

In vivo binding of circulating immune complexes by C3b receptors (CR1) of transfused erythrocytes

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SUMMARY The effects of packed erythrocyte transfusion with high CR1 activity on circulating immune complex concentrations were studied in 14 transfusion experiments involving 12 patients with immune complex related diseases. Before erythrocyte transfusion circulating immune complex concentrations ranged from 8 to 128 µg/ml. After transfusion (2-3 units) immune complex concentrations decreased depending on the levels of CH₅₀ titres in the recipients. In 11 experiments, in which the patients' CH₅₀ titres ranged from 21 to 44, immune complex concentrations decreased by 75-100% within five days. The CH₅₀ titres were also decreased after erythrocyte transfusion but subsequently increased to initial ranges within 6-35 days. In three patients with low CH₅₀ titres (1.0-10.0) decreases in immune complexes were not observed. Direct Coombs' tests for IgG and C3 were performed before and after erythrocyte transfusion to determine potential in vivo binding of circulating immune complexes. Thus in eight of 14 experiments, in which erythrocytes carried no IgG before packed erythrocyte transfusion, seven became Coombs' positive for IgG after the transfusion. In seven of 14 experiments, in which erythrocytes were negative for complement before transfusion, five became positive afterwards. Moreover, in 12 instances slight increases of CR1 activity of patients' erythrocytes were observed within eight days, which improved further within 35 days after erythrocyte transfusion. These studies suggest that transfusion of erythrocytes with high CR1 activity results in the removal of circulating immune complexes and that this process is dependent on complement consumption. These experiments support the hypothesis that erythrocyte-CR1 has a functional role in the removal of circulating immune complexes and may thereby inhibit the deposition of immune complexes within body tissue constituents.

A number of previous studies have shown that erythrocytes play a major part in the removal of circulating immune complexes via immune adherence receptors (C3b receptor, CR1). Medof *et al* reported that immune complexes interact preferentially with erythrocytes in vitro rather than with other peripheral cells bearing CR1.^{1, 2} Furthermore, CR1 isolated from human erythrocytes inhibited the complement cascade in both classical³ and alternative⁴ pathways. As 95% of CR1 in humans are found on erythrocyte surfaces a number of investigators have suggested a major immunomodulating function for erythrocytes via CR1.

Recently, several studies concerning CR1 of erythrocytes in patients with immune complex related diseases were reported.⁵⁻¹⁰ Thus two reports on patients with systemic lupus erythematosus showed a decrease in CR1 binding activity associated with an increase in disease activity.^{8, 9} This suggests that a loss of CR1 activity may be acquired. These observations have led to the suggestion that the major function of erythrocyte-CR1 is to bind and inactivate potentially pathogenetic circulating immune complexes and shuttle them to mononuclear phagocytic cells located primarily in the spleen and liver. Our previous observations that patients with active acute autoimmune diseases usually have low or absent erythrocyte-CR1 activity suggest that saturation of the erythrocyte-CR1 system is a step preceding the inflammatory flare up of a clinical

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exacerbation in these diseases. In an elegant study with baboons Cornacoff *et al* reported that the uptake of circulating immune complexes bearing C3b molecules with delivery to the mononuclear phagocyte system is a possible function of erythrocyte-CR1.¹¹ If erythrocyte-CR1 is indeed involved in such an immune complex clearing mechanism we reasoned that transfusion of erythrocytes with high CR1 functional activity might have therapeutic potential in the clearance and immobilisation of circulating immune complexes.

In this study we report the potential value of packed erythrocyte transfusion for the removal of immune complexes from the circulation as a method of treatment. It appears that packed erythrocyte transfusion participates in the removal of soluble immune complexes from the blood stream in the presence of appropriate concentrations of total complement. The results of this report support our previous observations.¹²

Materials and methods

CLINICAL MATERIALS

Packed erythrocyte transfusion was studied in 14 experiments involving 12 patients as follows: seven with systemic lupus erythematosus, four with rheumatoid arthritis, and one with thrombotic thrombocytopenic purpura. All patients were anaemic. Serum samples were collected before erythrocyte transfusion and five days and within six to 35 days after (first and second follow up period). Erythrocytes were obtained from heparinised blood.

