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BSA–anti-BSA immune complexes formed in the presence of human complement do not bind to autologous red blood cells

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SUMMARY

The binding of ^{125}I-BSA–anti-BSA immune complexes (IC) formed in the presence of complement (nascent IC) and those that reacted with complement after their formation (preformed IC) to human erythrocytes was studied. The effect of antigen–antibody ratios and serum dilutions on IC binding to red blood cells (RBC) were also investigated. A dramatic difference was found between the behaviour of the two types of complexes: while preformed IC bound effectively to human RBC after their interaction with complement, no efficient RBC-binding was observed with nascent BSA–anti-BSA complexes formed in the presence of complement. Changing the antigen–antibody ratio or dilution of serum did not affect markedly the extent of difference between the binding of preformed and nascent IC. Our findings do not support the essential role of red blood cells in the elimination of each type of IC.

INTRODUCTION

The binding of IC to different blood cells after their interaction with the complement system has been studied extensively. Experimental IC formed *in vitro* and incubated in fresh complement-containing serum have been shown to bind efficiently to erythrocytes (Nelson, 1963), macrophages (Onyewutu, Holborow & Johnson, 1974), PMN (Henson & Oades, 1975), some types of lymphocytes (Eden, Miller & Nusenzweig, 1973) and to platelets of non-primate mammals (Ginsberg & Henson, 1978). Under optimal circumstances in the presence of complement, more than 50% of the IC binds to the human red blood cells (RBC) (Medof & Oger, 1982; Jepsen *et al.*, 1986a). Since human RBC carry only one type of complement receptor, the C3b/C4b receptor (CR1), complement-reacted IC most probably bind to these receptors. The significance of CR1 on primate RBC *in vivo* in the binding and elimination of preformed antigen–antibody (Ag–Ab) complexes has been proved by Cornacoff *et al.* (1983). Moreover, it has been calculated that 90% of the total amount of CR1 present on blood cells can be found on RBC (Siegel, Liu & Gleicher, 1981). Based on these findings, the essential role of RBC in the elimination of immune complexes has been accepted (Fearon, 1986; Sim & Walport, 1987).

Abbreviations: BSA, bovine serum albumin; CIC, circulating immune complex; CPD, citrate phosphate dextrose solution; CR1, complement receptor, type 1; IC, immune complex; NHS, serum from a healthy blood donor; RBC, red blood cells.

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The experiments mentioned above were performed mainly with IC which were interacted with the complement system only after their formation. *In vivo*, however, the formation of circulating immune complexes (CIC) occurs in the presence of complement. That is why artificial complexes prepared in the complement-sufficient serum are better models for CIC studies than immune aggregates or preformed Ag–Ab complexes (Schifferli & Peters, 1983).

In the present study we have investigated whether IC formed in the presence of complement can also bind efficiently to autologous human erythrocytes. Using bovine serum albumin (BSA)–rabbit anti-BSA complexes we found no efficient binding of IC to the RBC, although a strong binding was observed under identical circumstances with preformed IC containing the same amounts of Ag and Ab.

MATERIALS AND METHODS

Antigen (Ag)

Bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO) was used as antigen. It was radiolabelled with ^{125}I with the chloramine T method of McConahey & Dixon (1966).

Antibody (Ab)

The IgG fraction of a high titre rabbit antiserum to BSA was obtained from Phylaxia Co. (Budapest). The same preparation was used throughout.

Preparation of preformed IC

BSA–anti-BSA immune complexes (IC) were made at equivalence, at five times Ag, five times and 10 times Ab excess. Point of equivalence was determined as described in our previous work

(Varga *et al*., 1987). Preparation of preformed IC was carried out exactly as outlined in the paper by Jepsen *et al*. (1986b): 10 μl BSA (200 μg/ml), containing ^{125}I-BSA as tracer ($1–1·2 \times 10^4$ c.p.m.), were added to 1 ml of diluted rabbit–anti-BSA. The final volume was adjusted to 3 ml with PBS and the IC preparation was incubated at 37° for 60 min.

