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The role of hypocomplementaemia and low erythrocyte complement receptor type 1 numbers in determining abnormal immune complex clearance in humans

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SUMMARY

Defective clearance of immune complexes (IC) may contribute to the pathogenesis of diseases such as SLE. We studied the effect of hypocomplementaemia and the influence of erythrocyte complement receptor type 1 (CR1, CD35) number on the clearance of radiolabelled tetanus toxoid (TT)-anti-TT IC from the circulation. These were injected intravenously into 9 normal subjects and 15 patients with diseases characterized by IC formation and/or hypocomplementaemia, including 2 with hereditary complement deficiency. IC were found to bind to erythrocyte CR1 in a complement-dependent manner and their degree of uptake was directly correlated with CR1 numbers. Two phases of IC clearance were identified. The first was rapid, occurring within 1 min. Since this phase might represent inappropriate deposition of IC in target organs we called it *trapping*. It was seen predominantly in subjects with low CR1, low complement, and low binding of complexes to red cells. The second phase was monoexponential with a mean elimination rate of 14·1%/min; it was inversely correlated with CR1 numbers and binding of complexes to red cells. In a second study each individual was injected with IC bound to autologous erythrocytes *in vitro* using normal serum so that the effects of complement deficiency were eliminated. Up to 81·4% of these bound IC were released *in vivo* from erythrocytes in 1 min, and the proportion was inversely correlated with CR1 numbers. Only five patients showed trapping, and these had low CR1 numbers and high percentage release of IC. The second phase of elimination was inversely correlated with CR1 numbers and the proportion of IC remaining bound to red cells at 1 min. The two complement-deficient patients had normal CR1: when IC were injected, trapping and very fast clearance rates were observed; however complexes that had been opsonized and bound to erythrocytes were cleared at a slower rate without evidence for trapping. These studies show that complement and erythrocyte CR1 may determine the physiological clearance of certain types of IC and suggest that this system may function abnormally when CR1 number or complement function are reduced.

Keywords complement receptor type 1 complement immune complexes

INTRODUCTION

The majority of circulating IC are cleared by the fixed mononuclear phagocytic system (Wilson & Dixon, 1971). Failure of this clearance mechanism may be a pathogenic factor in diseases such as systemic lupus erythematosus (SLE). Several mechanisms have been investigated which may cause impaired removal of IC from the circulation by the fixed mononuclear phagocytic system. IC themselves may overload the capacity of tissue macrophages to remove further IC from the circulation—'saturation of the mononuclear phagocytic system'. There is evidence from animal models showing that, under conditions of extreme experimental IC overload, saturation of the mononuclear phagocytic system can occur (Haakenstad & Mannik, 1974). Erythrocytes coated with antibodies have been used to investigate this phenomenon in humans. Such IgG-coated cells show impaired clearance by the spleen in patients with SLE and related diseases, and this has been interpreted to reflect mononuclear phagocytic system saturation (Frank *et al.*, 1983). Similarly the transient trapping in the liver of IgM-coated erythrocytes may be impaired in disease (Frank *et al.*, 1983).

The relevance of these models using particulate IC to understanding the clearance system for soluble IC is doubtful. In particular, it is clear that exogenously administered soluble IC are irreversibly cleared from the circulation of animals largely by the liver, with only a small splenic contribution (Mannik & Arend, 1971). Recently Hebert and colleagues have

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studied the clearance of large IC from the circulation of baboons (Cornacoff *et al.*, 1983; Waxman *et al.*, 1984; 1986). They found these complexes fixed complement *in vivo*, bound to erythrocyte C3b receptors, and were cleared from the circulation mainly by the fixed mononuclear phagocytic system of the liver. When IC were injected into animals that had been rendered hypocomplementaemic, or when IgA-containing IC were injected that fixed complement poorly, the complexes were cleared more rapidly from the circulation and a small percentage of the complexes were cleared in organs outside the fixed mononuclear phagocytic system where they had the potential to cause inflammation.

