J. Hyg., Camb. (1976), **76**, 109
Printed in Great Britain

Specific immunoglobulins in infants with the congenital rubella syndrome

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(*Received* 5 *June* 1975)

SUMMARY

The indirect immunofluorescent technique has been used to detect and titrate the specific immunoglobulins in serum specimens from 154 infants with confirmed or suspected congenital rubella. IgM antibody was stained more efficiently in sucrose density gradient fractions than in whole serum and was detected in this way in 27 out of 40 patients with confirmed congenital rubella at ages ranging from birth to 2 years. It was present in 48 out of 50 serum specimens during the first 6 months of life and in 11 out of 38 specimens obtained at ages between 6½ months and 2 years. IgM antibody was therefore estimated to persist for about 6 months in the majority of cases and up to 2 years in a few individuals. IgM antibody was also detected by this method in 11 out of 114 infants with suspected but unconfirmed congenital rubella at ages up to 5 months. The total concentrations of IgM were above the normal range in nearly all sera taken from confirmed cases during the first 3 months of life and in half the specimens obtained between the ages of 3 and 6 months.

IgG antibody was detected by fluorescent staining of whole serum in all patients with congenital rubella. Geometric mean titres increased during the first 3 months of life and then declined slowly. IgA antibody was not detected, except in two patients in whom traces were present at the age of 6 months, and the total concentrations of IgA were usually within normal limits.

Fluorescent staining of fractions showed that the sedimentation characteristics of rubella IgG and IgM antibodies were the same in infants as in adults. The peak IgM fractions never contained IgG antibody, and the presence of specific IgM in these fractions could usually have been safely inferred from their HAI titres. Fluorescent staining, however, was more sensitive and frequently detected IgM antibody in fractions which had no definite HAI activity.

Fluorescent staining of whole serum for IgM antibody was less distinct, and often unsuccessful, even in specimens in which specific IgM was detected in the fractions. The addition of IgG- to IgM-containing fractions caused depression of IgM staining and suggested that failure to detect IgM antibody in whole serum was partly due to competitive inhibition by specific IgG.

INTRODUCTION

The total concentration of IgM in the serum is normally low at birth, increases to half the adult level by the age of 4–5 months and reaches the full adult value at about the age of 1 year (Allansmith, McClellan, Butterworth & Maloney, 1968, 1969; Buckley, Dees & O'Fallon, 1968; Hardy *et al.* 1969; Sever *et al.* 1969). In infants with congenital rubella the IgM concentration is usually increased at birth and may remain above normal for at least the first 6 months of life (Stiehm, Amman & Cherry, 1966; Soothill, Hayes & Dudgeon, 1966; Hayes, Dudgeon & Soothill, 1967; Dudgeon, Marshall & Soothill, 1969). IgM does not normally cross the placenta and the presence of increased concentrations suggests that IgM antibody specific for rubella virus may be produced by the fetus and neonate in response to intra-uterine infection persisting into the first few months of life.

Several workers, using various techniques, have found evidence of this class of antibody in infants with congenital rubella. Alford (1965) and Bellanti *et al.* (1965) examined serum fractions obtained after centrifugation on sucrose density gradients and by gel filtration through Sephadex G-200; they detected neutralizing activity in the IgM-containing fractions at ages up to 10 months. Vesikari, Vaheri, Pettay & Kunnas (1969) examined density gradient fractions by the haemagglutination inhibition (HAI) test and found evidence of IgM antibody at ages up to $6\frac{1}{2}$ months. Indirect immunofluorescence was used by Baublis & Brown (1968) and by Cohen, Ducharme, Carpenter & Deibel (1968), who observed IgM staining in unfractionated serum at ages up to 4 months and 17 months respectively.

The concentration of IgA increases more slowly than that of IgM or IgG and reaches only a quarter of the adult value by the age of 1 year (Allansmith *et al.* 1968, 1969; Buckley *et al.* 1968). There is little evidence that IgA production is increased in infants with congenital rubella. McCracken *et al.* (1969) found that mean IgA concentrations were significantly greater than normal between the ages of 2 and 6 months, but about three-quarters of the values were within the normal range. Dudgeon and his colleagues found no consistent increase in IgA concentration in infants with congenital rubella during the first year of life. The possibility that such infants may produce specific IgA antibody has received little study, probably because of the difficulty of separating IgA from IgG. However, Baublis & Brown (1968), using immunofluorescence, reported the presence of IgA antibody in nine infants with the rubella syndrome at ages ranging from 2 days to 7 months.

