A New Complement Function: Solubilization of Antigen–Antibody Aggregates (immune-complexes/immune-complex diseases)

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ABSTRACT Antigen–antibody aggregates are solubilized when incubated with fresh serum at 37°, yielding immune-complexes of relatively small molecular weight which contain antigen, antibody, and complement (C3) determinants. Solubilization is complement-dependent, requires free Mg++, but not Ca++, and proceeds in sera from C4- or C5-deficient animals. It is accelerated in the presence of Ca++ in normal or C4-deficient guinea pig serum, suggesting involvement of the Cl-bypass activation of the properdin system. Immune precipitates can also be solubilized by monovalent fragments (Fab) of antibodies directed against determinants of the antibody molecules included in the antigen–antibody lattice. Similarly, it is suggested that complement-mediated solubilization might be induced by the combination of a complement fragment with the antibody in the immune-aggregate.

The classical papers of Heidelberger et al. (1, 2) demonstrated that complement (C) components may be incorporated into the lattice formed when antigen (Ag) and specific antibody (Ab) combine. Subsequent investigations have shown that fresh serum may actually facilitate aggregation of soluble Ag–Ab complexes (3, 4). A widely accepted explanation for this aggregating activity of C is the further crosslinking of complexes by C1q, which is multivalent and has binding activity for the Fc region of some classes of immunoglobulin molecules (5, 6).

We show here that the complement cascade, and in particular, the properdin pathway, can also have the opposite effect; that is, to solubilize large Ag–Ab aggregates and lead to the formation of complexes of relatively small molecular weight containing antigen, antibody, and C3. This provides an explanation for the previously described phenomenon (7) of “release” of membrane-bound immune complexes from the cell surface through complement activity (CRA), which we showed to be dependent on C3 (but not C5) and on factor B (or C3PA) of the properdin pathway.

MATERIALS AND METHODS

Materials. The sources of the materials used here have been described (7). Bovine serum albumin was labeled with 125I by the method of McConahey and Dixon (8), extensively dialyzed against saline, and centrifuged at 20,000 × g for 30 min. It had a specific activity of 1 to 2 × 10⁶ cpm/μg of protein.

Antisera. Mouse antiserum to bovine serum albumin was obtained by injecting CBA/J mice with bovine serum albumin as described (9); rabbit antiserum to bovine serum albumin was similarly produced. Rabbit antiserum to guinea pig C3 was obtained by injecting a rabbit with 15 μg of purified guinea pig C3, given to us by Dr. Manfred Mayer, and it precipitated only C3 among serum proteins, as shown by immunoelectrophoretic analysis. Rabbit antiserum to mouse Ig was obtained by injection of purified mouse IgG (10); this antiserum contained precipitating antibodies to mouse γ₁, γ₂, and κ Ig chains. The antigens were emulsified in complete Freund’s adjuvant.

Purified IgG fractions were prepared from the rabbit antibodies against bovine serum albumin and against mouse Ig. Pepsin [F(ab')2] fragments of the former and papain (Fab) fragments of the latter were prepared as described (11).

Quantitative precipitations were performed for the preparations of Ab against bovine serum albumin [mouse Ab against bovine serum albumin, and IgG and F(ab')2 fragments of rabbit Ab against bovine serum albumin], as described (12), with ¹²⁵I-labeled as antigen.

Immune Precipitates. These were prepared by mixing the undiluted preparations of Ab against bovine serum albumin (each containing about 1.7 mg of precipitating Ab per ml) with equal volumes of a solution of ¹²⁵I-labeled bovine serum albumin, in the presence of 0.01 M EDTA adjusted to pH 7.4. Amounts of ¹²⁵I-labeled bovine serum albumin were calculated to yield complexes at equivalence or in the regions of Ag or Ab excess. After 30 min at 37°, the complexes were left overnight at 4°. The resulting precipitates were washed three times by centrifugation at 4°, resuspended in cold saline to a concentration of about 200 μg of protein per ml, and always thoroughly dispersed by passage through a syringe with a 25-gauge needle before use in the solubilization assay.