We present here the results of studies of the kinetics of clearance from the circulation of intravenously injected TT-anti-TT IC. We selected subjects in order to assess the role of variation in complement activity and erythrocyte complement receptor type 1 (CR1, i.e. the C3b receptor) numbers in determining the rate of in-vivo processing and clearance of these IC. Our evidence suggests that IC may indeed be processed abnormally in the presence of deficiencies of complement or of erythrocyte CR1.

MATERIALS AND METHODS

Subjects

The nine normal subjects were males, aged 26 to 39 years, and were selected on the basis of erythrocyte CR1 number in order to include subjects with a range of CR1 numbers. The 15 patients were selected for disease processes in which complement and IC are known to be involved: four patients with active SLE satisfying the modified criteria of the American Rheumatism Association (Tan *et al.*, 1982); two patients with rheumatoid arthritis (normal complement, rheumatoid factor titre: 1/640 and 1/1280 by latex agglutination); four patients with essential cryoglobulinaemia (three of whom had mixed cryoglobulinaemia (monoclonal IgM-κ with rheumatoid factor activity and polyclonal IgG), and one had a monoclonal IgM-κ cryoglobulin); two patients with nephritis, partial lipodystrophy and circulating nephritic factor; one patient with hypocomplementemic cutaneous vasculitis; and two patients with inherited complement deficiency (one with C2 deficiency, the other with C1q deficiency).

Preparation of TT- anti-TT complexes

These IC were prepared as described previously (Schifferli *et al.*, 1988). In brief, purified TT (Institut sérothérapique et vaccinal suisse, Bern-CH) was radiolabelled with ^{125}iodine using iodogen (Biorad, Richmond, CA). The antibody source was a human IgG preparation (120 mg/ml) containing a high and standardized titre of anti-TT antibodies (Tetuman, Berna, Bern-CH). The equivalent point of the antigen/antibody reaction was 1 mg TT for 1 ml of anti-TT IgG, with a molar antigen/antibody ratio of 1/10. The anti-TT IgG was dialysed against phosphate-buffered saline (pH 7·2, conductance 14 mmho/s), and aggregates were removed by ultracentrifugation at 120 000 *g* for 2 h. This anti-TT IgG preparation was first immobilized on a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden) and ^{125}I-tetanus toxoid was applied so that only antigen capable of being recognized by the fixed antibody was retained in the column. The complexes were eluted with HCl-glycine (pH 2·8 in 0·2 M glycine and 0·5 M NaCl), the eluate was immediately neutralized with Tris, and dialysed against a neutral phosphate buffer containing 0·5 M NaCl. The procedure was done under sterile conditions and standardized to form complexes in antibody excess (20-fold excess as compared with the point of equivalence). Aliquots containing 2–3 μg of TT in the IC were prepared and stored at 4°C until used (< 6 weeks). The complexes were diluted with water before injection to physiological ionic strength. These IC were soluble when centrifuged at 10 000 *g* for 10 min, and were 45 S in size, measured by isokinetic sucrose gradient ultracentrifugation experiments. Seventy-five per cent of these complexes incorporated complement when exposed to serum *in vitro*, judged by increase in size of the complexes and binding to CR1 on erythrocytes.

Clearance studies

These studies were approved by the Hammersmith Hospital ethical committee. Each patient and normal subject was studied twice. Two millilitres of 5% potassium iodide were given orally to each subject at least 2 h before each study.

Study 1. Direct injection of ^{125}I-TT-anti-TT complexes: the IC (containing 2–3 μg TT labelled with 2–4 μCi ^{125}I) were mixed with ^{99m}Tc-labelled erythrocytes as a blood volume marker and were injected into an antecubital vein as a bolus. These IC were shown not to bind to the ^{99m}Tc-labelled erythrocytes prior to injection *in vitro*.

