Clin. exp. Immunol. (1982) **47**, 563–569.

Complement-mediated inhibition of immune precipitation. II. Analysis by sucrose density gradient ultracentrifugation

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(Accepted for publication 25 September 1981)

SUMMARY

The factors influencing the ultracentrifugation characteristics of immune complexes generated in the presence of fresh normal human serum have been analysed. In the absence of alternative pathway factors B, D or Properdin, the size of complexes was increased. When classical pathway function was blocked, in C1q deficient serum or in the presence of Mg EGTA, although the proportion of complexes remaining in solution were reduced their size was similar to those formed in normal human serum. In C2 deficient serum, a heterogeneous population of complexes was generated. In all instances repletion with the appropriate missing complement component reversed the abnormality. We conclude that there is normally a rapid sequential process of classical followed by alternative pathway activation leading to stable soluble complexes. In the absence of C1 activation the alternative pathway process requires precipitation of the antigen–antibody aggregates whereas in normal serum these events occur in the fluid phase. We suggest that in C2 deficient serum the C1 and/or C4 reacted complexes fail to activate the alternative pathway efficiently.

INTRODUCTION

The size of soluble BSA-rabbit anti-BSA immune complexes formed at equivalence in the presence of complement is approximately 25S when analysed by sucrose density-gradient ultracentrifugation (Schifferli, Bartolotti & Peters, 1980). This represents aggregates of Ab–Ag and complement fragments of more than 10^6 molecular weight. Takahashi, Takahashi & Hirose (1980) analysed the size of complexes released during solubilization of an immune precipitate (BSA-rabbit anti-BSA) by complement and reported that they form soluble complexes of heterogenous size starting from 11S to much larger (probably >25S) complexes. The pattern of solubilized complexes was not influenced by the Ab/Ag ratio of the precipitates, by re-exposure of solubilized complexes to fresh serum or by solubilization via alternative pathway activity only in the presence of Mg EGTA. Since the mechanisms of complement activation involved are different it was of interest to compare the physical characteristics of complexes held in solution by complement activation and complexes solubilized from immune precipitates by complement. In this study we present the results of analysis by sucrose density ultracentrifugation of the size of soluble complexes formed under various conditions during inhibition of immune precipitation.

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0009-9104/82/0300-0563$02.00

MATERIALS AND METHODS

Rabbit anti-BSA Ab, sera depleted of complement components (RB, RD, RP) and purified components (B, D, P, C1q, C1 and C2, were prepared as reported previously (Schifferli, Woo & Peters, 1982) and the same C1q and C2 deficient sera were used.

Sucrose density gradient ultracentrifugation. Radiolabelled BSA ^{125}I-rabbit anti-BSA Ab complexes were formed in the presence of sera as previously reported (Schifferli *et al.*, 1982), and from similar experiments 100 μl aliquots were removed and layered on sucrose density gradients (10/50% sucrose w/w) in CFD (complement fixing diluent, Oxoid). When not otherwise stated the experiments were performed at equivalence after 1 hr incubation. Ultracentrifugation was carried out at 80,000 ***g*** for 14 hr and 30 min at 4°C using an MSE prespin 65 ultracentrifuge. Forty-eight fractions were collected from each tube and radioactivity was measured. Pellets in the polystyrene ultracentrifuge tube were counted as well. Results were expressed as percentage of total counts per minute (c.p.m.) recovered from the fractions and pellet.

All mixtures had a final dilution of 1:1·5 of initial serum to avoid different patterns of ultracentrifugation related to dilution. The difference in size between two populations of immune complexes was deemed significant when their peaks were consistently separated by at least one fraction.

RESULTS

Role of Ab/Ag ratio

Ab and Ag were mixed at different ratios (fixed quantities of ^{125}I-BSA of 1 μg/100 μl of serum) in the presence of normal human serum (NHS) or EDTA (ethylene-diamine tetraacetate, 10 mM) serum as a control (Fig. 1).

