JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1976, p. 86-90 Vol. 3, No. 2
COPYRIGHT © 1976 American Society for Microbiology Printed in U.S.A.

Evaluation of the Hemolysis-in-Gel Test for the Screening of Rubella Immunity and the Demonstration of Recent Infection

LENA GRILLNER* AND ÖRJAN STRANNEGÅRD

Department of Virology, Institute of Medical Microbiology, University of Göteborg, Göteborg, and Department of Virology, University of Umeå, Umeå, Sweden

Received for publication 15 October 1975

The hemolysis-in-gel method for detection of antibodies to rubella virus gave results which correlated well with results of hemagglutination inhibition and neutralization tests. With a diffusion time of 24 or 48 h, a linear correlation was obtained between the logarithm of antibody concentration and the diameter of the hemolytic zone. Fourfold, and even twofold, differences in serum antibody concentrations were shown to give statistically significant differences in hemolytic zone diameters. It could therefore be concluded that the hemolysis-in-gel test is well suited for the serological diagnosis of primary rubella infection, as well as of reinfection. The sensitivity of the hemolysis-in-gel test was comparable to that of the hemagglutination inhibition test. Pigeon erythrocytes were superior to sheep erythrocytes for use in the test. Studies of the antibody response after natural rubella infection or vaccination showed that the appearance and persistence of antibodies measured by hemolysis in gel is similar to that of hemagglutination inhibition antibodies.

Hemolysis-in-gel (HIG), or single radial hemolysis, has recently been described for detection of antibodies against influenza and rubella viruses (10–12). The results obtained by this method correlated well with those obtained by hemagglutination inhibition (HI), and this suggested that the HIG test may be of great value for routine screening of large numbers of serum samples.

In the present study we have further evaluated the use of this technique for diagnosis of rubella infection and for determination of serological response to rubella vaccination and have studied the correlation between HIG, HI, and neutralization (NT) tests for screening for rubella immunity. A comparative study was also made of the use of sheep and pigeon erythrocytes in the test.

MATERIALS AND METHODS

Patients. Sera were obtained from women vaccinated against rubella postpartum. All women had been tested during pregnancy in antenatal clinics and those who were susceptible to rubella, i.e., had an HI titer of 10 or less, were vaccinated within 4 days after delivery. Blood samples were obtained 8 weeks and 2 years after vaccination. Sera were inactivated and stored at −20 C until tested.

Rubella vaccines. The Cendehill (Smith, Kline and French Laboratories), the HPV-77 DE-5 (Merck, Sharp and Dohme) or the RA 27/3 (Burroughs Wellcome) rubella vaccine strains were injected subcutaneously. Twenty-one women received the Cendehill, 18 received the HPV-77 DE-5, and 16 received the RA 27/3 vaccine.

Rubella antigen. Rubella hemagglutination (HA) antigen from Wellcome laboratories was used throughout the study (lot K 0516).

Agarose. Agarose from Behringwerke (Marburg/Lahn, West Germany) was used at a 1.6% concentration in Roswell Park Memorial Institute medium (RPMI 1640, Flow laboratories, Irvine, Scotland). In some of the experiments Indubiose A-37 (L'Industrie Biologique Francaise) was used dissolved in phosphate-buffered saline (PBS), pH 7.2, to give a final concentration of 1.5%. Both types of agarose were dissolved at 100 C for 30 min and then cooled to 46 to 47 C.

Erythrocytes. Pigeon or sheep erythrocytes were collected in Alsevers solution and washed three to six times in PBS before use. A 50% erythrocyte suspension in PBS was used for incubation with antigen. In some experiments trypsin-treated sheep erythrocytes were prepared as described by Quirin et al. (9).

