

Erythrocyte CR1 Determination Using Monoclonal Antibody in a Microtiter Plate ELISA; Receptors Are Not Masked by Immune Complexes

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The microtiter plate ELISA using monoclonal antibody is a specific, sensitive and quantitative technique for measuring CR1 on human erythrocytes. The present investigations established that receptor occupancy by immune complexes did not affect the measurements. The monoclonal anti-CR1 antibody To5 bound unimpeded to receptors that had reacted with an excess of complement-opsonized tetanus toxoid anti-tetanus toxoid complexes prepared at antigen:antibody ratios between 32:1 and 1:8. The CR1 levels on erythrocytes from 11 patients with systemic lupus erythematosus (SLE) were not increased ($P > 0.30$) after release of CR1-bound immune complexes by incubation with factor I. Neither did the serum from these patients contain blocking anti-CR1 activity ($P > 0.10$). Additionally, the number of antigenic CR1 sites in 10 normals and in the 11 patients with SLE was well correlated with the number of functional receptor sites as assessed by binding of soluble complexes ($P < 0.001$). These data establish that the true CR1 levels are determined using the microtiter plate ELISA for quantitation of CR1 in patients with diseases involving immune complexes and/or autoantibodies.

Key words: autoantibody; C3b receptor (CR1); ELISA; human erythrocyte; immune complexes; monoclonal antibody.

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CLINICAL ASPECTS

Complement C3b receptor (CR1) deficiencies of erythrocytes are supposed to be involved in the pathogenesis of immune complex-induced disease manifestations. The present results establish that the microtiter plate ELISA is applicable for determination of CR1 in patients even if the receptors are occupied with *in vivo* bound immune complexes. Further, it appears that potentially blocking autoantibodies to CR1 are rare, and that the number of CR1 is correlated with the functional receptor capacity. Valid conclusions may therefore be drawn from clinical investigations focusing on a low CR1 level as a predisposing and/or disease-associated factor.

Recent evidence indicates that the C3b receptors (CR1) on erythrocytes may have a major role in the processing and clearance of circulating immune complexes (IC) (25, 36), and the CR1 deficiencies observed in patients with systemic lupus erythematosus (SLE) (8, 9, 10, 11, 12, 13, 17, 22, 23, 24, 26, 27, 29, 33, 34, 35), rheumatoid arthritis (10, 17, 29, 34) and prim-

ary Sjögren's syndrome (31) are supposed to be involved in the pathogenesis of these diseases.

The CR1 levels on red blood cells (RBC) have been measured by immune adherence haemagglutination (11, 12, 13, 17, 22, 23, 24, 27, 33), by binding of dimeric C3b (35, 38), ¹²⁵I-aggregated IgG (24) or immune complexes containing ³H-dsDNA (9, 29), as well as by

radioimmunoassays involving polyclonal (8, 35) or monoclonal (10, 22, 26, 34) anti-CR1 antibodies and by an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody (30, 31).

Qualitative data obtained by haemagglutination assays indicate that IC bound to CR1 on RBC *in vivo* are detectable, and that the reduced CR1 levels observed by the use of indicators coated with C3b might at least in part be due to blockade of receptors on test cells by IC bound *in vivo* (11, 12, 13). One of the polyclonal antibodies was found to block the function of CR1 as assessed by C3b dependent rosetting (5), but bound to receptors occupied by aggregated IgG (15). The monoclonal anti-CR1 antibody E11 did not interfere with C3b dependent rosetting and the receptors remained accessible to the antibody after binding of IC (26, 34). Other monoclonal antibodies to some extent inhibited C3b dependent rosette formation, but it has not been examined whether the accessibility of the receptors to the antibodies might be changed by bound IC. Autoantibody to CR1 seems to be an exception in patients with SLE (1, 35, 37).

We report here that the monoclonal anti-CR1 antibody To5, used for quantitation of CR1 on RBC in the microtiter plate ELISA (30), binds to receptors occupied by IC, that the CR1 levels on RBC from patients with SLE are not increased after incubation with factor I, and that serum from these patients does not alter the CR1 level of normal RBC. Additionally, the number of antigenic CR1 sites are shown to correlate with the number of functional receptor sites.

