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Hemolysis-In-Gel and Neutralization Tests for Determination of Antibodies to Mumps Virus

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A hemolysis-in-gel test for the demonstration of antibodies to mumps virus is described. The results were compared with those of neutralization tests using a modified microtechnique. In the neutralization test viral replication was demonstrated by the hemadsorption of guinea pig erythrocytes, the visibility of which could be further enhanced by the use of *o*-tolidine. Good correlation was found between the results of the two techniques. The hemolysis-in-gel test was simple to perform, rapid, sensitive, and shown to be a useful test for the demonstration of mumps antibodies.

Neutralization (NT) and hemagglutination-inhibition (HI) tests are the most commonly used methods for determination of antibodies to mumps virus. The most reliable and sensitive of these two is the NT (1). However, it is not well suited for screening of large numbers of serum samples, since it is rather laborious and time consuming. Recently hemolysis-in-gel (HIG) or single radial hemolysis tests have been described for demonstration of antibodies to rubella (16, 17), influenza (14), parainfluenza viruses (13), and chlamydia (Lycke and Peterson, in press). This technique is based on the principle that erythrocytes coated with antigen are lysed by specific antibodies in the presence of complement. The HIG technique has been shown to be a sensitive method well suited for screening of large numbers of serum samples for antibodies. HIG tests for rubella and influenza virus antibodies are as sensitive as HI and have the advantage of being unaffected by nonspecific inhibitors (14, 16, 17). We have applied this technique for the detection of antibodies to mumps virus and have compared it with NT tests using a modified microtechnique in which macroscopic reading is possible.

MATERIALS AND METHODS

Patients. Serum samples were obtained from 40 blood donors. In addition 83 individuals were bled prior to vaccination against mumps, and blood samples were drawn from 15 of the vaccinees 4 weeks after immunization. Acute- and convalescent-phase serum samples were obtained from 25 clinical cases of mumps infection. All serum samples were inactivated and stored at −20°C until tested.

Virus. A mumps virus strain (SBL 1/67) obtained from the Department of Virology, National Bacteriological Laboratory, Stockholm, Sweden, was used for preparation of antigen for NT, HIG, and complement fixation (CF) tests. Mumps virus-infected allantoic fluid was harvested after 3 days at 33°C followed by a 24-h incubation at 4°C. The antigen preparation for the HIG test was clarified by centrifugation at 3,000 rpm for 15 min and had a hemagglutination titer of 64/0.025 ml in the microtiter system. The CF antigen was prepared by centrifugation of infected allantoic fluid at 15,000 rpm for 60 min and by resuspension of the pelleted virus in phosphate-buffered saline, pH 7.2 (PBS).

Agarose. Indubiose A-37 (L'industrie Biologique Francaise) was dissolved in PBS to give a concentration of 1.5% (wt/vol). The agarose was dissolved by heating at 100°C for 30 min and then cooled to 45°C.

HIG test. Erythrocytes (RBC), collected in Alsever solution, were washed three to six times in PBS before incubation with antigen. Sheep RBC were generally used but chicken RBC were also tested. For coating of RBC, about 300 hemagglutination units of antigen were used per 25 μl of a 50% RBC suspension. After incubation for 30 min at 4°C and centrifugation, the RBC was resuspended in 1.75 ml of PBS heated to 45°C. The resuspended RBC were then thoroughly mixed with 1.5 ml of agarose and poured into petri dishes (50 mm; Nunc, Roskilde, Denmark) placed on a level surface. After solidification, 3-mm holes were punched in the gel. The holes were filled with 5 μl of inactivated serum which was allowed to diffuse into the gel for 24 h at 4°C. One milliliter of normal guinea pig serum diluted 1:6 in barbital buffer (pH 7.2) was then poured on top of each plate. The plates were then incubated at 37°C for 2 h after which the zone of hemolysis was measured to the nearest 0.1 mm. As a control for nonspecific lysis, plates with uncoated RBC were always used in parallel.

CF test. The CF method used was that of Sever (15) with minor modifications. Four units of antigen and two units of complement were employed in the test.

Sucrose density gradient centrifugation. Serum was adsorbed with sheep RBC and layered on top of a 10 to 50% sucrose gradient. After centrifugation in

a Spinco SW 50.1 rotor at 34,000 rpm for 18 h, 10 to 12 fractions were collected dropwise through the bottom of the tube. The fractions were then tested by HIG and on immunodiffusion plates for identification of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies (Tri-Partigen IgG, Tri-Partigen IgM, Behringwerke, Marburg/Lahn).

