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A lysine-binding protein in SLE sera inhibits the binding of immune complexes to normal erythrocyte CR_1 (complement receptor type 1)

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SUMMARY

The binding of ^{125}I-labelled immune complexes (IC) to normal human erythrocyte CR1 (complement receptor type 1) by sera from patients with SLE was found to be significantly decreased compared to normal sera. In 13/29 patients, there was an inhibitor which decreased the binding of opsonized IC in normal sera to normal erythrocytes. It was found in each of the nine patients who had clinically active disease. The inhibitor was shown to be a globulin that was labile at 56°C and bound to lysine; low concentrations of tranexamic acid and of lysine abolished the effects of the inhibitor which suggests that it possesses lysine-binding sites: these may block the CR_1-binding site on IC opsonized with complement. This inhibitor may decrease the efficiency of IC carriage by erythrocyte CR_1.

Keywords immune complex erythrocyte CR_1 inhibitor SLE

INTRODUCTION

Studies of the mechanisms of immune complex (IC) transport and disposal are relevant to the understanding of some of the factors that contribute to the pathogenesis of immune complex disease (Schifferli, Ng & Peters, 1986). There is recent evidence to suggest that the erythrocyte complement receptor, CR_1, in man and other primates is important in the transport of IC. IC that have fixed complement may bind to CR_1 via the ligands C3b, iC3b, C4b and iC4b. Although CR_1 is an important opsonic receptor on phagocytic cells, 90–95% of circulating CR_1 in primates is located on erythrocytes (Siegal, Liu & Gleicher, 1981). Direct evidence that erythrocyte CR_1 may participate in the transport of IC has come from studies of the fate of iodinated IC in the circulation of baboons (Cornacoff *et al.*, 1983). Under circumstances in which IC failed to bind efficiently to CR_1 on erythrocytes, a small percentage of these complexes were deposited in tissues outside the reticulo-endothelial system (Waxman *et al.*, 1984; 1986). A second function of CR_1 is as a cofactor for factor 1 in the cleavage of C3b to iC3b (Iida & Nussenzweig, 1981) and then to C3dg (Ross *et al.*, 1982; Medof *et al.*, 1982a). Since C3dg has no affinity for CR_1, the IC are released from the erythrocytes. These IC have decreased ability to bind to fresh erythrocytes in serum *in vitro*, presumably due to decreased complement activation (Medof, Prince & Oger, 1982). It is possible that failure of IC binding to erythrocyte CR_1 may contribute to the deposition in tissues of inflammatory IC. Several examples where this may occur in patients with IC disease have been identified: first, erythrocyte CR_1 in patients with SLE are significantly decreased (Miyakawa,

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Yameda & Kosaka, 1981; Iida, Mornaghi & Nussenzweig, 1982; Wilson *et al.*, 1982; Walport *et al.*, 1985); this may be expected to reduce their capacity for IC transport. Second, hypocomplementaemia results in decreased opsonization of IC and hence decreased binding to CR_1 (Jepsen *et al.*, 1986). The aim of this study was to investigate a third possible mechanism, namely the inhibition by SLE sera of binding of normally opsonized IC to erythrocytes. We report the finding of such an inhibitor which we partially characterized as a globulin with lysine-binding properties.

MATERIALS AND METHODS

Patients. All patients had SLE by the ARA revised criteria (Tan *et al.*, 1982). Two patient groups were studied. For the measurement of IC binding to erythrocytes, the sera examined were within the normal range for serum concentrations of C3 and C4 and total haemolytic complement (CH50). C3 and C4 were measured by radial immunodiffusion and CH50 by a haemolytic plate technique (Lachmann & Hobart, 1978). To ascertain the prevalence of the inhibitor of IC binding to erythrocytes in patients with SLE, serum and EDTA plasma samples were taken from a series of 29 unselected patients.

Sera and EDTA (10 mM, pH 7·2) plasma samples were separated within 1 h and stored at −70°C until assayed.

Disease was considered to be active if there were symptoms or signs of organ involvement as defined by the ARA classification criteria for SLE.

