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A micro immune haemolysis test for rubella antibody screening

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SUMMARY

A micro test for the detection of rubella IgG antibody by passive haemolysis in free solution (MIH) is presented. The technique is reproducible, sensitive, simple to perform and economical in the use of reagents. Identical results were obtained in all but one of 408 tests on serum specimens screened for rubella antibody at 15 i.u./ml by the MIH and haemagglutination inhibition (HI) techniques. The quantitative potential of the MIH test and the relative merits of the MIH, HI and radial haemolysis (RH) procedures for rubella screening are discussed.

INTRODUCTION

The significance of maternal rubella as a cause of human embryopathy is well documented and numerous tests have been developed to determine an individual's immunity to the disease. Until recently the haemagglutination inhibition (HI) test was regarded generally as the procedure of choice. However, the single radial haemolysis (RH) technique, first applied to the measurement of influenza virus antibody (Schild, Pereira & Chakraverty, 1975) and subsequently to rubella antibody, (Skaug, Orstravik & Ulstrup, 1975; Strannegard, Grillner & Lindberg, 1975) is now preferred by many workers because it is simpler to perform than the HI test and, more importantly, the reaction is not affected by non-specific inhibitors of rubella haemagglutinin.

These advantages are shared by an alternative micro-immune haemolysis (MIH) technique described in this paper. The new micro test is more sensitive than the radial haemolysis test and much more economical in the use of reagents.

MATERIAL AND METHODS

Sera

One hundred serum samples were obtained from residual amounts of sera examined in a W.H.O. Collaborative Study to establish a recommended rubella HI test. Twenty-two of the sera had been shown to be free of detectable rubella antibody by HI and radioimmunoassy (RIA) techniques. Serum samples from women attending antenatal clinics were kindly provided by Dr M. H. Hambling of the Leeds Public Health Laboratory and Dr M. S. Shafi of the Central Middlesex Hospital Public Health Laboratory. Sera were stored at −20 °C and thawed at

37 °C before testing. The freeze-dried anti-rubella reference reagent 80/1 prepared from a pool of recalcified human plasma contained 15 international units (i.u.) of rubella antibody per millilitre.

Antigens

The rubella haemagglutinating (HA) antigen (DMRQC HA 23/80) used in all the MIH antibody tests was prepared by alkaline extraction from BHK21 monolayer cell cultures infected with rubella virus (strain Judith). After treatment with Tween 80 and ether (Norrby, 1962) the HA was freeze-dried in the presence of a stabilizing agent.

A control antigen was made by subjecting BHK cells, not infected with rubella virus, to the same preparative procedures as the infected cells.

Complement

Either frozen (−70 °C) guinea-pig complement from which antibodies to sheep erythrocytes had been removed (Northumbria Biologicals Ltd) or commercial freeze-dried guinea-pig complement in 20% Richardson's preservative (Tissue Culture Services Ltd) was used in the MIH tests. When the preserved complement was used, one part of the reconstituted reagent was mixed with seven parts of distilled water and one part of a 30% suspension of sheep erythrocytes in veronal buffer (complement fixation buffer, Oxoid BR16, containing penicillin 100 units/ml and streptomycin 100 μg/ml). The mixture was held at 4 °C and gently shaken every 15 min for 90 min. After centrifugation at 4 °C the supernatant was regarded as a 1 in 10 dilution of complement. Further dilutions were made in veronal buffer.

Pretreatment of serum

Patient's serum (10 μl) was transferred to an Eppendorf tube containing 30 μl of veronal buffer and the 1 in 4 dilution inactivated by heating in a water bath at 60 °C for 20 min. After cooling, 10 μl of a 15% suspension of sheep erythrocytes in veronal buffer was added, the contents of the tube mixed in a vortex mixer and held at 4 °C overnight. After centrifugation the supernatant constituted a 1 in 5 pretreated serum sample.

Sensitization of sheep erythrocytes

Fifty microlitres of a 1·5% suspension of sheep erythrocytes in veronal buffer was mixed with 50 μl of rubella HA and held at 4 °C for 30 min. The antigen-coated cells were washed in 2 ml of veronal buffer and the pellet resuspended in 500 μl of complement. A control erythrocyte suspension in which the cells were treated with control antigen was similarly prepared. The working dilutions of antigen and complement were determined in preliminary titrations as described below under standardization of the test.

Test procedure

Two microlitres of each pretreated serum sample was transferred to each of two wells of a Terasaki plate by means of an Eppendorf or Oxford semi-automatic pipette. The negative control serum and three samples of the rubella reference reagent containing 15, 3 and 1 i.u./ml of rubella antibody were similarly dispensed.

