

Relationships between C3b receptor (CR1) activity of erythrocytes and positive Coombs' tests

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SUMMARY Although positive direct Coombs' tests occur in most patients with active systemic lupus erythematosus (SLE), haemolytic anaemia associated with antibody to erythrocytes (E) occurs in less than 10%. Our studies show an association between positive direct Coombs' tests and both the presence of circulating immune complexes and diminished activity of the C3b receptor (CR1) of E. Data presented in this report suggest that *in vivo* binding of immune complexes and complement by the CR1 of E results in positive direct Coombs' tests in the absence of antibody to E. These observations explain the low frequency of haemolytic anaemia compared with the high frequency of direct positive Coombs' tests in patients with SLE.

Key words: circulating immune complexes.

Anaemia is common in systemic lupus erythematosus (SLE). Its features and multiple causes have been reviewed.¹ Although positive direct Coombs' tests have been reported in 18-65% of these patients, haemolytic anaemia occurs in less than 10%.² A reasonable explanation for this discrepancy appeared during our studies of the complement receptor (CR1) function of erythrocytes (E).

Human E possess, on their surfaces, receptors to the third complement component. In 1981 we reported a new method for measuring circulating immune complexes (CIC) derived from the immune adherence haemagglutination (IAHA) phenomenon.³ Subsequently we reported a method of measuring CR1 binding activity of E, which involved the inhibition of IAHA.⁴ In the application of these methods to clinical situations we observed that both the presence of CIC and blunting of CR1 activity were consistently associated with direct positive Coombs' tests. With one exception in our studies there was no association of positive Coombs' tests with haemolytic anaemia.

In this paper we report our observations on the

relation between types of positive direct Coombs' tests and the presence of CIC and diminished CR1 activity in patients with SLE and related CIC mediated syndromes.

Materials and methods

CLINICAL MATERIALS

Three hundred and eleven venous blood samples were obtained from 205 patients. Heparinised blood for E isolation and untreated blood for serum were collected. Patient populations were as follows: 164 blood samples from 103 patients with SLE, 46 from 26 patients with rheumatoid arthritis, 33 from 18 patients with vasculitis, nine from six patients with nephrotic syndromes, eight from six patients with chronic active hepatitis, six from four patients with idiopathic pulmonary fibrosis, four each from two patients with dermatitis herpetiformis and three with mixed connective tissue disease, two each from patients with Sjögren's syndrome, sarcoidosis, and thrombotic thrombocytopenic purpura, and one each from patients with Behçet's syndrome, breast cancer, acquired immune deficiency syndrome, hyperglobulinaemic purpura, meningitis, and macrogammaglobulinaemia. Also there were 25 samples from patients with unknown diseases who had CIC or defective CR1 activity of E, or both. Only one

Accepted for publication 30 September 1985.

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patient with SLE had haemolytic anaemia by generally accepted clinical and laboratory criteria.

ANTISERA

Rabbit antihuman gamma chain specific antibody, antihuman C3c, and antihuman C4 were purchased from Calbiochem Laboratories (San Diego, California, USA). An A Ripley serum (sample of 24 September 1974, 1:10 dilution) was used as an incomplete anti-Rh(D) antibody, which has been found to fix complement.⁵ Lyophilised guinea pig serum (Eisai Co., Tokyo, Japan) was used as a source of natural antihuman E antibody requiring complement and of whole complement (250 CH₅₀ units/ml).

DETECTION OF CIC

The immune adherence haemagglutination (IAHA) method was applied for the detection of complement dependent circulating immune complexes.³

MEASUREMENT OF CR1 BINDING ACTIVITY OF ERYTHROCYTES

A method involving competitive inhibition of IAHA was applied as previously described.⁴ In summary, the positive IAHA pattern between sheep erythrocyte antibody complement (EAC) and CR1 of indicator E was competitively inhibited by CR1 of the test E lysate, depending on the CR1 activity and on the number of CR1.

DIRECT COOMBS' TESTING

Patient E suspensions (1.0% in ethylenediamine-tetra-acetic acid-gelatin veronal buffer (EDTA-GVB)) were mixed with serially diluted antisera in a microtitre plate, as mentioned above. The presence of IgG, C3c, and C4 on E surfaces was estimated by the degree of the haemagglutination patterns rated from 0 to 4+.

