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IgG, IgA and IgM responses in acute rubella determined by the immunofluorescent technique

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SUMMARY

The indirect immunofluorescent technique has been used to study the specific immunoglobulin responses in twelve adult cases of acute uncomplicated rubella. IgG, IgA and IgM antibodies increased virtually simultaneously. IgG antibody persisted throughout the period of study but showed a slight tendency to fall in titre after 7 months. IgM antibody was detected in nine cases. In these patients it was present in high titre 5–15 days after the rash but was not detected after 20 days. IgA antibody was detected in all cases. It was present in high titre 5–20 days after the rash but was no longer detectable after 29 days except in one patient who had a very low titre at 78 days. The presence of specific IgA and IgM indicates recent rubella in uncomplicated cases, and if the immunofluorescent method is used both types of antibody should be sought.

INTRODUCTION

A rise in antibody titre detected by haemagglutination-inhibition (HAI), neutralization or complement fixation is the most satisfactory serological proof of the diagnosis of acute rubella. Unfortunately this is often impossible to demonstrate in those cases which present more than 4 to 5 days after the onset of the rash, when the initial rise in titre has already occurred. In such patients, and in many symptomless contacts, only serum of high titre may be available. The importance of confirming rubella in a woman who has developed a rash in early pregnancy has led many workers to devise tests which would identify antibody formed in response to acute infection and distinguish it from antibody resulting from infection in the more distant past.

Schluederberg (1965), studying the serological response to acute infection with mumps, measles and Coxsackievirus, showed that the initial antibody contained

a proportion of IgM which was not detectable in late convalescent sera taken after several weeks, nor in sera from healthy adults with no history of recent infection.

Subsequent workers have shown that a temporary IgM response occurs also in rubella and that the presence of rubella-specific IgM indicates recent infection. Banatvala *et al.* (1967) and Best, Banatvala & Watson (1969) demonstrated rubella-specific IgM in early convalescent sera by observing a fall in HAI titre after treatment with 2-mercaptoethanol which reduces IgM. Vesikari & Vaheri (1968), Best *et al.* (1969) and Desmyter, South & Rawls (1971), demonstrated HAI activity in the IgM-containing fractions obtained from early convalescent sera by centrifugation on sucrose density gradients. The indirect fluorescent antibody technique has been used to study immunoglobulin responses in acute rubella by Baublis & Brown (1968), Cohen, Ducharme, Carpenter & Deibel (1968) and Haire & Hadden (1970), all of whom showed that the IgM response was temporary in uncomplicated cases. The presence of HAI activity has also been demonstrated in the IgM fractions obtained from early convalescent sera by gel filtration through Sephadex G-200 (Gupta, Peterson, Stout & Murphy, 1971) and agarose (Bürgin-Wolff, Hernandez & Just, 1971). More recently Ogra *et al.* (1971) have combined density gradient centrifugation with the technique of radio-immunodiffusion in order to measure the immunoglobulin responses in acute rubella and following the administration of attenuated rubella vaccine.

The evidence from several techniques indicates that IgG and IgM antibodies in acute rubella appear rapidly within a few days of the rash and reach their highest titres within 1 or 2 weeks. IgG antibody persists, but IgM rapidly disappears and its detection after more than a month becomes increasingly unlikely in uncomplicated cases.

Virus-specific IgA was demonstrated in acute rubella by Baublis & Brown (1968) and was still present 22 days after the rash, but no subsequent sera were tested. Bürgin-Wolff and her colleagues showed that IgA followed the same course as IgM in acute rubella and was likely to be useful as an index of recent infection. However, Ogra and his colleagues showed that IgA appeared later and increased more slowly than IgG and IgM and that it persisted without appreciable loss for at least a year.

We have used the indirect immunofluorescent technique, based largely on the methods of Haire & Hadden (1970), to study the specific IgG, IgA and IgM levels in patients with acute rubella and in patients with no recent history of the disease. Because of the demonstration by Fraser, Shirodaria & Stanford (1971) that the presence of rheumatoid factor with anti-IgG activity can cause apparent staining of IgM by the fluorescent method we have also studied a small number of patients with rheumatoid arthritis.