Reagents and methods. Bovine serum albumin (BSA), ovalbumin (OVA) DFP (di-isopropylfluorophosphate), PMSF (phenylmethylsulphonylfluoride) and EACA (E-amino-caproic acid) were purchased from Sigma, Poole, United Kingdom. Other reagents purchased were streptokinase from Behringwerke AG, Marburg, Germany, aprotinin from Bayer, Germany, tranexamic acid from Kabivitrum, Sweden, protein A-Sepharose from Pharmacia AB, Uppsala, Sweden, CFD (complement fixing diluent) from Oxoid Ltd, Basingstoke, UK.

Purified human factor H was a gift from Dr M. C. Venning. Monoclonal IgM rheumatoid factor was separated from the cryoprecipitate from a patient with mixed essential cryoglobulinaemia (Schifferli *et al.*, 1981). Polyclonal IgM rheumatoid factor from a patient with rheumatoid arthritis was a gift from Dr A. Gharavi. Plasminogen was prepared by the method of Deutsch & Mertz (1970). Human tissue plasminogen activator was prepared by the method of Rijken & Collen (1981). Rabbit antiserum to human lys-plasminogen was raised by Dr S.A. Cederholm-Williams. Rabbit antiserum to CR_1 was a gift from Dr G. D. Ross (Dobson, Lambris & Ross, 1981).

Proteins were labelled with ^{125}I by the iodogen method (Fraker & Speck, 1978) except for staph protein A (Pharmacia, Uppsala, Sweden) which was iodinated by the method of Bolton & Hunter 1973).

Antigen-antibody complexes. ^{125}I-BSA-antiBSA IC were made according to the method described by Medof *et al.* (1982b) with minor modifications. ^{125}I-BSA (specific activity 5·25 μCi/μg), 0·124 μg, was incubated with a 4-fold excess of rabbit anti-BSA (heat inactivated and diluted 200-fold in 0·2% OVA in CFD) in a total volume of 250 μl at 37°C for 30 min and the IC mixture diluted 4-fold in OVA/CFD before use. The IC were non-precipitating.

^{125}I-tetanus toxoid (TT)-anti TT IC were a gift from Dr J. A. Schifferli and were prepared in 20-fold antibody excess. IC were non-precipitating.

Experiments were performed with BSA-anti-BSA IC unless otherwise stated.

Normal erythrocytes. Normal erythrocytes were obtained from healthy laboratory workers. Heparinized blood (10 U/ml preservative-free heparin, Weddel Pharmaceuticals, Wrexham, UK) was separated on Ficoll Hypaque (Pharmacia, Sweden) by centrifuging at 800 *g* for 15 min at room temperature. The erythrocyte pellet was washed three times in PBS and resuspended in an equal volume of 0·2% OVA/PBS.

IC binding to erythrocytes. Ten microlitres of radiolabelled IC were incubated with 10 μl of test serum at 37°C for 5 min, the tubes transferred to ice and normal human red cells (approximately 2×10^9) were added; under these conditions titration demonstrated that an excess of CR_1 was present so that the majority of the IC bound to the erythrocytes. The erythrocytes were centrifuged

at 400 ***g*** for 3 min, washed three times in 0·2% OVA/PBS and the radioactivity in the pellet counted. This represented IC bound to erythrocytes.

Co-precipitation with anti-C3. Ten microlitres of radiolabelled IC were incubated with 10 μl of test serum at 37°C for 5 min and then transferred to ice. Twenty microlitres of a polyclonal anti-C3 antiserum was added in the presence of 10 mM EDTA, pH 7·4, incubated at 4°C overnight. The IC were centrifuged at 1200 ***g*** for 30 min, washed twice in PBS, and the radioactivity in the pellet counted.

SLE inhibitor. Ten microlitres of ^{125}I-IC were opsonized with 10 μl of normal human serum (NHS) by incubation at 37°C for 5 min then incubated with 20 μl of test serum for 30 min on ice before binding to normal red cells (approximately $1{\cdot}5 \times 10^9$) was measured. Certain SLE sera inhibited the binding of normally opsonized IC to red cells, hereafter referred to as 'SLE inhibitor'.

Inhibition of immune precipitation and solubilization of immune aggregates. These were performed by the method of Schifferli *et al.* (1981).