RBC

Blood was collected in 1·5-volume percentage of CPD from healthy volunteers, centrifuged at 150 *g* at 4° for 10 min, and the buffy coat and plasma removed. After washing three times with RPMI and counting the cell number, the RBC suspension was adjusted to $7·3 \times 10^9$ cell/ml and kept at 4° until used (Jepsen *et al*., 1986b). Autologous serum was obtained from the same donor on the same occasion.

CR1-binding assay with preformed IC in a one-step procedure

One-hundred and fifty microlitres of NHS (in various dilutions) were added to 150 μl RBC and incubated at 37° for different time periods with 150 μl ^{125}I-BSA–anti-BSA complexes. The reaction was stopped by the addition of 2 ml ice-cold RPMI and samples were centrifuged at 150 *g* at 4° for 10 min. The supernatant was discarded, and the cells washed three times with RPMI. Binding on RBC was calculated from the determination of the c.p.m. on RBC expressed in the percentage of the total c.p.m. added. Heat-inactivated normal human serum was used as background (< 10% of total c.p.m.). All tests were run in triplicate. The mean values are shown in the figures.

Cr1-binding assay with nascent IC in a one-step procedure

Seventy-five microlitres of BSA (0·1 μg) containing ^{125}I-BSA as tracer were incubated with 150 μl NHS in various dilutions and 150 μl of erythrocytes from the same donor ($1·1 \times 10^9$ cells) at 37° for 5 min, and 75 μl prewarmed rabbit anti-BSA (in a dilution corresponding to the point of equivalence, five times antigen excess or to five or 10 times antibody excess) were added at zero time. Calculations were made as in the case of preformed IC.

CR1-binding assay with performed IC in a two-step procedure

One-hundred and fifty microlitres of NHS were prewarmed at 37° for 5 min, 150 μl labelled IC added and allowed to be solubilized at 37° for 2 or 60 min. The reaction was stopped by the addition of 600 μl ice-cold RPMI and by transferring the sample to an ice-bath (5 min). One-hundred and fifty microlitres of washed autologous erythrocytes ($1·1 \times 10^9$) were added and the samples incubated at 37° for 2–32 min. The reaction was stopped by transferring the tubes to an ice-bath. The samples were centrifuged at 150 *g* at 4° for 10 min, the supernatant discarded, the RBC washed three times and the radioactivity measured and expressed as a percentage of the total c.p.m. added (Jepsen *et al*., 1986b).

CR1-binding assay with nascent IC in a two-step procedure

Seventy-five microlitres of ^{125}I-BSA (containing 0·1 μg) were incubated with 75 μl of prewarmed anti-BSA (10 times antibody excess to the equivalence) in the presence of 150 μl NHS at 37° for 2 or 60 min. Subsequent procedure was equal to that described in the case of preformed IC in a two-step procedure.

RESULTS

Binding reaction to autologous human RBC with preformed BSA–anti-BSA complexes

Preformed IC preparations prepared at five times antibody excess were incubated with autologous human erythrocytes. Our experimental procedure was designed on the basis of the methods described by Jepsen *et al*. (1986b). The findings here were similar to those of these authors (Fig. 1). Maximal binding of IC to RBC was observed in the first minutes of incubation. When the incubation period was increased further, the amount of IC bound to RBC dropped to half of the maximal value.

Reaction of preformed and nascent BSA–anti-BSA IC prepared at different Ag:Ab ratios to RBC in a one-step procedure

In contrast to the results of the previous experiment, no efficient binding to autologous human erythrocytes was found with the BSA–anti-BSA complexes formed in the presence of fresh human serum at a Ag:Ab ratio corresponding to five times antibody excess (Fig. 2). Similar results were observed at a Ag:Ab ratio corresponding to the point of equivalence (Fig. 3), 10 times Ab excess and five times Ag excess (data not shown). By contrast, preformed IC prepared at the same Ag:Ab ratios bound effectively to the autologous RBC, except those prepared at five times Ag excess where only weak (maximally 20%) binding was found.

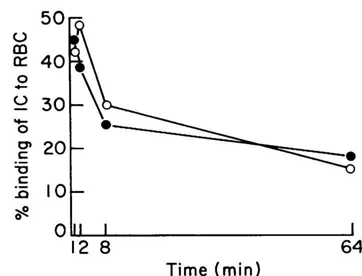

Figure 1. Binding of preformed IC (prepared at five times Ab excess) to human RBC in the presence of NHS (1:2) at 37°. Background binding (< 10% of total c.p.m.) when IC was incubated with heat-inactivated NHS has been subtracted. Results from Jepsen *et al*. (1986b), (●); those from the present study, (○).

