APPLIED MICROBIOLOGY, Mar. 1972, p. 485-489

Vol. 23, No. 3
Printed in U.S.A.

Further Studies on the Anti-Immunoglobulin G Hemagglutination-Inhibition Test for Influenza

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Received for publication 4 November 1971

Incorporating species-specific anti-immunoglobulin G (IgG) serum into the hemagglutination-inhibition (HI) test for influenza markedly increased sensitivity without loss of specificity. The effectiveness of anti-IgG serum for augmenting antibody titers may be influenced by the variable potency of commercial anti-IgG preparations. Maximal enhancement of HI titers was achieved when anti-IgG serum and virus-antiserum mixtures were incubated at 23 C for 10 min. Precision and reproducibility of the test were within acceptable limits. Other conditions likely to affect the test were investigated.

The efficacy of antiglobulin serum in enhancing the sensitivity of serological tests has been demonstrated in agglutination, precipitin, and virus neutralization reactions (1, 3, 5, 8, 15, 16; H. Daugharty, Bacteriol. Proc., p. 169, 1971). Recently, we reported that the sensitivity of the hemagglutination-inhibition (HI) test for influenza was markedly increased by the addition of anti-gamma globulin (IgG) serum (7). Titers were increased by as much as 32-fold when compared to the conventional HI test. Enhancement was effected by species-specific anti-IgG serum and limited to immunoglobulins of the IgG class. In this report, we attempt to evaluate some of the conditions that may influence the augmentation of influenza HI titers by anti-IgG serum.

MATERIALS AND METHODS

Viruses. Strains of human influenza employed in this study were AO/PR8, A_1/Ann Arbor/1/57, A_2/Hong Kong/8/68, A_2/Japan/62, B/Lee, and B/Great Lakes/1739/54. All strains were obtained from the American Type Culture Collection, Rockville, Md. Viruses were grown in the allantois of 11-day-old chick embryonated eggs. After incubation at either 35 or 37 C for 48 hr, eggs were chilled for 5 or more hr. Allantoic fluids were then harvested and individually titered for hemagglutinin, and those fluids having the highest titer were pooled. Stock preparations of viruses were distributed in 1-ml amounts and stored at −70 C.

Antisera. Antiserum to each virus strain was prepared in rabbits by an initial intramuscular injection of an equal quantity of virus suspension and Freund complete adjuvant. At weekly intervals, thereafter, animals were twice injected intravenously; they were exsanguinated 10 days after the last injection. Acute and convalescent human sera were obtained through the courtesy of Walter Dowdle, Center for Disease Control, Atlanta, Ga., and Bernard Portnoy, USC-Los Angeles County Medical Center, Los Angeles, Calif. Routinely, all rabbit and human antisera were treated with receptor-destroying enzyme (RDE). Goat anti-rabbit or anti-IgG serum and goat anti-rabbit or anti-human whole serum were obtained from the following sources: Microbiological Associates, Bethesda, Md.; Hyland Laboratories, Los Angeles, Calif.; Miles Laboratories, Kankakee, Ill.; Cappel Laboratories, Downingtown, Pa.; and Meloy Laboratories, Springfield, Va. Normal goat serum was purchased from Microbiological Associates, Bethesda, Md. All goat sera were heat-inactivated at 56 C for 0.5 hr. For experimental purposes, anti-IgG sera were treated with kaolin (14), trypsin-periodate (12), or RDE (11).

Diluents. Although 0.01 M phosphate-buffered saline (PBS), *p*H 7.2, was routinely used to prepare dilutions of test reagents, other solutions tested were tris (hydroxymethyl) aminomethane (Tris) and the dipolarionic buffers HEPES (*N*-2 hydroxyethylpiperazine-*N*'-2'-ethanesulfonic acid), tricine [*N*-tris (hydroxymethyl) methylglycine], MES [2-(*N*-morpholino) ethanesulfonic acid], bicine [*N*, *N*-bis (2-hydroxyethyl) glycine], TES [*N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid], and BES [*N*, *N*-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid] obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. The concentration of these buffer reagents was 0.025 M in normal saline; each solution was adjusted to *p*H 7.1 ± 0.1.

Erythrocyte suspension. Blood obtained from the wing vein of white leghorn chickens was mixed with an equal volume of Alsever's solution, and the cells were washed three times in PBS. The final washing was carried out in a graduated centrifuge tube at the recommended conditions of sedimentation (2). Packed cells were resuspended in PBS to

make a 10% suspension that was calculated by volume. For each day's test, the 10% cell suspension was diluted with PBS to yield a 0.5% erythrocyte suspension.