Study 2. Injection of pre-opsonized ^{125}I-TT-anti-TT complexes bound to erythrocytes *in vitro*: IC were bound to autologous ^{99m}Tc-labelled erythrocytes *in vitro* using autologous serum for the normal subjects and serum obtained from a normal individual (blood group AB with negative serology for HBsAg and HIVAb) for the patients. The erythrocytes were washed three times in ice-cold phosphate buffer/saline by centrifugation at 500 *g* for 3 mins. These erythrocyte-bound IC were then injected intravenously. Before injection, more than 96% of the complexes were shown to be bound to erythrocytes in all studies.

Blood samples (5 ml) were collected during 60 min, starting at 1 min, in 5 mM ethylene diamine tetracetate (pH 7·2) and immediately cooled in a melting ice waterbath. Radioactivity was measured on 1·5 ml of whole blood and on 1·5 ml of plasma obtained by centrifugation (within 5 min of sampling) of the remaining 3·5 ml of blood. The haematocrit was measured in triplicate and used to calculate the radioactivity remaining attached to erythrocytes. This indirect approach to calculating erythrocyte-bound counts was taken in order to overcome the problem of dissociation *in vitro* of IC from erythrocytes by Factor I whilst erythrocytes were being washed free of plasma. There was no significant drop in the fraction of IC bound to erythrocytes when the samples were kept up to 1 h at 4°C. In one patient with cryoglobulinaemia, the fraction of IC attached to erythrocytes could not be determined because the monoclonal IgM-κ rheumatoid factor induced precipitation of all complexes *in vitro* as soon as the temperature was below 36 °C. The initial elimination rates of the IC were calculated from the ^{125}I/^{99m}Tc ratios (the ratio of the injected mixture being 1·00). This correction was necessary for the first three samples, due to incomplete mixing of the injected sample in the whole blood volume until 4 min after injection. From this point onwards the absolute disappearance rate of protein-bound ^{125}I was used.

The model for analysis of IC clearance was defined from our initial studies of three normal subjects and three patients with

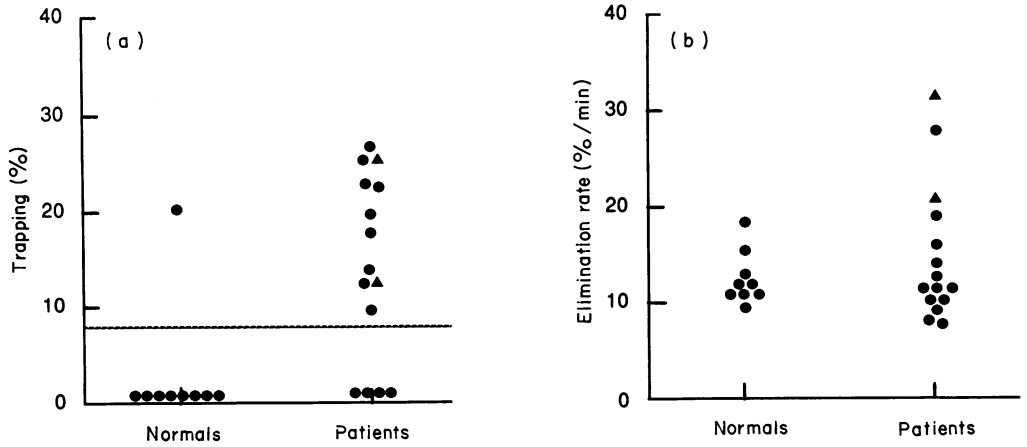

Fig. 1. (a) Percentage of trapping of immune complexes at 1 min after their i.v. injection in normal subjects and patients (the C1q- and the C2-deficient patients in all figures: ▲). Trapping was considered significant above 8% (—). (b) Monoexponential elimination rates of IC in normal subjects and patients.