The size of the soluble complexes generated by complement activation increased from Ag-excess complexes to Ab-excess complexes with minor changes in the total amount of precipitating

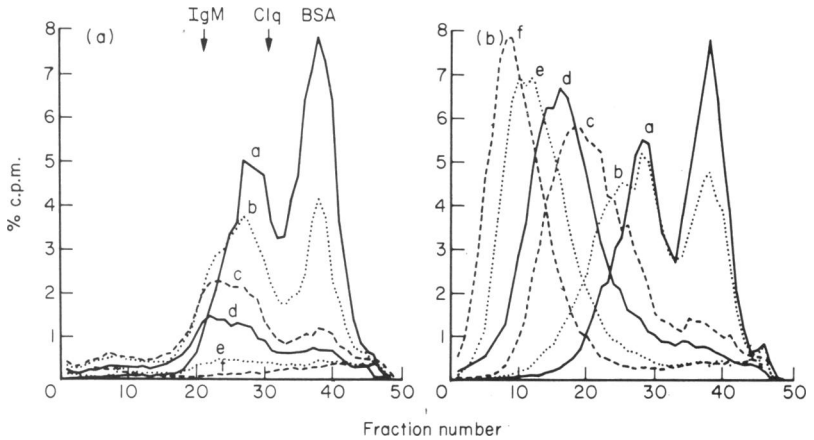

Fig. 1. Influence of Ab/Ag ratio on the size of soluble immune complexes formed during immune precipitation in serum. Ab/Ag ratio expressed in equivalent units, from Ag excess to Ab excess (a = 1/4, b = 1/2, c = 3/4, d = equivalence = 1, e = 3/2, f = 3). Markers: IgM = 19S, C1q = 11·5S, free BSA = 4·5S. (a) In the presence of EDTA free Ag and small soluble complexes present in Ag excess disappeared in Ab excess and a proportional increase in per cent counts per min (c.p.m.) was recovered in the pellet (not shown): from < 1% in Ab/Ag ratio = 1/4 to > 98% in Ab/Ag ratio = 3. (b) In normal human serum a new fraction of complement reacted soluble immune complexes was formed in Ab excess, at equivalence and in slight Ag excess (but not in 4 × Ag excess). Less than 8% of the total c.p.m. were found in the pellets at all ratios. The size of the new fraction of complexes was dependent on the Ab/Ag ratio—increasing from Ag excess to Ab excess.

complexes formed (< 8% of the count per min were recovered in these pellets). In the presence of Mg EGTA, (2 mM Mg^{++}, 10 mM EGTA), similar results were observed, but more complexes were recovered in the pellets in Ab excess (not shown). The complexes formed in the absence of complement were of two types: small soluble complexes of 11S and precipitating complexes found in the pellets. The small complexes were seen in Ag excess and the proportion of precipitating complexes increased from Ag to Ab excess (< 1% in four-fold Ag excess to > 98% in three-fold Ab excess).

Size of soluble complexes formed by alternative pathway activation only

In the presence of Mg EGTA initial precipitation was followed by solubilization of the complexes by alternative pathway activation. The proportion of soluble complexes generated was less than in normal serum, but their size was unchanged (Fig. 2). This was best demonstrated when the fraction of free Ag and small complexes formed in the absence of complement was taken off (Fig. 2b).

