New Tests for Characterization of Mumps Virus Antibodies: Hemolysis Inhibition, Single Radial Immunodiffusion with Immobilized Virions, and Mixed Hemadsorption

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Hemolysis inhibition (HLI), single radial immunodiffusion (SRID) with immobilized virions, and mixed hemadsorption tests were used for measuring antibodies against mumps virus. Rabbit hyperimmune sera against mumps and early and late human convalescent sera were analyzed. All three tests identified antibodies against both hemagglutinin and the second major envelope component, hemolysin (fusion factor). The sensitivity of the HLI test corresponded to that of the hemagglutination inhibition (HI) test, but in some sera HLI antibodies occurred in greater quantity than HI antibodies. The SRID test readily identified rises in antibody titers in connection with acute infection. Due to its simplicity and lack of sensitivity to nonspecific inhibitors, it is recommended for use in this context. The mixed hemadsorption test showed a high sensitivity for specific identification of mumps antibodies. It therefore may be suitable for use in screening for immunity to mumps.

Primarily, three techniques, neutralization, hemagglutination inhibition (HI), and complement fixation (CF), are available for determination of antibodies to mumps virus (8). For the serological diagnosis of a mumps infection, CF and HI tests are usually satisfactory but, particularly in the latter test, nonspecific inhibitors may cause certain problems. In some cases the neutralization test can indicate antibody rises not detectable by the other two tests. However, the technical complexity of the neutralization test limits its usefulness. Identification of low titers of antibodies as an indicator of immunity to mumps infections presents considerable problems. The neutralization test gives the most reliable results, but it is not practically applicable.

Recently, a single radial hemolysis-in-gel technique was developed for demonstration of mumps virus antibodies (P. Väänänen, T. Hovi, E.-P. Helle, and K. Penttinen, *Arch. Virol.*, in press). This test was shown to be somewhat more sensitive than the HI test, and it was not sensitive to nonspecific inhibitors. A fairly good correlation between the diameter of the hemolysis ring and the antibody titer measured by the CF technique was found.

The aim of the present study was to develop simplified and reliable techniques for serological diagnosis of mumps virus infections and for screening immunity to mumps. For these pur-

poses hemolysis inhibition (HLI), single radial immune diffusion (SRID), and the mixed hemadsorption test were tried. The SRID test was performed with immobilized virions in agarose gels and thus detected antibodies against the surface of virus particles. This technique has previously been successfully applied to the characterization of antibodies against the neuraminidase and hemagglutinin of influenza virus (15), antibodies to different antigens on the surface of adenovirus (7), and poxvirus particles (V. J. Prakash and E. Norrby, *J. Gen. Virol.*, in press). The mixed hemadsorption technique is a variant of the mixed antiglobulin reaction adapted by Fagraeus and Espmark (5) for the demonstration of surface antigens in monolayer tissue cultures. The technique has been used for titration of antibodies against membrane-associated cellular and viral antigens (1, 3-6).

MATERIALS AND METHODS

Virus and cell cultures. The strain of mumps virus used was isolated by the Department of Virology, National Bacteriological Laboratory, Stockholm. The virus was propagated by allantoic sac inoculation into 7- to 9-day-old chicken embryos. The virus-containing allantoic fluid was collected after 5 days by using standard techniques (8). The virus was purified by differential centrifugation at 10,000 rpm for 45 min in the GSA rotor of a Sorvall superspeed ultracentrifuge. Further purification of

the pelleted material was obtained by centrifugation in discontinuous sucrose gradients to an interphase between 50 and 20% sucrose, as has been previously described for measles virus (12).

Conventional serological tests. HI tests were performed by using standard techniques in microplates. Tween 80-ether-treated virus material was used as antigen (2). Sera to be tested were preabsorbed with the type of erythrocytes used in the test. HLI tests were performed as previously described for measles virus (10, 11), using green monkey erythrocytes. CF tests were performed as a standard microassay, using allantoic fluid from infected eggs as antigen. This preparation contains mainly V antigen.

