

Complement mediated inhibition of immune precipitation and solubilization generate different concentrations of complement anaphylatoxins (C4a, C3a, C5a)

J. A. SCHIFFERLI, GERTRAUD STEIGER & J.-P. PACCAUD *Clinique Médicale, Hôpital Cantonal Universitaire, Geneva, Switzerland*

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SUMMARY

Complement prevents the formation of insoluble immune complexes (inhibition of immune precipitation (IIP)), and solubilizes preformed immune aggregates (solubilization (SOL)). Since the mechanism of complement activation differs in these two reactions, it is possible that they differ also in the amount of complement fragments released, in particular the anaphylatoxins C3a, C5a and C4a. We measured C4 and C3 consumption, and the formation of complement anaphylatoxins during IIP and SOL using two different immune complex models (BSA, rabbit anti-BSA; tetanus toxoid (TT), human anti-TT). At equal immune complex concentrations in both models, SOL was more efficient than IIP at cleaving C3, and more C3a and C5a was released. Comparing the two reactions, C3a formation was followed by more C5 cleavage (C5a) during SOL. Similarly C4a formation (classical pathway activation) was followed by more C3 cleavage (C3a: classical and alternative pathway activations), during SOL. It is suggested that *in vivo* SOL of insoluble complexes is rapidly accompanied by a damaging phlogistic reaction, whereas IIP produces less inflammation.

Keywords complement activation complement anaphylatoxins immune complexes

INTRODUCTION

Complement brings about the formation of soluble immune complexes (IC) either by inhibiting immune precipitation at the time of the antigen-antibody reaction (IIP) or by solubilizing preformed immune aggregates (SOL) (Schifferli, Bartolotti & Peters, 1980; Miller & Nussenzweig, 1975). Whereas SOL is dependent on activation of the alternative pathway of complement, IIP requires the classical pathway (Takahashi *et al.*, 1978; Schifferli, Woo & Peters, 1982). However, in both reactions the formation of stable soluble IC is due to the covalent binding of C3 fragments (Czop & Nussenzweig, 1976; Takata, Tamura & Fujita, 1984; Hong *et al.*, 1984). A C5 convertase is formed on IC during the solubilization process, however, neither C5 activation nor assembly of the membrane attack complex are necessary for SOL and IIP (Takahashi *et al.*, 1978; Schifferli *et al.*, 1985a). Little is known about the comparative potency of these two reactions to activate complement and to release the various fragments which induce inflammation. Indeed if the physiological role of SOL and IIP is to produce soluble complexes capable of diffusing away from their site of tissue formation, then these complement functions should not produce excessive local inflammation (Schifferli & Peters, 1983).

Correspondence: Dr J. A. Schifferli, Clinique Médicale, Hôpital Cantonal Universitaire, 1211 Geneva 4, Switzerland.

In this study we compared complement activation in human serum at the time of IIP and SOL by BSA-rabbit anti-BSA IgG complexes. We then performed similar studies using an IC model made with IgG of human origin, i.e. tetanus toxoid (TT)-human anti-TT IgG complexes. Complement activation was assessed by haemolytic C4 consumption, C3 conversion and release of the complement anaphylatoxins C4a, C3a, and C5a (Gorski, Hugli & Müller-Eberhard, 1979; Hugli & Chenoweth, 1980).

MATERIALS AND METHODS

Immune complex models. (Bovine serum albumin (BSA, Armour Ltd.) was radiolabelled with ^{125}I by the chloramine T method (McConahey & Dixon, 1966). Rabbit anti-BSA antibodies (IgG fraction) were prepared as previously described (Schifferli *et al.*, 1985a). Immune complexes used in the different assays were formed at equivalence, i.e. 1 mol. BSA for 5 mol antibody (Schifferli, Steiger & Schapira, 1985c).

Partially purified (TT) was a gift from Dr E. Furer (Institut Sérothérapique et Vaccinal Suisse, Berne), and was further purified by gel filtration on Sepharcyl S 300. TT had an apparent mol wt of 150 kD by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (Laemmler, 1970), and no contaminant was detected. TT was radiolabelled with ^{125}I using *N*-chloro-benzenesulphonamide (sodium salt) derivatized uniform non-porous polystyrene beads (Iodo-Beads, Pierce) according to the manufacturer's notice. Human anti-TT immunoglobulins containing more than 95% IgG were obtained from Berna (Tetuman), dialysed against veronal buffered saline containing 0.15 mM Ca^{2+} and 0.5 mM MgCl_2 , pH 7.2, conductance 14 mmho/s (VBS), and aggregates were removed by ultracentrifugation at 120,000 *g* for 2 h. The equivalence point was determined (Hudson & Hay, 1980) and immune complexes used in the assays were formed at equivalence, i.e. 1 mol TT for 18 mol antibody.