Performance of the HIG test. The following procedure was used. Rubella antigen was diluted to correspond to 4 HA units/0.025 ml in the hemagglutination test, which corresponded to a ratio of about 300 HA units/0.025 ml of a 50% erythrocyte suspension. RPMI medium was used as diluent and 1.75 ml of the antigen dilution was incubated with 25 μl of a 50% erythrocyte suspension for 30 min at +4 C. After centrifugation the erythrocytes were resuspended in RPMI heated to +47 C, mixed with 1.5 ml of agarose, and poured onto small petri dishes

(Nunc, Roskilde, Denmark) which were placed on a level surface. In some experiments large square plates, 1012 Intergrid petri dishes, (Falcon Plastics, Los Angeles) were used. In these experiments 7 ml of the antigen dilution was incubated with 0.1 ml of 50% erythrocytes, and after centrifugation and resuspension of the erythrocytes in 7 ml of RPMI they were mixed with 6 ml of agarose. From this mixture, 11 ml were poured onto the plate. In all experiments plates with uncoated erythrocytes were used in parallel as a control for nonspecific hemolysis. After solidification 3-mm holes were punched in the gel and 5 μl of serum or serum dilution was added to the holes. The small plates could be used for testing seven sera and the large plates for testing 36. After incubation at +4 C for 24 or 48 h, 1 and 4 ml, respectively, of normal guinea pig serum diluted 1:4 in barbital buffer (pH 8.2) was poured on top of each plate and the plates were incubated for 2 h at 37 C. Two perpendicular diameters were measured on each zone of lysis, with the aid of a precision calliper, to the nearest 0.1 mm.

HI test. The HI tests were performed according to the method of Halonen et al. (5) with slight modifications. Pigeon erythrocytes and kaolin were used in the tests. An HI titer of 20 was considered as positive, indicating immunity.

NT. The RK13 rabbit kidney cell line was used (3) and twofold dilutions of sera were incubated with equal volumes of 50 to 100 mean tissue culture infective doses of rubella virus per 0.1 ml for 2 h at room temperature. To enhance the neutralizing capacity, 4% unheated guinea pig serum was added to the serum diluent (7). After incubation each serum-virus mixture was inoculated into three tube cultures and incubated in rollers for 7 to 10 days at 34 C. The cultures were observed for cytopathic changes between days 7 to 10. The final titer was expressed as the serum dilution that inhibited the cytopathic effect of rubella virus in two of three tubes. A titer of 2 was considered as positive.

RESULTS

Relation between antibody concentration and size of hemolytic zone. Two sera with HI titers of 80 and 40, respectively, were tested by HIG in the dilutions given in Fig. 1. Each serum dilution was tested in six replicates. The relation obtained between the square radius of the zone and the relative antibody concentration after a diffusion time of 24 and 48 h is shown. The relation was not linear but there was a good correlation between HI titer and square radius. Plotting of the log of the antibody concentration of the same sera against diameter of the hemolytic zone gave straight lines with correlation coefficients close to 1 (Fig. 1). Figure 2 shows the results obtained when five sera with different HI titers were tested in twofold dilutions and the regression lines were calculated from six replicates per serum dilution. As seen in Fig. 2, the slopes for different sera were almost parallel and fourfold and even twofold titer increases could easily be detected. Since the slope value should be related to the concentration of antigen in the gel (8), the slope of the line could be assumed to vary with different batches of plates. When two of the sera shown in Fig. 2 were tested in another batch of plates, slightly different slope values were obtained.

The results obtained in different plates from the same batch were shown to be almost equal and the standard deviation of the mean was about 3.5% in all experiments. When different sera were tested in dilutions, it was shown that the minimum dose of antibody that gave a positive HI test was in most cases also able to produce a measurable hemolytic zone. Thus,