ELUTION OF IgG FROM E SURFACES

One part of a 10% E suspension, washed and resuspended in physiological saline, was mixed with

two parts of ether in a 10 ml glass test tube and vigorously shaken for two minutes. The mixture was then incubated in a water bath at 37°C for 30 min with shaking.⁶ After incubation the sample was spun for 15 min at 2500 rpm and the eluate (aqueous phase) was removed to another test tube by a Pasteur pipette and incubated for 30 min at 37°C in a water bath without a top to evaporate the rest of the ether. The eluate was stored at -70°C before being used in the indirect Coombs' test. To obtain a positive control eluate the above procedures were performed on normal E coated with anti-Rh(D) antibody.

To determine whether IgG had been eluted from E eluates were assayed by the immune adherence haemagglutination method with rabbit antihuman gamma chain specific serum.⁷

INDIRECT COOMBS' TESTING

25 µl of a 1% E suspension in EDTA-GVB from a normal healthy donor was incubated with either patient serum or eluate from E which had been serially diluted with EDTA-GVB at 37°C for 60 min. The cells were washed five times with EDTA-GVB and resuspended in 25 µl of EDTA-GVB. Appropriately diluted rabbit antihuman gamma chain serum (1:30) was added to each well. The presence of IgG anti-E antibody was estimated by the grade of the haemagglutination patterns.

Results

DIRECT COOMBS' TESTS IN PATIENTS WITH AND WITHOUT CIC OR CR1 ACTIVITY

As shown in Table 1 183 serum specimens were found to contain CIC ranging from 4 to 512 µg/ml (equivalent heat aggregated human IgG). One hundred and seventy two E samples (94%) from these 183 serum donors showed absent or impaired CR1 activity (0-75% of the activity of normal control E). The other 11 E samples had normal CR1 activity. Ninety nine E samples from 128 serum

Table 1 *CR1 binding activity in relation to the presence of CIC and positive Coombs' tests*

No of blood samples	Presence of CIC	CR1 activity	Positive Coombs' tests		
			Type I	Type II	Type III
311*	(+) 183	(-) 172	21	61	24
		(+) 11	0	1	0
	(-) 128	(-) 99	13	26	15
		(+) 29	0	2	0

*Three hundred and eleven samples were obtained from 205 patients.

donors having no CIC showed absent or impaired CR1 activity.

No significant differences were observed in the frequency of positive Coombs' tests between patients with and without immune complexes in their sera. One hundred and seven of 183 E samples (58%) from patients with CIC showed positive direct Coombs' tests as follows: type I (IgG only) 21, type II (IgG and complement) 62, and type III (complement only) 24 samples. Fifty six of 128 E samples (44%) from patients without CIC showed positive direct Coombs' tests as follows: type I 13, type II 28, and type III 15.

In relation to CR1 activity of E, however, significant differences were found in the frequency of positive direct Coombs' tests. In 271 E samples (172+99 samples) with absent or impaired CR1 activity 59% (160/271) of the samples showed positive direct Coombs' tests. There were 34 samples of positive direct Coombs' tests. In 271 E samples type III. In contrast, only 7% of 40 E samples (11+29 samples) with normal CR1 activity showed positive direct Coombs' tests and of type II only.

EFFECTS ON ERYTHROCYTE CR1 ACTIVITY OF ANTI-E ANTIBODIES

As shown in Table 2 non-treated normal E lysates had inhibitory activity in the IAHA system. On the other hand, normal E bearing immune complexes formed in vitro (heat aggregated human IgG) did not inhibit the IAHA system as previously we had reported.⁴ The lysates of normal E coated with anti-Rh(D) antibody showed normal CR1 activity. The immune lysates of normal E coated with natural pre-existing antihuman E antibody of guinea pig serum (without heat inactivation) in the presence of complement also had normal receptor activity, as did the aqueous lysates of E coated with natural

antihuman E antibody of heat inactivated guinea pig serum.

INDIRECT COOMBS' TEST

One hundred and three of 163 serum specimens from patients showing positive direct Coombs' tests were subjected to indirect Coombs' testing against normal type O human E with and without Rh(D) antigen. Only one serum sample from an SLE patient having haemolytic anaemia had IgG anti-E antibody against both O type E. This patient had immune complexes in her serum, and her E had no CR1 activity.

Twenty two eluate specimens from patients with SLE were assayed for their antibody activity against normal E as mentioned above. None of these 22 eluates reacted with control E. Thus anti-E antibody could not be eluted from the E even with positive IgG direct Coombs' test, except from positive control E coated with anti-Rh(D) antibody.

