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Hemolysis-in-Gel Test for the Demonstration of Antibodies to Rubella Virus

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A rapid and simple method for the determination of rubella immunity is described. The method, which employs passive hemolysis in agarose gel, is sensitive and reproducible and does not require prior absorption of test sera to remove inhibitors. Immunoglobulin G, but not immunoglobulin M, antibodies were regularly found to give demonstrable reactions in the test. It is concluded that the hemolysis-in-gel test may provide a valuable tool, in particular for mass screening for rubella immunity.

The methods most commonly used for the diagnosis of rubella infection are the hemagglutination inhibition (HI) and the complement fixation tests. For the purpose of screening sera to demonstrate immunity to rubella, the HI test is preferentially used. This test is rather simple to perform, but requires titration of sera and absorption to remove nonspecific inhibitors and also, when nonhuman sources of erythrocytes are employed, absorption to remove erythrocyte (RBC) agglutinins. The present report describes a hemolysis-in-gel (HIG) method which is similar to that used by Schild et al. (G. C. Schild, J. S. Oxford, and J. L. Virelizier, in press) for the detection of antibodies to influenza virus. This method is extremely simple to perform, is reproducible and sensitive, and appears to be well suited for determining rubella immunity.

MATERIALS AND METHODS

Rubella antigen. One batch of rubella hemagglutination antigen was obtained from Wellcome Laboratories (lot K8973). Another batch was made in our laboratory employing an established line of monkey kidney (Vero) cells. The antigen preparations had hemagglutination titers of 1:128 and 1:32, respectively, when tested in conventional hemagglutination tests in microtrays.

Agarose. Agarose was obtained from Behringwerke (Marburg/Lahn, West Germany). Agarose (960 mg) in 60 ml of Roswell Park Memorial Institute medium (RPMI 1640, Flow Laboratories, Irvine, Scotland) was dissolved at 100 C for 30 min and then cooled to 47 C.

RBC. RBC of pigeon, goose, sheep, ox, or human origin were mixed with Alsevers solution and then washed three times with phosphate-buffered saline, pH 7.2. The RBC were kept as 50% suspensions for up to 6 days at +4 C before being used. Treatment of RBC with trypsin was performed as described by Quirin et al. (7).

HI tests. HI tests were performed in microtrays after absorption with kaolin and pigeon RBC, essentially as described by Halonen et al. (2).

Sucrose density gradient centrifugation. The procedure recommended by the Center for Disease Control (6) was followed. Serum was adsorbed with pigeon RBC and layered on top of a 10 to 50% sucrose gradient. After centrifugation in a Spinco SW50.1 rotor at 34,000 rpm for 18 h, 12 to 15 fractions were collected dropwise through the bottom of the tube. The different fractions were then tested for HI antibodies.

Description of the HIG test. After a series of experiments in which dilution of antigen, pH and composition of buffers, RBC concentration, and incubation times were varied, the following procedure was adopted for performance of the HIG test. Rubella antigen was diluted in RPMI medium to correspond to about 4 U in the hemagglutination test. After addition of 25 μl of 50% RBC to 1.75 ml of rubella antigen the mixture was kept at +4 C for 30 min and then centrifuged, and the RBC were resuspended in 1.75 ml of RPMI medium which had been heated to 47 C. The resuspended RBC were then mixed with 1.5 ml of 1.6% agarose, heated to 47 C, and then poured onto small petri dishes (Nunc, Roskilde, Denmark). After solidification in the cold, 3-mm holes were punched out in the gel, and each hole was filled with 5 μl of serum or serum dilution. After the plates were held in the cold for 7, 24, or 72 h, 1 ml of normal guinea pig serum, diluted 1:4 in barbital buffer, pH 8.2, was poured on top of each plate. The plates were incubated at room temperature for 15 min and thereafter at 37 C for 2 h. During incubation hemolytic zones gradually appeared, and clear zones were easily discernible after the end of the incubation (Fig. 1). Hemolysis first became visible at the periphery of the zone, and in some cases, in particular when RBC from species other than pigeon were employed, an unhemolyzed central zone persisted.

