Modified Staphylococcal Absorption Method Used for Detecting Rubella-Specific Immunoglobulin M Antibodies During a Rubella Epidemic

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A recently described method for detecting rubella-specific immunoglobulin M (IgM) antibody based on absorption of IgG by *Staphylococcus aureus* strain Cowan I has been applied to 198 sera collected during a recent rubella epidemic in Israel. Modification of the original method introduced for the present study includes treatment with 2-mercaptoethanol of antibody remaining after absorption by staphylococci. This treatment confirms that the residual antibody is IgM (sensitive to 2-mercaptoethanol) rather than IgG (2-mercaptoethanol resistant). None of the 67 control patients (seropositive for rubella but without history of recent illness or contact) had specific IgM when tested by this method, though 15 showed some residual antibody after staphylococcal absorption. A total of 125 of 131 rubella convalescents (95%) were positive 4 to 49 days after onset of the clinical symptoms. Six patients had no IgM antibodies when tested by the method described, and all were convalescents tested late in relation to onset of clinical symptoms (beyond 3 weeks). When density gradient centrifugation was applied to clarify some results, 2 of 3 convalescents classified as IgM negative by the staphylococcal absorption method did in fact possess IgM antibody. None of 10 controls tested by density gradient centrifugation was IgM positive. This combination of staphylococcal absorption and 2-mercaptoethanol treatment is recommended as a screening test for selection of IgM positives, in addition to the use of a more sensitive method (such as density gradient centrifugation) on at least some samples classified as IgM negative.

During the recent rubella epidemic in Israel, about 12,000 pregnant women sought laboratory advice concerning the continuation of their pregnancies. In over 700 cases no definite conclusion could be drawn from results obtained by conventional serological tests, hemagglutination inhibition (HI), complement fixation, and 2-mercaptoethanol (2-ME) treatment (12). The laborious and expensive methods recommended for detecting rubella-specific immunoglobulin M (IgM) as an indicator of primary infection were not feasible for the large number of samples that arose under epidemic conditions.

In 1966, some strains of *Staphylococcus aureus* were reported to combine with the FC portion of human IgG through their cell wall substance (protein A) (13). In 1974, the staphylococcus Cowan I was used successfully to reduce the amount of IgG in testing for both rubella (2) and herpes (20) IgM's. Ankerst et al. (2), using radiolabeled human IgG, have shown that 92 to 95% of this globulin (fractions 1, 2,

and 4) can be absorbed from the serum with a formalinized staphylococcal suspension. IgM, IgA, and IgD showed only slight reductions after this procedure (2.5, 4.1, and 4.0%, respectively). These authors tested the results of staphylococcal absorption of 80 sera, about half of which were recent rubella cases. They found that, whereas in cases of proven old infection this treatment removed all antibody activity, in sera taken 7 to 14 days after recent rubella infection there was only a slight drop in titer.

The present study was undertaken to evaluate the possible use of this method for routine tests on large numbers of patients during epidemic conditions.

MATERIALS AND METHODS

Patients. Cases of clinical rubella confirmed by conventional methods provided sera, which had been stored at -20°C since the 1972 rubella epidemic. Control sera were obtained from seropositive patients in whom no recent rubella infection or contact was reported.

HI tests were performed by a microtiter technique with locally produced antigens, as described previ-

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ously (12). Density gradient centrifugation was performed according to the method described by Vesikari and Vaheri (21), with minor modifications. Briefly, 1 ml of a 1:2 dilution of tested serum absorbed previously with chicken erythrocytes was layered on top of a linear (37 to 10%, wt/vol) sucrose gradient and centrifuged in an MSE SW-30 rotor at 29,000 rpm for 18 h. Twenty fractions were collected dropwise from the bottoms of the tubes. Linearity of the gradients was checked with a Bausch & Lomb refractometer. By this method, IgM sedimented at a concentration of sucrose between 33 and 23%, with a peak at 26 to 28%, as checked by immunodiffusion with standard anti-IgM and anti-IgG antisera (Hyland). Fractions of the gradient were tested in a routine HI test without further treatment.

