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An Improved Diluent for Rubella Hemagglutination and Hemagglutination-Inhibition Tests

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Rubella hemagglutinating (HA) antigen was prepared in BHK-21 tissue as 5% cell suspensions and from unconcentrated and 20× concentrated infected supernatant fluids. In some instances, unconcentrated fluids were treated with Tween 80 and ether; cell suspensions were treated with ether alone. Preparations were tested for HA activity in dextrose-gelatin-Veronal (DGV) buffer solutions; 0.85% NaCl; Sorenson's phosphate-buffered saline, *p*H 7.2; and a diluent of 0.9% NaCl, 0.1% $CaCl_2$ (anhydrous), and 0.1% $MgSO_4 \cdot 7H_2O$. HA titers were consistently two- to fourfold higher in the saline with added Ca^{++} and Mg^{++} than in DGV. Hemagglutination-inhibition titers of paired human sera were the same in either diluent. It is suggested that the interaction between rubella HA antigen and the red cells of young (less than 1-day-old) chicks may be at least partially ion dependent and that titers are enhanced by increased quantities of divalent cations.

The development of hemagglutination (HA) and hemagglutination-inhibition (HAI) tests for rubella virus provided valuable tools for the detection of antibody to this virus (10). These tests are more rapid and easier to perform than the neutralization test or the detection of antibody by fluorescent staining, and provide more reliable information on susceptibility than does the complement-fixation test. The antigen prepared for this test generally has a low titer. For this reason, a method to improve the antigen titer would be of considerable value.

This report describes an improved diluent for use in the rubella hemagglutination and hemagglutination-inhibition tests.

MATERIALS AND METHODS

RV strain (9) rubella virus in its 9th to 15th passage in baby hamster kidney (BHK-21) cells was used for antigen preparation. Previously, it had been grown in African green monkey kidney tissue and in RK-13 tissue culture cells. The tissue culture was grown in Eagle's minimal essential medium in Hank's balanced salt solution supplemented with 10% heat-inactivated fetal bovine serum, 100 μg of streptomycin, and 100 units of penicillin per ml. Cells were grown in 32-oz prescription bottles and were inoculated when they were 48 to 72 hr old.

Preparation of fluid HA antigen. Fluid HA antigen was prepared from infected supernatant fluids of stationary cultures inoculated with 2.0 ml of undiluted seed virus with infectivity titers of 6 to 7 $\log_{10}$ TCIND$_{50}$/ml. Virus was allowed to adsorb for 1 to 3 hr at 35 C. The tissue was maintained with 30 ml of Eagle's basal medium in Earle's salt solution (EBME) supplemented with 2% heat-inactivated, kaolin-treated fetal bovine serum and antibiotics. The incubation temperature was 35 C. Fluids were harvested when sampling revealed the presence of antigen, generally between days 4 and 8 after inoculation.

Antigen preparations were used with no further treatment or were treated with Tween 80 and ether according to Norrby (6).

Preparation of cell-associated antigen. Cell-associated HA antigen was prepared as a 5% cell suspension of infected tissue. Tissue cultures were maintained with EBME supplemented with 3% heat-inactivated agamma calf serum, 100 μg of streptomycin, and 100 units of penicillin per ml. The cells were inoculated with 1.0 ml of undiluted seed virus with infectivity titers of 5 to 7 $\log_{10}$ TCIND$_{50}$/ml. Four days after inoculation, the cultures were washed three times in ethylenediaminetetraacetic acid (EDTA) and were removed from the glass by means of a rubber policeman. Cells were centrifuged at 500 × *g* for 10 min and were resuspended in EDTA to a 5% concentration. Suspensions were frozen and thawed three times and were used as HA antigen.

Preparation of concentrated sonic-treated antigen. As a third method of antigen preparation, fluids were concentrated to 20 times their original volume by centrifugation at 105,536 × *g* for 120 min in a no. 40 rotor on a model L ultracentrifuge (Spinco Division, Beckman Instruments, Palo Alto, Calif.). Cell sheets were inoculated as in the preparation of fluid HA antigen and were overlaid with 30 ml of EBME. On day 7, the cells and fluid were frozen and thawed three times and centrifuged. Pellets were resuspended in EBME and were sonically treated at 1,000 kc for 10 min.

Each lot of antigen was accompanied by a matching control antigen, prepared in the same manner and at the same time as the test antigen.