NT test. This was performed in sterile 96-well Cooke Microtiter plates with flat bottoms (no. M 29 ART), prefilled with 0.025 ml of Eagle minimal essential medium. A 0.025-ml portion of the serum to be tested was added to an edge well with a 0.025-ml microdiluter that had been modified to prevent scratching the plastic (Titertek, no. 77-024-02).

The same microdiluter was then used for diluting the serum from 2^{-1} to 2^{-8} or,, using both 12-row edges, from 2^{-1} to 2^{-4}. Thus, with one instrument both addition and dilution of the serum could be accomplished. For each serum, two parallel rows of dilutions were made. Consequently, 6 or 12 sera/plate could be tested, depending on the dilution scheme used. When 12 sera were tested, those exceeding or equaling 2^{-4} in titer had to be retested at higher dilutions. However, this occurred relatively infrequently.

Approximately 25 median tissue culture infective doses of mumps virus in 0.025 ml were then added to all wells containing diluted sera. In one plate, a virus titration of four parallel rows of dilutions from 2^{0} to 2^{-7} and two rows of uninfected cells were included.

After 30 min of absorption at room temperature, 50 μl of a suspension of approximately 10^5 GMK AH 1 cells/ml in Eagle minimal essential medium containing streptomycin, penicillin, and 2% calf serum was added.

The plates were wrapped in polyethylene film and incubated at 37°C in a humidified 5% CO_2 atmosphere.

After 5 days, 0.025 ml of a 1% guinea pig erythrocyte suspension in PBS was added to each well and allowed to adsorb at 4°C for 15 min followed by washing with PBS. Macroscopically visible hemadsorption was seen at the sites of viral replication. The macroscopic reading was as accurate as the microscopic reading of cytopathogenic effects or hemadsorption.

The macroscopic visibility of the hemadsorption could be further enhanced by adding 25 μl of a freshly prepared solution of *o*-tolidine and hydrogen peroxide in acetic acid to each well. This solution was made from equal amounts of two stock solutions: (i) 4% (wt/vol) *o*-tolidine in acetic acid and (ii) 10% hydrogen peroxide in PBS. After 1 to 2 min at room temperature a dark-green color developed in the wells containing erythrocytes (Fig. 1). As *o*-tolidine is considered to be carcinogenic, other redox indicators such as guaiacol or tetramethyl benzidine could probably be substituted.

RESULTS

Relation between size of hemolytic zone and antibody concentration. In Fig. 2 the di-

