

Use of Trypsin-Modified Human Erythrocytes in Rubella Hemagglutination-Inhibition Testing

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Received for publication 21 April 1972

A method of treating human erythrocytes with trypsin has been modified and found to be an efficient and practical indicator system for the rubella hemagglutination-inhibition test. Both the trypsin-treated human cells and the widely used, newborn chicken erythrocytes were used in comparative testing of 464 selected diagnostic rubella serums. Results with each cell system were essentially the same. The trypsin treatment procedure has been found to be relatively simple, and with our limited testing has not presented any problems with reproducibility. Other advantages include the ready availability of human cells, greater intralaboratory standardization of the test by using the same donors over a long period of time, and elimination of adsorption of test sera with red blood cells.

Since the introduction of the rubella hemagglutination-inhibition test in 1967 (9), erythrocytes of several animal species, primarily avian, have been recommended for use in the test (4, 6, 9). The most commonly employed have been cells from newly hatched chickens. These cells can be used for only a period of 2 weeks after collection. Laboratories desiring to offer the test on routine basis must, therefore, either purchase cells or make some arrangement to have newly hatched chickens available on a regular weekly or biweekly schedule. Many small laboratories, with little initial demand for the test, find this economically impractical.

One commercial laboratory, Abbott Diagnostic Laboratories, has for some time been marketing stabilized avian cells, with a usable life of 60 days. A method for the production of stable, formalinized sheep cells has been published (3). The stabilized avian cells are only available commercially, and the sheep cell technique is quite time consuming.

In 1971, Biddle (1) reported the use of trypsinized human cells in rubella hemagglutination and hemagglutination-inhibition. The ready availability of human cells to all laboratories would make their use preferable to those cell systems currently available. This paper reports our experience in applying human cells to routine rubella testing and our modification of this reported technique.

MATERIALS AND METHODS

Alsever's solution, dextrose-gelatin-Veronal (DGV) buffer, 5.0% dextran SO_4 , 1.0 M calcium chloride, and *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (HEPES)-saline-albumin-gelatin (HSAG) buffer (pH 6.2) were all made in this laboratory as previously recommended (2) except that bovalbumin was used in a final concentration of 0.5% instead of 1.0%. Phosphate-buffered saline (PBS), pH 7.2, was used as described (5). (Both the HEPES and the dextran sulfate were from Nutritional Biochemicals Co., Cleveland, Ohio.)

Trypsin, lyophilized (Difco) or 3× crystalline trypsin, 100 mg/ampoule (lyophilized; Nutritional Biochemicals Co.) was used for treatment of the red cells.

Human blood was collected from five volunteers as a source of fresh cells and was mixed with an equal volume of Alsever's solution. In addition, three units of packed red cells were obtained from the Badger Regional Blood Center, American National Red Cross, Madison, Wis., on the date of expiration for transfusion purposes (21 days after collection). The cells were held at 4 C in the anticoagulant used for collection until trypsinization. All were Rh-positive cells except one of the units from the blood bank.

Newly hatched 2-day-old chickens' blood was collected in Alsever's solution on a weekly basis. These cells were washed three times in DGV immediately following the bleeding. They were then suspended at 10% concentration and held at 4 C in DGV.

Sera tested were received for routine rubella serology and represent the spectrum of ages with which most laboratories performing this test would

be concerned. The majority, 357 of 464, were from young adult women who were possibly exposed to rubella during their first trimester of pregnancy, and 27 sera were from children ranging in age from newborn to 14 years of age.

Antigens were prepared in this laboratory in either Vero or BHK-21 tissue culture and extracted with Tween 80 and ether. All were used in concentrations to give 4 hemagglutinating units per 0.025 ml.

Treatment of sera for removal of nonspecific inhibitors was by the dextran sulfate-calcium chloride procedure as previously described in earlier work from this laboratory (*manuscript submitted for publication*). To a tube (12 by 75 mm) containing 0.2 ml of serum and 0.35 ml of HSAG, 0.05 ml of 5.0% dextran SO₄ and 0.1 ml of 1.0 M CaCl₂ were added using microtiter droppers. The tubes were held at 4 C for 30 min. After precipitation, those sera to be adsorbed with red blood cells received 0.1 ml of a 10% cell suspension of chicken red blood cells and were incubated at 4 C for 30 min. After adsorption, 0.8 ml of HSAG was added, to bring the total dilution at this point to 1:8. Those sera not to be adsorbed with erythrocytes received 0.9 ml of HSAG to complete the 1:8 initial dilution. After the addition of HSAG, the tubes were centrifuged for 10 min at 900 × *g*. The clear supernatant fluid was then ready to test.