We have used the indirect immunofluorescent technique to study the specific immunoglobulins, particularly IgM, in infants suffering from congenital rubella. First, we wished to discover whether a conjugate prepared against adult IgM would stain fetal and neonatal IgM, since it has been reported that some conjugates have failed to detect IgM antibody in infants suffering from congenital toxoplasmosis (Remington, 1969). Secondly, we wished to study the sedimentation characteristics of the immunoglobulins in cord and neonatal sera, since it has been reported that IgM-containing fractions from cord sera may be contaminated with

IgG (Newman, Horta-Barbosa & Sever, 1969). Finally, we wished to observe the incidence and duration of IgM antibody formation in infants with congenital rubella in order to assess the value of detecting this class of antibody as an aid to diagnosis.

In previous work in which we used immunofluorescence to detect rubella IgM antibody in adults we were sometimes unable to stain IgM in whole serum, particularly when the titre was low, but detected it easily when we stained fractions obtained from the same serum after centrifugation on a sucrose density gradient (Cradock-Watson, Bourne & Vandervelde, 1972; Cradock-Watson *et al.* 1974). We attributed the difficulty of detecting IgM in whole serum to the presence of IgG antibody which it was thought might compete with IgM for the same antigenic sites during staining. We expected that any such competition would seriously depress IgM staining in sera from cases of congenital rubella if high titres of IgG antibody were present with relatively low titres of IgM. We therefore centrifuged all sera on density gradients and stained the fractions as well as the whole serum.

MATERIALS AND METHODS

Infants 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 HAI antibody after the age of 6 months. Congenital infection causes the infant to produce HAI antibody which persists after maternally acquired antibody has disappeared. Acute infection is uncommon before the age of 4 years; the presence of antibody between the ages of 6 months and 4 years may therefore be evidence of congenital rubella. Eighty-eight specimens of serum were examined from 40 confirmed cases, at ages ranging from birth to 3 years. In 24 patients one or more abnormalities were apparent at birth or during the first few weeks of life. The other 16 patients were apparently normal at birth but were found after 8 months or more to be suffering from defects such as deafness, speech difficulty and mental or physical retardation.

Infants with suspected congenital rubella

One hundred and twenty specimens of serum were examined from 114 infants with suspected but unconfirmed congenital rubella, at ages ranging from birth to 20 months. These patients covered a wide clinical range. Forty-seven had clinical abnormalities which strongly suggested an intra-uterine infection; in the others the clinical findings were progressively less suggestive. Seven patients had no significant abnormalities but were found to possess rubella HAI antibody at ages ranging from 6 to 20 months.

Immunofluorescent technique

Rubella-specific immunoglobulins in serum or serum fractions were titrated by the indirect immunofluorescent technique, using the methods described in our previous work. Serum titrations were started at 1/16 because of the difficulty of

distinguishing between specific and non-specific fluorescence at lower dilutions. Cover-slip cultures of BHK21 cells infected with rubella virus were treated with dilutions of serum or density gradient fractions and were then stained with fluorescein-labelled globulins prepared against adult human IgG, IgA or IgM (Wellcome Reagents Limited). The anti-IgM conjugate used throughout this work was prepared against IgM from normal adults. Fractions from some sera were additionally tested with a separate conjugate prepared against IgM from patients with macroglobulinaemia. The stained cover-slips were examined in a Reichert microscope, using quartz-halogen dark-ground illumination and an interference exciter filter.

Sucrose density gradient centrifugation

Sera were initially diluted 1/2 and absorbed with chick red cells for 1 hr. at 4° C. A volume of 0·5 ml. of this dilution was layered on top of a gradient extending from 12·5 to 37·5% (w/v) which was then centrifuged at 40,000 rev./min. for 17 hr. Twelve fractions were collected after piercing the bottom of the tube. The separate immunoglobulin classes in the fractions were detected by double diffusion in agar, using antisera specific for human IgG, IgA and IgM (Wellcome Reagents Limited). Rubella-specific immunoglobulins in the fractions were titrated by the immunofluorescent technique and HAI activity was titrated in microtitre trays.