MATERIAL AND METHODS

Erythrocyte and serum specimens

Whole blood was drawn into glass tubes with citrate-phosphate-dextrose-adenin solution (CPDA formula 1: 3.27 g citric acid, 26.3 g sodium citrate, 2.22 g monosodium phosphate, 29.0 g dextrose, and 0.28 g adenin per litre; one part CPDA to seven parts blood) and stored at 4°C for less than 1 week.

Before use RBC were washed 3 times with PBS (0.01 M phosphate-buffered saline, pH 7.2), carefully removing the buffy coat after each centrifugation. RBC from the same healthy donor of blood group AB Rh pos was used as standard at CR1 determination and throughout the experiments, with the exception of investigations involving incubation of normal RBC with serum from patients with SLE; in these cases a blood group 0 Rh neg donor was used.

Serum from the AB Rh pos donor was separated by centrifugation after clotting at room temperature for 2 h, stored in aliquots at -70°C and used as a source of complement. The serum had a normal C3 concentration and did not contain irregular RBC antibodies.

Likewise, RBC from 10 normals and RBC and sera from 11 patients with at least four of the revised ARA criteria for SLE (28) were obtained.

Quantitation of CR1

The microtiter plate ELISA using the monoclonal anti-CR1 antibody To5 was performed in quadruplicate as described in detail earlier (30), with some minor modifications of the standard procedure: Automatic washing (Titertek® multiwash, Teknunc, Roskilde, Denmark) was employed. To prevent non-specific binding the concentration of Tween 20 was increased to 0.1%, the wells were postcoated with 300 µl 1% bovine serum albumin, and the alkaline phosphatase-conjugated rabbit anti-mouse antibody was diluted in PBS containing 5% heat-inactivated (56°C for 30 min) normal rabbit serum. The enzymatic reaction was performed with 250 µl substrate solution for 60 min, and 200 µl of the reaction product was transferred.

The calibration curve obtained by titration of standard RBC expressed a detection limit of about 10% and discrimination between levels of CR1 in the interval of about 10-140% of the standard erythrocytes. The between-day determinations of the standard RBC gave a coefficient of variation of about 11%, and the mean CR1 value for a panel of eight staff members varied by less than 1% through a period of 1 year.

*Tetanus toxoid anti-tetanus
toxoid complexes (TT-aTT)*

Since the CR1 has been shown to bind various types of antigen-antibody complexes, indicating that the phenomenon is a general one (25), a well-characterized system (7, 20) involving homologous IgG was chosen.

TT-aTT were prepared from a purified tetanus toxoid preparation (No. XXVII, 3 mg/ml) and human tetanus immunoglobulin (250 IU/ml) from Statens Seruminstitut, Copenhagen, Denmark. A turbidimetric precipitation test (16) was carried out, and TT-aTT were formed at different antigen:antibody ratios in PBS containing 5% human serum albumin (ORHA 20, Behringwerke AG, Marburg, W. Germany) by varying the concentration of TT. The mixtures were incubated at 37°C for 1 h, and the complexes were stored in aliquots at -70°C. The concentrations of TT-aTT dilutions at a given ratio are given in terms of μg TT/ml. The complexes formed at equivalence contained 40 μg TT/ml.

Binding of TT-aTT to erythrocytes

Two hundred μl PBS⁺⁺ (PBS with 1.5×10^{-4} M Ca⁺⁺ and 5×10^{-4} M Mg⁺⁺), containing 6×10^7 RBC, were delivered to a 12 $\times$ 75 mm glass tube. The tubes received 100 μl serum and 100 μl TT-aTT. They were gently shaken and incubated in a water bath at 37°C with constant agitation. The reaction was stopped (at the indicated times) by cooling and dilution with 5 ml PBS (4°C). RBC were spun down at 1000 g for 5 min, and the supernatant was discarded. After 4 washings at 4°C the RBC were resuspended in 950 μl PBS transferred to microtiter plates (5.5×10^6 cells in 100 μl PBS/well; 8 wells/sample). Coating of the microtiter plates was performed as described for CR1 determinations (30).

