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Comparison of immunofluorescence and radioimmunoassay for detecting IgM antibody in infants with the congenital rubella syndrome

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SUMMARY

Immunofluorescence (IF) and radioimmunoassay (RIA) have been compared as methods for detecting IgM antibody in 124 infants with confirmed or suspected congenital rubella. IF was used to test sucrose density gradient fractions and RIA to test fractions and whole serum.

When fractions were tested IF and RIA were equally specific and distinguished clearly between IgM and IgG, but RIA was the more sensitive method. The RIA titre in whole serum was always greater than in the peak IgM fraction and there was no evidence that testing the serum, rather than the fraction, could result in failure to detect IgM. With some sera RIA gave low titres which became negative after absorption with IgG-coated latex beads. The mechanism of this 'false positive' effect, which may have been due to IgM with anti-IgG activity, was not investigated, but if it can be removed by absorption it need not reduce the specificity of the test.

During the first 6 months of life IgM antibody was detected by RIA in 30 out of 32 unfractionated sera and by IF in fractions from 28 of these. After the age of 6 months IgM was found progressively less frequently and the greater sensitivity of RIA became a more obvious advantage: 17 out of 60 specimens were positive by RIA and 11 of these were negative by IF.

RIA testing of whole serum appears to be an economical, specific and sensitive method for detecting IgM antibody in congenital rubella, of particular value when the titre of antibody is low.

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INTRODUCTION

Congenital rubella can be diagnosed by detecting specific IgM antibody in the serum of infected infants. Pattison *et al.* (1978) compared six methods for detecting this class of antibody in babies with congenital rubella: fractions obtained by centrifugation on sucrose density gradients or by gel filtration through Sephadex G-200 were tested by haemagglutination-inhibition (HI), using both long and short periods of incubation, and by indirect immunofluorescence (IF). Titres were higher in sucrose gradient fractions than in gel filtration fractions, and IF was the most sensitive and specific method of detection. By applying the IF technique to sucrose gradient fractions Cradock-Watson, Ridehalgh & Chantler (1976) detected specific IgM in 48 out of 50 sera taken from 19 infants with congenital rubella at various times during the first 6 months of life. Fluorescent staining of whole serum was less reliable, possibly because of competitive inhibition by IgG antibody.

Recently radioimmunoassay (RIA) – a method potentially more sensitive than HI or IF – has been used to detect rubella-specific immunoglobulins in adults. A solid-phase technique, with antigen adsorbed onto polystyrene balls, was used by Kalimo *et al.* (1976) to detect IgG antibody, and by Meurman, Viljanen & Granfors (1977) to detect IgM in patients with acute infection. Kangro, Pattison & Heath (1978) used an analogous technique, with antigen fixed to the wells of microtitre trays, to detect IgG and IgM antibodies in whole serum and in Sephadex fractions from adults with recent rubella. When fractions were tested their method distinguished clearly between IgG and IgM and gave IgM titres 10–150 times greater than those obtained by HI. There was no evidence that IgM detection was influenced by the simultaneous presence of IgG antibody, since addition of IgG did not depress the IgM titres of fractions and removal of IgG did not affect the IgM titre of whole serum. Kangro and his colleagues showed that RIA was a sensitive and usually specific method for detecting IgM antibody in unfractionated serum, although, like Meurman *et al.*, they found that sera containing rheumatoid factor occasionally gave false positive results.

We have compared IF and RIA for detecting IgM antibody in infants with confirmed and suspected congenital rubella, using IF to demonstrate antibody in sucrose gradient fractions and RIA to detect antibody in fractions and whole serum. Because of the possibility suggested by Reimer *et al.* (1975) that IgM from an infant congenitally infected with one pathogen might become attached to preparations of another, provided that maternal IgG specific for the latter were present, we have also examined sera from infants with congenital toxoplasmosis and cytomegalovirus (CMV) infection.

