Epidem. Inf. (1988), **100**, 443–454
Printed in Great Britain

Specific IgG subclass antibody in rubella virus infections

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(*Accepted 14 December 1987*)

SUMMARY

A solid-phase antigen enzyme-linked immunosorbent assay (ELISA) was developed for the detection of rubella-specific IgG subclasses. For rubella-specific IgG_1 and IgG_3 sera were quantitated in arbitrary units (au) by comparison with standard curves. A concentration of 3 au was taken as that indicating positivity for specific IgG_1 and specific IgG_3. No sera reactive for specific IgG_2 and IgG_4 have been found, and thus the assay reagents were controlled by testing dilutions of a standard calibrant serum containing known concentrations of the specific IgG subclasses.

Of 105 unselected sera negative for rubella antibody by radial haemolysis (RH), two gave concentrations of specific $IgG_1 > 3$ au and both were positive by rubella latex agglutination (LA). The sensitivity of the assay for specific IgG_1 was confirmed by examining 25 selected sera negative by RH but reactive by LA. Twenty-one gave concentrations > 3 au. None of these 130 was positive for specific IgG_3. All 63 sera containing > 15 international units rubella antibody by RH from cases of rubella in the remote past contained specific IgG_1 and eight contained specific IgG_3.

In 79 cases of primary rubella, specific IgG_1 developed in all cases by day 8. Specific IgG_3 became detectable in all cases except one by day 16. Serum taken on day 21 from one case was negative for specific IgG_3 but the absence of later sera precluded further investigation. One case had become negative for specific IgG_3 by day 56.

Sera from 24 cases of rubella reinfection were examined and all contained specific IgG_1. In three cases of symptomatic reinfection, specific IgG_3 was detectable in two but not in the remaining case. In 2 of the 21 cases of asymptomatic reinfection only a very early or a very late serum was available. Of the remaining 19 cases, 7 had detectable specific IgG_3. However, only one of 9 sera collected 30–50 days after contact contained specific IgG_3. Thus for the asymptomatic patient for whom other serological tests suggest a recent rubella infection, the failure to detect specific IgG_3 in sequential sera collected after contact suggests reinfection rather than primary rubella. The detection of specific IgG_3 did not correlate with the presence of specific IgM.

Sera collected 6–8 weeks after rubella vaccination had detectable specific IgG_1 in 32 of 33 cases and specific IgG_3 in 9 of 33. The remaining vaccinee was seronegative.

INTRODUCTION

Asymptomatic infections with rubella are diagnosed serologically when a seroconversion or rising titre of total antibody or rubella-specific IgG is demonstrated following contact. A primary infection may be distinguished from reinfection by the former having no detectable rubella antibody in an early serum and by patients with the latter having had rubella antibody demonstrated in sera taken prior to contact. If previous results or early sera are not available, primary rubella may be diagnosed by detecting substantial concentrations of specific IgM, whereas in reinfection specific IgM is usually undetectable or present in only low concentration. However, the recent demonstration of higher concentrations of specific IgM in reinfection means that occasionally it may be impossible to distinguish reinfection from asymptomatic primary infection using currently available tests for rubella-specific IgG and IgM (Morgan-Capner *et al.* 1985).

Distinguishing asymptomatic primary rubella from asymptomatic reinfection is of critical importance to the management of the pregnancy if the gestation is less than 16 weeks. Primary rubella in early pregnancy is of established risk to the fetus (Miller, Cradock-Watson & Pollock, 1982), whereas asymptomatic reinfection is of minimal risk (Cradock-Watson *et al.* 1981; Morgan-Capner *et al.* 1985). Thus there is a need to investigate other serological approaches to making the distinction.

Symptomatic reinfections are rare (Morgan-Capner, 1986), and as they are considered to present a risk to the fetus, making a serological distinction from primary infections is not critical to the management of pregnancy. However, to distinguish the two serologically would be of value in investigating rubella illnesses in people who have been previously immunized.