IC-associated C3 fragments. Opsonized IC were incubated with SLE serum or normal serum, then absorbed on Sepharose coupled to anti-C3g (clone 9, a gift from Professor P. J. Lachmann). After extensive washing, the anti-C3g-Sepharose bound material was separated by polyacrylamide gel electrophoresis using a 10% gel and reducing conditions (Laemmli, 1970). Western-blotting was performed by the method of Kyhse-Anderson (1984). Blots were probed with rabbit polyclonal anti-C3d (Dako, Denmark) which had been absorbed on human IgG-sepharose and diluted 1/50. After washing, this was followed by ^{125}I-Staph Protein A (0·5 mg in 15 ml, specific activity 12 μCi/mg) and developed by autoradiography.

RESULTS

IC binding to erythrocytes. The binding of IC to erythrocytes was dependent on complement and CR_1; binding of complexes opsonized by normal human serum (NHS) was reduced from 85% of offered counts to 5% with 10 mM EDTA-NHS and heat inactivated NHS, and was blocked by 8 μl of polyclonal anti-CR1 antibody.

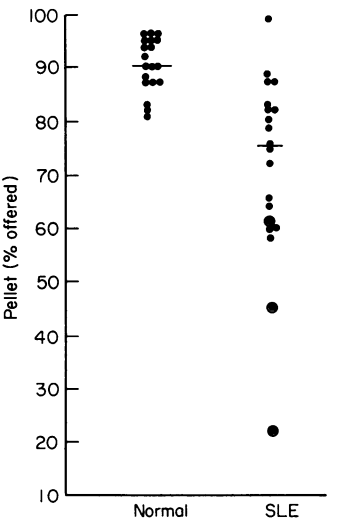

Fig. 1. Binding to normal human erythrocytes of ^{125}I-BSA anti-BSA IC incubated at 37°C 5 min with 10 μl serum: normal subjects (median = 89·4) compared with SLE sera (median = 75·0). Circles around dots show clinically active patients. Binding expressed as % offered counts.

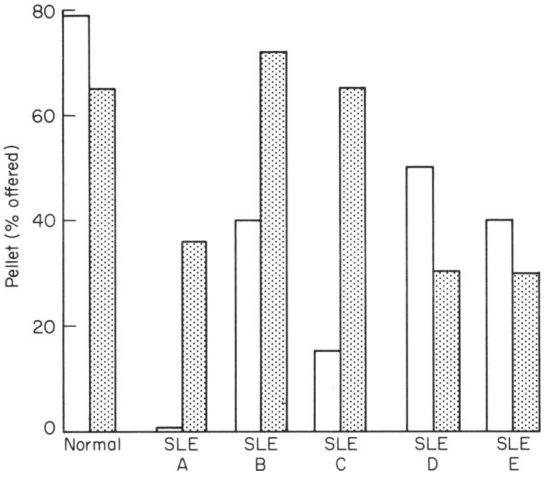

Fig. 2. Sera from a normal subject and SLE patients (A, B, C, D & E): comparison of anti C3 coprecipitation (filled bars) with binding to normal erythrocytes (white bars).

The SLE patients were clinically inactive except for three (Fig. 1) and all had normal serum concentrations of C3, C4 and CH50. The SLE sera had a significantly decreased ability to bind IC to normal human erythrocytes (Fig. 1) ($P = 0{\cdot}0004$, Mann Whitney U-test). These sera were normal in their capacity to solubilize pre-formed IC. Some sera were defective in their ability to inhibit immune precipitation whereas others were normal (Table 1). However there was no correlation between their ability to inhibit immune precipitation and to bind IC to normal erythrocytes.

