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Tetanus toxoid–anti-tetanus toxoid complexes: a potential model to study the complement transport system for immune complex in humans

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SUMMARY

Complement and its receptor on erythrocytes appears to play a physiological role in the elimination of large immune complexes (IC) in monkeys, and a similar system is likely to work in humans. Here we define a safe IC model which is suitable for clinical investigations. Soluble tetanus toxoid (TT)–human anti-TT (IgG) antibody complexes were prepared in large antibody excess. The size of the complexes was approximately 45 S. When incubated in normal human serum, 50% of the IC increased further in size, but remained soluble, and bound rapidly to human erythrocytes *in vitro*. This binding was shown to require intact classical pathway function. When injected into normal guinea-pigs a comparable proportion of IC bound immediately to blood cells (mainly to platelets). No platelet binding of IC occurred in C4-deficient guinea-pigs, but this binding was restored when C4 was supplied. Initial immune complex elimination was faster in C4 deficient than in C4-supplemented and normal guinea pigs. Thus classical pathway function appeared to be necessary for the normal processing, transport and elimination of TT–anti-TT complexes.

Keywords complement classical pathway CR1,C3b receptor immune complex

INTRODUCTION

Under normal circumstances large immune complexes (IC) are cleared rapidly from the blood stream. The mechanisms responsible for the safe delivery of IC to the fixed macrophage system include a complement-dependent transport system by blood cells (Siegel, Liu & Gleicher, 1981). This transport is due to the binding of C3 bearing IC to C3b receptors (CR1) present on various circulating cells (Fearon, 1980; Ross & Medof, 1985): in mice, rabbits and guinea-pigs most CR1 are located on platelets (Miller *et al.*, 1975; Taylor *et al.*, 1985), whereas in primates the bulk of CR1 is on erythrocytes. Cornacoff *et al.* (1983) have established that, in monkeys, the overall clearance of poorly soluble IC is slowed by this transport; in the absence of C3, as obtained by depleting C3 with cobra venom factor (CVF), IC are cleared at a higher rate but a fraction ends up trapped in the capillaries of various organs like lung and kidney (glomeruli) where they could induce inflammation and tissue destruction (Waxman *et al.*, 1984).

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In humans different mechanisms could account for a deficient complement-mediated IC transport system, in particular a complement depleted or deficient state, or a low CR1 number as observed in patients with SLE (Miyakawa *et al.*, 1981; Taylor *et al.*, 1983; Ross *et al.*, 1985). The study of the handling of IC *in vivo* in humans awaits the development of a simple and safe IC model. Here we define such a model that has many of the characteristics of putative pathogenic large circulating IC.

MATERIALS AND METHODS

Immune complex model. Soluble TT–anti-TT complexes were prepared as previously described (Schifferli, Steiger & Paccaud, 1986). Briefly, purified TT (kindly provided by Dr Fürher) was radiolabelled with ^{125}I using Iodo-Beads (Pierce). Human anti-TT IgG immunoglobulin preparation (Tetuman Berna) was added in large excess to the labelled TT, i.e. 500 μg anti-TT for 1 μg TT (25 × equivalence). This reaction mixture was incubated 1 h at 37°C and 1 h at 4°C. IC were snap-frozen in liquid nitrogen and stored at −80°C until used. The IC produced were ultracentrifuged on isokinetic and linear sucrose density gradients (SDGU) to asses their size (Johns & Stanworth, 1976; Schifferli *et al.*, 1985).

Immune adherence assay. This assay measured the capacity of human erythrocytes to bind IC previously exposed to normal or pathological sera. IC containing 64 ng of TT were added to 25 μl of serum prewarmed at 37°C and adjusted to a final volume of 100 μl with veronal-buffered saline (VBS). After various intervals of time 12 μl samples were added to 100 μl of a 25% suspension of erythrocytes in VBS at 4°C for 10 min; 900 μl of cold VBS was added, the mixtures were centrifuged and the percentage of binding determined. All assays were done in duplicate.