MATERIALS AND METHODS

Children with confirmed congenital rubella

The diagnosis of congenital rubella was regarded as confirmed if the patient had compatible clinical abnormalities combined with laboratory evidence in the form

of virus isolation or the presence of circulating antibody between the ages of 6 months and 4 years. Ninety-two serum specimens, taken from 70 children at ages from birth to 4 years 7 months, were tested for IgM antibody by RIA, without prior fractionation. Selected sera were fractionated on sucrose gradients and the peak IgM fraction (fraction 2 or 3) was tested by RIA and IF or, in a few instances, by IF only.

Other children with congenital abnormalities or neonatal disease

This group comprised 54 infants, aged up to 12 weeks. Some had neonatal disease or abnormalities suggestive of congenital rubella, including purpura (8), microcephaly (12), cataract (12) and congenital heart disease (9). Others had miscellaneous congenital abnormalities of various kinds and six had only prolonged neonatal jaundice. Two babies were stillborn and five died in early infancy. In its final form this group contained no confirmed cases of congenital rubella, although some children who had initially been placed in this group became recognized as confirmed cases and were removed from it during the course of this study. One serum from each child was fractionated on a sucrose gradient. The peak IgM fraction was tested by RIA and IF, and the whole serum by RIA only.

Normal newborn infants

Single specimens of cord serum were obtained from 28 healthy babies whose mothers had not had rubella, or contact with rubella, during pregnancy. Whole serum was tested for IgM antibody by RIA only.

Infants with congenital toxoplasmosis and CMV infection

Single specimens of serum were tested from ten infants aged between 1 day and 15 weeks with congenital CMV infection, diagnosed by isolating virus within 2 weeks of birth. Eight of these sera contained rubella-specific IgG antibody, presumably of maternal origin. CMV-specific IgM was detected in five sera by the IF method of De Silva *et al.* (1977).

A single specimen was tested from each of two infants, aged 1 day and 3 weeks, with congenital toxoplasmosis. Both contained rubella-specific IgG antibody. IgM specific for the parasite was detected in both sera by the IF method of Karim & Ludlam (1975).

Sucrose gradient fractions from these 12 sera were tested for rubella antibody by IF and RIA. Whole serum was tested by RIA only.

Sucrose density gradient centrifugation

Serum was diluted 1/2 and centrifuged on a 12·5–37·5 % (w/v) sucrose gradient as previously described (Cradock-Watson *et al.* 1976). Usually the starting volume of diluted serum was 0·6 ml, and 12 fractions, each of about 0·6 ml, were collected. When the volume of serum was insufficient for this procedure the gradient was made in a smaller tube which fitted into an adaptor; the serum and fraction volumes were then 0·2 ml. The separate immunoglobulin classes in the fractions were detected by double diffusion in agar, using antisera specific for human IgG

and IgM (Wellcome Reagents Limited). Rubella-specific immunoglobulins in the fractions were titrated by IF and RIA.

Immunoflorescent technique

Staining was carried out as previously described (Cradock-Watson, Bourne & Vandervelde, 1972). Briefly, cover-slip cultures of BHK 21 cells infected with rubella virus were treated with dilutions of serum fractions and were then stained with fluorescein-labelled globulins prepared against human IgG or IgM (Wellcome Reagents Limited). The cover-slips were finally mounted in glycerol and examined by dark-ground illumination from a quartz-halogen lamp.

