

Influenza Antihemagglutinin and Antineuraminidase Activity of IgG and IgM in Reference Chicken Antisera (36660)

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Chickens have been extensively used for a number of years as a source of reference antisera for antigenic characterization of influenza virus hemagglutinins. Two major advantages are usually cited. First, since most influenza type A strains are isolated and grown in embryonated eggs, the use of chickens avoids the problem of antihost antibodies. And second, the antibody response of chickens is rapid, with peak HI titers occurring 10 days after a single injection of crude infected allantoic fluid. Little is known, however, about the immunoglobulin class distribution of antiviral activity found in these sera. Such information is particularly relevant since chicken antisera are often thought to demonstrate closer antigen relationships between influenza A strains than those observed with antisera from other animals.

Previous studies have shown the development of two and perhaps three immunoglobulin classes in chicken antisera (1, 2) with IgG predominating by day 10 after injection of antigen. In this report we describe the fractionation of chicken antisera prepared against recombinants possessing the hemagglutinin (H3) and the neuraminidase (N2) of the A/Hong Kong/68 strain. We also determine and present the homologous and heterologous [A/Japan/57(H2N2)] antibody activity of each immunoglobulin class.

Materials and Methods. Influenza viruses. A/Hong Kong/8/68 (H3N2) and A/Japan/305/57 (H2N2) were from the collection of the WHO International Influenza Center for the Americas. Recombinant viruses A/Hong Kong/16/68 (H3)-NWS/33 (N1) and A/equine/Prague/1/56 (Heq1)-Hong Kong/16/68(N2) were received from Drs. J. L. Schulman and E. D. Kilbourne, Mount Sinai

School of Medicine, New York, as HKe and X15-HK, respectively.

Antisera. White Rock laying hens were injected intravenously with 5 ml of egg allantoic fluid containing 200 hemagglutinating units of influenza virus. Chickens were bled prior to and on day 10 after inoculation.

Antisera to normal chicken sera and immunoglobulin fractions were prepared in rabbits. On the first day rabbits were injected intravenously with an aqueous suspension of 6 mg of immunoglobulin and intramuscularly with 8 mg mixed with Freund's complete adjuvant. Additional intravenous injections of 6 mg were given every 4 days for 20 days. Animals were bled 12 days after the final injection.

Hemagglutination inhibition (HI) tests. Sera and immunoglobulin fractions were treated with 100 units of receptor destroying enzyme (RDE) to remove nonspecific inhibitors. HI tests were performed using 0.5% chicken erythrocytes in the standardized microtiter procedure (3).

Neuraminidase inhibition (NI) tests. Sera and immunoglobulin fractions were tested for NI activity according to the method of Webster and Pereira (4). Values were adjusted to 1 ml volumes.

Separation of immunoglobulins. The two major immunoglobulin classes of chicken sera were separated by molecular sieve chromatography after sodium sulfate precipitation of the immunoglobulins from defatted sera. Defatting was accomplished by adding a saturated solution of sodium bromide to raise the density of whole serum to 1.2 g/ml (5). After 1 hr at room temperature the treated sera were centrifuged at 8000g for 1 hr at 4° and lipids were removed from the surface

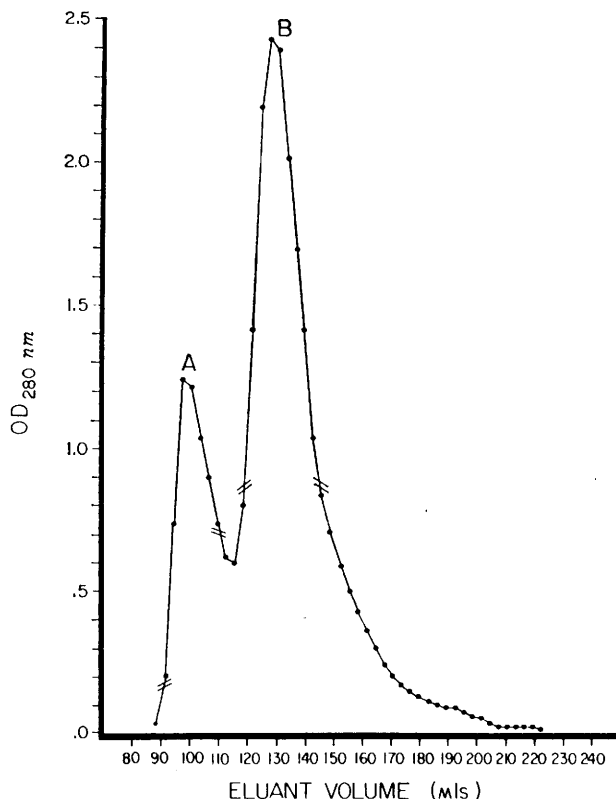


FIG. 1. Elution profile of chicken immunoglobulins precipitated with 18% sodium sulfate and eluted from a column of Bio-gel P-200. Double bars designate the fractions in pool A and pool B.

with a curved spatula. The sera were dialyzed at 4° for 4 hr against several changes of a 0.32 *M* saline solution containing 0.001 *M* sodium borate, pH 8 (0.32 S-B).