Staphylococcal absorption. *S. aureus* strain Cowan I was obtained through the courtesy of D. Sempolinsky, Department of Bacteriology, Bar Ylan University, Tel Aviv. Preparation of bacteria and absorption of sera were performed essentially according to the method described by Ankerst et al. (2), with some modifications. The stock of staphylococcus was kept on agar slants at room temperature and passaged at 3- to 4-week intervals. To prepare bacterial suspensions for absorption experiments, freshly prepared (24 to 48 h previously) agar slants were inoculated into a casein-casein yeast medium (3) and cultivated at 37°C in a roller drum (two slants were used per 300 ml of medium). After 18 h of cultivation, the bacterial suspension was passaged into larger volumes of casein-casein yeast medium (about 3 liters of medium was inoculated in 6- by 500-ml bottles, using a 1:10 inoculum). After incubation for 18 h, the final suspension was centrifuged for 15 min at 8,000 rpm. After washing in phosphate-buffered saline (PBS) and additional centrifugation, the bacteria were treated with 0.5% formaldehyde in PBS with frequent shaking for 30 min at room temperature. After three additional washings and centrifugation in PBS, a 10% suspension of bacteria containing 0.1% sodium azide in PBS was prepared and kept at 4°C until used (between 40 and 50 ml of such a 10% suspension was obtained, usually, from 3 liters of casein-casein yeast medium used for cultivation). Before use the bacterial suspension was washed in PBS and centrifuged as described above.

Absorption of sera. The sera were pretreated with $MnCl_2$, heparin, and chicken erythrocytes in the same manner as for a routine HI test (9). The optimal conditions of absorption, as determined by preliminary tests, were 0.8 ml of serum diluted 1:8 plus 0.2 ml of packed bacteria. Absorption was performed at room temperature for 30 min with frequent shaking. The bacteria were then sedimented by centrifugation for 20 min at 3,000 rpm, and the supernatant fluid was carefully harvested with a Pasteur pipette.

2-ME treatment. The concentration of 2-ME used by other authors for detection of rubella-specific IgM antibody has varied from 0.1 to 1.0 M (9, 21). In preliminary experiments, the optimal final concentration was found to be 0.167 M, prepared for the test in the following manner: 0.1 ml of 2-ME solution (0.5 M, made fresh before the test) plus 0.2 ml of serum diluted 1:8. A control serum sample was treated

with dextrose-gelatin-Veronal buffer, and both samples were incubated for 1 h at 37°C and tested for HI antibody without further treatment.

RESULTS

Table 1 summarizes the results obtained by absorption of 67 sera designated negative controls (patients with rubella HI antibody but without a history of recent rubella or contact). Fifty-two of these sera were reduced significantly in titer by staphylococcal absorption (postabsorption titers, $\leq 1:8$). Fifteen showed the presence of antibody after absorption (titers of 1:16 or 1:32), and all these 15 sera initially had high HI titers, of 1:256 or more.

Tables 2 through 4 show the results of similar experiments performed on rubella convalescents 4 to 30 days after clinical rubella was confirmed by conventional laboratory methods (HI or complement fixation rises). Forty-four

TABLE 1. Rubella HI antibody titers before and after staphylococcal absorption in 67 sera of control subjects

Preabsorption titer ^a	No. of samples	Postabsorption titer			
		<8	8	16	32
64	4	4			
128	9	9			
256	26	18	5	3	
512	22	12	2	7	1
1,024	6	2		1	3

^a Reciprocal.

TABLE 2. Results of staphylococcal absorption in 44 sera of rubella convalescents 4 to 10 days postinfection

Preabsorption titer ^a	No. of samples	Postabsorption titer				
		32	64	128	256	512
64	2	2				
128	6	4	2			
256	19	1	10	7	1	
512	14		7	5	2	
1,024	3				2	1

^a Reciprocal.

TABLE 3. Results of staphylococcal absorption in 46 sera of rubella convalescents 11 to 20 days postinfection

Preabsorption titer ^a	No. of samples	Postabsorption titer				
		32	64	128	256	512
128	2	2				
256	10	3	5	2		
512	25	8	8	8	1	
1,024	9	1	4	2	1	1

^a Reciprocal.

TABLE 4. Results of staphylococcal absorption in 33 sera of rubella convalescents 21 to 30 days postinfection

Preabsorption titer ^a	No. of samples	Postabsorption titer						
		<8	8	16	32	64	128	256
128	1				1			
256	10	1	2	1	3	3		
512	14			2	6	3	3	
1,024	7				1	3	2	1
2,048	1						1	

^a Reciprocal.

sera of convalescents 4 to 10 days postinfection showed quite different results after staphylococcal absorption, compared to the results presented in Table 1. All 44 sera showed residual antibody at titers of 1:32 or more, including those that had an initial low antibody titer of 1:64 or 1:128. Similarly, none of the sera taken 11 to 20 days after clinical rubella showed complete loss of antibody after absorption (Table 3).