Inhibition of immune precipitation (IIP) and solubilization (SOL) assays. These two assays have been described for BSA-anti-BSA antibody complexes (Schifferli *et al.*, 1985a). The assays were identical for TT-anti-TT antibody complexes. In brief, insoluble IC (SOL assay), or antigen followed by antibody (IIP assay) were added to 100 μl of normal human serum preincubated at 37°C for 5 min (final volume 150 μl with VBS). After an incubation at 37°C for 60 min the reactions were stopped by 1 ml of cold buffer (phosphate buffered saline (PBS) Oxoid). The percentage soluble complexes was determined after centrifugation at 3,000 *g* for 15 min at 4°C, by counting pellets and supernatants.

Normal human sera (NHS). NHS was collected from healthy controls; the NHS used in the TT-anti-TT assays was obtained from a control who had not been immunized with TT: ^{125}I -TT (3 ng) mixed with serum (50 μl) did not produce precipitating or soluble IC as determined by precipitation at 3,000 *g* and by sucrose density gradient ultracentrifugation (80,000 *g* for 14.5 h at 4°C).

Complement measurements. Antigenic C3, C4, and C5 concentrations were measured by single radial immunodiffusion using monospecific antisera (Miles) (Mancini, Carbonara & Heremans, 1965). The results were expressed in $\mu\text{g/ml}$ using a control plasma as standard (Hyland, Travenol). C3 conversion was determined by crossed immunoelectrophoresis (Laurell, 1966). Haemolytic C4 titre was calculated according to Cooper & Müller-Eberhard (1968) using C4 deficient guinea-pig serum (Gaither, Alling & Frank, 1974). The formation of C4a, C3a, and C5a was measured with radioimmunoassay kits provided by Upjohn (Kalamazoo) (Gorski, 1981; Hugli & Chenoweth, 1980). From the concentrations of C4, C3 and C5 in the NHS used, it was possible to estimate the maximum formation of their respective fragments; in the serum incubated with BSA-anti-BSA complexes, maximum C4a: 10.8 $\mu\text{g/ml}$, C3a: 36.8 $\mu\text{g/ml}$; C5a: 4.29 $\mu\text{g/ml}$; in the serum incubated with TT-anti-TT complexes: maximum C4a, 11.3 $\mu\text{g/ml}$; C3a, 43.7 $\mu\text{g/ml}$; C5a, 5.37 $\mu\text{g/ml}$.

Complement consumption in NHS. To minimize the formation of complement fragments in NHS, blood was allowed to clot for 15 min at 22°C, and was centrifuged at 4,000 *g* for 15 min at 4°C. The sera were kept at 4°C until used within the next 30 min. The SOL and IIP assays were performed with radiolabelled antigens in the initial experiments when C4 titre and C3 conversion were determined after 15 and 60 min. Unlabelled antigens were used when complement anaphylatoxins

were measured. In all experiments further complement activation was blocked by adding 10 mM EDTA and cooling the samples to 4°C before performing the complement assays. The experiments comparing SOL and IIP in a given serum were done in duplicate and twice. For each assay, the variation in the duplicate experiment was never more than 10%. However, there was a day-to-day variation of basal C3 conversion, C4 haemolytic activity and C4a, C3a and C5a measurements in the freshly collected sera. Thus the experiments which were done twice provided always similar but not identical results. Only one set of each experiment is shown in Results. Controls included NHS incubated without IC, with antigen or with antibody at concentrations corresponding to the highest IC concentration used.

RESULTS

The capacity of normal human serum to mediate IIP and SOL was determined for BSA-anti-BSA and TT-anti-TT complexes at various IC concentrations (Table 1). After an incubation period of 60 min, IIP was preserved at all concentrations tested for both IC models; in contrast SOL was concentration dependent, being nearly absent at the highest IC concentrations. Thus, complement activation was due to soluble complexes during IIP and to insoluble complexes during SOL, at least during the initial part of the SOL reaction.

