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Erythrocyte Binding Preference of Avian Influenza H5N1 Viruses▽†

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Five erythrocyte species (horse, goose, chicken, guinea pig, and human) were used to agglutinate avian influenza H5N1 viruses by hemagglutination assay and to detect specific antibody by hemagglutination inhibition test. We found that goose erythrocytes confer a greater advantage over other erythrocyte species in both assays.

Endemicity of H5N1 avian influenza in Southeast Asian countries since late 2003 has led to the application of various techniques to diagnose the disease. For the isolation technique, influenza virus progenies released from the infected cells are primarily recognized by hemagglutination (HA) test. In addition, HA is employed to quantify the amount of hemagglutinin antigen used in HA inhibition (HI) assay (7). Importantly, the World Health Organization (WHO) has specified the presence of a horse erythrocyte HI titer of ≥160 in adjunct with a microneutralization (microNT) antibody titer of ≥80 in a single serum collected at day 14 or later as one among other criteria for a confirmed case of H5N1 infection (8).

Agglutination of erythrocytes by influenza viruses is mediated by the interaction between the receptor binding site (RBS) in hemagglutinin molecule and the sialyl receptor. Human influenza H1N1 and H3N2 viruses preferentially bind to a sialic acid receptor, the oligosaccharide side chain of which is linked with α2,6-galactose linkage (SAα2,6Gal), while avian and equine influenza viruses prefer an α2,3-galactose linkage (SAα2,3Gal). Horse and cow erythrocytes contain mainly an SAα2,3Gal linkage but no SAα2,6Gal (1). Chicken and goose erythrocytes contain more SAα2,3Gal linkage than SAα2,6Gal, while this is reversed with human O cells and pig, guinea pig, and turkey erythrocytes (1, 2).

Herein, five erythrocyte species (horse, goose, chicken, guinea pig and human O cells) were tested by HA assay against 14 H5N1 clade 1 isolates from Thailand, including five from humans, seven from wild and domestic birds, one from a tiger and, one from a clouded leopard, together with one human H1N1 isolate and five H3N2 isolates. Final concentrations of 0.5% goose, 0.5% chicken, 0.75% guinea pig, 0.75% human group O, and 1% horse erythrocytes were used. Except for those from the horse, erythrocytes were suspended in phosphate-buffered saline, pH 7.2. Horse erythrocytes were suspended in phosphate-buffered saline plus 0.5% bovine serum albumin. These protocols were followed as described previously (6, 7). A reaction well, consisting of 50 μl of diluted virus and 50 μl of erythrocyte suspensions, was incubated for 1 h at 4°C before the agglutination pattern was read. One HA unit is defined as the highest virus dilution that yields complete HA.

The experiments demonstrated that 13 of 14 H5N1 isolates could agglutinate erythrocytes from all five species with a statistical difference in the extent of titer (Friedman test, $P < 0.05$). Interestingly, an isolate from the clouded leopard could not agglutinate horse erythrocytes (Table 1). The result was consistent, as repeatedly tested with erythrocytes from three donors within one species. Goose erythrocytes yielded the highest HA titer, followed in order of sensitivity by chicken, guinea pig, human, and horse erythrocytes (Wilcoxon's signed-rank test, $P < 0.005$).

Hemagglutinin amino acid sequences of our H5N1 isolates were compared with those of A/Goose/Guangdong/1/96 (the ancestor) and with Hong Kong virus 1997 (5) (see Fig. S1 in the supplemental material). No change in RBS was found, except for one isolate, A/Thailand/676(NYK)/05, which contained a mutational change A134V in RBS. However, this mutational change did not relate to erythrocyte binding preference. It remains to be elucidated why the isolate from the clouded leopard could not agglutinate horse erythrocytes while there were no change in RBS and no difference in the deduced amino sequence of hemagglutinin. Receptor specificity of influenza viruses is influenced by both the galactose linkage and species of sialic acid: *N*-acetylneuraminic acid (NeuAc) or *N*-glycolylneuramic acid (NeuGc). Horse erythrocytes contained only NeuGcα2,3Gal (1, 3). Therefore, loss of the ability to agglutinate horse erythrocytes may be related to loss of the ability to recognize either NeuGc or galactose linkage (1, 3).