CR1 on erythrocytes ($2{\cdot}7 \times 10^9$ cells/ml in PBS) were digested with trypsin ((Cooper, Malvern, PA) 0·1% final concentration) for 15 min at 37°C, and the reaction was stopped by adding soya bean trypsin inhibitor (Sigma) to a final concentration of 0·15% for 5 min. Such cells did not bind significant amounts of opsonized IC and were used as negative controls in immune adherence experiments. The binding of opsonized IC was also blocked by pretreatment of erythrocytes with anti-CR1 mouse monoclonal antibody (Dakopatts).

Sera, complement measurements and proteins. The presence of anti-TT antibody in all sera was detected by linear SDGU: 64 ng ^{125}I-TT was added to 25 μl EDTA-sera, incubated for 10 min at 37°C and ultracentrifuged (a shift of the TT towards the bottom of the gradient indicating the presence of anti-TT antibodies). All sera contained a low or undetectable concentration of anti-TT antibodies, i.e. producing no, or an incomplete, shift of the TT (anti-TT concentration below the equivalence point). The antibody/antigen ratio of the preformed IC was not significantly modified (from 25 to maximum 26 × equivalence) when they were added to the tested sera. The relevant complement measurements in normal and the pathological sera were performed as previously reported (Schifferli *et al.*, 1985). Results are expressed in % of a pool of 25 normal human sera.

Fresh human serum (5 ml) was depleted of factor D (RD) by two consecutive cycles on a Sephadex G-75 column (Pharmacia). The AP50 of RD was abolished, and restored to 80% by purified factor D. Depletion of C3 in normal human serum (NHS) was achieved by incubating 100 μl for 1 h at 37°C with CVF (0·5 units) (Cordis, Miami, FL), or purified IgG (100 μg) from a patient with Nef. C1q, C4 and properdin were purified according to methods described by Medicus *et al.* (1980), Tenner, Lesavre & Cooper (1981) and Hammer *et al.*, (1981), and functional human C2 was obtained from Cordis.

Experiments in guinea-pigs. Fresh guinea-pig blood was obtained by cardiac puncture. Platelets were isolated according to the method of Adlie, Packham & Mustard (1970). Immune complexes containing 20 ng of TT were incubated at 37°C for various periods of time in 25 μl of different human and guinea-pig sera; 15 μl samples were added to 100 μl of a solution of 150,000 platelets/μl, incubated for 20 min at 20°C, centrifuged at 10,000 ***g*** for 5 min, and the binding was then determined.

Normal and C4-deficient guinea-pigs (C4defGP) (Porcellus) were used for *in vivo* studies. Under anesthaesia a catheter was introduced in the right jugular vein. Immune complexes containing 5 μg of TT were injected via the catheter, which was immediately flushed with isotonic saline. 'Zero' time

was defined as the end of the 20 s injection period. Blood samples (300 μl) were collected at various time intervals in 2 U of heparine and transferred immediately to an ice-bath until processed. Total circulating IC was measured by counting the radioactivity of 100 μl of whole blood in 900 μl of VBS. In initial experiments the value obtained in the first measurement (30 s) was considered to represent 100% of the injected dose. In later experiments IC were injected simultaneously with ^{131}-I labelled BSA (Armon Pharmaceutical Company Ltd), which was used as a marker to determine the initial clearance of IC in the first 30 s after injection, i.e. when 100% of the BSA was still intravascular. To determine the amount of cell-bound IC, 50 μl of blood was mixed with 950 μl of ice-cold VBS, and centrifuged for 30 min at 3500 *g* at 4°C; supernatant and pellet (cell bound) radioactivity was measured. Initial experiments using Ficoll-Hypaque gradient centrifugation (Pharmacia) showed that more than 90% of the cell bound IC were also located in the platelet-rich plasma. All values were corrected for free iodine.

Experiments in rabbits. Normal white rabbits were immunized with TT (once in CFA and twice in IFA, at 2 week intervals). Normal and immunized rabbits were injected with IC (containing 5 or 50 μg TT), or I^{125}-TT. Elimination and platelet binding of IC were determined as for guinea-pig experiments.