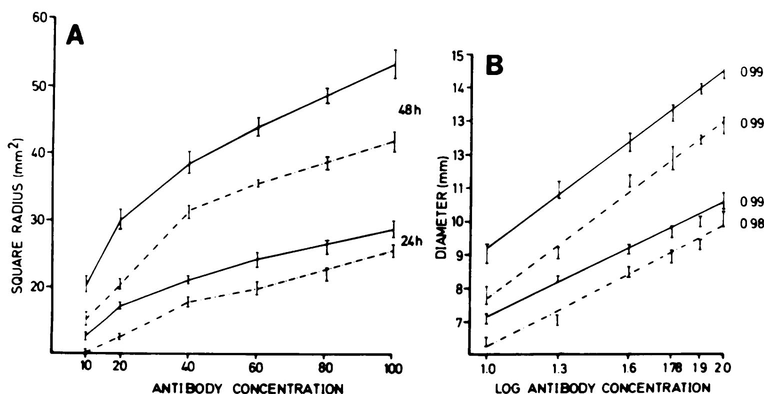

FIG. 1. *Relation between antibody concentration and the diameter of hemolytic zone for two serum samples with HI titers of 80 (O——O) and 40 (O----O) after a diffusion time of 24 and 48 h. Each serum dilution was tested in six replicates on the same plate. Mean values and standard deviations are given in the figure. The relations between antibody concentration and square radius (A) as well as that between the log antibody concentration and the diameter of hemolytic zone (regression lines, B) are indicated in the figure. The correlation coefficients (r) for the regression lines are given.*

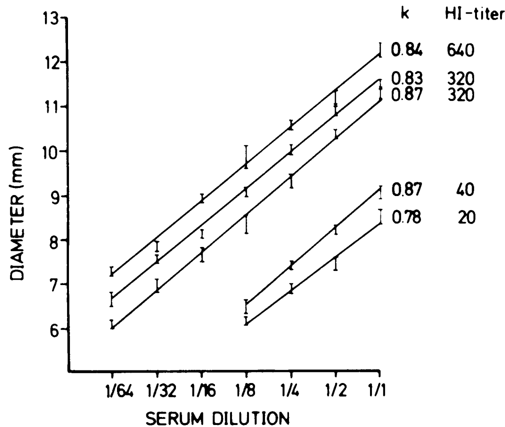

FIG. 2. *Regression lines for five different serum samples tested in twofold dilutions on the same day. The mean diameter and the standard deviation for six replicates are indicated. The slope value (k) of the regression line and the HI titer for each serum sample are given.*

one HIG antibody unit corresponded to 1 to 2.5 HI antibody units.

To evaluate the sensitivity of the technique for detecting differences in antibody concentration, a number of sera were tested undiluted and in twofold and fourfold dilutions under code. The paired serum samples with a twofold difference in antibody concentration were tested in triplicate, whereas those with a fourfold difference were assayed as a single specimen, and in addition tested by HI. As shown in Table 1, twofold and fourfold titer differences could be detected with high accuracy by the HIG test. The HI test gave more scattering of the results than did HIG and also showed a tendency towards exaggerated titer increases. The latter fact could possibly be explained by the use of an automatic diluter for the HI test.

Statistical analysis of one of the regression lines in Fig. 1 (HI = 80) showed that a difference in diameter of 0.6 mm between two serum samples after 24 h of diffusion gave a probability of 0.95 for a positive difference in antibody concentration. If a difference of 0.8 mm or more was found, the probability level was 0.99. Thus all differences obtained for the 78 paired sera in the HIG test could be considered as significant. In the HI test two sera showed no difference in titer and 10 showed only a twofold difference, which is not considered significant.

Comparative use of pigeon and sheep erythrocytes in the HIG test. Thirty-two sera which were all positive by NT and HI were assayed by HIG using both pigeon and sheep RBC. The sera had HI titers between 20 and 40. The HIG results using pigeon erythrocytes correlated well with the results of HI and NT, whereas, when sheep erythrocytes were used, only 75% of the sera gave positive HIG reactions (Table 2). The sensitivity of sheep erythrocytes could not be increased by using a higher concentration of antigen for coating or by treating the erythrocytes with trypsin.

Sheep erythrocytes were also found to give a greater number of nonspecific reactions than did pigeon erythrocytes, but in all cases this could be abolished by prior absorption of sera by sheep or pigeon erythrocytes, respectively.

Correlation between results of HIG, HI, and NT. Ninety-three prevaccination sera with low or no antibody content as previously measured by HI were selected and tested by HIG and NT. Thirty-three sera which were HI positive were also NT and HIG positive. As seen in Table 3, 60 sera had an HI titer of 10 or less and, of these, 57 were negative in HIG and 58 in NT. Two sera were HIG and NT positive but negative in HI, and one serum was positive in HIG and had an HI titer of 10 but an NT titer of less than 2.