MATERIALS AND METHODS

Patients with acute rubella

An outbreak of rubella affecting eleven university students aged 19 to 22 years occurred in February and March, 1971. Ten students were male, nine of whom lived in the same hall of residence. Prodromal symptoms were more severe than is usual in rubella and included headache, malaise, running nose, sore throat, pain on movement of the eyes, conjunctivitis and aches in the joints and limbs. They were followed after 1–5 days by the appearance of the rash which was finely maculopapular or erythematous and involved the face, trunk, arms and legs. Most patients had enlargement of the lymph nodes, particularly in the occipital and cervical groups, which persisted in some cases for 7 days after the appearance of the rash. Of the seven patients who were seen within 3 days of the onset of the rash all had rises in HAI titre of at least four-fold. Rubella virus was isolated from the throats of four of these. Sera from the first four cases were tested for liver function and for the presence of heterophile antibody, but showed no abnormality.

Twenty-three specimens of serum were taken from the eleven cases at times ranging from the day of the rash to the twenty-ninth day thereafter. Twenty-one further specimens were taken between 45 and 256 days after the rash.

Nine specimens of serum were also taken from a medical colleague who developed rubella, at times ranging from two to 153 days after the rash.

None of these patients with acute rubella possessed rheumatoid factor detectable by the latex agglutination test.

Patients with no history of recent rubella

Serum samples were obtained from 30 pregnant women who were attending the antenatal clinic and were undergoing routine screening for rubella antibody. Sera containing rheumatoid factor were obtained from seven out-patients with rheumatoid arthritis and one patient with suspected lupus erythematosus. The ages of these eight patients ranged from 37 to 75 years.

Absorption of sera

All sera were absorbed at least once with washed BHK21 cells in order to reduce non-specific fluorescence. At a dilution of 1/4 0·8 ml. of serum was absorbed with about 50 million cells at 4° C. overnight. Absorption was particularly necessary for sera with low fluorescent titres. Some sera required two absorptions and a few required three separate absorptions before nonspecific fluorescence had been reduced sufficiently for a final reading of the IgM or IgA titre to be made. Conjugates were absorbed once only, usually at a dilution of 1/10.

Rubella virus

The Judith strain of rubella virus was grown at 35° C. in monolayers of Vero cells in rotating 500 ml. flat glass prescription bottles. Vero cells used for this purpose were grown in medium 199 containing 7% (v/v) fetal calf serum and maintained in the same base with 1% serum. The tissue culture fluid was harvested

4–8 days after infection and yielded virus titres of 10^5 to 10^6 infectious particles per ml. when titrated in RK13 cells.

Cover-slip preparations for fluorescent staining

Cover-slip cultures were made with BHK21 (clone 13) cells and with LLC.MK2 cells. BHK21 cells, free from mycoplasma, were grown in minimum essential medium (as modified by Macpherson & Stoker, 1962) containing 10% (v/v) fetal calf serum and 10% (v/v) tryptose phosphate broth. They were maintained in the same base with 1% serum and 5% tryptose phosphate broth. LLC.MK2 cells were grown in Eagle's basal medium (Grand Island Biological Company) containing 2% (v/v) calf serum and were maintained in the same base without serum.

Fragments of cover-glass measuring 22 × 5 mm. were inserted into 4 × ½ in. tissue culture tubes to which 1 ml. volumes of growth medium containing between 50,000 and 90,000 cells were then added. The tubes were incubated at 37° C. in a sloped position for 24–48 hr., when they were infected by replacing the growth medium with tissue culture fluid from the Vero bottles diluted with an equal volume of the appropriate maintenance medium. The tubes were re-incubated at 30° C. for 3 days and the medium was replaced by fresh maintenance medium on the day after infection. Uninfected cover-slip cultures were prepared for use as controls. Preliminary experiments showed that incubation of infected cover-slips for 3 days at 30° C. was the optimum combination of time and temperature. Fluorescence was less bright when higher temperatures were used and at 37° C. was often barely visible. After incubation the cover-slips were removed, rinsed in phosphate-buffered saline (PBS), fixed in acetone for 5 min. at room temperature and then allowed to dry in air for at least half an hour. For storage the cover-slips were fixed to microscope slides with adhesive tape so that about 12 mm. of each cover-slip protruded over the edge of the slide. Up to six cover-slip fragments were attached to each slide in this way, in the form of a rake. The slides with cover-slips attached were then stored in airtight polythene containers at −20° C. until required.

Fluorescent staining

Fluorescein-conjugated globulins prepared against individual classes of human immunoglobulin were obtained from commercial sources. The anti-IgG conjugate (Behringwerke*) used for staining LLC.MK2 cell preparations was used at a dilution of 1/80. The conjugates prepared against human IgG, IgA and IgM (Wellcome Reagents Limited) which were used to stain BHK21 cell preparations were used at dilutions of 1/160, 1/25 and 1/15 respectively. A single batch of each type of conjugate was used throughout. The cover-slips, still attached to slides, were stained by applying the reagents to their protruding portions. Serum dilutions were allowed to act for one hr. at 37° C. After three 10 min. washes in separate changes of PBS the conjugate dilution was applied and allowed to act for 45 min. at 37° C. After three more washes the preparations were counterstained in

* Obtained from Hoechst U.K. Limited, Hoechst House, Salisbury Road, Hounslow, Middlesex.