Radioimmunoassay procedure

RIA was carried out as described by Kangro *et al.* (1978). Briefly, rubella haemagglutinating antigen (Wellcome Reagents Limited) or control antigen prepared from uninfected BHK 21 cells was fixed to the wells of polyvinyl chloride microtitre trays (Dynatech Laboratories Limited). Duplicate wells containing test and control antigen were treated first with serum or serum fractions and then with ^{125}I-labelled globulins prepared against human IgG or IgM. Individual wells were clipped from the plates and the bound radioactivity was measured in a gamma counter. The specific binding of labelled antibody was calculated as a binding ratio (BR). This is the mean counts/min. in wells exposed to antibody divided by the mean counts/min. in wells exposed to assay diluent. For each serum or fraction dilution a BR was calculated for wells coated with rubella antigen and for control wells. A specific binding index was then calculated as follows:

$$\text{binding index} = \frac{\text{BR of test antigen}}{\text{BR of control antigen}}.$$

The titre was determined as the highest dilution of serum or fraction with a specific binding index of 2. It is necessary to take account of binding to the control antigen since individual sera vary greatly in this respect; binding is usually minimal with sera from children under the age of 6 months but becomes progressively greater with increasing age. Sucrose gradient fractions were screened at a dilution of 1/40 and unfractionated sera at 1/100. Positive sera were titrated after absorption with IgG-coated latex as follows: 10 μl of serum in 1 ml of assay diluent were reacted for 1 h at 37 °C with 25 μl of a 5 % suspension of latex coated with human IgG. This latex suspension was that described by Chantler *et al.* (1976) and was kindly supplied by Dr Shireen Chantler, Wellcome Research Laboratories. The latex was sedimented by centrifugation (3000 rev./min for 30 min) and the supernatant assayed by RIA.

RESULTS

Antibodies in sucrose gradient fractions

The sedimentation pattern of immunoglobulins from a child aged 5 weeks with congenital rubella is shown in Table 1. IgM and IgG were detected by gel diffusion

Table 1. *Antibody titres determined by immunofluorescence (IF) and radioimmunoassay (RIA) in sucrose density gradient fractions from serum from an infant aged 5 weeks with congenital rubella*

	IgG			IgM		
Fraction no.	Gel diffusion	IF	RIA	Gel diffusion	IF	RIA
1	–	< 1	< 40	+	64	28000
2	–	< 1	< 40	++	256	56000
3	–	< 1	< 40	++	256	34000
4	–	2	90	trace	8	2000
5	+	64	630	–	< 1	160
6	++	1024	2500	–	< 1	< 40
7	++	1024	4300	–	< 1	< 40
8	++	256	1400	–	< 1	< 40
9	+	32	250	–	< 1	< 40
10	–	16	⩾ 40	–	< 1	< 40
11	–	8	⩾ 40	–	< 1	< 40
12	–	16	⩾ 40	–	< 1	< 40

in fractions 1–4 and 5–9, respectively. Rubella-specific IgM was detected by IF in fractions 1–4 and by RIA in fractions 1–5. RIA gave higher titres, with a gain in sensitivity that ranged from 133- to 437-fold. Specific IgG was detected in fractions 4–12 by both methods; RIA gave higher titres, but the gain was less than in the case of IgM. The radioactive IgG and IgM conjugates were evidently highly specific, since they gave negative results with the peak IgM and IgG fractions, respectively.

Titres of IgM antibody determined by IF and RIA in sucrose gradient fractions from children with congenital rubella

The titres of peak IgM fractions from 57 sera, taken from 36 children at ages from birth to 18 months, are shown in Fig. 1. IF revealed antibody in 37 fractions, in titres ranging from 2 to 256. Thirty-six of these were also positive by RIA, which gave titres ranging from 100 to 20000 and a gain in sensitivity of 12- to 700-fold. One fraction with an IF titre of 2 was negative by RIA (< 40). The 20 fractions which were negative by IF (< 2) were all from different children who had previously been positive: 12 were positive by RIA (titres 40–400) and 8 were negative.