The availability of monoclonal IgG subclass antibodies in the 1980s has led to the investigation of IgG subclass profiles for a number of viruses including rubella (Skvaril, 1983; Doerr, Fleischer & Wiesman, 1984; Skvaril & Schilt, 1984; Linde, 1985; Sarnesto *et al.* 1985; Stokes, Mims & Grahame, 1986; Lehtinen, 1987). However, the studies on rubella have examined small numbers of sera from patients with rubella in the remote past, recent primary rubella, recent rubella immunization and infants with congenital rubella, but have not examined sera from cases of reinfection. Therefore, we have developed assays for rubella-specific IgG subclasses and applied them to sera from proven cases of primary infection and rubella reinfection (both asymptomatic and symptomatic) to ascertain whether they may be differentiated by the specific IgG subclass profile. In addition we have examined sera from rubella antibody-negative patients and patients having had natural rubella in the remote past or immunization.

MATERIALS AND METHODS

Sera

The sera examined for rubella-specific IgG subclasses were as follows.

(*a*) A total of 130 sera in which rubella-specific IgG had not been detected by radial haemolysis (RH) (Kurtz *et al.* 1980). Of these, 105 were sera submitted for routine antenatal testing and had not been examined by another technique. Twenty-five sera were specially selected as they had previously been shown to be positive by latex agglutination (Rubalex, Orion Diagnostica, SF02101, Finland).

(*b*) Sixty-three sera with rubella-specific IgG detectable by RH at a concentration > 15 international units (iu) per ml. All were received for rubella-antibody screening with no history of a recent rubelliform illness being given. Forty-four were from men and the rubella-specific IgG almost certainly must have been a result of natural rubella. Nineteen were from women whose immunization history was not known, and therefore their rubella-specific IgG may have been a consequence of natural rubella or immunization.

(*c*) One hundred and eighteen sera from 79 patients with symptomatic primary rubella. The date of onset of a rubelliform illness was known. Primary rubella had been diagnosed by detection of elevated concentrations of rubella-specific IgM by M-antibody capture radioimmunoassay (MACRIA) (Mortimer *et al.* 1981) with, in some cases, further confirmation from the demonstration of seroconversion.

(*d*) Thirty-nine sera from 24 patients with symptomatic primary rubella but for whom the date of onset of illness was not known. All patients had elevated concentrations of rubella-specific IgM detectable by MACRIA.

(*e*) Forty-two sera from 24 cases of rubella reinfection were tested. Diagnosis of reinfection rather than primary infection was based on detection of rubella antibody prior to or on the day of contact, and a consideration of the serological profile including the concentration of rubella-specific IgM when this was detected. The majority had no clinical illness, but three cases had had a rubelliform illness with detectable specific IgM and two have been previously reported (Morgan-Capner *et al.* 1983; 1984). Many of the asymptomatic reinfections examined have also been reported previously (Cradock-Watson *et al.* 1981; Morgan-Capner *et al.* 1985). In five sera from four cases of asymptomatic reinfection rubella-specific IgM had been detected by MACRIA (all < 10 au).

(*f*) Forty-two sera collected after rubella immunization of women who had no rubella-specific IgG detectable by RH. Thirty-three sera had been collected 6–8 weeks and the remaining nine had been collected 24–28 months after immunization.

ELISA method

A solid-phase antigen immunoassay was developed. As we had no individual subclass preparations available, the SPS-01 standard calibrant serum (SCS) (Supra-Regional Protein Reference Unit, Royal Hallamshire Hospital, Sheffield, UK) containing known concentrations of IgG subclasses was used for determining the optimum concentration of reagents. Wells of flexible polyvinyl microtitre plates (Falcon Microtitre Test III; Becton Dickinson, Oxnard, CA 93030, USA) were coated with 100 μl of chicken-anti-human IgG (Sera-Lab Ltd, Crawley Down, Sussex RH10 4FF, UK) at a dilution of 1 in 200 in carbonate/bicarbonate coating buffer, pH 9·6. The plates were covered and incubated in sealed moist boxes overnight at 4 °C before washing three times with phosphate-buffered saline containing 0·05% Tween 20 (PBST). Wells were blocked by adding 100 μl of PBST containing 5% normal goat serum (5% NGS/PBST) and incubating for 1 h at room temperature. The SCS was diluted from neat to 1 in 500000 in NGS

