

Release of Immune Complexes Bound to CR1 on Erythrocytes; Suramin Inhibits Factor I in the Presence of EDTA

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An experimental model was established in order to study the release of immune complexes (IC) bound by complement C3b receptors (CR1) on human erythrocytes (RBC). Soluble tetanus toxoid anti-tetanus toxoid complexes were incubated with RBC in the presence of autologous serum at optimal conditions for binding. The RBC carrying complement-opsonized complexes were incubated with appropriate serum reagents, and it was shown that factor I was required for release of the complexes, which occurred without loss of CR1. Suramin was, irrespective of factor I, found to induce release of CR1-bound IC in the absence of EDTA, whereas factor I-mediated release was inhibited by suramin in the presence of EDTA. EDTA probably interfered through a charge-dependent interaction. These observations are decisive for the interpretation of *in vitro* experiments involving these reagents. The combination of EDTA and suramin was found inappropriate for use in quantitative determination of *in vivo* CR1-bound IC.

Key words: C3b receptor (CR1); factor I; human erythrocyte; immune complexes; suramin.

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CLINICAL ASPECTS

Complement C3b receptors on human erythrocytes bind complement-fixing, potentially pathogenic immune complexes. Immune complexes bound to CR1 *in vivo* are qualitatively detectable, but attempts to characterize these complexes further demand inactivation of factor I in blood samples, which has so far been universally unsuccessful.

The complement C3b receptors (CR1) on human erythrocytes bind complement-fixing immune complexes (IC) and may play a key role in the normal removal from the circulation of such potentially pathogenic complexes (25, 32).

CR1 binds C3b, iC3b and C4b reversibly, the greatest affinity being for C3b. Under physiological conditions the fragments C3b and C4b are considered to be the main ligands (20, 25). Native C3 does not bind CR1 and the affinity of fluid-phase monomers C3b and C3c to CR1 is very low, whereas effective binding between dimeric C3b or complement-activating IC and CR1 takes place under isotonic conditions (1,

2). Once bound to the red blood cells (RBC), the release of IC from CR1 is mediated essentially by interaction between CR1 and factor I, and is associated with degradation of IC-C3b into IC-C3d and fluid-phase C3c (15, 16, 18, 19). Moreover, under physiological conditions, CR1 serves as the essential cofactor for I-mediated enzymatic breakdown of membrane-bound C3b (and iC3b) into C3c and C3dg as well as C4b into C4c and C4d (20, 25). The release of IC from CR1 *in vivo* seems to take place during passage of RBC through the liver (3).

Despite this comprehensive knowledge concerning interaction of IC with CR1 *in vitro* and qualitative data obtained by haemagglutination

assays, which indicate that IC bound to CR1 on RBC *in vivo* are detectable (10, 11, 12), attempts to quantify *in vivo* CR1-bound IC have so far been almost unsuccessful (21, 27, 29, 31). Five samples of RBC from patients with systemic lupus erythematosus were found to have elevated IgG, probably representing bound IC (29). The collection and processing of blood samples seem to have a significant influence on the amount of IC released from RBC (29), pointing to the need of blocking the reaction between CR1, factor I and IC immediately at venipuncture.

Specific inactivation of factor I is not possible. Factor I is not inhibited by protease inhibitors or chelating agents such as 50 mM EDTA (23) but a selective inhibiting effect has been obtained by the use of suramin (15, 24) or K76 monocarboxylic acid (7, 8, 15, 16) at appropriate concentrations. However, it has recently been reported that suramin can cause release of IC bound to CR1 on RBC as well as block the binding of IC to the receptors (28).

Here we present an experimental model suitable for studies of factor I-mediated release of CR1-bound IC. It is shown that the factor I inhibitor suramin inhibits release in the presence of EDTA. The implications as regards *in vitro* and *in vivo* investigations are discussed.