Incubations of the RBC coated plates with optimal concentrations of antibodies (proportionality between quantity of IC added and absorbance values was secured) were performed in quadruplicate to detect bound complexes. To quantitate IgG 100 μl alkaline

phosphatase-conjugated F(ab')₂ fragments of goat anti-human IgG (Sigma No. A-5403, Sigma Chemical Co., St. Louis, MO) diluted 1:200 in PBS were added to each well. After incubation for 60 min at 22°C the wells were washed. To quantitate C3c 100 μl rabbit antibody to human C3c (that also bound to C3b) (Dakopatts, Copenhagen; code No. A062) diluted 1:5000 in PBS was added to each well and incubated for 60 min at 22°C, the wells were washed and subsequently incubated for 60 min at 22°C with alkaline phosphatase-conjugated swine antibody to rabbit immunoglobulins (Dakopatts; code No. D306) diluted 1:200 in PBS containing 5% heat-inactivated (56°C for 30 min) normal swine serum. Finally, the wells were washed.

The colorimetric reaction was carried out as described for CR1 determination, except that it was allowed to take place for 50 min. The results were expressed by mean absorbances.

*Assay for release of blocking
CR1-bound immune complexes*

Washed RBC (6×10^7 in 200 μl PBS) were incubated with 200 μl PBS containing purified factor I (final concentration 50 U/ml) at 37°C for 30 min in order to induce release of blocking CR1-bound IC, if any (32). After washing, the cells were added to microtiter plates (5.5×10^6 /well) and the levels of CR1 were measured. Assays were performed in duplicate.

Assay for anti-CR1 activity in serum

Two hundred μl PBS containing 6×10^7 RBC (0 Rh neg, CR1 70%) were delivered to a 12 $\times$ 75 mm glass tube, and 200 μl of patient serum containing 10 mM (final concentration) EDTA (ethylenediamine tetraacetate) were added. The tubes were incubated at 37°C and 22°C, respectively, for 1 h with constant agitation, the cells were washed 4 times with PBS, resuspended in 950 μl PBS, transferred to the plates (5.5×10^6 cells/well) and examined for CR1 as well as for bound IgG. Assays were performed in duplicate.

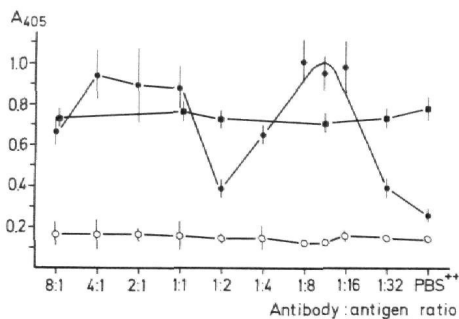


Fig. 1. Analysis of RBC for C3c (●) and CR1 (■), in parallel, after incubation at 37°C for 15 min with an excess of TT-aTT of different antigen: antibody ratios and serum diluted 1:8. ○ denotes C3c on RBC reacted in the presence of 10 mM EDTA. The results are expressed in absorbances using rabbit anti-human C3c (and C3b) and the mouse monoclonal anti-CR1 To5 as detecting antibodies, respectively. Each point represents the mean of five experiments, and the error bars denote SEM.

RESULTS

Binding of immune complexes to CR1

Studies were initially performed in order to optimize the binding of TT-aTT to RBC. The experiments were carried out in accordance with the detailed descriptions given by others, emphasizing that the kinetics of interaction between CR1 and IC (14, 19) and the serum dilution optimal for binding (18) do not change with antigen:antibody ratio, and also that sustained, but not decreased, binding of IC is achieved by dilution of serum (18, 19, 20).

TT-aTT preparations formed at both 2 times antibody excess and 12 times antigen excess were used. The binding was examined at serial 2-fold dilutions of serum and employing times of incubation between 1 and 60 min. Optimal binding was seen at a serum dilution of 1:8. The time required for optimal binding was 10 min. At later times the binding was unchanged. Unless otherwise stated 15 min of incubation and the 1:8 dilution of serum were chosen. Negligible binding of the developing antibodies was observed when the reaction mixtures contained 10 mM EDTA (see below concerning

anti-human IgG), and when uncomplexed TT or aTT in appropriate concentrations were substituted for TT-aTT. Further, a concordant loss of CR1 antigenicity and capacity for binding of IC was seen when the receptors were removed by dithiothreitol (30).