1/100,000 Evans Blue for 20–30 sec., passed briefly through distilled water and allowed to dry in air at 37° C. The cover-slips were finally mounted in glycerol buffered at pH 8·5.

Microscopy

A Reichert Zetopan microscope with a toric lens glycerol-immersion dark-ground condenser was used throughout. LLC.MK2 preparations were illuminated by ultraviolet light from an HBO 200 mercury vapour lamp, using a UG1/1·5 mm. exciter filter and GG13/2 mm.+Wratten 2B barrier filter. BHK21 preparations were illuminated by a 100 watt quartz-halogen bulb, using an interference exciter filter of the type described by Rygaard & Olsen (1969) and a matched OG 530 barrier filter.*

Haemagglutination-inhibition titrations

Sera were inactivated at 56° C. for 30 min., absorbed with kaolin, and titrated in plastic trays by the method in routine use in this laboratory (Thompson & Tobin, 1970). When the endpoint appeared to fall between two doubling dilutions the titre was recorded as the arithmetic mean.

Sucrose density centrifugation

A density gradient was prepared consisting of five layers of sucrose solution, each of 0·9 ml. volume, ranging in concentration from 12·5 to 37·5% (w/v). The gradient was left to stand for 5 hr. at 4° C. A 1/2 dilution of serum was absorbed with chick red cells for at least 1 hr. at 4° C, and 0·5 ml. was layered on the top of the gradient which was then centrifuged overnight at 35,000 rev./min. in the SW 50 rotor of a Beckman 'Spinco L' centrifuge. No absorption with kaolin was done because the non-specific inhibitors, which are β-lipoproteins, remain at the top of the gradient. About 12 fractions consisting of five drops each were collected after piercing the bottom of the tube. The HAI titres of the fractions were determined in microtitre trays, starting with undiluted material. The presence of separate classes of immunoglobulin in the fractions was detected by double diffusion in agar gel, using antisera specific for human IgG, IgA and IgM (Wellcome Reagents Limited).

RESULTS

Microscopic appearances

In positive preparations finely granular cytoplasmic fluorescence was seen in a variable proportion of cells. Nuclear fluorescence was not observed. In serum titrations the number of fluorescent cells diminished with increasing dilution of the serum, as did the brightness of individual cells. The end-point was taken as the last dilution in which specific fluorescence could clearly be seen. In the early stages of this work we used cover-slip cultures of LLC.MK2 cells, because these cells had previously been used successfully by Baublis & Brown (1968). In preparations of

* The interference and barrier filters were obtained from Polaron Equipment Limited, 4 Shakespeare Road, Finchley, London, N3 1XH.

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these cells stained for IgG and illuminated by ultraviolet light the fluorescent material occupied a hemispherical position adjacent to the nucleus. In such preparations we obtained titres up to 1024 with early convalescent sera (Table 1). However, we were unable to obtain satisfactory staining of IgM by the use of this system. Considerably better results were obtained by the use of BHK21 cells illuminated by a quartz-halogen lamp. Although the proportion of fluorescent cells appeared to be somewhat less than with LLC.MK2 cells the contrast between the fluorescence and the background was superior. Intracellular fluorescence was more easily observed, finer detail could be seen and higher end-points in titrations were obtained (Table 1). Examples of specific staining of IgG, IgA and IgM using the latter system are shown in Plates 1 and 2. In BHK21 cells stained for IgG the fluorescent material showed a tendency to be concentrated near the nucleus, whereas in preparations stained for IgA and IgM it was usually distributed more uniformly throughout the cytoplasm. However, this difference was not consistently observed in all preparations.

Immunoglobulin responses in patients with acute rubella

The titres of rubella antibody in the IgG, IgA and IgM classes of immunoglobulin in eleven students with acute rubella are shown in Table 1, together with the HAI titres. All three classes of antibody increased virtually simultaneously within three days of the rash. In only one patient in this group (case 1, 2 days after the rash) was IgM detected without IgG, and then only in low titre. IgG antibody persisted throughout the period of study, but in some cases declined after 7 months. HAI antibody followed a similar course. IgA antibody was demonstrated in all patients and reached titres of 128 to 2048 between 5 and 20 days after the rash. It then rapidly declined, and was not detected after 29 days except in case 6 in whom it was still present 78 days after the rash in very low titre. IgM antibody was demonstrated in titres of 64 or more in seven cases, and reached maximum levels 5–15 days after the rash. It then rapidly disappeared and was not detected in sera taken after 15 days. In one patient (case 2) very little specific IgM was detected, and in three patients (cases 3, 6 and 10) none at all. No IgM response was detected in case 6, but no serum was available between the day of onset and the twentieth day thereafter.