RESULTS

Immune complexes model. TT–anti-TT antibody complexes prepared in large antibody excess were soluble and their size remained unchanged when prepared at antibody/antigen ratios between 20 and 100 × equivalence (around 45 S as estimated by isokinetic SDGU). A ratio of 25 × equivalence was chosen for all experiments: these IC did not aggregate at 4°C over 5 days; freezing in liquid nitrogen, storage at −80°C and thawing at 37°C did not alter their size and reactivity. IC remained soluble when incubated in NHS for various periods of time; however 50% of the IC had enlarged significantly already after an incubation of 3 min at 37°C (Fig. 1a). This enlargement was dependent on complement i.e. it was abolished in EDTA-treated or heat-inactivated serum, and was identical in various NHS with different concentrations of anti-TT antibody.

Immune adherence assay. Binding of IC to erythrocytes required complement (Fig. 2a), and was abolished when erythrocyte CR1 were destroyed or blocked (see Materials and Methods). Opsonization of IC in NHS was rapid; the binding reached maximum within 1 to 5 min, and diminished slowly thereafter. Only IC which had enlarged upon incubation with serum were capable of binding to erythrocytes as indicated by the following experiments: (1) absorption of IC exposed to NHS for 3 min with excess erythrocytes selectively removed the enlarged IC; (2) the 25 fractions obtained from a linear SDGU run of complement-reacted IC were exposed to erythrocytes and the enlarged IC bound almost completely whereas smaller IC had little capacity to bind ($< 10\%$ binding); (3) complement-reacted IC were first bound to erythrocytes, then released by incubation with heat inactivated serum for 10 min at 37°C: their size indicated that they had enlarged (Fig. 1b).

Role of complement components in promoting IC binding. The binding was directly related to the presence of functional C3 concentration: it was abolished in CVF-treated serum (no remaining functional C3), and was only 5% in Nef treated serum (containing 10% functional C3). Sera deficient in different classical pathway components (C1q, C2, C4) were unable to promote IC binding (C1q deficient serum, see Fig. 2b). By restoring C1q, C2 or C4 in the respective deficient serum it was possible to restore IC binding, as well as the kinetics of the reaction: lowering the C1q, with respect to the C4 concentration, indicated that 10% normal C1q and 1% C4 were sufficient to normalize IC binding. In contrast properdin deficiency and D-depleted sera did not delay nor significantly reduce the binding reaction. However repletion by the missing component enhanced the maximum binding slightly ($\leq 5\%$). Deficiency of C7 or C8 had no effects on the binding reaction. In all these sera, binding of IC correlated with a change in IC size: no enlarged IC were formed in C1q, C4 and C2 deficient sera in contrast to the other sera in which IC binding was normal (not shown).

Binding of immune complexes was measured in various pathological sera; the results, summarized in Fig. 3, indicated that depletion of C1 to C3 prevented the rapid binding reaction.