SRID test. The SRID test was performed as described previously (7; Prakash and Norrby, *J. Gen. Virol.*, in press). For certain technical considerations, see Results.

Mixed hemadsorption. The previously described technique (4, 6) was adopted. Monolayer cultures of Vero cells (a green monkey kidney cell line) in milk bottles were infected with mumps virus at a multiplicity of 1 to 10. Two to 3 days later, the cultures were used for the test. Uninfected cultures were used as controls. Antiglobulin sera were prepared by immunization with ammonium sulfate-precipitated human or rabbit globulins. The test system, therefore, indicates primarily immunoglobulin G (IgG) but may also demonstrate IgM. Certain modifications of the test had to be introduced as described below.

Serum samples. Three different sets of serum samples were studied.

Rabbit hyperimmune sera against (i) purified whole virus, (ii) isolated hemagglutinin obtained by isoelectric focusing of Tween 20-alkali-treated virions, and (iii) projection-less particles prepared by Pronase treatment of virions, previously used in studies of surface antigens of mumps virus (13), were used. The two latter types of sera were included because they contain antibodies predominantly against the hemagglutinin and hemolysin of the virus, respectively.

Six pairs of sera from patients with mumps showing a significant antibody rise in CF tests were selected from the routine diagnostic activity of the Virus Department of the National Bacteriological Laboratory. Acute sera were collected in connection with appearance of clinical symptoms, and convalescent sera were taken 10 to 30 days later.

Twenty human sera with no or only marginal titers of mumps antibodies in conventional HI and CF tests were selected for serological analysis of immunity to mumps.

RESULTS

SRID: technical considerations. The SRID test required a high concentration of virus in the agarose gel. A minimum hemagglutinin titer of 640 U of purified and concentrated mumps virions mixed with an equal amount of 2% agarose was needed to yield detectable zones. Most virion-containing gel plates were used for immediate experimentation but, simi-

lar to experiences with other systems, it was found that plates could be stored for several weeks at 4°C before use. After addition of sera to wells in the plates, opalescent zones started to develop within 1 h. The main increase in size of zones occurred during 7 to 8 h of incubation, and final readings were taken at 24 h if not otherwise stated. Zones obtained usually had sharp margins, but they were somewhat more dense in tests with rabbit hyperimmune sera than in tests with human convalescent sera (cf. Fig. 3). Further, some rabbit sera gave multiple zones, whereas this was not clearly distinguishable with the human sera studied.

A dose-response analysis was performed with a rabbit hyperimmune serum against whole virus and a human convalescent serum known to give a strong reaction in the SRID test (Fig. 1). A linear relationship between the relative area of zones formed and serum dilutions was found with diluted samples, similar to findings in other systems (9). However, undiluted serum gave a somewhat smaller zone area than that expected theoretically.

The diameter of zones never varied more than 2 mm in comparisons of tests with different plates prepared on the same or different days. A difference between zone diameters of 3 mm or more was therefore considered significant. With the linear relationship illustrated in Fig. 1, this corresponds to about a threefold titer difference with diluted serum samples.

Whether addition of anti-human IgG or anti-rabbit IgG could enhance the visualization of zones was determined. However, no significant improvement in distinctness of zones was found.

FIG. 1. Relationship in SRID tests between relative area of zones and concentration of antibodies in rabbit hyperimmune serum against whole virus (Δ) and in human mumps convalescent serum (2c in Table 2; $\circ$). Readings were taken after incubation for 24 h at room temperature.

