An alternative method for inactivating heteroagglutinins in human sera applicable to rubella haemagglutination inhibition testing at low dilutions

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SYNOPSIS Serum agglutinins of chick and pigeon cells are predominantly immunoglobulin M and can be inactivated by 2 mercaptoethanol. 2 Mercaptoethanol is more effective than strong suspensions of red cells in removing the agglutinins of indicator cells from sera being prepared for HAI tests. In rubella HAI tests from a dilution of 1 in 10 dilute 2 mercaptoethanol offer a convenient method of removing agglutinins, and, at a higher concentration, allow dilutions of sera from 1 in 2.5 to be tested without significant interference by heteroagglutinins. HAI titres are comparable when red cell absorbed and 2 mercaptoethanol treated sera are tested in parallel.

Human sera contain agglutinins to the erythrocytes of a wide range of animals and birds. In haemagglutination inhibition (HAI) tests these 'hetero'-agglutinins produce a pattern of agglutination of indicator red cells similar to specific haemagglutination and often mask inhibition by low titres of antibody. Although heteroagglutinins may be removed from sera by treatment with strong suspensions of cells absorption is slow and incomplete. It has also been suggested that the incubation of red cells with sera releases significant amounts of cell membrane lipoprotein which may act as a non-specific inhibitor (Saeed and Murray, 1975).

In rubella HAI tests pigeon and day-old chick cells are most commonly used as indicator cells, though agglutinin to the red cells of both these species is present in human sera. Cell agglutinin can be partially removed from sera by absorption, but at dilutions of sera below 1 in 20 residual agglutinins, particularly to pigeon cells, often make the detection of rubella haemagglutination inhibition impossible. At dilutions below 1 in 10 non-specific agglutination is also caused by divalent cations added to precipitate non-antibody inhibitors.

Biano *et al* (1974) used strong cell suspensions to absorb heteroagglutinins, and sodium carbonate to precipitate excess manganese ions, and were thus able to read rubella HAI tests from a dilution of 1 in 4. In this paper alternative methods

for removing agglutinins by treating sera with 2 mercaptoethanol (2 ME) are described, and applied to rubella HAI testing from serum dilutions of 1 in 10 and 1 in 2.5.

Material and Methods

SERA

Sera were collected from women attending antenatal clinics and were stored at -20°C until used.

SERUM TREATMENT

Non-antibody inhibitors of rubella haemagglutination were precipitated by adding to sera the same volume of a freshly prepared mixture of equal proportions of molar manganese chloride and heparin. Heparin was diluted in dextrose gelatin veronal buffer (DGV) from a stock solution in distilled water of 25000 units per ml for use at a strength of 5000 units per ml. Agglutinins were removed either by absorbing sera with concentrated suspensions of red cells for 2 h at 4°C or by adding diluted 2 ME and incubating sera for 30 min in a 37°C waterbath. Dilutions of 2 ME were prepared in DGV from a stock molar solution. The following methods of serum preparation were used:

Method A (chick cells)

Final serum dilution 1 in 5

Mix	$\left\{ \begin{array}{l} 0.10 \text{ ml serum} \\ 0.10 \text{ ml heparin/MnCl}_2 \\ 0.20 \text{ ml DGV} \end{array} \right\}$	Incubate at 4°C for 20 min.
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Centrifuge at 2000 rev/min for 20 min and remove 0.20 ml of supernatant.

Add 0.05 ml 0.02 molar 2 ME, incubate at 37°C for 30 min, and test.

Add 0.05 ml 20% chick cells and incubate at 4°C for 2 h. Centrifuge at 900 rev/min for 10 min and test supernatant.

Method B (pigeon cells)

Final serum dilution 1 in 5

Mix { 0.10 ml serum
0.10 ml heparin/MnCl₂
0.20 ml DGV } Incubate at 4°C for 20 min.

Centrifuge at 2000 rev/min for 20 min and remove 0.20 ml of supernatant.

Add 0.05 ml 0.2 molar for 2 ME, incubate at 37°C 30 min, and test.

