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Complement-mediated inhibition of immune precipitation. I. Role of the classical and alternative pathways

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SUMMARY

This study has examined the mechanisms involved in complement-mediated inhibition of immune precipitation using radiolabelled BSA and rabbit anti-BSA. Purified proenzyme C1 was capable of maintaining the complexes in soluble form during the first few minutes of reaction whereas immune precipitation was immediate in the presence of purified C1q or C1 + EDTA (ethylenediamine tetra-acetate). In C1q deficient serum, initial immune aggregation was followed by partial solubilization of the formed precipitate similar to that obtained with normal human serum in the presence of Mg EGTA. In C2 deficient serum precipitation occurred at a slow rate. Repletion of the deficient component (C1q or C2 respectively) restored fully inhibition of precipitation. These experiments establish a critical role for the classical pathway, in this phenomenon. By contrast the role of the alternative pathway in maintaining complexes in solution was less important: only partial and delayed precipitation occurred in sera depleted of factor D (RD) or factor B (RB). B and D restored normal complement activity to depleted sera. No precipitation was detected in a reagent depleted of properdin (RP). The mechanisms of inhibition of precipitation are therefore distinct from those responsible for solubilization of an immune precipitate, which is largely dependent on the alternative pathway.

INTRODUCTION

We have shown that complement inhibits immune precipitation of bovine serum albumin (BSA) rabbit anti-BSA complexes in normal human serum at 37°C (Schifferli, Bartolotti & Peters, 1980). Our experiments showed that initial inhibition was mediated by the classical pathway of complement but suggested that at later stages activation of the alternative pathway contributed to this process. C4 and C3 fragments are bound to the soluble complexes and they probably interfere with lattice formation. A comparable phenomenon is complement-mediated solubilization of a preformed immune precipitate (Miller & Nussenzweig, 1975) where massive C3 deposition is thought to break the lattice (Takahashi, Tack & Nussenzweig, 1977). The mechanism of complement activation causing solubilization is now understood (Czop & Nussenzweig, 1976; Takahashi *et al.*, 1977; Takahashi *et al.*, 1978). Whereas all the alternative pathway components (B, D, P, C3) are required for solubilization, the classical pathway accelerates the initiation of alternative pathway activity and enhances the amount of soluble complexes formed, but is itself unable to effect solubilization. The purpose of this paper is to establish more precisely the requirements for inhibition of precipitation; we show that there are striking differences in complement mechanisms of inhibition of precipitation and solubilization of a preformed precipitate.

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MATERIALS AND METHODS

Assay for inhibition of immune precipitation. The assay system was based on the inhibition of precipitation of chloramine T-I^{125}-radiolabelled BSA (McConahey & Dixon, 1966) as previously reported (Schifferli *et al.*, 1980) with the modification that the IgG fraction of a serum of a rabbit hyperimmunized against BSA was used. This was obtained by sodium sulphate salt precipitation (Heide & Scwick, 1978), dialysed against PBS; aggregates were removed by centrifugation at 40,000 ***g*** for 2 hr. This IgG contained 5 mg/ml of anti-BSA Ab. Except where specified, 1 μg of ^{125}I-BSA and appropriate reagents were preincubated at 37°C with 100 μl of serum in a final volume of 150 μl. After 5 min an equivalent amount of rabbit anti-BSA Ab was added. Aliquots of serum were removed after various intervals, diluted in cold PBS and the supernatant and the pellets were separated by centrifugation at 3,000 ***g*** for 10 min at 4°C

Solubilization assay. The same BSA and rabbit anti-BSA were used to form an immune precipitate at equivalence. One microgramme of aggregate was added to 50 μl of serum at 37°C. All reaction mixtures had a final dilution of 1 : 2 of the original serum. The percentage of solubilization was measured as reported by Miller & Nussenweig (1975). Both assays were also performed in the presence of Mg EGTA (2 mM Mg^{++}, 10 mM EGTA (ethyleneglycol tetra-acetate) to block classical pathway activity.

Sera deficient or depleted of complement components

C1q deficient serum. This was obtained from a patient belonging to a family with homozygous C1q deficiency (Thompson *et al.*, 1980). Antigenically abnormal C1q was present in this serum, but had no functional activity.

C2 def serum. This was obtained from a patient with total C2 deficiency but without any other abnormality of complement components.

Factor B depleted sera. (i) Fresh human serum was heat-inactivated at 50°C for 20 min. (ii) Immunochemical depletion of factor B (RB) was carried out using a monospecific F(ab')2 rabbit anti-human B according to the method of Lachmann (1971). At equivalence this antibody precipitated 95% of radiolabelled B added to EDTA human serum.