Positive HIG reactions were also obtained with goose RBC, untreated and trypsin-treated sheep RBC, and trypsin-treated ox RBC. Positive reactions were not obtained with untreated or trypsin-treated human group O RBC. In none of these cases were the results

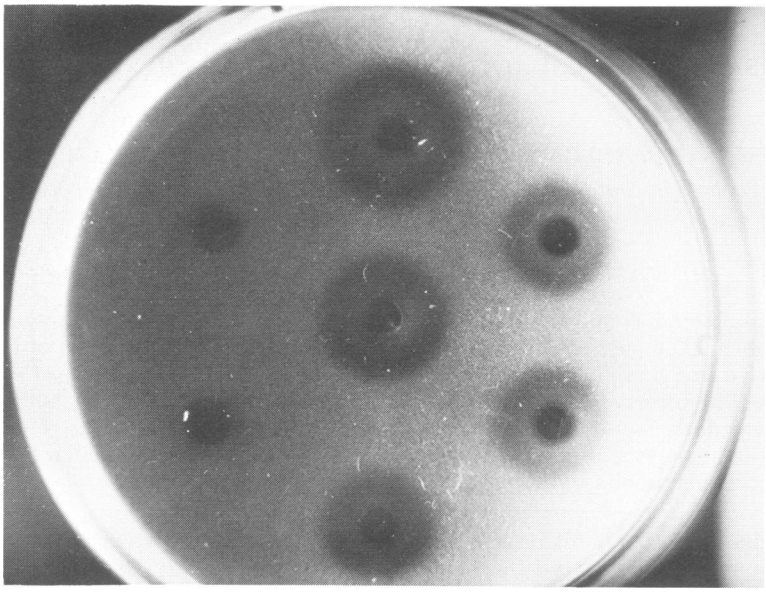

FIG. 1. *Hemolytic zones obtained with sera having different HI antibody titers.*

as clearcut as those obtained with pigeon RBC, and the experiments described below were all performed using the latter type of RBC.

RESULTS

Reproducibility of test. Six sera were tested by the HIG test on three occasions using two different antigen batches. The results shown in Table 1 demonstrate the reproducibility of the test and the independence of antigen concentration.

Kinetics of the development of hemolytic zones. HIG tests were performed on sera with varying HI titers, using varying diffusion times. Positive HIG tests were obtained in most cases when complement was added 7 h after application of antisera (Fig. 2). When sera with low HI titers were tested, however, positive HIG reactions were obtained after 24 but not after 7 h of diffusion. Reactions obtained after 48 and 72 h of diffusion were difficult to read, possibly because of the occurrence of spontaneous hemolysis of the RBC. The results shown in Fig. 2, however, indicate that differences in antibody content of the sera were more easily demonstrable when the time of diffusion was increased.

Relationship between results of antibody determinations with the HI test and the HIG test. Ninety heat-inactivated sera were tested on the same day in HI and HIG tests. There was a very good correlation between the results obtained with the two tests (Fig. 3). None of 22 sera which had a titer of <20 in the HI test were clearly positive (i.e., hemolytic zone diameter ≥ 6 mm) in the HIG test. One HI-positive serum (titer = 20), however, was negative in the HIG test. In another series of 47 sera, none gave false-positive or false-negative reactions in the HIG test. All sera that were negative in the HIG test were tested for presence of agglutinins to pigeon RBC. All sera were found to contain such agglutinins, in titers of 1:4 to 1:32, when tested in a microtitration system.

Effect of inactivation and absorption of sera. Six sera with high HI titers were tested before and after heating at 56 C for 30 min and after absorption with kaolin and pigeon RBC. None of these treatments had any demonstrable effect on the reactivity of these sera in the HIG test. Of 21 sera which were negative by the HI test, 14 gave weakly positive reactions when tested by HIG in the native state. These reactions were no longer demonstrable after heat inactivation of the sera.