HA and HAI tests. Comparative HA tests were performed with four diluents: (i) saline with added Ca^{++} and Mg^{++} [consisting of (w/v) 0.9% NaCl, 0.1% $CaCl_2$ (anhydrous), and 0.1% $MgSO_4 \cdot 7H_2O$]; (ii) dextrose-gelatin-Veronal (DGV; 3) buffer; (iii) Sorsenson's phosphate-buffered saline, *p*H 7.2, and (iv) normal saline. HAI tests were performed in: (i) saline with added Ca^{++} and Mg^{++} ions; and (ii) DGV only.

Red blood cells were obtained by cardiac puncture from unfed 24-hr-old chicks or by wing vein puncture from adult chickens. Cells were stored as 20% suspensions in Alsever's solution. Red blood cells from young chicks were used for only 5 days. Cells from adult chickens were kept until hemolysis was evident. For the tests in saline with added Ca^{++} and Mg^{++}, Sorenson's buffer, and normal saline, red cells were washed three times in normal saline and were resuspended to a 20% (v/v) suspension in the appropriate diluent. For the tests in DGV, the cells were washed three times in DGV and were resuspended to a 20% (v/v) suspension in DGV. Paired human and animal sera were used in the HAI tests.

For HAI tests, pretreatment of the sera was carried out with each of the two diluents, saline with Ca^{++} and Mg^{++} or DGV. In each case, a 1:5 dilution of serum in the diluent was mixed with an equal volume of 25% kaolin in the same diluent and was allowed to incubate, with occasional mixing, for 1 hr at room temperature. The mixture was centrifuged for 20 min at 1,000 × *g*, and 0.1 ml of 50% baby chick red blood cells in the same diluent was added to the tube without removing the supernatant fluid. The serum was incubated for 1 hr at 4 C. The cells were sedimented by centrifugation, and the serum was removed and inactivated at 56 C for 30 min. The final product was considered to be a 1:10 dilution in the HAI tests.

For comparison in several studies, the sera were treated with kaolin and 50% red blood cells in borate saline, *p*H 9.0, according to the method of Halonen (4). The sera were diluted 1:5 with borate saline, *p*H 9.0; an equal volume of 25% kaolin, also in borate saline *p*H 9.0, was added to each tube. The mixture was incubated for 20 min at room temperature and then was centrifuged at 1,000 × *g* for 20 min; 0.1 ml of 50% baby chick red blood cells in borate saline was added to each tube without removing the supernatant fluid. The serum was incubated in the cold for 30 min and centrifuged; the supernatant fluid, now considered to be diluted 1:10, was removed and used in the HAI test. Sera treated in this manner were tested in saline with Ca^{++} and Mg^{++} and in DGV.

For the HA test in tubes (100 × 13 mm), serial twofold dilutions were made and 0.2 ml of red blood cells was added to 0.4 ml of antigen dilution. Red cells were used as 0.25% suspensions in saline with Ca^{++} and Mg^{++}, in DGV, in Sorenson's phosphate buffer, or in normal saline. HAI tests were performed with 0.2 ml of serum dilution, 0.2 ml of antigen, and 0.2 ml of red blood cells.

HA tests were performed in microtiter by use of disposable U or V plates and spiral loops (Microbiological Associates, Inc., Bethesda, Md.), with 0.025 ml of antigen and 0.025 ml of diluent for each dilution. For tests with the saline containing Ca^{++} and Mg^{++} diluent, 0.025 ml of a 0.32% suspension of red cells was added to each dilution. For tests with DGV diluent, the red cells were used at a 0.16% concentration. Microtiter tests with Sorenson's buffer or normal saline were not performed because tube tests with these diluents did not demonstrate HA activity. For a few comparative tests, bovine plasma albumin (Difco) was added to some red blood cell suspensions to a final concentration of 0.25%. Each test included red blood cell-diluent controls and a control antigen prepared from uninfected BHK-21 cells in order to detect nonspecific agglutination of the red blood cells by tissue antigens.

For the HAI tests, 0.025 ml of antigen, diluted to give 4 HA units (HAU), was incubated for 1 hr at 4 C or at room temperature with 0.025 ml of serum which had been treated and diluted with either saline with Ca^{++} and Mg^{++} or DGV. HA titers were determined independently in each system immediately before the HAI test was performed. Red blood cells were added at the concentration given above, and the cells were allowed to settle at 4 C for 1 to 1.5 hr.

