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# Trypsinised human O erythrocytes in the detection of rubella-specific IgM by sera fractionation on sucrose density gradient and absorption with staphylococcal protein A

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SUMMARY Detection of rubella virus-specific IgM employing trypsin-treated human group O erythrocytes was evaluated using the method of sera fractionation on sucrose density gradients (SDG) and that of sera absorption with staphylococcal protein A. The former method proved to be highly specific and sensitive in confirming or excluding rubella by demonstration of specific IgM. In contrast, the latter method provided comparable results in only 71·43% of specimens tested by both methods while false-positive or -negative IgM results were obtained in the remaining 28·57% of specimens. In view of these results, therefore, it is recommended that all those specimens found positive for specific IgM by the protein A method must be confirmed by another procedure, possibly that of specific IgM reduction with 2-mercaptoethanol.

The use of trypsin-treated human group O erythrocytes instead of chick erythrocytes in the standard rubella haemagglutination-inhibition (HAI) test, has been shown to provide not only a more sensitive, less time consuming, and more economical test but also a procedure which is as specific as that employing chick erythrocytes.[1] Recently, trypsinised human O erythrocytes for rubella HAI testing have become available commercially (Calbiochem-Behring, Corp.). It is therefore likely that these erythrocytes would become more widely available in due course. In a comparative study (as yet unpublished) of all commercially-available rubella antibody testing kits marketed in the USA including virus haemagglutination-inhibition and enzyme-linked immunosorbent-assays (ELISA), kits using trypsinised human O cells have been shown to be among the most specific methods in accurately detecting "true" rubella antibody-positive and -negative sera.[2]

However, the application of trypsinised human O erythrocytes for HAI testing after serum fractionation on sucrose density gradient or after absorption with protein A for the detection of rubella-specific IgM has yet to be evaluated. Our paper describes the detection of rubella-specific IgM by SDG and protein A absorption using trypsin-treated human group O erythrocytes.

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## Material and methods

### SERA

Sera were obtained from women who were exposed to or who had developed rubella-like illness and children with rashes who were referred for diagnosis to the Virus laboratory at the Department of Microbiology. Blood was collected (5-10 ml) and allowed to clot overnight at room temperature. Sera were separated by centrifugation at 900 *g* for five minutes and stored at −20°C until tested.

### DETECTION OF RUBELLA-SPECIFIC IGM

Sera were pre-treated with heparin/$MnCl_2$ before centrifugation on SDG to remove the "heavy" non-specific inhibitors of HAI which may sometimes be present in the IgM-containing fractions and which become detectable when the "long-incubation" procedure of HAI (when sera were incubated with antigens at 4°C overnight) is employed.[3] Sera were fractionated on SDG using an MSE Superspeed 75. Fractions (0·2 ml) were collected and tested by the HAI method of Vesikari and Vaheri,[4] except that trypsin-treated human group O erythrocytes were

substituted for day-old chick cells.[1] This also made sera absorption with erythrocytes before SDG fractionation unnecessary.

### QUANTITATIVE ESTIMATION OF IMMUNOGLOBULINS

This was conducted as an additional procedure to detect the presence of IgG and IgA in the IgM and other fractions of several sera that had been fractionated on SDG. Thus, Tri-partigen and the more sensitive S-partigen and LC-partigen plates (Behringwerke, AG) were used for this purpose.

### PROTEIN A TREATMENT OF SERA

Staphylococcal protein A was obtained from Institute Virion, Rüshlikon, Switzerland. Removal of IgG was conducted according to the method of Ankerst *et al.*[5] Only those sera that showed rubella HAI antibody activity of $\geq 1/16$ after absorption with protein A were considered positive for rubella IgM.

## Results

### EFFICIENCY OF SEPARATION OF IGM FROM IGG AND IGA

Our results show that in 176 of 177 (99·44%) fractionated sera, IgM was completely separated from IgG and IgA in at least two to five fractions. Furthermore, with the more sensitive LC-Partigen and S-Partigen immunodiffusion plates (Behringwerke, AG), the presence of IgM, IgG and IgA was investigated in the various fractions of a number of sera. IgG and IgA were undetectable in the IgM-containing fractions, thus providing further evidence for the efficiency of separation.

To investigate whether rubella HAI antibody activity in the IgG fractions might "spill-over" and precede the IgG into the IgM fractions, sera that were devoid of rubella-specific IgM antibody (titre $< 1/4$) but had rubella IgG antibody ranging from 1/4 to 1/512 were fractionated on SDG and the position of the rubella HAI antibody activity in the various IgG fractions was observed. As shown in Table 1, in 48 of 131 (36·64%) and 56 of 131 (42·75%) specimens, the rubella HAI antibody activity was visible in the first and second IgG fractions, respectively. Moreover, in 25 of 131 (19·08%) specimens the rubella HAI antibody activity started in the third to the fifth IgG fraction. In contrast, in only 2 of 131 (1·53%) specimens, "trace reactions" of rubella HAI activity were detected in one fraction ahead of the IgG fractions (Table 1).