MATERIAL AND METHODS

Erythrocytes and serum reagents. RBC and serum were obtained from a healthy AB Rh pos donor as described elsewhere (30).

Since factor I, in contrast to classical and alternative pathway complement function, is stable to heating at 56°C for 30 min but not for 120 min (15, 18, 19, 23), portions of serum were heat-inactivated at 56°C for 30 min (SΔ30) or for 120 min (SΔ120) and stored in aliquots at -70°C.

Immune complexes. Tetanus toxoid anti-tetanus toxoid complexes (TT-aTT) were prepared at 12 times excess of antigen (480 µg/ml) (30).

Suramin. Germanin® Bayer 205 (Antrypol) was purchased from Bayer AG, Leverkusen, W. Germany.

Factor I. C3 Complement Inactivator Human (Cordis No. 750-530, Cordis Laboratories, Miami, FL) containing 500 units per ml was used.

Release assay. TT-aTT were first allowed to bind to RBC at optimized conditions (30). Briefly, 200 µl PBS⁺⁺, containing 6×10^7 RBC, were incubated at 37°C for 15 min with 100 µl human serum diluted 1:8 in PBS⁺⁺ and 100 µl TT-aTT diluted 1:2 in PBS⁺⁺. The reactions were stopped by cooling and dilution, and the RBC were washed. Subsequently, the RBC bearing complement-opsonized TT-aTT were resuspended in 150 µl PBS⁺⁺ or PBS and incubated with 200 µl of different serum reagents with or without suramin and EDTA (ethylenediamine tetraacetate) at the indicated times and temperatures. Again, the reactions were stopped and the RBC washed. Non-released IC were quantitated by the microtiter plate enzyme-linked immunosorbent assay (ELISA) using F(ab')₂ fragments of goat anti-human IgG and rabbit anti-human C3c (that also bound to C3b) as detecting antibodies (30).

Quantitation of CR1. The CR1 levels were determined by the microtiter plate ELISA (30).

RESULTS

Factor I-mediated release of CR1-bound TT-aTT

Experiments were first performed to establish that I is the serum factor mediating release. RBC bearing CR1-bound complexes were resuspended in PBS⁺⁺ and incubated for 20 min at 37°C in sequentially diluted (in PBS⁺⁺) serum, or alternatively in SΔ30, SΔ120, or SΔ120 supplemented with normal serum concentration of factor I (100 U/ml). The amounts of complexes remaining bound were quantitated using both anti-human C3c (Fig. 1) and anti-human IgG (not shown) as detecting antibodies. The release of TT-aTT at incubation in SΔ30 and serum decreased in parallel at dilution as measured by the amount of bound IgG, while the use of anti-human C3c as detecting

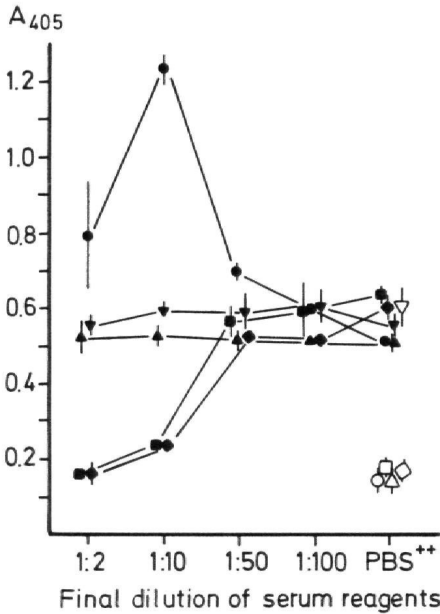


Fig. 1. CR1-bound TT-aTT following incubation at 37°C for 20 min of RBC, bearing prebound complexes, with dilutions of serum (●), SΔ30 (■), SΔ120 (▲), or SΔ120 supplemented with normal serum concentration of factor I (◆) using rabbit anti-human C3c (that also bound to C3b) as detecting antibody. CR1 determinations (▼), performed in parallel with measurements of bound complexes, are shown for RBC corresponding to ◆. The open symbols indicate control experiments using RBC without prebound complexes (PBS⁺⁺ substituted for TT-aTT in the binding step). Mean and range of two experiments.