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Table 1. *Monoclonal anti-human IgG subclass antibodies used*

Subclass	Clone	Source	Dilution for use
Anti IgG_1	NL 16	Unipath/Oxoid Ltd, Wade Road, Basingstoke RG24 0PN, UK	1 in 2000
Anti IgG_2	AC3-AA11	Dr C. Reimer, CDC, Atlanta, GA30 333, USA (Now available from: Unipath/Oxoid Ltd)	1 in 1000
Anti IgG_3	SJ33	ICN Biomedicals, Free Press House, Castle Street, High Wycombe HP13 6RN, UK	1 in 300
Anti IgG_4	RJ4	Unipath/Oxoid Ltd	1 in 2000

and 50 μl of a 1 in 50 dilution in 2% NGS/PBST added to duplicate wells. Plates were incubated for 1·5–2 h at room temperature before washing.

Such plates were used to determine the optimal concentrations for use of the monoclonal anti-human IgG subclass antibodies and the peroxidase-conjugated anti-mouse IgG+IgM (Tago Inc., Burlingame, USA). Using this system, the lowest concentrations of the four IgG subclasses detectable were: IgG_1, 0·2 ng/ml; IgG_2, 6·3 ng/ml; IgG_3, 1·2 ng/ml; IgG_4, 1·4 ng/ml. As no serum has been discovered reactive for rubella-specific IgG_2 or IgG_4, SPS-01 calibrant serum plates were used to control each batch of assays for rubella-specific IgG_2 and IgG_4 subclasses.

For determination of rubella-specific IgG subclasses, microtitre plates were coated with rubella haemagglutinating antigen and control antigen (Wellcome Reagents Ltd, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, UK) in alternate columns by overnight incubation with 100 μl of a 1 in 200 dilution in carbonate/bicarbonate coating buffer, pH 9·6 at 4 °C. The optimum dilutions of rubella and control antigen were determined by chessboard titration with rubella-specific IgG-positive and -negative sera and a peroxidase-conjugated polyclonal goat anti-human IgG antibody (Miles Scientific, Stoke Poges, Slough SL2 4LY, UK). The plates were blocked with 5% NGS/PBST by adding 100 μl to each well and incubating for 1 h at room temperature. Fluid was then aspirated from the wells. Separate plates were used for each subclass. Sera being tested were diluted 1 in 50 in 2% NGS/PBST and 50 μl applied to duplicate test antigen and control antigen wells of each of four coated plates. After incubation for 1·5–2 h at room temperature plates were washed and 50 μl mouse monoclonal anti-human IgG subclass antibodies diluted in 2% NGS/PBST added to appropriate wells. The monoclonal anti-human IgG subclass antibodies and their dilution of use are shown in Table 1. The monospecificity of these subclass antibodies has been demonstrated by a World Health Organisation Collaborative Study (Jefferis *et al.* 1985).

After incubation for 1·5–2 h at room temperature the plates were washed and 50 μl of a 1 in 1000 dilution in 2% NGS/PBST of peroxidase-conjugated goat

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anti-mouse IgG + IgM was added to each well. After 1·5–2 h at room temperature the plates were washed and 50 μl of orthophenylenediamine/H_2O_2 (OPD) substrate was added to all wells. The reaction was stopped after 20 min incubation at room temperature in the dark by adding 50 μl of 4N-H_2SO_4 to each well. The optical density (OD) at 492 mm was read using an MR600 spectrophotometer (Dynatech Laboratories Ltd, Billinghurst, Sussex RH14 9SJ, UK). The mean of the OD on the control antigen-coated wells was subtracted from the mean of the OD on the rubella antigen-coated wells to give the final result.

