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The avidity of specific IgM detected in primary rubella and reinfection

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SUMMARY

An IgM capture enzyme-linked immunosorbent assay for rubella-specific IgM was used to assess the avidity of specific IgM by comparing the results obtained with and without a mild protein denaturant in the washing fluid used after incubation of IgM with rubella haemagglutinating antigen. An avidity index (AI) was calculated with AIs $< 50\%$ considered to indicate low avidity. Sera from recent primary rubella, rubella reinfection and from patients persistently reactive for specific IgM were tested. Urea and diethylamine (DEA) were compared as the protein denaturants. Twenty-six of 28 sera from cases of primary rubella gave an AI $< 50\%$ with DEA, compared with 25 of 28 with urea. Seventeen of 20 sera from cases of reinfection gave an AI $> 50\%$ with DEA whereas only 14 of 20 had a similarly high avidity with urea. Eight of 10 sera from 4 cases of persistent specific IgM reactivity gave AIs $> 50\%$ with DEA, although this was reduced to 5 when urea was used. Thus a difference has been demonstrated between the avidity of specific IgM in primary infection from that demonstrated after a secondary antigenic challenge (reinfection). This may help in serologically distinguishing primary infection from reinfection.

INTRODUCTION

Distinguishing primary rubella serologically from reinfection continues to be a problem [1]. Assays which detect specific IgG subclasses [2] only occasionally help but determination of specific IgG avidity has proved of great value [3, 4]. It has been shown that for the month after onset of the rash, rubella-specific IgG is of low avidity compared with specific IgG of longer duration and hence found in reinfection. Cases of possible recent rubella are investigated occasionally, however, in whom serological results are difficult to interpret and it was considered appropriate to ascertain whether the avidity of specific IgM detected after recent primary rubella differed from that detected after reinfection.

In addition, cases have been reported [5, 6] in which specific IgM has persisted for many months or longer, but such persistence only becomes apparent when the patient has been serologically followed over that duration. When the patient first presents, it is easy to misinterpret the detection of specific IgM in such a patient as indicating recent rubella. We had available sera from a few such patients to test for specific IgM avidity.

The individual monomeric components of the IgM molecule are thought to have low intrinsic affinity. However, because of the pentavalent structure of the molecule, it may be that IgM has a high functional affinity or avidity [7]. Most studies on IgM avidity have been done on the primary immune response in animals [8–10]. It has been claimed that there is no maturation of the IgM response [10, 11] although others [9] have claimed that there is but that the maturation is antigen-dose dependent. Specific IgM is either not produced in secondary responses or is produced in such small amounts that it has generally not been possible to draw conclusions about the avidity of IgM in the secondary response. However Webster [12] found a slight increase in the avidity of IgM produced in one rabbit following a secondary immunization with influenza virus.

The availability of sera containing reasonable amounts of specific IgM from proven cases of reinfection, which means that the patient has, by definition, had a previous antigenic challenge, enabled us to examine whether the rubella-specific IgM antibodies in rubella reinfection were of a higher avidity than those in primary rubella.

The method we used was an adaptation of the M-antibody-capture ELISA (MACELISA) [13] with the incorporation of a mild protein denaturant, diethylamine (DEA) or urea, into the washing fluid used after the reaction between the captured IgM and the rubella antigen. The reactivity of sera tested in this way was compared with reactivity without such an additive.

MATERIALS AND METHODS

Sera

Twenty-eight sera from 26 cases of symptomatic primary rubella were examined. All contained specific IgM detected by M-antibody capture radioimmunoassay (MACRIA) [14], 17 at concentrations > 30 arbitary units (a.u.), the remainder varying between 5 and 26 a.u.

Twenty sera from 14 cases of confirmed asymptomatic reinfection were tested. All contained specific IgM, 6 sera from 4 cases at concentrations of > 30 a.u., the remainder ranging from 4·6–29 a.u.

Ten sera were available from 4 patients who were persistently reactive for rubella-specific IgM by MACRIA. The patients had been reactive for between 2 and 7 months with MACRIA results of 6·8–27 a.u. In none of these asymptomatic adult patients was the reactivity thought to indicate recent primary rubella or reinfection when the clinical details and the results of other serological tests were considered.