Haemagglutination-inhibition titrations

Sera which were sent directly to the Manchester Public Health Laboratory were absorbed with kaolin and titrated in WHO trays by the routine method (Thompson & Tobin, 1970). The majority of sera were sent from other Public Health Laboratories where they had already been titrated and the volume available was often too small for the test to be repeated. Because HAI titres can vary widely in different laboratories we have standardized the results by converting them into 'units/ml.'. Public Health Laboratories which carry out rubella HAI titrations employ a local standard serum which has been calibrated against a reference serum supplied by the Standards Laboratory of the Central Public Health Laboratory. The HAI titre of an unknown serum can be expressed in 'units/ml.' by comparing it with the local standard and hence with the reference serum. The latter has a nominal titre of 100 units/ml. and gave actual titres ranging from 64 to 256 in the various laboratories from which specimens were obtained.

Total immunoglobulin concentrations

The total concentrations of IgM and IgA in serum were measured by single radial diffusion in commercial immuno-plates (Hyland Laboratories). The results have been expressed in International Units per ml. (i.u./ml.) as recommended by Rowe, Grab & Anderson (1972). To obtain the results in mg./100 ml. the IgM values should be multiplied by 0·847 and the IgA values by 1·42. As well as examining sera from infants with confirmed and suspected congenital rubella we also tested serum specimens which had been taken from normal children for serological tests before adoption.

Table 1. *Rubella antibodies in sucrose density gradient fractions from a serum from an infant aged 1 day with confirmed congenital rubella*

Fraction no.	HAI titre	Immunoglobulin detected by gel diffusion			Immunofluorescent titre of rubella-specific immunoglobulin		
		IgG	IgA	IgM	IgG	IgA	IgM
1	<1	–	–	–	<1	<1	8
2	2	–	–	tr	<1	<1	32
3	4	–	–	+	<1	<1	256
4	2	–	–	tr	<1	<1	8
5	1	–	–	–	<1	<1	<1
6	8	+	–	–	64	<1	<1
7	16	+	–	–	128	<1	<1
8	8	+	–	–	128	<1	<1
9	4	+	–	–	8	<1	<1
10	2	tr	–	–	2	<1	<1
11	2	–	–	–	1	<1	<1
12	4	–	–	–	1	<1	<1
Whole serum	80*	.	<4†	120†	1024	<16	64

* Standardized titre = 50 units/ml. † International units per ml.

RESULTS

IgM antibody in infants with confirmed congenital rubella

When whole serum was examined by immunofluorescence for IgM antibody, specific staining was frequently weak, undetectable, or difficult to distinguish from non-specific fluorescence. With some sera there appeared to be a prozone and staining was more distinct at dilutions from 1/32 to 1/128 than at 1/16. The end-point was often indefinite. When density gradient fractions were stained the detection of IgM antibody was much improved and good cytoplasmic fluorescence was obtained with a conjugate prepared against normal adult IgM. Not only was fluorescence brighter and clearer but in many specimens IgM was detected only when fractions were stained and not when whole serum was tested.

The sedimentation pattern of the immunoglobulins in cord and neonatal sera was similar to that obtained with adult serum. Specific IgM antibody was found in fractions 1–4 (rarely in fraction 5), with a peak in fraction 3. IgG antibody was present in fractions 5–12 (rarely in fraction 4), with a peak in fraction 7 or 8. The results from a representative case are given in Table 1. The anti-IgG and anti-IgM conjugates were evidently highly specific, since they never caused staining in the peak IgM and IgG fractions, respectively. Overlap between IgG and IgM antibodies was slight and never occurred in more than one fraction.

Eighty-eight sera were examined from 40 confirmed cases of congenital rubella at ages ranging from birth to 3 years. IgM antibody was detected by immunofluorescence in 59 sera, obtained from 27 cases at ages from birth to two years. In 28 of these specimens IgM antibody titres ranged from 16 to 256 in unfractionated serum and from 8 to 512 in fraction 3, but no consistent relationship was observed between the titre in this fraction and the titre in whole serum. In 31 specimens IgM

https://doi.org/10.1017/S0022172400055005 Published online by Cambridge University Press

Table 2. *Presence of IgM antibody and peak fluorescent titres in density gradient fractions from sera obtained at various ages from 40 infants with confirmed congenital rubella*

Age group	No. of patients tested	No. of sera with antibody/no. tested	Range of peak titres	Median titre
Birth to 7 days	13	13/13	8–256	32
8 days to 4 weeks	10	11/11	4–256	128
5–13 weeks	11	14/14	8–512	64
14 weeks to 6 months	10	10/12	<1–64	11
6½–11 months	20	8/21	<1–64	<1
1–3 years	16	3/17	<1–16	<1

antibody was not detected in whole serum in dilutions of 1/16 or greater, but was found only in the fractions in titres ranging from 2 to 256. No significant differences in titre were noted when fractions from nine sera were additionally tested with a conjugate prepared against IgM from patients with macroglobulinaemia.