To determine the consumption of C4 and C3 by similar concentrations of IC during IIP and SOL, haemolytic C4 and C3 conversion were measured after an incubation time of 15 and 60 min.

BSA-anti-BSA complexes produced a dose-dependent decrease in haemolytic C4 and increase in C3 conversion after 15 min (Fig. 1a, b). However, when comparing the two reactions, IIP (open circles) led to more C4 consumption than SOL (solid circles), whereas SOL led to more C3 conversion at the lower IC concentrations tested. Results at 60 min were similar with little additional drop in haemolytic C4 (not shown) and comparable differences in C3 consumption between IIP and SOL (Fig. 1b). In the human IC model, i.e. TT-anti-TT complexes, SOL produced

Table 1. Solubilization (SOL) and inhibition of immune precipitation (IIP) of BSA-anti-BSA and TT-anti-TT complexes.

| Immune complex concentration ($\mu\text{g/ml}$ serum)* | SOL† (% soluble complexes) | IIP† (% immune complexes) |
|---|----------------------------|---------------------------|
| BSA-anti-BSA | | |
| 6, 7 | 90 | 96 |
| 20 | 68 | 95 |
| 60 | 17 | 95 |
| 180 | 9 | 95 |
| TT-anti-TT | | |
| 5 | 90 | 95 |
| 10 | 83 | 94 |
| 20 | 55 | 95 |
| 40 | 40 | 96 |
| 80 | 10 | 96 |

* $\mu\text{g/ml}$ of antigen + antibody.

† Incubation for 60 min at 37°C.

Controls: SOL in EDTA (10mM)-serum: BSA-anti-BSA, < 7%; TT-anti-TT, < 5%; IIP in EDTA-serum: BSA-Anti-BSA, < 10%; TT-anti-TT, < 43%.

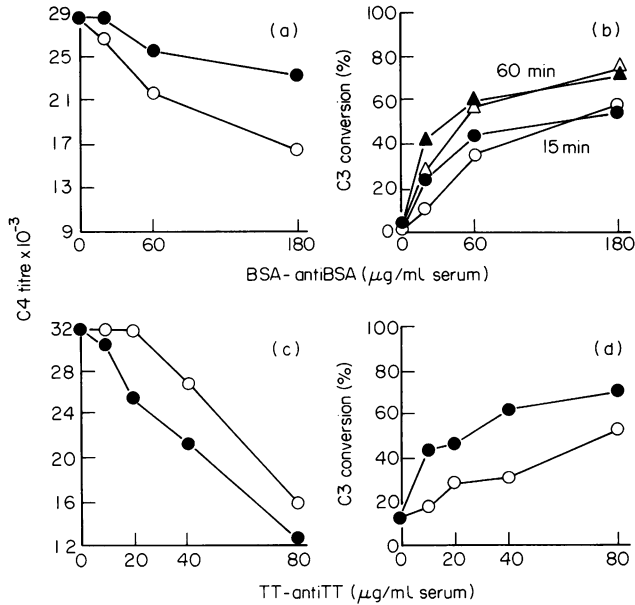


Fig. 1. Consumption of haemolytic C4 and C3 conversion by SOL and IIP of BSA-anti-BSA and TT-anti-TT complexes after 15 min. (a-c): C4 consumption; (b-d): C3 conversion. (a-b): BSA-anti-BSA complexes; (c-d): TT-anti-TT complexes. SOL (●) (▲ after 60 min); IIP: (○) (△ after 60 min).

more C4 and C3 activation than IIP at all concentrations tested at 15 min (Fig. 1c, d) and at 60 min (not shown). Thus the comparative C4 consumption by IIP and SOL differed between the two IC models, however, in both SOL produced more C3 conversion.

To determine the release of complement anaphylatoxins at these and at lower IC concentrations, the experiments were repeated in a similar way and C4a, C3a and C5a measured. In both IC models the dose-dependent increase in C4a and C3a release corresponded to the drop in haemolytic C4 and to C3 conversion (Fig. 2 a, b, d, e): more C4a was formed by IIP than by SOL of BSA-anti-BSA complexes, whereas the reverse was true for TT-anti-TT complexes; in both IC models more C3a was formed by SOL than by IIP. Of particular interest was the cleavage of C5: there was more C5a formed by SOL than by IIP in both IC models and at all IC concentrations tested (Fig. 2c, f). In particular in the TT-anti-TT complex model IIP produced little C5a release.