Immunoglobulins were precipitated at room temperature from defatted sera by adding crystalline sodium sulfate to a final concentration of 18%. Immunoglobulins were dissolved in distilled water and reprecipitated in the same manner. Final precipitates were dissolved in a minimum of distilled water and dialyzed for 8 hr against 0.32 S-B. Immunoglobulins were separated chromatographically with Bio-gel P-200 equilibrated in 0.32 S-B. Samples were applied to the column at a ratio of 1 mg protein to 4 cm³ of column bed volume. Protein concentrations in the eluate were monitored spectrophotometrically at 280 nm. Fractions were concentrated in an Amicon ultrafiltration device fitted with an XM-50 Diaflo membrane.

Analyses of immunoglobulin. Immunoglobulins were tested for purity by immunoelectrophoresis on 12 × 5.7 cm glass plates coated with 0.85% agarose in 0.05 *M* sodium barbital buffer, pH 8.0. A constant current of 6 mA was applied for 120 min. Electrophoretic examinations of immunoglobulin fractions were performed on cellulose acetate membranes in 0.05 *M* barbital buffer (pH 8.6) with a constant current of 0.4–0.5 mA/cm strip width for 2.5 hr. Sedimentation coefficients were determined by analyzing the immunoglobulins in a Beckman Model E analytical ultracentrifuge.

Results. Two major protein peaks were demonstrated by molecular sieve chromatography of chicken immunoglobulins precipitated by sodium sulfate (Fig. 1). Fractions from each of these peaks were collected as indicated in Fig. 1, and concentrated to a range of 2–3 mg/ml protein in pool A and

7–10 mg/ml protein in pool B.

The first peak (pool A) was shown by ultracentrifugal analysis to consist of a single protein with a sedimentation coefficient (S_{20w}) of 19.5 S. Ultracentrifugal analysis of this protein in 6.7 guanidine hydrochloride confirmed its unit molecular structure. Further evidence that peak A was IgM and consisted largely of a single protein was seen by electrophoretic analysis and immunoelectrophoresis with rabbit anti-chicken immunoglobulins. A single line was observed by both techniques.

The second peak (pool B) consisted of a single protein having a sedimentation coefficient of 7.5 S. Evidence of purity of the IgG was also obtained by electrophoretic mobility and immunoelectrophoresis.

Sera from five chickens injected with A/Hong Kong(H3)-NWS(N1) were each fractionated into the two immunoglobulin

classes. The average IgM:IgG protein ratio calculated from the elution profile of each serum was 1:3.5, which was roughly equivalent to the actual ratios in whole sera. Homologous Hong Kong/68(H3N2) and heterologous Japan/57(H2N2) HI titers were determined for whole sera and for each immunoglobulin class (Table I). Homologous HI titers were highest with IgG. The geometric mean homologous HI titer for the IgG fractions was five times that for IgM. This appeared to be related both to the larger quantity of IgG in whole serum and to the apparent fact that its antibody activity per unit of protein was nearly twice that for IgM. The reactive quantity of protein required for IgG was only 60% of that required for IgM.

Heterologous Japan/57 HI titers were more nearly equivalent in each class. This, in effect, reflects the higher degree of cross reactivity of IgM antibodies. The Hong

TABLE I. Hemagglutination Inhibition (HI) Titers of Recombinant Influenzavirus A/Hong Kong/16/68(H3)-NWS/33(N1) Chicken Antisera and Immunoglobulin Fractions.

Antiserum no.	Immunoglobulin		HI titer		Least reactive quantity of protein (μg) ^a	
	Class	Protein (mg/ml)	Hong Kong ^b	Japan ^c	Hong Kong	Japan
285	Serum		320	20		
	IgM	2.7	80	10	0.84	6.75
	IgG	6.4	160	5	1.0	32
286	Serum		640	80		
	IgM	1.5	40	10	0.94	3.75
	IgG	3.6	320	20	0.28	4.5
289	Serum		320	80		
	IgM	2.0	40	20	1.25	2.5
	IgG	5.9	160	20	0.92	7.38
292	Serum		320	20		
	IgM	2.6	40	10	1.63	6.5
	IgG	6.4	160	5	1.0	32
293	Serum		320	0		
	IgM	1.0	20	0	1.25	—
	IgG	4.2	320	0	0.33	—
Av	Serum		364 ^d	23 ^d		
	IgM	1.96	40	9	1.18	4.9
	IgG	6.63	209	8	0.71	19

^a Protein (mg/ml) \times initial test volume (ml) \times 1000 \div dilution factor.