The immunoglobulin responses are typified by the results from a colleague who experienced an attack of rubella and from whom it was possible to obtain numerous specimens of serum. These results are shown separately in Table 2. In this case low titres of rubella IgA and IgM were found on the second day in the absence of detectable IgG.

Immunoglobulins in sera from patients with no history of recent rubella

Serum samples were examined from 30 pregnant women who were attending the antenatal clinic and who gave no history of recent rubella or recent contact with the disease. In eleven consecutive sera without rubella antibody (HAI < 20) no specific IgG, IgA or IgM was detected at a serum dilution of 1/4. In 19 consecutive sera with HAI titres ranging from 20 to 480 IgG antibody was detected

Table 1. *Haemagglutination-inhibition and immunoglobulin antibody titres in 44 sera from 11 patients with acute rubella*

Case no.	Days* after onset	HAI titre	Immunoglobulin titre obtained by immunofluorescence: LLC.MK2† preparations IgG	BHK21 preparations‡ IgG	BHK21 preparations‡ IgA	BHK21 preparations‡ IgM
6	0	< 20	< 4	< 4	< 4	< 4
2	1	< 20	< 4	< 4	< 4	< 4
5	1	< 20	< 4	< 4	< 4	< 4
7	1	< 20	< 4	< 4	< 4	< 4
10	1	120	32	128	64	< 4
1	2	< 20	< 4	< 4	< 4	16
9	3	320	64	128	64	64
4	8	1280	16	128	512	512
11	5	2560	256	2048	128	128
10	8	1280	256	4096	512	< 4
9	10	1280	128	2048	512	512
7	11	⩾ 2560	64	2048	2048	128
8	11	⩾ 2560	128	2048	2048	2048
5	13	480	64	2048	512	256
2	14	960	128	1024	1024	8
1	15	320	64	2048	512	1024
3	16	⩾ 2560	256	2048	2048	< 4
6	20	640	128	⩾ 8200	512	< 4
4	26	1280	64	4096	< 4	< 4
7	26	⩾ 2560	256	4096	128	< 4
5	28	1280	128	1024	4	< 4
1	29	960	180	4096	< 4	< 4
3	29	⩾ 2560	256	⩾ 8200	256	< 4
3	45	⩾ 2560	256	1024	< 4	< 4
11	56	320	512	1024	< 4	< 4
10	57	1280	1024	⩾ 8200	< 4	< 4
9	68	320	512	2048	< 4	< 4
8	77	640	512	⩾ 8200	< 4	< 4
6	78	640	512	⩾ 8200	8	< 4
5	79	1280	512	1024	< 4	< 4
2	80	320	256	1024	< 4	< 4
4	84	160	.	512	< 4	< 4
7	84	1280	512	⩾ 8200	< 4	< 4
1	87	⩾ 1280	128	⩾ 8200	< 4	< 4
3	95	1280	512	⩾ 8200	< 4	< 4
9	221	320	.	4096	< 4	< 4
11	227	80	.	1024	< 4	< 4
5	239	320	.	2048	< 4	< 4
2	240	160	.	256	< 4	< 4
6	243	320	.	4096	< 4	< 4
4	244	160	.	256	< 4	< 4
1	246	120	.	512	< 4	< 4
10	247	⩾ 1280	.	1024	< 4	< 4
3	256	640	.	1024	< 4	< 4

* Day 0 = day of onset of rash.
† LLC.MK2 cell preparations were examined by ultraviolet illumination.
‡ BHK21 cell preparations were examined by quartz-halogen illumination.

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Table 2. *Serial haemagglutination-inhibition and immunoglobulin antibody titres in a patient with acute rubella*

Days after onset	HAI	Immunoglobulin titre obtained by immunofluorescence on BHK21 cell preparations		
		IgG	IgA	IgM
2	20	< 4	16	8
4	160	512	128	128
6	960	1024	256	128
10	1280	1024	512	512
12	960	2048	256	512
20	640	2048	64	16
33	960	2048	< 4	< 4
75	960	2048	< 4	< 4
153	480	2048	< 4	< 4

by immunofluorescence in all cases in titres ranging from 8 to 2048. The number of cases in this group was too small for any consistent relationship to be detected between the HAI and IgG titres. In 18 cases in this group no IgA or IgM was detected at a serum dilution of 1/4. One patient with an HAI titre of 120 and no detectable IgM showed IgA staining at a dilution of 1/4, which was not removed after three successive absorptions with BHK21 cells.