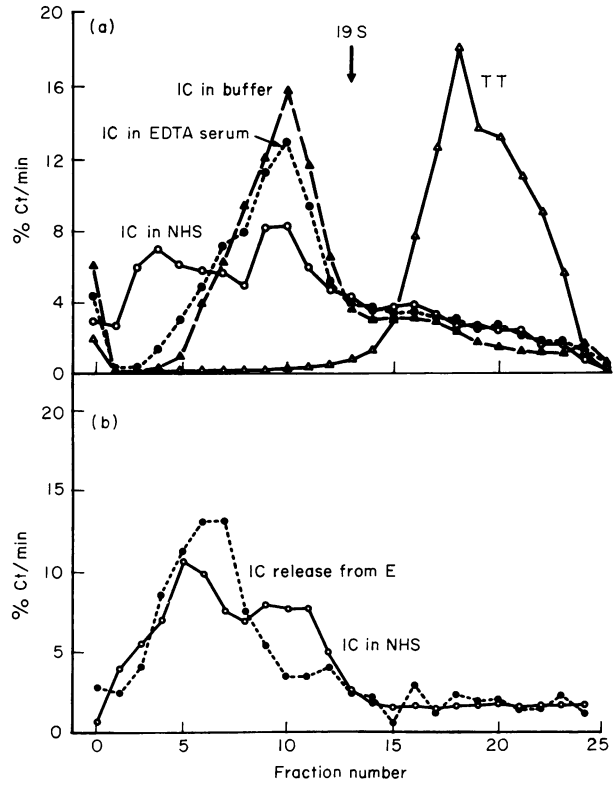

Fig. 1. (a) SDGU of preformed TT–anti-TT complexes. Twenty-five fractions were collected and the results are expressed in percentage of total ct/min recovered. The bottom of the gradient is on the left. IgM served as 19S marker. (△—△) TT in buffer; (▲- -▲) IC in buffer; (○—○) IC incubated 5 min in NHS; (●- -●) IC incubated 5 min in NHS with 10 mM EDTA. b. Complement-reacted IC bound to erythrocytes were released by incubation in heat inactivated serum for 10 min at 37°C. Such released IC (●- -●) were analysed by SDGU, and compared to complement reacted IC (○—○). To note is that released IC are very large, comparable to the fraction of IC which had enlarged upon exposure to complement.

Binding and clearance of preformed IC in normal and C4-deficient in vivo *in guinea-pigs.* This was done after having determined that similar classical pathway dependent reactions occurred in guinea-pig blood: despite being of human origin, TT–anti-TT complexes were also modified by guinea-pig serum: approximately 50% of IC increased in size, and a similar proportion bound to isolated guinea-pig platelets (not shown).

Soluble IC were injected i.v. into normal guinea-pigs and C4defGP. The binding varied from 40 to 60% according to the animal and the preparation of the IC; it was maximal after 4–8 min and was maintained for about 30 min (Fig. 4). Immune complexes bound mainly to platelets (see Material and Methods). In contrast IC did not adhere to platelets in C4defGP over 2 h. This lack of binding was due to the absence of classical pathway function since it was possible to restore binding by an injection of normal guinea-pig plasma before IC administration: in this experiment, the amount of C4 injected corresponded to 1% of the normal C4 concentration in normal guinea-pigs. C4 repletion was also done after the IC injection: binding did not occur in the first 4 min before plasma infusion, but rose sharply afterwards.

The rate of IC elimination was significantly faster in C4defGP than in normal guinea-pigs (Fig. 5). Restored C4defGP had an IC clearance comparable to that of normal animals. Restoring C4 in C4defGP even 4 min after IC injection temporarily modified the rate of IC elimination. Identical results where obtained in two more series of clearance experiments in which initial blood volume