SLE, all of whom are included in the present series (Schifferli *et al.*, 1988). In these studies we found that the clearance kinetics of IC in normal subjects were well described by a single monoexponential elimination rate constant. However, in patients it was found that a variable percentage of the IC was rapidly cleared from the circulation during the first minute after injection. After the first minute a monoexponential rate constant again fitted the measured data closely. We called the fraction which was removed within the first minute 'initial trapping', and showed that this phase of clearance occurred before IC had time to enter the mononuclear phagocytic system of liver and spleen by external scanning using the simultaneously-injected ^{99m}Tc-labelled erythrocytes. The two parameters, initial trapping (expressed as percentage injected dose) and rate constant of monoexponential clearance (expressed as percentage IC cleared per min), were calculated from each data set using a computer program. Built into the computer-fitted analysis was an estimate of the error of fit of the calculated to the observed values. This gave an average standard deviation of $\pm 4\%$ of the initial time point. Using this value we took values of initial trapping of greater than 8% as being significantly abnormal.

Other measurements

CR1 number per erythrocyte was measured using the monoclonal antibody, E11 (kindly donated by Dr N. Hogg, ICRF Laboratories, Lincolns Inn Fields, London), by radioligand binding assay as described previously (Ross *et al.*, 1985). C3, C4 and Factor I were quantified by single radial immunodiffusion using monospecific antisera (Serotec, UK; Cappel, Malvern, PA), and CH50 by a haemolytic plate assay (Harrison & Lachmann, 1986). Results were expressed as a percentage of a normal human pool of serum. Anti-TT antibody titre was measured using a standard ELISA (Nakamura, Voller & Bidwell, 1986), the anti-human IgG being a peroxidase-conjugated rabbit IgG (Diagnostic Pasteur, Marne La Coquette, France); a standard curve was defined using the anti-TT IgG preparation.

Statistical analysis

When appropriate we used the Fisher exact test, the Wilcoxon signed rank, the Kendall correlation (tau), and the Mann–Witney *U* (MW-*U*: one-tailed for comparisons between individuals with or without trapping).

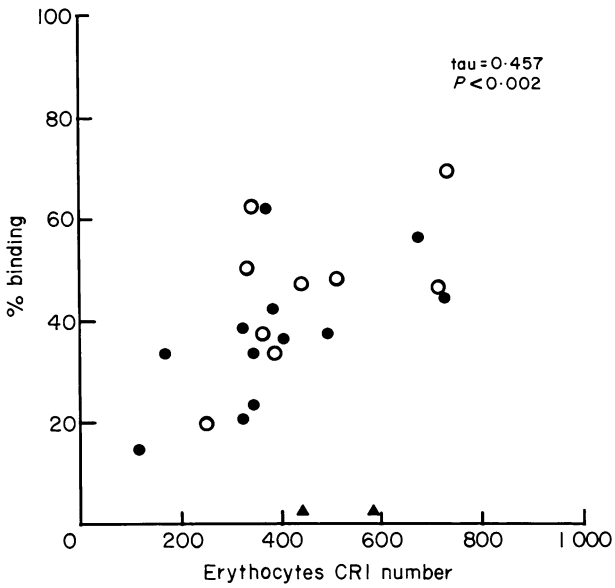

Fig. 2. Relationship of erythrocyte CR1 number to the percentage of injected IC bound to erythrocytes at 1 min. Normal subjects (○), patients, (●).

RESULTS

Clearance of TT-anti-TT complexes

The IC were cleared in two distinct phases. The first occurred very rapidly during the first minute after injection ('initial trapping' before IC had time to enter the mononuclear phagocytic system of liver and spleen); it was only seen in some subjects. The second could be described by monoexponential kinetics. Initial trapping was only seen in one out of nine normal subjects in comparison with 11/15 patients ($P < 0{\cdot}025$) (Fig. 1a). The second phase of elimination also occurred rapidly and between 9·9 and 18·7% injected complexes/min were cleared in