Solubilization Assay. Serum-mediated solubilization of immune precipitates was assayed kinetically by addition at 0° of 25-μl aliquots of finely suspended immune precipitate to tubes containing 0.2–0.5 ml of normal serum diluted in Dulbecco’s phosphate-buffered saline, containing Ca++ and Mg++ (13). Contents of the tubes were mixed and placed into a water bath at 37°. At selected times 25-μl samples were taken, and immediately mixed into tubes containing 2 ml of ice-cold saline, together with 0.05% sheep erythrocytes serving as an inert carrier. The diluted samples were centrifuged in the cold for 10 min at 1400 × g and carefully decanted; supernatants and pellets were assayed for ¹²⁵I activity. In experiments with EDTA or EGTA added to the nor-
The diagram shows the solubilization of immune precipitates by normal serum. An immune precipitate was prepared from 125I-labeled bovine serum albumin and mouse Ab against bovine serum albumin at equivalence, and 25 μl of the suspension was added to 200 μl of 1:2, 1:4, 1:8, and 1:16 dilutions of normal mouse serum, and to 200 μl of 1:2 dilution of heat-treated mouse serum (56°, 30 min). Samples were taken from the mixtures after various times at 37°, diluted, centrifuged, and counted.

Normal serum, veronal-buffered saline, pH 7.6, was added to a final veronal concentration of 15 mM to maintain the pH; concentrations much in excess of this were inhibitory. The control tubes contained heat-treated serum (30 min, 56°), zymosan-treated serum, or cobra venom factor-treated serum. Zymosan-treated serum was prepared by incubation of 1 ml of serum for 30 min at 37° with 5 mg of boiled washed zymosan, cobra venom factor-serum by incubation of 1 ml for 30 min at 37° with the minimum dilution of cobra venom factor (Cordis Corp.) capable of inactivating C3. Splitting of C3 by zymosan and cobra venom factor was ascertained by use of different dilutions of treated sera as a source of C in hemolytic or immune-adherence assays. The treated sera had less than 2% of their original activity.

RESULTS

Solubilization of Immune Precipitates by Normal Serum. A precipitate of 125I-labeled bovine serum albumin and mouse Ab against bovine serum albumin, prepared at equivalence, was mixed with several dilutions of normal mouse serum at 37°, and its solubilization was assayed kinetically as described (Fig. 1). Solubilization approaches 100%, and its rate is highly dependent upon the dilution of the serum. The precipitate was solubilized at a similar rate by fresh rabbit serum, whereas the same amount of precipitate prepared at equivalence with rabbit Ab against bovine serum albumin and bovine serum albumin was solubilized much more slowly by fresh rabbit serum; however, solubilization of rabbit complexes also approaches 100% if sufficient time is allowed (see Fig. 5).

Effect of Antigen: Antibody Ratio. Immune precipitates of 125I-labeled bovine serum albumin and mouse Ab against bovine serum albumin were prepared at various antigen:antibody (Ag:Ab) ratios, ranging from a 3 X Ag excess to an 8 X Ab excess; their solubilization by normal mouse serum was measured against time (Fig. 2). The rate of solubilization clearly increased with the Ag:Ab ratio, but even the precipitate in 8 X Ab excess was largely solubilized. A similar effect of changes in the Ag:Ab ratio was found with precipitates from the IgG fraction of rabbit Ab against bovine serum albumin when solubilized with rabbit serum.

Ion Requirements. Fig. 3a shows the results of an experiment in which a precipitate of 125I-labeled bovine serum albumin and mouse Ab against bovine serum albumin was incubated with normal mouse serum in the presence or absence of free Mg++ or Ca++ ions. The top curve shows the solubilization with serum containing optimal amounts of both ions. Addition of sufficient EGTA to chelate Ca++, in the presence of excess Mg++, caused a marked decrease in the rate and extent of solubilization; addition of sufficient Ca++ restored the activity almost to the control level. Thus, Ca++ is not essential for solubilization, but enhances it. Solubilization is completely inhibited in the presence of 0.01 M EDTA, showing that Mg++ is necessary for the effect. In a similar experiment in which mouse complexes were solubilized using C4-deficient guinea pig serum, the ionic requirements appeared the same (Fig. 3b).