Sera of convalescents drawn later after onset of illness showed more variable results. Three of 33 samples of convalescents 21 to 30 days postinfection showed significant reductions in titer after absorption (HI, 1:8), and an additional three sera were reduced to a titer of 1:16 (Table 4).

The results presented in Tables 1 through 4 show an apparent difference in reaction of control and convalescent sera to staphylococcal absorption. To further characterize the antibody remaining after absorption, all sera with residual titers of 1:16 or more were treated with 2-ME as described in Materials and Methods. It has been generally accepted that an IgM-positive serum should be reduced in titer by 2-ME treatment at least fourfold (9). In our experience, some sera of convalescents (those taken later than 2 weeks after clinical rubella) showed consistently only slight reduction ($\times 2$) in titer when treated with 2-ME after staphylococcal absorption. To further clarify the results obtained, some of these sera were tested by density gradient centrifugation. The results are summarized in Table 5. This table includes the results of all tests performed on eight sera taken 31 to 49 days after clinical rubella and on those samples of convalescents taken 11 to 30 days after illness that presented difficulties in antibody classification by our method of staphylococcal absorption and 2-ME treatment. Only 12 of a total of 21 samples could be tested by density gradient centrifugation. The remaining nine were unavailable for retesting. Four of eight samples of late convalescent sera (no. 14–21) were clearly IgM positive by staphylococcal

absorption and 2-ME treatment (no. 15, 16, 18, 21). Six sera (no. 10, 12, 13, 17, 19, 20) were reduced significantly in titer by staphylococcal absorption (residual titer, $\leq 1:8$) and could not be further tested by 2-ME. When three of these six were tested by density gradient centrifugation, two were found to contain IgM antibodies, apparently proving a false-negative result of the staphylococcal absorption method. Eleven sera were reduced by 2-ME only twofold; 8 of these 11 were available for testing by density gradient centrifugation, and all eight were found to be IgM positive. On the basis of these results, all 11 sera showing only twofold reduction by 2-ME treatment were classified as IgM positive.

The total results obtained by the combined method of staphylococcal absorption and 2-ME treatment on 198 sera are summarized in Table 6. A total of 125 of 131 samples of rubella convalescents (95%) showed the presence of IgM antibody by the combined method of staphylococcal absorption and 2-ME treatment. Six sera were negative by this method, and some of these might be false-negatives, as verified by density gradient centrifugation. On the other hand, none of 67 control samples, cases of proven old-

TABLE 5. Results of staphylococcal absorption, 2-ME treatment, and density gradient centrifugation in 21 selected convalescents following clinical rubella

Case no.	Days after onset of illness	HI titer ^a		Reduction by 2-ME	Density gradient ^b
		Before absorption	After absorption		
1	18	512	32	$\times 2$	ND ^c
2	15	256	64	$\times 2$	+
3	15	256	32	$\times 2$	+
4	20	512	64	$\times 2$	+
5	14	1,024	32	$\times 2$	+
6	21	512	16	$\times 2$	ND
7	28	512	32	$\times 2$	+
8	25	256	64	$\times 2$	+
9	30	512	16	$\times 2$	+
10	22	256	<8	ND	+
11	24	1,024	32	$\times 2$	ND
12	21	256	8	ND	+
13	28	256	8	ND	ND
14	36	256	16	$\times 2$	+
15	38	256	32	$\times 4$	ND
16	37	1,024	512	$\times 16$	ND
17	49	256	8	ND	ND
18	40	512	16	$\times 4$	+
19	35	512	8	ND	ND
20	36	512	8	ND	–
21	34	1,024	32	$\times 4$	ND

^a Reciprocal.^b +, Specific antibody present in IgM fraction; –, negative for IgM antibody.^c ND, Not done.

TABLE 6. Summary of tests based on staphylococcal absorption and 2-ME treatment for detection rubella-specific IgM antibody

Patients	No.	Sera with residual antibody after staphylococcal absorption ^a		Total % IgM:	
		No. tested	Reduced further by 2-ME ^b	Positive	Negative
Convalescents					
4-10 days ^c	44	44	44	100	0
11-20 days	46	46	46	100	0
21-30 days	33	30	30	91	9 ^d
31-49 days	8	5	5	62.5	37.5
Total	131	125	125	95	5
Controls ^e	67	15	0	0	100

^a Residual antibody after staphylococcal absorption $\geq 1:16$.

^b Reduced by 2-ME $\geq \times 2$ or to undetectable level in sera with residual titer of 1:16.

^c From the onset of clinical rubella.

^d Three samples false-negative (see Table 5).