HI tests. The standard microtiter system (13) using rigid plastic microtitration plates containing 96 "V" bottom wells was employed (Cooke Engineering Company, Alexandria, Va.). In a typical test, each well received 0.025 ml of PBS; test serum in 0.025-ml amounts was serially diluted in twofold steps. All serum dilutions then received 0.025 ml of 4 virus hemagglutinin units. After incubation at room temperature (22 to 24 C) for 1 hr, 0.025 ml of a 1:40 dilution of either goat anti-rabbit IgG or normal goat serum was added to each well. These were incubated at room temperature for 15 min at which time each well received 0.05 ml of 0.5% chick erythrocyte suspension to make a total test volume of 0.125 ml. Appropriate controls consisted of combinations of the different reagents with erythrocyte suspension. Plates were sealed and then agitated on a Micro-Mixer (Microbiological Associates, Bethesda, Md.). Test patterns were read after incubation at room temperature from 1 to 2 hr. The HI titer was the reciprocal of the highest test serum dilution that completely inhibited hemagglutination.

RESULTS

HI titers with different buffer systems. Tests were performed with the addition of either anti-IgG or normal goat serum to determine the effect of different buffer systems, *p*H 7.1 ± 0.1, on influenza HI titers. Standard buffers such as phosphate and Tris were evaluated as well as six dipolarionic buffers, HEPES, tricine, MES, bicine, TES, and BES (4). All reagents, e.g., virus, sera, and erythrocytes, were diluted in the appropriate solution to be tested. Comparable HI titers resulted with all buffer systems at the *p*H and temperature conditions of the test. Hemagglutination was inhibited when either 0.2% bovine serum albumin or 0.2% gelatin or both were added to phosphate or HEPES buffers. For routine performance of HI tests, PBS was adopted as the diluent for reagents.

Time relationship for enhancement of HI titer by anti-IgG serum. The neutralization of infectious virus-antibody complexes by anti-IgG serum was shown to be complete within 1 min at 35 C (6). To determine the length of incubation time between influenza virus-antiserum mixtures and anti-IgG serum for optimal enhancement of HI titers, mixtures of virus and antiserum dilutions were first incubated at 23 C for 1 hr. Normal goat or goat anti-IgG serum was added to mixtures, which were then incubated at 23 C for designated intervals. Immediately thereafter, normal serum was added to bind unreacted anti-IgG molecules. Ten minutes later, chick erythrocyte suspension was added. Results show that maximal enhancement of HI titer of antiserum occurred within 10 min after the addition of anti-IgG serum (Table 1). An incubation time of 15 min between anti-IgG serum and virus-antiserum mixtures was employed in subsequent tests.

Inhibitors of hemagglutination in anti-IgG serum. The possibility that anti-IgG serum may contain inhibitors of hemagglutination which could account for the observed enhancement of HI titers was investigated. HI tests were carried out with Lee, Great Lakes, and PR8 antisera. Anti-IgG serum that was untreated or treated with RDE, trypsin-periodate, or kaolin was then diluted 1:40 and reacted with each of the virus-antiserum mixtures. HI titers were unchanged for either untreated or treated anti-IgG serum. These findings indicate that inhibitors of hemagglutination were not present in the dilution of anti-IgG serum employed in the tests.

Enhancement of HI titers by anti-IgG and anti-whole sera. To determine whether anti-whole serum is as effective an anti-IgG serum in enhancing HI titers, rabbit and human anti-IgG and anti-whole sera were tested with their respective species-specific antisera. Normal goat serum served as a control. Equivalent enhancement of HI titers was obtained when either anti-IgG or anti-whole serum was used.

TABLE 1. *Length of incubation time between influenza virus-antiserum mixtures and anti-IgG serum for enhancement of HI titer*

Incubation time[a] (min)	HI titer of rabbit antiserum[b]	
	Normal goat serum	Goat anti-rabbit IgG serum
1	4,000	8,000
3	4,000	8,000
5	4,000	8,000
10	4,000	32,000
20	4,000	32,000
30	4,000	32,000
60	4,000	32,000

[a] Mixtures of Great Lakes strain of influenza virus and twofold dilutions of rabbit antiserum were incubated at 23 C for 1 hr; a 1:40 dilution of normal goat or goat anti-rabbit IgG serum was then added to each mixture and incubated at 23 C for designated intervals. Immediately thereafter, normal rabbit serum was added to bind unreacted anti-IgG antibodies. Ten minutes later, 0.5% chick RBC suspension was added.