### SPECIFICITY OF TRYPSIN-TREATED HUMAN GROUP O ERYTHROCYTES IN RUBELLA-SPECIFIC IGM DETECTION

Our preliminary study[1] on the use of trypsin-treated human group O erythrocytes in the HAI test following SDG fractionation of sera has been extended to include tests on specificity. Thus, Table 2 shows that rubella-specific IgM could not be detected in any seropositive (HAI $\geq$ 1/8) patients

Table 1 *Starting position of the rubella HAI antibody, within the IgG fractions among rubella-specific IgG positive activity rubella-specific IgM negative sera after fractionation on SDG*

| | *Starting position of rubella HAI antibody activity among IgG fractions* | | | | |
|---|---|---|---|---|---|
| | *One fraction ahead of IgG fractions* | *1st IgG fraction* | *2nd IgG fraction* | *3rd IgG fraction* | *4th or 5th IgG fractions* |
| No of sera/total fractionated | 2*/131 | 48/131 | 56/131 | 16/131 | 9/131 |
| % of the total fractionated | 1·53 | 36·64 | 42·75 | 12·21 | 6·87 |

*These two cases gave "trace reactions" in one fraction (therefore not even clear inhibition of rubella haemagglutination).

Table 2 *Specificity of SDG/HAI technique with trypsin-treated human group O erythrocytes in detecting rubella-specific IgM*

| *Group* | *No of cases tested* | *No of cases with rubella-specific antibody* | |
|---|---|---|---|
| | | *IgM* | *IgG & IgA* |
| (1) Rubella-contact mothers without evidence of clinical rubella | 67 | 0/67 | 67/67 |
| (2) Rubella seropositive cases without evidence of recent infection (HAI titre $\geq$ 1/8) | 131 | 0/131 | 131/131 |
| (3) Rubella seronegative cases (HAI titre $<$ 1/8) | 33 | 0/33 | 0/33 |
| (4) Patients with postnatally or congenitally acquired rubella | 16 | 16/16 | 12/16 |
| Total | 247 | 16/247 (6·48%) | 210/247 (85·02%) |

without evidence suggestive of a recent rubella infection. All the patients had rubella HAI antibody in only the IgG and IgA containing fractions, this being indicative of an old infection. Moreover, when sera from rubella seronegative cases (HAI antibody titre < 1/8) were fractionated on SDG and tested for rubella antibody, no HAI activity could be found in any of the IgM, IgG or IgA fractions. In contrast, all of 16 (100%) sera from patients with documented evidence of clinical or congenital rubella demonstrated specific rubella HAI antibody activity in the IgM fractions.

### DETECTION OF RUBELLA-SPECIFIC IgM USING SERA ABSORPTION WITH STAPHYLOCOCCAL PROTEIN A

Table 3 shows that 20 of 28 (71·43%) sera tested for rubella-specific IgM by both SDG/HAI and by HAI before and after absorption with staphylococcal protein A, gave comparable results. However, of the eight sera in which discordant results were obtained, four were positive by protein A/HAI and negative by SDG and four were positive by SDG and negative by protein A/HAI. Since the four sera positive by protein A/HAI and negative by SDG were from patients without any evidence of a recent rubella infection and the four which were negative by protein A but positive by SDG, were obtained from patients with a recently documented postnatally acquired or congenitally acquired rubella, our investigation suggests that pre-absorption with protein A does not provide a reliable method for the diagnosis of rubella.

Table 3 *Comparison of sensitivity and specificity of protein A/HAI and SDG/HAI in the detection of rubella-specific IgM*

| *Specimen No* | *Protein A/HAI* †*Titre* | | ‡*IgM* | *SDG/HAI* ‡*Titre* | |
|---|---|---|---|---|---|
| | *Before treatment* | *After treatment* | | ‡*IgM* | *IgG & IgA* |
| *1 | 256 | 32 | + | <4 | 64 |
| 2 | 16 | <8 | − | <4 | 4 |
| 3 | <8 | <8 | − | <4 | <4 |
| 4 | >1024 | 512 | + | 32 | 256 |
| *5 | 16 | <8 | − | 8 | <4 |
| 6 | 64 | <8 | − | <4 | 16 |
| 7 | 32 | <8 | − | <4 | 64 |
| 8 | >1024 | 128 | + | 128 | >256 |
| 9 | 512 | <8 | − | <4 | >256 |
| *10 | 512 | 32 | + | <4 | 64 |
| *11 | 128 | 16 | + | <4 | 512 |
| 12 | 128 | <8 | − | <4 | 512 |
| 13 | 128 | <8 | − | <4 | 512 |
| *14 | 128 | <8 | − | 128 | 128 |
| 15 | 256 | <8 | − | <4 | 128 |
| *16 | 64 | <8 | − | 16 | 16 |
| *17 | 512 | 32 | + | <4 | 512 |
| 18 | 64 | 8 | − | <4 | 64 |
| 19 | 64 | <8 | − | <4 | 64 |
| 20 | 32 | <8 | − | <4 | 16 |
| 21 | 4096 | 256 | + | 128 | 1024 |
| 22 | 256 | 8 | − | <4 | 256 |
| 23 | 256 | 8 | − | <4 | 512 |
| 24 | 256 | <8 | − | <4 | 512 |
| 25 | 32 | <8 | − | <4 | 16 |
| 26 | 32 | <8 | − | <4 | 32 |
| 27 | >4096 | 64 | + | 128 | 256 |
| *28 | 32 | 8 | − | 16 | 8 |