No IgM antibody was detected in fractions from the remaining 29 sera, which were obtained at ages ranging from 6 months to 3 years. Sixteen of these specimens were taken at ages between 6 months and three years from patients in whom IgM had previously been detected on one or more occasions; 13 were single specimens taken from 13 patients at ages ranging from 8 to 31 months.

The presence of IgM antibody in fractions from sera obtained at various ages is shown in Table 2. Up to the age of 6 months IgM was detected in varying amounts in 48 out of 50 serum specimens taken from 19 different patients. After the age of 3 months titres were lower, and beyond the age of 6 months IgM antibody was detected progressively less frequently. After the first year of life it was present in only 3 out of 17 sera, obtained from two patients who still possessed antibody at the ages of 16 months and two years. The presence of specific IgM in nearly all specimens taken during the first 6 months of life suggests that the 13 patients in whom it was never detected had previously possessed IgM antibody which had disappeared before they were tested.

In order to assess the relative sensitivities of the HAI test and the immunofluorescent technique for detecting IgM antibody in gradient fractions we compared the fluorescent and unstandardized HAI titres in fractions 2 and/or 3 from 58 sera which contained IgM. In one fraction the immunofluorescent titre was half the HAI titre, in six fractions the titres were the same by both methods and in 89 fractions the fluorescent titres were 2- to 64-fold greater than the HAI titres. In 13 fractions (from seven sera) the fluorescent method gave titres ranging from 2 to 32 in the absence of any definite HAI activity and in three confirmed cases IgM antibody would not have been detected at all if reliance had been placed solely on the HAI activity of the fractions. Immunofluorescence was therefore usually more sensitive than the HAI test for measuring IgM antibody and in 96 fractions in which both titres could be measured the median gain in sensitivity was eightfold.

The total concentrations of IgM in sera obtained during the first year of life from normal infants and from patients with congenital rubella are given in Table 3. The values for normal children covered approximately the same range as those

Table 3. *Total IgM concentrations (i.u./ml.) at various ages in sera from normal infants and from infants with confirmed congenital rubella*

	Normal infants (149)			Congenital rubella (28 patients)			
		IgM concentration				IgM concentration	
Age group	No. of sera	Range	Median	No. of sera	No. of patients	Range	Median
Birth to 4 weeks	31	5–50	13	22	20	34–432	219
5–13 weeks	79	11–120	50	13	12	132–288	186
14 weeks to 6 months	20	31–155	79	9	8	86–432	156
6½–11 months	19	40–270	120	19	18	60–345	156

obtained by Allansmith and his colleagues. In patients with congenital rubella the IgM concentrations during the first thirteen weeks of life were considerably increased, and all the values except one (34 i.u./ml.) were above the normal range. There was no correlation, however, between the total IgM concentration and the peak titre of IgM antibody in the fractions. Between the ages of 14 weeks and 6 months half the values were above the normal range and the median was double the value derived from normal infants. Between the ages of 6½ and 11 months the majority of values were within the normal range and the difference between the median values was small.

IgM antibody in infants with suspected congenital rubella

IgM antibody was detected by fluorescent staining of fractions in 15 sera obtained from 11 patients at ages which ranged from birth up to 5 months. In 8 of the 15 sera IgM was detected only in the fractions, in titres ranging from 4 to 64, and was not detected in whole serum. In six sera IgM was detected by immunofluorescence in the fractions, in titres ranging from 4 to 64, in the absence of any definite HAI activity. In four patients IgM would not have been detected at all if reliance had been placed solely on the HAI activity of the fractions. Ten of the patients with IgM antibody had clinical evidence strongly suggestive of congenital rubella, and three of them died. Five of the mothers of these ten patients had suffered from confirmed rubella in early pregnancy, two reported uninvestigated rashes during the first trimester and one had been in close contact with rubella. It is likely that most, probably all, of these 11 infants were suffering from congenital infection but the diagnosis could be neither confirmed nor excluded because no virus isolations were made and no further sera which might have demonstrated persistence of antibody were available.