Sera tested with human type O cells were not routinely adsorbed; those tested with type A, B, or AB cells were adsorbed with a 10% concentration of trypsinized cells of the same blood type for 30 min at 37 C.

The previously cited paper (1), reporting trypsin treatment of cells, used crystalline trypsin at the rate of 1 mg for each ml of a 0.5% red blood cell suspension in PBS. We found working at that cell concentration (0.5%) inconvenient because large volumes had to be handled for washings. Preliminary studies showed no appreciable difference in effect, using 1 mg of trypsin/ml of cell concentrations at 0.5, 1.5, 3.0, 6.0, and 10%. We therefore used a 10% cell concentration throughout these studies.

Since trypsin (Difco) is a readily obtainable type of trypsin, studies were undertaken to evaluate this material. However, this introduced another problem, in that the activity of this material is standardized in terms of proteolytic activity rather than in milligrams of purified trypsin. Tests using the reconstituted trypsin undiluted, 1:2, 1:4, 1:8, and 1:16, at a ratio of 0.1 ml/ml of 10% cells showed that the first three dilutions gave essentially the same effect as the crystalline trypsin. Greater dilutions resulted in reduced antigen titers. To use the most effective concentration which might be expected to give minimal cell damage, the 1:4 dilution was chosen.

The trypsinization procedure was as follows. (i) Erythrocytes were washed three times in PBS by centrifuging at 600 × *g* in a 165-mm centrifuge head. (ii) Concentration was adjusted to 10% by reading the packed-cell volume and adding PBS. (iii) Either 0.1 ml of crystalline trypsin aqueous solution containing 10 mg/ml or 0.1 ml of a 1:4 dilution of

trypsin in water for each milliliter of cells was added. (iv) Incubation was at room temperature for 1 hr after mixing by gently inverting the tube. (v) The trypsinized cells were washed in PBS as in step i, and the cells were made to a 10% final concentration and stored at 4 C until used.

The procedure using newly hatched chicken erythrocytes was similar to that previously described (2). For the procedure with trypsinized human cells, two changes were introduced. The cell concentration was increased to 0.3%, and the final incubation of the tests after adding cells was at 37 C. These changes were made since agglutination patterns were observed to be best at these values. Antigen titers run simultaneously at 4 C, room temperature, and 37 C showed no difference in levels of sensitivity, but the settling patterns of the cells were best at 37 C.

Microtitration U-plates were used throughout all tests.

RESULTS

Freshly drawn human erythrocytes of each blood type collected in Alsever's solution were washed and treated with trypsin as described. Freshly drawn cells from newly hatched chickens were tested both with and without trypsin treatment. As shown in Table 1, trypsin treatment had no effect upon the chicken cells, whereas in each case the trypsinized human cells showed antigen titers equal to or greater than the chicken cell titer. Later tests using another source of fresh type O, Rh-positive cells and three units of outdated type O blood, all 21 days old from the blood bank, showed similar results. All of the freshly drawn human cells could be held at 4 C in Alsever's solution for a minimum of 4 weeks after collection. During the first 3 weeks, these cells could be trypsin-treated and held for 3 to 5 days be-

TABLE 1. Rubella hemagglutination titers of treated and untreated erythrocytes

Cell treatment	Type of red blood cell				
	0- to 2-day-old ^a chicken	Hu-man ^b O	Hu-man A	Hu-man AB	Hu-man B
No trypsin	1:128	<1:8	1:16	<1:8	<1:8
Trypsinized with 1:4 trypsin	1:128	1:256	1:256	1:128	1:128
Crystalline trypsin	1:128	1:128	1:256	1:128	1:256

^a Test run at 4 C. Cell concentration = 0.25%.

^b All titers with human cells were similar at 4 C, room temperature, and at 37 C. Most uniform pattern of agglutinated cells was seen at 37 C. Cell concentration = 0.3%.

fore objectionable hemolysis could be seen. After 3 weeks, best results were obtained by processing the cells to be used either the morning of the test or the day prior to testing. One of the three bloods obtained from the blood bank was usable for only 1 week. Demonstrable agglutination without trypsin treatment was seen in only one sample of human blood, type A, and the antigen titer was lower than after treatment.

