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Influence of aggregate size on the binding and activation of the first component of human complement by soluble IgG aggregates

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Summary. The interaction between small aggregates of human IgG and the first component of human complement was studied. Stabilized soluble IgG aggregates of restricted size were prepared by heat aggregation of human IgG, followed by sucrose-density ultracentrifugation. Human C1 was isolated in its precursor form by euglobulin precipitation, followed by gel filtration and immunoadsorption. A C1 preparation was obtained of which more than 90% was still in its unactivated form.

Soluble aggregates containing 20, 10 or 5 molecules IgG, and monomeric IgG were tested for their ability to bind and to activate C1. The binding of C1 was determined by C1 consumption, whereas the activation of C1 was measured as the increased ability of the C1 preparation to consume purified human C4 after the incubation with the aggregates. The three aggregates tested and monomeric IgG were all able to bind and to activate C1, but the efficiency of both processes markedly increased with increasing aggregate-size. Furthermore, it was found that all four preparations activated an appreciable amount of C1 at concentrations that did not result in any detectable C1 fixation. These results confirm earlier suggestions that C1 can be activated during a short, transient binding to small aggregates or immune complexes that have a low avidity for C1, after which the activated form, $C\bar{1}$, is released into the medium.

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INTRODUCTION

The classical pathway of complement can be activated by immune complexes containing IgG- or IgM-antibodies (Müller-Eberhard, 1975; Porter & Reid, 1978). The initial step of this process consists of activation of the first complement component (C1), which is a Ca^{++}-dependent complex of the subcomponents C1q, C1r, and C1s (Lepow, Naff, Todd, Pensky & Hinz, 1963), presumably with the molecular formula $C1q(1r\text{-}1s)_2$ (Ziccardi & Cooper, 1977). C1 binds via its C1q subcomponent to the Fc parts of the immunoglobulins in an immune complex (Müller-Eberhard & Kunkel, 1961). This can result, possibly through physicochemical alterations in the C1q molecule, in conversion of C1r to its enzymatic form $C\bar{1}r$, which in turn activates C1s (Valet & Cooper, 1974a; Ziccardi & Cooper, 1976; Porter & Reid, 1978). Although the biochemical events occurring upon activation of C1 are relatively well understood, there is little agreement about the molecular size and composition of immune complexes required for efficient activation. Insoluble immune complexes such as antibody-coated erythrocytes require the presence of at least two adjacent IgG molecules or a single IgM molecule on their surface for

C1 activation (Borsos & Rapp, 1965; Linscott, 1970). Recently, immunoprecipitates have been shown to activate C1, but an analysis of the composition of the precipitates was not reported (Folkerd, Gardner & Hughes-Jones, 1980). On the other hand, the capacity of soluble immune complexes to activate the classical pathway of complement, has received limited attention, although several assays for their detection in the sera of patients are based on this ability (Theofilopoulos & Dixon, 1979). The use of several models to study the binding and/or activation of C1 has led to conflicting results. Thus, binding of C1 has been found with immune complexes containing at least one (Goers, Ziccardi, Schumaker & Glovsky, 1977), two (Wright, Tschopp, Jaton & Engel, 1980) or four (Hyslop, Dourmashkin, Green & Porter, 1970) IgG molecules, while others have reported C1 binding by non-complexed, monomeric IgG (Augener, Grey, Cooper & Müller-Eberhard, 1971). However, the binding of C1 is not necessarily followed by its activation (Colten, Borsos & Rapp, 1969). It is possible that some immune complexes, due to their composition and physical state, can bind C1q, but fail to activate C1 (Füst, Medgyesi, Rajnavölgyi, Ccécsi-Nagy, Czikora & Gergely, 1978; Daha & van Es, 1979). We have studied the binding and activation of purified human C1 by stabilized soluble aggregates of human IgG, which can be used as standards in immune complex assays (Kauffmann, van Es & Daha, 1979). Aggregates of restricted size, containing 20, 10 or 5 molecules IgG and monomeric IgG were used. The experiments were done with purified complement components, to rule out stimulatory or inhibitory effects of other serum proteins. It was found that C1 could be bound and activated by all four preparations tested.