No IgM antibody was detected in sera obtained from the other 103 suspected cases at ages ranging from birth to 20 months. Ninety-six infants in this group were in the first 5 months of life and all possessed HAI and IgG antibody to rubella, but no further specimens were available and the disappearance or persistence of antibody could not be observed.

No IgM antibody was detected in the seven patients without significant symptoms who possessed HAI and IgG antibody at ages ranging from 6 to 20 months.

https://doi.org/10.1017/S0022172400055005 Published online by Cambridge University Press

Table 4. *Total IgA concentrations (i.u./ml.) at various ages in sera from normal infants and from infants with confirmed congenital rubella*

	Normal infants (150)			Congenital rubella (26 patients)			
		IgA concentration				IgA concentration	
Age group	No. of sera	Range	Median	No. of sera	No. of patients	Range	Median
Birth to 4 weeks	31	<4–18	<4	22*	20	<4–30	<4
5–13 weeks	79	<4–29	6	12	11	<4–44	11
14 weeks to 6 months	20	<4–62	13·5	8	8	6–88	21
6½–11 months	20	5·5–81	19	17	17	5·5–62	17

* In 20 sera the total concentration was <4 i.u./ml. Values of 30 and 13 i.u./ml. were present in two patients, in cord serum and at the age of 3 weeks respectively.

Two of them had low HAI titres of 12·5 and 25 units/ml. at the ages of 6 and 7 months, respectively, and had only traces of IgG antibody which may possibly have been maternal in origin. The other five, who were aged from 9 to 20 months, had HAI titres ranging from 50 to 400 units/ml., but whether this antibody resulted from postnatal or prenatal infection is uncertain.

In three sera HAI titres ranging from 1 to 4 were obtained in heavy fractions which gave negative results by immunofluorescence. The total concentrations of IgM were normal in these sera and it is probable that the HAI activity was non-specific. No further specimens were available from these three infants, who had only minor symptoms.

The total concentrations of IgM were measured in sera obtained during the first month of life from 66 infants with suspected congenital rubella. Seven infants who possessed specific IgM antibody had greatly increased IgM concentrations which were in the same range as those occurring in confirmed cases of similar age. Most of the other 59 infants, in whom specific IgM was not detected, had much lower total IgM concentrations, and about half of them had values within the normal range.

IgA antibody in infants with confirmed congenital rubella

Traces of specific IgA were detected in serum fractions obtained from two patients at the age of 6 months, but IgA antibody was not detected in these two patients earlier in life nor was it found in any other case of congenital rubella at any age.

The total concentrations of IgA in sera from normal infants and from patients with congenital rubella are given in Table 4. The normal values covered a wide range which was approximately the same as that obtained by Allansmith and his colleagues. In patients with congenital rubella the IgA concentration during the first month of life was sufficiently high to be measured in only two cases, who had values of 30 and 13 i.u./ml. in cord serum and at the age of 3 weeks respectively. The significance of an increased IgA concentration in cord serum is uncertain because it may be due to leakage from the maternal to the fetal circulation.

Table 5. *IgG and standardized HAI antibody titres (units/ml.) in serum specimens obtained at various ages from 40 confirmed cases of congenital rubella*

	IgG			HAI		
Age group	No. of sera	Range of titres	GMT	No. of sera	Range of titres	GMT
Birth to 4 weeks	23	128–4096	1172	23	25–800	292
5–13 weeks	14	512–8200	4096	14	50–800	362
4–6 months	11	512–4096	2719	11	50–800	151
6½–11 months	21	256–8200	2224	21	25–800	224
1 year to 21 months	9	256–8200	1024	10	< 12·5–800	123
2–3 years	9	64–2048	532	9	12·5–800	76

GMT = geometric mean titre.

Between the ages of 5 and 13 weeks most of the values were in the upper half of the normal range but only one patient had an IgA concentration (44 i.u./ml.) above normal limits. Between the ages of 14 weeks and 6 months two patients had raised IgA concentrations of 64 and 88 i.u./ml., but in the remaining six patients the values were normal. Out of 24 patients whose total IgA concentrations were measured at various ages during the first year of life only four had values which appeared to be above normal limits and in none of these specimens was rubella-specific IgA antibody detected.