Add 0.075 ml 33% pigeon cells and incubate at 4°C for 2 h. Centrifuge at 900 rev/min for 10 min and test supernatant.

Method C (chick cells)

Final serum dilution 1 in 2.5

Mix { 0.2 ml serum
0.2 ml heparin/MnCl₂ } Incubate at 4°C for 20 min.

Centrifuge at 2000 rev/min for 20 min and remove 0.20 ml of supernatant.

Add 0.025 ml 0.2 molar 2 ME and incubate at 37°C for 30 min. Add 0.025 ml 1.0 molar Na₂CO₃ and shake vigorously for 1 min. Centrifuge at 2000 rev/min for 20 min and test supernatant.

Add 0.05 ml 50% chick cells and incubate at 4°C for 2 h. Centrifuge at 900 rev/min for 10 min, remove supernatant, and add 0.025 ml 1.0 molar Na₂CO₃. Shake vigorously for 1 min and centrifuge at 2000 rev/min for 10 min. Test supernatant.

Centrifugation procedures were carried out at 4°C on the multiple swing-out head of an MSE mistral 4L centrifuge.

SERUM FRACTIONATION

Density gradients of 15.0% to 37.5% sucrose were prepared in 5 ml centrifuge tubes, and 0.4 ml volumes

of serum diluted with 0.2 ml DGV were layered onto them. The tubes were centrifuged at 4°C on a 50L swing-out rotor at 35 000 rev/min (100 000 g) for 17 h in a Beckman L3-50 centrifuge. Twelve fractions were collected and tested for chick and pigeon cell agglutinins with and without treatment with molar 2 ME, and for immunoglobulin class by double diffusion in 1% agarose against antihuman immunoglobulin M (IgM) and immunoglobulin G (IgG) sera (Wellcome Laboratories).

HAEMAGGLUTINATION INHIBITION AND CELL AGGLUTINATION TESTS

A conventional three-volume test in microtitre plates was used. The diluent was DGV, pH 7.2. Red cells of day-old chicks and pigeons were prepared as 0.25% and 0.20% suspensions respectively. Rubella antigen was kindly supplied by the Standards Laboratory, Central Public Health Laboratories, Colindale. It was used diluted in DGV with 0.2% bovine plasma albumin (BPA) to a dose of 6 haemagglutination (HA) units. In cell agglutination tests DGV with 0.2% BPA was substituted for antigen. Serum dilutions were incubated with antigen for 1 h at room temperature and, after the addition of cells, for 2 h at 4°C. Plates were left on the bench for 15 min and read. Titres of HAI antibody and of red cell heteroagglutinin were taken as the highest dilution at which agglutination was completely absent. In serum controls degrees of agglutination were recorded as 'Ag' (agglutination which would interfere with test reading), 'sl' for slight, and 'tr' for trace agglutination.

Results

EFFECT OF 2 ME ON SERUM AGGLUTINATION TITRES TO CHICK AND PIGEON CELLS

Twenty-four unselected sera from adult patients were tested untreated, and treated with 1/10 volume of 0.1 molar, 0.2 molar, and 1.0 molar 2 ME (table I). Agglutinin of chick cells was present at relatively low titres, and could be lowered to below the serum dilutions used in rubella HAI tests by 1/10 volume of 0.1 molar 2 ME. Pigeon cell agglutinin titres were higher, but fell below the working serum dilutions after treatment with 1/10 volume of 1.0 molar 2 ME. However they were not adequately removed by weaker solutions of 2 ME.

