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Immune adherence and clearance of hepatitis B surface Ag/Ab complexes is abnormal in patients with systemic lupus erythematosus (SLE)

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SUMMARY

Complement levels and complement receptor 1 (CR1) on erythrocytes (E) are reduced in systemic lupus erythematosus (SLE). To see whether these abnormalities are responsible for defective transport and elimination of immune complexes (IC) from the circulation, patients with active SLE (14) and normal volunteers (14) were injected with preformed IC (hepatitis B surface Ag/Ab). Two minutes after injection only $25 \cdot 9 \pm 19 \cdot 1\%$ (mean $\pm$ 1 s.d.) of the circulating IC were bound to E in the SLE patients as compared to $63 \pm 3 \cdot 7\%$ in the normal subjects ($P = 0 \cdot 0001$). For SLE patients, the reduced immune adherence was best explained by a combination of complement depletion and low CR1 binding capacity ($\tau = 0 \cdot 80$, $P = 0 \cdot 0001$).

The disappearance of IC as estimated from the area under the elimination curve was faster in SLE than in controls ($P = 0 \cdot 02$), and correlated with CR1 ($\tau = 0 \cdot 54$, $P = 0 \cdot 0001$) and immune adherence observed *in vivo* ($\tau = 0 \cdot 33$, $P = 0 \cdot 013$). Finally, immune adherence was absent and IC disappeared very rapidly in a patient with C2 deficiency and an SLE-like disease. These observations suggest that in SLE the defective immune adherence reaction might be responsible for the accelerated disappearance of IC from the circulation.

Keywords systemic lupus erythematosus immune complexes erythrocyte complement receptor 1 (CD35) immune adherence immune elimination

INTRODUCTION

In normal subjects, when antigen (Ag) and antibody (Ab) react to form immune complexes (IC), complement is activated by the classical pathway resulting in the covalent attachment of C3b (Hong *et al.*, 1984). Such complexes are soluble, and the largest of them bind to erythrocytes (E) via the C3b receptor (immune adherence receptor, CR1, CD35) (Schifferli *et al.*, 1988). Patients with systemic lupus erythematosus (SLE) demonstrate major defects in these reactions (Naama *et al.*, 1983; Ng *et al.*, 1987). First the opsonization of IC is decreased in lupus sera. Complement is very often depleted, but additional factors may play a role: SLE sera may contain inhibitor(s) of complement activation (Waldo *et al.*, 1985), and a lysine-binding protein has been shown to inhibit the binding of IC to normal E (Ng *et al.*, 1987). Finally, E from SLE patients have a low number of CR1 (Miyakawa *et al.*, 1981; Wilson *et al.*, 1982), which might be responsible for reduced immune adherence as well.

Over recent years, Hebert and co-workers have analysed how, in monkeys, IC are transported by E CR1 in the circulation (Cornacoff *et al.*, 1983; Waxman *et al.*, 1984, 1986). This transport prevented deposition of IC in lung and kidney. Recently we have shown that preformed tetanus toxoid Ag/Ab complexes injected intravenously bind to E in the circulation of humans (Schifferli *et al.*, 1988). The binding required complement and correlated with CR1 number on E in normal individuals and in patients with various defects in complement function (Schifferli *et al.*, 1989). In addition, disappearance of IC was accelerated in those with defective immune adherence reaction, suggesting that normal complement and CR1 function to prevent IC trapping outside of the fixed macrophage system. Only few patients with SLE have been studied but the preliminary observations made on three patients suggested that IC did not bind well to E and disappeared too promptly (Schifferli *et al.*, 1988).

The aim of the present study was to analyse immune adherence and clearance of IC in a large group of patients with active SLE, using hepatitis B surface Ag/Ab (HBsAg/Ab) complexes.