ELUTION OF IgG FROM ERYTHROCYTES

Erythrocytes with positive IgG direct Coombs' test from 22 SLE patients were chosen for the elution of IgG antibody against normal E. Sixteen out of 22 had detectable levels of IgG in their eluates.

Discussion

The customary application of the Coombs' tests in SLE has been for the purpose of detecting antibodies to E and of complement components activated by the interaction between antibodies and E as an antigen.¹ It has become apparent that the results of the direct Coombs' tests may not be related to the presence of haemolytic anaemia. In these studies we have provided an explanation for this historical discrepancy. We have shown that direct Coombs' tests can be related to absent or

Table 2 Inhibition of IAHA system by human erythrocyte lysates as a measure of CR1 binding activity

Inhibitor (water lysate)	Erythrocyte lysate added (%)				Buffer control
	6.0	3.0	1.5	0.75	
Normal human E	-†	-	±	+++	++++
Human E coated with anti-Rh(D) antibody	-	-	+	+++	++++
Human E coated with haemolytic antibody and complement (1)*	-	-	+	++/+++	++++
Human E coated with haemolytic antibody and without complement (2)*	-	-	+	+++	++++
Human E bearing in vitro formed immune complexes	+++	++++†	++++	++++	++++

*Undiluted guinea pig serum with (2) and without (1) heat inactivation at 56°C for 30 min was mixed with human E at 37°C for 30 min. †(++++) pattern represents no inhibition of IAHA system, i.e., CR1 activity is defective. (-) pattern represents inhibition of IAHA system, i.e., CR1 activity is normal.

impaired CR1 activity of E, but not necessarily to the presence of CIC. This could be explained by our previous observations that CR1 activity was still absent or impaired for a period after the disappearance of CIC due to treatment, particularly with corticosteroids.⁴

C3b molecules activated by antibodies to E adhere to C3b acceptors.⁸ Rothman *et al* also reported that CR1 was not required for the alternate pathway fixation of C3b molecules on the E membranes.⁹ These two reports support our conclusions that C3b molecules, activated by the reaction between antibody and E, do not adhere to CR1 of E. Accordingly, in such cases CR1 activity of E will not be decreased, but positive direct Coombs' test (type II) will occur by adding anti-immunoglobulins and anticomplement reagents (Fig. 1A). In contrast, when antigen-antibody reaction not involving E as

an antigen occurs in the vicinity of E membranes C3b molecules of antigen-antibody-complement complexes will adhere to CR1 of E by the immune adherence phenomenon. In this case positive direct Coombs' tests will be evident, but CR1 activity will be absent (Fig. 1B). As we previously reported, these E might have high membrane osmotic fragility resulting in anaemia without antibody to E.¹⁰ We assume that there are different binding mechanisms of C3b molecules onto E surfaces depending on the antigen-antibody reaction, whether or not E is involved as an antigen. Accordingly, the characteristics of binding of C3b molecules can be distinguished by the presence of CR1 activity of E.

C3 inactivator is present in human serum.¹¹ Antigen-antibody-complement complexes on E via CR1 are released by C3 inactivator in normal human serum heated at 56°C for 30 min, and C3c fragments