DETECTION OF CIRCULATING IMMUNE COMPLEXES IN PATIENTS' SERA

Immune adherence haemagglutination was used for the measurement of complement dependent circulating immune complexes.¹³

MEASUREMENT OF CR1 BINDING ACTIVITY OF ERYTHROCYTES

A semiquantitative method involving competitive inhibition of immune adherence haemagglutination, as described previously, was used for estimating erythrocyte-CR1 function.⁸ In summary, the positive immune adherence haemagglutination pattern between sheep erythrocyte-antibody-complement complexes and the CR1 of intact indicator erythrocytes (1.5% suspension) was competitively inhibited by the CR1 of the test erythrocytes (patient erythrocytes) lysate depending on its CR1 activity and on the concentration of the test erythrocyte lysate. Erythrocyte lysate was obtained as follows: 60 μ l of packed erythrocytes, washed with edetic acid-gelatin veronal buffer, was lysed in 660 μ l of distilled

water and reconstituted in 1 ml of 0.01 M edetic acid-gelatin veronal buffer, which was prepared by addition of 0.1 ml of 0.1 M edetic acid solution, 180 μ l of five times concentrated veronal buffer and gelatin. This resulted in a 6% erythrocyte lysate, which was serially diluted to obtain lysate concentrations of 3, 1.5, and 0.75% to compete with the 1.5% indicator erythrocyte suspension. Thus we were able to estimate test erythrocyte binding activity of test erythrocytes ranging from 1/16 the activity to twice the activity of control erythrocyte-CR1. A 0+ haemagglutination pattern (inhibition of haemagglutination) was interpreted as indicative of high CR1 activity of the test erythrocyte sample.

DIRECT COOMBS' TESTING

An erythrocyte suspension (1.0% in edetic acid-gelatin veronal buffer) was mixed with serially diluted antisera such as rabbit antihuman γ chain specific antibody (Calbiochem-Behring, San Diego, CA) and rabbit antihuman C3 (Pel Freeze, Rogers, Arkanso) in a microtitre plate. The presence of IgG and C3 on erythrocyte surfaces was estimated by the degree of the haemagglutination patterns ranked from 0 to 4+. Erythrocyte suspensions from both blood bank bloods to be transfused and from patients' bloods were assayed. None of the erythrocytes from the blood bank had a positive direct Coombs' test.

INDIRECT COOMBS' TEST

A 1% erythrocyte suspension (25 μ l) in edetic acid-gelatin veronal buffer from a normal healthy donor (type O, Rh positive) was incubated with patients' serum which had been serially diluted with edetic acid-gelatin veronal buffer at 37°C for 60 minutes. The cells were washed five times with the edetic acid-buffer and resuspended in 25 μ l of edetic acid-buffer. Appropriately diluted rabbit antihuman γ chain serum (1:30) was added to each well. The presence of IgG anti-erythrocyte antibody was estimated by graded haemagglutination patterns. Furthermore, 1% erythrocyte suspensions of blood units to be transfused were assayed for an antibody cross match test with the patient's serum before transfusion at our blood bank, and the serum specimen after erythrocyte transfusion (first period) was assayed for IgG antibody to erythrocytes transfused which had been kept at 4°C. Eluates from patients' erythrocytes after erythrocyte transfusion were also tested for their anti-erythrocyte activity.¹²

MEASUREMENT OF TOTAL HAEMOLYTIC COMPLEMENT IN SERUM

The total haemolytic complement (CH₅₀ units/ml) of serum samples was assayed by Mayer's method.¹⁴

PACKED ERYTHROCYTE TRANSFUSION

Before packed erythrocyte transfusion several stored units of packed erythrocytes were screened for CR1 binding activity and also the presence of IgG and C3 on erythrocyte surfaces. Packed erythrocyte units with activity lower than the control erythrocytes or positive direct Coombs' tests, or both, were eliminated. Two to three units of properly matched packed erythrocytes (one unit of erythrocytes with a 0.80 packed cell volume) were infused in all patients.