Each test included positive (1:160 or greater) and negative (1:10) rubella serum. Red blood cell-serum controls, a simultaneous antigen titration, and red blood cell-diluent controls were also included.

In two instances, five serum samples with known titers were tested by a slight modification of this routine. The serum was diluted and 4 HAU of antigen was added to each dilution; red blood cells were immediately added, and the test was incubated at 4 C for 1 hr.

The final *p*H of the red cell-antigen suspension in the Ca^{++} and Mg^{++} diluent was 7.1; the *p*H of the red-cell antigen suspension in DGV was 7.0 to 7.1.

RESULTS

HA tests. Fluid antigens, cell-associated antigens, and concentrated sonic-treated antigens were tested with four diluents. None of the preparations exhibited HA activity when tested in Sorenson's phosphate-buffered saline, *p*H 7.2, or normal saline. More than 50 antigen preparations were tested in saline with Ca^{++} and Mg^{++} and in DGV. Titers were consistently two- to fourfold higher in the saline diluent with added Ca^{++} and Mg^{++} than in DGV. The results of representative tests with each diluent are shown in Table 1. With very few exceptions, preparations which showed no HA activity in DGV had no activity in saline with Ca^{++} and Mg^{++}. In general, an initial HA titer of 1:4 was necessary before an increase in titer was brought about by a diluent. Antigen titers in either diluent were generally two- to fourfold higher in tubes than in microtiter.

Treatment of the antigen with Tween 80 and ether did not always result in greater antigen titers. Often, a preparation which possessed little or no activity before treatment showed little increase after treatment. On occasion, a preparation which had an HA titer before treatment was negative after treatment. In those instances, the diluent had no effect upon the titer (Table 1).

TABLE 1. *Rubella HA titers in saline with added Ca^{++} and Mg^{++} and in dextrose-gelatin-Veronal (DVG) buffer*

Antigen[a]	Treatment	Titer in saline[b]	Titer in DGV[b]
Fluid antigen			
1		≥1:128	1:32
2		1:16	1:4
3		1:128	1:32
4		1:128	1:32
5		1:64	1:16
6		1:128	1:32
7		1:64	1:8
8		<1:2	<1:2
8	Tween 80-ether	<1:2	<1:2
9		<1:2	<1:2
9	Tween 80-ether	<1:2	<1:2
10		1:32	1:8
10	Tween 80-ether	<1:2	<1:2
11	Tween 80-ether	1:8	<1:2
12	Tween 80-ether	1:32	1:8
13	Tween 80-ether	1:64	1:8
14	Tween 80-ether	1:16	1:2
15	Tween 80-ether	1:128	1:32
16	Tween 80-ether	1:32	1:8
17	Tween 80-ether	1:32	1:8
18	Tween 80-ether	1:128	1:32
Cell-associated antigen			
1		1:64	1:32
2		1:128	1:32
3		1:64	1:16
4		1:32	1:8
4	Sonically oscillated	1:128	1:32
Concentrated sonic-treated antigen			
1		1:256	1:64
2		1:32	1:16

[a] None of the antigens tested exhibited activity in Sorenson's phosphate buffer, *p*H 7.2, or in normal saline.

[b] All titers are based upon microtiter test results with baby chick red blood cells.

Cell-associated antigens were sonically oscillated and the HA titer was determined. The HA titer increased from two- to fourfold in each instance. The increase was apparent in both diluents.

Preparations which showed a high degree of HA activity when titered in saline with Ca^{++} and Mg^{++} with red blood cells from young chicks were tested in both diluents with red blood cells from adult chickens. Titers were from four- to eightfold lower than they had been with the red blood cells from baby chicks. Titers in the Ca^{++} and Mg^{++} enhanced diluent ranged from 1:2 to 1:32; titers in DGV ranged from 1:2 to 1:16. Antigen titers in DGV must be based upon partial agglutination, as complete agglutination occurred only at dilutions of 1:2. Tests performed in saline with increased ion concentrations showed complete agglutination to 1:16.