Rubella-specific IgG_1 and IgG_3 in test sera were quantitated in arbitrary units (au) by comparison with standard curves prepared from sera containing rubella-specific IgG_1 and IgG_3. For rubella-specific IgG_1 a pool of sera containing rubella-specific IgG as a consequence of natural rubella in the remote past was doubly diluted in rubella antibody-negative serum from neat to 1 in 1024 and tested for rubella-specific IgG_1. Six rubella antibody-negative sera (negative by RH, latex agglutination and a commercial rubella-specific IgG ELISA assay (Enzygnost Rubella; Behringwerke AG, Marburg D-3550)) were tested simultaneously, and that dilution of the control positive serum which gave an OD reading just greater than the mean plus two standard deviations of the negative sera was allotted an arbitrary unitage of one. Dilutions of the standard positive serum were then prepared in rubella antibody-negative serum to give a range of au from 100 down to 1. A similar positive standard serum dilution series was established for IgG_3, except that serum from a case of recent primary rubella was used. On the basis of the results with the test sera a concentration of > 3 au was taken as indicating positivity (see Discussion).

RESULTS

Occasional sera gave reactivity with the control antigen, and thus it was essential that test sera were assayed on both rubella and control antigen. None of the sera showed detectable levels of rubella-specific IgG_2 and IgG_4, with no serum giving an OD greater than the mean plus two standard deviations of six rubella antibody-negative sera.

Of the 105 unselected sera negative by RH, 103 had concentrations of rubella-specific IgG_1 < 3 au (Table 2). The remaining two had concentrations of 25 au and 42 au. These were positive on testing by latex agglutination. Of the 25 selected sera nine were definitely positive by latex agglutination. Eight had a specific IgG_1 concentration > 3 au (range 19 to ⩾ 100 au) and one had a concentration of 3 au. Sixteen were weakly positive or equivocal by latex agglutination and had concentrations of > 3 au (13 sera), 3 au (2 sera) and 2 au (1 serum).

All 63 sera from the cases of rubella in the remote past and having a rubella-specific IgG concentration of > 15 iu by RH had a rubella-specific IgG_1 concentration of > 3 au (range 8 to ⩾ 100 au with 39 ⩾ 100 au).

None of the sera negative by RH gave a specific IgG_3 concentration > 3 au, whereas eight of the RH-positive sera had detectable specific IgG_3 at a concentration > 3 au (Table 2). However, the concentration in these eight sera were low; all < 20 au (range 4–18 au). No difference was observed between males and females in this group with 2 of 19 males (11 %) and 6 of 44 females (14 %) having specific IgG_3.

Table 2. *Rubella-specific IgG subclasses in various categories of rubella infection*

Category	No. of sera	No. of patients	Rubella-specific IgG_1 > 3 au	Rubella-specific IgG_1 ⩽ 3 au	Rubella-specific IgG_3 > 3 au	Rubella-specific IgG_3 ⩽ 3 au
Rubella antibody screening						
Unselected sera						
RH negative	105	105	2*	103	0	105
RH positive (> 15 iu)	63	63	63	0	8	55
Selected sera						
RH negative, LA positive	9	9	8	1	0	9
RH negative, LA weak positive/equivocal	16	16	13	3	0	16
Primary rubella						
Date of onset known	118	79	86	32	75	43
Date of onset not known	39	24	29	10	31	8
Rubella reinfection	42	24	42	0	13	29
Post immunization						
6–8 weeks	33	33	32	1	9	24
24–28 weeks	9	9	9	0	1	8

*, Positive by latex agglutination; RH, radial haemolysis; LA, latex agglutination; au, arbitrary unit. No serum was reactive for rubella-specific IgG_2 or IgG_4.