State of Solubilized Complexes. The serum-solubilized immune complexes from several different experiments (including some stored at 4° for 2 days) were precipitated with 50% saturated ammonium sulfate to determine whether or not the 125I-labeled bovine serum albumin and the Ab against bovine serum albumin were still associated; in all cases the bovine serum albumin label was more than 95% precipitable, showing that solubilization is not a result of dissociation of most of the Ag from Ab. Controls showed that the complexes were not centrifuged down in the absence of the ammonium sulfate, and that free 125I-labeled bovine serum albumin was not precipitated.
Fig. 3. Ca\(^{++}\) requirement for optimal serum-mediated solubilization of immune precipitates. Precipitates were prepared with mouse Ab against bovine serum albumin and \(^{125}\)I-labeled bovine serum albumin in 3 × Ag excess, in the presence of 10 mM EDTA, washed, and solubilized with normal mouse serum diluted to a final volume of 0.5 ml. Zymosan-inactivated sera served as controls. Each tube received various amounts of EGTA, MgCl\(_2\), and CaCl\(_2\), calculated as follows: the serum was assumed to have a [Ca\(^{++}\)] of about 3 mM and a [Mg\(^{++}\)] of about 1 mM. To each tube MgCl\(_2\) was added to give a final [Mg\(^{++}\)] of between 1 and 2 mM; a preliminary titration showed that the solubilization rate does not change over a [Mg\(^{++}\)] range of 0.5–3 mM. Some tubes received sufficient EGTA to give a 2-fold molar excess over the [Ca\(^{++}\)] in the serum (EGTA + Mg\(^{++}\)). Selected tubes also received sufficient CaCl\(_2\) to overcome the EGTA, and give final Ca\(^{++}\) concentrations of 0.6 mM (EGTA + Mg\(^{++}\) + Ca\(^{++}\)). Veronal buffer (pH 7.6) was added to a final concentration of 15 mM to control pH changes resulting from chelation of cations. Finely suspended immune precipitate (0.05 ml) was added to each tube. (a) Solubilization by normal guinea pig serum, 1:5. (b) Solubilization by C4-deficient guinea pig serum, 1:2.

Centrifugation at 1400 × g for 10 min was routinely used to separate the solubilized complexes from any remaining insoluble precipitate. The effect of stronger centrifugation was also examined. For example, in the experiment-exploring solubilization of complexes with various Ag:Ab ratios (Fig. 2), the 20-min supernatants were subjected to further centrifugation at 11,000 × g for 60 min. The percentage of each complex remaining in the supernatant was as follows: 3 × Ag excess, 88%; 1.5 × Ag excess, 85%; 2 × Ab excess, 67%; and 8 × Ab excess, 56%. Thus, the complexes prepared at different Ag:Ab ratios were all soluble, but had discernible differences in their degree of aggregation. All of these centrifuged complexes were completely precipitated by 50% saturated ammonium sulfate, showing that the above differences were not due to different degrees of spontaneous dissociation of bovine serum albumin from the antibody.

The range in size of mouse complexes solubilized with normal mouse serum after passage through a column of Sepharose 6B is shown in Fig. 4. A small proportion of the label appeared in the void volume (molecular weight greater than 2,000,000), but most of it was eluted from the column after the IgM marker.

![Graph](image)

**Fig. 4.** Elution profile of solubilized immune complexes on Sepharose 6B (2 × 50 cm). An \(^{125}\)I-labeled bovine serum albumin-mouse Ab against bovine serum albumin immune precipitate made in 3 × Ag excess was solubilized with normal mouse serum diluted 1:2, for 30 min at 37°. The mixture was centrifuged for 1 hr at 10,000 × g, and 77% of the radiolabel was found in the supernatant, which was then added to the Sepharose 6B column. Column fractions were assayed for radioactivity, and their optical absorbances were measured. The albumin in the mouse serum served as one of the size markers. A human IgM myeloma protein was run through the same column under identical conditions, as was blue dextran, to determine the void volume.

