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Quantitative Assessment of Hemadsorption by Myxoviruses: Anti-Immunoglobulin G Hemadsorption-Inhibition Test

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A quantitative hemadsorption-inhibition test was developed to estimate myxovirus serum antibodies within 24 h by determining the serum dilution inhibiting hemadsorption in 50% of the infected cells. The test depends on the interactions of virus-infected cell monolayers with antiviral serum and of the resultant complexes with antiimmunoglobulin G serum. The incorporation of species-specific anti-immunoglobulin G serum into the test significantly increased sensitivity.

The discovery that erythrocytes adsorb to myxovirus-infected cell cultures (12) and that the phenomenon is inhibited by specific viral antiserum provided the basis for a hemadsorption-inhibition (Had-I) test that proved useful for the detection and measurement of myxovirus serum antibodies (3, 11). In comparison to the widely used hemagglutination-inhibition test for detection of myxovirus antibodies, the Had-I test has a serious disadvantage as it is generally performed. Several days are required to obtain results because the test is dependent on the utilization of infected-cell cultures in which virus has propagated for several generations (4). In addition, the virus dose cannot be accurately controlled in the conventional Had-I test. Recently, a quantitative assay of myxoviruses, based on the enumeration of individual infected cells exhibiting hemadsorption, was developed that is sensitive, rapid (less than 24 h), and reproducible (9). The applicability of this assay was extended to the measurement of myxovirus serum antibodies. Preliminary tests indicated that the serum dilution end point which inhibits hemadsorption in 50% of the infected cells may be quantitatively determined within 24 h. Furthermore, the incorporation of anti-immunoglobulin G (IgG) serum was found to enhance the sensitivity of the test.

This report describes the standardization and evaluation of an Had-I test for myxovirus antisera that depends on both the interactions of virus-infected cells with antiserum and of the resultant complex with anti-IgG antibodies.

MATERIALS AND METHODS

Viruses. Strains of human influenza and parainfluenza viruses employed in this study were A_0/PR8/8/34, A_2/Japan/8/62, A_1/Ann Arbor/1/57, A_2/Hong Kong/8/68, B/Lee/40, B/Great Lakes/1739/54, and parainfluenza 3 (C 243). They were obtained from the American Type Culture Collection, Rockville, Md. Stock virus pools were prepared in the manner described elsewhere (9).

Cell culture. Clone 1-5C-4 cells were propagated on cover slips as described earlier (9).

Erythrocyte suspension. The preparation of 0.4% guinea pig erythrocyte suspensions has been reported previously (9).

Antisera. Antiserum to each virus strain was prepared in rabbits as described previously (8). Acute and convalescent human sera were obtained through the courtesy of Walter Dowdle, (Center for Disease Control, Atlanta, Ga.) and Bernard Portnoy (University of Southern California-Los Angeles County Medical Center, Los Angeles, Calif.). Goat anti-rabbit and anti-human IgG sera were obtained from Meloy Laboratories, Springfield, Va. Normal goat serum, goat anti-monkey, anti-mouse, anti-guinea pig, and anti-bovine IgG sera were obtained from Microbiological Associates, Bethesda, Md. All sera were heat-inactivated at 56 C for 0.5 h.

Had-I test. To initiate infection of cells, virus attachment onto cover slip cell monolayers was augmented by centrifugation (9). The dilution of virus inoculum in a 0.2-ml volume was such that an average of 4 to 10 infected cells per microscopy field was obtained after incubation from 22- to 24-h at 35 C. The general procedure for the anti-IgG Had-I test consisted of adding appropriate twofold dilutions of test serum in a 0.25-ml volume onto duplicate, infected, cover slip cell cultures. After incubation at

35 C for 30 min, cell monolayers were rinsed twice with phosphate-buffered saline, and a 1:10 dilution of either normal goat, goat anti-human, or anti-rabbit IgG serum was added in a 0.25-ml volume. Cell monolayers were then incubated at room temperature (23 C) for 10 min and again rinsed with phosphate-buffered saline. Appropriate controls consisting of normal serum dilutions and normal goat or anti-IgG serum were included. A 0.4% suspension of guinea pig erythrocytes was added in a 0.5-ml volume onto each cell monolayer which was then held at 6 C for 20 min. Cell monolayers were rinsed twice with PBS and examined microscopically for hemadsorption. The procedure for enumerating cells has been described previously (9). For each cover slip cell monolayer, 30 microscope fields were examined.