antibody revealed that incorporation of C3 and possibly rebinding and/or fixation of bystander C3 also took place in serum (18, 26). The release following incubation in SΔ120 supplemented with factor I paralleled that in SΔ30. No release occurred following incubation in SΔ120. The time of incubation was varied between 5 and 60 min; no further release was seen beyond 20 min (not shown).

RBC were tested for CR1 in parallel with analysis for TT-aTT. The results obtained using SΔ120 supplemented with factor I as releasing reagent are shown in Fig. 1. Determination of CR1 on RBC following incubation in serum,

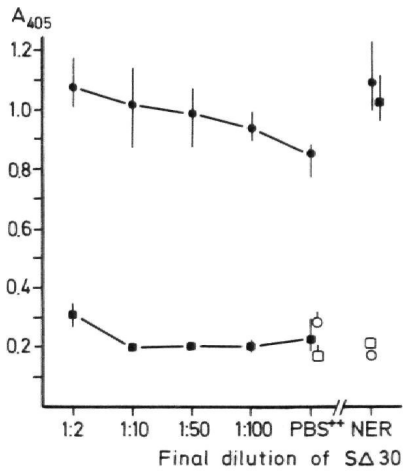


Fig. 2. Effect of suramin (final concentration 5 mg/ml) incubated for 60 min at 22°C with RBC, bearing prebound TT-aTT, and SΔ30 in the presence (●) and absence (■) of 20 mM EDTA. The amount of non-released complexes were quantitated using rabbit anti-human C3c (that also bound to C3b) as detecting antibody. The corresponding results for RBC not exposed to release (NER) are shown to the right. The open symbols denote control experiments using RBC without prebound complexes (PBS⁺⁺ substituted for TT-aTT in the binding step). Mean and range of three experiments.

SΔ30 and SΔ120 gave identical results (not shown).

Inhibited release of CR1-bound TT-aTT by suramin

Studies were next carried out to provide measures for blockade of release of RBC-bound IC as well as of classical and alternative pathway function (binding and rebinding). To simulate an easily attainable arrangement for blood sampling in a hospital clinic, the subsequent incubations were carried out for 60 min at 22°C. As expected, experiments showed that factor I mediated release at these conditions (18), and that CPDA (30), in contrast to 10 mM EDTA (18, 30), did not prevent complement activation at the concentration used for anticoagulation (9).

The introductory studies also showed that a

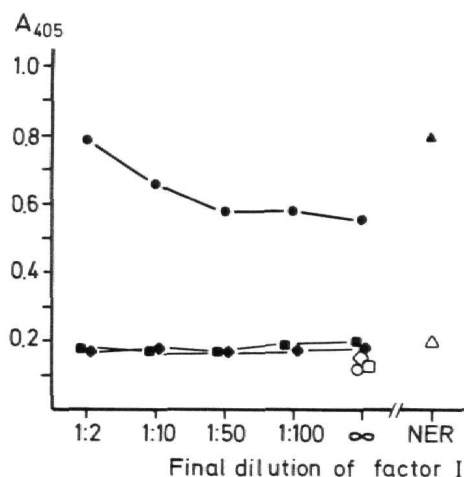


Fig. 3. Effect of suramin (final concentration 5 mg/ml) incubated for 60 min at 22°C with RBC, bearing prebound TT-aTT, and purified factor I in the presence (●) and absence (■) of 20 mM EDTA using PBS-buffer, as well as in the absence of EDTA using PBS⁺-buffer (◆). The amount of non-released complexes were quantitated using rabbit anti-human C3c (that also bound to C3b) as detecting antibody. The corresponding results for RBC not exposed to release (NER) are shown to the right. The open symbols denote control experiments using RBC without prebound complexes (PBS⁺ substituted for TT-aTT in the binding step). The dilution of factor I refers to normal serum concentration (100 U/ml).