Centrifugation on sucrose density gradients

Because of the failure to detect specific IgM in cases 3 (day 16) and 10 (day 8) and the low titre in case 2 (day 14) we centrifuged these three sera on sucrose density gradients together with three other sera which showed high titres of IgM by the fluorescent method (case 1, day 15; case 8, day 11; case 9, day 10). In all six sera rubella HAI activity was detected in similar amounts in the IgM-containing fractions. The three sera in which the fluorescent method had detected little or no specific IgM in fact contained as much IgM as those which had shown high fluorescent titres. The fractions were then tested for the presence of rubella-specific immunoglobulin by the fluorescent method. The fractions were applied undiluted for 1 hr. to infected BHK21 cover-slip preparations which were then washed with PBS and stained with conjugates in the normal manner. All six sera gave similar results and the findings from two sera are shown in Tables 3 and 4.

IgM antibody was detected by immunofluorescence in the heavy fractions from all six sera, and there was very little overlap with IgG which was present in the lighter fractions. IgA antibody was present in the IgG-containing fractions, but was also detected in lesser amounts in the IgM-containing fractions, although the latter fractions showed no detectable IgA by gel diffusion.

Competition between IgM and other immunoglobulins

In three cases the fluorescent method had failed to detect specific IgM in unseparated serum but had demonstrated it successfully in the heavy fractions obtained by ultracentrifugation. We therefore tried to detect blocking of IgM

Table 3. *Rubella antibodies in serum fractions obtained by centrifugation on a sucrose density gradient*

Patient no. 10, 8 days after rash

Fraction no.	HAI titre of fraction	Immunoglobulin detected in fraction by gel diffusion			Rubella-specific immunoglobulin detected in fraction by immunofluorescence		
		IgG	IgA	IgM	IgG	IgA	IgM
1	4	−	−	+	−	+	++
2	4	−	−	+	−	+	++†
3	4	−	−	+	−	++	++
4	8	tr*	+	−	+	+++	+
5	32	+	+	−	++	++	−
6	⩾ 64	+	+	−	++	++	−
7	⩾ 64	+	+	−	++	++	−
8	16	+	+	−	++	+	−
9	4	tr	−	−	++	+	−
10	8	−	−	−	+	tr	−
11	32	−	−	−	.	.	.
Titre in unseparated serum	1280				4096	512	< 4

* tr = Trace.
† Fluorescent titre of this fraction = 32.

Table 4. *Rubella antibodies in serum fractions obtained by centrifugation on a sucrose density gradient*

Patient no. 8, 11 days after rash

Fraction no.	HAI titre of fraction	Immunoglobulin detected in fraction by gel diffusion			Rubella-specific immunoglobulin detected in fraction by immunofluorescence		
		IgG	IgA	IgM	IgG	IgA	IgM
1	4	−	−	−	−	tr	++
2	8	−	−	tr	−	+	+++
3	32	−	−	+	−	+	+++†
4	8	−	−	tr	−	+	++
5	4	−	−	−	tr	++	+
6	16	+	+	−	++	+++	−
7	32	+	+	−	++	++	−
8	⩾ 64	+	+	−	++	+	−
9	⩾ 64	+	+	−	+++	+	−
10	⩾ 64	+	+	−	+++	+	−
11	4	tr*	−	−	.	.	.
12	2	−	−	−	.	.	.
Titre in unseparated serum	⩾ 2560				2048	2048	2048

* tr = Trace.
† Fluorescent titre of this fraction = 64.

Table 5. *Fluorescent staining reactions in rubella-infected BHK21 preparations treated with immunoglobulin-containing fractions applied together or sequentially and stained with anti-IgM conjugate*

Case no.	Days after rash	Fraction (or mixture of fractions) used for staining: IgG+IgA+IgM together	IgG+IgA together, followed by IgM	IgM alone
3	16	tr*	+	++
2	14	tr	++	++
10	8	+	+	++
1	15	+	+	+++
8	11	+	++	++
9	10	+	++	++

* tr = Trace.