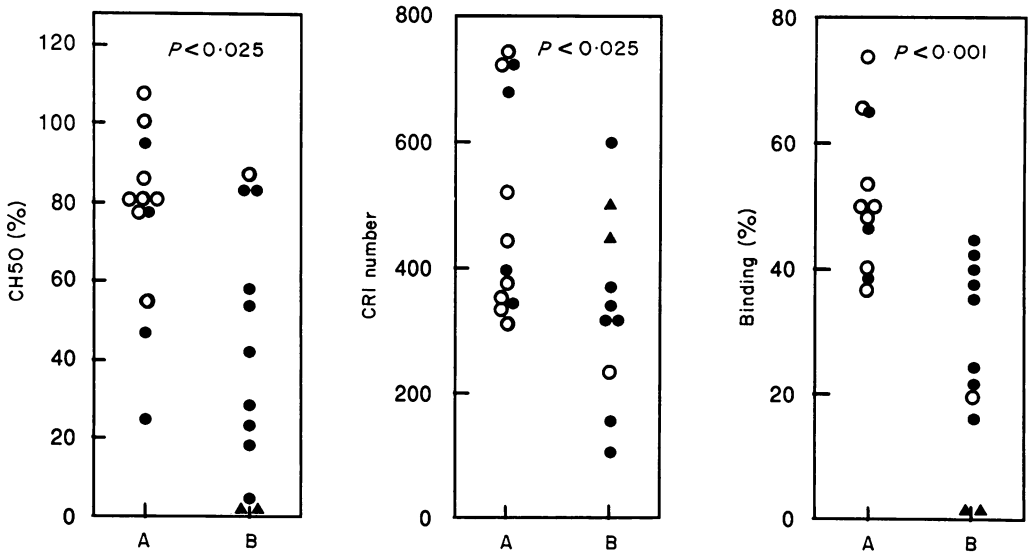

Fig. 3. CH50, erythrocyte CR1 number, and percentage immune complex binding to erythrocytes at 1 min in individuals without (A) and with (B) significant initial IC trapping. Normal subjects; (○) patients; (●).

normal subjects compared with 8·6 and 32·2%/min amongst the patients (Fig. 1b). Only four patients had elimination rates that were faster than the fastest normal subject.

After intravenous injection a fraction of the IC bound rapidly (by 1 min) to erythrocytes (between 0 and 71·5%). Since complement and CR1 have been shown to be essential for this binding reaction *in vitro* and in primates, we compared CH50 levels and erythrocyte CR1 number with the percentage of IC bound *in vivo* to erythrocytes at 1 min. CH50 levels did not influence binding, except for the two complement-deficient patients in whom IC did not bind at all. Erythrocyte CR1 number correlated with binding of IC at 1 min (Kendall tau 0·457, $P<0·002$) (Fig. 2), excluding the two patients with hereditary complement deficiency whose sera were unable to opsonize IC for binding to CR1. The patient with C1q deficiency was infused with 300 ml of fresh frozen plasma. After this infusion, which reconstituted his functional C1q levels from 0% to 5% of a normal pool, 29·8% of IC bound to his erythrocytes, but there was no significant change in the overall IC elimination rate.

We analysed the role of complement function, CR1 number, and IC binding to erythrocytes in determining IC clearance reactions. The two patients with hereditary complement deficiency showed differences in both phases of IC clearance from normal subjects; they showed significant initial trapping and had faster monoexponential clearance phases (Fig. 1). Subjects who showed initial trapping of IC at 1 min were compared with those who did not. These individuals had significantly lower CH50, CR1 number and IC binding than subjects without initial trapping of IC (Fig. 3). The rate of monoexponential clearance of IC did not show any correlation with CH50, but was inversely correlated with erythrocyte CR1 numbers, excluding the two complement-deficient patients, (Kendall tau $-0·256$, $P<0·05$), and showed a weak inverse correlation with IC binding (Kendall tau $-0·202$, $0·1<P<0·05$).