SEPARATION OF HETEROAGGLUTININS IN SUCROSE DENSITY GRADIENTS

Three sera with high agglutinin titres to both chick and pigeon cells were fractionated in sucrose density gradients, and fractions were tested with and without

Serum	Chick Cell Agglutinin (reciprocal titre)				Pigeon Cell Agglutinin (reciprocal titre)			
	Untreated	0.1M 2ME	0.2M 2ME	1.0M 2ME	Untreated	0.1M 2ME	0.2M 2ME	1.0M 2ME
1	16	8	4	2	64	128	32	8
2	8	4	2	2	256	128	8	4
3	4	4	N	2	32	16	2	4
4	4	4	N	N	32	16	4	4
5	4	2	N	N	256	256	8	4
6	4	2	N	N	64	32	2	2
7	4	2	2	2	256	64	4	2
8	8	8	2	N	32	16	4	4
9	4	2	N	N	128	64	16	8
10	2	2	2	N	256	128	8	4
11	4	2	N	N	256	128	8	2
12	4	N	N	N	64	16	4	4
13	2	N	N	N	128	64	32	16
14	32	4	2	N	128	32	16	8
15	8	4	N	2	256	64	32	16
16	4	2	2	N	256	16	2	4
17	8	4	N	N	16	8	2	2
18	32	8	2	N	256	256	16	8
19	2	2	N	N	32	16	4	2
20	4	4	2	N	16	16	8	2
21	2	N	N	N	128	32	16	16
22	4	2	N	N	16	16	4	2
23	4	2	2	N	128	32	8	8
24	16	2	2	N	256	64	16	4
Geometric mean	5.3	2.7	1.4	1.2	93.0	42.0	7.0	4.5

Table 1 Effect of varying concentrations of 2 ME on serum agglutinin of chick and pigeon erythrocytes

N = neat

treatment with 1/10 volume of 1.0 molar 2 ME (figure). Double diffusion tests of fractions with anti IgM and anti IgG sera showed that the peak agglutinin activity in fraction 4 corresponded to the highest concentration of IgM, and that there was no separate peak of agglutinin between fractions 6 and 9 where IgG was concentrated. Almost all chick cell agglutinin and most pigeon cell agglutinin in the

first six fractions were inactivated by treatment with molar 2 ME.

COMPARISON OF RUBELLA HAI TITRES OF 23 POSITIVE SERA TREATED WITH 2 ME AND ABSORBED WITH RED CELL SUSPENSIONS
Twenty-three sera from antenatal patients and a high titred control serum were titrated against 6 HA units

Figure Reciprocal of red cell agglutinin titres of fractions of three sera taken from sucrose density gradients.

Serum	Method A: Chick Indicator Cells						Method B: Pigeon Indicator Cells					
	2 ME Treated Serum Control			Chick Cell Absorbed Serum Control			2 ME Treated Serum Control			Pigeon Cell Absorbed Serum Control		
	HAI Reciprocal Titre	1/10	1/20	HAI Reciprocal Titre	1/10	1/20	HAI Reciprocal Titre	1/10	1/20	HAI Reciprocal Titre	1/10	1/20
25	80	—	—	40	—	—	80	sl	—	80	sl	—
26	160	tr	—	160	sl	—	160	sl	—	160	sl	—
27	40	sl	—	40	—	—	80	Ag	—	80	Ag	—
28	80	—	—	40	—	—	80	—	—	80	sl	—
29	40	—	—	40	—	—	80	Ag	sl	80	tr	—
30	80	—	—	80	—	—	160	sl	—	80	tr	—
31	160	—	—	160	—	—	320	sl	—	320	tr	—
32	80	—	—	80	—	—	80	Ag	—	80	Ag	—
33	40	—	—	40	—	—	80	sl	—	40	tr	—
34	40	Ag	sl	40	sl	—	80	sl	—	80	Ag	tr
35	80	—	—	80	—	—	80	tr	—	40	Ag	tr
36	40	—	—	40	—	—	80	tr	—	80	Ag	—
37	40	sl	—	20	—	—	40	tr	—	40	Ag	—
38	80	—	—	80	—	—	160	Ag	tr	80	Ag	tr
39	80	—	—	40	Ag	sl	160	Ag	—	80	Ag	tr
40	80	—	—	40	—	—	160	Ag	sl	160	Ag	tr
41	160	sl	—	160	—	—	160	Ag	—	80	Ag	—
42	40	—	—	40	tr	—	40	sl	—	40	Ag	—
43	80	—	—	80	—	—	80	sl	—	80	Ag	—
44	40	tr	—	20	—	—	20	Ag	—	20	—	—
45	80	—	—	80	—	—	80	Ag	—	80	sl	—
46	80	—	—	160	—	—	160	tr	—	160	tr	—
47	80	tr	—	80	—	—	80	sl	—	80	Ag	—
Positive control	320	—	—	640	—	—	320	sl	—	320	Ag	—