BINDING CAPACITY OF NORMAL CR1 OF ERYTHROCYTES

To determine the continuing ability of erythrocyte-CR1 to bind immune complexes we determined the binding capacity of CR1 after repetitive binding and releasing of in vitro formed immune complexes to CR1 of erythrocytes as follows: an equal volume of heat aggregated human IgG (20 µg/ml) was incubated with 25 µl of human serum complement (2.5 CH₅₀ units/ml, approximately 1:15 dilution) at 37°C for 40 minutes in a microtitre plate. Then 25 µl of indicator human erythrocytes (1.5% suspension) was added to the reaction mixture. A 4+ immune adherence haemagglutination pattern develops and if dithiothreitol solution (20 mM) is added at the same time the pattern does not dissociate.¹⁵ When a completely negative pattern was obtained, without addition of dithiothreitol solution (complete dissociation of aggregation by C3 inactivator derived from human serum for complement source), the erythrocytes were collected, washed five times, and resuspended in edetic acid-gelatin veronal buffer at a concentration of 1.5%. These erythrocytes were repeatedly exposed to newly prepared heat aggregated human IgG-complement reaction mixtures. At each exposure dithiothreitol solution was added to one or two wells of a 120 well microtitre plate to observe the CR1 activity of the erythrocytes.

Results

CIRCULATING IMMUNE COMPLEX LEVELS BEFORE AND AFTER PACKED ERYTHROCYTE TRANSFUSION

Before packed erythrocyte transfusion all serum samples from 12 patients contained circulating immune complex concentrations ranging from 8 to 128 µg/ml equivalent heat aggregated human IgG before transfusion (Fig. 1). After packed erythrocyte transfusion the circulating immune complexes decreased according to the levels of total haemolytic complement titres present before transfusion. In 11 experiments, in which CH₅₀ titres ranged from 21 to

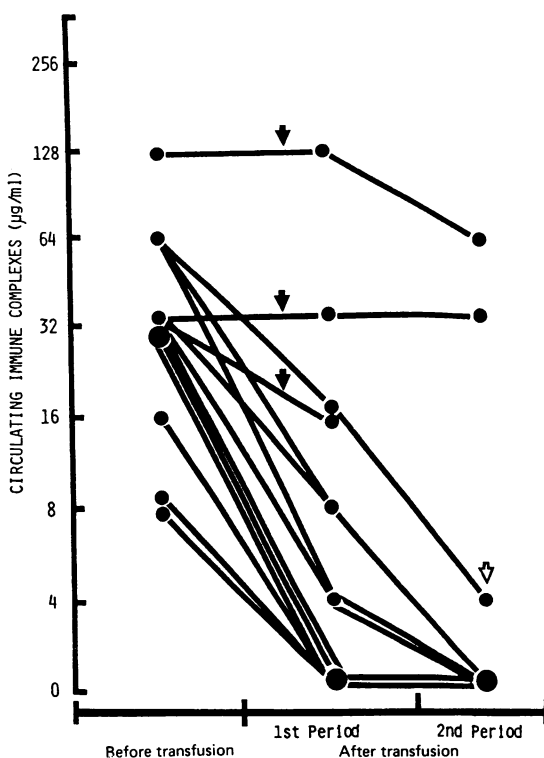


Fig. 1 Effects of packed erythrocyte transfusion in relation to the concentrations of circulating immune complexes. No significant decreases of immune complexes were observed in three cases (closed arrows) having low titres of total haemolytic complement (corresponding to closed arrows in Fig. 2).

44 units/ml, the circulating immune complexes decreased 75–100% within the first five days. Two patients in whom the circulating immune complexes disappeared in the first five days continued to be negative for these complexes even in the second period (six to 35 days). Circulating immune complexes did disappear in the second period in most patients. In three experiments, however (closed arrows, corresponding to Fig. 2), where levels of CH₅₀ titres were low (ranging from 1 to 10 units/ml), such decreases in circulating immune complexes could not be shown either in the first or second period after packed erythrocyte transfusion.

