1% chicken erythrocytes were added into the wells. Human serum samples were started at a 1:100 dilution and bird serum samples were started at a 1:10 dilution.

2.6. Data analysis

The Students' t-test was utilized for statistical evaluation of the difference among sera and rHAs from three independent experiments.

3. Results

3.1. Neutralization activity of serum samples against four pseudotype H5N1 viruses

To investigate the recent strain-specific and cross-strain nAb response against H5N1 pseudotype viruses and HA proteins, 16 human and 4 bird serum samples were collected for the analysis of their neutralization and binding activities using 4 H5N1 HAs (Table 1). All human and avian serum samples were able to neutralize these four H5N1 pseudotype viruses, but varied in efficiency. All human sera had effective neutralization activity against all four pseudotype H5N1 viruses in the indicated dilutions (Fig. 1A). The avian sera had the strongest neutralization activity against HK (A/Hongkong/213/03) pseudotype virus and the weakest neutralization activity against AH (A/Anhui/2/2005) pseudotype virus (Fig. 1B).

Healthy human serum and bird H5N-negative serum had the strongest neutralization activity against HK pseudotype virus (Fig. 1C, 1D). Healthy human serum contained some cross-reactive neutralization antibodies against the HK H5N1 strain. Human serum samples exhibited high cross reactivity among these four H5N1 strains in the neutralization assay at these dilutions. These results suggested that human sera contained more nAbs than bird sera against the H5N1 strains. Our results demonstrate that the sera have varying neutralization activities against H5N1 strains belonging to different clades.

3.2. Binding ability of serum samples to four H5N1 rHAs

Next we examined the binding ability of the sera to four different HA proteins. Both vaccinated and infected human sera showed the weakest binding ability to QH-HA (A/Bar-headed Goose/Qinghai/1/05) protein (Fig. 2A). Consistent with the neutralization results, the binding ability of avian sera was weak to XJ (A/Xinjiang/1/2006) and AH HA proteins, but was strong to HK HA (Fig. 2B). The binding ability of healthy human serum was weak to all four HAs (Fig. 2C). As shown in Fig. 2D, b2, 3, 4 had higher binding titer to HK HA and lower binding titer to XJ HA. Interestingly, the serum sample b4, which was deemed as negative control, showed significantly high binding ability to HK-HA ($P < 0.05$).

3.3. HI test among serum samples and pseudotype H5N1 viruses

HI titers of human and wild bird sera are summarized in Table 2. The reactivity of human sera against pseudotype H5N1 viruses was higher than bird sera. More importantly, there was a difference in reactivity among HK and QH, XJ, AH pseudotype viruses. Human vaccinated serum samples had higher reactivity to HK pseudotype virus. We believe that this occurred because the HK H5N1 and H5N1 vaccine strain belong to the same clade. Bird serum samples had a lower HI titer against XJ and AH pseudotype viruses, but higher HI titer against QH and HK pseudotype viruses. These results were consistent with the neutralization and binding assays. Taken together, these results demonstrate that strain-specific and cross-reactive nAbs were produced in both humans and avians following natural avian influenza infection.

4. Discussion

Continuous evolution and co-circulation of multiple genetic H5N1 lineages in broad regions have generated diverse antigenic variants that could cause more antigenic drift variants to emerge. In this study, we used human and bird serum samples to demonstrate the immunogenicity and serologic cross-reactivity of H5N1. Two important points regarding avian influenza infection may be concluded from this study: birds, as the natural host of avian influenza, contained a large amount of cross-reactive neutralizing antibodies (nAbs) against different clades. Second, humans, as the new host of avian influenza, mounted species specific nAbs after infection or vaccination.