Detection of IgM antibody by RIA in unfractionated sera from children with congenital rubella

When 92 unfractionated sera were tested by RIA, 47 gave positive results (titres 100–70000) and 45 were negative (< 100). We fractionated all the positive and 25 of the negative sera on sucrose gradients, and in Fig. 2 we have compared the RIA titres of these sera with the IF titres of their peak IgM fractions. In 34 out of 35 sera whose fractions were positive by IF (titres 2–256) RIA was also positive (titres 600–70000). One serum, whose fraction had a titre of 2 by IF, was

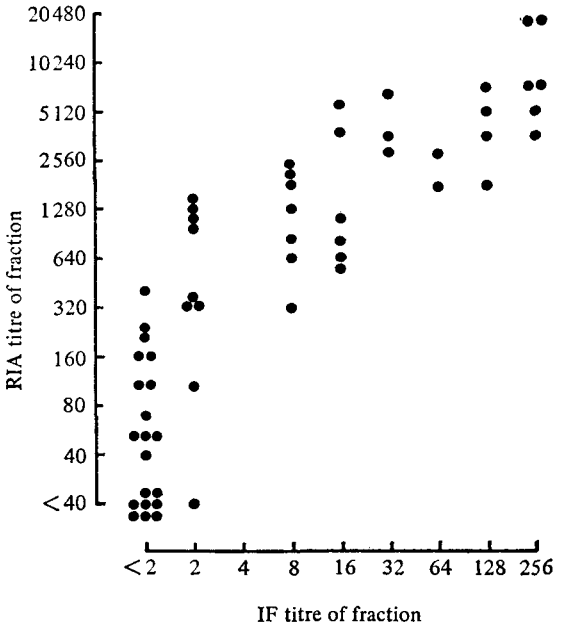

Fig. 1. Peak IgM antibody titres in sucrose gradient fractions from 57 sera from 36 children with congenital rubella. Abscissa = titre determined by immunofluorescence (IF). Ordinate = titre determined by radioimmunoassay (RIA).

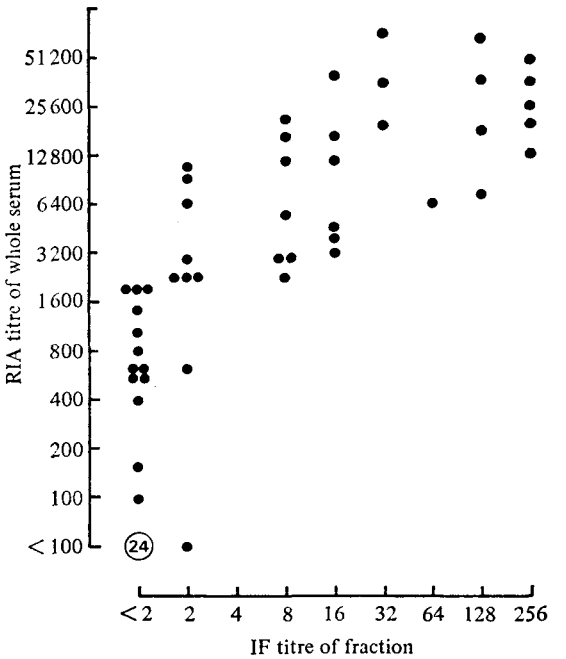

Fig. 2. Rubella IgM antibody titres in 72 sera from 50 children with congenital rubella. Abscissa = titre of peak IgM fraction determined by immunofluorescence (IF). Ordinate = titre of whole serum determined by radioimmunoassay (RIA).

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Table 2. *Presence of IgM antibody at various ages in sera from 70 children with congenital rubella, when tested by immunofluorescence or radioimmunoassay*

Age group	No. of patients tested	No. of positive sera/no. tested		Range of RIA titres	Median RIA titre
		IF	RIA		
Birth to 4 weeks	10	10/10	10/10	5200–70000	18200
5–13 weeks	12	13/14	14/14	100–60000	16000
14 weeks to 6 months	8	5/8	6/8	< 100–12800	2700
6½–11 months	16	4/16*	9/17	< 100–35000	160
1 year to 23 months	24	3/16	6/24	< 100–6400	< 100
2 years to 35 months	12	0/6	1/12	< 100–600	< 100
3 years to 47 months	6	0/3	1/6	< 100–560	< 100
4 years, 7 months	1	.	0/1	< 100	< 100