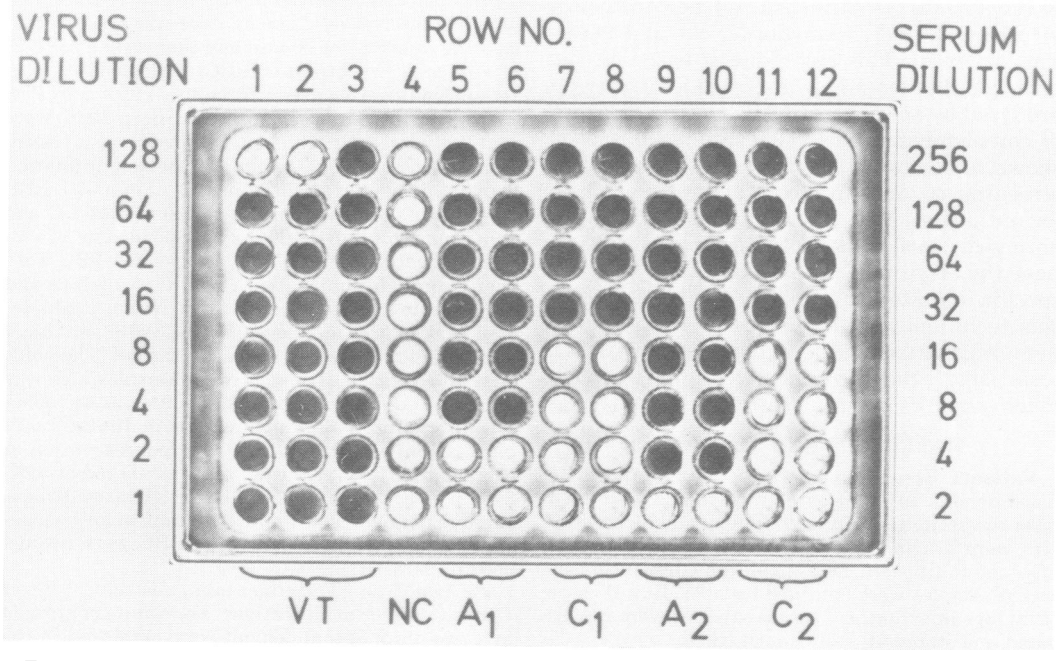

FIG. 1. *Microneutralization test for mumps antibodies. Abbreviations: VT, Virus titration 2^{0} to 2^{-7} from bottom to top; NC, cell control wells; A_1, C_1, A_2, C_2, acute- and convalescent-phase sera from two patients with mumps. The sera were diluted 2^{-1} to 2^{-8} from bottom to top. o-Tolidine reagent has been added to enhance the visibility of viral hemadsorption.*

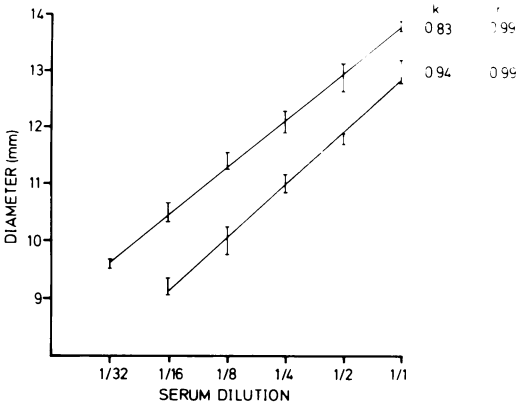

FIG. 2. *Relationship between the diameter of hemolytic zone and the log antibody concentration. Regression lines for two serum samples tested in twofold dilutions. The vertical bars represent the mean ± standard deviation of six replicates. The slope values (k) of the regression lines and the correlation coefficients (r) are given in the figure.*

ameter of the hemolytic zone is plotted against the antibody concentration on a log scale for two serum samples tested in twofold dilutions. Each serum dilution was tested in six replicates. The regression line for these values and the mean diameter ± standard deviation are indicated in the figure. A linear relationship existed between these parameters, and fourfold, and even twofold, differences in antibody concentration could be detected. The standard deviation of the mean was found to be around 2% and in no experiments exceeded 6%.

Effect of antigen concentration in the gel. Different concentrations of antigen were used for coating of RBC. Six serum samples were tested against different antigen concentrations. No differences in results were seen when concentrations between 250 and 2,000 hemagglutination units per 0.025 ml of a 50% RBC suspension were used. Thus, the results of HIG seem to be less dependent on the antigen concentration than are those of HI.

RBC. Both sheep and chicken RBC gave reproducible results, but since sheep RBC are more readily available they were used in most experiments. The plates with sheep RBC were also more stable and could be used after storage for 3 to 4 weeks at 4°C. Plates with chicken RBC were only stable for 3 to 4 days. Nonspecific hemolysis sometimes occurred but usually gave small zones and could always be abolished by prior absorption of serum with RBC.

Comparison of HIG and NT for screening of antibodies to mumps virus. Serum samples (122) were tested in HIG and NT for antibodies to mumps. The results are shown in Table 1. In 83 cases antibodies were detected by both methods; i.e., NT antibody titers of 2 or more and hemolytic zone diameters larger than 5 mm were demonstrable. Twenty-six samples gave NT titers less than 2 and no hemolysis in HIG. Ten serum samples gave a zone diameter of more than 5 mm in the HIG test but had an NT titer less than 2. However, four of these serum samples were found to neutralize the virus when tested undiluted. Finally, of three serum samples with NT antibody titers of 2, two gave a hemolytic zone 5 mm in diameter and one was negative in HIG. When the NT titers were compared with the diameters in HIG a general correlation was found, although the titers for a given diameter sometimes varied more than twofold. For routine use, a zone diameter of 6 mm was considered to indicate the presence of antibodies. Very few of the NT-positive sera gave smaller zone diameters in HIG, and when nonspecific hemolysis occurred the zones were usually in the range of 4 to 5 mm.

Among the blood donors only 5% lacked antibodies to mumps as determined by HIG. In comparison 28% of the vaccinees who were not aware of any mumps infection earlier in life had no demonstrable antibodies.