antibody by IgG or IgA which may have been competing for the same antigenic sites. Coverslips were stained with separate fractions, applied in mixtures or sequentially, from the six sera which had been centrifuged. For each serum one cover-slip was treated with a mixture of fractions designed to include IgG, IgA and IgM in approximately equal amounts. A second cover-slip was treated with a mixture of IgG and IgA, washed, and then treated with IgM alone. A third cover-slip was treated with IgM only. The final dilution of each fraction was kept constant in the staining mixtures by appropriate dilution with PBS. All cover-slips were finally stained with anti-IgM conjugate. The results are shown in Table 5. As is usual in blocking experiments the results were not clear-cut. Nevertheless, there was a marked reduction in the intensity of staining of IgM when IgG, IgA and IgM were applied together, as compared with control preparations which were treated with IgM alone (contaminated only with small amounts of IgA). This reduction in the intensity of staining either did not occur, or was less marked, when treatment with IgG and IgA together was followed in a separate stage by treatment with IgM. In a corresponding experiment in which cover-slips treated with IgG, IgA and IgM together were then stained with anti-IgG and anti-IgA conjugates, no evidence of blocking of IgG or IgA by IgM was obtained.

Immunoglobulins in sera containing rheumatoid factor

Sera were examined from seven patients with rheumatoid arthritis and one patient with suspected lupus erythematosus. None of the patients gave any history of recent rubella or recent contact with the disease. All eight sera contained rheumatoid factor, with high titres in the sensitized red cell agglutination test or the latex agglutination test or both. All contained rubella antibody from past infection, with HAI titres from 40 to 480 and fluorescent IgG titres from 64 to 2048. Only one serum failed to show staining when tested for rubella-specific IgA and IgM. Three sera showed apparent rubella IgM titres of 32 but no detectable IgA at a dilution of 1/4. The remaining four sera showed apparent IgM titres from 64 to 256 and IgA titres from 16 to 64.

DISCUSSION

Our results by the fluorescent method confirm that the IgM response is temporary in acute rubella and that if only a single serum is available the presence in it of specific IgM should indicate recent infection. Our results also confirm the findings of Bürgin-Wolff and her colleagues by showing that IgA antibody follows a course similar to that of IgM and may be equally valuable as an index of recent infection. Our results differ, however, from those of Ogra and his colleagues who used density gradient centrifugation followed by radio-immunodiffusion and found that IgA appeared in only about 50 % of children with acute rubella but persisted for at least a year.

In three of our cases little or no specific IgM was detected in whole serum by immunofluorescence although centrifugation on sucrose density gradients showed that it was present. Occasional failure of the fluorescent method has also been reported by Vesikari, Vaheri & Leinikki (1971) who were unable to demonstrate specific IgM by fluorescence in six out of 22 early convalescent sera, although they detected it by centrifugation in all cases. When they examined sera which were taken after the administration of attenuated vaccine and which had HAI titres much lower than those occurring after natural disease, they detected specific IgM by centrifugation in 50 % of cases but could demonstrate only traces of IgM in occasional sera by fluorescence. Vesikari and his colleagues considered the fluorescent method to be less sensitive than the density gradient technique, and attributed their failures to the relative insensitivity of the method and the difficulty of distinguishing weak specific fluorescence from the background. In our work occasional failure to demonstrate IgM by fluorescence could not have been due to insensitivity of the method because the latter was able to detect IgM in the heavy fractions obtained by centrifugation, even after considerable dilution (see Tables 3 and 4). It is possible that failure to detect IgM in the unseparated sera may have been due to blocking of IgM by other immunoglobulins. Competition between IgG and IgM for antigenic sites has been demonstrated by Cohen, Norins & Julian (1967) who studied IgG and IgM antibodies to *Neisseria gonorrhoeae* by the immunofluorescent technique. Our experiments suggest that blocking may occur in the rubella system described here, but there was no conspicuous difference in this respect between the sera in which the fluorescent method failed to detect IgM and those sera in which high fluorescent IgM titres were obtained. Because of the failure to detect specific IgM in some cases it seems advisable, if the fluorescent method is used, to test for IgA in addition.

Although the presence of specific IgM indicates recent infection in uncomplicated acute cases, positive fluorescent staining of IgM may occur in the absence of recent infection if a serum containing virus-specific IgG also contains IgM globulins such as the rheumatoid factor (RF) with anti-IgG activity (Fraser *et al.* 1971). Fraser and his colleagues distinguish between 'primary' staining, which is directly due to virus-specific IgM, and 'secondary' staining which is due to IgM anti-globulins and which can be eliminated by previous treatment of the serum with aggregated IgG.