The percent inhibition of hemadsorption of infected cells by each serum dilution was computed from normal (control) serum counts as follows: percentage inhibition = 1 − (no. of hemadsorption cells with test serum/no. of hemadsorption cells with control serum × 100. Inhibition percentages were then plotted against the logarithm of the corresponding final dilutions of test serum on probability paper. A linear relationship was obtained over a critical range. By interpolation, the 50% Had-I serum titer was determined.

RESULTS

Parameters related to the enhancement of Had-I serum titers by anti-IgG. Preliminary tests were performed to determine the 50% Had-I titer of human anti-influenza serum in the presence of either normal goat serum or goat antiserum to human IgG. Essentially, these tests were conducted in the manner described earlier. The cell monolayers employed were infected with influenza PR8 virus. Results of a representative test (Fig. 1) show a linear function over a critical range of serum dilutions when inhibition percentages of hemadsorption were plotted against the corresponding final dilutions of test sera. The 50% Had-I titer of serum in the presence of normal goat or anti-IgG sera were 1:59 and 1:230, respectively. The serum Had-I activity was approximately fourfold higher when anti-IgG serum was incorporated into the test.

The effect of time and temperature of incubation on the binding of serum antibodies to influenza virus-infected cells was investigated. Test serum dilutions were incubated with infected cells at 23 and 35 C for 0.5, 1, and 2 h. Results (Table 1) show that the maximal Had-I serum titer was achieved within 0.5 h at 35 C incubation. At incubation temperature of 23 C for 2 h, the Had-I serum titer was markedly lower than that noted at 35 C.

The time relationship for enhancement of Had-I titer by anti-IgG serum was determined

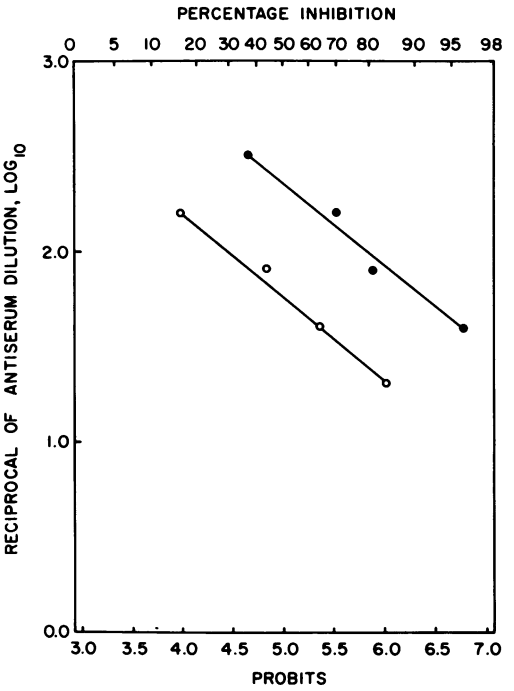

FIG. 1. *Comparative determination of influenza (PR8) Had-I human serum titer in the presence of normal goat* (○) *and goat anti-human IgG* (●) *sera. The dilution of test serum for 50% inhibition of hemadsorption was obtained by interpolation.*

TABLE 1. *Effect of incubation time and temperature for influenza (PR8)-infected cells and antiserum on hemadsorption-inhibition (Had-I) serum titer*

Incubation conditions		Reciprocal of 50% Had-I serum titer[a]
Temp (C)	Time (h)	
23	0.5	1,300
23	1	2,500
23	2	2,700
35	0.5	4,000
35	1	3,800
35	2	4,000

[a] Rabbit anti-influenza (PR8) serum.

by first reacting influenza virus-infected cells with rabbit anti-virus serum dilutions at 35 C for 0.5 h. After washing cell monolayers, a 1:10 dilution of goat anti-rabbit IgG serum was added to cell monolayers which were then incubated at 23 C for designated times. Results show that maximal enhancement of Had-I serum titer occurred within 1 min after the addition of anti-IgG serum (Table 2). Similar results were obtained at 35 C. For routine testing, anti-IgG serum was incubated with

infected cell-serum complexes at 23 C for at least 5 min.