IF = immunofluorescent staining of the peak IgM fraction from a sucrose density gradient.
RIA = radioimmunoassay of whole serum.
* One serum was positive by IF but negative by RIA.

negative by RIA; this was the same specimen whose fraction was IF-positive but RIA-negative and it seems likely that the IF staining was non-specific. Thirteen RIA-positive sera (titres 100–1800) yielded fractions that were IF-negative: 10 of these came from children who had been tested at an earlier age (8 by both methods and 2 by IF only) and had been found to be positive. The remaining 24 IF-negative fractions were all from RIA-negative sera, four of which gave low titres (140–560) before absorption with latex-IgG.

The titre of IgM antibody, measured by RIA, was always greater in whole serum than in the peak fraction, by a factor that ranged from 1·28 to 12 (median = 6·7). Only one positive serum, with a low titre of 160, yielded fractions that were RIA-negative.

Detection of IgM antibody at different ages by IF and RIA

The presence of IgM antibody at different ages is shown in Table 2. Up to the age of 6 months RIA detected antibody in 30 out of 32 sera from 25 out of 27 patients, but failed to detect antibody in 2 infants, both aged 5 months. One of these infants had previously been IF-positive at the age of 7 days, but this serum was no longer available. IF was almost as successful as RIA, detecting antibody in 28 sera, but failed with two additional specimens from patients aged 11 weeks and 6 months who had previously been positive by both methods. After the age of 3 months titres were generally lower, and after the age of 6 months IgM was found progressively less frequently. Between the ages of 2 and 4 years it was detected by RIA in only 2 out of 18 patients. One child who was RIA-negative at the age of 4 years 7 months had previously been positive at the age of 13 months.

Detection of IgM antibody by RIA in other infants with congenital abnormalities or neonatal disease

Rubella IgM antibody was detected by both IF and RIA in 5 out of 54 infants. One of these five was stillborn and three died in early infancy. Of the three who

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died, one had meningitis and two had serious congenital abnormalities. No further specimen was available from the fifth infant, who had cataracts and congenital heart disease. All five were probably cases of congenital rubella, but the diagnosis could be neither confirmed nor excluded.

In three infants, all of whom had serious abnormalities, RIA gave positive results with whole serum (titres 400–1500) and peak IgM fractions, although the latter were negative by IF. We were unable to follow up two of these babies, one of whom died in early infancy. The third had a negative HI test and no detectable IgG antibody when retested at the age of 2 years. The positive RIA result at the age of 6 weeks is therefore difficult to interpret: either it was non-specific, or the child was genuinely infected but was unusual in showing loss of IgG antibody at the age of 2 years.

In four children RIA gave low titres (100–500) which became negative after absorption with latex-IgG. In the remaining 42 children RIA was clearly negative.

Infants with congenital toxoplasmosis and CMV infection

Whole serum from one infant with congenital CMV infection gave a titre of 100 when tested for rubella-specific IgM by RIA, becoming negative after absorption with latex-IgG. This serum contained rubella-specific IgG, but no CMV-specific IgM. The remaining nine CMV sera, and all the peak IgM fractions, were clearly negative. Negative results were also obtained with whole serum and fractions from two infants with congenital toxoplasmosis.

Normal newborn infants

Rubella IgM antibody was not detected by RIA in any of the 28 cord sera from normal newborn infants.