In this study, the QH H5N1-negative bird serum showed higher reactivity to HK H5N1. This may be explained by the large number of viral subtypes and the high frequency of genetic mixing in wild birds. It is believed that waterfowl and shorebirds maintain separate reservoirs of viral gene pools, from which new virus subtypes could emerge. It is important to note that the virulence of a virus cannot be determined by the bird's antigenic subtype [11,12]. It was difficult for birds to produce the specific reactivity against different H5N1 strains, since they were the reservoir of influenza and presented an extensive spectrum of sera recognition. The human results differed from those obtained from bird samples. Both infected and vaccinated human serum samples had higher binding activity and HI titer to XJ, AH and HK H5N1 strains (which were isolated from humans), while they had lower binding activity and HI titer to QH H5N1 strain (which was isolated from birds). Humans, as the

new host of avian influenza, mounted species specific nAbs following infection or vaccination. Thus, immunoprophylaxis of avian influenza is more suitable for humans than birds.

A single amino acid substitution can induce avian influenza and break the species barrier, thus making interspecies transmission possible. For instance, according to the comparison of H5N1 HA sequences isolated from human to QH HA, some H5N1 HA strains present a striking similarity to QH HA with only one amino acid difference. P251S, N198S and S239N appeared in HAs of A/Djibouti/5691NAMRU3/2006, A/human/Iraq/207-NAMRU3/2006 and A/Turkey/12/2006 H5N1 respectively. These cases seemed to inform us that the species barrier could not efficiently stop avian influenza from spreading.

Preventing the entry of newly emerged influenza virus into poultry flocks might be important measures to defend the avian influenza interspecies transmission. HPAI occurred primarily in domestic birds, which acted as a bridge for avian influenza to infect humans. Experimental infections of domestic birds with viruses from wildlife do not cause mortality. Likewise, virulent viruses or viruses that cause disease in the domestic fowl do not cause mortality in the wild waterfowl. In the domestic birds, the signs of disease have not been determined [13]. Thus, more research should be focused on the domestic birds.

In conclusion, this study provided substantial evidence for the cross reactivity of H5N1 on the sera and protein level. Application of immunoprophylaxis of avian influenza in humans might be a feasible approach to prevent infection.

Acknowledgments

We thank Professor Yuelong Su at CCDC and Professor George F. Gao at CAS for providing the human and bird sera. This work was funded by the National Institutes of Health (NIH) (U19 AI051915-05S1).

References

1. Peiris JS, de Jong MD, Guan Y. Avian Influenza Virus (H5N1): a Threat to Human Health. *Clinical Microbiology Reviews*. 2007; 20(2):243–267. [PubMed: 17428885]
2. Wang H, Feng Z, Shu Y, Yu H, Zhou L, Zu R, et al. Probable limited person-to-person transmission of highly pathogenic avian influenza A (H5N1) virus in China. *Lancet*. 2008; 371(9622):1427–34. [PubMed: 18400288]
3. Ungchusak K, Auewarakul P, Dowell SF, et al. Probable person-to-person transmission of avian influenza A (H5N1). *N Engl J Med*. 2005; 352(4):333–40. [PubMed: 15668219]
4. Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature*. 2009; 459(25):1122–1126. [PubMed: 19516283]
5. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, et al. Antigenic and Genetic Characteristics of Swine-Origin 2009 A(H1N1) Influenza Viruses Circulating in Humans. *Science*. 2009; 325(5937):197–201. [PubMed: 19465683]
6. Chen Y, Qin K, Wu WL, Li G, Zhang J, Du H, et al. Broad Cross-Protection against H5N1 Avian Influenza Virus Infection by Means of Monoclonal Antibodies that Map to Conserved Viral Epitopes. *The Journal of Infectious Diseases*. 2009; 199(1):49–58. [PubMed: 19032063]
7. Butler D. Politically correct names given to flu viruses. *Nature*. 2008; 452(24):923. [PubMed: 18441546]
8. Hu YC. Baculovirus as a highly efficient expression vector in insect and mammalian cells. *Acta Pharmacol Sin*. 2005; 26(4):405–416. [PubMed: 15780188]
9. Wang K, Holtz KM, Anderson K, Chubet R, Mahmoud W, Cox MM. Expression and purification of an influenza hemagglutinin-one step closer to a recombinant protein-based influenza vaccine. *Vaccine*. 2006; 24(12):2176–2185. [PubMed: 16310896]