When testing large numbers of sera, it is an economical advantage to use the highest dilution of anti-IgG serum that gives maximal enhancement of Had-I serum titers. To determine this, twofold dilutions of anti-IgG serum were reacted with influenza virus-infected cell-serum complexes at 23 C for 10 min. Results indicate that maximal enhancement of Had-I serum titer could be achieved with a 1:10 dilution of anti-IgG serum (Table 3). Because the potency of anti-IgG serum from different lots and sources may vary (8), preliminary determinations are requisite to assess the effectiveness of anti-IgG serum.

An experiment was performed to determine the capability of goat antiserum to IgG of different animal species (human, monkey, mouse, guinea pig, and bovine) to enhance the Had-I titer of human serum. Influenza virus-infected cell-serum complexes were treated with different anti-IgG sera and incubated at 23 C for 10 min, and then cell monolayers were tested for reduction of hemadsorption. The reactivity of anti-IgG serum in the test was species specific, but some cross-reactivity between anti-monkey IgG and the human test serum was evident. This reactivity may be attributable to a common primate antigenic determinant.

When different classes of human immunoglobulins—anti-immunoglobulins G, M, A, D, and E—were introduced into the test, only anti-IgG serum significantly enhanced the Had-I serum titer.

To estimate the precision of the anti-IgG Had-I test, 10 determinations were made under identical conditions with a human serum sample and influenza virus-infected cell monolayers. Reciprocal 50% Had-I serum titers ranged from 110 to 120 with a mean of 115 and standard deviation of ±4.8. Expressed as a percentage, the standard deviation was 4.1% of the mean which was comparable to that noted with anti-IgG serum-neutralization tests for viruses (7, 14).

TABLE 2. *Length of incubation time between influenza (PR8)-infected cell-antiserum complex and anti-IgG serum on hemadsorption-inhibition (Had-I) serum titer*

Incubation time[a] (min)	Reciprocal of 50% Had-I serum titer
1	1,600
5	1,600
10	1,500
15	1,400
30	1,500
60	1,600

[a] Influenza-infected cell monolayers were treated with twofold serial dilutions of rabbit anti-virus serum for 0.5 h at 35 C; monolayers were washed twice with phosphate-buffered saline, and a 1:10 dilution of goat anti-rabbit IgG serum was then added to each cell monolayer. At designated intervals of incubation at 23 C, cell monolayers were washed with phosphate-buffered saline, and tested for hemadsorption.

TABLE 3. *Effect of anti-IgG serum dilutions on the hemadsorption-inhibition (Had-I) titer of influenza (PR8) serum antibodies*

Dilution of goat anti-human IgG serum	Reciprocal of 50% Had-I titer of human serum
1:5	250
1:10	240
1:20	210
1:40	120
1:80	52
1:20 (Normal goat)	52

Cross-reactivity determination. Reciprocal anti-IgG Had-I tests were carried out with antisera from rabbits previously immunized with representative influenza A_0, A_1, A_2, B, or parainfluenza 3 virus strains. Antiserum reacting with homologous virus-infected cells gave the highest Had-I responses (Table 4). Reciprocal inhibition of hemadsorption was only noted between A_2 strains of influenza virus.

Comparative serological determinations of Had-I serum titers. Thirteen pairs of human sera, consisting of acute and convalescent specimens, were assayed for influenza and parainfluenza 3 antibodies by the Had-I test in the presence of normal goat (conventional) or goat anti-human IgG serum. With the exception of two paired sera tested by the conventional method, all convalescent sera showed at least a threefold rise in Had-I titers when assayed by either procedure (Table 5). However, Had-I titers determined in the presence of anti-IgG serum showed an average rise in titer that was twice that obtained by the conventional method. In one instance, paired influenza sera that were tested by the conventional procedure showed no rise in titer. When tested in the presence of anti-IgG serum, a threefold or greater rise in titer was noted. It is evident from these results that the sensitivity of the Had-I test employing anti-IgG serum is greater than that of the conventional procedure.