Factor D depleted serum. This was prepared by two cycles of Sephadex G-75 chromatography, excluding fractions coming after factor B (Lachmann & Hobart, 1978).

Properdin (P) depleted serum. This was obtained by incubation with Zymosan (50 mg/ml of serum) at 16°C for 60 min according to Todd, Pillemer & Lepow (1959). After centrifugation at 3,000 ***g*** for 60 min at 1°C no antigenic P was detected by single radial immunodiffusion using monospecific anti-properdin (Nordic Laboratories).

Purified complement components from normal human serum. (a) C1q was purified by the method of Zubler *et al.* (1976), modified by Pussell *et al.* (1978). (b) Proenzyme C1 was partially purified by euglobulin precipitation in the presence of calcium and 10 mM DFP followed by Sepharose 6B chromatography in the presence of calcium and 5 mM DFP (Gigli, Porter & Sim, 1976). The fractions containing C1 activity by haemolytic assay (Lachmann & Hobart, 1978) were pooled. (c) Factor B, D and C2 were purified from the supernatant of a globulin precipitation step (20 g anhydrous Na_2SO_4/100 ml serum, in the presence of EDTA). After a second step of ion exchange chromatography on CM cellulose C2 and B were further separated by DEAE cellulose chromatography and D by Sephadex G-75 gel filtration (Lachmann & Hobart, 1978). Only fractions containing C2 or B activity were pooled and concentrated to form the C2 and B preparations respectively; Factor B gave a single band on SDS polyacrylamide gel and the C2 contained trace amounts of contaminant of a molecular weight similar to albumin. The preparations were functionally pure in assays for B, D and C2. (d) Partially purified activated properdin ($\bar{P}$) was prepared according to Todd *et al.* (1959).

Table I summarizes the functional activity of the various deficient and depleted sera and the effect of addition of purified complement components on classical pathway (CH50) and alternative pathway activity by haemolytic plate assays (Lachmann & Hobart, 1978) and on the capacity to increase or restore solubilization.

B conversion. B conversion was measured by immunoelectrophoresis using a monospecific rabbit anti-human factor B.

RESULTS

C1 and C1q deficient serum

Whole C1, in a Ca^{++} containing buffer, (CFD, complement fixing diluent, Oxoid) was able to delay precipitation of the BSA rabbit anti-BSA complexes in the absence of other complement components (Fig. 1). When sufficient EDTA to deplete Ca^{++} was added immediate precipitation occurred as in the absence of complement.

Table 1. Functional activity of the various complement deficient and depleted sera and the effect of addition of purified components.

Sera	CH50*	Alternative pathway* function	Solubilization* after 60 min
C1q def.	0	80	75
+C1q†	80	n.d.	63
C2 def.	0	120	51
+C2	100	n.d.	115
RD	80	0	0
+D	n.d.	75	80‡
RB (immunochemical)	70	0	0
+B	n.d.	70	70
Rp	70	90	0
P	n.d.	85	65

n.d. = not done.

* Results in % of pooled normal human serum for C1q and C2 deficient sera, in % of initial serum for RD, RB and RP. (−background solubilization).

† The purified components were added in the minimum concentration that will allow maximum restoration of complement function.

‡ Solubilization tested after 90 min because of a delayed start in the reaction.

In C1q deficient serum initial fast precipitation was followed by solubilization of the already formed precipitate as occurs with normal human serum treated with Mg EGTA (Fig. 2a). The addition of Mg EGTA to the C1q deficient serum did not change the reaction. The addition of purified C1q restored the normal, total inhibition of precipitation. These experiments established that purified C1q is needed for inhibition of precipitation in this system. However, solubilization of a preformed immune precipitate was not enhanced by the addition of C1q to the deficient serum (Fig. 2b).

C2 deficient serum

This serum handled immune precipitation differently from the C1q deficient serum (Fig. 3a). No immediate fast precipitation was seen but slow aggregation of immune complexes proceeded for about 60 min (as in the presence of C1 only), when further precipitation was blocked. To restore full inhibition of precipitation only 50% of the concentration of C2 necessary for the restoration of total haemolytic activity was needed. Similarly solubilization was greatly accelerated and enhanced in the

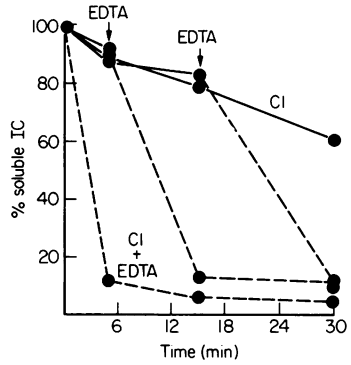

Fig. 1. Effect of C1 on immune precipitation. One microgramme of ^{125}I-BSA was preincubated in 100 μl of CFD for 5 min at 37°C with purified C1 at a concentration equivalent to normal serum. Anti-BSA Ab was added at time 0. The percentage of soluble immune complexes (IC) remaining in the supernatant was calculated after centrifugation of aliquots of the mixture at various times (see Materials and Methods). Note the very delayed precipitation in the presence of C1 and the effect of EDTA added at various times causing immediate precipitation.