10. Hoffmann E, Lipatov AS, Webby RJ, Govorkova EA, Webster RG. Role of specific hemagglutinin amino acids in the immunogenicity and protection of H5N1 influenza virus vaccines. *Proc Natl Acad Sci*. 2005; 102(36):12915–12920. [PubMed: 16118277]
11. Wu WL, Chen Y, Wang P, Song W, Lau SY, Rayner JM, Smith GJ, et al. Antigenic Profile of Avian H5N1 Viruses in Asia from 2002 to 2007. *Journal of Virology*. 2008; 82(4):1798–1807. [PubMed: 18077726]
12. Milton, F.; Christian, FJ. *Field Manual of Wildlife Diseases*. USGS-National Wildlife Health Center; 1999. p. 181-184.
13. Easterday, BC.; Hinshaw, VS. *Influenza, Diseases of Poultry*. Calnek, BW., editor. Iowa State University Press; Ames Iowa: 1991. p. 532-552.

Fig 1.

Serologic neutralization assay. (A) Neutralization of 10 infected and vaccinated human sera and 6 health human sera was analyzed against QH (A/Bar-headed Goose/Qinghai/1/05), XJ (A/Xinjiang/1/2006), AH (A/Anhui/2/2005), HK (A/Hongkong/213/03) H5N1 pseudotype viruses at the indicated dilutions. Data is shown in a box and whiskers graph. The box extends from the 25th percentile to the 75th percentile, with a line at the median. The whiskers above and below the box indicate the 95th and 5th percentiles. Serum samples from two H5N1 negative healthy humans were used in this assay. (B) Neutralization of four wild bird sera against QH, XJ, AH and HK H5N1 pseudotype viruses is shown. (C) Neutralization of individual human serum sample against these four pseudotype viruses is presented. Vaccinated samples 1 and 2, one healthy human serum sample with high binding activity to these HA proteins is shown here as instance. (D) Neutralization of four avian serum samples against these four pseudotype viruses is presented. B4 was QH-H5N1 negative avian serum sample. Neutralization assays were performed in duplicate.

Fig 2.

Serologic binding assay. (A and B) The binding activities of 14 human serum samples and 4 avian serum samples to recombinant HA proteins are summarized. Dilution of serum was 1:5000. (C and D) Binding of individual serum sample is shown as instance. Unpaired Students' t-test was used for statistical analysis. In fig. 2C, the binding affinity to QH HA of Vaccinee 2, Patient 1 and Patient 2 were significantly lower than to XJ, AH and HK HAs. ($P < 0.01$) In fig. 2D, the binding affinity to HK HA of b4 was significantly higher than b1, b2 and b3. ($P < 0.01$) The binding affinity to XJ HA of b4 was significantly lower than b1, b2 and b3. ($P < 0.01$) Data of binding assay were the mean $\pm$ s.d. of triplicate experiments.

Table 1

H5N1 viruses used in this study

Source	Virus strains	Accession No.	HA phylogeny	HA _s
wild bird	A/Bar-headed Goose/Qinghai/1/05	AAZ17522	Clade 2.2	QH
human	A/Xinjiang/1/2006	ACJ68614.1	Clade 2.2	XJ
human	A/Anhui/2/2005	ABD28181	Clade 2.3.4	AH
human	A/Hongkong/213/03	AB212054	Clade 1	HK

Table 2

HI titer of human sera samples

Sera	Pseudotype H5N1 viruses			
	QH	XJ	AH	HK
Vaccinee 1	3200	1600	6400	3200
Vaccinee 2	1600	3200	1600	800
Vaccinee 3	3200	1600	3200	1600
Vaccinee 4	1600	6400	1600	800
Vaccinee 5	800	6400	1600	1600
Vaccinee 6	400	3200	1600	200
Patient 1	12800	12800	12800	12800
Patient 2	12800	12800	12800	6400
b1	640	320	160	640
b2	1280	640	320	1280
b3	1208	320	160	1280
b4	<40	<40	<40	80