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Radial haemolysis for the detection of rubella antibody in acute postnatal rubella

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SUMMARY

One hundred and forty-eight sera from 101 cases of acute postnatal rubella were examined by the radial haemolysis (RH) technique currently recommended by the Public Health Laboratory Service of the United Kingdom. In 75 cases the date of onset of rash was known. No sera were RH positive until the third day after the onset of rash and one serum was still negative at 9 days. By 10 days all sera gave a zone of at least 10 mm. The antibody response detected by RH appears later than that detected by haemagglutination inhibition (HI). Thirty-nine of the 101 cases could be diagnosed by seroconversion or a significant rise in titre using the HI test (diagnosis of the remainder depending upon rubella-specific IgM tests). Using the RH test this figure rises to 48 but in ten of these cases reliance on RH would have meant a delay in diagnosis. The results also indicate that many more sera would need to be tested for rubella-specific IgM if the RH test was used instead of the HI test for evaluating possible cases and contacts of rubella.

INTRODUCTION

Radial haemolysis (RH) has proved to be a specific and sensitive assay for the detection of rubella-specific IgG (Kurtz *et al.* 1980). It has previously been shown that RH does not detect rubella-specific IgM (Strannegard, Grillner & Lindberg, 1975) or rubella-specific IgA (Kurtz *et al.* 1980). All these classes of rubella-specific antibody can be detected by the haemagglutination inhibition (HI) test. IgM antibodies appear before IgG antibodies during the immune response in acute rubella. Therefore there may be a delay in the appearance of rubella-specific antibodies detectable by RH compared to those detectable by HI (Champsaur, Dussaix & Tournier, 1980). It has been suggested that this delayed response measured by RH may be of help in situations where acute sera are received some days after the onset of the clinical illness. Seroconversion may be demonstrated by RH in pairs of sera with the same HI titres.

Although the constituents and methods of preparation of rubella RH gels may vary, the technique has been widely adopted for the determination of rubella-immune status. Indeed, many laboratories which previously did not perform rubella serology have introduced this method for the determination of immune status as the technique is simple to perform and requires no specialized equipment. Some laboratories which previously performed the HI test for rubella antibody screening have replaced this test with RH. Laboratories using only RH for rubella serology have to forward sera from suspected cases of acute postnatal rubella to laboratories which perform the HI test and which have facilities for the detection of rubella-specific IgM. It would be useful for such laboratories if RH could be applied to the diagnosis of acute rubella, if only to select those sera which require a rubella-specific IgM determination.

The immune response in acute postnatal rubella as determined by RH has been previously investigated by Strannegard, Grillner & Lindberg, 1975; Grillner & Strannegard, 1976; Harnett, Palmer & Mackay-Scollay, 1979; Forger & Gilfillan, 1979; Väänänen & Vaheri, 1979, and by Champsaur, Dussaix & Tournier, 1980. However, these studies have not made firm recommendations for the interpretation of results, whereas such recommendations are available for the interpretation of the HI test (Morgan-Capner & Pattison, 1981; Public Health Laboratory Service Monograph, 1982).

In this study we have evaluated, for the diagnosis of acute rubella, the RH technique currently used by the Public Health Laboratory Service, United Kingdom, for rubella antibody screening. The aim was to determine whether RH could be a satisfactory substitute for HI in the initial investigation of cases and contacts of acquired rubella.

MATERIALS AND METHODS

Sera

The sera evaluated are listed in Table 1. In all, sera from 101 cases were studied. Thirty-five paired sera and 40 single sera were available from confirmed cases of primary postnatal rubella. The date of onset of rash in these 75 cases of rubella had been stated on the request form. Twelve paired sera and 14 single sera from cases where the date of onset of rash was not stated were also tested. Recent rubella in the 54 cases where only a single serum was available and in the eight cases showing an elevated, non-rising HI titre in paired sera, was confirmed by detecting rubella-specific IgM using HI tests on gel filtration fractions (Morgan-Capner, Davies & Pattison, 1980). Sixteen of the 39 cases which showed a seroconversion or a four-fold rise by HI were also examined for the presence of rubella-specific IgM. All 16 were positive.