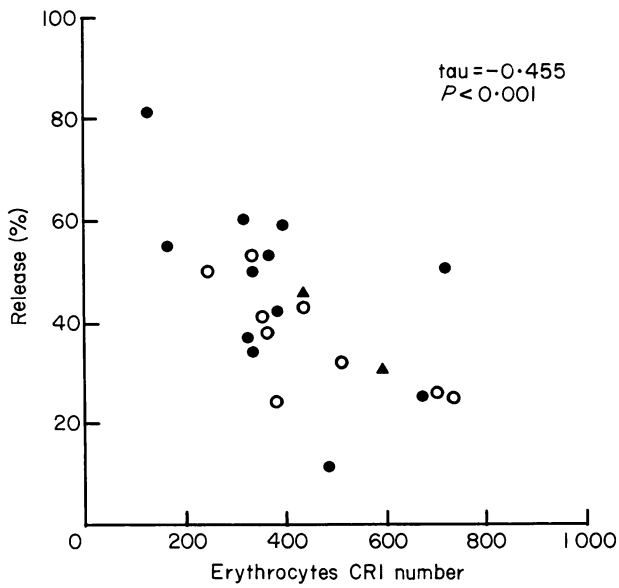

Fig. 4. Relationship of erythrocyte CR1 number to the percentage of the injected erythrocyte-bound IC released from erythrocytes at 1 min. Normal subjects, (○), patients, (●).

Clearance of erythrocyte-bound TT-anti-TT complexes

We opsonized the IC with serum and bound them to erythrocytes *in vitro* in order to assay IC elimination when the role of complement *in vivo* was bypassed. After injection of these erythrocyte-bound IC, there was rapid partial release of the complexes from the erythrocyte surface which occurred before the first blood sample was taken (< 1 min). The extent of this release reaction was significantly inversely correlated with erythrocyte CR1 number (Kendall tau $-0·455$, $P<0·001$) (Fig. 4).

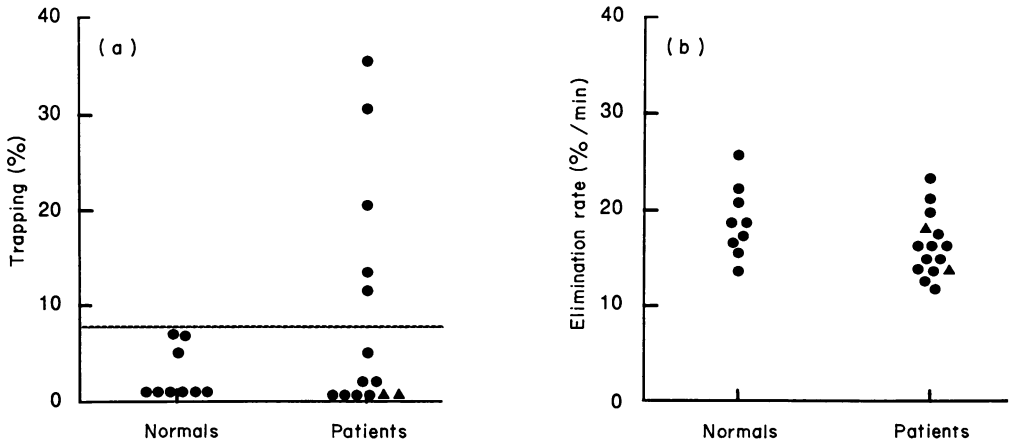

Fig. 5. (a) Percentage of trapping of IC at 1 min after their injection in the form of erythrocyte-bound IC in normal subjects and patients. (b) Monoexponential elimination rate of IC that were injected in the form of erythrocyte-bound IC in normal subjects and patients.

Initial trapping of IC was only seen in five patients (Fig. 5a); the two patients with hereditary complement deficiency no longer showed initial trapping when the IC were attached to their erythrocytes *in vitro* using normal serum. The percentage IC release at 1 min was determined in four out of five of these patients (the fifth had cryoglobulinaemia which caused IC precipitation *in vitro* which interfered with measurement of released IC). These four patients showed greater release of IC at 1 min than those in whom no significant trapping was demonstrated ($P<0.001$) and had lower erythrocyte CR1 numbers ($P=0.05$).

Following injection of erythrocyte-bound IC the monoexponential elimination rates were very similar in normals and in patients (Fig. 5b). These rates correlated inversely with the percentage of IC released at 1 min (Kendall tau -0.289, $P<0.05$) and with erythrocyte CR1 numbers (Kendall tau -0.291, $P<0.025$), but did not correlate with CH50.