The suspension of control cells in complement was throughly mixed by alternately aspirating and discharging it through a gauge-18 canula attached to a 2 ml 'Plastipak' syringe. The canula was replaced with a gauge-27 canula (Hamilton KF 727) and one drop of the suspension was added to one of each pair of wells containing the test serum. Similarly one drop of antigen-coated erythrocytes resuspended in complement was added to the duplicate wells containing the test sera and to the wells containing the positive and negative serum controls. The Terasaki plate was held at 37 °C overnight in a humidifier. Condensation was prevented by replacing the lid with a disc of filter paper. After 16–22 h the plate was removed from the incubator and the contents of the wells examined with an inverted or a conventional microscope equipped with a ×2·5 or ×4 objective. The presence or absence of intact erythrocytes was recorded. With the concentration of reagents chosen for use in the test (see Results) complete haemolysis denoted a concentration of antibody equal to or greater than 15 i. u. rubella antibody per ml serum.

Rubella HA and HI tests

Rubella HA was assayed in V-well polystyrene Microtiter plates. Twenty-five microlitre serial twofold dilutions of each antigen from 1 in 8 to 1 in 2048 were made in CFT diluent, Oxoid Code BR16, containing 0·2% bovine albumin Fraction V, Armour (CFTA).

Fifty microlitres of a 0·2% suspension of day-old chick erythrocytes in CFTA diluent was added to each well, the plate was shaken in a Titertek shaker and the test read after overnight incubation at 4 °C. The HA titre of the antigen was taken as the reciprocal of the highest dilution showing complete haemagglutination.

Rubella HI antibody was assayed by the technique recommended by the Public Health Laboratory Service Standing Advisory Committee on Viral Reagents (Report, 1978) except that CFTA diluent was used instead of DGVA and the titration end-point was read as the highest serum dilution showing complete inhibition of haemagglutination. The tests were done in V-well Microtiter plates; pigeon erythrocytes were used.

RESULTS

Standardization of the test

Absorption of serum

In the course of preliminary work it became evident that a proportion of sera gave equivocal or false positive results if absorption with sheep erythrocytes was omitted. Eighteen serum specimens, including 10 known to be without antibody to rubella virus, were screened by the MIH technique with and without prior absorption with sheep erythrocytes. When absorption was not carried out, seven of the 10 antibody negative sera gave false reactions in the MIH test. These false reactions were not found after preliminary treatment of the sera with sheep erythrocytes.

Antigen

Aliquots (50 μl) of a 1·5% sheep erythrocyte suspension in veronal buffer were mixed in conical centrifuge tubes with 50 μl of antigen at the following dilutions:

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Table 1. *Rubella antigen titration*

Rubella antigen dilution	Rubella antibody (i.u./ml)					
	15	7·5	3·8	1·9	0·9	0·5
1 in 2·5	0*	0	0	1	1	1
1 in 3·5	0	0	0	2	2	2
1 in 5·0	0	0	0	2	<3	<3
1 in 7·0	0	0	1	2	3	3
1 in 10	2	2	2	2	3	3
1 in 13	2	2	2	2	3	3
No antigen	3	3	3	3	3	3

* 0, Complete lysis; 3, no lysis; 1 and 2, indicate varying degrees of partial lysis.

1 in 2·5, 1 in 3·5, 1 in 5, 1 in 7, 1 in 10 and 1 in 13. After 30 min incubation at 4 °C with intermittent shaking the antigen-coated cells in each tube were washed and resuspended in a 1 in 10 dilution of complement as described under Methods. One drop of each suspension was added through a 27-gauge canula to each of six serial twofold dilutions of the anti-rubella reference reagent (15–0·5 i.u./ml) in a Terasaki plate. Sensitized erythrocytes suspended in buffer only and unsensitized cells in the presence and absence of complement were included as controls. The test was read after 18 h incubation at 37 °C. The antigen potency was defined as the reciprocal of the highest dilution showing complete haemolysis to the titre of the rubella antibody preparation (Table 1). An antigen concentration 1·5 times greater than the potency was used in the MIH test. In the example in Table 1 the potency of the antigen is 5 and this antigen should be used at a concentration of 1 in 3·3.

Although one batch of antigen (DMRQC HA 23/80) was used in all the screening tests reported, antigens of different provenance were used in the early stages of development of the technique. Their potencies were low but the transition from lysis to non-lysis was always sharp.

Complement

The complement dilution used in the MIH test was based on replicate titrations of a pretreated human serum carried out with optimally sensitized erythrocytes resuspended in 1 in 10, 1 in 20, 1 in 30, 1 in 40 and 1 in 50 complement dilutions respectively. In the example (Table 2) a dilution of 1 in 15 was chosen for use in the test.