DISCUSSION

By testing sucrose gradient fractions from infants with congenital rubella we confirmed that RIA is as specific as IF for detecting IgM antibody, and many times more sensitive. Fractionation, however, is a laborious preparative procedure which requires an ultracentrifuge and causes some dilution of antibody; testing whole serum is simpler, quicker, and requires less material. Unfortunately there are two main limitations to testing whole serum by any indirect labelling technique, whether this be IF, RIA or enzyme-linked assay. The first difficulty is that IgG antibody may interfere with the detection of IgM by competing for the same antigenic sites. This type of inhibition is particularly troublesome when whole serum is tested for rubella IgM antibody by IF, and we therefore employed the more reliable procedure of staining the fractions (Cradock-Watson *et al.* 1976). In order to discover whether RIA is similarly affected we applied this method both to fractions and to whole serum. The RIA titre was always greater in whole serum than in the peak IgM fraction, but by a factor which varied widely from 1·28 to 12. This variation probably arises from differences in the distribution of immunoglobulins in the fractions after ultracentrifugation. It may also, perhaps, reflect different degrees of competition from IgG, but in no case was a positive fraction

obtained from a 'negative' serum and therefore, unlike the position with IF, there was no evidence that testing whole serum might result in failure to detect specific IgM.

The second limitation to testing whole serum arises from the occasional presence of IgM with anti-IgG activity, such as rheumatoid factor. This IgM, even if not virus-specific, can still cause 'secondary' staining with a fluorescein-labelled anti-IgM conjugate in the presence of specific IgG (Fraser, Shirodaria & Stanford, 1971). Antiglobulin activity of this type is not uncommon in adults and may give false positive results in RIA tests with whole serum (Meurman *et al.* 1977; Kangro *et al.* 1978). It may also occur in infants: Urquhart, Logan & Izatt (1971) detected it by agglutination of sensitized group O Rh positive red cells in cases of sudden unexplained death in infancy, and Reimer *et al.* (1975) demonstrated it by means of the fluorescent treponemal antibody test in two babies with suspected congenital syphilis. Reimer and his colleagues suggested that IgM of this type might be formed as an immune response to complexes of maternal IgG with the infecting agent. Using a modified latex agglutination test they found antiglobulin activity in numerous infants with congenital syphilis, toxoplasmosis, CMV and rubella virus infections, and they warned that serum from an infant infected with one of these pathogens might give IgM staining with any of the others, provided that maternal IgG specific for the latter were present.

False positive results due to antiglobulin activity can be recognized because they are abolished by prior absorption with aggregated IgG (Fraser *et al.* 1971) or IgG-coated latex beads (Chantler *et al.* 1976). We detected low-titre activity that was removed by latex-IgG in nine infants, of whom four had confirmed congenital rubella, four had abnormalities or neonatal disease probably due to other causes, and one had congenital CMV infection. We did not investigate the mechanism of this activity, but evidently it need not give misleading results provided that sera which are positive on screening are retested after absorption.

In 13 children, aged 11 weeks or more, with congenital rubella RIA gave serum titres of 100–1800 after absorption, although the fractions were negative by IF. Since ten of these specimens were from children who had been shown to have IgM antibody when they were younger it is probable that the results were specific and that RIA was revealing low titres of IgM that IF could not detect. The results shown in Fig. 2 suggest that IF will detect antibody in the peak IgM fraction when the RIA titre of whole serum is greater than about 1800 but is unlikely to succeed when the titre is less than this.

The IF and RIA techniques described here for detecting IgM antibody are probably of similar specificity, but RIA is clearly the more sensitive method, particularly when applied to whole serum. During the first 6 months of life titres were usually sufficiently high for IgM to be detected by both procedures, and only two sera were RIA-positive but IF-negative. After the age of 3 months, however, titres were generally lower, and after the age of 6 months they had fallen sufficiently for the greater sensitivity of RIA to become apparent: 11 of the specimens taken after this age were RIA-positive but IF-negative, and 1 was RIA-negative but IF-positive. After the age of 1 year the ability to detect low titres of IgM in a

minority of patients is only of occasional value, since congenital infection can usually be inferred from the presence of HI antibody. But between 6 months and 1 year the presence of a low HI titre is difficult to interpret since some normal infants still have maternal antibody at the age of nine months (Cloonan, Hawkes & Stevens, 1970). In this age group RIA has a clear advantage as a method for detecting IgM since in our hands it was successful with 9 out of 17 sera (53 %) whereas IF gave positive results with only 4 out of 16 (25 %) and one of these was probably non-specific.