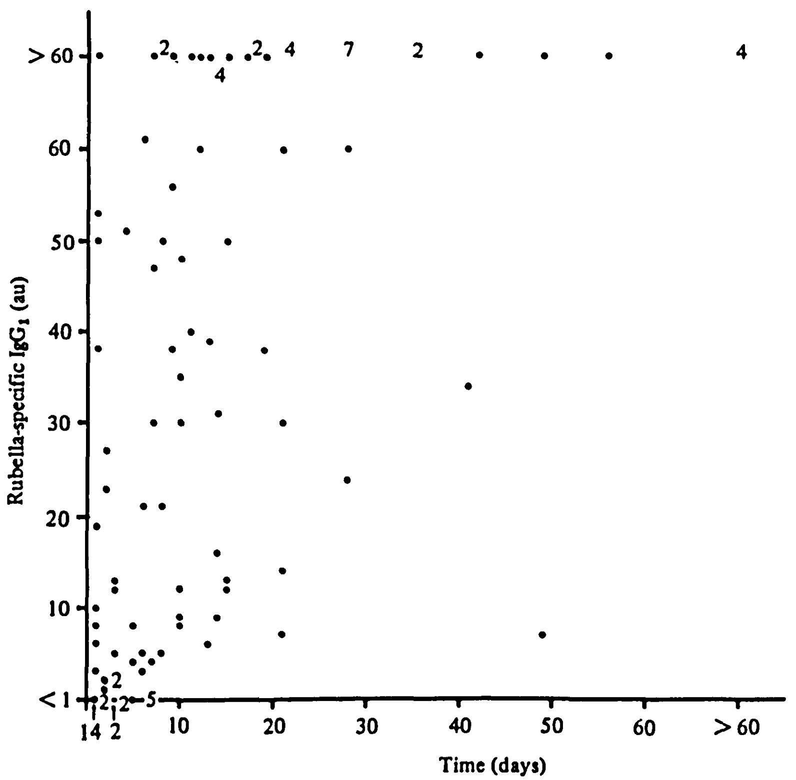

Fig. 1. Rubella-specific IgG_1 development in relation to time in primary rubella.

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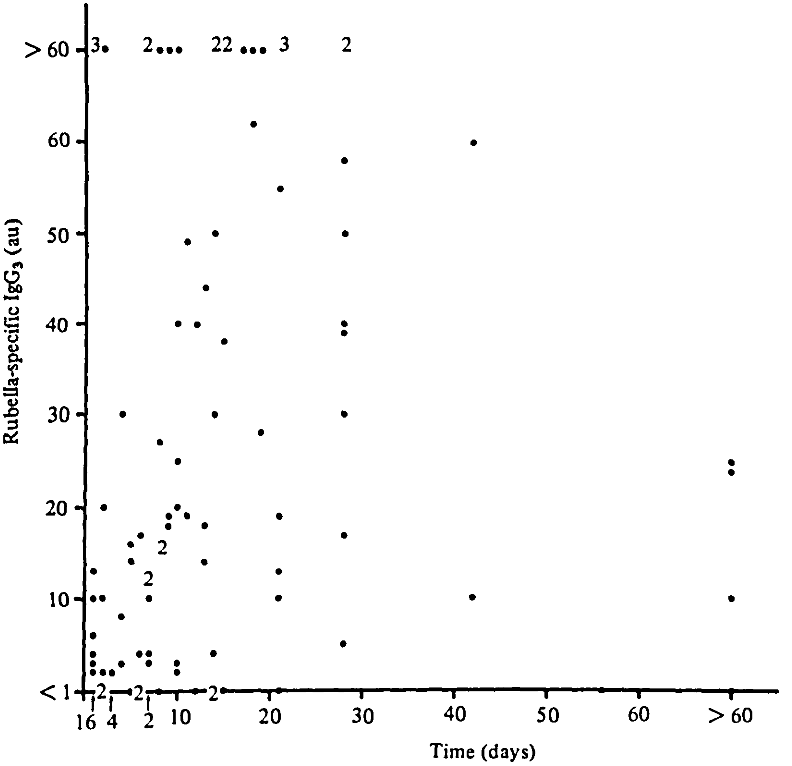

Fig. 2. Rubella-specific IgG_3 development in relation to time in primary rubella.

In 157 sera from cases of primary rubella, 115 had detectable specific IgG_1, whereas 42 did not. Thirty-two of these 42 sera were from cases where the date of onset was known, and they were all collected within 8 days of the onset of symptoms (Fig. 1). Subsequent sera examined from all these cases contained specific IgG_1.