Mixed hemadsorption: technical considerations. The mixed hemadsorption technique required a careful calibration of various reagents to be used in the test (4, 6). However, once this was performed, the technique was highly reproducible and very sensitive for detection of antibodies. When the test was adopted for determination of antibodies to mumps virus, it was observed that indicator sheep erythrocytes coated with rabbit or human hemolysin and anti-human or anti-rabbit globulin showed a general tendency to adsorb to the monolayer of infected cells. This appeared to be due to the availability of receptors on the coated erythrocytes, which could react with virus hemagglutinin on the surface of infected cells. Two techniques were tried to eliminate the panadsorption of erythrocytes. One technical modification was to increase the concentration of hemolysin used for coating of erythrocytes. This was found to reduce the tendency for panadsorption of cells but had the drawback of consuming large quantities of the hemolysin reagent. The alternative procedure, which was later generally applied, was to pretreat the erythrocytes in a final concentration of 10% with a preparation of receptor-destroying enzyme (*Vibrio cholera* filtrate; N.V. Philips-Roxane, The Netherlands) diluted 1:3 in phosphate-buffered saline, pH 7.4, for 3.5 h at 37°C. After the treatment, erythrocytes were washed twice in phosphate-buffered saline and then, using the regular procedure, incubated with human or rabbit hemolysin and the corresponding antiglobulin serum. This treatment completely abolished the tendency for erythrocytes to adsorb non-specifically to the infected-cell monolayer.

A dose-response analysis was performed with twofold dilutions of a mumps hyperimmune globulin (The National Bacteriological Laboratory, Stockholm) and a mumps convalescent serum known from preliminary tests to give a distinct zone of specific hemadsorption. It was found that there was a linear relationship between the diameter of hemadsorption zones and the dilution of serum plotted on a logarithmic scale (Fig. 2). A similar relationship was found in other mixed hemadsorption systems (4). By use of this linear relationship, diameters of zones can be readily converted into absolute titers.

Mumps virus antibodies in rabbit hyperimmune sera characterized by SRID and mixed hemadsorption tests. The three kinds of rabbit hyperimmune sera all gave visible reactions in both SRID and mixed hemadsorption tests (Table 1, Fig. 3). However, the antiserum against purified mumps hemagglutinin gave a rela-

FIG. 2. Relationship between diameter of hemadsorption zones and reciprocals of dilutions (log scale) of human mumps hyperimmune globulin (○) and human mumps convalescent serum (2c in Table 2; △).

tively weak reaction in the SRID test in spite of an HI titer of 320. In contrast, the antiserum against Pronase-treated, projection-less virions, which contained a relatively high titer of HLI antibodies as compared to HI antibodies, gave strong reactions in both SRID and mixed hemadsorption tests. This shows that both kinds of tests detect antibodies against the two different major surface components of mumps virus, i.e., hemagglutinin and hemolysin.

Mumps virus antibodies in paired human sera characterized by HLI, SRID, and mixed hemadsorption tests. Six pairs of human sera were analyzed both in conventional serological tests and by the HLI, SRID, and mixed hemadsorption tests (Table 2). All sera showed antibody rises of varying orders of magnitude in the HI and CF tests. Similarly, antibody rises were demonstrable in HLI tests with all sera. The HLI titer in convalescent sera corresponded to the HI titer in two cases, but in four cases it was four to eight times higher. The SRID test gave readily identifiable zones (see Fig. 3). Significant increases in diameter of zones were found in all six pairs of sera. Similarly, the mixed hemadsorption test also revealed antibody rises in all six pairs of sera. High antibody titers were found in all convalescent sera, and due to its sensitivity the test also revealed antibodies in some of the acute sera. The hemadsorption zones formed by human sera were less distinct than those formed by rabbit hyperimmune sera. However, diameters could be readily measured, and the appearance of differences

TABLE 1. *Antibody titers in rabbit hyperimmune sera against mumps virus products*

Virus prepn used for immunization	HI	HLI	CF	SRID zone diam in mm (dilution)	Mixed hemadsorption	
					Zone diam in mm (dilution)	Absolute titer
Purified whole virus (A)	1,600	1,600	1,600	10 (1/2)	24 (1/5)	5,070
Isolated hemagglutinin (B)	320	160	160	5 (1/2)	18 (1/5)	1,350
Projection-less particles, Pronase treated (C)	40	1,280	640	9 (1/2)	22 (1/5)	3,200

FIG. 3. Appearance of an SRID test with different serum samples. Samples 1, 2, and 6, a and c, correspond to paired sera in Table 2. r, Mumps-positive reference serum; g, regular human gamma globulin. Remaining sera are of rabbit origin; A-C correspond to hyperimmune sera in Table 1, and D is a serum from a nonimmunized rabbit.

between acute and convalescent sera is shown in Fig. 4.