The results provided additional information. When the formation of C4a was correlated with C3a, it was evident that SOL produced more C3 cleavage than IIP for any given level of C4a formation, suggesting more efficient alternative pathway activation by SOL (Fig. 3a, c). In this respect both IC models produced similar results. A similar observation was made for C5 cleavage; SOL produced more C5a formation than IIP for any given level of C3a formation. These differences were only marginal using BSA-anti-BSA complexes, however, they were evident in the TT-anti-TT complex model (Fig. 3b, d).

In the control experiments, both antigens (BSA, TT) only or antibodies only did not lead to complement activation (haemolytic C4, C3 conversion) or to significant release of anaphylatoxins, when compared to serum incubated alone.

DISCUSSION

The two major points emerging from this study were: (1) IIP and SOL differed in their capacity to consume complement, in particular they brought about the release of different concentrations of the complement anaphylatoxins; (2) SOL caused the release of more C3a and C5a than IIP at

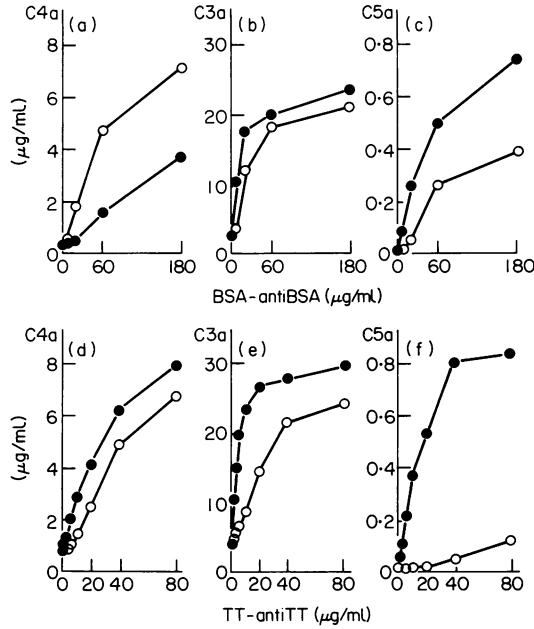


Fig. 2. Formation of C4a, C3a, and C5a by SOL and IIP of BSA-anti-BSA and TT-anti-TT complexes after 15 min. (a-d): C4a; (b-e): C3a; (c-f): C5a. (a-b-c): BSA-anti-BSA complexes; (d-e-f): TT-anti-TT complexes. SOL: (●); IIP (○). In both immune complex models and at all concentrations tested, SOL produced more C3a and C5a.

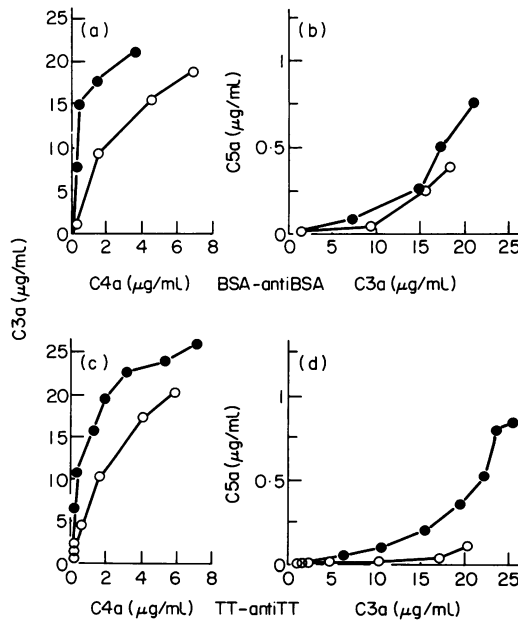


Fig. 3. Correlations between C4a and C3a formation (a-c), and C3a and C5a formation (b-d), for SOL and IIP, in both immune complex models. (a-b): BSA-anti-BSA complexes; (c-d): TT-anti-TT complexes. SOL: (●); IIP: (○). In both immune complex models IIP led to less C3a formation for any given C4a formation, and led to less C5a formation for any given C3a formation.

equivalent IC concentrations in both IC models. Thus it is suggested that SOL is likely to be a reaction producing more inflammation than IIP *in vivo*. These and related points merit attention.