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TABLE 1. HA titers of influenza A viruses as assayed with erythrocytes from different species

Influenza A virus and HA GMT	Passage history	Accession no.	HA titer by erythrocyte source[a]				
			Goose	Chicken	Guinea pig	Human	Horse
H1N1 human influenza virus							
A/New Caledonia/20/99-like virus (Siriraj 07/00)	MDCK7	EF568930	128	64	128	64	<2
H3N2 human influenza viruses							
A/Sydney/05/97-like virus (Siriraj 08/98)	MDCK4	EF568929	64	64	128	64	<2
A/Fujian/411/02-like virus (Siriraj 03/04)	MDCK6	EF568924	32	<2	64	64	<2
A/Fujian/411/02-like virus (Siriraj 01/03)	MDCK3	EF568925	32	<2	32	32	<2
A/Fujian/411/02-like virus (Siriraj 02/03)	MDCK2	EF568926	4	<2	32	32	<2
A/California/07/04-like virus (Siriraj 12/04)	MDCK4	EF568927	4	<2	16	32	<2
H5N1 influenza viruses							
A/Thailand/1(KAN-1)/04	LLC-MK2, MDCK8	AY555150	512	512	128	128	128
A/Thailand/2(SP-33)/04	MDCK6	AY555153	64	64	64	32	16
A/Thailand/3(SP-83)/04	MDCK5	AY577314	64	64	16	32	16
A/Thailand/5(KK-494)/04	MDCK4	AY627885	128	64	32	64	32
A/Thailand/676(NYK)/05	MDCK9	DQ360835	256	256	512	256	32
A/Great Barbet/Thailand/VSMU-2-CBI/2005	MDCK2	EF206697	128	128	64	64	32
A/Green Peafowl/Thailand/VSMU-3-CBI/2005	MDCK2	EF206700	256	128	64	64	64
A/Gray-Crowed Crane/Thailand/VSMU-4-CBI/2005	MDCK2	EF206696	512	256	128	128	128
A/Tree Sparrow/Thailand/VSMU-16-RBR/2005	MDCK4	EF178506	64	64	32	32	16
A/Golden Pheasant/Thailand/VSMU-21-SPB/2005	MDCK1	EF178517	128	64	32	16	32
A/Pigeon/Thailand/VSMU-25-BKK/2005	MDCK3	EF206698	64	64	32	16	16
A/Chicken/Thailand (Suphanburi)/137/05	MDCK4	EF568922	512	512	64	64	128
A/Tiger/Thailand/VSMU-11-SPB/2004	Egg 1	EF178531	512	512	256	256	256
A/Clouded Leopard/Thailand (Chonburi)/AI-1216A/2004	Egg 1	EF568923	512	512	256	256	<2
HA GMT of H5N1 viruses			190.21	156.03	74.25	67.25	33.62

[a] The most frequent titer obtained from three erythrocyte donors within the same species is presented.

The study of human influenza H1N1 and H3N2 viruses showed that all six isolates could not agglutinate horse erythrocytes (Table 1). An A/Sydney/05/97(H3N2)-like isolate could agglutinate chicken erythrocytes, while all three A/Fujian/411/02(H3N2)-like isolates and one A/California/07/04(H3N2)-like isolate could not. This result supported previous findings that current H3N2 isolates have lost their agglutinating activity with chicken erythrocytes (2, 4). However, all of the data, including ours, were discrete and could not conclude that there was a correlation between certain mutational positions and loss of the agglutinating activity (data not shown).

Fourteen serum samples from seven H5N1 patients, including the survivors, were assayed for H5N1 antibody. Two H5N1 strains, A/Thailand/1(KAN-1)/04 which was the first human isolate from the country (5), and A/Thailand/676(NYK)/05, as described above, were selected as the test antigens. This study was approved by the Institution Ethical Committee for Human Research.

