AN AGGREGATE-HEMAGGLUTINATION TEST FOR ANTIERYTHROCYTIC ANTIBODIES

A. M. Olovnikov, M. M. Koifman, L. G. Gorina, and L. I. Idel'son

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An aggregate-hemagglutination test for antierythrocytic antibodies is suggested. In one of its forms it is three times as sensitive as the indirect Coombs' test. The test is based on agglutination of the erythrocytes under investigation on treatment with test-erythrocytes coated with aggregated antiglobulin serum proteins.

KEY WORDS: aggregate-hemagglutination; antierythrocytic antibodies; autoimmune hemolytic anemia; Coombs' test.

The antiglobulin method of determination of antierythrocytic antibodies, suggested by Coombs et al. [12, 13], has been widely used because of its high sensitivity. However, the direct Coombs' test is negative in a large proportion of patients with autoimmune hemolytic anemia (AIHA) [1, 18], evidently because of the inadequate sensitivity of the Coombs' test [15, 16].

In this connection it was decided to try to develop a more sensitive method of detecting antierythrocytic antibodies by the use of the aggregate-hemagglutination test developed previously [7, 8] for the detection of soluble antigen.

A new test based on combined agglutination of the erythrocytes for investigation, carrying the required antierythrocytic antibodies on their surface, and test erythrocytes coated with aggregated antiglobulin serum is suggested in this paper. Test erythrocytes, stabilized and activated with glutaraldehyde and coated with aggregated protein of an immune serum, are used in the test. Aggregation of the protein enables the active centers of the antiglobulin antibodies to be removed from the surface of the test erythrocyte, so that the active centers of the antibodies are made accessible to the determinant of the antigen, in this particular case the antigenic determinant of the specific immunoglobulin located on the surface of the erythrocyte of the patient under investigation.

The following antisera were used: donkey's antiserum against rabbit IgG, and rabbit antiserum against total human globulin and sheep's globulin, produced by the N. M. Gamaleya Institute of Epidemiology and Microbiology and the Central Blood Transfusion Station, Ministry of Communications; sheep's antiserum against rabbit IgG, kindly provided by P. Z. Budnitskaya and A. I. Gusev, and rabbit and sheep antisera against human immunoglobulin of the G-, M-, and A-classes respectively, kindly provided by E. V. Chernokhvostova.

Erythrocytes from a blood donor of group O(I), Rh'_0"-(negative), stabilized and activated by glutaraldehyde (GLA) [2, 9, 10, 17] in the following modifications, were used as test erythrocytes to be sensitized with the aggregated protein of the antiglobulin serum. One volume of washed residue of erythrocytes was treated with two volumes physiological saline and 0.03 volume of a 25% solution of GLA (Merck), incubated from 3 h at 37°C, washed with physiological saline, and stored as an 8% suspension in 0.25% GLA in the

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presence of 1:10,000 merthiolate in physiological saline at 4°C, and washed with physiological saline before sensitization.

To neutralize the antierythrocytic antibodies present in the antiglobulin sera, instead of ordinary absorption by erythrocytes, in most investigations the sera were treated with amniotic fluid (AF) material, lyophilized without preliminary dialysis, containing O, A, and B group antigenic determinants [5, 19]. AF was added in the following amounts: 5 mg of each of the three groups (O, A, and B) to 1 ml rabbit antiserum, 15 mg to 1 ml donkey antiserum, and 25 mg to 1 ml sheep antiserum. The AF does not dissolve completely in the sera, but this does not affect their subsequent aggregation, which may begin immediately after the addition of AF. Insoluble material was removed from the serum after completion of aggregation by centrifugation for 15 min at 8,000 rpm on the TsLN-2 centrifuge.

To aggregate the antiserum proteins a 2.5% solution of GLA was added in the following proportions: 0.08 ml GLA to 1 ml rabbit serum and 0.1 ml GLA to 1 ml sheep or donkey serum; incubation followed for 1 h at 37°C.

To sensitize the test erythrocytes, the residue from 1 ml of 8% suspension of stabilized erythrocytes was treated with 1 ml aggregated antiserum and 20 mg $NaHCO_3$ and incubated for 18-20 h at 37°C or for 90 min at 56°C. The residue was washed with physiological saline, suspended in 10 ml of the same solution, preserved with 1:10,000 merthiolate or with sodium azide up to a final concentration of 0.02%, or with 1-2 drops chloroform, and kept at 4°C without loss of activity for 1 month. The direct and indirect Coombs' test was performed in the usual manner [12,13].