Determination of immunity to mumps by using the SRID and mixed hemadsorption tests. Twenty human sera containing HI antibodies in titers of less than 40 were studied. The mumps history of individuals from whom the sera were derived was not available. Eight sera contained antibodies detectable in HI, CF, and mixed hemadsorption tests. Antibody titers demonstrated in the remaining 12 sera are summarized in Table 3. Five and 4 out of 12 sera contained antibodies in a titer of 5 or

higher in HI and CF tests, respectively. In contrast, 9 out of 12 sera were distinctly positive in the mixed hemadsorption test. The three sera that were negative in the latter test also lacked antibody activity in HI and CF tests, with the exception of one serum that had a marginal HI titer of 5. SRID tests were performed with 10 out of the 20 sera, and barely detectable zones were found in 5 sera.

Regular gamma globulin, representing a concentrate of late convalescent mumps sera, was analyzed for comparison. It had an HI and HLI titer of 320 and gave a weak zone in the SRID

TABLE 2. Antibody titers in paired human sera from cases of mumps

Sample ^a	HI	HLI	CF	SRID zone diam (mm) ^b	Mixed hemadsorption	
					Zone diam in mm (dilution)	Absolute titer
1a	<5	<5	<5	<4	8 (1/5)	125
1c	160	160	80	8	19 (1/5)	1,600
2a	20	40	<5	5	8 (1/5)	125
2c	3,200	1,600	1,280	12	24 (1/5)	5,070
3a	<5	<5	<5	<4	6 (1/5)	80
3c	80	640	80	8	23 (1/5)	4,030
4a	<5	<5	<5	<4	0	0
4c	20	160	20	7	21	510
5a	<5	20	<5	<4	7	20
5c	80	320	40	8	19	320
6a	<5	<5	<5	<4	7 (1/5)	100
6c	160	640	320	9	20 (1/5)	2,000

^a a and c denote acute and convalescent sera, respectively.
^b All tests were performed with sera diluted 1:2.

FIG. 4. Appearance of mixed hemadsorption zones obtained with: (A) serial twofold dilutions (1:25, 1:50, 1:100, 1:200) of a human mumps hyperimmune globulin; and (B) paired serum samples 1a, 1c, 3a, 3c (cf. Table 2) diluted 1/5, undiluted sera 1 and 6 for screening of mump immunity (cf. Table 3), and mumps-positive reference serum (r) diluted 1:40.

test, but it gave a pronounced zone when diluted 1:10 in the mixed hemadsorption assay.

DISCUSSION

All three serological tests recently used to determine the immune response to mumps virus have a potential for identifying antibodies against the surface components of the virus. Mumps virus, like other paramyxoviruses, carries two major surface components, hemagglutinin and hemolysin (the fusion factor). In the HI test only antibodies against the hemaggluti-

nin can be detected, but these antibodies also have the capacity to block the hemolytic activity of the virus. The mumps HLI test as used in this study appears to have a sensitivity comparable to that of the HI test, similar to the experience with measles virus serology (10, 11). However, the HLI test possesses the advantage over HI tests of detecting antibodies to both major surface antigens of the virion. Therefore, sera that contain undetectable or low titers of HI antibodies but relatively higher titers of antibodies reacting directly with the hemolysin

TABLE 3. *Determination of mumps immunity by conventional HI and CF tests and by SRID and mixed hemadsorption tests*