In seven out of eight sera containing rheumatoid factor we obtained positive IgM staining which we assume is secondary, although we have not treated the sera with aggregated IgG. In four of these specimens we also obtained positive IgA staining. Rheumatoid factor is predominantly IgM, but some anti-IgG activity may occur in other immunoglobulin classes and may have accounted for the IgA staining in these cases. The results of IgM and IgA staining on single specimens of serum should therefore be interpreted with caution, and consideration should be given to possible causes of antiglobulin activity in the serum such as rheumatoid arthritis, infectious mononucleosis and hepatitis. The early convalescent sera studied in this work contained no RF and there seems little doubt that the IgM and IgA-staining in these specimens was primary.

Immunofluorescence is more sensitive than gel diffusion and our results therefore provide some information on the purity of the fractions obtainable by sucrose density gradient centrifugation. The latter method was criticized by Newman, Horta-Barbosa & Sever (1969) who found when studying cord sera from congenitally infected infants that the heavy fractions were consistently contaminated with IgG which they thought might contribute to the HAI activity. In the sera from acute adult cases described here there was negligible overlap between IgG and IgM and no IgG could be detected by fluorescence in the heavier fractions which were richest in IgM. Possibly the globulins in cord sera may behave differently from those in adult sera on centrifugation. However, the IgM-containing fractions did contain IgA, presumably in polymeric form, which was detectable by fluorescence although not by gel diffusion. The work of Bürgin-Wolff and her colleagues shows that IgA may contribute to the HAI activity, but if the IgA response is temporary, as our work indicates, then its presence in the IgM fractions obtained by centrifugation need not interfere with the diagnosis of recent infection.

All methods in current use for the detection of rubella-specific immunoglobulins require specialized apparatus and expertise, the availability of which is likely to determine the choice of technique in any individual laboratory. Each method has certain shortcomings and it is possible that a combination of methods, such as fluorescent staining of serum fractions, might be the most sensitive technique for the study of immunoglobulins in rubella and other viral infections, although such a combination would probably be too laborious for routine use.

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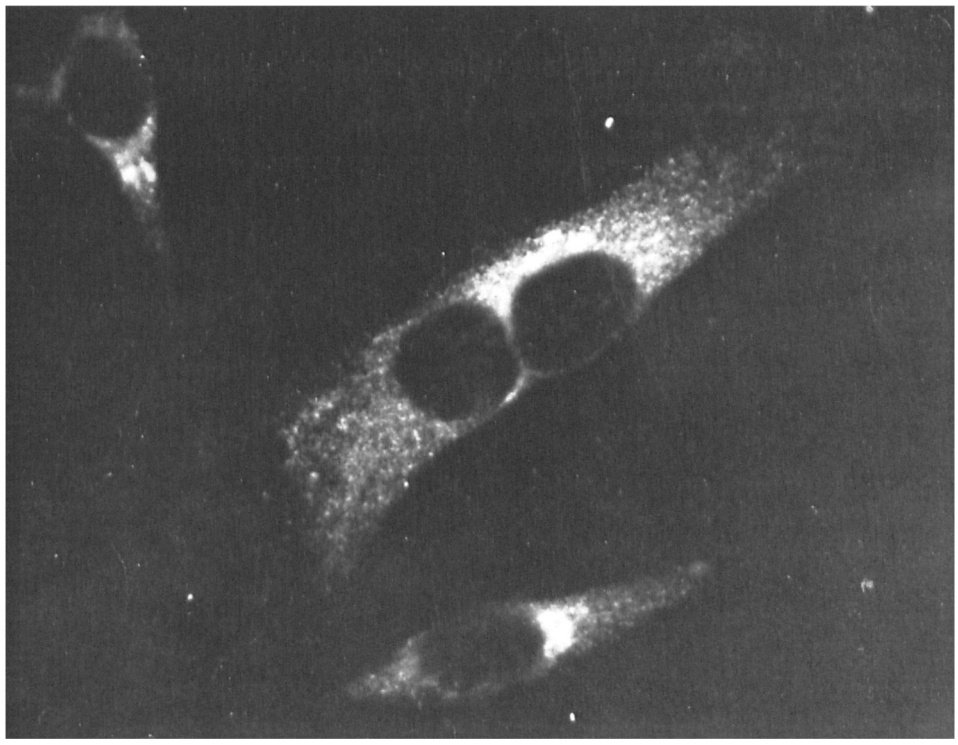