IgG and HAI antibody in infants with confirmed congenital rubella

IgG antibody was detected in all cases in every specimen of serum. IgG and HAI titres in whole serum were measured in 88 specimens obtained from 40 confirmed cases at ages ranging from birth to 3 years, and are given for different age groups in Table 5. In each age group the IgG and HAI titres covered a wide range, but the geometric mean titres showed a downward trend after the age of 13 weeks. The difference between the first and last age groups is significant ($P < 0{\cdot}01$) for both IgG and HAI antibody. The mean IgG antibody titre showed a significant rise in the 5 to 13-week age group before declining steadily.

In the majority of specimens the IgG titre, determined by immunofluorescence, was greater than the HAI titre in units/ml. Up to the age of 6 months, when HAI activity was attributable to both IgG and IgM antibodies, the IgG titre was lower than the HAI in a minority of specimens. After the age of 1 year, when IgM antibody was absent, or present only in small amounts, the IgG titre always exceeded the HAI by a factor which varied but had a median value of tenfold. The greater sensitivity of immunofluorescence was particularly noticeable when antibody titres were low. HAI titres of 25 units/ml. or less were present in 12 sera (from 12 patients), 10 of which were obtained at ages greater than one year. In one serum HAI activity was undetectable (< 12·5 units/ml.). IgG antibody was detected in all these specimens in titres which ranged from 64 to 1024.

Immunofluorescence was also found to be more sensitive when IgG and HAI titres were compared in fractions 6–8 which contained IgG antibody but no IgM,

https://doi.org/10.1017/S0022172400055005 Published online by Cambridge University Press

Table 6. *Fluorescence obtained when serial dilutions of an IgM-containing fraction were mixed with equal volumes of other fractions (undiluted) from the same serum and were then stained for IgM antibody*

Fraction mixed with serial dilutions of fraction 3	IgG titre of fraction	Final dilution of peak IgM fraction (fraction 3) 4	8	16	32	64	128	256	512	IgM titre
5	1	+	+	+	+	+	+	+	−	256
6	32	+	+	+	+	−	−	−	−	32
7	64	±	±	−	−	−	−	−	−	<4
8	128	+	±	−	−	−	−	−	−	4
9	16	+	+	+	±	−	−	−	−	16
10	4	+	+	+	+	+	−	−	−	64
11	2	+	+	+	+	+	−	−	−	64
12	2	+	+	+	+	±	−	−	−	32
Saline	.	+	+	+	+	+	+	±	−	128

IgA or non-specific inhibitors. In 46 such fractions (from 16 sera) immunofluorescence showed a median gain in sensitivity of eightfold over the actual (unstandardized) HAI titres.

Depression of IgM staining by fractions containing IgG antibody

The unreliability of trying to detect IgM antibody by immunofluorescent staining of whole serum suggested that other constituents of serum might interfere with staining. We therefore fractionated a serum in which staining had been poor and observed the effect on IgM staining of mixing the IgM fraction with other fractions from the same specimen. Serial dilutions were made from the peak IgM fraction (fraction 3). Undiluted samples of each of the other fractions (from 5 to 12) were then mixed with equal volumes of these dilutions. The mixtures were used to treat cover-slips, which were finally stained with anti-IgM conjugate. The IgG titres of fractions 5–12 were also determined. The results are given in Table 6, in which the rows can be regarded as titrations of IgM antibody in the presence of constant amounts of other fractions from the same serum. IgM staining was greatly depressed in those mixtures which contained high titres of IgG antibody. Qualitatively similar results were obtained with sera from four other patients.

In order to study the mutual interaction of IgG and IgM antibodies we made a chess board titration of the two fractions containing the highest titres of IgM and IgG antibody (fractions 3 and 8 respectively). Serial dilutions of fractions 3 and 8 were mixed in equal volumes; the mixtures were used to treat duplicate sets of cover-slips, which were then stained with anti-IgM and anti-IgG conjugates. The results of IgM staining are given in Table 7, in which the rows can be regarded as titrations of IgM antibody in the presence of different concentrations of IgG. As the concentration of IgG increased, IgM staining was depressed and the end-point became less definite. The results of IgG staining of individual cover-slips are not shown, but the IgG titre is given at the bottom of each column. There was no significant variation in this titre and therefore no evidence that the presence of IgM antibody depressed IgG staining.