Radial haemolysis

The method described by Kurtz *et al.* 1980, was used. Sera were inactivated at 60 °C for 20 min so minimizing the problems which may be encountered with zones in the control gels. RH gels were made in 100 mm × 100 mm Petri dishes (Sterilin Ltd., Teddington, U.K.) and contained 15 ml agarose (Indubiose A37, Pharm-

Table 1. *Sera from acute post-natal rubella tested for rubella-specific antibody by radial haemolysis*

	Result of HI tests (Rubella-specific IgM positive)			
Date of rash	Seroconversion	⩾ 4-fold rise	Non-rising titre (⩾ 200 i.u.)	Total
Known				
Paired sera	24* (9)	6 (4)	5 (5)	35 (18)
Single serum	—	—	40 (40)	40 (40)
Not stated				
Paired sera	4 (1)	5 (2)	3 (3)	12 (6)
Single serum	—	—	14 (14)	14 (14)

* Includes five cases where the first serum was collected prior to the rash.

industrie, Clichy, France), 0·3 % sheep red blood cells, 0·3 ml rubella haemagglutinin (HA), titre 128 (Public Health Laboratory Service, Colindale, U.K.) and 0·5 ml of undiluted Richardson's preserved complement (LIP, Shipley, U.K.). For control gels, the rubella HA was omitted. Sixty-three, 3 mm wells were cut in each gel. Gels were used within 3 days of being made. Sera were added to corresponding wells in test and control gels using microcapillary pipettes. Each plate included a 15 international unit (i.u.) standard in two wells and a known negative serum in one well. Plates were incubated in a humidified box overnight at 35 °C. Zones of haemolysis were measured to the nearest 0·5 mm by marking spots on the edges of the zone with a fine felt tip marker whilst transilluminating the gel and then reading the diameter with a calibrated eyepiece.

Rubella HI test

Non-specific inhibitors of rubella haemagglutination were removed by treatment with 25 % kaolin for 20 min at room temperature (RT). The rubella HI test incorporated 4–8 HA units of antigen (Flow Laboratories, Irvine, U.K.) and a serum/antigen incubation of 1 h at RT before the addition of 0·3 % day-old chick cells (Tissue Culture Services, Slough, U.K.). Results were reported in i.u. by comparison with a reference positive control serum.

Gel filtration

Gel filtration was performed on Sephacryl S-300 as described by Morgan-Capner, Davies & Pattison, 1980. Immunoglobulin-containing fractions were titrated from undiluted to a dilution of 1 in 64 and the rubella-specific antibody titre determined after overnight fixation at 4 °C with Flow Laboratories HA antigen before the addition of day-old chick red cells. Amounts of rubella-specific IgM and IgG were estimated by taking the highest titres of fractions contributing to the appropriate immunoglobulin peaks. This method of assessing relative IgM/IgG concentrations is only an approximation as rubella-specific IgA contributes to the detectable HI activity and the total rubella-specific immunoglobulin is represented not only by the height of the HI peak but also by its spread.

Sucrose density gradient centrifugation

Non-specific inhibitors were removed by treatment with a manganous chloride: heparin mixture. A volume (0·5 ml) of a 1 in 2 dilution of serum was layered onto a 4 ml 10·7–30 % sucrose gradient in a polypropylene tube and centrifuged at 32000 rev./min for 15 h in a swing-out rotor in an MSE Prepspin 75 ultracentrifuge. Twelve equal fractions were obtained by puncturing the bottom of the tube and collecting by downward displacement. The presence of IgM and IgG in the fractions was confirmed by immunodiffusion against rabbit anti-human IgM or IgG (Dako, Copenhagen, Denmark). Rubella-specific antibody within the fractions was determined by HI using 4 units Flow Laboratories rubella HA, chick red blood cells and an antigen/antibody incubation of 1 h at RT.

Rubella complement fixation test (CFT)

A standard microtechnique CFT was used with rubella CF antigen supplied by the Public Health Laboratory Service, Colindale, U.K. An overnight antigen/antibody/complement incubation at 4 °C was used prior to the addition of sensitized sheep red blood cells.