ELISA for specific IgM avidity

A previously described M-antibody capture ELISA (MACELISA) [13] was adapted to measure the avidity of rubella-specific IgM. The wells of three flexible polyvinyl microtitre plates (Falcon Microtitre Test III; Becton Dickinson, USA) were coated overnight at 4 °C with 100 μl of rabbit anti-human IgM (Dako Ltd, High Wycombe, Bucks.) at a dilution of 1 in 500 in carbonate/bicarbonate coating buffer pH 9·6. After washing with phosphate-buffered saline containing 0·05% Tween 20, pH 7·3 (PBST), 100 μl of a 1 in 50 dilution of serum in PBST was added

to duplicate wells on each of three plates. Incubation for 3 h at room temperature (RT) was followed by washing three times with PBST and adding to each well 100 μl of a 1 in 10 dilution of rubella haemagglutinating antigen (HA) (Division of Microbiological Reagents and Quality Control, Central Public Health Laboratory, London) in PBST. The plates were incubated at 4 °C overnight. The plates were then washed once with PBST before treating each of the three plates differently. The wells of the first plate were filled with 150 μl PBST, the second with 150 μl of 8 M urea (Analar) in PBST and the third with 150 μl of 35 mM-DEA (BDH Ltd, Poole, UK) in PBST. The plates were left for 5 min before washing thoroughly with PBST. Bound rubella HA was detected by adding to each well 100 μl of a 1 in 50 dilution of peroxidase-conjugated mouse monoclonal anti-rubella antibody (Northumbria Biologicals Ltd, Cramlington, Northumberland) in PBST containing 1% bovine serum albumen and incubated at RT for 3 h. After washing with PBST 100 μl of orthophenylenediamine/H_2O_2 in citric acid buffer was added and incubated for 20 min in the dark at RT. The reaction was stopped by adding 100 μl 2 M-H_2SO_4 to each well and the optical density (OD) at 490 nm measured. The mean OD of duplicate wells was calculated and the avidity of the specific IgM for rubella HA estimated by comparing the OD with PBST wash only (A) with the OD after urea wash (B) or DEA wash (C). An avidity index (AI) [3] was calculated for urea wash as $(B/A) \times 100$ and for DEA wash as $(C/A) \times 100$.

RESULTS

The 17 sera from cases of primary rubella which had elevated concentrations of specific IgM (> 30 a.u.) all gave an AI of < 50% when either DEA or urea was in the wash fluid (Figs. 1, 2). Indeed, when DEA was used, all gave AI < 40% (Fig. 1), whereas 15 gave an AI < 40% with urea (Fig. 2). For these 17, using DEA, the mean AI was 28% (range 18–39%), and for urea the mean was 33% (range 19–42%).

Of the 11 sera from cases of primary rubella which had specific IgM concentration < 30 a.u., 9 had an AI of < 50% when DEA was used (mean 38%; range 21–61%) (Fig. 1). Using urea, 8 had an AI of < 50% (mean 42%; range 22–56%). The 2 sera having an AI > 50% with DEA were the same as 2 of the 3 sera having an AI > 50% with urea. One serum was the first of a pair of sera and was taken on the day of onset of the rash (DEA AI 61%, urea AI 51%). It had a specific IgM concentration of 14 a.u. and had no detectable specific IgG by ELISA [13]. The other serum was also the first of a pair and was taken 3 days after onset of rash and had a low specific IgM concentration of 5 a.u. and no specific IgG. The AI was 52% with DEA and 56% with urea. The serum which had an AI > 50% by urea only (AI 53%) gave an AI of 33% with DEA. This serum was collected 5 weeks after the onset of the rash, gave a specific IgM concentration of 9 a.u., and was from the same patient who was the source of the second serum noted above. When all the sera from cases of primary rubella were considered together, there was no significant difference in AI between using DEA or urea as denaturant ($P = 0{\cdot}09$; Student's t test). There was a difference, however, when only those sera containing > 30 a.u. specific IgM were considered, with DEA giving significantly lower AIs ($P = 0{\cdot}02$; Mann–Whitney Rank Sum test).