[b] Numerical values are the reciprocal of HI titer of virus antiserum.

This is concordant with results obtained on the neutralization of infectious virus-antibody complexes by anti-IgG and anti-whole sera (9).

Relative potency of anti-IgG serum from different sources. When testing large numbers of sera, it is an economic advantage to use the highest dilution of anti-IgG serum that gives maximal enhancement of HI titers. Based on this criterion, the relative potency of anti-IgG serum from five different commercial sources was evaluated by reacting different dilutions of anti-IgG serum with PR8 virus-antiserum mixtures. Results show that the highest dilution of anti-IgG serum capable of enhancing HI titers maximally varied with each serum tested (Table 2). The prozone of hemagglutination encountered with high concentrations of anti-IgG serum was noted previously (7). This finding and the variable potency of anti-IgG serum from different sources emphasize the need to assess the effectiveness of the anti-IgG serum to be employed in tests.

Precision and reproducibility. To estimate the precision of the anti-IgG HI test, 10 determinations were made with the same reagents under identical test conditions. That similar HI titers were obtained in 9 of the 10 determinations attests to the precision of the test. The one determination that varied from the others showed a twofold difference in titer.

The reproducibility of titers with four paired human sera was determined by making daily tests for 5 consecutive days under similar conditions. That only one determination showed a twofold difference in titer from the others attests to the reproducibility of the test.

Cross-reactivity determination. Reciprocal HI tests were carried out with antisera from rabbits previously immunized with representative influenza virus strains AO, A1, A2, and B. Normal goat or goat anti-rabbit IgG serum was added to virus-antiserum mixtures; the former exemplified a conventional HI test. Antiserum reacted with homologous virus gave the highest HI responses; in the presence of anti-IgG serum, however, titers were as much as 64-fold higher than those obtained with normal goat serum (Table 3). Strong reciprocal reactions were noted between both A_2 virus strains and between both B virus strains, respectively. In all instances, cross-reactivity was highest in the presence of anti-IgG serum. Reciprocal reactions between influenza A and B virus strains did not occur.

Comparative serological diagnosis of influenza. Fourteen pairs of human sera, consisting of acute and convalescent specimens, were titers for influenza HI antibodies with three representative A strains and one B virus strain. Tests were performed by the conventional test and with the addition of anti-human IgG serum. Based on a fourfold or greater increase in titer, A_2 influenza comprised the majority of serologically positive sera (Table 4). One serum was positive for influenza B. That the sensitivity of the HI test employing anti-IgG serum was greater than that of the conventional test is indicated by the significantly higher titers and the greater number of positive responses of sera obtained with the former.

DISCUSSION

Previous studies demonstrated that the enhancement of influenza HI titers by anti-immunoglobulins was limited to the IgG class (8). An attempt was made to recognize and characterize the conditions that favor the potentiation of HI titers by anti-IgG serum. On examining the relative potency of five anti-IgG sera obtained from different commercial sources, we found that the highest dilution of anti-IgG serum capable of augmenting HI titers maximally varied with each anti-IgG serum tested.

TABLE 2. *Relative potency of anti-IgG sera from different commercial sources for enhancement of influenza HI titers*[a]

Commercial source of anti-IgG serum	Dilutions of goat anti-human IgG serum[b]					
	1:10	1:20	1:40	1:60	1:80	1:100
A	320	1,280	1,280	1,280	1,280	640
B	640	1,280	1,280	1,280	640	320
C	320	1,280	1,280	1,280	320	320
D	320	1,280	1,280	320	320	320
E	320	1,280	320	320	320	320
Normal goat serum (control)	320	320	320	320	320	320

[a] HI tests were performed with PR8 virus and human antiserum.
[b] Reciprocal of HI titer of virus antiserum.

Each product, however, increased HI titers to the same level. A comparative evaluation of commercial anti-human immunoglobulins indicated that there is considerable variability in antibody potency and specificity from that which is claimed (10). Our findings confirmed the variability of antibody potency of anti-IgG preparations. As a prerequisite to employing anti-IgG serum in HI tests, the relative potency of each serum should be assessed.

A phenomenon related to the use of anti-IgG serum was the manifestation of a prozone of hemagglutination, noted in this study (Table 2) and reported earlier (7), when low dilutions of anti-IgG serum were used. That virus is freed from its union with specific antibody by the action of excess anti-IgG serum may account for the phenomenon. There was no evidence that inhibitors of agglutination (11) were present with the dilution of anti-IgG serum routinely employed in tests.