Use of the HIG test for diagnosis of mumps infection and determination of serological response to vaccination. Twenty-five acute- and convalescent-phase sera were tested in HIG, NT, and CF tests. In all cases a significant titer rise could be shown in CF and NT antibodies, and in the HIG test an increase in zone diameter of at least 2 mm was seen between acute- and convalescent-phase sera.

Of 15 vaccinees with sera collected before and after vaccination, six were found to be negative by HIG prior to vaccination. All of these developed a positive HIG reaction by 4 weeks after vaccination. In the group that showed a positive reaction in HIG before immunization, only one vaccinee responded with an increase in zone diameter of 1.5 mm.

To determine whether antibodies to parainfluenza virus could cross-react with mumps virus in the HIG test, nine acute- and convales-

TABLE 1. *Results of NT and HIG tests for detection of antibody to mumps virus*

NT titer	HIG zone (≥6 mm)	HIG zone (<6 mm)
≥2	83	3[a]
<2	10[b]	26

[a] Two sera gave a hemolytic zone diameter of 5 mm.

[b] Four sera were NT positive when tested undiluted.

cent-phase sera showing a significant titer rise in CF antibodies to a parainfluenza virus were tested by the HIG and NT tests. Seven of the paired serum samples demonstrated a CF titer rise to parainfluenza virus type 3, one to parainfluenza type 1, and one to parainfluenza type 2. In these cases mumps antibody titer rises were not demonstrable by HIG and NT tests.

Reactivity of IgM and IgG antibodies in the HIG test. Immunoglobulin separations were performed by sucrose density gradient centrifugation of three serum samples obtained 10 to 14 days after onset of clinical mumps infection. Both IgM and IgG antibodies gave hemolytic reactions (Fig. 3). However, it should be noted that the hemolysis produced by IgM antibodies was less clear than that induced by antibodies of the IgG class.

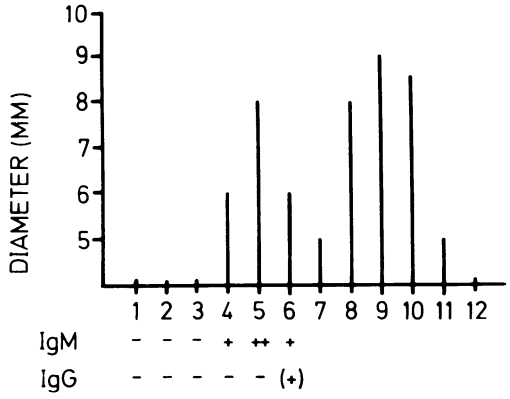

FIG. 3. *Sucrose density gradient centrifugation of an early convalescent-phase mumps antiserum. The diameters of hemolytic zones are indicated for the different fractions. The six first fractions were tested for IgM and IgG antibodies by immunodiffusion.*

DISCUSSION

In the HIG test, according to the principles of the single radial immunodiffusion method of Mancini et al. (10), a linear relationship between the area of the hemolytic zone and the antibody concentration is obtained after the end of diffusion, i.e., after 5 to 6 days for antibodies of the IgG class. Before this time a linear relationship exists between the diameter of the zone and the log antibody concentration (5). This was also found to apply in this study, and fourfold, and even twofold, differences in antibody concentrations could be detected. It has earlier been shown that twofold differences in rubella antibody concentrations give statistically significant differences in zone diameters in HIG (6).

A relative inability of antibodies of the IgM class to react in HIG has been demonstrated with the techniques used for rubella and influenza (14, 17). However, in the present study mumps IgM antibodies were shown to give lytic reactions, although they were less clear than those obtained with antibodies of the IgG class. This may be due to differences in the reactivity with IgG and IgM antibodies of antigens bound to avian (rubella, influenza) and sheep (mumps) RBC. Thus, there may be differences in membrane properties such as the ability to bind and activate complement or the steric arrangement of the viral antigens on the RBC surface. From work on blood group typing (12) or heterophile agglutination (3) a strong dependence on the RBC surface structure for reactivity with antibodies is well recognized.

The results obtained with HIG correlated well with those of NT. An NT titer of 2 or more is generally considered to provide immunity to mumps (7). The NT procedure presented is simpler than those previously published (2, 8, 9). The enhanced visibility of hemadsorption by the use of *o*-tolidine should be valuable in all hemadsorption techniques (e.g., 4) and is especially suited for automation (11).

The antigenic relationship between mumps and parainfluenza viruses may result in cross-reaction in CF and HI tests (18). With the limited number of sera tested, cross-reactions did not seem to be a major problem in HIG, but this must be further evaluated.