Table II Comparison of rubella HAI and cell agglutinin titres of sera treated with 2 ME and absorbed with strong suspensions of indicator cells

of rubella antigen using methods A and B. The results are presented in table II. Tests on sera treated with 2 ME and with concentrated cells gave comparable titres. Chick cell agglutinin was removed from 1 in 10 dilutions of serum by either method, but pigeon cell agglutinin often obscured test reading at the 1 in 10 dilution whichever method of serum treatment was used.

COMPARISON OF RUBELLA HAI TITRES OF 26 LOW POSITIVE AND NEGATIVE SERA TREATED WITH CONCENTRATED CHICK CELLS AND 2 ME

Low titred sera were titrated against 6 HA units of rubella antigen using serum treatment with 0.2 molar 2 ME and with 50% chick cell absorption (method C). The HAI and chick cell agglutinin titres are recorded in table III. HAI titres varied within the limits of experimental error. In some sera heteroagglutinin interfered with the reading of the low titres, and 2 ME was more effective than 50% chick cells in removing this agglutinin.

When 2 ME was used at the higher concentrations recommended in methods B and C there was some distortion of the pattern of specific haemagglutination in serum dilutions up to 1 in 10. Instead of a shield pattern haemagglutinated cells formed coarse clumps. There was usually no difficulty in recog-

nizing this appearance but at dilutions of 1 in 2.5 and 1 in 5 of a few sera these clumps settled as a loose button in the well. This loose button could be distinguished from the compact button of haemagglutination inhibition by reference to the serum control and by examination with a hand lens. This tendency of haemagglutinated cells to settle at the low dilutions was also seen in sera absorbed with concentrated cells.

Discussion

2 ME disrupts pentameric IgM molecules into inactive fragments and has been widely used to remove IgM activity from serum. The results presented in table I and the figure show that heteroagglutinins of chick and pigeon cells in human sera are present mainly in the IgM fractions, and may also be inactivated by 2 ME. It is possible that even the small amount of agglutinin found in the IgG containing fractions of sera is IgM which has 'trailed' in the gradient, and the absence of IgG peaks in the three specimens tested supports this view.

As well as removing heteroagglutinins 2 ME inactivates specific IgM. IgM class antibody does not therefore contribute to HAI titres in tests on 2 ME treated sera although it forms, in any case, a negligible proportion of the total specific antibody in sera from

Serum	2 ME Treated				Concentrated Chick Cell Treated			
	Reciprocal of Rubella HAI Titre	Serum Control			Reciprocal of Rubella HAI Titre	Serum Control		
		1/2.5	1/5	1/10		1/2.5	1/5	1/10
48	2.5	—	—	—	2.5	tr	—	—
49	10	—	—	—	5	Ag	tr	—
50	2.5	—	—	—	<10	Ag	Ag	tr
51	2.5	tr	—	—	2.5	—	—	—
52	2.5	—	—	—	2.5	—	—	—
53	2.5	—	—	—	<10	Ag	Ag	tr
54	20	—	—	—	40	Ag	sl Ag	—
55	2.5	—	—	—	2.5	—	—	—
56	2.5	—	—	—	2.5	—	—	—
57	2.5	—	—	—	2.5	—	—	—
58	20	—	—	—	20	—	—	—
59	10	—	—	—	20	Ag	Ag	sl Ag
60	2.5	—	—	—	2.5	—	—	—
61	20	—	—	—	40	—	—	—
62	20	—	—	—	20	—	—	—
63	2.5	—	—	—	2.5	—	—	—
64	40	—	—	—	20	—	—	—
65	2.5	—	—	—	2.5	tr	—	—
66	2.5	—	—	—	2.5	—	—	—
67	2.5	—	—	—	2.5	—	—	—
68	2.5	—	—	—	2.5	—	—	—
69	20	—	—	—	20	—	—	—
70	2.5	—	—	—	2.5	—	—	—
71	10	—	—	—	10	—	—	—
72	2.5	—	—	—	5	—	—	—
73	10	—	—	—	10	—	—	—
Negative control	2.5	—	—	—	2.5	—	—	—
Low titre positive control	10	—	—	—	10	sl	—	—
Positive control	320	—	—	—	320	—	—	—