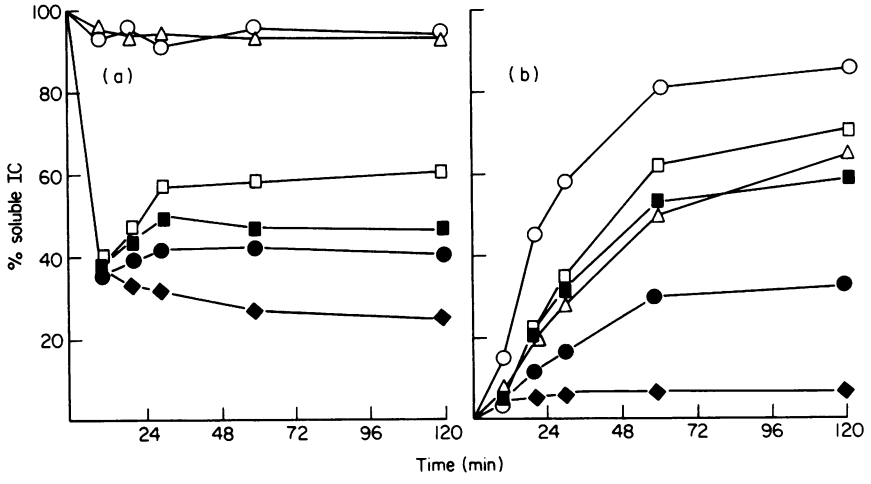

Fig. 2a. Immune precipitation in C1q deficient serum. Note the control precipitation curves: no precipitation in normal human serum (NHS), precipitation followed by partial solubilization in the presence of Mg EGTA which blocks classical pathway activity (NHS + Mg EGTA), normal precipitation in the presence of EDTA. Note the similarity between the kinetics of precipitation and solubilisation in C1q deficient (C1q def), in C1q def with Mg EGTA and NHS Mg EGTA. Normal inhibition of precipitation is restored by the addition of C1q. (In this experiment all reagents were diluted 1/2 accounting for the diminished precipitation in EDTA). (○——○ = NHS; ●——● = NHS + Mg EGTA; ♦—— ♦ = NHS + EDTA; □——□ = C1qdef; ■——■ = C1qdef + Mg EGTA; △——△ = C1qdef + C1q)

Fig. 2b. Solubilization by C1q deficient serum. One microgramme of ^{125}I BSA-anti BSA Ab precipitate was incubated at 37°C with 50 μl of serum. Aliquots of the mixtures were removed at various times and the percentage of soluble complexes measured as described in Methods. Note the control solubilization curves: fast solubilization which started after 10 min already in normal human serum (NHS), partial and slightly delayed solubilization in Mg EGTA NHS and no solubilization in EDTA (background). Note also that the efficient solubilization of the C1q deficient serum was not significantly altered by the presence of Mg EGTA or purified C1q in optimal concentration. Note also that in the presence of Mg EGTA C1q deficient serum solubilized complexes more efficiently than normal human serum. (Symbols same as for Fig. 2a).