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MATERIALS AND METHODS

Normal subjects and patients

The IC clearance studies were performed in 14 normal subjects (11 men and three women; age 18–45), in 14 patients with active

Table 1. Clinical and laboratory data in SLE patients and the C2 deficient patient

Patient	ARA criteria*	CH50 (%)†	C3 (%)	C4 (%)	CR1/E‡	C3dg/E	Anti-dsDNA§	ANA
1	7	73	75	75	412	132	1:80	1:5000
2	5	125	110	77	827	130	0	1:1280
3	4	75	108	41	595	152	0	1:2560
4	5	72	58	39	608	136	1:80	1:2560
5	5	33	36	13	1060	146	1:40	1:1280
6	7	61	61	42	794	170	1:640	1:1280
7	7	26	10	5	377	147	1:320	1:1280
8	5	113	113	121	488	161	0	1:2560
9	8	102	88	75	174	173	1:160	1:5000
10	4	103	113	104	512	66	0	1:1280
11	4	114	120	100	520	50	0	1:2560
12	5	27	20	19	386	86	1:40	0
13	7	68	73	50	319	83	1:40	1:2560
14	6	67	58	55	273	149	1:20	1:640
C2def	—	18	94	71	1105	130	0	0
14 normal controls (±1 s.d.)¶	—	103±16	97±18	110±42	849±220	40±6	—	—

* Number of ARA criteria for SLE.
† Per cent of 25 pooled normal sera.
‡ CR1 and C3dg: number of molecules per cell.
§ Dilution of positive fluorescence.
¶ CR1 measurements were missing in two normal controls.

SLE satisfying ≥4 of the revised criteria of the American Rheumatism Association (ARA) (Tan *et al.*, 1982) (see Table 1), and one 19-year-old woman with inherited complete C2 deficiency and a lupus-like syndrome who was in remission. The diagnosis of SLE was established between 5 years and 1 month preceding the study. All had nephritis except one patient who had no kidney biopsy.

Complement, IC and CR1 measurements

The methods for measuring CH_{50}, antigenic C3, C4, I, H and C3d, and IC using radiolabelled C1q have been described (Schifferli *et al.*, 1987). Purified C2 was obtained from Cordis (Miami, FL). Erythrocyte CR1 numbers were measured as previously described (Ross *et al.*, 1985) using a mouse monoclonal anti-CR1, E11 (kindly provided by Dr N. Hogg) radiolabelled to a specific activity of 1·03 μCi/μg using the iodogen method (Fraker & Speck, 1978).

Hepatitis B surface Ag/Ab complexes

Hepatitis B surface antigen (HBsAg) was dialysed against phosphate buffered saline (PBS) pH 7·4, and radiolabelled with the Bolton and Hunter reagent (N-succinimidyl-3-(4-hydroxy, 5-(^{125}I) iodophenyl) propionate) (Amersham, Amersham, UK) to a specific activity of 3·6 μCi^{125}Iodine/μg HBsAg. Immune complexes were formed in large antibody excess, by mixing 41·3 μg of ^{125}I-HBsAg with polyclonal IgG containing 937·5 U of anti-HBsAb (Swiss Red Cross, Bern). After an incubation of 90 min at 37°C, no significant precipitation was obtained and the reaction was stopped by freezing aliquots of IC in liquid nitrogen. By trichloro-acetic acid precipitation (final concentration 10%) more than 95% of the iodine was protein-bound. The IC was larger than 45 S by isokinetic sucrose gradient ultracentrifugation, although a small fraction (between 5% and 10%) of the radioactivity remained in small molecular weight fractions below the size of the Ag (i.e. smaller than 3·000 kD). Each IC precipitation was pyrogen-free when tested in two white rabbits. Three separate batches of IC were used; their immune adherence and clearance were similar when injected into rabbits.

In vitro *studies*

Role of complement and CR1 on immune adherence reactions. The immune adherence of HBsAg/Ab IC opsonized with each patient's serum was studied using E from a normal subject (525 CR1/E). In short, 15 μl of IC were incubated for 5 min at 37°C with 5 μl of serum. An E suspension (300 μl, haematocrit 22%) in PBS-10 mM ethylenediamine tetracetic acid (EDTA) was then added to the opsonized IC and incubated for 15 min at 37°C. The E were spun through oil, and counts measured in the pellet and supernatant.

The immune adherence capacity of the different E populations were measured using preopsonized IC. Such IC were obtained by incubating 200 μl of HBsAg/Ab complexes with 100 μl of normal human serum for 15 min at 37°C, and separating the IC from serum by sucrose density gradient ultracentrifugation (Schifferli *et al.*, 1988). Erythrocyte suspensions (300 μl, at a final concentration of 5×10^8 E/ml) were mixed with 20 μl of preopsonized IC and incubated for 2 h at 4°C. Immune adherence was measured as described above. All assays were done in duplicate.