The decreased ability of SLE sera to bind IC to normal red cells could have been due either to reduced C3 deposition on the IC or to the presence of an inhibitor which interfered with the binding of normally opsonized IC to red cells. To distinguish between these two possibilities, the C3 bound to IC was quantified in a fluid phase coprecipitation assay and compared with IC binding to erythrocytes in individual serum samples (Fig. 2). In two SLE patients (D and E) the decreased binding of IC to red cells was associated with decreased C3 deposition on IC, suggesting poor opsonization as the explanation for the poor erythrocyte binding. In contrast, in three SLE patients (A, B and C) binding to erythrocytes was disproportionately poor for the amount of C3 on IC

Table 1. ^{125}I BSA-anti-BSA IC binding to normal erythrocytes compared with inhibition of immune precipitation (11P) by 20 SLE sera

	Binding to erythrocytes	
	Normal	Abnormal
11P		
Normal	7	6
Abnormal	3	4

Binding to erythrocytes: Normal = 75% bound.
11P: Normal = 10% precipitate at 15 min.
Results expressed as % offered counts.
$P = 1{\cdot}0$ (Fisher's exact test).

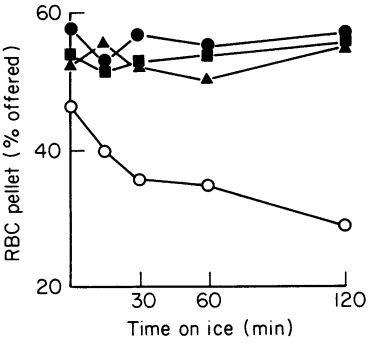

Fig. 3. SLE inhibitor: effect of SLE serum on opsonized IC incubated on ice with PBS (●), NHS (▲,■) and SLE serum (○). At intervals, binding of IC to normal erythrocytes was measured.

detected, suggesting the presence of an inhibitor that was interfering with the binding of opsonized IC to red cells.

SLE inhibitor. To test the hypothesis that SLE sera contained an inhibitor of IC binding to erythrocytes, SLE sera were incubated with IC pre-opsonized with NHS. Figure 3 demonstrates the inhibitory effect of an SLE serum on the binding of normally opsonized IC to red cells, an effect that increased with time of incubation on ice. There was no difference in behaviour of EDTA plasma compared with serum. Pre-incubation of patient serum with the red cells before addition of normally opsonized IC did not reproduce the effect, demonstrating that the SLE effect occurred on the opsonized IC and not on erythrocyte CR_1. Plasma from 29 SLE patients was examined, and the inhibitor was detectable in 13. All nine patients with clinically active disease possessed the inhibitor.

Further studies on the nature of this inhibitor showed it to be contained in the pseudoglobulin fraction of serum. It was precipitated by 50% ammonium sulphate but not at low ionic strength (achieved by dialysis against water) and had a molecular weight greater than 10 kD (determined by dialysis). The inhibitor was undetectable at serum dilutions of greater than one in three. Attempts to identify its molecular weight by gel filtration were unsuccessful since the inhibitor could not be recovered after concentration of the fractions. Its activity was significantly reduced following heating at 56°C for 30 min (Fig. 4).

Two possible explanations for the inhibitory activity were considered (i) IC in the SLE sera; (ii)

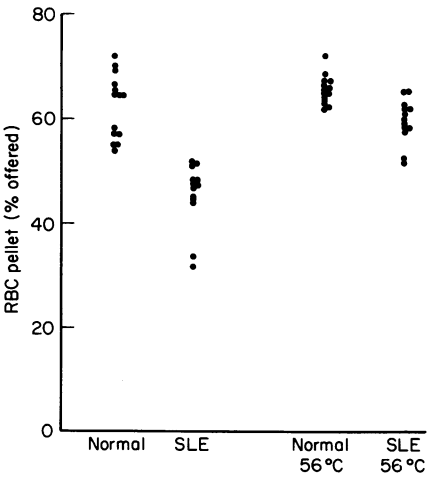

Fig. 4. SLE inhibitor: Heat lability. The effect of heating normal sera and SLE sera at 56°C for 30 min prior to assay.

immunoconglutinins binding to the C3 on the IC blocking its binding to CR_1. The inhibitor was not removed by adsorption on Protein A-Sepharose under conditions which depleted IgG detectable by Ouchterlony suggesting that the effect was unlikely to be due to IgG-containing IC. Further, opsonized unlabelled BSA-anti-BSA IC (made in 4-fold antibody excess) added in 50-fold excess failed to significantly inhibit the binding to red cells of opsonized, radiolabelled IC. Taken together with the heat lability, persistence of the inhibitor after Protein A absorption makes IgG immunoconglutinins unlikely mediators of the effect.