*Sera showing disagreement in results between the two procedures.
†Reciprocal of rubella HAI antibody titre.
‡For protein A/HAI titre ⩾ 1/16 are positive for specific IgM. For SDG/HAI titre ⩾ 1/4 are positive for specific IgM and IgG.

## Discussion

The data presented in this paper clearly show that excellent separation of IgM from IgG and IgA can be achieved after fractionation on SDG with the system and conditions as reported. This is in contrast to the experience of Caul *et al.*[6 7] who appear to have failed to obtain good separation and therefore suggested 2-mercaptoethanol treatment of fractions to confirm the presence of specific IgM. Failure to achieve good separation of IgM from IgG or IgA may be due to several factors—for example, the use of an inappropriate buffer to make the various sucrose concentrations, possible heat-inactivation of sera before fractionation on SDG, or the fact that the volumes of fractions collected may be too large.

We have shown that detection of rubella-specific IgM in fractionated sera with trypsin-treated human O erythrocytes is highly specific. Thus, HAI antibody activity was detected in the IgG and IgA fractions and not the IgM fractions of sera from patients with rubella antibodies of long duration. Furthermore, no rubella antibodies were detected in the IgM, IgG and IgA-containing fractions of seronegative persons, although rubella-specific IgM was detected in all of 16 (100%) cases with evidence of postnatally-acquired or congenital rubella.

Absorption of sera with staphylococcal protein A to remove IgG followed by HAI has been proposed as a simple method for the detection of rubella-specific IgM in whole sera.[5] Our data which compared the sensitivity and specificity of the protein A/HAI method to our standard and well-established SDG/HAI technique, show that there was a 71·43% agreement between the two procedures. However, in 14·29% of specimens the protein A/HAI method gave false-positive rubella-specific IgM results and in another 14·29% of the cases it gave false-negative rubella-specific IgM results. Some investigators have shown that absorption with protein A fails to remove IgG 3.[8] Therefore, sera with an initally high rubella HAI antibody titre, may still retain titres of ⩾ 1/16 after treatment with protein A. In our study, all the sera that were rubella-specific IgM positive by the protein A/HAI

technique had high rubella HAI antibody titre (1/128 to ⩾ 1/4096) (Table 3). Indeed, in only four of eight (50%) of these sera (Nos 4, 8, 21, and 27) could the presence of rubella-specific IgM be confirmed by SDG/HAI. Furthermore, protein A not only removes IgG but also a proportion of IgM.[9 10] Should there be only low concentrations of rubella-specific IgM in a particular specimen, it is possible that it might be partially or completely removed, giving rise to false-negative results. As shown in Table 3, three of the four specimens (Nos 5, 16, and 28) that were negative for specific IgM according to protein A criterion (titre < 1/16) but were positive for specific IgM as examined by the SDG/HAI method, had initial rubella HAI antibody titres of 1/16 to 1/64. In view of these findings, it would be unwise to rely wholly on protein A/HAI results in the diagnosis of recent rubella infections in early pregnancy especially if a decision as to whether to conduct a therapeutic abortion or not will be based on the results of such a procedure. Our conclusion agrees with similar conclusions obtained by other investigators who have also evaluated the protein A/HAI technique.[11] Perhaps coupling of protein A/HAI with 2-mercaptoethanol treatment of sera as suggested by Handsher and Fogel[11] would be a viable alternative for those laboratories with no ultracentrifuge facilities.

Although it would be desirable to have a procedure capable of detecting specific IgM in whole serum, such a procedure must be at least as sensitive and specific as that of SDG/HAI. At present other established techniques such as immunofluoresence (IF),[12] or the recently documented radioimmunoassays (RIA)[9 13] and enzyme-linked-immunosorbent assays (ELISA)[14] which have been applied in rubella-specific IgM diagnosis, seem to have a common limitation namely interference by rheumatoid factor[12–14] although a recently described RIA[9] provided encouraging results. However, RIA systems cannot be applicable on large scale due to the complexity of the procedure including the preparation of highly-purified and highly-specific anti-human IgM $^{125}$I-label and because of the requirement for complex, expensive equipment such as a gamma-counter. In view of this, therefore, it appears that the SDG/HAI method should remain for the time being as the most applicable reference technique for the specific detection of rubella IgM.

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