CH₅₀ LEVELS BEFORE AND AFTER PACKED ERYTHROCYTE TRANSFUSION

We observed that the initial concentrations of serum complement correlated with the degree of circu-

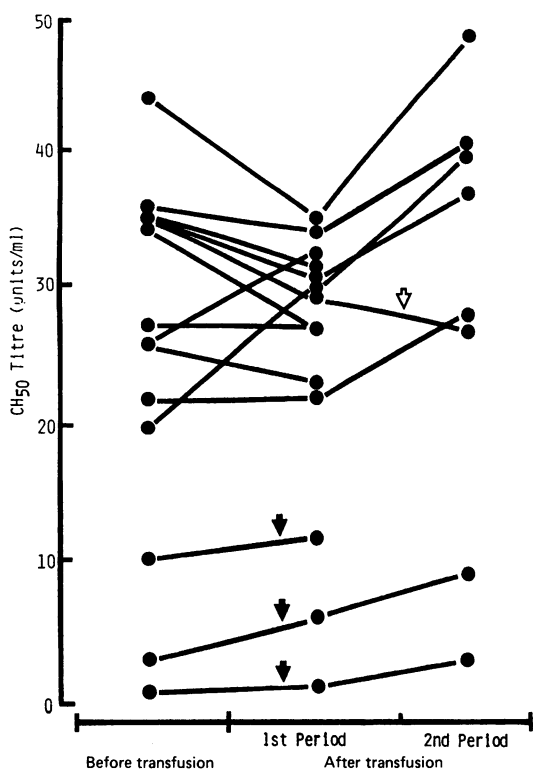


Fig. 2 Levels of total haemolytic complement before and after transfusion. Rebound phenomenon was observed in patients with complement titre higher than 30 CH₅₀ units/ml before transfusion. One patient (open arrow, corresponding to open arrow in Fig. 1) showed continuous decrease of the CH₅₀ titre and this patient's serum contained circulating immune complexes even in the second period.

lating immune complex decrease in 11 instances with initial CH₅₀ titres ranging from 21 to 44 units/ml (Fig. 2). Striking decreases were observed in patients with CH₅₀ titres greater than 35 CH₅₀ units/ml. On the other hand, no decreases of complement concentration occurred in three patients with low CH₅₀ titres ranging from 1 to 10 units/ml (closed arrows) at the onset.

After the disappearance of the circulating immune complexes complement concentrations increased to the initial ranges or higher during the second period. In one experiment, in which the circulating immune complexes persisted into the six to 35 day period (open arrow, Fig. 1), the complement concentration continued to decrease.

CR1 ACTIVITY

The CR1 binding activity of patients' erythrocytes

was measured before and after packed erythrocyte transfusion (Fig. 3). In all 14 experiments abnormal CR1 binding activity was present before erythrocyte transfusion. Receptor activity was absent in four and the others had defective activity ranging from + to ++ (normal CR1 activity is ++++).

After erythrocyte transfusion we observed slight increases of CR1 activity within the first five days. Three of 11 patients with relatively high CH₅₀ titres showed increased receptor activity from + (before) to ++ (after) (two patients) and from ++ to +++ (one patient) after transfusion. No increases were observed in the remaining eight patients. During the six to 35 day period four of six patients with relatively high CH₅₀ titres showed further increases in CR1 activity. Two of three patients with significantly low CH₅₀ showed no increases after receiving