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**Table 1. Presence of C3 determinants on solubilized immune complexes**

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Antiserum</th>
<th>% cpm precipitated</th>
</tr>
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<tbody>
<tr>
<td>Solubilized</td>
<td>Anti-egg albumin</td>
<td>7.0</td>
</tr>
<tr>
<td>Solubilized</td>
<td>Anti-C3</td>
<td>76.5</td>
</tr>
<tr>
<td>Control</td>
<td>Anti-egg albumin</td>
<td>8.6</td>
</tr>
<tr>
<td>Control</td>
<td>Anti-C3</td>
<td>11.3</td>
</tr>
</tbody>
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Egg albumin was mixed with \(^{125}\)I-labeled bovine serum albumin-mouse Ab against bovine serum albumin complexes solubilized with C4-deficient guinea pig serum. Excess rabbit Ab against guinea pig C3, or Ab against egg albumin were added to aliquots of the mixture. Controls contained soluble complexes obtained after incubation of the same bovine serum albumin-Ab against bovine serum albumin aggregates with zymosan-treated, C4-deficient guinea pig serum.
The presence of a fragment of C3 on solubilized complexes was shown as follows: an immune precipitate of 125I-labeled bovine serum albumin and mouse Ab against bovine serum albumin was incubated with C4-deficient guinea pig serum, or with zymosan-treated serum as a control. After centrifugation at 11,000 × g for 1 hr, 62.5% of the complexes treated with C4-deficient serum were in the supernatant (solubilized complexes), but only 12% of the complexes treated with zymosan-treated serum. An excess of rabbit Ab against guinea pig C3 was added to an aliquot of each mixture to precipitate all of the guinea pig C3 present. To other aliquots equivalent amounts of egg albumin and excess rabbit Ab against egg albumin were added to produce an unrelated precipitate. To prevent C fixation, both antisera had been heated at 56° for 60 min and treated with EDTA (final concentration: 0.01 M). The results are shown in Table 1. The solubilized complexes were mostly precipitated by Ab against C3 and not by Ab against egg albumin, but the soluble complexes that had been obtained with the zymosan-treated serum were not precipitated by either antiserum.

**Importance of Fc.** The possible involvement of the Fc portion of the IgG antibody in the solubilization was tested as follows: immune precipitates were made in slight antigen excess with either the IgG fraction of rabbit Ab against bovine serum albumin or F(ab')2 fragments from the same IgG. The Ag:Ab ratio in the washed immune precipitate was approximately the same for the F(ab')2 as for the native IgG. Each precipitate was then solubilized with normal rabbit serum or zymosan-treated serum as a control (Fig. 5). Solubilization of the complexes prepared with F(ab')2 fragments was much slower than for the complexes prepared with native IgG, indicating that the Fc portion of the Ig molecule influences the rate of solubilization.

**Solubilization by Fab of Ab Against Ig.** An immune precipitate from mouse Ab against bovine serum albumin was treated with various dilutions of Fab fragments of rabbit Ab against mouse Ig (Fig. 6). This solubilized the precipitate very rapidly, and the rate was dependent upon the amount of Fab antibody against Ig used; a nonspecific rabbit Fab fragment had no effect. The complexes solubilized in this manner were almost completely precipitated by 50% saturated ammonium sulfate, showing that little dissociation of Ag and Ab occurred.

**DISCUSSION**

Our findings demonstrate that bovine serum albumin–Ab against bovine serum albumin precipitates are solubilized through the activity of complement. The solubilized complexes, which contain antigen, antibody, and C3, or most likely a fragment of C3, are heterogeneous in size. Part of the complexes have relatively small molecular weight and do not come out of solution even after incubation for several days at 4° or centrifugation at 10,000 × g for 1 hr. Although the present experiments were performed only with bovine serum albumin as antigen, recent observations indicate that serum-mediated solubilization also occurs with immune precipitates prepared with other antigens, such as ovomucoid and F- (ab')2 fragments of guinea pig γ-2 globulin (J. Csop and V. Nussenzweig, unpublished observation).