RESULTS

Character of zone

Some of the acute phase sera gave indistinct zones of haemolysis when tested initially. However, repeat testing showed that this was not a consistently reproducible feature; all grades of haemolysis could be produced by a single serum even though the RH gel constituents remained the same. The rings of incomplete lysis reported by Champsaur, Dussaix & Tournier (1980) to be characteristic of acute rubella were not observed.

RH response in known date of rash

Fig. 1 shows the diameter of the zone of haemolysis of 105 sera from 75 cases of acute rubella related to the number of days after the onset of the rash. The earliest time at which antibody was detectable by RH was 3 days after the onset of the rash. Four sera collected on day 3 had RH zones ranging from 8 mm to 12·5 mm, whereas three sera gave no zone. These three RH negative sera had HI titres of 1600 i.u., 100 i.u. and < 12 i.u. Two sera collected on day 4 were RH negative and were also seronegative (< 12 i.u.) by HI. The two RH negative sera on day 7 had HI titres of 50 i.u. and 200 i.u. and the RH negative serum on day 9 was also seronegative by HI. No sera were seen which gave an RH zone but were seronegative by HI.

All sera taken 10 days or longer after the onset of the rash gave RH zones, the diameter varying from 10–15 mm.

RH evaluation of paired sera

Table 2 shows the RH results of paired sera from 47 cases, the date of onset of rash being known in 35 cases. Acute rubella was diagnosed by HI seroconversion in 28 cases (with first serum being collected prior to the onset of the rash in five

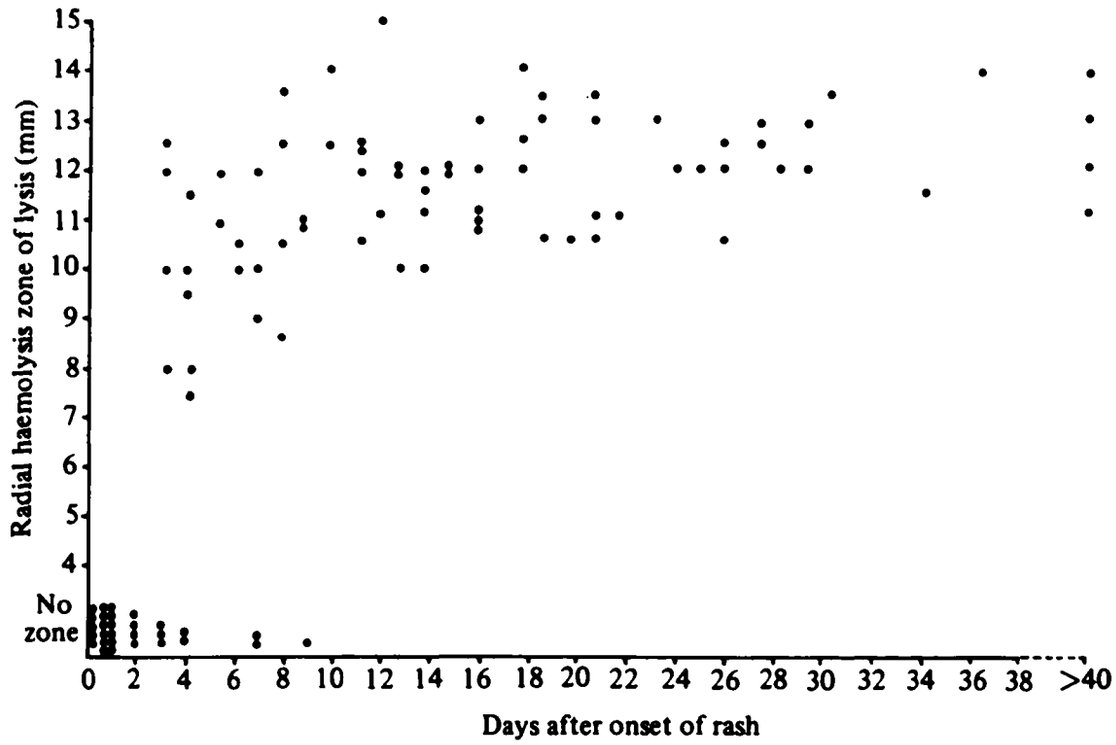

Fig. 1. RH zones in relation to days after onset of rash in 75 cases of confirmed rubella.