^e Seropositive for rubella antibody but without a history of recent illness or contact.

standing immunity, showed a positive result by this method, although 15 had residual antibody after staphylococcal absorption. None of these 15 samples could be further reduced by 2-ME treatment; 10 of these 15 sera were available for testing by density gradient centrifugation, and all were IgM negative (results not shown).

DISCUSSION

The method described, based on staphylococcal absorption of sera and modified by the addition of 2-ME treatment, combines sufficient sensitivity and simplicity to be recommended as a routine test for detection of rubella-specific IgM antibody. Among other methods described so far, none could be considered satisfactory for diagnostic purposes. Direct treatment of serum with 2-ME shows a very low sensitivity, and only high titers of IgM antibody can be detected, mainly within a week from the onset of illness (9, 12).

IgM antibody, when present in lower titers, can be measured after its separation from IgG by different methods. For this purpose, density gradient centrifugation in sucrose presents a sensitive and reliable method (5, 11, 18, 21). Its main limitation for large-scale use is high expense, involving use of an ultracentrifuge. In addition, some authors have expressed reservations concerning this method, stemming primarily from frequently observed contamination of IgM by IgG (8, 18).

Gel filtration on Sephadex (15) or agarose (6) has also been applied successfully for separation of rubella-specific IgM, but this procedure cannot be used for mass screening of sera, and its use is complicated by the necessity for subsequent concentration of diluted fractions. Several authors have used a fluorescent antibody technique for the detection of rubella-specific IgM (4, 10, 16). In this kind of test some false-negative results may be observed, due to the blocking of IgM by IgG (10, 17). False-positive results also have been described for this method, owing to the presence of a "rheumatoid factor" in the serum (14).

Some laboratories have tried to develop simplified tests by a combination of two or more methods. Caul et al. (7) used density gradient centrifugation and tested only one fraction of the gradient in an HI test in parallel both with and without a 2-ME treatment. These authors were able to confirm recent rubella in all 51 patients tested, whereas 16 cases of known old infection were negative. Hornsleth et al. (17) applied a fluorescent antibody technique after reducing IgG levels in sera by filtration on Sephadex and found this method as reliable as density gradient centrifugation. Whereas both these combined techniques are simpler than other methods, they do not lend themselves to routine tests on a large number of samples.

Staphylococcal absorption modified by the addition of 2-ME treatment provides a simple and convenient test for mass screening of sera for the presence of rubella-specific IgM. A positive result obtained by this method seems reliable, since no false-positives have been found among 67 cases of known old infection. Without 2-ME treatment, 15 samples in this control group showed some residual antibody after staphylococcal absorption, and they could have been misclassified as IgM positive. On the other hand, a negative result obtained by our combined method is not conclusive, particularly in cases analyzed with some delay after suspected rubella. No false-negatives could be detected among 90 samples taken from convalescents 4 to 20 days after clinical rubella, providing that a twofold reduction of residual antibody by 2-ME treatment was considered significant (5 of 90 showed only twofold reductions; see Table 5). In sera taken later than 20 days after clinical rubella, 6 of 30 IgM-positive sera showed only twofold reduction of residual antibody by 2-ME, and an additional three samples behaved as IgM negatives, although two were in fact IgM positive by density gradient centrifugation. This possibility of false-negatives should be kept in mind in all cases of retrospective diagnosis when pregnant women are concerned,

since misclassification in such cases may lead to tragic consequences.

Apparently, the kinetics of appearance and persistence of specific IgM antibody differs individually and depends on several unknown mechanisms. Some examples of such individual differences in response are presented in Table 5. Cases 10 and 12 were reduced significantly in titer by staphylococcal adsorption 22 and 21 days after clinical rubella; in contrast, case 16 was strongly IgM positive 37 days after illness.

Such individual differences in the persistence of IgM antibodies after primary rubella infection are detected more frequently when a recently described method that substitutes overnight incubation for the standard 1-h incubation in HI testing is used (1, 19). The long-incubation method is apparently more sensitive, since in selected cases IgM antibodies can be detected as late as 10 months after illness (19). In our opinion, however, this extra sensitivity is not relevant to the pregnant patient who routinely applies for diagnostic testing within days or weeks after illness or contact with rubella.

We recommend the simplified method described in this paper as a screening test for detecting IgM-positive patients. Samples classified as negative or doubtful by this short-incubation method should be interpreted cautiously; when a pregnant patient reports a clinical history suggestive of recent rubella infection or contact with a laboratory-confirmed case of rubella, the more sensitive tests for the detection of rubella-specific IgM, such as density gradient centrifugation or the long-incubation method, should be applied.

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