Analysis of complexes formed during the first 30 min of reaction

Analysis of complexes held in solution by complement (those generated in the first few minutes of the reaction between Ag and Ab in fresh normal serum) revealed two populations of complexes (Fig. 3). An insoluble fraction and a stable soluble fraction of 25S with little in between. Within 15

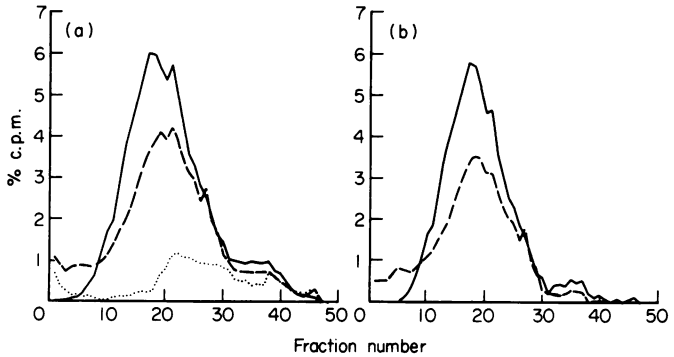

Fig. 2. (a + b) Size of immune complexes formed in NHS (———) or Mg EGTA (2 mM Mg^{++}, 10 mM EGTA) serum (– – – –) and EDTA serum (. . . . .) at equivalence. In NHS, 6% of the counts offered were recovered in the pellet compared with 25% in Mg EGTA and 77% in EDTA. Note only a small difference in size of soluble complexes generated in NHS and in Mg EGTA serum (a) which disappears when background counts, (EDTA serum) are subtracted (b).

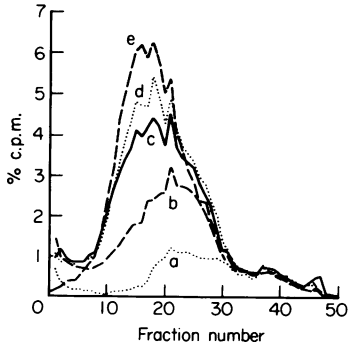

Fig. 3. Kinetics of formation of soluble complexes during the initial minutes of the reaction. (a = NHS-EDTA control; incubation time in NHS: b = 1 min, c = 3 min, d = 5 min, e = 15 min.) Pellets: a = 76%, b = 41%, c = 14%, d = 7%, e = 4%. Note the paucity of complexes intermediate in size between those in the pellet and those solubilized by complement.

min about 95% were in solution attesting to the very fast handling of complexes by complement. However, no change in the size of the soluble fraction was found, giving the impression of sudden jump from aggregated complexes to complement solubilized complexes of a well defined size.

Dilution of reactants

Dilution of serum to 1/4 reduced the proportion but not the size of the soluble complexes formed. Similarly, BSA and anti-BSA were added at equivalence at different concentrations (0·25–1·5 μg of Ag) without any effect on their size (not shown).

Complexes found in sera deficient of classical pathway components and in the presence of C1 only

Whereas complexes formed in C1q deficient serum were identical to those formed in Mg EGTA sera (normal human serum + Mg EGTA or C1q deficient + Mg EGTA—not shown), in C2 deficient serum when tested after 60 min (Fig. 4) a heterogenous population of complexes was generated. This heterogeneity was lost after Mg EGTA was added, suggesting that it was due to binding of C1 and C4. The repletion of the deficient components (C1q or C2) restored to normal the capacity of these sera to generate soluble complexes (Fig. 4 shows C2). Purified C1 was unable, in itself to produce, stable soluble complexes (not shown).

The effect of B depletion

Complexes formed in the absence of factor B (immunochemical RB) were of increased size and were more widely distributed inside the gradient (Fig. 5). Purified factor B restored the normal handling

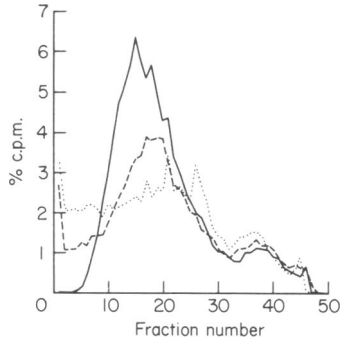

Fig. 4. Soluble complexes formed in C2 deficient serum. C2 deficient (. . . . . .), C2 def + C2 (———), C2 def + Mg EGTA (– – – – –). Note that a homogenous population of complexes was not formed in C2 deficient serum. The addition of Mg EGTA restored a clear peak, similar to NHS treated with Mg EGTA, and repletion with purified C2 allowed soluble complexes to be formed as in NHS.