Use of HIG test for the determination of serological response to rubella vaccination and primary rubella infection. Sera from 55 women who were vaccinated against rubella after delivery were tested before and 8 weeks and 2 years after vaccination. All 55 had an HI titer of 10 or less in prevaccination sera and all showed seroconversion as measured by HI after vaccination.

As shown in Table 4, 95% of these women were HIG positive 8 weeks postvaccination, compared to 72% as determined by NT. After 2 years two women had HI titers of less than 20 and one of these was negative in the HIG test. In NT, on the other hand, 92% of the women were positive after 2 years. The geometric mean titers at 8 weeks and 2 years for HI were 31 and 37, respectively, and in HIG the mean diameters were 8.2 and 8.9. The median titers in NT were 4 and 8, respectively.

To evaluate the response in HIG to primary rubella infection, acute- and convalescent-phase sera from 49 patients with clinical rubella were tested by HI and HIG. Forty-seven of the serum samples showed a significant titer rise in HI and gave negative HIG reaction with the acute-phase sera, whereas positive reactions were obtained with the convalescent-phase sera. Sixteen of the acute-phase sera contained low levels of HI antibodies but all were negative in HIG. In two patients acute-phase sera were obtained late after onset of disease and no titer increase could be shown either by HI or HIG. However, a fourfold titer in-

TABLE 1. *Detection of differences in antibody concentration with the HIG and HI methods.*

Difference in antibody concn	No. of paired sera	HIG				HI		
		Mean difference in diam (mm) ± SD[a]	Difference in diam (mm)[b]			Difference in titer[b]		
			<0.6	0.6 – 0.9	≥1	≤Twofold	Fourfold	≥Eightfold
Twofold	25	0.8 ± 0.2	1	17	7			
Fourfold	78	1.9 ± 0.5		3	75	12[c]	46	20

[a] SD, Standard deviation.
[b] Figures indicate number of paired sera showing the indicated differences in diameter or titer.
[c] Two paired sera did not show any increase in titer.

TABLE 2. *Comparison between results of HIG test obtained with sheep and pigeon erythrocytes*

Erythrocyte species	Antigen units HA/0.025 ml of 50% RBC	No. of positive sera
Pigeon	300	32/32[a]
Sheep	300	24/32[a]
Sheep	700	22/32[a]
Pigeon	None	6/144
Sheep	None	31/95

[a] All 32 sera were positive in HI and NT tests.

TABLE 3. *Correlation between results obtained by HI, HIG, and NT tests*

Titer	No. of sera	NT ≥ 2	NT < 2	HIG zone ≥ 6 mm	HIG zone ≤ 5mm
HI ≥ 20	33	33	0	33	0
HI < 20	60	2	58	3	57

crease was seen when the complement fixation test was used.

DISCUSSION

In the single radial diffusion method of Mancini et al. (8) a linear relationship is obtained between the area of the zone and the antibody concentration when equilibrium is reached, i.e., when antibodies are no longer available to react with the antigen in the gel. For antibodies of the immunoglobulin G class this will not be achieved within a diffusion time of 5 to 6 days. However, before this time a linear relationship exists between the diameter of the zone and the log antibody concentration (2). This was also found to be valid for the HIG test, where the diffusion time usually could not be extended beyond 48 h due to the instability of the erythrocytes. It was shown that fourfold and even lower titer increases could be detected with high accuracy after a diffusion time of 24 h. An increase in diffusion time up to 48 h gave a higher slope value, and thus larger differences in diameter. However, the standard deviation increased and the accuracy of the test is probably not favorably influenced by a longer diffusion time than 24 h. The variation of results between different plates made from the same gel was small.

TABLE 4. *Results of HI, HIG, and NT tests in 55 nonimmune women 8 weeks and 2 years after vaccination against rubella.*

Method	8 weeks			2 years		
	No. of positive/no. tested	%	Mean titer[a]	No. of positive/no. tested	%	Mean titer[a]
HI	55/55	100	31	53/55	96	37
HIG	52/55	95	8.2	54/55	98	8.9
NT	38/53	72	4	47/51	92	8

[a] Indicates geometric mean titer for HI, mean diameter for HIG, and median titer for NT.