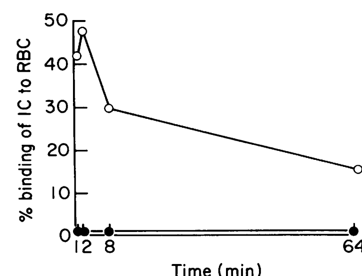

Figure 2. Binding of nascent (●) and preformed (○) BSA–anti-BSA IC (prepared at five times Ab excess) to human RBC in the presence of NHS (1:2) at 37°.

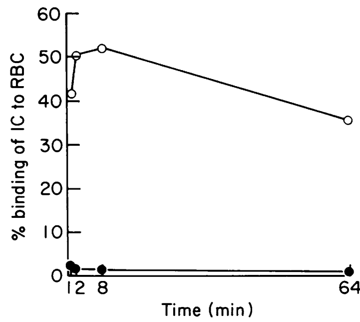

Figure 3. Binding of nascent (●) and preformed (○) BSA–anti-BSA IC (prepared at equivalence) to human RBC in the presence of NHS (1:2) at 37°.

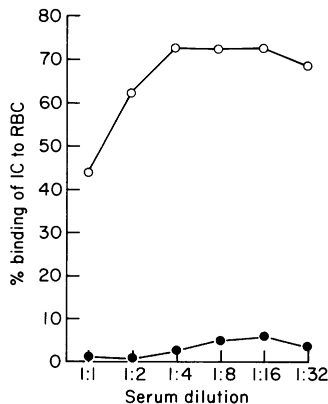

Figure 4. Binding of nascent (●) and preformed (○) BSA–anti-BSA IC (prepared at equivalence) to human RBC in the presence of different dilutions of NHS at 37° for 8 min.

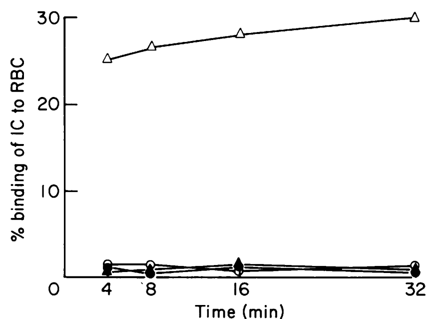

Figure 5. Binding of preformed BSA–anti-BSA presolubilized in NHS (1:2) for 2 min (△) or 60 min (○) and BSA–anti-BSA complexes formed and incubated in NHS (1:2) for 2 min (▲) or 60 min (●) to autologous RBC at 37°. Antigen and antibody interacted with each other at 10 times Ab excess.

Influence of serum dilution on the binding of preformed and nascent IC to RBC

When serum diluted 1:1–1:32 was used in the experiments, a weak (<5%) binding of nascent IC was found at serum dilutions of 1:4–1:32, whereas strong (>40%) binding was observed in the case of preformed IC with maximal values with the same serum dilutions (Fig. 4).

Binding reaction of preformed and nascent RBC in a two-step procedure

In the two-step procedure, nascent IC formed in the presence of serum for 2 or 60 min, and preformed IC presolubilized in serum for the same time, were incubated with RBC. Binding on RBC was found only with preformed IC presolubilized for 2 min, whereas preformed IC presolubilized for 60 min and nascent IC preparations did not bind to the autologous RBC. In the experiments when antigen, antibody and serum were incubated simultaneously for 2 or 60 min, followed by the addition of RBC, binding of nascent IC did not differ from that measured in control samples containing heat-inactivated serum (Fig. 5).

DISCUSSION

No effective binding to autologous human erythrocytes (RBC) was found in the present study with BSA–anti-BSA immune complexes formed in fresh human serum in the presence of complement. Neither complexes formed in the presence of RBC nor those formed before the addition of RBC and preincubated in serum bound to these cells. The same lack of binding was observed with a broad range of antigen:antibody ratios from five times antigen excess to 10 times antibody excess or with undiluted or diluted serum as a complement source.