Anti-Rh_0(D)- and anti-Rh'_0(DC)-sera obtained from donors immunized with the corresponding antigen were used as the source of incomplete antibodies, not agglutinating in physiological saline, in model experiments. In the model investigations three drops of each of a series of double dilutions of each anti-Rh serum was incubated for 30 min at 37°C with one drop washed residue of erythrocytes from a blood group O(I) Rh+ (positive) donor. The erythrocytes were washed and tested in parallel series in the aggregate-hemagglutination test and the Coombs' test.

EXPERIMENTAL RESULTS

In order to use the advantages both of the Coombs' test and of the aggregate-hemagglutination method, two alternative forms of the new test, called the aggregate-hemagglutination test for antierythrocytic antibodies, were developed.

The following conditions were found to be optimal. The first, or antiglobulin, form of the aggregate-hemagglutination test: one drop of a 50% washed suspension of erythrocytes for investigation (cells sensitized with the antibodies for investigation either in vivo, in the case of AIHA, or in vitro, for the model investigations) is mixed with a glass rod on a white tile with one drop of the working suspension of test erythrocytes, sensitized by aggregated rabbit antiserum against human globulin and the appearance of agglutination is watched for in the course of 10 min at room temperature.

The second, or anti-antiglobulin, form of the aggregate-hemagglutination test: one drop of washed residue of the erythrocytes for investigation is incubated for 30 min at 37°C with three drops of antiglobulin solution (in the model investigation 3 drops of rabbit antiserum against human immunoglobulins, taken in a dilution equal to the working titer of antiglobulin in the Coombs' test); the residue is washed to remove protein not bound with the erythrocyte and one drop of a 50% suspension of cells is mixed with a glass rod on a white tile with one drop of working suspensin of test-erythrocytes sensitized with aggregated anti-antiglobulin serum (in the model experiment, sheep or donkey serum against rabbit IgG) and agglutination is read over a period of 10 min at room temperature.

Comparison of the sensitivity of these two forms of the aggregate-hemagglutination test with the sensitivity of the indirect Coombs' test was carried out in model investigations with anti-Rh sera not agglutinating under the conditions specified. The results are given in Table 1.

As Table 1 shows, the sensitivity of determination of anti-Rh antibodies when the first form of the aggregate-hemagglutination test was used was on the average 16-30 times greater than the sensitivity of the indirect Coombs' test. When the second form of the test was used, with test erythrocytes coated with aggregated sheep antiserum, the sensitivity of determination of anti-Rh antibodies was three orders of magnitude greater (Table 1) than the sensitivity of the indirect Coombs' test. The sheep serum against rabbit IgG used was approximately four times stronger than the donkey serum, whether the test erythrocytes were

TABLE 1. Determination of Anti-Rh Antibodies in Model Experiments

No. of anti-Rh sera	Indirect Coomb's test	First form of aggregate-hemagglutination test	Second form of aggregate-hemagglutination test		Test with unaggregated antisera against rabbit IgG	
			test erythrocytes with sheep antiserum against rabbit igG	test erythrocytes with donkey antiserum against rabbit IgG	sheep antiserum	donkey antiserum
1	2—4	—	2 048 (1024—512)	512 (256—128)	64 (32—16)	16 (8—4)
2	4	—	4 096 (1024)	1 024 (256)	64 (16)	16—32 (4—8)
3	8—16	—	32 768 (4096—2048)	40 96 (512—256)	128—256 (16—8)	64 (8—4)
4	16	512 (32)	65 536 (4096)	—	—	—
5	16—32	1 024 (64—32)	131 072 (8192—4096)	—	—	—
6	16	512 (32)	16 384 (1024)	—	—	—
7	16	—	32 768 (2048)	8 192 (512)	256 (16)	64 (4)
8	16—32	—	65 536 (4096—2048)	16 384 (1024—512)	512 (32—16)	128 (8—4)
9	32	—	131 072 (4096)	32 768 (1024)	1 024 (32)	128 (4)
10	32	1 024 (32)	131 072 (4096)	—	—	—
11	32	1 024 (32)	131 072 (4096)	—	—	—
12	64	—	131 072 (2048)	32 768 (512)	1 024—2048 (16—8)	256 (4)
13	64	4 096 (64)	1 048 576 (16384)	—	—	—
14	64	2 048 (32)	524 288 (8192)	—	—	—
15	64	2 048 (32)	524 288 (8192)	—	—	—
16	128—256	—	65 536—131 072 (512)	16 384 (128—64)	2 048 (16—8)	512 (4—2)
17	128	4 096 (32)	2 097 152 (16384)	—	—	—
18	128	2 048 (16)	131 072 (1024)	—	—	—
19	256	—	131 072 (512)	32 768 (128)	1 024—2 048 (4—8)	1 024 (4)
20	256	—	262 144 (1024)	32 768 (128)	1 024 (4)	1 024 (4)
21	256	4 096 (16)	1 048 576 (4096)	—	—	—
22	256	4 096 (16)	131 072 (512)	—	—	—
23	512	—	262 144 (512)	65 536 (128)	2 048—4 096 (4—8)	2 048 (4)
24	512	—	262 144 (512)	65 536 (128)	4 096 (8)	2 048 (4)
25	512	4 096 (8)	262 144 (512)	—	—	—
26	512	4 096 (8)	262 144 (512)	—	—	—