Reactivity of 7S and 19S antibodies in the HIG test. Experiments were undertaken to evaluate the efficiency of the HIG test in detecting changes in antibody concentration. Acute- and convalescent-phase sera from six patients with rubella were tested by HIG. In all cases the test changed from negative to positive. However, four acute-phase sera negative by HIG were positive by the HI test (titers, 20 to 40). Sucrose density gradient centrifugation showed that these antibodies demonstrable by HI belonged to the immunoglobulin M (IgM) class. Further experiments using sucrose density gradient centrifugation confirmed that,

TABLE 1. *Reproducibility and antigen dependence of the HIG test*[a]

Serum no.	HI titer	Mean of 6 tests ± SD	Diam of hemolytic zone (mm)				
			Antigen dilution				
			1:2	1:4	1:16	1:64	1:256
1	20	7.5 ± 0.5	7	7.5	7.5	7.5	<4
2	640	10.5 ± 0.8	12	11.5	11	11	<4
3	80	8.8 ± 0.4	8	10	9.5	9.5	<4
4	<20	<4	<4	<4	<4	<4	<4
5	640	11.2 ± 0.4	12	11.5	11.5	10	<4
6	160	10.5 ± 0.8	10	10	10.5	10	<4

[a] The results of testing six sera on three different occasions using two antigen batches, as well as the results of varying the concentration of the antigen preparation used in the test (Wellcome rubella antigen; hemagglutination titer, 1:128), are indicated in the table. SD, Standard deviation.

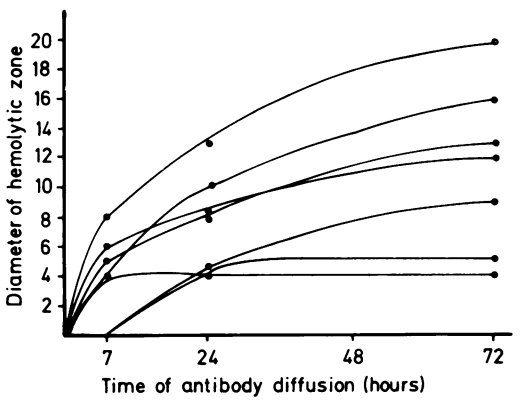

FIG. 2. *Influence of time of antibody diffusion on the size of hemolytic zones. Seven sera with various HI titers were subjected to HIG tests using 7, 24, or 72 h of incubation of the plates at +4 C. Lines represent the kinetics of development of hemolytic zones for each serum.*

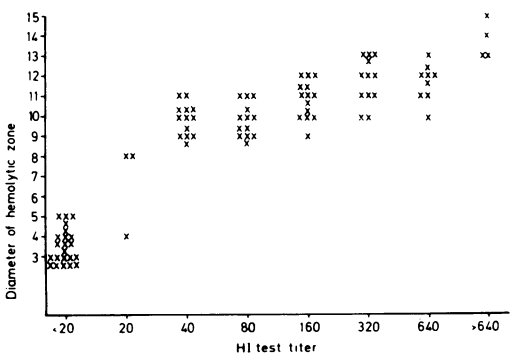

FIG. 3. *Relationship between results of HI and HIG tests. The time of antibody diffusion in the HIG test was 24 h for all sera.*

with the antibody concentrations used, 7*S* (immunoglobulin G [IgG]) but not 19*S* (IgM) antibodies gave demonstrable reactions in the HIG test (Fig. 4).

HIG test using whole blood instead of serum. Blood was drawn from individuals possessing rubella antibodies demonstrable by the HI test. Small amounts of heparin (250 IU/10 ml of blood) were added to the blood specimens. HIG tests performed on whole blood gave somewhat smaller hemolytic zones than did the corresponding serum, but positive results were obtained even when blood specimens from individuals with low serum HI titers (20 or 40) were used.

DISCUSSION

Passive hemolysis in agarose as described by Hall (1) appears to be an easy and rapid method for testing large numbers of sera. The method has been adapted to assay immunity to *Toxoplasma gondii* (3) and has recently also been used for the detection of antibodies to influenza virus (Schild et al., in press).