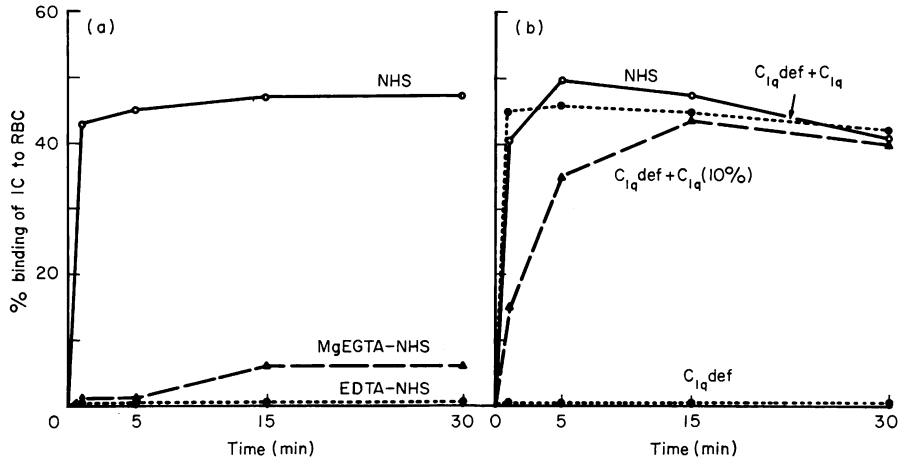

Fig. 2. (a) Binding of TT–anti-TT immune complexes to erythrocytes. IC containing 64 ng of TT were added to 25 μl of NHS and incubated at 37°C. At various time intervals samples containing 8 ng of TT were added to a 25% solution of washed erythrocytes and incubated on ice for 10 min. Cold buffer was added up to 1 ml, the samples centrifuged at 200 *g* for 7 min, half the supernatant removed and the percentage of bound IC was determined. (○—○) IC in NHS; (●– –●) IC in NHS chelated with 10 mM of EDTA; (▲– –▲) IC in NHS containing 2 mM MgCl + 10 mM EGTA. (b) (○—○) IC in NHS; (●– –●) IC in C1q deficient serum; (●– –●) IC in C1q deficient serum restored to physiological concentration with purified C1q; (△– –△) IC in C1q deficient serum repleted with 10% of the physiological concentration of C1q.

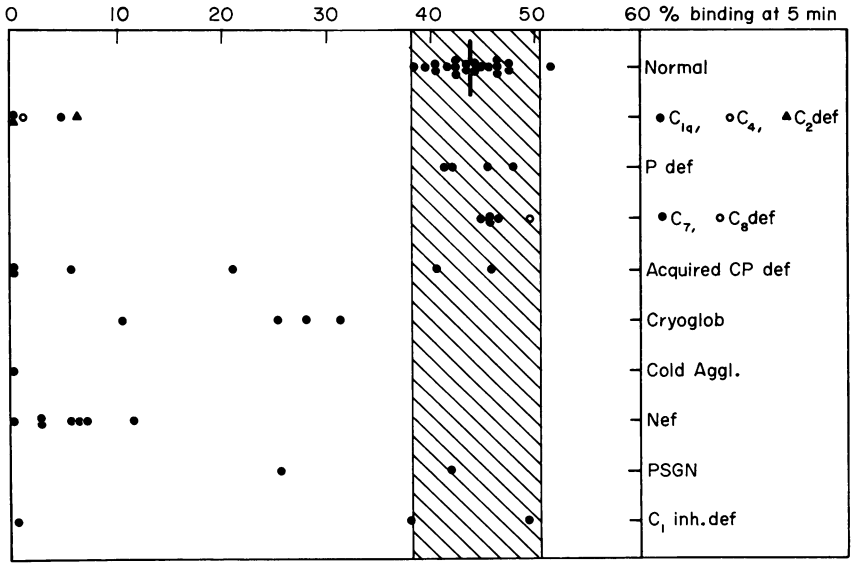

Fig. 3. Capacity of hypocomplementaemic sera to promote IC binding to erythrocytes after an incubation of 5 min. The normal range of binding (dashed area = ± 1 s.d.) was determined from 20 normal sera. P def, properdin deficient; acquired CP def, acquired classical pathway deficiency associated with paraproteinemia (C1q ≤ 25% and /or C4 ≤ 5%; Cryoglob, cryoglobulinaemia (C1q ≤ 45% and/or C4 ≤ 16%); cold Aggl, cold agglutinin disease (10% C3, undetectable C4); Nef, nephritis with nephritic factor (C3 ≤ 10%). PSGN, post streptococcal glomerulonephritis, binding reduced in the patient with 15% C3 (40% C3 for the other patient); C1 inh. def, C1-inhibitor deficiency, binding reduced in the patient with depleted C4 (≤ 1%), the two others were treated with danazol/stanazol (normal C4 and C2).