MATERIALS AND METHODS

Materials

Benzamidine HCl (Aldrich, Beerse, Belgium), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), Biogel-A5 and A-1·5 (Biorad Labs, Pleuger, Amsterdam), Sephadex-G200, Sephacryl-S300, SP-Sephadex-C50 (Pharmacia Fine Chemicals, The Hague). Trypsin (Bovine Pancreas, type XI), trypsin inhibitor (Ovomucoid, type II-0) and DEAE-Sephacel (Sigma Chemicals, St Louis, Mo.) were purchased as indicated. Outdated human plasma was obtained from the Blood Bank of the Leiden University Hospital, and fresh human plasma from laboratory personnel. Isotonic veronal-buffered saline (VBS, pH 7·5) containing 0·1% gelatin, 0·15 mM $CaCl_2$ and 0·5 mM $MgCl_2$ (GVB^{++}), half isotonic veronal-buffered saline, containing 0·1% gelatin, 3% dextrose, 0·15 mM $CaCl_2$ and 0·5 mM $MgCl_2$ ($DGVB^{++}$) and half isotonic GVB without divalent cations, containing 0·04 EDTA (GVB-EDTA) were used as diluents.

Preparation of aggregates

Isolation and radiolabelling of normal human IgG, and preparation of stabilized IgG aggregates of restricted size were done essentially as described by Kauffmann *et al.* (1979). To obtain relatively small aggregates, however, heat aggregation at 63° was shortened to 10 min (the IgG concentration was 17·4 mg/ml) and the sucrose-density ultracentrifugation was prolonged to 6 hr. The sedimentation rate and molecular size of material in the fractions of the gradient were calculated as described previously (Kijlstra, Knutson, van der Lelij & van Es, 1977). In each run, two gradients contained unlabelled heat-aggregated IgG, one gradient contained ^{125}I-labelled non-aggregated IgG and the fourth gradient contained IgG, that was aggregated in the presence of a trace amount of ^{125}I-IgG. The distribution of ^{125}I-radioactivity in the fourth gradient was used to calculate the IgG concentrations in the fractions of the first two gradients, after determination of the concentration in the 7S fraction by radial immunodiffusion. The unlabelled aggregates were frozen at −70° immediately after fractionation and were used within 3 months.

Isolation of precursor C1

Precursor human C1 was isolated from ACD plasma, that had been stored at −20° in the presence of 1 mM benzamidine. One hundred millilitres of this plasma were recalcified with 20 mM $CaCl_2$ and allowed to clot for 2 hr at room temperature. An euglobulin precipitate of the resulting serum was prepared by dialysis (4 hr at 0°–2°) against 2 litres of 5 mM phosphate buffer, pH 7·4, containing 0·3 mM $CaCl_2$ and 2 mM benzamidine. The precipitate was centrifuged (15 min, 25,000 *g*), washed with 25 ml dialysis buffer, centrifuged again and dissolved in 5 ml VBS, containing 0·15 M NaCl, 5 mM $CaCl_2$ and 2 mM benzamidine. After removal of non-dissolved material by centrifugation (15 min, 1800 *g*), a volume of 2 ml of the supernatant was further purified by gel filtration on a Biogel A-1·5 column in VBS, containing 0·15 M NaCl, 5 mM $CaCl_2$ and 2 mM benzamidine. The fractions containing peak

C1 haemolytic activity were pooled and treated batch-wise with a solid phase immunoadsorbent of rabbit antibodies against human IgG, IgA, β_1H, C3 and C4 (see below). Because dilution during this procedure was unavoidable, the final material was concentrated by ultrafiltration to approximately 5 ml. After 1:1 dilution with GVB^{++}, it was stored in a glass tube on ice. With this method of storage the preparation retained its C1 haemolytic activity and remained in its precursor form for more than 2 months. Before dilution a sample, which contained 0·75 mg/ml, was taken to assess the purity of the preparation by immunoelectrophoresis, Ouchterlony analysis and SDS-PAGE.

Preparation of an anti-human IgG, IgA, β_1H, C3 and C4 immunoadsorbent

Analysis of early C1 preparations after the gel filtration step demonstrated contamination with IgG, IgA, β_1H, C3 and C4. Therefore, we prepared an immunoadsorbent of agarose-coupled rabbit antibodies against these proteins. Monospecific rabbit antisera against human IgG, IgA, β_1H, C3 and C4, produced in our laboratory, were mixed (20 ml of each antiserum), and the IgG fraction was isolated by ammonium sulphate precipitation, followed by DE-52 chromatography. To prevent C1 activation during immunoadsorption, the IgG preparation was pepsin-digested (Lachmann, 1971) and the $F(ab')_2$ fragments were coupled to CNBr-activated Biogel-A5 (March, Parikh & Catrecasas, 1974). The immunoadsorbent was stored in borate-buffered saline (BBS) containing 0·02% azide and was extensively washed with VBS buffer before use during the C1 isolation.