sufficient reduction of release was unattainable solely by dilution of serum (containing EDTA). Suramin caused a dose-dependent release of complexes when the experiments were performed without EDTA and using PBS⁺ for resuspension of RBC and dilution of serum. But this effect of suramin was reversed by the use of an adequate concentration of EDTA (20 mM). Therefore, and because SΔ30 permits investigation of the effects of factor I without addition of EDTA to inactivate classical and alternative pathway functions, the interaction between CR1-bound IC, factor I and suramin was examined in experiments using SΔ30 and 20 mM EDTA.

As appears from Fig. 2, using rabbit anti-human C3c as detecting antibody, suramin (5 mg/ml) inhibited release of the complexes in the presence of EDTA, but caused release in the

absence of EDTA at dilutions of SΔ30 (1:50 and 1:100) and in the buffer, which did not show I-mediated release (Fig. 1). A final suramin concentration of 2 mg/ml gave identical results, but about 30% of the complexes were lost at 1 mg/ml, while both concentrations were sufficient for unchanged release in the absence of EDTA (not shown). Identical results were obtained using F(ab')₂ fragments of goat anti-human IgG as detecting antibody.

The effect of EDTA was not due to chelation of Ca⁺⁺ and/or Mg⁺⁺, since similar results were obtained when the RBC bearing CR1-bound IC were incubated with purified factor I and suramin in the presence or absence of EDTA using PBS-buffer, and as release of IC took place in the absence of EDTA using both PBS and PBS⁺ (Fig. 3). Identical results were obtained using rabbit anti-human C3c and F(ab')₂ fragments of goat anti-human IgG as detecting antibodies.

DISCUSSION

The purpose of the present study was to develop an experimental model suitable for investigation of the requirements for release of CR1-bound IC, and to evaluate measures for blockade of the reaction between CR1 and IC.

Our findings concerning the experimental system using TT-aTT (Fig. 1) showed that factor I, and not intact classical or alternative pathway complement function, was essential for the release of CR1-bound, complement opsonized TT-aTT. The consistency with data reported on TT-aTT (18) and other IC-systems (9, 13, 14, 15, 16, 17, 18, 26, 28, 29) demonstrated that the TT-aTT used were not unique in their binding (30) and release properties. Further, in accordance with previous studies regarding antigenicity (26) and function of CR1 (3, 17), the receptors were found to be antigenically unchanged by the binding and release of TT-aTT.

As expected 10 mM EDTA abolished activation of the classical and alternative pathway functions that mediate binding and rebinding of IC (18).

The described suramin-induced release of

CR1-bound IC (28) was reproduced, but it was also shown that this effect could be reversed by EDTA irrespective of Ca^{++} and Mg^{++} (Figs. 2 and 3). Since the interaction of C3b with factor I is charge-dependent (24), and the polyanionic suramin is supposed to bind directly to complement components (5, 28) EDTA might interfere through a charge-dependent interaction. This observation is decisive for interpretation of *in vitro* studies involving EDTA and suramin (28).

Suramin is a potent, rapidly acting and selective (non-specific) but also reversible and firmly protein-bound inhibitor of factor I (4, 5, 6, 22). The reversibility together with the presence of unspecific binding of IgG to RBC at increasing concentrations of suramin rendered application of a low but still sufficient concentration of suramin necessary, which was difficult to obtain during sampling and washings of RBC because of changing protein concentrations (data not shown). Therefore, the combination of suramin and EDTA was found inappropriate for blockade of the reaction between CR1 and IC at sampling and washings of RBC with a view to investigate *in vivo* bound IC. The irreversibly acting factor I inhibitor, K76 monocarboxylic acid (7, 8), might have advantages in this context.

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