Although one batch of complement was used in the screening tests reported here, experience was gained with several batches of local and commercial complements. They varied in potency and non-specific activity. Failure to absorb the complement with sheep erythrocytes invariably resulted in an unacceptable degree of non-specific haemolysis.

Erythrocyte species

In contrast to sheep erythrocytes, pigeon and trypsin-treated human O erythrocytes, though eight times more susceptible to haemagglutination by rubella antigen, were found to be relatively insusceptible to lysis in the MIH test.

Table 2. *MIH titrations of an anti-rubella serum carried out with sensitized and control erythrocytes in various dilutions of complement*

Complement dilution	Reciprocal serum dilutions: Test 10	20	40	80	160	320	Control 10	20	40	80	160	320
1 in 10	0	0	2	2	3	3	<2	2	2	2	2	2
1 in 20	0	0	2	3	3	3	<3	3	3	3	3	3
1 in 30	0	0	2	<3	3	3	<3	3	3	3	3	3
1 in 40	<2	<2	2	3	3	3	<3	3	3	3	3	3
1 in 50	<2	<2	2	3	3	3	<3	3	3	3	3	3
No complement	3	3	3	3	3	3	<3	3	3	3	3	3

For details of scoring system see Table 1.

Effect of erythrocyte concentration on the sensitivity of the MIH test

Fifty microlitre volumes of six sheep erythrocyte suspensions in veronal buffer varying in concentration from 0·5 % to 3 % were sensitized with antigen (optimized for 1·5 % cells), washed and resuspended in complement. Triplicate MIH assays of the anti-rubella reference reagent were made with each of the sensitized cell suspensions in three Terasaki plates. The end-point of each titration was taken as the reciprocal serum dilution corresponding to the well showing approximately 50 % lysis of control cells at the particular erythrocyte concentration used. Except for the titrations involving the lowest initial erythrocyte concentration (0·5 %), which were difficult to read, the mean end-point of each set of three titrations was plotted graphically against the relevant erythrocyte concentration (Fig. 1). Thus the minimum detectable rubella MIH antibody concentration was found to vary inversely with the sensitized erythrocyte concentration over the range of cell concentrations tested. An initial erythrocyte concentration of 1·5 % was adopted for use in the routine test because at this concentration the end-points were easily read and by virtue of the inherent sensitivity of the technique it was possible to adjust the antibody screening cut-off to conform with the currently accepted criterion of immunity (15 i. u./ml) by incorporating a serum dilution of 1 in 5 as a result of the pretreatment procedure (see Materials and Methods).

Sensitization of erythrocytes

To determine the duration of incubation at 4 °C required to coat the sheep erythrocytes with antigen, five duplicate MIH titrations of the anti-rubella reference reagent were made with five sensitized sheep cell suspensions prepared as described under Methods except that the cells were incubated with antigen at 4 °C for 12, 30, 60, 120 and 240 min respectively before washing and resuspension in complement. The same titre was obtained in each of the titrations.

Duration of incubation

Quadruplicate titrations of the anti-rubella reference reagent in serial twofold dilution steps were made in four Terasaki plates. The plates were removed from the 37 °C incubator and read after 15, 18, 21 and 23 h respectively. The same titre was obtained in all four assays.

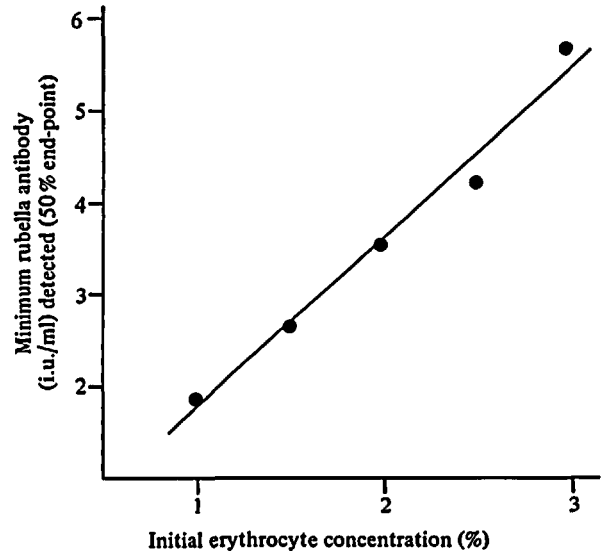

Fig. 1. Influence of erythrocyte concentration on sensitivity of MIH technique.