cases) and 25 of these cases also showed seroconversion by RH. In the remaining three cases both sera had no zone by RH. In two of these the second serum was taken 3 days and 7 days after the onset of the rash, the first serum having been taken prior to the onset of the rash. In the third case the date of the rash was not stated.

Eleven pairs showed a $\geqslant$ fourfold rise in HI titre and eight of these showed RH seroconversion. Of the remaining three pairs, one pair showed an increasing zone of lysis (8 mm–12·5 mm). One pair gave no zone with either serum but showed a rise in HI titre from 100 to 400 i.u., a rise in CF titre from 8 to 32 and both sera contained rubella-specific IgM. The onset of the rash was not stated in this case. The other pair gave two zones of equal diameter (12 mm) and were collected 3 and 15 days after the onset of the rash. This pair showed a rise in HI titre from 200 to 800 i.u. but insufficient of this pair of sera remained to perform further evaluation.

Paired sera which showed a high non-rising HI titre but which contained rubella-specific IgM were available from eight cases. Four of these showed an increasing zone of at least 2 mm by radial haemolysis. The first serum of these four pairs was collected 4, 6 and 8 days after the onset of the rash in the three cases where the date was known. With the remaining four pairs both sera had equal zones. The date of onset of rash was known in two cases and the first serum was collected 7 and 21 days after onset. Rubella CFTs were also performed on three of the four pairs of sera which showed a high stable HI titre but an increasing RH zone. All three showed a rise in CF antibody titre. Two of the four pairs showing a stable HI titre and a stable RH zone were evaluated by CFT. One pair, with RH zones of 12 mm, showed a rise from 8 to 64 and with the other pair both sera had CFT titres of 32 and RH zones of 12 mm.

Table 2. *RH zones in paired sera from cases of acute rubella*

	Radial haemolysis				
HI result	Seroconversion	Increasing zone (⩾ 2 mm)	Stable zone	No zone (both sera)	Total
Seroconversion	25	0	0	3	28
⩾ Fourfold rise	8	1	1	1	11
Stable high titres (⩾ 200 i.u.)	0	4	4	0	8
Total	33	5	5	4	47

Thus, of the total of 47 paired sera tested, the HI test was diagnostic in 39 with the remaining eight giving stable high antibody titres. The RH test was diagnostic in 38 cases, stable zones in both sera were found in five cases and no zone in either serum in the remaining four.

RH evaluation of single sera

The 40 single sera from patients with a known date of rash were collected 1–70 days after the onset. Three sera collected 1, 4 and 7 days after the onset gave no zone by RH but had antibody detectable by HI. The remaining 37 sera gave zones from 7·5 mm (at 4 days post onset of rash) to 15 mm. Three of the 14 sera from patients whose date of onset was not stated gave no zone by RH but had detectable HI antibody. The remaining 11 sera gave zones of 8 to 13 mm.

Relationship between RH zone and early convalescent IgG antibody

In this study a total of 148 sera were tested. Nineteen sera from 18 patients had a demonstrable HI titre but gave no zone by radial haemolysis. Fourteen of these sera had HI titres of 100 i.u. or less and fractionation by gel filtration of nine of these sera indicated that IgM antibody represented 50–90% of the HI activity. One serum contained 800 i.u. of rubella HI antibody but gel filtration indicated that 90% of the HI activity was in the IgM fractions. Insufficient serum remained to evaluate this serum further. Two sera contained 200 i.u. of rubella HI antibody but insufficient remained for fractionation . The remaining two contained 400 and 1600 i.u. of rubella HI antibody and fractionation by gel filtration suggested that sufficient rubella-specific IgG was present to be detectable by RH.