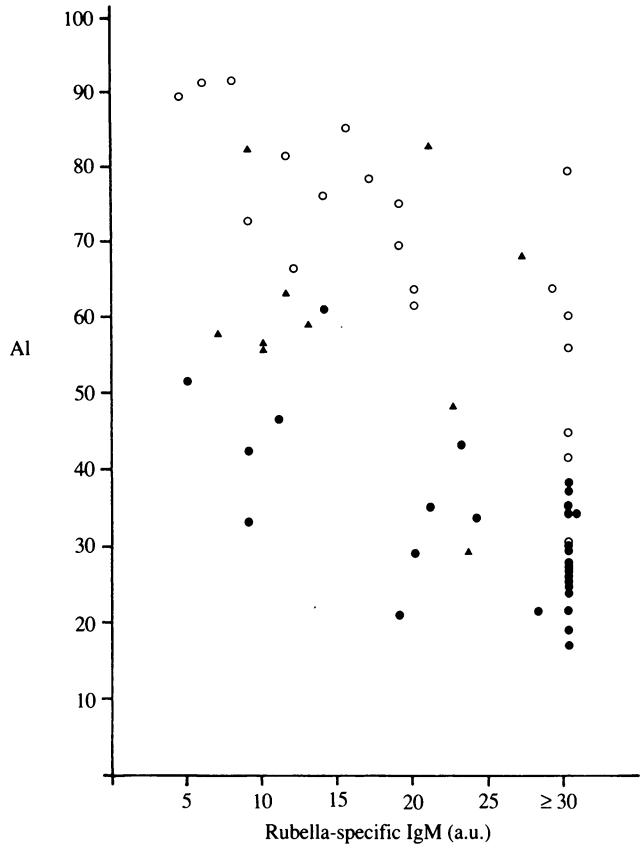

Fig. 1. Comparison between the avidity index for rubella-specific IgM and the total rubella-specific IgM using diethylamine in the washing fluid. AI, avidity index; ●, primary rubella; ○, rubella reinfection; ▲, persistent rubella IgM reactivity.

Only 3 of the 6 sera from cases of reinfection which had concentrations of specific IgM > 30 a.u. gave an AI of < 50% using DEA (Fig. 1), whereas 5 had an AI of < 50% with urea (Fig. 2). With DEA the mean AI was 52% (range 31–79%) compared with 48% (range 35–79%) for urea. All 14 of the sera from cases of reinfection with concentrations of specific IgM < 30 a.u. had AIs > 50% (mean 76%, range 62–91%) with DEA (Fig. 1). With urea, 1 of the 14 gave an AI of 49%, the remainder being > 50% (mean 65%; range 49–74%) (Fig. 2). There was no significant difference between using DEA or urea as a denaturant when all sera from cases of reinfection were considered ($P = 0{\cdot}07$; Student's t test), but when only those sera containing < 30 a.u. specific IgM were considered, DEA gave higher AIs than urea ($P = 0{\cdot}001$; Student's t test).

Whichever denaturant was used, for sera having a specific IgM concentration > 30 a.u. or < 30 a.u., the AIs of sera from cases of primary rubella were significantly lower than those from cases of reinfection ($P = 0{\cdot}001$; Student's t test and Mann–Whitney Rank Sum test).

There was no significant correlation between the AI of specific IgM antibodies and the concentration of specific IgM.

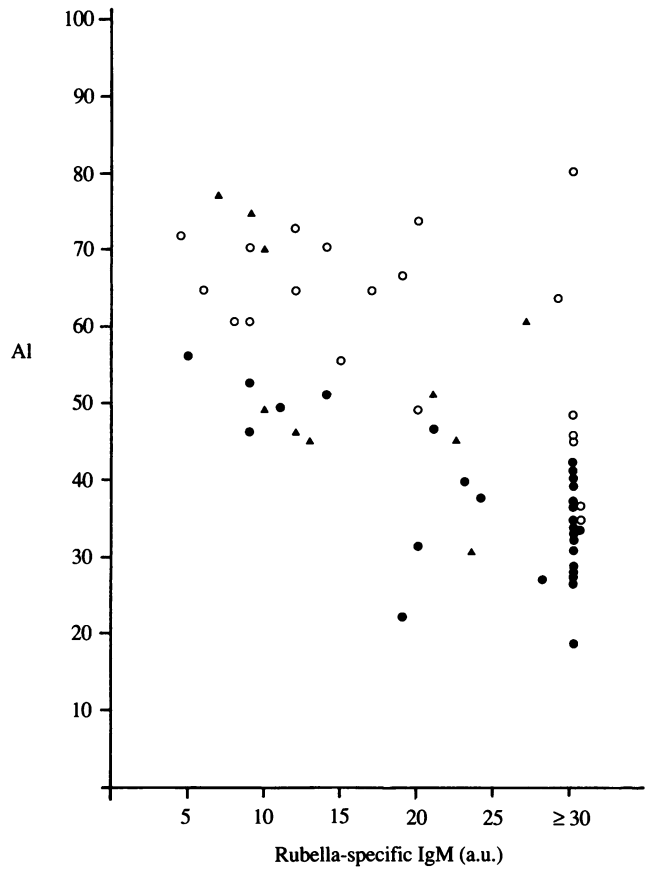

Fig. 2. Comparison between the avidity index for rubella-specific IgM and the total rubella-specific IgM using urea in the washing fluid. AI, avidity index; ●, primary rubella; ○, rubella reinfection; ▲, persistent rubella IgM reactivity.