Human studies

Erythrocytes were labelled with ^{99m}Tc *in vitro* using Glucoscintimed solution (stannous gluco-ene-diolate) (Banna Laboratories, Geneva, Switzerland). Two hours before the studies the

subjects were tested for immediate hypersensitivity reaction by an intradermal injection of 50 μl of an IC suspension (1 : 1000 in 0·9% NaCl), and received 1·5 ml of 5% Lugol. They were injected intravenously in less than 5 s with a mixture containing 1·52–4·4 μCi of ^{125}I-HBsAb/Ag IC and 5·3–35 μCi of ^{99m}Tc-labelled autologous E. Blood samples (5 ml) were drawn from an indwelling canula in a peripheral vein in the opposite arm at different time intervals and immediately processed at 4°C. Radioactivity was measured in samples of whole blood (2 ml) and of E (2 ml) that had been washed twice with 10 ml of cold PBS. TCA-precipitable counts were determined in each blood sample. Immune adherence and IC disappearance were calculated as previously reported (Schifferli *et al.*, 1988), using ^{99m}Tc as a blood volume marker in the initial samples, and taking into account only protein-bound ^{125}I. This study was approved by the Ethical Committee of the Department of Medicine, Hôpital Cantonal Universitaire, Geneva. All subjects gave a written informed consent.

Mathematical model for IC clearance

The kinetics of disappearance could be explained by a simple compartmental model after removal of the fraction of non-specific radioactive protein-bound material remaining 60 min after injection (5·9%). A fraction of the IC disappeared immediately (initial disappearance), the remaining complexes were cleared at a monoexponential rate (elimination rate). We analysed these data with a computer program previously described (Schifferli *et al.*, 1988) which calculated the best fit for each data set. The correspondence between the experimental values and the mathematical model was assessed by simple inspection, and by the satisfaction of numerical criteria of convergence towards an optimum. The area under the curve was used to define disappearance of IC (area = (1 − initial disappearance)/elimination rate).

Statistics

When appropriate, the Mann–Whitney U-test or the Kendall rank correlation coefficient (τ) were used.

RESULTS

HBsAg/Ab complexes were injected intravenously and their binding to E in the circulation was followed over 60 min. In the 14 normal individuals, a large fraction of the IC bound rapidly to E, the maximum adherence being observed after 2 min (Fig. 1). The mean binding at 2 min was 63·0 ± 3·7% (1 s.d.) of the circulating IC. This immune adherence was reduced in most of the 14 patients with SLE (mean binding at 2 min: 25·9 ± 19·1%, $P = 0·0001$), and not detectable in the patient with C2 deficiency (< 3% binding).

To see whether, in SLE patients, defective adherence was due to hypocomplementemia or low CR1, the percentage of adherence observed *in vivo* was correlated with complement and CR1 (Table 2). Both C4 and CR1 correlated with immune adherence, although not significantly. A better relation was found when C4 and CR1 measurements were combined (C4 × CR1). The significant correlation with CH50 was also improved when CH50 was combined with CR1, suggesting that both complement and receptors contributed independently to immune adherence.

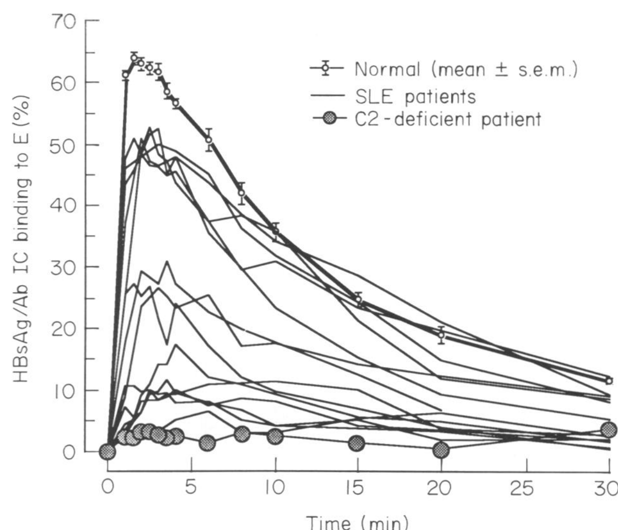

Fig. 1. Immune adherence *in vivo* in normal individuals, SLE patients, and the C2-deficient patient. The percentage of circulating IC bound to erythrocytes was followed over 60 min. Mean binding in normal controls (± s.e.m.).