Sample	HI	CF	SRID zone diam (mm)	Mixed hemadsorption	
				Zone diam in mm (dilution)	Absolute titer
1	<5	10	<4	15	160
2	10	<5	5	17	220
3	10	<5	<4	19	320
4	<5	10	<4	16	190
5	<5	10	5	18	270
6	<5	<5	<4	17	220
7	<5	<5	<4	0	0
8	<5	<5	— ^a	0	0
9	5	<5	—	0	0
10	10	<5	—	15	160
11	10	<5	—	13	105
12	<5	5	—	17	220
Fraction of positive sera	5/12	4/12		9/12	
Gamma globulin	320	AC ^b	5	19 (1/10)	3,200

^a —, Not tested.^b AC, Anti-complementary.

preferably should be examined in the HLI test. In a study of a large group of patients with multiple sclerosis and healthy control individuals, it was found that about 10% of all sera had a predominance of measles HLI antibodies over HI antibodies (14). The possible occurrence of a similar preferential antibody response to the hemolysin components of mumps virus has not been described. However, it is of interest that in four out of the six convalescent sera, titers measured by the HLI test were four to eight times higher than in the HI test. A similar excess of HLI over HI antibodies was found in rabbit hyperimmune sera against Pronase-treated whole virus, as previously shown (13). Further studies of the antibody response to the mumps virus hemolysin in regular infections and of the capacity of these antibodies to neutralize the virus would be of interest.

The SRID test and the mixed hemadsorption test both appear to give an efficient indication of the two surface antigens. It is of interest that the titers in these tests were relatively higher in rabbit hyperimmune sera against Pronase-treated, projection-less virions than against isolated hemagglutinin (predominately HLI antibodies). Although the two tests are capable of detecting antibodies against both major surface virion antigens, they differ markedly with regard to their applicability to diagnosis of mumps infections and in determination of immunity to mumps.

The SRID test does not hold any promise for use in screening for mumps immunity. However, it can be conveniently used for serological

analysis of paired serum samples. The test is simple to perform, rapid (it can be read within 6 to 8 h), and reliable. Further, preformed plates containing virions can be stored at 4°C, and nonspecific inhibitors in sera do not pose any problems. The only drawback is the relatively large quantities of virions needed in the gel to give visible reactions. However, mumps virus grows efficiently in the chorioallantoic membrane of embryonated eggs, and the amount of virus produced readily suffices to provide the proper concentration of virions. Comparison of the sensitivity of the SRID test with the single radial hemolysis-in-gel technique (Väänänen et al., Arch. Virol., in press) for detection of antibody rises in acute mumps infections remains to be done.

The dose-response analysis of SRID data showed a linear relationship between the surface area of the zones formed and the amount of antibodies added to the well, except for a somewhat reduced zone size when undiluted serum was used. From a practical point of view, it appears preferable to test sera diluted about 1:2 and to use a 3-mm difference in diameter as a criterion for significant difference between antibody titers of two serum samples.

The mixed hemadsorption test shows a much higher degree of sensitivity than the SRID test. The high sensitivity of the former test has been experienced in studies of other virus systems, e.g., measles (1, 5), distemper, herpes (3), and rubella viruses (M. Grandien, submitted for publication). This test, therefore, is attractive to use for screening of immunity to mumps, but

it can also be used for detection of antibody rises in connection with acute infections. The test is somewhat cumbersome to set up and to standardize, but if properly applied, it gives highly reproducible results. In the present study it was found important to use erythrocytes pretreated with receptor-destroying enzyme to prevent virus-specific panadsorption of erythrocytes. In preliminary experiments, whether antibody titers could be determined by a direct analysis of inhibition of virus-specific hemadsorption was determined. It was found, however, that large quantities of antibodies were needed to give a detectable inhibition.

In conclusion, it seems preferable from a practical point of view to use the SRID test for diagnosis of acute infection, whereas for screening of immunity to mumps the mixed hemadsorption test might be more useful. In the present study, only a limited number of late convalescent sera were examined by the latter test, and there is a need for a wider application of this test before the reliability of a positive result with regard to protection against disease can be finally decided. This might be determined by following the antibody response, after immunization with live vaccine, of a group of individuals who lack detectable antibodies by the mixed hemadsorption test.

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