Fig. 3 shows that there was no apparent maturation of the IgM response to rubella in primary infections over a period of up to 6 weeks. Unfortunately it was not possible to relate the AI to time after contact in the majority of the reinfection cases.

Of the 10 sera from 4 patients persistently reactive for specific IgM, 2 (from the same patient) gave an AI of $< 50\%$ (48 and 29%) with DEA (Fig. 1). The remaining 8 gave AIs of 56–83%. However, when urea was used, 5 sera (from 2 patients) gave AIs $< 50\%$ (31–49%) (Fig. 2). The remaining 5 ranged from 51–77%. Overall, for this group of sera the mean AI was 60% for DEA and 55% for urea.

DISCUSSION

A number of reports have shown the value of measuring the avidity of specific antibody as a marker for recent primary infection [3, 4, 15]. These have generally considered the avidity of specific IgG. Little work has been done on the relative avidity of specific IgM and most has been done on animals and for primary

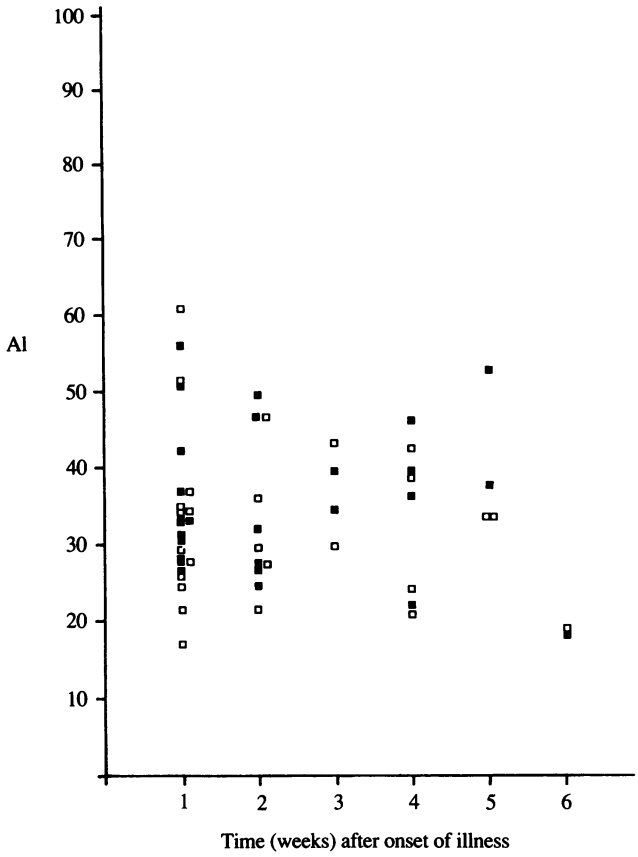

Fig. 3. Relationship between the avidity index of rubella-specific IgM and the time (weeks) after onset of illness in primary rubella. AI, avidity index; ■, urea in the washing fluid; □, diethylamine in the washing fluid.

responses only [8–10]. We know of only one report [15] of studies on the avidity of human rubella-specific IgM. The authors used regression analysis of the dose–response curves to assess the avidity of rubella-specific IgM. They presented results of their analyses of sequential sera taken from 14 patients over a period of about 100 days after onset of primary rubella and reported an increase in high avidity specific IgM in the first 6–7 days in 10 of these patients. The high avidity antibodies then decreased rapidly. We did not have sequential sera from patients to study, but found no apparent maturation of the IgM response over a period of up to 6 weeks in the group of sera we examined.

Initial studies (5 sera from cases of primary rubella; 4 sera from cases of reinfection) demonstrated that a 1 in 50 dilution of patient's serum was suitable for use, and results lay on the linear part of the dilution curve. However, because of the nature of the technique (IgM capture), the dilution of the serum does not become crucial until higher dilutions where some of the populations of antibody molecules within the whole of the specific IgM complement may be diluted out and their effects on the average avidity lost.