Legend. Reciprocals of titers given; enhancement factor compared with indirect Coombs' test given in parentheses.

TABLE 2. Detection of Antierythrocytic Antibodies in Patients (number of cases)

Diagnosis	Direct Coombs' test					First form of aggregate-hemagglutination test, given negative results of the direct Coombs' test				Second form of aggregate-hemagglutination test, given negative results of first form				
	negative results	positive results				negative results	positive results			negative results	positive results			
		G+M	G	M	A		G	M	A		G+A	G	M	A
		immunoglobulins					immunoglobulins				immunoglobulins			
AIHA, idiopathic form	76	5	120			52	22		2	4		47		1
Partial red-cell aplasia (AIHA with antibodies against bone marrow normoblasts)	23					22			1	1	2	5		14
Symptomatic forms of AIHA in diseases:														
Chronic lymphatic leukemia	43		34			36	5		2	7		24	1	4
Acute leukemia	8					6	2			1		5		
Systemic lupus erythematosus	6		8			5	1					5		
Chronic lymphatic leukemia without symptomatic forms of AIHA	66					66				66				
Acute leukemia » » »	28					28				28				
Systemic lupus erythematosus » » »	12					12				12				
Familial microspherocytosis	33					33				33				
Paroxysmal nocturnal hemoglobinuria	49					49				49				
Hypoplastic anemias	21					21				21				
Deficiency of glucose-6-phosphate dehydrogenase activity	2					2				2				
Paraproteinemic hemoblastoses (multiple myeloma, Waldenstrom disease)	17					71				17				

used or the work was carried out with unaggregated serum. The sensitivity of determination was 32-128 times greater when the test erythrocytes were used in the second form of the test compared with when native unaggregated sheep or donkey serum was used (Table 1). The prozone phenomenon was not observed during the performance of the aggregate-hemagglutination test.

In subsequent investigations the specificity of the test and its possible diagnostic role were studied.

False positive results were absent during the investigation of 329 donors by the aggregate-hemagglutination test in the first form and 708 donors in the second form.

The results of investigation of certain categories of patients are given in Table 2. In all patients with positive results of the test the diagnosis of AIHA was confirmed clinically. Althogether 24 patients in whom the antierythrocytic antibodies belong entirely to class A immunoglobulins were discovered. In the world literature, incidentally, only a few cases of the discovery of such patients by means of the Coombs' test are described [14, 20].

Several methods of increasing the sensitivity of the Coombs' test are known [3, 4, 6], in which the antibodies located on the erythrocytes to be investigated are bound by a free valency with the antigenic determinant of the immunoglobulin, whether free or fixed on the erythrocytes. By using one of these methods [6], antibodies were found in 17 of 30 cases when the results of the direct Coombs' test were negative but the results of the first form of the aggregate-hemagglutination test were positive. In no case of AIHA, when the results of the first form of the aggregate-hemagglutination test were negative and the results of the second form were positive, could antibodies be found by the method of Zotikov et al. [6]. The use of polyvinylpyrrolidone in the Coombs' test in order to determine anti-Rh antibodies [11] increases its sensitivity by not more than 40 times. The aggregate-hemagglutination test, which in one form is about 1000 times more sensitive than the Coombs' test, will presumably be useful as a method of detecting not only antierythrocytic antibodies but also antiplatelet, antiallotypic, and other types of antibodies.

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