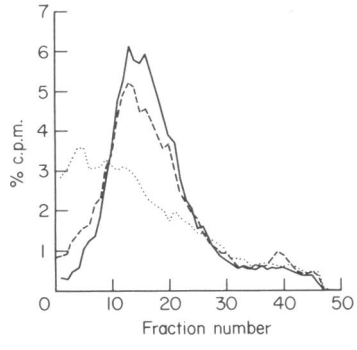

Fig. 5. Soluble complexes formed in the absence of factor B (immunochemical RB). NHS (———), RB (. . . . . .), RB + B (– – – – –). Note a broad peak of very large complexes in the absence of factor B and normal handling of complexes in the restored reagent (RB + B).

of complexes. In the presence of Mg EGTA no soluble complexes were formed in the RB reagent whereas in RB + B, their size and proportion was similar to those formed in normal human serum (not shown).

The effect of D depletion

Complexes prepared in RD were significantly and reproducibly bigger than in normal human serum and formed a clearly defined population. However these complexes started to aggregate after 150 min (Fig. 6). The addition of purified factor D restored haemolytic activity, B cleavage and solubilization to the depleted serum but B cleavage and solubilization were delayed, for reasons that are uncertain (Schifferli *et al.*, 1982). This provided an opportunity to study the dissociated activity of classical and alternative pathway on inhibition of precipitation. Sucrose density gradient ultracentrifugation runs were done after three different periods of incubation (Fig. 6), at 30 min before B cleavage, at 90 min after full activation of the alternative pathway and at 150 min when some aggregation was seen in the absence of factor D. The purified D was able to partially normalize the size of complexes formed in the RD (Fig. 6a, b, c), however this occurred after a lag phase. In the presence of Mg EGTA, solubilization of the recently formed precipitate was also delayed (Fig. 6d,

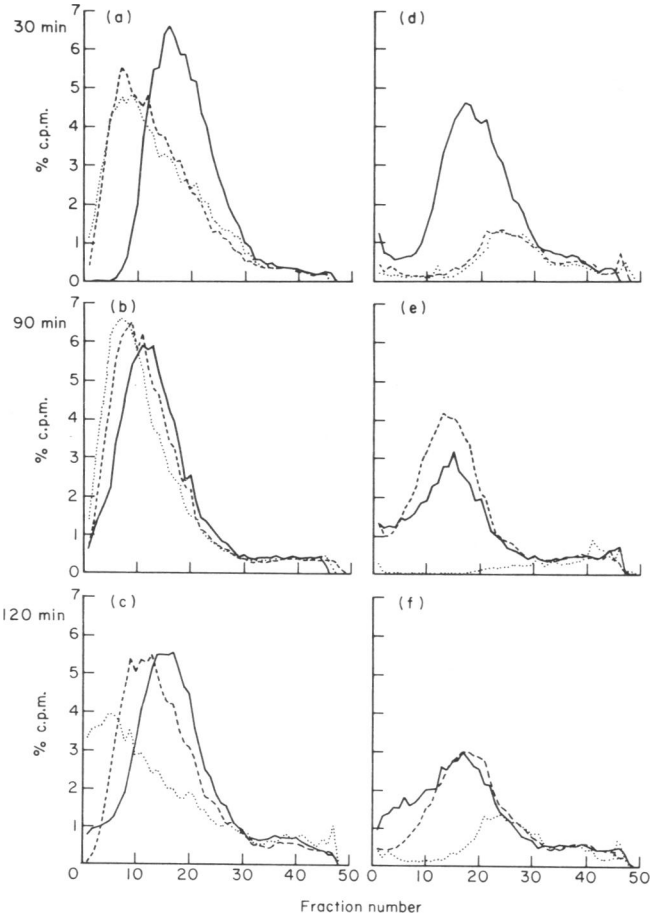

Fig. 6. Soluble complexes formed in the absence of factor D (RD) after 30 min (a, d), 90 min (b, e) and 120 min (c, f). NHS (———), RD (. . . . .), RD + D (– – – –); a, b and c in the presence of Ca^{++} and d, e and f in Mg EGTA. In a, b and c note the increased size of complexes formed in RD and the delayed and partial normalization of their size by purified factor D. In d, e and f note the delayed solubilization in RD + D and the normal size of the complexes formed. (The 90 min experiment was done in slight Ab excess).