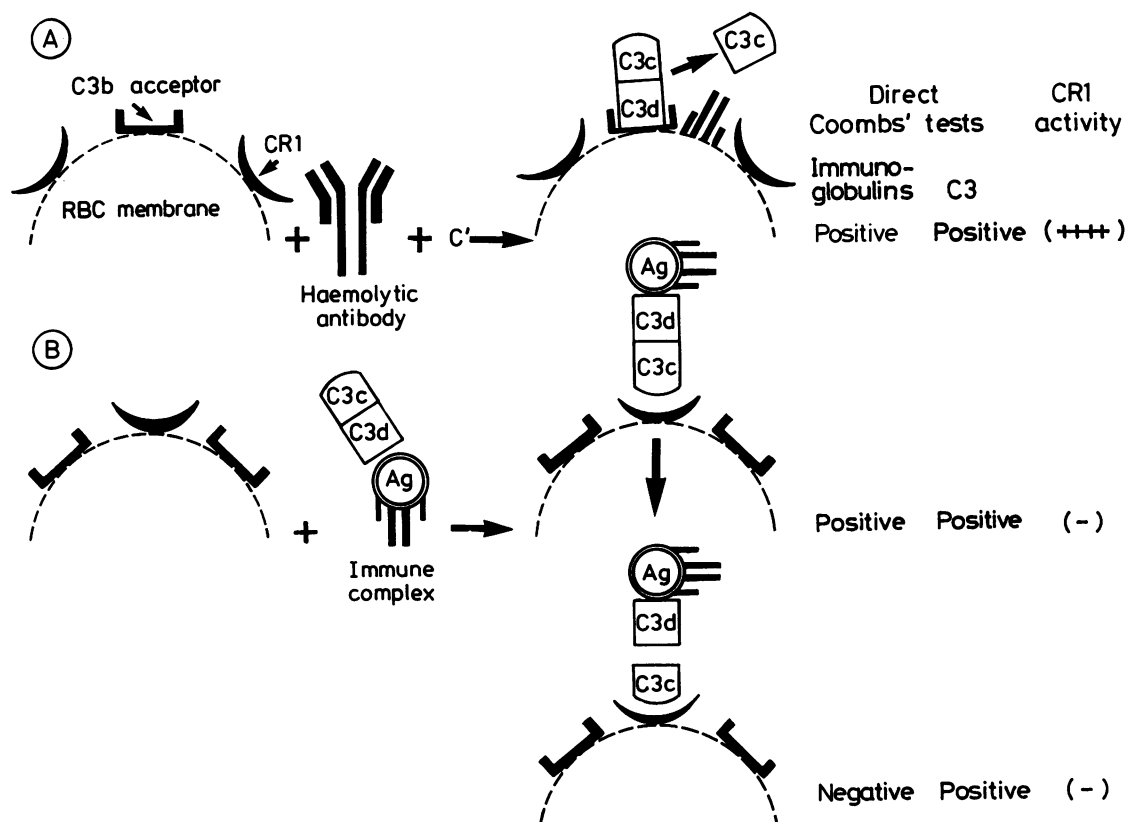


Fig. 1 A schematic drawing of the genesis of positive direct Coombs' tests in relation to CR1 binding activity of erythrocytes. If CR1 are defective and immunoglobulins or C3b molecules, or both, are detected on erythrocyte surfaces by direct Coombs' tests it can be concluded that a positive direct Coombs' test is due to *in vivo* binding of immune complexes and complement activated by antigen-antibody reaction without involvement of erythrocytes as an antigen.

of C3b molecules are generated into the fluid phase.¹² In the mixing experiments heat inactivated serum from SLE patients did not inhibit the normal serum supported binding of in vitro formed immune complexes to normal cells.¹³ The removal of bound E-IgM-C3b complexes from mononuclear cell surfaces was found to be dependent on the number of C3b molecules per E and on the concentration of C3 inactivator.¹⁴ Thus it is suggested that immune complexes on E via CR1 are not released in vivo in SLE patients and remain on E surfaces, resulting in positive direct Coombs' tests (type II). Even if C3c are generated by C3 inactivator into the fluid phase C3c molecules inhibit the IAHA phenomenon between EAC1423b and human E.^{15 16} This suggests that a portion of C3c molecules on CR1 could remain after the cleavage of C3b molecules of antigen-antibody-complement-E reactants by C3 inactivator. This could result in impaired CR1 activity by occupation with complement and also in a type III positive Coombs' test (especially with antihuman C3c reagent). In contrast, cell bound C3b molecules (EAC1423b) release C3c molecules after cleavage by C3 inactivator, β 1H, and other proteolytic enzymes. Such E were not aggregated by anti-C3c reagent, i.e., a negative type III Coombs' test.^{17 18}

From the results of type I Coombs' tests it seemed possible that a small amount of C3b molecules, undetectable by conventional serological techniques, was responsible for the negative Coombs' test with antihuman C3c reagent. Other possibilities are immune adsorption phenomena with E. In most experimental instances of immune adsorption¹⁹ the first step is fixation of antigen on cells, followed by attachment of antibodies directed only against the adsorbed foreign antigen: for example, adsorption of many bacterial polysaccharides on untreated E leading to positive type I Coombs' test, followed by the binding of antibacterial antibody to antigen.

It was necessary to determine whether IgG had been eluted from E. Sixteen of 22 eluates contained detectable IgG levels. This result suggests that the elution method is appropriate for dissociation of antibody from antigen.

Circulating anti-E antibody in one patient with SLE reacted not only with type O, Rh(D) positive but also with type O, Rh(D) negative E, suggesting that the specificity of the IgG is directed against a non-Rh(D) determinant of E as previously reported.^{1 20} Negative indirect Coombs' tests of sera and eluates from the other patients indicate that sera contain no antibody against E and that IgG in eluates are not capable of binding to E and may be IgG dissociated from immune complexes deposited on E surfaces via CR1.