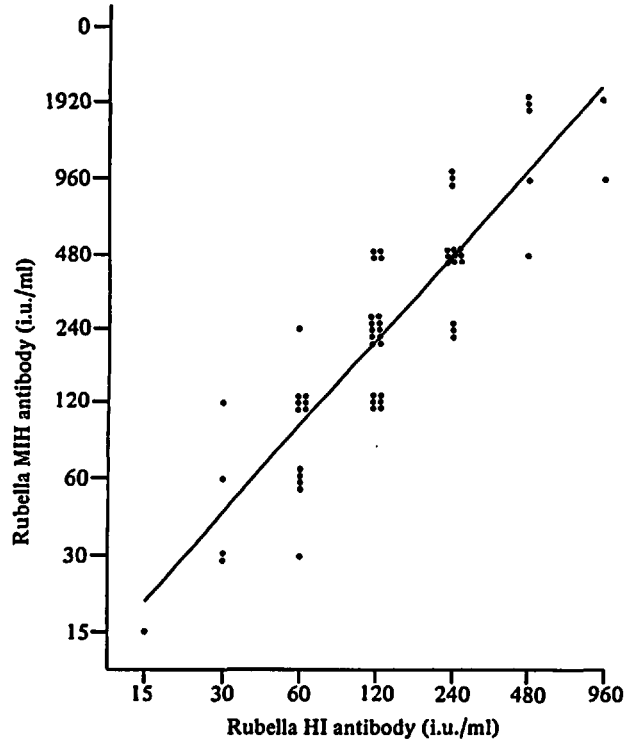

Fig. 2. Correlation of rubella antibody levels obtained by HI and MIH techniques in 59 sera.

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Sensitivity and reproducibility

Numerous titrations of the anti-rubella reference reagent in serial twofold dilution steps over a period of 2 years consistently gave a titre (100% end-point) representing a rubella antibody concentration of 3·8 i.u./ml. Several titrations of the reference reagent in suitably graded dilutions showed the limit of sensitivity of the prescribed technique to be 3 i.u./ml.

Quantitation of rubella IgG

The results of rubella antibody titrations of 59 sera by HI and MIH techniques are shown in Fig. 2.

Comparison of MIH and HI screening test results

Samples from 100 serum pools and 308 serum specimens from ante-natal patients were screened for rubella antibody by the MIH and HI techniques. Eighty-two specimens were found to contain less than 15 i.u. rubella antibody per ml in both tests. No discrepancies were found. Twenty-two of the serum pools, rubella antibody negative by RIA, were retested by MIH at a 6 i.u./ml screening cut-off level, all 22 sera were antibody negative at this level.

DISCUSSION

Antibody-dependent complement mediated haemolysis in free solution on a micro scale provides an economical alternative to haemolysis-in-gel (HIG; synonym, radial haemolysis, RH) for rubella antibody screening. Like the RH procedure the MIH test is simple to carry out and read and non-specific inhibitors of rubella haemagglutinin are irrelevant. The removal of antibody to sheep erythrocytes, essential in the MIH technique, may be expected to preclude the equivocal results given by the small proportion of sera which react in the control plate of the RH test. Notwithstanding the extra step entailed in absorbing the serum the MIH technique is no more laborious than the RH procedure and does not require the prior preparation of gels with their inherent short shelf-life. Although developed primarily as a screening test the MIH technique may be used to quantify rubella IgG by testing patients' serum dilutions in Terasaki plates. Titration end-points were reproducible and a good correlation between HI and MIH antibody titres was demonstrated (Fig. 2). Like RH, the MIH test does not detect IgM antibody (unpublished observations). A rubella antibody concentration of 15 or more i.u. per millilitre is generally regarded as evidence of infection with rubella virus at some time. This criterion of immunity was chosen because lower antibody concentrations to rubella could not be detected reliably by the HI technique. With the advent of more sensitive rubella antibody assays it is likely that the criterion of immunity could be reduced below 15 i.u./ml without loss of specificity (Mortimer *et al.* 1981), a view supported by Pattison in a recent review of rubella screening (Pattison, 1982). In the MIH test optimally sensitized sheep erythrocytes (1·5%) appear to be uniformly susceptible to lysis by as little as 3 i.u. rubella antibody per ml in the presence of complement, therefore the cut-off level could be adjusted to accommodate a revised criterion by lowering the serum dilution in pretreatment.

Forger III and Gilfillan (1979) have shown that erythrocytes, antigen and complement are the most expensive components of the RH test. One-sixtieth fewer cells and one-tenth to one-twentieth less antigen and guinea-pig complement were required in the MIH technique per serum tested. If extended experience of the MIH test confirms the virtual absence of reactions between patients' sera and the control antigen the omission of the control antigen might be justified. This would effect a further economy and simplification of the MIH test procedure.

The MIH technique may be regarded as a valid alternative to the RH test for rubella screening that may be preferable when economy is an important consideration.

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