Skaug et al. (11), who used chicken erythrocytes in the HIG test, obtained reproducible results and good correlation with results of HI. Sheep erythrocytes were shown in the present study to be less sensitive than pigeon erythrocytes. Pigeon erythrocytes also gave fewer nonspecific hemolytic reactions as compared to sheep erythrocytes. Although nonspecific lysis is rather rare when using pigeon erythrocytes, it is still necessary to use control plates, i.e., plates with uncoated erythrocytes.

Determination of immunity against rubella has usually been made by the HI test; the NT has been assumed to be more reliable, but not suitable for screening of large numbers of serum samples. A good correlation was found between the results of HIG and NT when testing sera with negative or low HI titers and in only one case was there disagreement between the results of HIG and NT.

Rubella vaccination is followed by the devel-

opment of HI antibodies in most cases, whereas the development of NT antibodies has been shown to be a later event and dependent on the vaccine used for immunization (4). In 95% of the women in this study antibodies were demonstrable by the HIG test 8 weeks after vaccination, and after 2 years all but one woman had antibodies as measured by HIG. The reason why only 95% were positive in the HIG test after 8 weeks might be explained by the relative inability of rubella immunoglobulin M antibodies to react in this test (12).

The HIG technique has been shown to be well suited for screening of immunity against rubella and to detect serological response to vaccination and to natural infection. We have also found HIG to be superior to HI for the detection of titer increases. Thus it appears that HIG is useful for the demonstration of reinfection. This seems to be important since the reinfection rate in individuals with vaccine-induced immunity is high (6) and reinfection has been associated with transmission to the fetus (1). It may be concluded that the HIG test is a good alternative to the HI test for the serological diagnosis of rubella, since it is easier to perform and has equal or higher sensitivity than the HI test. The only limitation of the HIG test seems to be its inability to detect rubella-specific immunoglobulin M antibodies.

ACKNOWLEDGMENTS

We are grateful to Agnete Lundmark for skilful technical assistance and to Anders Odén for help with the statistical analysis.

LITERATURE CITED

1. Eilard, T., and Ö. Strannegård. 1974. Rubella reinfection in pregnancy followed by transmission to the fetus. J. Infect. Dis. 129:594–596.
2. Fahey, J. L., and E. M. McKelvey. 1965. Quantitative determination of serum immunoglobulins in antibody agar plates. J. Immunol. 94:84–90.
3. Furesz, J., P. Moreau, and W. Yarosh. 1969. A micro tissue culture test for the titration and neutralization of rubella virus. Can. J. Microbiol. 15:67–71.
4. Grillner, L. 1975. Neutralizing antibodies after rubella vaccination of newly delivered women. A comparison between three vaccines. Scand. J. Infect. Dis. 7:169–172.
5. Halonen, P. E., J. M. Ryan, and J. A. Stewart. 1967. Rubella hemagglutinin prepared with alkaline extraction of virus grown in suspension culture of BHK-21 cells. Proc. Soc. Exp. Biol. Med. 125:162–165.
6. Horstmann, D. M., H. Liebhaber, G. L. Le Bouvier, D. A. Rosenberg, and S. B. Halstead. 1970. Rubella: reinfection of vaccinated and naturally immune persons exposed in an epidemic. N. Engl. J. Med. 283:771–778.
7. Leerhøy, J. 1968. Rubella virus neutralization in heated sera. Acta Pathol. Microbiol. Scand. 73:275–282.
8. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235–254.
9. Quirin, E. P., D. B. Nelson, and S. L. Inhorn. 1972. Use of trypsin-modified human erythrocytes in rubella hemagglutination-inhibition testing. Appl. Microbiol. 24:353–357.
10. Russell, S. M., D. McCahon, and A. S. Beare. 1975. A single radial hemolysis technique for the measurement of influenza antibody. J. Gen. Virol. 27:1–10.
11. 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.
12. 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.