Table III Comparison of rubella HAI titres of low titred sera treated with 2 ME and absorbed with a concentrated chick cell suspension

patients with past infection. When acute and convalescent sera are compared specific IgM limits the rise in titre between specimens because it reaches its highest concentrations in the early phase of illness and declines in convalescence. Therefore, neither in tests for immunity nor in the diagnosis of infection by measuring a rising titre is the loss of specific IgM through 2 ME treatment a disadvantage, though sera which are to be fractionated to demonstrate the presence of specific IgM must of course be absorbed with concentrated red cells. The results in table II show that at the concentrations used here 2 ME has no significant effect on IgG antibody.

In testing sera from dilutions as low as 1 in 2.5 inaccuracies may arise both because of loss of antibody absorbed or destroyed by reagents used to prepare the sera, and because of incomplete removal of non-antibody inhibitors. It should be remembered that these factors can be responsible for both false negative and false positive results. While sera that show no inhibition at a dilution of 1 in 2.5 and a clear button of cells in the control wells can only be read as negative, those few sera that show incomplete inhibition at the lowest dilutions should probably be checked by a flotation centrifugation technique to distinguish between the effect of low

levels of antibody and residual inhibitor. A method for separating lipoprotein from small samples of serum is referred to by Hatch (1968) and has been used to separate rubella non-specific inhibitors from antibody by Haukenes (1973).

Tests for low titres of rubella HAI antibody have proved useful in resolving puzzling findings in natural and vaccine infections. For instance, an anomalous situation in which a 'seronegative' nurse was immunized twice with rubella vaccine without serological response at a test dilution of 1 in 16 was clarified by titres of 1 in 5, 1 in 10, and 1 in 5 being found in sera taken before and after vaccinations. Adult women found to be negative at routine screening dilutions often have low levels of antibody, and if the interpretation of serological findings in natural and vaccine infections is to be correct these low titres must be identified. Their occurrence suggests that a recent primary infection should not be diagnosed by a fourfold rise in titre unless an early serum is negative at a low dilution, or IgM has been detected.

As well as resolving some of the problems that arise in cases and contacts of natural rubella, low titre testing will have an important role in assessing the duration of immunity after rubella vaccination.

At present 11·8% of adult women tested at 1 in 16 are seronegative (Hambling, 1975), but 20% of women negative at 1 in 16 are positive at lower titres (unpublished findings). Serological responses to rubella vaccines are about three times lower than to natural infection (Plotkin and Farquhar, 1972) so this suggests that up to 10% of children vaccinated at 12 to 13 years of age will have antibody at a titre of less than 1 in 16 in adult life, and that a more sensitive test than is generally used now will be needed in the future.

Although interference by residual non-specific inhibitors in sensitive rubella tests requires further investigation, the use of 2 ME to destroy serum agglutinins enables tests at low dilutions to be carried out without difficulty, as well as simplifying the preparation of sera for routine rubella HAI screening.

I wish to thank Dr M. P. Jevons for providing sera, and Dr M. S. Pereira for help in the preparation of this paper. Since it was written I have learnt that

Dr S. K. R. Clarke, of the Public Health Laboratory, Bristol, has been using 2 ME to remove agglutinins from kaolin treated sera in routine rubella tests with very satisfactory results.

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