Of the 157 sera, 106 had specific IgG_3 at a concentration > 3 au. Forty-three of the 51 specific IgG_3 negative sera were from patients with a known date of onset of symptoms. Thirty were collected within 7 days of onset of symptoms and 10 were collected 7–15 days after onset (Fig. 2). In 39 patients subsequent sera were available, and all contained detectable levels of specific IgG_3. Six early sera contained specific IgG_3 in the absence of specific IgG_1, and the reverse occurred with nine early sera. Two of the three remaining specific IgG_3 negative sera were collected approximately 8–9 weeks after the acute infection. The third serum was collected 21 days after a rubelliform rash in a child, and was positive for rubella-specific IgM by MACRIA (34 au) and weakly positive for specific IgG_1 (14 au), but negative by RH and by latex agglutination. Insufficient serum precluded further examination.

All 42 sera collected from cases of rubella reinfection contained specific IgG_1 (Table 2, Fig. 3). The 42 sera taken from 24 cases of rubella reinfection contained 8 sera from 3 symptomatic patients. Of the three clinically apparent reinfections

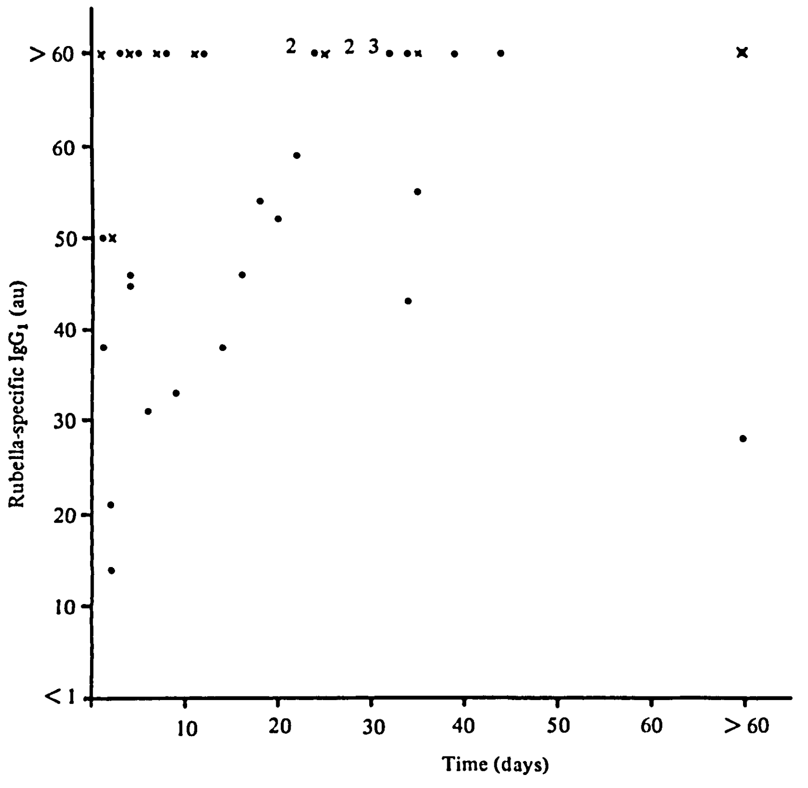

Fig. 3. Rubella-specific IgG_1 development in relation to time in rubella reinfection. For asymptomatic reinfection (●), time after contact. For symptomatic reinfection (×), time after onset of symptoms.

two had specific IgG_3 concentrations > 3 au (Fig. 3). The remaining case, a female with leukaemia (Morgan-Capner *et al.* 1983), did not have detectable specific IgG_3. All three cases had had detectable specific IgM in early sera.

Seven of the 21 cases of asymptomatic reinfection had specific IgG_3 concentrations > 3 au (Fig. 4) but 14 did not, although in one case only an early serum (8 days after contact) and in another case only a late serum (5 months after contact) was available. Of nine sera from nine cases collected 30–50 days after the estimated date of contact (i.e. approximately 15–35 days after the asymptomatic infection had it been primary rubella rather than reinfection) only one contained specific IgG_3 > 3 au (Fig. 4). Specific IgG_3 was detected at a concentration of > 3 au in three (15, 23 and 29 au) of the five sera containing specific IgM. Two patients produced detectable specific IgM but did not have a specific IgG_3 response.