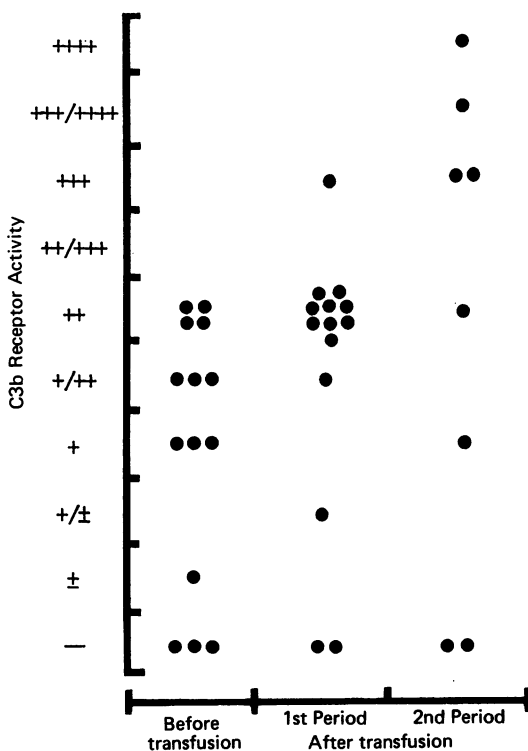


Fig. 3 Changes in C3b receptor (CR1) binding activity before and after transfusion. Three patients (closed arrows in Figs 1 and 2) had no receptor activity before transfusion. Two of these patients, who received two units of packed erythrocytes, had no increases of receptor activity after transfusion. The other patient, however, who received three units of packed erythrocytes, showed slightly increased receptor activity from (-) to (+/±).

two units of packed erythrocytes. A slight increase was observed in one patient receiving three units of packed erythrocytes, however.

DIRECT COOMBS' TESTING

To provide further evidence for the hypothesis that transfused erythrocytes are binding circulating immune complexes in vivo we carried out direct Coombs' tests before and after packed erythrocyte transfusion. Eight of 14 experiments showed positive direct Coombs' test before transfusion as follows: type I (IgG only): one, type II (IgG and C3): five, and type III (C3 only): two. Six experiments were negative for direct Coombs' tests before transfusion (Fig. 4).

Of eight patients whose erythrocytes were negative for IgG (two patients with type III and six patients with negative Coombs' tests), seven (two from type III and five from patients with negative Coombs' test) became positive for IgG after erythrocyte transfusion. None of these seven patients had any evidence for the presence of anti-erythrocyte antibody after transfusion. In seven of 14 experiments in which erythrocytes were negative for C3 (one patient with type I and six patients with negative Coombs' test) five of them became positive for C3 after packed erythrocyte transfusion. Of five with type II, no differences were observed after transfusion.

INDIRECT COOMBS' TEST

None of the serum specimens from patients before

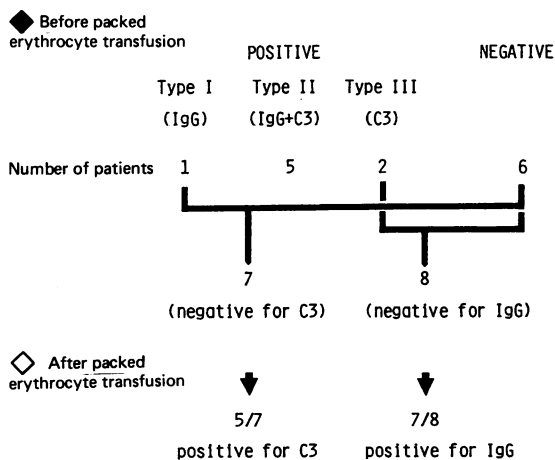


Fig. 4 Direct Coombs' test before and after transfusion. In advance of packed erythrocyte transfusion direct Coombs' tests were performed on erythrocytes to be transfused. None of the erythrocytes for transfusion showed positive direct Coombs' tests.

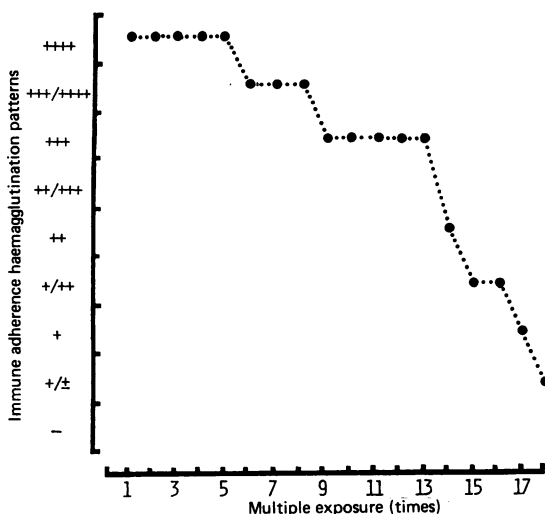


Fig. 5 Effects of the consecutive dissociation of in vitro formed immune complexes. Normal human serum was used as a source of total complement. A significant decrease of CR1 binding activity was observed after the 13th exposure.

and after erythrocyte transfusion in the first period was found to contain IgG antibody directed against transfused and control erythrocytes. We attempted to elute IgG from four erythrocyte specimens with positive IgG Coombs' test after erythrocyte transfusion. None of the eluates had any activity against either transfusion or control erythrocytes.