Fig. 1

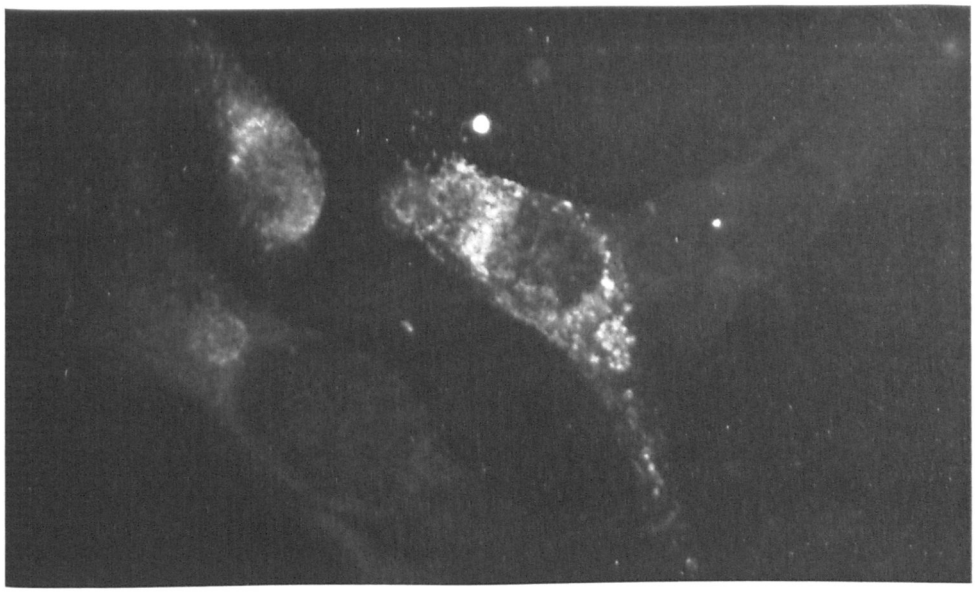

Fig. 2

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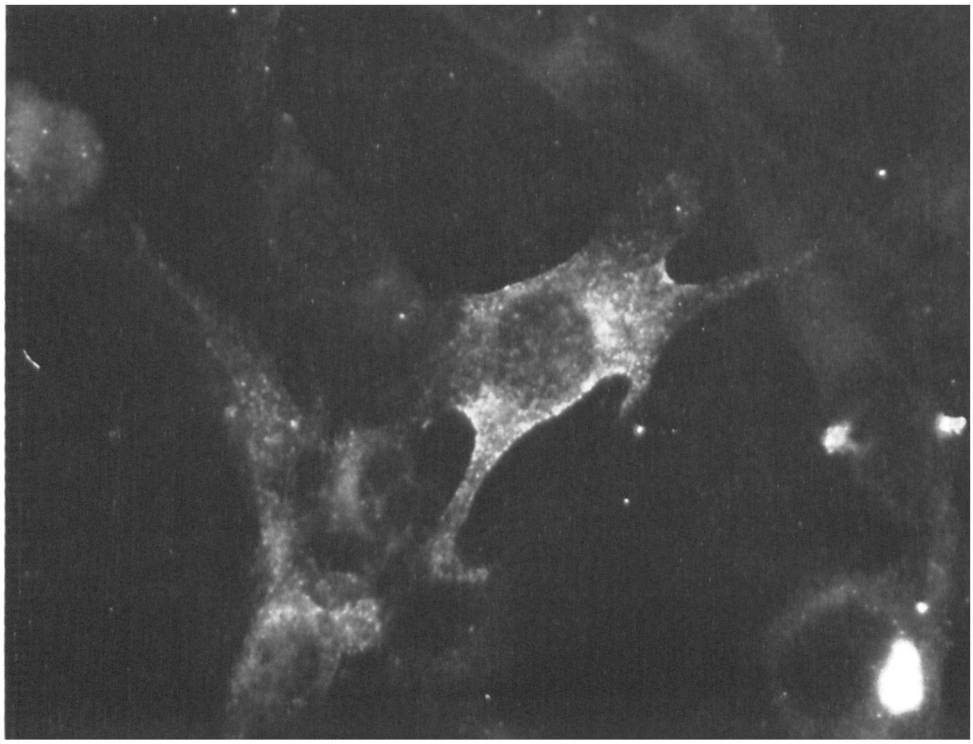

Fig. 3

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EXPLANATION OF PLATES

Fig. 1. Immunofluorescent staining of rubella-specific IgG. × 900. Serum from case 6, 20 days after the rash, at a dilution of 1 in 16.

Fig. 2. Immunofluorescent staining of rubella-specific IgA. × 750. Serum from case 8, 11 days after the rash, at a dilution of 1 in 20.

Fig. 3. Immunofluorescent staining of rubella-specific IgM. × 750. Serum from case 8, 11 days after the rash, at a dilution of 1 in 20.