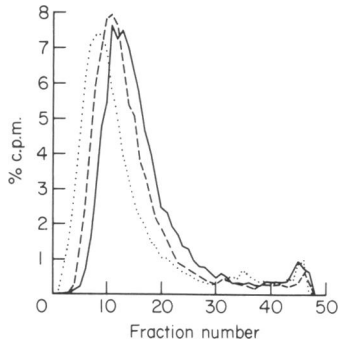

Fig. 7. Soluble complexes formed in the absence of Properdin (RP). NHS (———), RP (. . . . .), RP+P (– – – – –). Note the increased size of complexes formed in the absence of Properdin and the partial correction when purified P was added.

e, f) but the size and proportion of these complexes was normal, indicating normal alternative pathway function in this restored reagent.

The effect of Properdin depletion

Complexes formed in the absence of Properdin differed slightly but significantly in size from those prepared in normal human serum (Fig. 7). However they were stable and showed no tendency to aggregate even when tested after 150 min (not shown). The addition of $\bar{P}$ brought about a small reduction in complex size.

DISCUSSION

We have studied the ultracentrifugation characteristics of complexes formed in fresh serum and in reagents deficient in or depleted of components of the classical and alternative pathways. Our results indicate that after the reaction of antigen and antibody in normal serum a biphasic process follows: in the first phase, complexes are held in solution by activation of the classical pathway, and in the second by the alternative pathway. The eventual state of complexes is thus very similar if not indistinguishable from that generated by solubilization of a preformed immune precipitate.

In the absence of functional C1, for example in C1q deficient serum or in the presence of Mg EGTA, the proportion of complexes eventually held in solution was reduced, but their ultracentrifugation characteristics were the same as those in normal serum. By contrast when reagents deficient in alternative pathway function such as the RB, RD, or RP were studied, larger complexes were evident on ultracentrifugation; and in every case their size was reduced by reconstitution with the appropriate alternative pathway component. A number of interesting more specific findings also emerged. In the case of the C2 deficient serum, complexes of a broad range of size were generated. However when C1 function was blocked by Mg EGTA a homogeneous peak of complexes were generated similar to those seen in normal serum in the presence of Mg EGTA. These findings suggest that if no classical pathway C3 convertase is formed, the binding of C1 and/or C4 reduces the capacity of the immune aggregates to activate the alternative pathway and thereby to solubilize the complex.

The limited data available indicate that the alternative pathway dependent reaction occurs quickly, so that in normal serum it is not possible to identify complexes of an intermediate size of the kind that can readily be demonstrated using the alternative pathway depleted reagents. This suggests that the alternative pathway dependent events cause a rapid rearrangement of the lattice of the immune complex.

Not surprisingly the final size of complexes in normal serum was influenced by the proportion of antigen and antibody. In other experiments (Schifferli *et al.*, 1980) we showed that complement dependent inhibition of precipitation was more marked in the case of Ab-excess complexes.

However the addition of Mg EGTA had no effect on the ultimate size of complexes in solution suggesting that similar processes of alternative pathway activation are occurring over a range of antigen–antibody ratios.

J. A. Schifferli is a recipient of a grant from the Fond National Suisse de la Recherche Scientifique and from the British Royal Society. The work was supported by the Medical Research Council. We are indebted to J. MacAuley for art work and S. Goodwin for typing the manuscript.

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