The monoexponential elimination rates of the erythrocyte-bound IC were significantly faster than the monoexponential elimination rates of the free IC injected in the first study ($P<0.01$), with the exception of the two patients with hereditary complement deficiency in whom the clearance rate of opsonized red-cell-bound complexes was slower than the clearance of directly injected complexes.

The role of complement was demonstrated most clearly in these two patients with complement deficiency. IC injected directly into these subjects showed high initial trapping and rapid monoexponential clearance; both these parameters were restored to normal when IC were opsonised *in vitro* with normal serum and bound to the patients' erythrocytes.

Other studies

The anti-TT titre was significantly higher in normal subjects than in patients ($P<0.001$), however this titre did not correlate with IC binding to, or release from, erythrocytes, or with initial trapping or monoexponential elimination rates in either of the two studies. Factor I concentration had no influence on IC binding or release reactions.

DISCUSSION

Recent studies in non-human primates have indicated that large complexes fix complement, bind to C3b receptors on erythrocytes and are then removed from the circulation in a single passage across the hepatic circulation (Cornacoff *et al.*, 1983). The use of IC which fixed complement inefficiently, or injection into decomplemented animals, resulted in a smaller fraction of the complexes being cleared by the fixed mononuclear phagocytic system and rapid removal of a fraction of the complexes by other organs (Waxman *et al.*, 1984; 1986).

We investigated this system in humans using 45 S complexes of TT-anti-TT. These complexes were previously characterized *in vitro* and shown to be safe when injected into guinea-pigs (Paccaud *et al.*, 1987). In guinea-pigs these complexes incorporated C3 and bound to C3b receptors, which in non-primates is located on platelets (Taylor *et al.*, 1985). In C4-deficient animals the complexes did not bind to platelets and were eliminated at an accelerated rate (Paccaud *et al.*, 1987). Initial studies in humans similarly showed that these IC bound to erythrocytes and two phases of clearance were established (Schifferli *et al.*, 1988). The first was extremely rapid, occurring within the first minute, and was only seen in three patients with SLE; we called this 'initial trapping' of IC. This name reflects the observation that this phase of clearance occurred before IC had time to enter the mononuclear phagocytic system of liver and spleen, which we established by external scanning using simultaneously injected labelled erythrocytes. The second phase was a monoexponential clearance with an elimination rate of approximately 15%/min, similar to the elimination rate of soluble complexes in baboons (Cosio *et al.*, 1987).

In the studies described here we systematically measured these two phases of clearance in a larger series of normal subjects and patients with various diseases characterized by hypocomplementaemia and/or low erythrocyte CR1 number. IC bound to erythrocytes *in vivo* within a minute in a complement-dependent manner. Thus no complexes were seen to bind to erythrocytes of a C1q-deficient or a C2-deficient patient. Infusion of the C1q-deficient subject with only 300 ml of fresh frozen plasma allowed the partial restoration of binding of complexes to erythrocytes. Absence of IC binding in these two patients resulted in fast and presumably abnormal clearance. In both a significant fraction of the complexes were trapped within 1 min and the remaining complexes were cleared at a faster rate than in any of the normal subjects. In contrast, in the same two patients, when IC were bound to erythrocytes *in vitro* using normal human serum before intravenous injection, the appar-

ently normal in-vivo processing of these IC was restored, i.e. there was no initial trapping of IC, and the IC clearance rate was within the normal range. These findings may help to explain how hereditary complement deficiency predisposes patients to develop IC diseases. On the basis of our hypothesis, we would have expected that infusion of fresh plasma into the patient who was deficient in C1q would have restored normal IC clearance kinetics. A possible explanation is that the patient was only replenished with 5% of normal C1q levels, which only partially restored IC binding to erythrocytes. We have observed that, *in vitro*, such a low concentration is insufficient to restore fully the fast initial kinetic of IC opsonization (Paccaud *et al.*, 1987).