Since factor H, a physiological inhibitor of complement activation, is partly heat labile (Crossley, 1981) it was a possible mediator of the SLE effect; this was unlikely since the SLE effect was not reproduced by the addition to opsonized IC of purified factor H to a concentration of 2·45 mg/ml.

Rheumatoid factors (RF) by binding to IC, may interfere with binding to erythrocyte CR_1. Addition to opsonized IC of IgM monoclonal RF and IgM polyclonal RF at a concentration of 1 mg/ml had no detectable effect on binding to erythrocytes.

Experiments were performed to test the hypothesis that the inhibitor might be a proteolytic enzyme which cleaved iC3b on the IC to small fragments not recognised by CR_1. First protease inhibitors were added simultaneously with the test serum to normally opsonized IC. None of PMSF 2 mM, aprotinin 50 kIU/(kallikrein inhibitory units) per ml, or EDTA (pH 7·4) 10 mM or DFP 10^{-2} M blocked the SLE effect.

Second, opsonized IC treated with SLE inhibitor immunoblotted with anti-C3d showed no evidence of C3 fragments of decreased molecular weight. The C3 detected was the α-chain fragment of iC3b, mol. wt 68 kD. Taken together with the observation that dilution beyond 1/3 resulted in loss of activity, enzymatic proteolysis is an unlikely mechanism and it is likely that the inhibitor acts by interference with the CR_1-binding site on the IC.

However, tranexamic acid 5 mM abolished the SLE inhibitor effect. A dose response curve showed that tranexamic acid at a concentration of 1 mM was sufficient to produce a detectable effect on the SLE inhibitor (Fig. 5). At this concentration, the mechanism of action of tranexamic acid is likely to be that of competition at a lysine-binding site (Hoylaerts, Lijnen & Collen, 1981), rather than inhibition of active site and this suggested that the SLE inhibitor was capable of binding to lysine. This was confirmed by adsorption of serum on lysine-Sepharose (with Sepharose 4B as a control) before incubation with normally opsonized IC. The ratio of red cell binding assayed with lysine-Sepharose treated serum to Sepharose-treated serum was 1 for NHS and 1·67, 1·57 and 1·29 for sera from three SLE patients ie lysine-sepharose removed the SLE inhibitor. Unfortunately attempts to recover activity of the SLE inhibitor from the material adsorbed on lysine-Sepharose by elution with each of 2 mM tranexamic acid and 0·4 M EACA + 1 M NaCL have been unsuccessful. The inhibitor was blocked by lysine 1 mM in the fluid phase, confirming its action via a lysine-binding site. Plasminogen, a lysine-binding protein, was a possible mediator. However, depletion of SLE serum of plasminogen by a specific antibody adsorbed on Protein A-Sepharose did not remove the

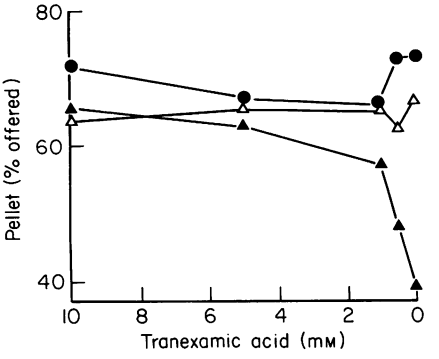

Fig. 5. SLE inhibitor: effect of different concentrations of tranexamic acid in SLE inhibitor assay on PBS (●), NHS (△) and SLE serum (▲).

inhibitor effect; conversely, adding purified plasminogen to a concentration of 2·5 mg/ml to NHS did not reproduce the SLE inhibitor effect. This was not reproduced either with tissue plasminogen activator (t-PA), another lysine-binding protein, added to a concentration of 3·3 μg/ml to NHS.

The SLE inhibitor effect was observed using IC made with human immunoglobulin viz, tetanus toxoid (TT)-anti-TT IC, demonstrating that it was not an artifact of heterologous systems.