Table 7. *Fluorescence obtained when serial dilutions of an IgM- and an IgG-containing fraction were mixed and stained for IgM antibody*

		Final dilution of IgM fraction (fraction 3)							
		4	8	16	32	64	128	256	IgM titre
Final dilution of IgG fraction (fraction 8)	2	±	−	−	−	−	−	−	<4
	4	±	+	−	−	−	−	−	8
	8	+	+	+	−	−	−	−	16
	16	+	+	+	±	−	−	−	16
	32	+	+	+	+	−	−	−	32
	64	+	+	+	+	+	−	−	64
	128	+	+	+	+	±	−	−	32
	256	+	+	+	+	+	−	−	64
	512	+	+	+	+	+	+	−	128
	Saline	+	+	+	+	+	+	−	128
	IgG titre*	128	128	128	128	128	256	256	

* The IgG titres were obtained by staining a duplicate set of cover-slips with anti-IgG conjugate. The individual results for each cover-slip are not shown.

Specific immunoglobulins in sera from mothers of confirmed cases of congenital rubella

Serum samples taken within 2 weeks of delivery were available from 14 mothers of congenitally infected infants in whom IgM antibody was detected. These sera, which all contained high titres of IgG antibody, were centrifuged on density gradients. In four specimens traces of IgA antibody were detected in the fractions, but in no instance was IgM antibody detected. From three of these mothers (and from three others who were not re-examined) sera were available which had been taken during pregnancy within 3 weeks of the onset of the rash. All contained specific IgA and IgM antibodies. No intermediate specimens were available and we were therefore unable to study the duration of IgA and IgM antibody responses in mothers carrying an infected conceptus, but we agree with Best, Banatvala & Watson (1969) and Desmyter, South & Rawls (1971), who found that IgM antibody is not usually present in maternal serum soon after delivery of an infected infant.

DISCUSSION

In laboratories in which density gradient centrifugation is used for the detection of rubella-specific IgM the presence of this class of antibody is usually inferred from the HAI activity of those fractions which can be shown to contain IgM by gel diffusion. This method was criticized by Newman *et al.* (1969), who found, when examining cord sera from infants with congenital rubella, that the IgM fractions were consistently contaminated with IgG which they thought might contribute to the HAI activity. Forghani, Schmidt & Lennette (1973) also detected IgG in the IgM fractions from some adult sera and suggested that this might be due to aggregation of IgG. Caul, Smyth & Clarke (1974) attempted to exclude false positive results due to contamination with IgG by titrating the HAI

activity before and after treatment with 2-mercaptoethanol (2ME) which reduces IgM. In our previous work, in which density gradient fractions from adult sera were stained with anti-IgG and anti-IgM conjugates, we found no significant overlap of IgG and IgM rubella antibodies and never found IgG in the peak IgM fractions (Cradock-Watson *et al.* 1972, 1974). In our present work we found the sedimentation characteristics of IgG and IgM rubella antibodies to be the same in infants as in adults, and in particular we found no evidence of the 7 S IgM which was detected by Perchalski, Clem & Small (1968) in normal cord serum. Immunofluorescence showed that the presence of IgM antibody in the heavy fractions could nearly always have been safely inferred from the HAI activity.

Fluorescent staining of fractions, however, offered the additional advantage of greater sensitivity. It confirmed the presence of antibody in fractions whose HAI titres were so low that any reduction produced by 2ME would have been difficult to interpret, and it often revealed antibody in fractions in which HAI activity was undetectable. In three confirmed and four suspected cases the presence of specific IgM would have been missed altogether if reliance had been placed solely on the HAI titres of the heavy fractions.

The greater sensitivity of immunofluorescence may indicate that neonatal IgM is relatively inefficient at combining with haemagglutinin, or it may be due to suboptimal conditions in the HAI test. Recent work by Caul *et al.* (1974) and by Pattison, Dane & Mace (1975) has shown that HAI titres may be increased 2- to 8-fold if the serum is allowed to react with the antigen overnight at 4° C. (long method) instead of for 1 hr. at room temperature (short method). In a limited number of comparisons (not recorded here) we found that the long method increased HAI titres up to fourfold and revealed low titres in some of the fractions which were negative by the short method. The long method may therefore be almost as sensitive as immunofluorescence, but the possibility that it may increase non-specific inhibition should be considered and further study of the long method in conjunction with fluorescent staining is desirable.