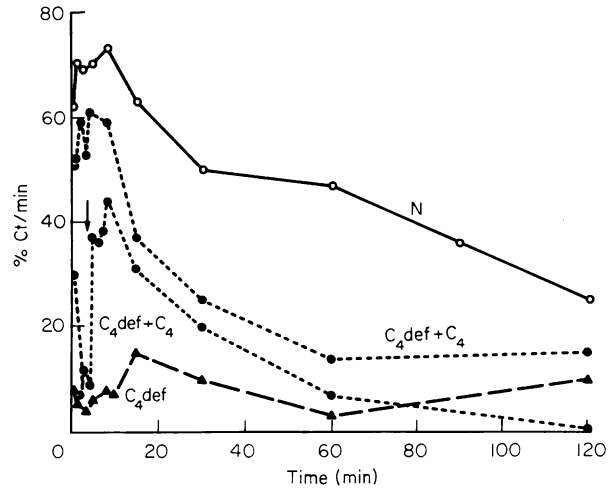

Fig. 4. Binding of IC to blood-cells in guinea-pigs after i.v. injection of TT–anti-TT complexes (expressed as the % of circulating IC bound to cells). Binding in NGP (○—○); in C4defGP, (▲- -▲); in C4defGP which had been supplemented with 1 ml of normal guinea-pig serum prior to IC injection, (●- -●); in C4defGP which had been repleted with 1 ml of NGP serum 4 min after IC injection: (●- - -●) (arrow).

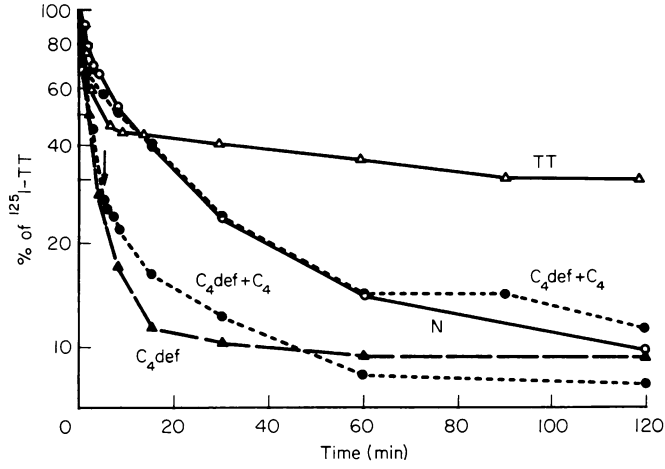

Fig. 5. Blood clearance of TT or IC injected into GP (expressed as the % of the injected dose of radiolabelled TT). TT, (△—△); IC in a NGP, (○—○); IC in a C4 deficient GP, (▲- -▲); IC in a C4 deficient GP repleted with 1 ml of NGP serum prior to IC injection, (●- -●); IC in a C4 deficient GP repleted with 1 ml of normal guinea-pig serum 4 min after IC injection, (●- -●) (arrow).

was determined by the concomitant injection of ^{131}-I-BSA and IC.

Injection of IC and ^{125}I-TT in normal and immunized rabbits. See Fig. 6. TT injected into a normal rabbit did not bind to platelets, whereas in the immunized rabbit the rapid formation of TT–rabbit anti-TT complexes was followed by very efficient and sustained binding. Preformed TT–human anti-TT complexes injected into a normal rabbit bound less well (but comparable to platelet binding in guinea-pigs *in vivo* and to human erythrocytes *in vitro*, i.e. around 50%). When injected into an immunized rabbit, the platelet binding of preformed TT–human anti-TT complexes was not

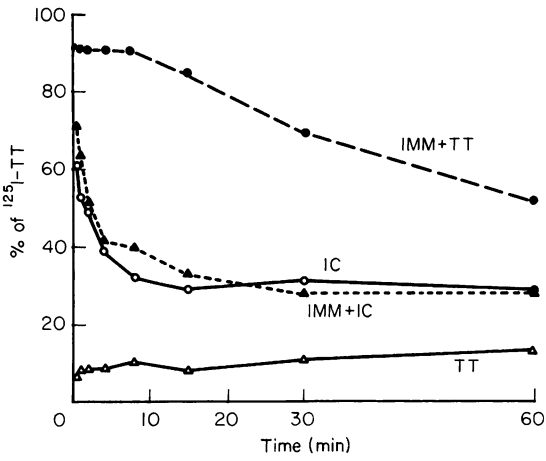

Fig. 6. Binding to blood-cells of TT or preformed IC in normal and immunized rabbits (expressed as the % of circulating radiolabelled TT). TT (△—△) and IC (▲- -▲) into normal rabbits; TT (●- -●) and IC (○—○) into immunized rabbits.

improved, suggesting that there was little exchange between human anti-TT and rabbit anti-TT in the complexes during the time of the experiment. Infusion of large quantities of IC (containing 50 μg TT) did not produce any detectable complement consumption (CH50 and functional C4), nor any alteration in the 'clinical status' of the animals (temperature, breath, skin colour, behaviour). Such animals remained well over an observation period of 2 weeks.