Table 2. Correlations between immune adherence *in vivo* and different complement measurements in the 14 SLE patients

Complement measurements	Kendall coefficient (τ)	*P*
Complement		
C4	0·39	0·0527
CH50	0·46	0·0208
CR1	0·33	0·0986
C4 × CR1	0·60	0·0030
CH50 × CR1	0·57	0·0042
Immune adherence assays		
Binding by sera*	0·60	0·0030
Binding by erythrocytes†	0·59	0·0033
(Binding by sera) × (Binding by erythrocytes)	0·80	0·0001

* Mean binding by sera, normals: 37 ± 3% (s.d.); SLE: 26 ± 13%; C2 def: 0%.

† Mean binding by erythrocytes: normals: 54 ± 10%; SLE: 43 ± 14%; C2 def: 82%.

This observation was confirmed using two separate assays which measured the capacity of the patients' sera to opsonize preformed HBsAg/Ab complexes or of patients' E to bind preopsonized complexes (Table 2). Again the combination of the two assays provided the best correlation with immune adherence observed *in vivo*. Thus, depending on the circumstances, complement and/or CR1 might be limiting in SLE patients. This was illustrated by analysing the cases separately. The patient with the highest CR1 number (1060 CR1) had low complement and very low adherence of IC *in vivo* (9%). At the opposite end, the patient with the lowest CR1 level (174) had

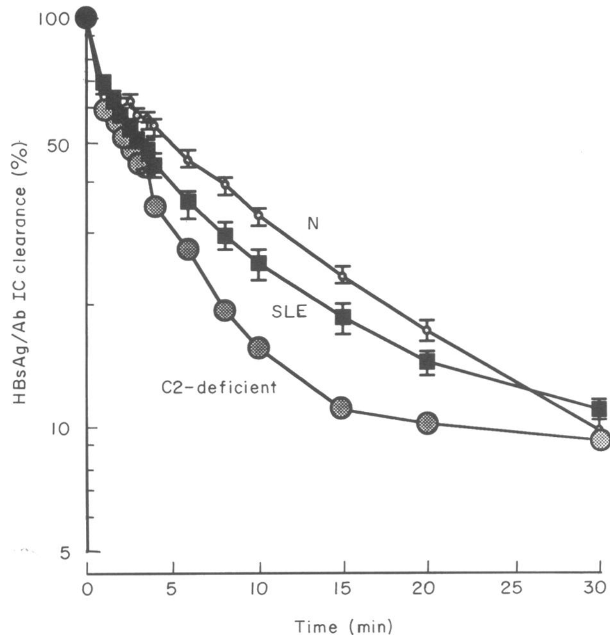

Fig. 2. Disappearance of IC in normal individuals, SLE patients, and the C2-deficient patient (expressed as the mean ± s.e.m. of the experimental data).

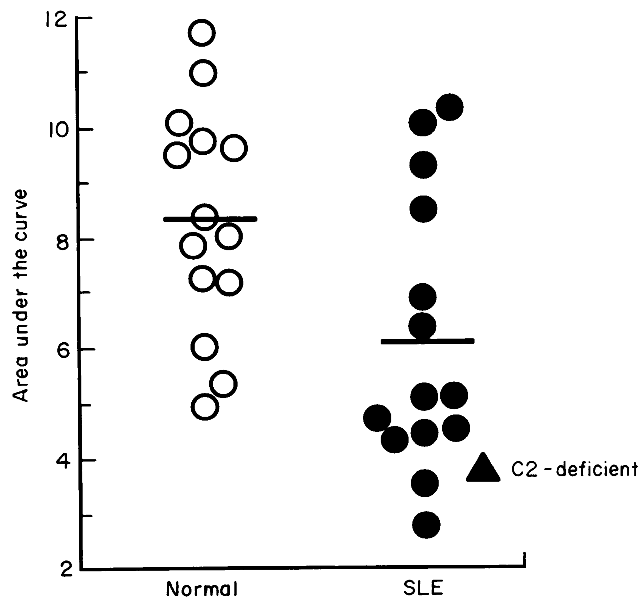

Fig. 3. Measure of IC disappearance by calculating the area under the curves for each individual. A small area corresponds to a fast disappearance of IC. Disappearance was faster in SLE patients.

normal complement levels but no significant binding of IC *in vivo* as well (3%).

As expected the serum of the C2 deficient patient was not capable of opsonizing IC *in vitro*, whereas her E were capable of binding preopsonized complexes. When supplemented with purified C2, the serum had a normal capacity to opsonize IC.