Trypsinized human type O cells were used in parallel with newly hatched chicken cells to test 282 sera. The results are shown in Table 2. In no cases was there more than a twofold variation in results, nor was there disagreement in results of negative (<1:8) titers. The same titer was obtained with 200 of the 282 sera (71.0%); 56 (19.8%) showed one twofold dilution higher titer with human cells, and 26 (9.2%) showed one dilution lower. Five sera showed nonspecific agglutinins using type O, Rh-positive cells without adsorption. All of these could be read following adsorption at 37 C for 30 min with 10% human type O, Rh-positive cells. Four of these five showed no agglutination when tested with Rh-negative cells. The fifth still required adsorption.

Comparative testing using type A, Rh-positive cells was done on 182 sera. As seen in Table 3, 53 of the 182 (29.1%) showed titers twofold higher with human A cells, and 21 (11.6%) showed one dilution lower. Identical titers were shown by 108 of the 182 serums (59.3%). Due to the expected high occurrence of anti-A isoagglutinins, these sera were routinely adsorbed with 0.1 ml of a 10% concentration of A cells for 30 min at 37 C. When

tested without adsorption, 97 of 182 (53.3%) showed agglutinins ranging from 1:8 to 1:256. In no case did the adsorption fail to remove the agglutinin.

To test the efficiency of removal of anti-Rh antibodies from sera by adsorption with Rh-positive cells, we obtained eight sera from the Immunology Section of the State Laboratory of Hygiene, with Coomb's titers of 1:4 through 1:2,048. The results of these tests are

TABLE 3. Comparison of rubella hemagglutination-inhibition titers on 182 sera by using trypsinized human type A and 0- to 2-day-old chicken cells

Titers with human A red cells	Titers with 0- to 2-day-old chicken cells								
	<1:8	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024
<1:8	37								
1:8		1							
1:16			7	3					
1:32			2	10	6				
1:64				11	22	6			
1:128					10	20	2		
1:256						13	7	1	
1:512							10	2	3
1:1,024								7	2

TABLE 4. Results with trypsinized human type O and chicken red blood cells by using sera with known Coomb's-positive reactions

Specimen no.	Coomb's titer	Rubella hemagglutination-inhibition titer			
		O Positive, not adsorbed	O Positive, adsorbed at 37 C	O Negative, not adsorbed	Chicken adsorbed at 4 C
927	1:4	1:256	1:128	1:128	1:256 (1:32) ^a
270	1:8	1:64	1:32	1:32	1:64
429	1:8	1:128 (1:32) ^a	1:128 (1:32) ^a	1:128	1:128
969	1:16	<1:8	<1:8	<1:8	<1:8
995	1:16	<1:8 (1:8) ^b	<1:8 (1:8) ^b	<1:8	<1:8 (1:8) ^b
717	1:32	NS ^c	NS ^c	1:32	1:32 (1:8) ^a
112	1:128	1:128 (1:32) ^a	1:128 (1:32) ^a	1:128	1:128
726	1:2,048	NS ^c	NS ^c	1:32	1:64 (1:8) ^a

^a Nonspecific agglutinin present at this level.

^b Partial nonspecific agglutinin at the 1:8 serum control.

^c Nonspecific agglutinin prevented reading of an endpoint.

TABLE 2. Comparison of rubella hemagglutination-inhibition titers on 282 sera by using trypsinized human type O and 0- to 2-day-old chicken cells

Titers with human O red cells	Titers with 0- to 2-day-old chicken red cells									
	<1:8	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1,024	1:2,048
<1:8	53									
1:8		2								
1:16			1	11						
1:32			16	26	4					
1:64			2	6	29	6				
1:128				13	27	27	1			
1:256					19	30	30			
1:512						10	12	2		
1:1,024							4	4	1	
1:2,048								2	1	1

shown in Table 4. Five of the eight could be read with or without adsorption with O positive cells, although two of these still contained agglutinins below the true antibody level. Adsorption of those sera showing nonspecific agglutination with type O, Rh-positive cells failed to remove or reduce the agglutinin. Using O negative cells and no adsorption, all eight could be read with no problem.

Another potential problem in using human cells is the possibility of cold agglutinins interfering with the test. As reported above, viral agglutination patterns were best at 37 C, and the antigen titers were essentially the same at 4 C, room temperature, and 37 C. It seemed likely that the use of incubation at 37 C after addition of the indicator cells to the plates would avoid this problem; however, to test the system, we obtained six sera from the Immunology Section with known cold agglutinin titers ranging from 1:16 through 1:4,096. The only serum that showed any nonspecific agglutinin was one with a cold-agglutinin titer of 1:4,096, and that could be removed by adsorption at 4 C, but not at 37 C.