IIP was complete in the two IC models at all IC concentrations tested so that the reaction of the antigen with its corresponding antibody never produced any insoluble aggregates. In contrast SOL did not produce complete fragmentation of the insoluble aggregates provided and SOL was only just detectable at the highest IC concentrations used. Thus in the different experiments, we compared in fact that same type of complex either in its soluble state or in its insolubilized form. That complement is capable of forming more soluble IC by IIP than by SOL has already been demonstrated (Schifferli, Bartolotti & Peters, 1980); this suggested that SOL is a process consuming more complement. Indeed at equal IC concentrations more C3 cleavage was produced by SOL than by IIP in both IC models (except for high concentrations of BSA-anti-BSA complexes). The mechanism of complement activation by IC has been studied extensively (see review: Theofilopoulos & Dixon, 1979), and the relative role of classical versus alternative pathway varies from one model to another depending not only on the antigen-antibody system studied but also on the solubility of the complexes as shown here. When, as in our models, the IC (soluble and insoluble) activate the classical pathway the initial C3 cleavage by the classical pathway C3 convertase is amplified several fold by alternative pathway activation (Medicus, 1978). However, many insoluble immune or immunoglobulin aggregates activate the alternative pathway directly, thus producing extensive C3 cleavage, without activating the classical pathway (Gadd & Reid, 1981). Indeed SOL is alternative pathway dependent and requires no classical pathway function (Takahashi *et al.*, 1978). In contrast IIP relies on classical pathway function which produces C3 binding to complexes, and does not need alternative pathway activation (Schifferli, Woo & Peters, 1982). These differences are highlighted in our studies by the comparative C4a (classical pathway activation) and C3a (mostly alternative pathway activation (Medicus, 1978)) production by soluble versus insoluble complexes. Indeed for a given level of classical pathway activation, soluble IC (IIP) produced less alternative pathway activation than insoluble complexes (SOL).

In addition insoluble complexes led to the formation of more C5a than soluble complexes. When C5 activation (C5a formation) was correlated with C3 cleavage (C3a formation), it became apparent that insoluble complexes produced more efficiency C5 activation as well, and this particularly so in the human IC model. This difference could be due to many mechanisms—differences in C3b binding and/or inactivation, in C5 convertases formation and/or inactivation. Whatever the reason for this observation, the more phlogistic potential of insoluble complexes is of biological interest.

Complement activation was not identical in the two IC models used. For instance studies on C4 cleavage provided opposite results. This could be considered as being of minor biological importance since C4a is not a major anaphylatoxin (Gorski, Hügli & Müller-Eberhard, 1979); however, such differences suggest as well that interactions between human complement and IC should be studied preferably with IC models of human origin. Furthermore, other human IC models should be investigated since the type of antigen and class and subclass of antibody determine complement activation as well.

The differences of complement activations observed, when comparing soluble (IIP) versus insoluble (SOL) complexes, could well be of biological significance. The release of different complement fragments increases the inflammatory reaction caused by IC, whether such complexes have formed *in situ* or have been deposited from the blood stream. The complement anaphylatoxins C3a and C5a produce pronounced microvascular alterations (Björk Hügli & Smedegard, 1985) and histamine release from basophils (Hartman & Glovsky, 1981; Kings, Nydegger & De Weck, 1984). In addition C5a induces significant chemotactic responses from monocytes and neutrophils (Hügli & Chenoweth, 1980). Thus in tissues, SOL induced by the formation or deposition of insoluble complexes could produce more inflammation than IIP of antigen-antibody complexes which have just formed. Indeed it has been known for a long time that the Arthus reaction depended on the presence of *precipitating* antibodies (Cochrane & Janoff, 1974). Furthermore alternative pathway activation by immune deposits accompanied by a severe inflammatory reaction has been observed in different human tissues (Uff, Evans & Bartolotti, 1979; Schifferli *et al.*, 1985b). Since soluble complexes can diffuse away from their site of formation it is evident that IIP in itself prevents the

localization of the inflammatory reaction to some extent (Schifferli & Peters, 1983); under such circumstances the attenuated formation of various proinflammatory complement fragments may well help the diffusion of IC into the blood stream from which they can reach the fixed macrophage system.

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