We propose that positive direct Coombs' tests are not always associated with antibodies to E, but may be associated with the binding of CIC to CR1 of E. Thus anaemia in patients with SLE could be an epiphenomenon secondary to the presence and binding of CIC rather than to the presence of autoantibodies to E. The presence of positive Coombs' tests in the absence of CIC or haemolytic anaemia, or both, may provide a simple method of extrapolating an immune complex induced disease phenomenon.

This work was supported by the Jacob Bleibtreu Foundation.

References

- Budman D R, Steinberg A D. Hematologic aspects of systemic lupus erythematosus. *Ann Intern Med* 1977; **86**: 220-9.
- Tan E M, Cohen A S, Fries J F, *et al*. The 1982 reviewed criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; **25**: 1271-7.
- Inada Y, Kamiyama M, Kanemitsu T, Clark W S. Detection of circulating immune complexes: a new application of immune adherence hemagglutination. *Ann Immunol (Paris)* 1981; **131C**: 181-90.
- Inada Y, Kamiyama M, Kanemitsu T, Hyman C L, Clark W S. Studies on immune adherence (C3b) receptor activity of human erythrocytes: relationship between receptor activity and presence of immune complexes in serum. *Clin Exp Immunol* 1982; **50**: 189-97.
- Waller M, Lawler S D. A study of the properties of the Rhesus antibody (Ri) diagnostic for the rheumatoid factor and its application to Gm grouping. *Vox Sang* 1962; **7**: 591-606.
- Vos G H, Kelsall G A. A new elution technique for preparation of specific immune anti-Rh serum. *Br J Haematol* 1957; **2**: 342-44.
- Mayumi M, Okochi K, Nishioka K. Detection of Australia antigen by means of immune adherence hemagglutination test. *Vox Sang* 1971; **20**: 178-81.
- Dierich M P, Bolkisch V A. Receptor-binding sites on C3 and C3b. *J Immunol* 1977; **118**: 2145-50.
- Rothman I K, Gelfand J A, Fauci A S, *et al*. The immune adherence receptor: dissociation between the expression of erythrocytes and mononuclear cell C3b receptors. *J Immunol* 1975; **115**: 1312-5.
- Inada Y, Kamiyama M, Kanemitsu T, Clark W S. Studies on immune adherence (C3b) receptor activity of human erythrocytes: Relationship between receptor activity and membrane osmotic fragility. *J Clin Lab Immunol* 1983; **12**: 97-103.
- Ruddy S, Austen K F. C3 inactivator in man. I. Hemolytic measurement by the inactivation of cell-bound C3. *J Immunol* 1969; **105**: 533-43.
- Medof M E, Prince G M, Mold C. Release of soluble immune complexes from immune adherence receptors on human erythrocytes is mediated by C3 inactivator independently of β 1H and accompanied by generation of C3c. *Proc Natl Acad Sci USA* 1982; **79**: 5047-51.
- Medof M E, Oger J J, Antel J P, Prince G M. Restoration of normal interactions between immune complexes and systemic lupus erythematosus blood cells by normal serum. *J Clin Lab Immunol* 1982; **7**: 93-103.
- Schreiber A D, McDermott P B. Effect of C3 inactivator on monocyte-bound C3-coated human erythrocytes. *Blood* 1978; **52**: 896-904.
- Ross G D, Polley M J. Specificity of human lymphocyte complement receptors. *J Exp Med* 1975; **141**: 1163-80.
- Tack B F, Segal D M, Schechter A N. Interaction of the third

- component of human complement (C3) with erythrocytes and leukocytes. *J Immunol* 1978; **120**: 1800-1.
- 17 Law S K, Fearon D T, Levine R P. Action of the C3-inactivator on cell-bound C3b. *J Immunol* 1979; **122**: 759-65.
- 18 Carlo J R, Ruddy S, Studer E J, Conrad D H. Complement receptor binding of C3b-coated cells treated with C3b inactivator, β 1H globulin and trypsin. *J Immunol* 1979; **123**: 523-8.
- 19 Shulman N R. A mechanism of cell destruction in individuals sensitized to foreign antigens and its implications in autoimmunity. *Ann Intern Med* 1964; **60**: 506-21.
- 20 Leddy J P, Peterson P, Ycaw M A, *et al.* Patterns of serologic specificity of human γ G erythrocyte autoantibodies. Correlation of antibody specificity with complement-fixing behavior. *J Immunol* 1970; **105**: 677-86.