All but one of the 42 sera taken after rubella immunization contained specific IgG_1 (Table 2). One serum taken 6–8 weeks after immunization contained neither specific IgG_1 nor IgG_3 and was negative by MACRIA (Table 2). Nine of the sera taken 6–8 weeks after immunization contained specific IgG_3, but all had concentrations < 18 au. One of the nine sera taken 24–28 months after immunization contained specific IgG_3.

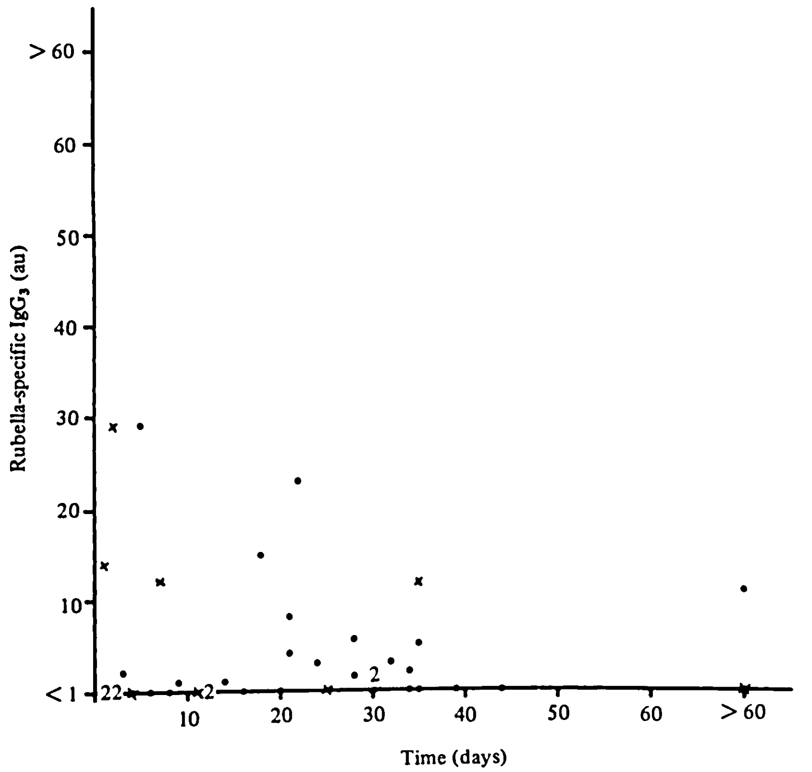

Fig. 4. Rubella-specific IgG_3 development in relation to time in rubella reinfection. For asymptomatic reinfection (●), time after contact. For symptomatic reinfection (×), time after onset of symptoms.

DISCUSSION

The use of standard curves in our assay enabled rubella-specific IgG_1 an(IgG_3 to be quantitated in au. The validity of the assay for detecting specifi IgG_1 was established by examining sera with no detectable rubella-specifi antibody and sera from cases of rubella in the remote past. All sera with greate than 15 iu of rubella antibody had a specific IgG_1 concentration > 3 au and 10: rubella antibody-negative sera a concentration < 3 au. The two sera from th unselected group of 105 which gave a concentration > 3 au were positive by late: agglutination, suggesting that the IgG_1 subclass result was correct. The sensitivit of the assay was confirmed by the examination of the selected group of 25 ser which had given conflicting results between RH and latex agglutination. Thu > 3 au was taken as the concentration of specific IgG_1 indicating positivity.

Similarly for rubella-specific IgG_3, 3 au was taken as the cut-off concentration although this concentration is less easy to justify. An arbitrary unitage of one wa established by assessing a positive serum in comparison with six rubella antibody negative sera, and thus a cut-off of 3 au may be considered conservative. Howeve with one exception, all sera from cases of primary rubella taken more than 15 day after the onset of the illness contained specific IgG_3 > 3 au. Conversely all ser