There are several advantages of the HIG assay over conventional techniques. It is easier to perform and less time consuming than HI, and is particularly less time consuming than NT. Compared to HI it has the advantage of being independent of nonspecific inhibitors and less dependent on the antigen concentration for reproducible results. In other techniques (HI, CF, and NT) the quantitation of antibodies is based on testing serial serum dilutions, which means that only fourfold increases in titer can be considered significant. In HIG, sera can be tested undiluted and titer increases less than fourfold are detectable with a high degree of accuracy. The present study suggests that the HIG test may be a valuable method for the demonstration of mumps antibodies.

LITERATURE CITED

1. **Buynak, E. B., J. E. Whitman, Jr., R. R. Roehm, D. H. Morton, G. P. Lampson, and M. R. Hilleman.** 1967. Comparison of neutralization and hemagglutination-inhibition techniques for measuring mumps antibody. Proc. Soc. Exp. Biol. Med. 125:1068–1071.
2. **Center for Disease Control.** 1972. Hemadsorption neutralization test for the serodiagnosis of mumps, p. 465–470. *In* T. B. Conrath (ed.), Handbook of microti-

ter procedures, Dynatech Co., Cambridge, Mass.
3. **Coombs, R. R. A., M. H. Gleeson-White, and J. G. Hall.** 1951. Factors influencing the agglutinability of red cells. II. The agglutination of bovine red cells previously classified as inagglutinable by the building up of an antibody: globulin lattice on the sensitized cells. Br. J. Exp. Pathol. 32:195–202.
4. **Espmark, A., and A. Fagraeus.** 1962. Detection of antigens in tissue culture with the aid of mixed hemadsorption. Acta Pathol. Microbiol. Scand. 154:258–262.
5. **Fahey, J. L., and E. M. McKelvey.** 1965. Quantitative determination of serum immunoglobulins in antibody agar plates. J. Immunol. 94:84–90.
6. **Grillner, L., and Ö. Strannegård.** 1976. Evaluation of the hemolysis-in-gel test for the screening of rubella immunity and the demonstration of recent infection. J. Clin. Microbiol. 3:86–90.
7. **Hilleman, M. R.** 1970. Mumps vaccination. Mod. Trends Med. Virol. 2:241–261.
8. **Karchmer, A. W., J. P. Friedman, H. L. Casey, T. C. Shope, and J. B. Riker.** 1971. Simultaneous administration of live virus vaccines. Am. J. Dis. Child. 121:382–388.
9. **Kenny, M. T., K. L. Albright, and R. P. Sanderson.** 1970. Microneutralization test for the determination of mumps antibody in Vero cells. Appl. Microbiol. 20:371–373.
10. **Mancini, G., A. O. Carbonara, and J. F. Heremans.** 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235–254.
11. **Mayner, R. E., D. J. McDorman, B. C. Meyer, and P. D. Parkman.** 1974. Automated microtransfer technique for the assay of poliovirus- and mumps virus-neutralizing antibodies. Appl. Microbiol. 28:968–971.
12. **Morton, J. A., and M. M. Pickles.** 1951. The proteolytic enzyme test for detecting incomplete antibodies. J. Clin. Pathol. 4:189–199.
13. **Probert, M., and S. M. Russel.** 1975. Measurement of parainfluenza-3 virus antibody by the single radial hemolysis technique. J. Clin. Microbiol. 2:157–161.
14. **Russel, S. M., D. McCahon, and A. S. Beare.** 1975. A single radial haemolysis technique for the measurement of influenza antibody. J. Gen. Virol. 27:1–10.
15. **Sever, J. L.** 1962. Application of a microtechnique of viral serological investigations. J. Immunol. 88:320–329.
16. **Skaug, K., I. Ørstavik, and J. C. Ulstrup.** 1975. Application of the passive haemolysis test for the determination of rubella virus antibodies. Acta Pathol. Microbiol. Scand. Sect. B 83:367–372.
17. **Strannegård, Ö., L. Grillner, and I.-M. Lindberg.** 1975. Hemolysis-in-gel test for the demonstration of antibodies to rubella virus. J. Clin. Microbiol. 1:491–494.
18. **van der Veen, J., and H. J. A. Sonderkamp.** 1965. Secondary antibody response of guinea pigs to parainfluenza and mumps viruses. Arch. Gesamte Virusforsch. 15:721–734.