The performance of the anti-IgG HI test for influenza is essentially similar to that of the conventional test. The interaction of anti-IgG serum with virus-antiserum mixtures only adds 10 to 15 min to the test. Inasmuch as anti-whole serum appears to effect enhancement of HI titers, it may be substituted for anti-IgG serum. The specificity, precision, and reproducibility of the test appear to be comparable to that of the conventional test. Sera containing antibodies to any of the major types of influenza virus were elevated in titer by the action of anti-IgG serum. Reciprocal HI tests carried out in the presence of anti-IgG serum showed stronger cross-reactions; the sensitivity of the test for diagnoses of influenza was greater than that of the conventional test.

TABLE 3. *Reciprocal HI of influenza virus strains in the presence of normal goat and anti-IgG serum*[a]

Virus strain	HI titer of antiserum[b]					
	PR8	Ann Arbor	Hong Kong	Japan	Great Lakes	Lee
PR8-AO	5,120/10,240	20/20	<20/<20	<20/<20	<20/<20	<20/<20
Ann Arbor-A_1	40/40	1,280/10,240	<20/<20	<20/<20	<20/<20	<20/<20
Hong Kong-A_2	40/640	<20/<20	1,280/40,960	320/5120	<20/<20	<20/<20
Japan-A_2	</220	<20/<20	160/1280	640/5,120	<20/<20	<20/<20
Great Lakes-B	<20/<20	<20/<20	<20/<20	2,560/40,960	<20/<20	80/5,120
Lee-B	<20/<20	<20/<20	<20/<20	<20/<20	20/40	320/2,560

[a] HI tests were performed with serum from rabbits immunized with different virus strains. Normal goat or anti-rabbit IgG serum was added to virus-antiserum mixtures.

[b] Reciprocal of HI titer of virus antiserum; normal goat serum/goat anti-rabbit IgG serum.

TABLE 4. *Serological diagnosis of influenza by the conventional and anti-IgG H-I tests*[a]

Serum sample	Virus							
	PR8-A0		Ann Arbor-A_1		Japan-A_2		Lee-B	
	NGS	Anti-IgG	NGS	Anti-IgG	NGS	Anti-IgG	NGS	Anti-IgG
1	20/20	20/20	40/80	40/80	160/160	640/640	80/320	80/1,280
2	<20/<20	20/<20	<20/<20	<20/<20	160/160	640/1,280	<20/<20	<20/<20
3	40/40	40/40	<20/<20	<20/<20	20/160	20/1,280	<20/<20	<20/<20
4	<20/<20	<20/<20	<20/<20	<20/<20	20/80	20/80	<20/<20	<20/<20
5	<20/<20	<20/<20	20/40	20/40	20/160	20/640	<20/<20	<20/<20
6	<20/<20	<20/<20	<20/<20	<20/<20	<20/160	<20/640	<20/<20	<20/<20
7	<20/<20	<20/<20	20/40	20/40	20/160	20/640	20/20	20/20
8	<20/<20	<20/<20	<20/<20	<20/<20	40/320	40/1,280	<20/<20	<20/<20
9	<20/<20	<20/<20	<20/<20	<20/<20	20/80	20/80	<20/<20	<20/<20
10	<20/<20	<20/<20	<20/<20	<20/<20	160/160	2,560/10,240	<20/<20	<20/<20
11	<20/<20	<20/<20	<20/<20	<20/<20	80/160	80/640	<20/<20	<20/<20
12	<20/<20	<20/<20	<20/<20	<20/<20	<20/80	<20/320	<20/<20	<20/<20
13	<20/<20	<20/<20	80/160	80/160	<20/320	<20/1,280	<20/<20	<20/<20
14	<20/<20	<20/<20	80/80	80/80	20/320	20/5,120	<20/<20	<20/<20
No. of sera with four-fold or greater rise in titer	0	0	0	0	10	12	1	1

[a] HI tests were performed with either normal goat serum (NGS) or goat anti-human IgG sera added to virus-serum mixtures. Numerical values are the reciprocal HI titers of acute/convalescent sera.

Anti-IgG serum incorporated into other serological reactions also increased sensitivity without loss of specificity (1, 3, 5; H. Daugharty, Bacteriol. Proc., p. 169, 1971). For detecting and estimating low concentrations of viral antibodies and in revealing subtle relationships among virus strains, the anti-IgG HI test may prove highly advantageous.

ACKNOWLEDGMENTS

We thank Janet Simpson and John Stewart for their excellent technical assistance.

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