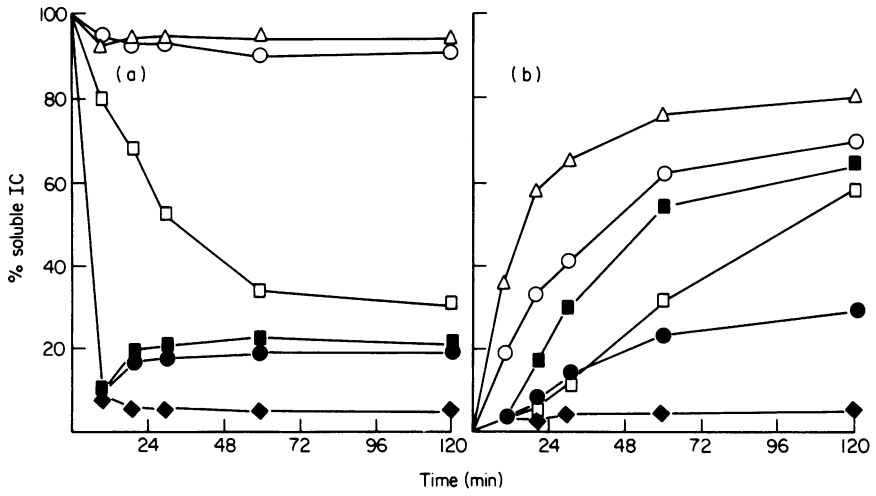

Fig. 3a. Immune precipitation in C2 deficient serum. Note the slow and incomplete precipitation in C2 deficient, restored by C2. Note also the difference between C2 deficient and C2 deficient + Mg EGTA. (o——o = NHS; ●——● = NHS + Mg EGTA; ♦—— ♦ = NHS + EDTA; □——□ = C2 def; ■——■ = C2 def + Mg EGTA; △——△ = C2 def + C2).

Fig. 3b. Solubilization by C2 deficient serum. Note the delayed solubilization in C2 deficient serum restored by C2. Note also that C2 deficient serum solubilized complexes more efficiently in the presence of Mg EGTA. (Symbols same as for Fig. 3a).

presence of C2 (Fig. 3b). When C1 function was blocked by Mg EGTA the C2 deficient serum solubilized complexes faster than in the presence of calcium (Fig. 3b). This raises the possibility that C1 and C4 may be partially inhibiting the process of solubilization.

Immune precipitation in B depleted serum (RB)
In the B depleted serum there was only a small difference from normal human serum, though after 120 min precipitation was becoming apparent. That this difference was significant, was confirmed by restoration of inhibition to full normal capacity by repleting the reagent with purified factor B (Fig. 4). Similar results were obtained using an RB prepared by heat-inactivation (50°C, 15 min) in which C2 was restored to normal by purified C2.

Immune precipitation in D depleted serum (RD)
The findings are essentially similar to RB. During the first 60 min of the reactions in the absence of D total inhibition of precipitation was preserved. However later in the reaction a small amount of aggregation occurred which could be reversed by the addition of the purified factor (Fig. 5).

Immune precipitation in $\bar{P}$ depleted serum (RP)
Depletion of Properdin had no effect on the capacity of serum to inhibit precipitation even after 180 min, nor did the addition of $\bar{P}$ have any effect (not shown).

Effect of depletion of calcium using Mg EGTA on various reagents (Fig. 4 & 5)
In the presence of Mg EGTA after a period of fast aggregation, the immune complexes were solubilized by activation of the alternative pathway and required factor B (Fig 4), factor D (Fig. 5), and properdin (not shown) as with solubilization of a preformed immune precipitate (see Materials and Methods). Repletion of RD, however, led to a reagent in which the solubilization was quantitatively normal, though this occurred at a reduced rate (Fig. 5).

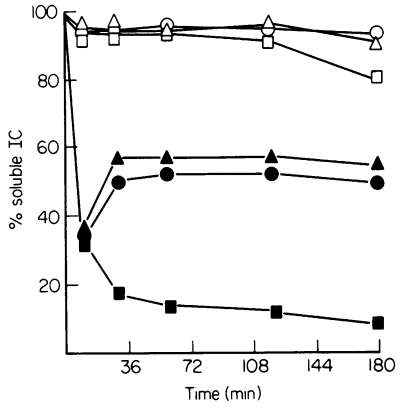

Fig. 4. Immune precipitation in B depleted serum (RB). Precipitation is reduced and greatly delayed. In the presence of Mg EGTA the second phase of solubilization was absent (RB+Mg EGTA). In this experiment all reagents were diluted 1/2. (o——o = NHS; Δ——Δ = RB+B; □——□ = RB; ▲——▲ = RB+B+Mg EGTA; ●——● = NHS+Mg EGTA; ■——■ = RB+MG EGTA).

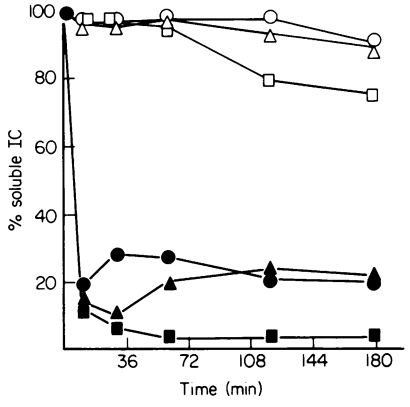

Fig. 5. Immune precipitation in D depleted serum (RD). Note similar findings to B depleted serum: delayed and partial precipitation blocked by the addition of purified Factor D (RD+D) and failure of the solubilization phase in RD+Mg EGTA. (o——o = NHS; Δ——Δ = RD+D; □——□ = RD; ▲——▲ = RD+D+Mg EGTA; ●——● = NHS+Mg EGTA; ■——■ = RD+Mg EGTA).