In view of the above findings five sera were fractionated in an attempt to correlate the amount of rubella antibody activity in the IgG fractions after serum fractionation with the presence or absence of an RH zone with whole serum. Sucrose density gradient centrifugation was used in order to minimize the dilution during fractionation. The results are presented in Table 3. Serum 1 is a rubella IgM negative control serum with 400 i.u. of rubella HI antibody. The unfractionated serum gives an RH zone of 13·0 mm diameter. After fractionation the peak HI activity of the IgG-containing fractions is 100 i.u. and these fractions give an RH zone of 9·0 mm. Serum 2 is another IgM-negative serum of lower potency which gives a similar pattern of results. Sera 3 and 4 are early convalescent sera

Table 3. *Rubella HI and RH antibody content of sera fractionated by sucrose density gradient centrifugation*

	Rubella antibody			
	Whole serum		Fractionated serum	
Serum	HI (i.u./ml)	RH zone (mm)	HI (i.u./ml), IgM/IgG fractions	RH zone (mm), IgM/IgG fractions
1	400	13·0	neg./100	neg./9·0
2	200	9·5	neg./25	neg./5·0
3	1600	8·0	25/100	neg./neg.
4	1600	8·0	6/50	neg./neg.
5	400	neg.	6/50	neg./8·5

containing 1600 i.u. of rubella HI antibody. However, the unfractionated sera give only small zones (8 mm diameter) when tested by RH. After fractionation the IgG-containing fractions have HI titres similar to sera 1 and 2 but no RH zones were given by these fractions. Serum 5 is the serum mentioned above with 400 i.u. of rubella HI antibody but no antibody detectable by RH. After fractionation HI activity similar in amount to that found with sera 1–4 was detected in the peak IgG fraction. However, in this case an RH zone of 8·5 mm diameter was given by this fraction. This result suggested that rubella-specific IgM or rheumatoid factor may have interfered with rubella-specific IgG when RH was performed on whole serum. Therefore, attempts were made to reproduce this apparent blocking effect by mixing IgM and IgG-containing fractions. However, when mixed in a ratio of 1:1 and tested by RH, no loss of RH zone could be demonstrated in excess of that resulting from the dilution involved.

DISCUSSION

There is a good correlation between the results of rubella HI and RH tests when they are used to screen for specific IgG antibody consequent upon a rubella virus infection months or years previously (Harnett, Palmer & Mackay-Scollay, 1979). However, there is a dissociation between the results of the two tests when they are used to detect early convalescent antibody. First, there is a delayed antibody response by RH compared with HI after acute rubella. In this series there were 19 sera from 18 patients in which there was a detectable HI titre but no RH zone. As RH fails to detect rubella-specific IgM it is not surprising that early acute-phase sera with HI titres of < 200 i.u., the majority of which is of the IgM class, do not give RH zones. Certainly 14, and possibly 16, of the 19 sera fall into this category. A further serum had a high HI titre (800 i.u.) but had only minimal HI activity in the IgG-containing fractions. Second, it is clear that, in some cases, the specific IgG antibody produced in the early convalescent phase reacts poorly in the RH test even when it is present to high titre. This may result in the production of a smaller than expected zone or no zone at all. Two of the 19 sera referred to above contained 400 and 1600 i.u. of rubella HI antibody, the majority fractionating in

the IgG fractions, but gave no RH zone. Interestingly, one of these sera contained antibody which fractionated as IgG and gave an RH zone after separation of serum proteins by density gradient centrifugation. The lack of a zone with the unfractionated serum could have been due to blocking of IgG reactivity by rubella-specific IgM or IgM-rheumatoid factor but the phenomenon could not be reproduced by mixing IgM and IgG-containing sucrose density gradient fractions.