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rubella antibody-negative by RH, even those that gave positive results by latex agglutination, had concentrations < 3 au. Using this cut-off concentration, rubella-specific IgG_3 could be detected in 13% of sera from cases of rubella in the remote past, although the concentrations observed were less than seen in most cases of primary rubella. Other investigators have reported conflicting data when examining sera from cases of rubella in the remote past. Skvaril & Schilt (1984) detected only specific IgG_1 whereas Skvaril (1983), Linde (1985) and Stokes, Mims & Grahame (1986) reported not only the presence of specific IgG_1 in all sera but also the occasional detection of specific IgG_2 and IgG_4. However, comparison of reports is difficult, as in some studies only a few sera were examined and assays differed particularly with regard to source of antigen, use of control antigen, specific anti-human IgG subclass antibodies used and the means of determining the cut-off point. A significant problem we encountered which not all previous investigators have addressed is controlling the assay when no sera are available containing a particular subclass, as determined by comparison with negative sera. We controlled our assay using plates coated with the SPS-01 calibrant serum, and at least this gave assurance that the reagents were working.

In primary rubella, specific IgG_1 developed in all cases by day 8 after the onset of the illness and persisted in all cases. Whereas for specific IgG_3 some cases were still negative up to day 15 and, in one case, day 21. Unfortunately, insufficient of the serum collected on day 21 and the failure to have any later sera precluded further examination of this case. A lack of sera taken late after infection prevented accurate assessment of the minimum duration of specific IgG_3 after primary rubella, but one serum taken 56 days after onset was negative. The specific IgG subclass response in primary rubella has also been reported. Sarnesto *et al.* (1985), Doerr, Fleischer & Wiesmann (1984) and Linde (1985) all found specific IgG_3 in addition to specific IgG_1, whereas Stokes, Mims & Grahame (1986) failed to detect specific IgG_3 although they found specific IgG_4 in two cases. Doerr, Fleischer & Weismann (1984) found that specific IgG_3 was the first to appear after primary infection wheres Linde (1985) found that it was specific IgG_1. Both patterns of response were found in the cases studied by us.

We had hoped that the specific IgG subclass profile would reliably distinguish primary rubella from reinfection, but this was not so. All post-contact sera from cases of reinfection had detectable specific IgG_1, as would be expected. In two of the three clinically apparent reinfections specific IgG_3 was detected, as occurred in seven of the asymptomatic reinfections, although the concentrations were generally lower than that observed in primary infection. In some cases of reinfection, however, specific IgG_3 was not detected, even when sera were appropriately timed. Thus if sera are obtained sequentially after a contact and other serological tests such as specific IgM indicate recent infection, the failure to demonstrate specific IgG_3 would be highly suggestive of reinfection rather than primary rubella. The detection of specific IgG_3 did not correlate with the detection of specific IgM.

One patient had not serologically responded 6–8 weeks after rubella immunization, but the remainder had produced specific IgG_1. It was surprising to find that sera collected 6–8 weeks after immunization did not all contain specific IgG_3, as might have been expected from the results obtained with sera from

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primary infections. Indeed, of the seroresponders, only 28% produced detectable specific IgG_3, and as for reinfections, the concentrations were generally lower than seen after primary rubella. This frequency of detectable specific IgG_3 conflicts with the reports of Doerr, Fleischer & Wiesmann (1984) and Lehtinin (1987), who suggested that specific IgG_3 appeared in all immunized persons. It has been observed that the rubella-specific IgG response after immunization is not as high as after natural rubella (Mortimer *et al.* 1981), and this may reflect the attenuated nature of the vaccine virus and the infection. Sera collected 24–28 months after immunization showed a similar subclass response (detectable specific IgG_1 and occasional specific IgG_3) to those collected from unimmunized males who had had natural rubella in the remote past, and thus the subclass profile cannot be used to discriminate someone whose antibody was a result of natural rubella from someone with rubella antibody as a consequence of immunization.

We gratefully thank Dr J. Cradock-Watson for providing many of the sera from cases of reinfection, Dr M. Clarke for providing the sera from vaccinees and Dr C. Reimer for the gift of anti-IgG_2. We also wish to thank Dr P. Johns for his helpful advice during the course of this work. Behring kindly provided rubella antigen-coated microtitre plates, which were used in the preliminary stages of this study.

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