RIA testing of whole serum thus appears to be a specific and sensitive method for detecting IgM antibody in infants with congenital rubella, provided that positive sera are retested after absorption with latex-IgG. It is simpler and quicker than fractionation methods, requires much less serum and is particularly useful when the titre of antibody is low.

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REFERENCES

CHANTLER, S., DEVRIES, E., ALLEN, P. R. & HURN, B. A. L. (1976). A rapid immunofluorescent procedure for the detection of specific IgG and IgM antibody in sera using *Staphylococcus aureus* and latex-IgG as absorbents. *Journal of Immunological Methods* **13**, 367.

CLOONAN, M. J., HAWKES, R. A. & STEVENS, L. H. (1970). Postnatal decline of maternally acquired rubella antibodies. *Journal of Hygiene* **68**, 461.

CRADOCK-WATSON, J. E., BOURNE, M. S. & VANDERVELDE, E. M. (1972). IgG, IgA and IgM responses in acute rubella determined by the immunofluorescent technique. *Journal of Hygiene* **70**, 473.

CRADOCK-WATSON, J. E., RIDEHALGH, M. K. S. & CHANTLER, S. (1976). Specific immunoglobulins in infants with the congenital rubella syndrome. *Journal of Hygiene* **76**, 109.

DE SILVA, L. M., KAMPFNER, G. L., LISTER, C. M. & TOBIN, J. O'H. (1977). Identification of pregnancies at risk from cytomegalovirus infection. *Journal of Hygiene* **79**, 347.

FRASER, K. B., SHIRODARIA, P. V. & STANFORD, C. F. (1971). Fluorescent staining of human IgM. *British Medical Journal* iii, 707.

KALIMO, K. O. K., MEURMAN, O. H., HALONEN, P. E., ZIOLA, B. R., VILJANEN, M. K., GRANFORS, K. & TOIVANEN, P. (1976). Solid-phase radioimmunoassay of rubella virus immunoglobulin G and immunoglobulin M antibodies. *Journal of Clinical Microbiology* **4**, 117.

KANGRO, H. O., PATTISON, J. R. & HEATH, R. B. (1978). The detection of rubella-specific IgM antibodies by radioimmunoassay. *British Journal of Experimental Pathology* **59**, 577.

KARIM, K. A. & LUDLAM, G. B. (1975). The relationship and significance of antibody titres as determined by various serological methods in glandular and ocular toxoplasmosis. *Journal of Clinical Pathology* **28**, 42.

MEURMAN, O. H., VILJANEN, M. K. & GRANFORS, K. (1977). Solid-phase radioimmunoassay of rubella virus immunoglobulin M antibodies: comparison with sucrose density gradient centrifugation test. *Journal of Clinical Microbiology* **5**, 257.

PATTISON, J. R., JACKSON, C. M., HISCOCK, J. A., CRADOCK-WATSON, J. E. & RIDEHALGH, M. K. S. (1978). Comparison of methods for detecting specific IgM antibody in infants with congenital rubella. *Journal of Medical Microbiology* **11**, 411.

REIMER, C. B., BLACK, C. M., PHILLIPS, D. J., LOGAN, L. C., HUNTER, E. F., PENDER, B. J. & MCGREW, B. E. (1975). The specificity of fetal IgM: antibody or anti-antibody? *Annals of the New York Academy of Sciences* **254**, 77.

URQUHART, G. E. D., LOGAN, R. W. & IZATT, M. M. (1971). Sudden unexplained death in infancy and hyperimmunization. *Journal of Clinical Pathology* **24**, 736.