The modified HIG test used in the present studies seems to be very well suited for determining immunity to rubella. Compared to the conventional HI test it is more rapid and appears equally sensitive and reproducible. It is advantageous in that only small amounts of antiserum and RBC are required for the test and, in contrast to the HI test, the concentration of antigen used in the test is not critical above certain limits. It is also a distinct advantage that for the HIG test no absorption of sera to remove lipoprotein inhibitors seems to be necessary. Why normally occurring agglutinins to pigeon RBC in heat-inactivated sera did not give false-positive reactions is unclear, but this may be due to the fact that "natural antibodies" to RBC usually belong to the IgM class. In our system IgM antibodies to rubella did not give positive reactions in the HIG test. If further studies show that agglutinins are indeed a source of error, it may be necessary to perform HIG tests on sera absorbed with RBC or to use

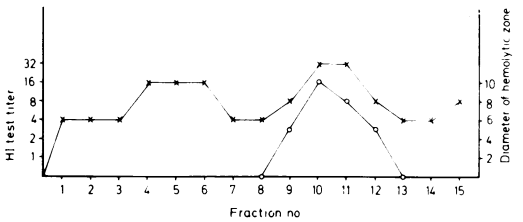

FIG. 4. *Sucrose density gradient centrifugation of acute-phase rubella antiserum. HI test titers (×) and diameters of hemolytic zones (O) are indicated. HI tests show antibody activity in the 19S as well as 7S region, whereas the HIG test only reveals antibody activity in the 7S region.*

control agarose plates containing uncoated RBC.

The failure of IgM antibodies to give positive HIG reactions is surprising in light of the fact that in the direct hemolytic plaque assay of Jerne et al (4) IgM, but not IgG, antibodies give positive reactions. It seems probable that this inability of the IgM antibodies is relative, and it remains to be shown if it also pertains to other systems, using RBC from other species. In any event, a failure of rubella IgM antibodies to react in the test may aid in the diagnosis of rubella infection. Thus, a positive HIG test might be a more reliable indication of rubella immunity than a positive HI test, since the latter might result from activity of IgM antibodies, which indicate recent infection rather than immunity to rubella. Moreover, the combined use of HI and HIG tests may reduce the need for immunoglobulin separation in rubella diagnosis. Using both these methods, more titer increases would presumably be demonstrated, and it might also be possible to indirectly demonstrate the occurrence of IgM antibodies in blood from newborns.

The HIG test is assumed to obey the laws that are valid for the single radial immunodiffusion method of Mancini (5). Thus, the area of the hemolytic zone should be proportional to the antibody concentration. To obtain equilibrium and thus linear relationships with sera with high antibody activity in the present study, the diffusion time had to be extended to 3 days or more. This presented problems, however, since results could scarcely be read after prolonged storage of the test plates. Thus, unless this difficulty can be overcome, the ability of the HIG test to detect differences in antibody concentrations between sera with high antibody activity would seem unsatisfactory. The difficulty can be circumvented by testing sera in varying dilutions. Seroconversions, however, would be easily detected with the HIG test. In fact, since the HIG test is very sensitive and the specificity of low HI titers is often doubtful, the HIG test might be better than the HI test for detecting seroconversions after rubella vaccination.

The present study suggests that the HIG test may be an excellent method for the screening of large numbers of sera to detect immunity to rubella. Comparative studies of the results of HIG and other rubella antibody tests will be necessary to reveal the value of the HIG test for the diagnosis of recent rubella infection.

ADDENDUM IN PROOF

After submission of this manuscript it has come to our attention that a similar study has been performed in Norway (K. Skaug, I. Ørstavik, and J. C. Ulstrup. Acta Pathol. Microbiol. Scand., in press). In this study chick erythrocytes coated with rubella antigen have been successfully employed and the authors conclude that the passive hemolysis test is as specific and sensitive as the HI test.

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