The striking change of phase that the serum-treated immune complexes undergo is dependent on complement. It is inhibited at 0°, and in the presence of chelating agents, by heating the serum at 56° for 30 min, or by preincubating the serum with cobra venom factor or zymosan. During the solubilization, C3 determinants are added to the complexes. In addition, we have previously demonstrated (7) that a similar phenomenon, the removal of aggregates of bovine serum albumin–Ab against bovine serum albumin from the membrane of lymphocytes (termed CRA) was dependent on

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**FIG. 5.** Requirement of Fe portion of IgG for efficient serum-mediated solubilization of immune complexes. Immune precipitates were prepared at 2 × Ag excess, with 125I-labeled bovine serum albumin and the IgG fraction of rabbit Ab against bovine serum albumin, or F(ab')2 fragments of the same. The precipitates were treated at 37° with a 1:2 dilution of normal rabbit serum, or with a 1:2 dilution of zymosan-inactivated serum as a control.

**FIG. 6.** Solubilization of immune precipitate by Ab against immunoglobulin. A precipitate of 125I-labeled bovine serum albumin and mouse Ab against bovine serum albumin was prepared in 3 × Ag excess, and treated at 37° with various amounts of Fab fragments of rabbit Ab against mouse Ig, or with nonspecific rabbit Fab fragment as a control.
both C3 and factor B of the properdin pathway, but not on C5. CRA experiments were similarly performed with complexes prepared at equivalence in the presence of complement, but the reagents were kept so dilute that the microaggregates did not precipitate spontaneously after a relatively short incubation time and centrifugation at 1200 × g for 20 min. However, part of the microaggregates was firmly bound to the cell membrane of B lymphocytes. These membrane-bound complexes were removed (10) by treating the cells with (a) excess antigen, (b) Fab fragments of rabbit antibodies to the mouse antibodies contained in the complexes, and (c) fresh serum as a source of complement. It is clear now that these different treatments led to a disaggregation of immune precipitates.

The kinetic curves of solubilization of complexes are sigmoidal and represent the end result of the multiple enzymatic reactions involved in the complement cascade. The velocity of solubilization is influenced by factors such as the Ag:Ab ratios of the immune aggregates, which may affect either the efficiency of activation of the complement system or the bonds that keep the immune complexes aggregated and out of the aqueous phase. On the other hand, precipitates prepared with rabbit 7S Ab against bovine serum albumin were more difficult to solubilize with complement from rabbit or mouse than those prepared with mouse 7S Ab against bovine serum albumin. In this case, the class of antibody or its binding affinity for the antigen may have influenced the dissociation. The finding that precipitates consisting of F(ab′)2 fragments of rabbit Ab against bovine serum albumin and bovine serum albumin are solubilized at a much slower rate than those prepared with native antibodies indicates the important role of the Fc region in this phenomenon.

The reaction proceeds in the presence of EGTA and Mg++, in C4-deficient guinea pig serum, or in C5-deficient mouse serum. These findings point to the role of the properdin pathway in solubilizing complexes. However, other pathways of complement activation may influence this reaction. For example, since Ca++ ions enhance the reaction mediated by C4-deficient guinea pig serum, the recently described Cl-shunt (14) may also participate in the process.

The mechanism of solubilization is not clear. Possibilities are (a) that a very efficient proteolytic activity is generated through the complement cascade leading to the fragmentation of the antibody, and (b) that during complement fixation a fragment of a complement component (C3b?) is intercalated in the complexes and disrupts the Ig–Ig and/or the Ag–Ab interactions which contribute to lattice formation and precipitation of Ag–Ab aggregates (15). That such a simple mechanism could work is supported by the observation that complexes can be similarly disaggregated by Fab of Ab against Ig, which can certainly do no more than bind onto the Ig in the complex.

Our observations indicate that the interaction between a soluble antigen and antibody in fresh serum may have two distinct phases, aggregation and disaggregation, both mediated through the complement cascade. It appears likely that, as in the case of lysis of red cells by antibody and complement, well-defined antigen–antibody–complement intermediates occur, perhaps with defined tridimensional arrangements that would account for distinct biological properties.

Should this be true, our findings will be of direct relevance to phenomena that depend on the interaction of fluid phase immune complexes with cell membranes, as well as to the antigen–antibody interactions that occur with peripheral or integral proteins of the cell membrane. Some implications for immunopathology are discussed elsewhere (16, 17).

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