DISCUSSION

The use of human erythrocytes for the rubella hemagglutination-inhibition test provides several advantages over the presently recommended systems. Availability of human cells eliminates one major problem. For the laboratory doing a small volume of tests, 10 to 20 ml

of whole blood from laboratory personnel or a local source is adequate for most requirements. Since the use of type O cells would virtually eliminate the need for adsorption of sera with concentrated cell suspensions, the necessity for large volumes of blood is reduced. For those laboratories doing larger volumes of testing, it would be practical to use packed cells from outdated blood-bank sources, if such cells could be available weekly or biweekly.

A further possible advantage to the use of human cells is the opportunity to standardize the source of cells by using blood from one individual, if not too large a volume of testing is to be done. Since fresh blood may be held for a minimum of 4 weeks, a small volume of blood could be drawn from the same person or persons, once each month. Tests in our laboratory show that, from a total volume of 3 to 4 ml of blood (50% in Alsever's), a total of about 200 sera may be tested through nine serum dilutions, if no adsorption is necessary.

The problem of anti-Rh antibodies may be eliminated, if encountered, by use of Rh-negative cells. None of the sera with known Coomb's-positive titers showed nonspecific agglutinins with type O, Rh-negative cells with no adsorption. Of the 282 clinical specimens tested with type O cells, 24 were done with Rh-negative cells. The five sera that did show nonspecific agglutinins with no adsorption were tested for anti-Rh antibodies and were found to be negative. Since only 5 of 258 (1.9%)

TABLE 5. Rubella hemagglutination-inhibition titers using trypsinized cells with homologous and heterologous sera

Blood type used	Serum from blood type							
	A		B		AB		O	
	Ads ^a	NA ^b	Ads	NA	Ads	NA	Ads	NA
Human A	1:64	1:32	1:256	1:128 (1:8) ^c	1:8	1:8	1:64	1:64 (1:32)
Human B	1:64	1:64 (1:8)	1:256	1:256	1:8	1:16	1:64	1:64 (1:16)
Human AB	1:64	1:64 (1:16)	1:256	1:256 (1:8)	1:8	1:8	1:64	1:128 (1:32)
Human O	1:32	1:32	1:256	1:128	1:8	1:8	1:64	1:64
Chicken	1:64	1:64 (1:16)	1:256	1:256	1:8	1:8	1:128	1:128

^a Serum adsorbed with 0.1 ml of a 10% concentration of trypsinized cells used for the test. Adsorption was done at 37 C for 30 min.

^b Serum not adsorbed.

^c Figures in parentheses indicate level of nonspecific agglutinin.

showed nonspecific agglutinins with type O, Rh-positive cells with no adsorption, we feel that the use of these cells is satisfactory, although in situations where Rh-sensitized women may be encountered, Rh-negative cells might be more ideal.

Although we have used only the most common blood types, A and O, with the HAI test, indications are that any human blood could be used as long as the test sera are absorbed with the specific cells used in the test (Table 5). The use of types A, B, or AB, however, would offer no advantage and would lengthen the processing time necessary, since adsorption would be needed.

One difficulty in working with human type O cells is the possibility of interference by cold agglutinins. This problem is eliminated by incubation at 37 C for the final incubation phase of the test.

A recent paper (8) reported the hemagglutination by rubella virus of red cells from a number of species previously reported not to be usable for rubella testing. Among these was a human type O, which gave titers slightly lower than newly hatched chicken cells. None were trypsin-modified. A more recent paper expanded upon the use of human O cells (7). In our experience, only one of eight human bloods showed any demonstrable hemagglutination with rubella antigen without trypsin modification, and this showed a titer eightfold lower than chicken cells. All eight human cells worked equally well following trypsinization. It is possible that human cells from certain individuals may be usable with no treatment, but, with this trypsin modification, we have had uniformly good results with all cells tested.

Most procedures recommend the use of microtiter V-plates for the rubella hemagglutin-

ation-inhibition test. We recommend V-plates for this procedure as well since on occasion tests run with U-plates were difficult to read and showed poor endpoints.

ACKNOWLEDGMENTS

We gratefully acknowledge the cooperation of R. Golub-jatnikov, Jean Koehler, and Margaret Nadeau of the Immunology Section of the State Laboratory of Hygiene in supplying the special sera for testing the effect of cold agglutinin and anti-Rh titers and their testing of the five sera that showed nonspecific agglutination for anti-Rh activity.

We also gratefully acknowledge the cooperation of P. Nowrasteh of the Badger Regional Blood Center for her help in supplying packed blood-bank cells and Donn D'Alessio, Herbert Brecht, and Lynn Strangstalien, who, along with two of us (E.P.Q. and D.B.N.), contributed the fresh cells used.

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