In contrast to the relative ease with which IgM antibody was detected in fractions, immunofluorescent staining of whole serum for specific IgM was unsatisfactory and frequently unsuccessful. In nine confirmed and six suspected cases IgM was detected only in the fractions and not in whole serum. Inability to detect IgM in whole serum could not have been due to lack of specificity of the conjugate, which was prepared against adult IgM but appeared to stain fetal, neonatal and adult IgM in fractions with equal facility. Failure is more likely to have been due to interference from other serum constituents, because IgM staining of fractions was depressed after the addition of other fractions from the same specimen. Depression of staining was approximately proportional to the amount of IgG added, and may have been caused by competition between IgG and IgM antibodies for the same antigenic sites. It is doubtful, however, if interference was solely due to a simple quantitative relationship between the titres of IgG and IgM antibodies since good staining in whole serum was sometimes obtained despite the presence of a high titre of IgG. Differences in the relative affinities of antibodies in individual patients may also have occurred, and it is possible that IgM

staining may have been inhibited by constituents other than IgG. Similar factors may possibly interfere with the fluorescent detection of IgM antibody following intra-uterine infection with other agents such as toxoplasma and cytomegalovirus.

The duration of the IgM response could not be determined in individual cases because serial specimens were not available. Our results suggest, however, that IgM antibody is formed for about 6 months in the majority of patients but may persist for more than a year in a few individuals. Our estimate of the duration of IgM formation agrees with the results obtained by other workers. We found, as others have done, that the total concentration of IgM is increased during this time, but there was no correlation between this and the peak titre of specific IgM in the fractions and we suspect, therefore, that not all the extra IgM, even at birth, is specific for rubella. Many workers have found that infectious virus can be recovered from 40 to 50% of infected infants at the age of 3 months and from about 20% at the age of 6 months (reviewed by Rawls, 1968). Although no correlation between virus shedding and IgM antibody formation in individual patients has yet been possible, our estimate of the duration of IgM production is similar to the period of virus shedding and fits the hypothesis proposed by Rawls that IgM antibody formation results from viral persistence.

The diagnosis in infants with suspected congenital rubella can be confirmed either by virus isolation, which is often unsuccessful, or by the demonstration of persistent HAI antibody beyond the age of 6 months, when maternal antibody would normally have disappeared. The detection of specific IgM antibody in a single serum should enable the diagnosis to be confirmed at any time during the first 3–6 months of life. It may also be of value in infants who are at risk from maternal rubella but are apparently normal at birth. The incidence of IgM antibody in such children and its correlation with the persistence of IgG antibody and the later appearance of symptoms is not at present known, but it is possible that the presence of IgM antibody soon after birth would prove to be a useful guide to prognosis.

In contrast with the consistent increase in IgM concentration and the presence of specific IgM antibody during the first few months of life there was little evidence of increased IgA synthesis and almost no evidence of specific IgA formation at the time when IgM antibody was being actively produced. Inability to detect IgA antibody was probably not due to lack of specificity of the conjugate, which stained IgA in some of the mothers of these patients and has been used successfully in other work to stain IgA antibody in infants suffering from infection with respiratory syncytial virus (unpublished). Recent work by Al-Nakib, Best & Banatvala (1975) suggests that radio-immunodiffusion may be superior to immunofluorescence for detecting serum IgA antibody, and the application of this technique to infants with congenital rubella would be of interest.

IgG in normal infants is mostly maternal in origin at birth and declines rapidly during the first 3 months of life. In infants with congenital rubella IgG antibody titres increased during this time, suggesting that active formation of antibody was occurring and may have started during the last few weeks of intra-uterine life. Several workers have shown that the production of HAI antibody in children

with congenital rubella is less well maintained than it is in adults after postnatal infection and that in children over the age of 4 years titres have fallen below detectable levels in a small proportion of cases (see Dudgeon, Marshall & Peckham, 1972). The onset of this decline no doubt varies in different individuals and cannot be defined without serial specimens, but our own results suggest that it is in progress after the first year of life. In sera with very low HAI titres we found that antibody, which at that age was all IgG, could be detected with greater sensitivity and confidence by immunofluorescence, and this method should prove useful in detecting low titres of antibody which may have fallen below the threshold of the HAI test in older children with congenital infection.

We would like to thank the Directors of the Public Health Laboratories at Chester, Leeds, Lincoln, Liverpool, Middlesbrough, Preston, Reading, Sheffield, Shrewsbury and Wolverhampton, and many paediatricians and family doctors for collecting and supplying sera. We thank Mrs Joyce Lomax, F.I.M.L.T., and her staff for carrying out the HAI tests, and Dr C. M. Patricia Bradstreet for supplying the standard serum. We are grateful to Mrs Hilary Tillett for statistical help.

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