These findings are in sharp contrast to the results of some previous works in which BSA–anti-BSA and other types of immune complexes were found to bind effectively to human RBC in the presence of human serum or after a preincubation in serum for a short period of time (Medof & Oger, 1982; Jepsen *et al.*, 1986b; Sherwood & Virella, 1986). Under optimal conditions, more than 50% of the complexes added were measured to be RBC-bound in the first minutes of incubation, after which a slow release was observed (Jepsen *et al.*, 1986a). Binding occurs through the interaction of C3b and/or C4b covalently bound to the complement-reacted IC on the one hand and CR1 on RBC on the other, whereas release is due to the cleavage of C3b as a result of the joint effect of CR1 and factor I (Jepsen *et al.*, 1986a).

The essential difference between the experiments of these authors and our present study was in the order of the addition of the reactants. While authors who observed an effective binding of IC to RBC used preformed immune complexes, we added antibody to the mixture of the antigen and serum. In the first case, IC were formed and precipitated in buffer and solubilized in the serum later on. By contrast, in the second case they were formed in the presence of complement. As a control, we also made experiments with preformed immune complexes and found binding to RBC to an extent comparable to that observed by other authors.

There could be three explanations for the difference between the behaviour of the preformed and nascent IC:

(i) No complement activation occurs and C3b does not bind to IC when their formation takes place in serum. We know, however, from the experiments of Schifferli, Bartolotti & Peters (1980) that this is not the case. Moreover, we also measured a marked complement consumption in sera in which IC formation occurred.

(ii) There could be significant differences in the kinetics of C3b binding and release between preformed IC incubated in

serum and IC formed in the presence of serum. If the cleavage of C3b leading to release of C3b-IC is faster in the latter case than in the former one, it is possible that most C3b-IC molecules release from the RBC in a very short period of time and that is why we did not find RBC-bound radioactivity at the first measurement performed 1 min after the beginning of the experiment. No observations, however, have been published indicating such a great difference in the cleavage rate of C3b bound to preformed or nascent IC. Experiments aiming to study this possibility are in progress in our laboratory.

(iii) According to the third and most probable explanation, the difference in the RBC-binding capacity of the nascent and preformed IC is due to a difference in their structure, e.g. density of accessibility of C3b molecules built in the lattice of the IC. Data on the comparison of the structure of the two types of complexes are scarce. According to the experiments of Schifferli *et al.* (1980), the size-spectrum of the preformed IC solubilized in complement and of those formed in the presence of complement is similar. By contrast, there is a significant difference in the molar ratio of C3b to IgG between these two types of complexes: it was found to be 2–5 in the case of IC formed in complement, that is approximately half that observed for IC which have been solubilized (Webb & Whaley, 1986). Previously, we have also found (Anh-Tuan *et al.*, 1984) marked differences in the precipitability and other properties between these two types of complexes. Moreover, it has been shown previously that inhibition of immune precipitation depends entirely upon the classical pathway (Naama *et al.*, 1984), whereas solubilization of preformed immune precipitates has an absolute requirement for the alternative pathway, but is the most efficient in the presence of an intact classical pathway (Takahashi, Tack & Nussenzweig, 1977; Takahashi *et al.*, 1978; Naama *et al.*, 1985). Clearly, more comparative studies are needed before this problem can be clarified definitely.

Irrespectively, however, of the explanation of the observed difference, our findings do not support the essential role of the erythrocytes in the elimination of circulating immune complexes. Since the formation of immune complexes in the blood takes place in the presence of complement (Schifferli *et al.*, 1980), it is probable that nascent IC similar to the BSA–anti-BSA IC used in our present study cannot bind effectively to RBC, and their elimination occurs directly by binding to CR1 and CR3 of the mononuclear phagocytic cells in the liver and other tissues. This assumption is in accordance with the results of Cornacoff *et al.* (1983) who, in contrast to the efficient RBC-binding of preformed IC infused to primates, did not find binding to erythrocytes if IC were formed in the circulation of animals. It cannot be excluded, however, that the RBC have an important role in the elimination of other types of IC, e.g. those of higher size or formed *in situ* within the tissues and solubilized by complement later on (Whaley, 1987).

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