DISCUSSION

The efficacy of antiglobulin serum for enhancing the sensitivity of serological reactions

TABLE 4. *Cross-reactivity determinations of myxovirus strains by the hemadsorption-inhibition (Had-I) test*

Virus strain	Reciprocal of 50% Had-I titer of viral antiserum[a]						
	PR8	Ann Arbor	Japan	Hong Kong	Lee	Great Lakes	Para-3
A_0/PR8/8/34	1,600	<10	<10	<10	<10	<10	<10
A_1/Ann Arbor/1/57	<10	250	<10	<10	<10	<10	<10
A_2/Japan/8/62	<10	<10	270	60	<10	<10	<10
A_2/Hong Kong/8/68	<10	<10	64	750	<10	<10	<10
B/Lee/40	<10	<10	<10	<10	230	<10	<10
B/Great Lakes/1739/54	<10	<10	<10	<10	<10	120	<10
Parainfluenza 3	<10	<10	<10	<10	<10	<10	1,150

[a] Virus antiserum from rabbits and goat anti-rabbit IgG serum were employed in tests.

TABLE 5. *Determinations of myxovirus antibodies in paired human serum samples by the conventional and anti-IgG hemadsorption-inhibition (Had-I) tests*[a]

Virus strain	Serum sample	Reciprocal of 50% Had-I titers of serum samples	
		Conventional	Anti-IgG
Influenza (PR8)	1	<10/78[b]	15/210
	2	17/36	40/360
	3	12/58	24/230
	4	<10/<10	<10/32
Influenza (Japan)	5	10/75	14/190
	6	<10/42	<10/90
	7	25/210	29/350
	8	34/370	35/600
	9	<10/90	20/240
	10	42/150	100/700
Parainfluenza 3	11	80/270	80/900
	12	11/30	26/120
	13	32/180	100/800

[a] Had-I tests were performed with either normal goat serum (conventional) or goat anti-human IgG serum.

[b] Acute/convalescent sera.

has been demonstrated in agglutination (1, 5, 10), precipitin (H. Daugharty, Bacteriol Proc., p. 169, 1971), hemagglutination-inhibition (8), and virus-neutralization tests (7, 13, 14). In this study, the applicability of the phenomenon was extended to the Had-I test. Myxovirus Had-I serum titers were significantly increased when anti-IgG serum was incorporated into the test. By employing a rapid and precise hemadsorption assay for myxoviruses, the test serum dilution that inhibits hemadsorption in 50% of the infected cells was quantitatively determined within 24 h. This is a singular advantage over current Had-I tests which require from 3 to 5 days before results are obtained (4).

Investigation of the relation of time and temperature to the interaction of test components revealed that maximal binding of serum antibodies to infected-cell monolayers was achieved within 0.5 h at a 35 C incubation. The reaction was less efficient at 23 C. In contrast to the temperature and time dependence of the latter reaction, the interaction of infected cell-antibody complexes with anti-IgG serum was complete within 1 min at 23 C. These findings are comparable to those noted in anti-IgG virus-neutralization tests (2, 7). That the capacity of anti-IgG serum to enhance Had-I titers, for the most part, is species dependent and that it is maximal with the IgG class of immunoglobulins are similar to results noted with other serological reactions (2, 7, 8). The mechanism by which increased inhibition of hemadsorption occurs when anti-IgG serum is added to a virus-infected cell-specific antibody complex is unknown. It is possible that anti-IgG antibodies may form bridges to cover the remaining critical sites of hemadsorption on infected cell membranes. In view of recent findings that anti-IgG serum may inhibit hemagglutination of influenza viruses in the presence of antibody specific for the neuraminidase (6), it is possible that neuraminidase antibodies may participate in the reactions described for the anti-IgG Had-I test. This may account for the cross-reactivity noted between the A_2/Japan/8/62 and A_2/Hong Kong/8/68 influenza viruses (Table 4).

The high precision of the Had-I test is a reflection of the hemadsorption assay of myxoviruses described earlier (9). The increased sensitivity is directly related to the incorporation of anti-IgG serum. In comparative tests with paired human sera, myxovirus Had-I serum antibodies determined in the presence of anti-IgG serum showed an average rise in titer that was twice that obtained by conventional means. For diagnostic purposes, the precision, sensitivity, and rapidity of the anti-IgG Had-I

test for detecting myxovirus serum antibodies offer distinct advantages over presently employed Had-I tests.

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