B conversion during inhibition of precipitation

In NHS or Mg EGTA NHS B conversion was apparent when measured after 30 min of incubation. In the RD reagent no B conversion was found even after 120 min. When purified D was added B conversion occurred but was delayed (negative at 30 min, positive at 120 min)

DISCUSSION

Our experiments have shown that the inhibition of precipitation of immune complexes, a property of fresh normal serum, is largely a function of the classical complement pathway. The alternative pathway becomes involved by solubilizing immune precipitates. This distinction was evident in the various deficient and depleted sera used in our experiments. The process of inhibition of precipitation and solubilization are therefore different. This distinction may have considerable implications for the unravelling of disease mechanisms associated with complement deficiencies.

It is worth discussing in more detail the relative contribution of the various degrees of activation of the two complement pathways in blocking immune precipitation and causing solubilization.

Purified C1 was able to block the initial aggregation of immune complexes. This aggregation is known to be mediated mainly by interactions between Fc portions of immunoglobulins (Möller, 1979; Möller & Stengaard, 1979; Rodwell, Lia-Hengtang & Schumaker, 1980). It is therefore probable that C1 interferes with these Fc-Fc interactions by steric hindrance as the C1 molecule is bound to Fc. When calcium was removed, the dissociation of the C1 molecule into its subcomponents led to immediate aggregation of the immune complexes, this is to be expected as only C1q remains fixed to the immunoglobulin and enhances interactions between complexes (Agnello, Winchester & Kunkel, 1970). These opposite functions of C1q and whole C1 have been found in other systems. C1q causes agglutination of IgG coated latex particles and enhances agglutination by rheumatoid factor whereas both reactions are abolished by purified whole C1 (Hällgren, Stalenheim & Venge, 1979; Hällgren, 1979). Kijlstra, van Es & Daha (1979) showed that C1 can dissociate guinea-pig IgG2 aggregates whereas C1q has an opposite effect. Wautier, Souchon & Reid (1978) reported interactions between platelets and immune complexes mediated by C1q but blocked by C1.

The slow aggregation in C2 deficient serum also pointed to the capacity of C1 and possibly C4 to delay interactions between complexes. However, C3b deposition by the classical pathway is needed for full inhibition of precipitation as indicated by the difference between the C2 deficient and normal serum.

It is well known that solubilization is greatly delayed in C2 deficient serum (Miller & Nussenzweig, 1975; Czop & Nussenzweig, 1976) and repletion of the deficient component restores this abnormality.

The findings in C1q deficient serum were less striking. Although the onset of solubilization was slightly delayed, the overall results fall within the normal range and repletion with C1q was without effect. It is worth pointing out that the inhibition of precipitation was greatly reduced in the same C1q preparation. A further finding of interest was that in the presence of Mg EGTA, solubilization was greater in the C1q deficient serum than in normal human serum. These results point to a role for C1q which tend to interfere with the process of solubilization, probably, as suggested by Rajnavölgy *et al.* (1978) by increasing cross-linking between immunoglobulin molecules. However, under physiological conditions this effect is not noticed because of the greatly enhanced efficiency of alternative pathway activity, resulting from the deposition of C3b on the immune precipitate by the activation of the classical pathway.

The difference observed between C2 deficient serum and C2 deficient serum in the presence of Mg EGTA, where solubilization was more efficient, also raises the possibility that the binding of C1 and C4 in some ways reduces the capacity of the immune precipitate to generate any alternative pathway convertase (that this difference might, alternatively, be accounted for on the basis of a Mg EGTA artifact would not explain the results in the C1q deficient serum where solubilization was slight reduced in the presence of Mg EGTA).

In experiments where longer periods of incubation were used a small but definite role for the alternative pathway was revealed. This was apparent in the reagents RB and RD, though the extent of precipitation was only 10–20% of the offered antigen. It is therefore not surprising that no differences could be detected in the RP since properdin plays a less central role in alternative pathway function (Müller-Eberhard & Schreiber, 1980). Again differences between solubilization and inhibition of precipitation have been found for properdin, B and D which have been shown to be essential for the former function of complement (Takahashi *et al.*, 1978).

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