^b A/Hong Kong/8/68(H3N2).

^c A/Japan/305/57(H2N2).

^d Geometric mean titer.

TABLE II. Neuraminidase Inhibition (NI) Titers of Recombinant Influenzavirus A/equine/Prague/1/56 (Heq1)-Hong Kong/16/68(N2) Chicken Antisera and Immunoglobulin Fractions.

Antiserum no.	Immunoglobulin		NI titer		Least reactive quantity of protein (μg) ^a	
	Class	Protein (mg/ml)			Hong Kong	Japan
			Hong Kong ^b	Japan ^c		
304	Serum		1320	200		
	IgM	4.1	250	28	16	146
	IgG	13.3	3560	200	4	67
306	Serum		159	28		
	IgM	3.0	32	<20	94	0
	IgG	8.5	440	20	19	425
307	Serum		1580	200		
	IgM	3.6	440	72	8	50
	IgG	8.7	1120	44	8	198
309	Serum		640	32		
	IgM	2.3	250	20	9	115
	IgG	8.1	320	<20	25	0
313	Serum		640	22		
	IgM	2.2	100	40	22	55
	IgG	13.3	640	<20	21	0
Av	Serum		660 ^d	58 ^d		
	IgM	3.0	150	28	30	92
	IgG	10.4	830	28	15	230

^a Protein (mg/ml) \times initial test volume (ml) \times 1000 \div dilution factor.

^b A/Hong Kong/8/68(H3N2).

^c A/Japan/305/57(H2N2).

^d Geometric mean titer.

Kong/68:Japan/57 ratio of geometric mean HI titers for IgM was 40:9 and for IgG it was 209:8.

Homologous Hong Kong/68 and heterologous Japan/57 NI titers were also determined for the two major immunoglobulin classes of sera from 5 chickens inoculated with A/equine/Prague (Heq 1)-HK (N2) (Table II). The homologous NI geometric mean titer of the IgG fraction was 5.5 times that of the IgM fraction. Much like the IgG homologous HI activity, the higher NI activity seemed to be related to both the larger amount of IgG in whole serum and its greater activity per unit of protein. Heterologous Japan/57 NI titers were not consistently found with either IgG or IgM, although the average titers for both classes were the same.

Discussion. In this report we have demonstrated that the predominant homologous antihemagglutinin and antineuramididase activ-

ities of chicken antisera collected 10 days after a single injection of influenza virus are associated with IgG. Low levels of IgM antibody were common although they were judged unlikely to influence the final homologous titers of whole sera. These findings are in contrast with earlier reports on at least one virus (bacteriophage) antigen where chicken antibody was found to be largely IgM (6).

Minor HI and NI cross-reactions in whole sera with the distantly related Japan/57(H2N2) antigens were associated equally with both immunoglobulin classes. Since IgG predominates in whole sera, these findings support general observations of greater cross-reactivity of IgM antibodies. Japan/57 N and H antigens were six times more closely related to Hong Kong/68 by titration with IgM than with IgG.

The Hong Kong/68 and Japan/57 strains were chosen for the study because they rep-

resent extremes in antigenic relatedness. In practice, when these chicken antisera are used for antigenic studies, the homologous Hong Kong/68 HI titers—which are due to IgG—are compared with heterologous Japan/57 titers—which may be due to either IgM, IgG, or both. The contribution of both classes to heterologous titers might lead to some inconsistency in the magnitude of cross-reactions observed between such distantly related strains. However, since cross-reactivity occurs in both immunoglobulin classes, there should be no problems in the recognition of common antigens.

Summary. Chicken antisera prepared against recombinants possessing the hemagglutinin (H3) and the neuraminidase (N2) of the A/Hong Kong/8/68 (H3N2) strains were fractionated and the homologous and heterologous [A/Japan/305/57(H2N2)] antibody activity of the 2 major immunoglobulin classes (IgG and IgM) was deter-

mined. Homologous hemagglutination inhibition (HI) and neuraminidase inhibition (NI) titers of whole sera were found to be predominantly IgG. Heterologous HI and NI titers of whole sera were associated with IgG, IgM, or both.

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