BINDING OR DISPOSING CAPACITY OF ERYTHROCYTE CR1

Our previous experiments suggested that erythrocytes with normal CR1 activity develop 4+ haemagglutination patterns as indicator cells in the presence of dithiothreitol in an immune adherence haemagglutination system.¹³ When dithiothreitol solution was omitted a negative pattern developed after the initial positive haemagglutination pattern as a result of the C3 inactivator in the human complement serum. No significant differences in haemagglutination patterns were observed until the eighth exposure. Decreased aggregation patterns (3+ pattern) were observed after the ninth exposure (Fig. 5). Furthermore, after the 14th exposure the aggregating activity became very weak (2+ pattern), and aggregating activity became unobservable after the 18th exposure. The time required for developing a negative pattern after addition of erythrocyte suspension without dithiothreitol solution became shorter with each additional exposure.

In contrast, in erythrocytes consecutively exposed to the same amount of heat aggregated human IgG in the absence of complement (human serum heated at 56°C for 30 minutes) haemagglutination patterns did not diminish even after the 18th exposure.

Discussion

The ability to remove soluble immune complexes by transfused blood products was first reported by Safai-Kutti *et al*, who showed that platelet transfusions resulted in the removal of soluble immune complexes from the circulation.¹⁶ This observation was attributed to the Fc receptors of the transfused platelets rather than to the dilution effects of transfusions. A number of previous studies have shown that erythrocyte-CR1 is able to bind circulating immune complexes *in vivo*.¹²⁻¹⁷ Such binding, which is complement dependent, results in diminished CR1 activity and in the appearance of positive direct Coombs' tests.¹²

Medof *et al* reported that erythrocytes isolated after the binding and release of immune complexes by C3 inactivator would again bind newly formed immune complexes with the same rapidity and capacity as fresh erythrocytes,¹ suggesting that receptor activity was not altered by the binding and subsequent release of immune complexes. These conclusions were based on observations using only one exposure of immune complexes to intact CR1, however. Our *in vitro* experiments showed that CR1 activity, although not altered by limited binding and release of immune complexes (Fig. 5), nevertheless gradually decreased after subsequent consecutive exposures to immune complex-complement complexes. The normal serum concentration of C3 inactivator has been determined as approximately 53 µg/ml.¹⁸ In another study only 2 µg/ml of purified C3 inactivator with purified CR1 (100 µg/ml:equivalent to 2.5×10^8 erythrocytes/ml) released approximately 75% of labelled C3 as the C3c fragment from erythrocyte-antibody-complement 1423b complexes.¹⁹ In our repetitive binding experiment a 1:15 dilution of human serum as a source of complement would have had no more than 3.5 µg/ml of C3 inactivator. This quantity appears to be enough to release C3c molecules from CR1 in the presence of 2.25×10^8 erythrocytes/ml (1.5% suspension in our experiment), and indicates that most heat aggregated human IgG bound to CR1 are released in our experimental condition. Although erythrocytes were always exposed to a certain amount of C3 inactivator in complement containing serum and to immune complexes bearing complement, the time taken for the formation of negative pattern without dithiothreitol solution was gradually shortened with