The role of erythrocyte CR1 in the normal processing of IC was suggested by a number of findings. In the first study the proportion of IC binding to erythrocytes correlated with CR1 numbers. Initial trapping of IC was seen more frequently in individuals with low IC binding to erythrocytes and low CR1 number, and the clearance rate of the remaining complexes was faster in those with low CR1 number. Similar observations were made in the second study in which we compenstated for *in vivo* hypocomplementaemia by injecting the complexes already bound to autologous erythrocytes. Within the first minute a percentage of these IC was released from erythrocytes and this percentage was inversely proportional to CR1 numbers. This observation was unexpected, since release by Factor I of IC already bound to CR1 would not be expected to be related to erythrocyte CR1 numbers. We formulated the hypothesis that this inverse relationship between percentage release and CR1 numbers was caused by rapid release of IC from CR1 on erythrocytes by Factor I with subsequent rebinding of the complexes to other erythrocytes according to their CR1 numbers. Following this observation we performed experiments *in vitro* which supported this idea (Ng, Schifferli & Walport, 1988). This suggests that rapid binding, release, and rebinding of these IC to erythrocyte CR1 occurs *in vivo*.

Significant trapping of IC in the second study was uncommon and was seen only in patients who had low CR1 numbers and high percentage IC release at 1 min. Thus low CR1 numbers appeared to be associated with abnormal disposal of these IC *in vivo*. The monoexponential clearance rate of IC in the second study was faster than in the first. In the second study, only the IC which had fixed complement and attached to CR1 on erythrocytes *in vitro* were injected.

What are the potential criticims of our model of IC clearance in humans? A specific criticism that may be levelled against the complexes that we used is that the majority of our subjects had circulating anti-tetanus antibodies which may have interacted with the injected complexes and modified their lattice. However we found no correlation between endogenous antibody levels and the fate of the injected complexes. Similarly the antigenic component of the complex may have played an important role in determining the clearance rate of the complexes as has been seen with DNA and certain carbohydrate-containing antigens (Emlen & Mannik, 1982; Finbloom *et al.*, 1981). A more general criticism concerns the size of the complexes which were large. Smaller complexes, or those fixing complement inefficiently, bind to erythrocyte CR1 poorly and may be cleared more slowly by different mechanisms from the circulation. This is illustrated by the recent report describing the clearance of heat-aggregated IgG from the circulation of humans in which a much smaller proportion of the complexes than we observed bound to erythrocytes and the clearance times of these complexes were much slower (Lobatto *et al.*, 1987; 1988). Finally, the system used is 'unphysiological' since IC are unlikely to form *in vivo* in the absence of complement. However similar complement- and CR1-dependent reactions have been demonstrated for IC that form directly in the circulation (Edberg, Kujala & Taylor, 1987).

In contrast to some previous studies with immune-coated erythrocytes in patients with SLE and related diseases, we saw no delay in the monoexponential clearance rate of IC in our patients (Frank *et al.*, 1983). However the two probes of the reticuloendothelial function are very different; immune-coated erythrocytes are cleared within the spleen whereas soluble IC are cleared predominantly within the liver (Mannik & Arend, 1971). Multiple mechanisms might have determined the monoexponential clearance phase in our studies: the function of the mononuclear phagocytic system, blood flow (IC elimination rates being very fast), and ongoing trapping of IC. Finally, we studied a heterogeneous population of patients whose common denominator was hypocomplementaemia and/or low CR1 number. Soluble IC and immune-coated erythrocytes provide different, but complementary information on immune elimination.

In conclusion, these studies demonstrate the involvement of complement and erythrocyte CR1 in the removal of large, soluble IC from the circulation of humans. The results illustrate the possible immunopathogenic role of deficiency of either or both of these systems in determining the organ localization of certain types of IC. Primary defects in the mechanisms that efficiently remove IC from the circulation may play a role in the development of disease mediated by circulating IC (Schifferli, Ng & Peters, 1986; Lachmann & Walport, 1987). An example of this may be the susceptibility to SLE in subjects with inherited deficiencies of individual proteins of the classical pathway of complement.

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