DISCUSSION

The biological role of the complement transport system for immune complexes has been well documented in various animal experiments. Because of the lack of immune complexes suitable for clinical investigation there are no data on the putative role of this system in humans. The main purpose of this work was to characterize an immune complex which would require the complement transport system for its safe elimination and would be acceptable for human studies. The TT–anti-TT complexes, as defined in this study, satisfy both requirements.

Before discussing the various results, we would like to review the reasons for choosing TT–anti-TT complexes. First, TT is a denatured protein which has been administered extensively to humans and can be regarded as an innocuous substance. Second high titre anti-TT IgG fraction has been prepared from immunized individuals and is commercially available. Third, TT–anti-TT complexes made in antibody excess are soluble but very large (size 45S); they are thus likely to require the complement receptor transport system to avoid being trapped in capillaries of various tissues. Fourth, IC made in antibody excess are relevant to human pathology, e.g. when overproduction of antibody is evident such as in chronic infections and autoimmune diseases (Horgan *et al.*, 1984). Last, these IC appear to be safe when injected into rabbits and initial experiments in humans confirm their innocuity.

The TT–anti-TT complexes were stable and not modified by additional anti-TT antibodies present in the different sera studied, as demonstrated by linear SDGU. Using normal and immunized rabbits we could not demonstrate a significant exchange of antibody bound to TT in the preformed complexes with excess autologous antibodies present in the circulation. The absence of such an exchange should not be surprising: antigen binding sites have been saturated in the IC, and high affinity antibodies have been selected by the preparation procedure. Thus excess autologous anti-TT antibodies should not influence the transport and fate of these preformed IC, i.e. their binding and clearance should be similar in individuals with various titres of anti-TT.

Studies *in vitro* demonstrated that a large fraction of TT–anti-TT complexes incubated in NHS reacted with complement and bound to human erythrocytes. Complement reacted complexes capable of binding to erythrocytes had enlarged considerably but remained soluble. The deposition of large numbers of C3b was probably responsible for this increase in size.

Because large soluble complexes are eliminated *in vivo* very rapidly, the kinetics of complement activation and binding to erythrocytes could determine their fate: if immediately bound to erythrocytes they could escape localization in various tissues other than the fixed macrophage system. Using different complement depleted and deficient sera, it was possible to show that: (1) C3 was the relevant opsonin. C4 had no demonstrable capacity to mediate IC binding to erythrocytes. (2) Classical pathway function was essential: the sera deficient in one of the components of the classical pathway leading to C3 activation were incapable of inducing IC binding.

It is worth emphasizing that there is no good evidence that stable soluble IC (not pelleted by a centrifugation of at least 3000 ***g*** for 10 min) can be opsonized with C3b in the absence of any classical pathway function (Yoshida, Yukiyama & Miyamoto, 1986; Jepsen *et al.*, 1986). Thus the physiological disposal of large soluble IC would be altered in classical pathway deficient states. *In vivo* experiments in guinea pigs provided additional evidence for the role of the classical pathway in IC elimination: IC binding to platelets, as observed in normal guinea-pigs abolished in C4defGP, and this defect correlated with fast IC clearance, possibly outside the FMS.

The initial studies performed in normal humans using TT–anti-TT complexes suggest that complement and erythrocyte CR1 participate in immune complex clearance reactions in humans as well: upon i.v. injection a significant fraction of such complexes bound to erythrocytes. It will be of particular interest to see whether IC clearance reactions are accelerated in patients with defective complement function, as observed in C4defGP, and in those with reduced CR1 number per erythrocytes; this work is in progress.

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