Experiments were next done at optimal conditions for binding of TT-aTT. The results obtained for TT-aTT formed at different antigen:antibody ratios (containing equal amounts of antibody including unreacted IgG), and using anti-human C3c as detecting antibody appear from Fig. 1. The properties of these TT-aTT preparations are known to be different (7): Fc density of the complexes decreases from left to right, while there are maxima for size at equivalence and in slight antibody excess and for reactivity with complement in both antibody and antigen excess.

Application of $F(ab')_2$ fragments of goat anti-human IgG as detecting antibody in parallel revealed that unbound IC sedimented together with the RBC at equivalence and in 2- and 4-fold antibody excess. In contradistinction to RBC-bound IC, these complexes, accounting for about 10–15% of the measured absorbances, were removed by centrifugation on Percoll as described by Cornacoff et al. (2). Otherwise, the results were identical to the data shown in Fig. 1.

RBC were incubated with 2-fold dilutions of TT-aTT prepared in 12 times antigen excess (480 µg TT/ml). As seen from Fig. 2, the amount of specifically bound TT-aTT increased by increasing concentration, reaching saturation at the 1:2 dilution (240 µg TT/ml), after which there was only increase with concordant increase in non-complement dependent binding. Saturation with TT-aTT prepared in 2 times antibody excess was achieved at the 1:4 dilution (not shown). These results indicate that the experiments shown in Fig. 1 were performed in excess of complement-opsonized TT-aTT.

Binding of monoclonal anti-CR1 to opsonized receptors

RBC were tested for CR1 in parallel with analysis for TT-aTT of different antigen:antibody

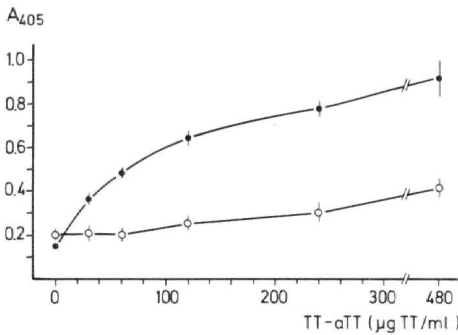


Fig. 2. Binding to RBC of TT-aTT prepared in 12 times antigen excess after incubation for 15 min at 37°C in serial 2-fold dilutions at the 1:8 dilution of serum without (●) and with (○) 10 mM EDTA. The amount of TT-aTT bound is expressed as absorbances using alkaline-phosphatase conjugated F(ab')₂ fragments of goat anti-human IgG as developing antibody. Mean and SEM of six experiments.

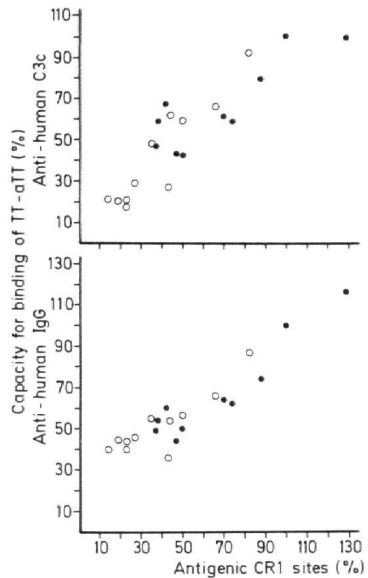


Fig. 3. Capacity of CR1 on erythrocytes from 10 normal individuals (●) and 11 patients with systemic lupus erythematosus (○) for binding of the monoclonal anti-CR1 antibody To5 and of soluble TT-aTT. Assays of TT-aTT-binding (see text) were performed in duplicate, using both alkaline-phosphatase conjugated F(ab')₂ fragments of goat anti-human IgG and rabbit anti-human C3c (and C3b) as detecting antibodies. The results are expressed in percentage of the standard RBC. The antigenic and functional capacities were significantly correlated ($P < 0.001$).

ratios bound at conditions optimal for saturation of CR1 (Fig. 1). The receptors remained accessible to the monoclonal antibody (To5) after binding of the complexes.