None of the sera from cases of rubella reinfection studied by Lehtonen and

Meurman [15] contained sufficient specific IgM for avidity studies. A major difficulty is obtaining sera containing reasonable concentrations of specific IgM from patients who have not had recent primary infection. Our interest in rubella reinfection has enabled us to collect a large group of sera from such patients. All these patients were asymptomatic and reinfection was confirmed from an assessment of historical aspects, such as previous testing and immunization, and results obtained on serological investigation.

We have shown that the avidity of specific IgM in primary rubella is less than that found after reinfection. The difference was apparent whether DEA or urea was used in the wash fluid. When sera with > 30 a.u. specific IgM were examined, in primary rubella the mean AI with DEA was 28% compared with 52% in rubella reinfection. With urea the figures were 33% and 48% respectively.

DEA gave significantly lower AIs in primary sera with > 30 a.u.-specific IgM than did urea and so would be the denaturant of choice when discrimination between primary rubella and reinfection is attempted in sera with high levels (> 30 a.u.) of specific IgM. In the groups of primary and reinfection sera with specific IgM concentrations < 30 a.u., DEA gave significantly higher AIs with reinfection sera than did urea. Thus, again, DEA would be the denaturant of choice. We chose an arbitrary AI of 50% as the cut-off point to discriminate between primary rubella and rubella reinfection. With DEA, only two sera from cases of primary rubella had an AI > 50%. Both were acute sera taken soon after the onset of the rash. It is difficult to explain why the specific IgM at the onset of the serological response should have such a relatively high avidity. It may be due to an imbalance of the distribution of the population of IgM antibodies in a serum with a low concentration of specific IgM antibodies (14 and 5 a.u. in these sera). It is known that the distribution of avidities within an antibody population is not a normal one [16] and that high avidity antibodies are produced early in an immune response as well as low avidity antibodies. It is the proportion of high and low avidity antibodies that changes (in the IgG response at least) over a period of time. Techniques such as ours measure the 'average' avidity of the antibody population [7].

Three sera from cases of primary rubella which had a low concentration of specific IgM also had AIs of > 50% when urea was used. Two were those which had AI's > 50% with DEA. The third was a late convalescent serum, also with a low specific IgM (9 a.u.).

For sera from cases of reinfection, with DEA in the wash fluid, 17 of the 20 gave an AI of > 50%, the exceptions being 3 sera with high concentrations (> 30 a.u.) of specific IgM. Thus assessing avidity of specific IgM using DEA in the wash fluid can assist in discriminating primary rubella from reinfection, although there is some overlap between the two groups.

It is interesting to speculate why some cases of reinfection respond with such an elevated concentration of specific IgM which is of low avidity. Perhaps it is a function of the immune system of the host, but it may be a consequence of their meeting certain rubella antigens for the first time, although there is no evidence to suggest antigenic difference between strains for rubella (J. M. Best, personal communication).

Patients who have been persistently reactive for rubella-specific IgM at

reasonable concentrations have been described infrequently [5, 6], although persistent low concentrations have been described after immunization [17]. The four cases we describe were all investigated after contact with a case of presumed rubella. None was symptomatic, all had high avidity IgG, and none had > 3 a.u. specific IgG_3. These results, together with their testing and immunization histories, excluded primary rubella and suggested the specific IgM reactivity was due to a reinfection. Follow-up between 2 and 7 months later revealed similar concentrations of specific IgM, unlike the fall in concentration which usually happens in the weeks following reinfection or primary rubella. Whether the specific IgM reactivity had preceded the contact investigated or was in response to the contact is obviously unknown. There must be some doubt as to whether the reactivity was or is a consequence of rubella antigenic stimulus, or whether it reflects an unrelated immunological abnormality. All these sera were negative for other potential complicating factors, such as rheumatoid factor (sheep cell agglutination test), infectious mononucleosis (Paul Bunnell), and were negative for other specific IgMs such as *Toxoplasma gondii* and coxsackievirus B. In three patients the AIs were > 50%, but the other patient gave AIs of 48 and 29% for two sera collected 4 months apart.

These last four patients demonstrate the caution with which serological results must be interpreted in possible cases of rubella and the help which may be obtained from serological investigations for subclass-specific IgG and avidity of specific IgG and IgM.

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