In the HI test, serum was pretreated with a receptor-destroying enzyme (Denka Seiken, Japan) at final dilution of 1:4 for 16 h at 37°C followed by heat inactivation for 30 min at 56°C and absorbed with a 50% erythrocyte suspension for 60 min at 4°C. A mixture of 25 μl of the diluted serum and 25 μl of the test virus at a concentration of 4 HA units was incubated for 30 min at room temperature before addition of 50 μl of erythrocyte suspension. The end result was read after incubation for 1 h at 4°C. HI antibody titer is defined as the final serum dilution that completely inhibits HA. Again, three donors from each of the five erythrocyte species were tested in separate runs with consistent results.

The results demonstrated that horse erythrocytes, which were the least sensitive in HA, gave the highest geometric mean titer (GMT) of antibody when A/Thailand/1(KAN-1)/04 was used as the test antigen (Wilcoxon's signed-rank test, $P < 0.005$). Goose erythrocytes were ranked second, followed by human, guinea pig, and chicken erythrocytes. In contrast, when A/Thailand/676(NYK)/05 was used as the test antigen, goose erythrocytes yielded the highest GMT, followed in order by chicken, horse, human, and guinea pig erythrocytes. However, a statistically significant difference was not found (Wilcoxon's signed-rank test, $P > 0.005$) (Table 2). Collectively, the level of HI antibody titer was dependent on both the erythrocyte species and the test antigen used. We also showed that HI is more sensitive for strain differentiation than microNT.

Our study proposes that goose erythrocytes confer a greater advantage for recognition of H5N1 viruses and HI antibody

TABLE 2. H5N1 HI antibody titers as tested by different erythrocyte species

Patient, sequential sample no., and HI antibody GMT	Time after onset of illness	A/Thailand/1(KAN-1)/04(H5N1)[a]						A/Thailand/676(NYK)/05(H5N1)[b]					
		HI antibody titer by erythrocyte source					NT antibody titer	HI antibody titer by erythrocyte source					NT antibody titer
		Goose	Chicken	Guinea pig	Human	Horse		Goose	Chicken	Guinea pig	Human	Horse	
Patient 1													
1	8 days	20	<20	20	<20	20	<5	20	<20	<20	<20	20	
2	17 days	640	640	640	640	1,280	1,280	1,280	1,280	1,280	1,280	640	
Patient 2	2 yr, 2 mo	20	<20	20	20	40	80	160	80	80	80	80	40
Patient 3	2 yr, 1 mo	20	<20	20	20	40	160	160	80	80	80	80	80
Patient 4													
1	10 days	20	<20	<20	20	20	<5						
2	12 days	20	<20	20	<20	20	5	20		20		20	
3	6 mo	40	20	20	40	80	160	160	320	160	80	320	
4	1 year	40	20	20	40	80	160	160	160	80	160	160	80
Patient 5	7 days	<20	<20	<20	<20	<20	<5	20	<20	<20	<20	20	
Patient 6													
1	4 days	<20	<20	<20	<20	<20	5	<20	<20	<20	<20	<20	
2	15 days	320	160	160	160	640	1,280	1,280	1,280	1,280	1,280	1,280	
3	21 days	160	80	80	160	320	640	640	1280	640	640	640	
4	5 mo, 9 days	20	20	20	20	40	80	160	80	80	40	40	80
Patient 7	5 days	80	40	80	160	320	320	640	640	320	320	640	
GMT of HI antibody		40.00	24.38	31.23	36.26	65.63		136.35	134.54	93.88	100.79	110.16	

[a] A/Thailand/1(KAN-1)/04 was isolated from patient 1.
[b] A/Thailand/676(NYK)/05 was isolated from patient 5.

assay. Whether this finding is also generalized for H5N1 clade 2 viruses need to be investigated.

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