Whatever the explanation of some aspects of the dissociation of HI and RH results with early convalescent, sera, the delay in appearance of strongly reactive RH antibody increases the potential value of the test for the diagnosis of recent rubella. Diagnostic seroconversion was found by HI with 28 pairs of sera and in 25 of these cases there was seroconversion by RH. In the other three pairs no RH zone was detectable with either serum. The date of the rash was known in two of these cases and the second serum was collected early after the onset of the rash, i.e. 2 and 7 days. Undoubtedly seroconversion by RH would have taken place eventually. The results reported here indicate that all sera taken 10 to 70 days after the onset of the rash had RH zones of > 10 mm diameter. It would be prudent to err on the side of caution and take a last serum 14 or more days after the onset of the rash before excluding seroconversion by RH. Thus, with sera taken at the appropriate time all 28 cases diagnosed by HI seroconversion and six diagnosed by HI and rubella-specific IgM determination could have been diagnosed by RH seroconversion although this would have led to a delay of 1–2 weeks in making the diagnosis in nine cases.

Of 11 paired sera showing a significant rise in HI titre recent rubella could be diagnosed by RH seroconversion in eight cases and by an increasing zone diameter (8·0–12·5 mm) in one case. In the tenth case neither serum gave an RH zone but again the assumption can be made that this case could have been diagnosed if a later serum was available. In the remaining case the pair of sera showed a stable RH zone in spite of an increasing HI titre. Thus, of the 39 cases diagnosed by seroconversion or a significant rise using the HI test, 37 could have been diagnosed by RH seroconversion and one by an increasing zone.

The remaining eight cases from which paired sera were available all had elevated (> 200 i.u.) stable concentrations of antibody by HI. Four of these could have been diagnosed as recent rubella on the basis of increasing RH zone diameter. The remaining four pairs showed a stable RH zone. Thus, of 47 paired sera the HI test alone was diagnostic in 39, whereas the RH test could have been diagnostic in 42 although delay in diagnosis would have occurred in four cases because of the necessity of waiting for later sera to demonstrate RH antibody.

A single serum was available from 54 cases diagnosed on the basis of the presence of rubella-specific IgM. In six of these cases the single serum had detectable HI antibody but no RH antibody and therefore in these cases diagnostic RH seroconversion could have been found if later sera were tested. Thus, considering all 101 cases tested, diagnostic seroconversion or a significant rise using the HI test was found in 39, whereas, using the RH test, diagnostic results could have been found in 48.

The problem with relying on RH for the diagnosis of recent rubella occurs in

those cases in which the first serum is taken relatively late (longer than 9 days) after the onset of the rash. The results described here show that the peak antibody response in the first month after acute rubella may give an RH zone of only 10 mm. Therefore, it would be necessary to examine for rubella-specific IgM any serum taken at this time which gives an RH zone of $\geqslant 9$ mm. As approximately 90% of ante-natal screening sera give such zones (Kurtz *et al.* 1980) the majority of sera would need to be screened for rubella-specific IgM. This would be greater than that necessary using the HI test since a rubella-specific IgM test is only required for sera with $\geqslant 200$ i.u./ml of HI antibody (PHLS Monograph, 1982). With the HI test used here only 50% of ante-natal screening sera have such an amount of HI antibody.

RH may also be applied to the investigation of patients having contact with rubella. The presence of antibody detectable by RH within 10–12 days of a defined date of contact would indicate immunity on the date of contact. If antibody is absent then rubella as a consequence of the stated contact can be confirmed or excluded by testing later sera in parallel with the first. Finally, if the patient presents more than 10–12 days after contact and rubella RH antibody giving a zone diameter of $\geqslant 9$ mm is present then recent rubella can only be excluded by tests for specific IgM.

In conclusion, it is clear that the RH test can be used for the diagnosis of rubella and the evaluation of patients in contact with the disease. All 101 cases of rubella diagnosed by HI and rubella-specific IgM tests in this series would have been diagnosed by RH plus specific IgM tests where indicated. However, there would be a delay in diagnosis in approximately 10% of cases if RH seroconversion were awaited and if rubella-specific IgM determinations were only performed on those sera having detectable RH antibody. This might be acceptable since it allows local laboratories to avoid referring all sera to reference laboratories for diagnosis although many virologists would recommend a confirmatory IgM test in all cases of rubella in pregnancy. In addition, reliance on RH would undoubtedly increase the number of specific IgM tests required in evaluating single late sera from cases and contacts.

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