Assays for release of blocking CR1-bound immune complexes and anti-CR1 activity in serum

RBC from 11 patients with SLE, whose cells had CR1 levels as shown in Fig. 3, were incubated with factor I. The CR1 levels remained unchanged and were identical with the levels of control cells incubated with PBS (Method of paired comparisons: $P > 0.30$). Incubation of normal RBC with serum from these patients did not change the CR1 levels ($P > 0.10$) or reveal IgG binding in excess of that observed in controls.

Correlation between antigenic and functional CR1 sites

RBC from 10 normal subjects and the 11 patients with SLE were assayed for the capacity to bind both monoclonal anti-CR1 and TT-aTT prepared at 12 times antigen excess and diluted 1:2 (Fig. 3). The standard RBC was included in each run, and the results obtained

by test cells were expressed by calculating the absorbance at 405 nm of each test sample as a percentage of the standard sample. The between-day determinations of TT-aTT binding to the standard RBC gave coefficients of variation of 18 and 21% using anti-human IgG and anti-human C3c, respectively, as detecting antibodies ($n = 23$). The antigenic and functional capacities were significantly correlated using both anti-human IgG (Pearson's correlation coefficient $r = 0.93$, $P < 0.001$) and anti-human C3c ($r = 0.89$, $P < 0.001$) as detecting antibodies. Similar figures were obtained by testing the patients and normals separately, except that the correlation in normals using anti-human C3c as detecting antibody was

slightly less pronounced ($r = 0.85$, $P < 0.01$). The binding of TT-aTT was not increased by extending the time of incubation to 30 min. Identical results were obtained using supernatants of TT-aTT preparations centrifuged at 1500 g for 30 min.

DISCUSSION

The microtiter plate ELISA and radioimmunoassays using monoclonal antibodies are specific, sensitive and quantitative techniques for measuring CR1 levels on RBC.

However, the presence in patient sera of immune complexes and autoantibodies introduces the question of false low results. The monoclonal anti-CR1 antibody To5 used in the microtiter plate ELISA (30) has been reported to inhibit the capacity to bind erythrocyte-antibody-complement complexes (EAC3b) of RBC (6) and B lymphocytes (21). The present results concerning the experimental system using TT-aTT are in accordance with previous findings as regards the kinetics and saturation of binding complement-opsonized complexes to erythrocyte CR1 (7, 14, 18, 19, 20). It was established that To5 binds to receptors saturated with IC representing a wide range of size and complement-fixing potential (Fig. 1). Therefore, quantitation of CR1 in patients can be made whether or not the receptors are occupied with IC. In accordance with this, the CR1 on RBC from patients with SLE drawn into CPDA-solution were found not to be masked by IC. The inability to detect antibody to CR1 in the serum of patients with SLE, as assessed by the lack of interference with determination of CR1 on normal RBC using the monoclonal antibody To5 or by uptake of IgG by these cells, is consistent with the observations that antibody to CR1 is an exception in these patients (1, 35, 37).

The different structural CR1 allotypes have been reported not to differ in affinity for dimeric C3b (38) or in antigenicity toward polyclonal (38) and monoclonal (3, 4) antibodies in normals. Neither have major differences in CR1 phenotypes been found between normal individuals and patients with SLE (3). Further, the numbers of binding sites for dimeric C3b

and for anti-CR1 were shown to correlate on RBC from both normal subjects and patients with SLE, and the affinity for dimeric C3b of CR1 did not differ on RBC from patients versus controls (35). The correlation between uptake of the monoclonal antibody To5 and soluble IC reported here (Fig. 3), adds to this evidence of a preponderance of low numbers of normal CR1 on RBC in patients, rather than non-functional, structurally altered forms that are antigenically intact. Nevertheless, a lowered number of CR1 may, in RBC excess, lead to a decrease in the kinetics of IC-binding, possibly because of slower receptor reorganization and clustering (9).

In conclusion, the reported data establish that the true CR1 level is determined using the monoclonal mouse anti-CR1 antibody To5 in the microtiter plate ELISA for quantitation of CR1 on RBC in patients with diseases involving immune complexes and/or autoantibodies. This is important in order to draw valid conclusions from investigations focusing on a low CR1 level as a predisposing and/or disease-associated factor in such patients.

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