The disappearance of HBsAg/Ab complexes from the circulation was rapid. Approximately 6% of protein-bound

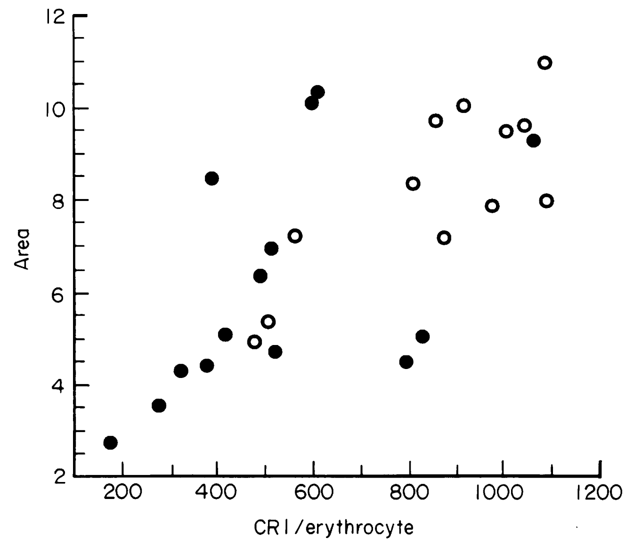

Fig. 4. Direct correlation between CR1 number per erythrocyte and disappearance of IC from the circulation (area under the curve), i.e. the lower the CR1, the faster the disappearance. The C2 deficient patient was not included. Normal subjects (○); SLE patients (●). For all studies, $\tau = 0{\cdot}54$, $P = 0{\cdot}0001$; for SLE patients only, $\tau = 0{\cdot}35$, $P = 0{\cdot}067$.

radioactivity remained circulating after 60 min. This material was shown to be smaller than radiolabelled HBsAg by sucrose gradients, and corresponded in size and percentage to the contaminant material detected in the HBsAg/Ab complexes that were used (see Materials and Methods). Thus, the analysis of the data was performed after removing this fraction of protein-bound radioactivity. The disappearance of IC was faster in SLE patients than in normal subjects, and was very fast in the C2 deficient patient (Fig. 2). Using the mathematical model described in Materials and Methods, we calculated the area under the curve, which was significantly larger in normal subjects than in SLE patients ($8{\cdot}34 \pm 2{\cdot}06$, versus $6{\cdot}16 \pm 2{\cdot}50$; $P = 0{\cdot}022$), and was very small in the C2 deficient patient (3·86) (Fig. 3).

Having analysed immune adherence and disappearance of IC injected into the circulation, it was of interest to see whether they might be related. Immune adherence observed *in vivo* correlated with IC disappearance (area under the curve), whether all subjects or only SLE patients were taken into account (respectively $\tau = 0{\cdot}43$, $P = 0{\cdot}026$, and $\tau = 0{\cdot}33$, $P = 0{\cdot}013$). Finally, the correlation between CR1 number and IC disappearance was very striking (Fig. 4).

The serological status for hepatitis B infection did not modify immune adherence and IC disappearance, since there was no correlation between titres of HBsAg or HBsAb, and the parameters studied in normal individuals and SLE patients. Similarly, the level of circulating IC measured by the C1q binding assay, the levels of C3d and factor I did not correlate with immune adherence or disappearance of IC.

DISCUSSION

A defective elimination of IC in SLE has been suggested by many studies using different substitutes for IC (Lockwood *et al.*, 1979; Hamburger *et al.*, 1981; Frank *et al.*, 1983; Kimberly, Merythew & Runquist, 1986; Lobatto *et al.*, 1988). The work of Frank and colleagues indicated defective Fc mediated clear-

ance of IgG-coated E by the spleen and C3b-coated E by the liver. Lobatto *et al.* (1988) have shown that the clearance of heat-aggregated IgG is impaired in SLE. However, little is known about the clearance of large complement activating IC except for the preliminary data obtained with tetanus toxoid Ag/Ab complexes (Schifferli *et al.*, 1988). The present study is the first to analyse the clearance of large complexes in a group of patients with active SLE. It indicates that the processing and elimination of IC is abnormal in such patients. First, the complexes used showed a reduced adherence to E, which was best explained by a combination of low complement function and a low binding capacity of E. Second, the disappearance of complexes from the circulation was faster in SLE than in normal individuals.