repeated exposures. These phenomena suggest that immune complexes adhere to erythrocytes via CR1 and that progressively smaller amounts of immune complexes adhere to erythrocytes via CR1 after each exposure. If the CR1 is occupied this could explain the cause of defective CR1 activity and also the cause of type III positive direct Coombs' tests. The exact mechanism of reduced binding activity of CR1 in systemic lupus erythematosus is unknown. It has been suggested that CR1 deficiency seen on erythrocytes from patients with systemic lupus erythematosus is due to the loss of CR1 molecules by proteolysis.²⁰⁻²¹ Monoclonal antibodies to CR1 will displace erythrocyte bound, complement coated immune complexes, suggesting that monoclonal antibodies are able to detect occupied CR1. In another study, however, monoclonal antibody was unable to detect all occupied CR1. This was shown when the CR1 of normal erythrocytes, after exposure to antigen-antibody-complement, was significantly decreased.²² In our continuing experiments (data not shown) we have examined five monoclonal antibodies (44D, To5, 57F, 57H, and 31D) and radiolabelled immune complexes formed *in vitro* to observe the effects of occupancy of CR1. Unlabelled immune complexes were bound to CR1, followed by the addition of labelled immune complexes or monoclonal antibodies. The binding first of unlabelled immune complexes to CR1 resulted in reduced binding of labelled complexes (-82%) as well as reduced monoclonal antibody binding (ranging from 20 to 40%), suggesting that immune complexes binding to CR1 interfered with the detection of CR1 antigen epitopes. Displacement of immune complexes by monoclonal antibody was observed in only 2-5%. Therefore decreased CR1 activity may in part be explained by an acquired deficiency of CR1 as a result of binding of circulating immune complexes or by the persistence of C3b molecules on immune complexes in CR1 owing to a defective mononuclear phagocytic system in immune complex related diseases such as SLE.

We estimate that the binding or disposing activity of CR1 is approximately 6000-8000 µg of equivalent heat aggregated human IgG per 5×10^9 erythrocytes. Therefore, transfusion of erythrocytes with high CR1 activity should assist in reducing circulating immune complexes, especially in patients with anaemia or defective CR1 activity of erythrocytes, or both. In our studies decreases of circulating immune complex concentrations correlated with initial concentrations of complement, suggesting that the removal of immune complexes requires the presence of a certain concentration of complement. This conclusion was supported by the observations of another investigator,² who reported that the

release of immune complexes from CR1 depended on a certain minimum level of CH₅₀ and native C3. These observations suggested that the transfused erythrocytes consumed complement as they captured and released circulating immune complexes and C3b molecules. We were able to observe this phenomenon in 11 of our patients who had adequate concentrations of complement. Immune complexes released from erythrocytes by C3 inactivator would not be detected in a CR1 dependent assay system but may, however, bind to the cells bearing C3d receptors—for example, in the spleen.^{23 24} In this phenomenon, total complement (particularly the first four components) is consumed. When hypocomplementaemia occurs during repetitive binding of circulating immune complexes to erythrocyte-CR1, the CR1 of transfused erythrocytes may not release their immune complexes.² CR1 activity of erythrocytes would thus diminish because the attached C3b molecules block CR1. When patients with low complement concentrations (three cases) were transfused, such decreases were not observed, presumably because complement concentrations were not sufficient to permit immune complexes-complement formation.

No significant increase in CR1 activity was observed in two patients who received two units of packed erythrocytes without decrease of circulating immune complex and complement concentrations. Therefore, the increases of erythrocyte-CR1 activity observed in the second period (six to 35 days) after transfusion may result from activity of new erythrocytes released from the bone marrow of patients. Increases of CR1 activity in patients with systemic lupus erythematosus have been reported by several investigators.^{6 8 9} In our study direct and indirect Coombs' tests demonstrated *in vivo* binding of circulating immune complexes to CR1 of transfused erythrocytes with the assistance of C3b molecules. This phenomenon was supported by another report.¹⁷ We could not detect anti-erythrocyte antibodies, suggesting that the presence of IgG on erythrocyte surfaces after transfusion is not due to the antibodies to erythrocytes. We conclude that transfusion of packed erythrocytes with high CR1 activity may be an alternative method for the clearance of circulating immune complexes and thus inhibit inflammatory properties of soluble immune complexes. It has been Rope's empirical impression that transfusions are beneficial in patients with systemic lupus erythematosus.²⁵ The explanation may be the addition of erythrocyte-CR1.²⁶

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