DISCUSSION

We have shown that sera from patients with SLE have decreased ability to bind immune complexes to normal human erythrocytes *in vitro*. Thirteen out of 29 patients had an inhibitor that interfered with the binding of normally opsonized IC to erythrocytes; it was found in each of the nine clinically active patients. The inhibitor was a globulin which was labile at 56°C and bound to lysine. Results of depletion and reconstitution experiments suggest that the inhibitor is unlikely to be IgG immunoconglutinin, IgM rheumatoid factor, circulating IC or factor H. The low concentrations of tranexamic acid and lysine needed to block the SLE inhibitor suggest that both these substances are acting at a lysine-binding site rather than protease active-site. The lack of effect of aprotinin and DFP on the SLE inhibitor is further evidence that the proteolytic active site of plasminogen, plasmin and tissue plasminogen activator (t-PA) are not involved. Of these three proteins bearing lysine-binding sites, plasmin is usually complexed to its inhibitors *in vivo* (Collen & Wiman, 1978) and t-PA is likely to be absorbed onto clot (Thorsen, Glas-Greenwalt & Astrup 1972) and unlikely to be present in serum. Furthermore addition of tPA to 3·3 μg/ml (1000 × concentration in normal plasma) did not reproduce the SLE inhibitor effect. Plasminogen was a likely candidate but depletion and reconstitution experiments failed to confirm it as the SLE inhibitor. The lack of effect of protease active-site inhibitors taken together with the loss of SLE inhibitor at dilutions greater than 1/3 (which makes an enzymatic reaction unlikely) and the failure to demonstrate smaller C3 fragments on opsonized IC treated with SLE inhibitor, all make proteolysis of C3b and iC3b an unlikely mechanism of the inhibitor; it probably interferes with the CR1-binding site on IC.

Two other mechanisms which may contribute to decreased IC binding to erythrocytes are (1) inhibition of complement activation and (2) complement deficiency. An inhibitor of complement activation would reduce opsonization by SLE sera. A circulating inhibitor of fluid phase complement amplification has been described in some SLE patients by Waldo *et al.* (1985). This molecule appears to potentiate the interaction of factor H with C3b, decreasing the efficiency of formation of the alternative pathway C3 convertase, C3bBb. It is a non-IgG molecule of molecular weight exceeding 12–14 kD and is probably distinct from the inhibitor described in this study since these SLE sera generated normal amounts of C3 on IC.

Deficiency of one or other complement component would result in decreased opsonization. Sera with obvious complement deficiencies were excluded from the IC binding experiments. Some SLE sera showed decreased opsonization of IC (as measured by anti-C3 coprecipitation) in spite of normal CH50. This disparity between opsonization and haemolysis is consistent with the different functional properties of the C4 isotypes, C4A and C4B; C4A binds preferentially to amino groups whereas C4B binds more easily to hydroxyl groups (Isenman & Young, 1984; Law, Dodds & Porter, 1984). Using sera from subjects with homozygous null alleles for either C4A or C4B, differences in function are demonstrable; C4A is relatively more efficient in IC handling (11P) whereas C4B is more efficient at haemolysis (Schifferli *et al.*, 1986).

Inefficient binding of IC to erythrocytes may have a number of important consequences *in vivo*. First, the transport of IC to the reticulo-endothelial system for safe disposal may be impaired: second there may be decreased factor I-mediated processing of IC to C3dg bearing complexes, whose activation of complement *in vitro* is decreased (Medof *et al.*, 1982b). The decreased efficiency of IC binding to CR_1 shown in these studies, has the potential consequence of increasing in the circulation the proportion of IC retaining their phlogistic properties. Clearly, the processes involved in IC clearance *in vivo* are complicated, with several factors determining the efficiency of CR_1-mediated transport of IC to the reticulo-endothelial system. These include the efficiency of the complement system, the presence of factors which interfere with opsonized IC binding to

erythrocytes such as described in this study, the number of erythrocyte CR_1 and the efficiency of the reticulo-endothelial system in removal of IC.

In summary, we have demonstrated that sera from patients with SLE have decreased ability to bind IC to normal human erythrocytes *in vitro*. In some cases this was associated with decreased C3 deposition on immune complexes, in others there was an inhibitor which prevented the binding of normally opsonized complexes to erythrocytes. These defects may contribute to the pathogenesis of immune complex disease by allowing persistence of IC in tissues.

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