Autoagglutination of the red cells in the saline with Ca^{++} and Mg^{++} diluent was occasionally observed. This phenomenon, which has been reported previously for cells in a nonproteinaceous diluent (1, 5), could be overcome by the addition of 0.2 ml of a 30% solution of bovine plasma albumin to each 25 ml of red cell suspension and by the incorporation of 1% dextrose into the diluent at the time of preparation. The incorporation of dextrose into the diluent resulted in longer stability of the washed red blood cell preparation; as a result of this, the cells did not require fresh preparation daily and could be used over a 2-day period with no loss of sensitivity. The 0.32% suspension of red cells was slow to settle in microtiter plates and, without the addition of bovine plasma albumin, required a minimum of 3 hr at 4 C. A 0.16% suspension of red blood cells in the saline with Ca^{++} and Mg^{++} did not settle in microtiter plates; the addition of bovine plasma albumin to the diluent did not enhance the settlement of the red blood cells at this dilution. The addition of the bovine plasma albumin had no apparent effect upon HA or HAI titers and reduced the incubation period by 50%.

TABLE 2. *Comparison of rubella HAI titers of human serum in saline with added Ca^{++} and Mg^{++} and in dextrose-gelatin-veronal (DGV) buffer*[a]

Serum	Titer in saline		Titer in DGV	
	Preserum	Postserum	Preserum	Postserum
BE	<1:10	1:4096	<1:10	1:4096
GI	<1:10	1:128	<1:10	1:128
BO	<1:10	1:5120	<1:10	1:5120
PH	<1:10	1:640	<1:10	1:640
20	1:10	1:160	1:10	1:80
ME	<1:10	1:640	<1:10	1:640
RAB	<1:10	1:1280	<1:10	1:1280
ML		1:1280		1:1280

[a] Sera were tested with fluid antigen diluted to give 4 HAU in an appropriate diluent.

HAI test. A total of 30 paired human and animal sera were tested for rubella antibody in the saline with added Ca^{++} and Mg^{++} and in DGV. Serum titers were the same or within twofold agreement in each instance. Table 2 gives the HAI titers of representative sera.

No difference in serum titers was observed when antigen and serum were allowed to incubate at room temperature and when plates were held at 4 C in an ice-water slurry as recommended by Halonen (4). There was also no difference in

titer when red blood cells were added immediately after serum and antigen were mixed together.

Discussion

The effect of hydrogen ion concentrations (4, 8, 11) and the absence or presence of divalent cations, such as Mg^{++} and Ca^{++}, upon virus cell interaction and hemagglutination (2, 8) has been demonstrated. In the present studies, higher titers were obtained with the saline with added Ca^{++} and Mg^{++}. These titers were two- to fourfold higher than those obtained with the DGV diluent. The interaction between rubella virus and the red cells of newly hatched chickens is apparently partially effected by the ion concentration of the diluent. The Ca^{++} and Mg^{++} ions in the diluent enhance the union of the antigen and red cells, perhaps by bringing available receptor sites into position or by providing holding forces which bind the antigen and red cell together to allow hemagglutination. Both the saline diluent and DGV contain Ca^{++} and Mg^{++}. However, the ion-enhanced saline contains 100 times as much Ca^{++} and 10 times as much Mg^{++} as does the DGV. Puck, Garen, and Cline (7) have postulated that, in the collision of virus and red cell, electrostatic forces which are mediated by ions, especially Ca^{++}, are responsible for bringing groupings into position and that this provides the holding forces to keep virus and red cell together. It may be that certain ion concentrations in the saline diluent increase the titer by providing for more receptor sites and firmer union between antigen and red cell. This increased ionic strength may also account for the more complete patterns of agglutination seen with adult chick red cells in the saline with Ca^{++} and Mg^{++} diluent.

Halonen (4) has reported increased rubella HA titers in borate-saline diluent which result in a final *p*H of 6.2 for the HA test. The final *p*H of the test in saline with Ca^{++} and Mg^{++} is 7.1. It would appear that a low *p*H is not essential for increased antigen titers.

If the antigen titers obtained with the saline diluent containing added Ca^{++} and Mg^{++} were a reflection of something other than rubella hemagglutination, this would be reflected in HAI test results. Sera known to possess rubella antibody would not show this in the HAI test, and the expected rise in antibody titer in paired serum samples would not be demonstrable. However, this does not occur and one must conclude that increased HA titers are the result of true rubella HA antigen-red cell interaction; saline with added Ca^{++} and Mg^{++} somehow makes more antigen available for reaction.

The use of saline with increased ionic strength as a diluent for rubella HA and HAI determinations has the advantage of increasing antigen titers, thus allowing greater use of an antigen preparation. At the same time, serum titers are not increased and the danger of detecting "false positive" serum titers is minimized.

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