Each IC has specific properties with regards to complement activation, binding of C3b fragments, and capacity to adhere to CR1 on E (Edberg, Kujala & Taylor, 1987; Lobatto *et al.*, 1987; Paccaud *et al.*, 1987; Edberg *et al.*, 1988; Halma *et al.*, 1989; Kimberly *et al.*, 1989; Taylor, Wright & Pocanic, 1989). The specific properties of HBsAg/Ab complexes have been studied (Madi *et al.*, 1989; Paccaud, Steiger & Schifferli, 1989). They acquire maximum adherence properties in minutes and bind efficiently to E. These observations corresponded to the results of immune adherence obtained *in vivo*, which indicated that maximum adherence was observed after 2 min, but was delayed in many patients. In addition, in SLE patients a direct correlation was found between complement levels and immune adherence *in vivo*, suggesting that reduced complement function may be responsible for diminished immune adherence of HBsAg/Ab complexes in the circulation.

CR1 has been shown to determine immune adherence *in vivo* for tetanus toxoid Ag/Ab complexes (Schifferli *et al.*, 1989). Although many SLE patients had reduced CR1 number, the correlation was not as evident. However, when CR1 and complement measurements were combined the correlation with immune adherence was better than for complement alone, indicating that CR1 determines immune adherence as well. The contribution of both complement and CR1 was further suggested by the assays performed *in vitro* which defined the contribution to immune adherence of patients' sera and erythrocytes independently. Again, it appeared that both serum and E determine immune adherence *in vivo*.

The clearance of HBsAg/Ab complexes was analysed similarly to that of tetanus toxoid Ag/Ab complexes (Schifferli *et al.*, 1988). The area below the elimination curve was smaller, i.e. the disappearance was faster in SLE patients than in normal subjects. Disappearance of IC might be due to two phenomena: deposition in tissues, and uptake by the fixed macrophages via several receptors which include Fc gamma, and C3 fragment receptors. The data collected here do not allow a differentiation between the two. However, the absence of immune adherence and fast disappearance of IC in the C2 deficient patient provides some clarification about the relative contributions of Fc and complement mediated clearance reactions. First, this patient was well at the time of study, thus abnormalities cannot be explained by an active process. Second, in two other complement deficient patients, defective C3b coating of IC has been shown to be directly responsible for the accelerated clearance of IC (Schifferli *et al.*, 1989). Thus it appears that the presence of complement reduces the rate of IC elimination. Complexes which form in the absence of complement enlarge rapidly, a reaction that might accelerate deposition in tissues like the mesangium of renal glomeruli (Cameron & Clark, 1982; Wener & Mannik, 1986). It is, however, unlikely that the differences in elimination rates were due to such tissue deposition only, since in comparable animal models only a very small fraction of IC (< 1%) localize in tissues (Waxman *et al.*, 1984). In addition, in a separate study using ^{123}Iodine-labelled complexes, fast disappearance appeared to correspond essentially to rapid localization in the liver and spleen without evidence for uptake in other organs (Davies *et al.*, 1990). The aggregation of IC, as observed in the absence of complement, may also enhance recognition by Fc receptors of the fixed macrophage system. In addition, the coating of IC with C3 fragments appears to be responsible for reducing the rate at which IC are recognized by Fc receptors (Daha & Van Es, 1983). Thus, *in vivo*, Fc mediated clearance reactions might be most efficient when no complement fragments are present on IC. The findings in the SLE patients also favour this hypothesis: opsonization of IC by SLE sera was poor as previously reported (Ng *et al.*, 1987). Thus, large aggregates may form, and Fc mediated clearance reactions may predominate.

The inverse correlation between immune adherence and elimination of the IC suggests the CR1-dependent binding of IC to E functions as a buffer system, which also allows complete inactivation of IC-bound C3b with loss of their phlogistic potential (Medof *et al.*, 1982). In addition, their uptake by fixed macrophages might be reduced, since the C3dg remaining attached to the IC is not likely to mediate immune elimination (Frank *et al.*, 1983). Since CR1 is the essential cofactor for the inactivation of IC-bound C3b (Medof *et al.*, 1982), it was of interest to find that there was a significant correlation between CR1 and the area under the immune elimination curve, i.e. an inverse correlation with immune elimination. Thus, inactivation of IC-bound C3b may also be more efficient in individuals with a high CR1 number, and the resulting complexes may be dead end complexes with a reduced capacity to induce inflammation or bind to fixed macrophages.

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