Isolation of C4

C4 was isolated from fresh human plasma by a three step procedure, involving anion and cation exchange chromatography and gel filtration. Briefly, 200 ml of fresh EDTA plasma containing 1 mM benzamidine was applied to a 2 × 25 cm column of DEAE-Sephacel, which was equilibrated with 0·01 M phosphate buffer, pH 7·5 containing 2 mM EDTA and 1 mM benzamidine, and NaCl to yield a conductivity of 6 mS. After thoroughly washing with the starting buffer, C4 was eluted with a linear (6–30 mS) NaCl gradient and was found in fractions with conductivity between 11 and 16 mS. After dialysis this pool was subjected to cation exchange chromatography on Sephadex SP-C50 in 0·01 M acetate, pH 6·0 containing 2 mM EDTA and 1 mM benzamidine; C4 was eluted from this column with a linear (3–25 mS) NaCl gradient and was found in fractions with a conductivity between 4 and 8 mS. Finally, the concentrated C4 pool was gel filtrated on a Sephacryl-S300 column. The peak fractions after the gel filtration were pooled and stored at −70°. Immunoelectrophoresis, Ouchterlony analysis and PAGE showed that the preparation, at a concentration of 0·34 mg/ml, contained only one protein with C4 haemolytic activity.

Determination of the percentage precursor C1 in C1 preparations

The determination of the relative amount of isolated C1, which was still in its precursor form, was based on the method of Valet & Cooper (1974b) for the detection of proenzyme C1s. Two 38 μg samples of purified C1 in 100 μl GVB^{++} were incubated for 2 min at 37° in the presence or absence of 1 μg trypsin; the reaction was stopped by the addition of 2 μg trypsin inhibitor. Purified C4 (136 ng in 100 μl GVB^{++}) was incubated for 30 min at 30° with 100 μl of two-fold dilutions of both trypsin-treated and untreated C1, after which the residual C4 haemolytic activity was determined. The difference between the two dose–response curves for C4 consumption was used to calculate the amount of activated C1 in the untreated sample, assuming that trypsin treatment resulted in 100% activation.

Fixation and activation of C1

Fixation of C1 was performed essentially as described by Augener *et al.* (1971). Portions of 9·5 ng C1 in 100 μl GVB^{++} were incubated (30 min, 30°) with 10 μl of two-fold dilutions of AIgG, after which the residual C1 haemolytic activity was determined in appropriate dilutions.

The activation of C1 by IgG aggregates was assessed in a three-step assay. Firstly, portions of 233 ng C1 in 100 μl GVB^{++} were incubated (30 min, 30°) with 10 μl diluted aggregates. Secondly, 100 μl of a six-fold dilution of the reaction mixtures were mixed with 100 μl of a C4 dilution (1·36 μg/ml) and incubated 30 min at 30°. Finally, the residual C4 haemolytic activity was measured in such a dilution that buffer-treated C4 showed a Z value of approximately 1. Controls consisted of C4 incubated with buffer, C4 incubated with buffer-treated C1, and C4 incubated with trypsin-treated C1. Except in the final haemolytic assays, GVB^{++} was used as the diluent.

Assays

C1 haemolytic activity was measured with $EAC4^{hu}$ (Borsos & Rapp, 1963). C4 was assayed with C4-deficient guinea-pig serum (Gaither, Alling & Frank, 1974), except in the C1-activation experiments, where we used $EAC1^{bov}$ (Rapp & Borsos, 1970), because the presence of minute amounts of AIgG in the final assay was found to cause inappropriate C4 consumption via C1 in the C4-deficient serum.

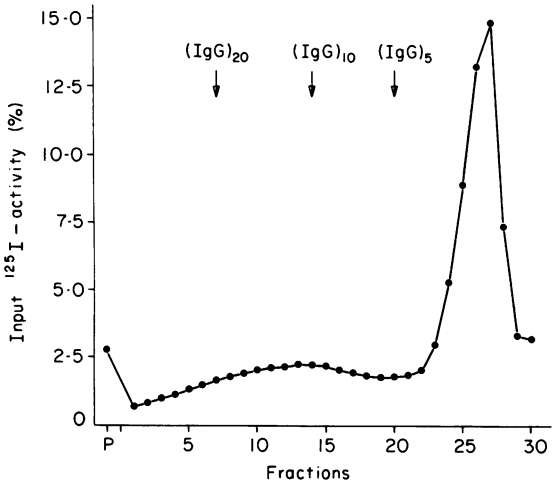

Figure 1. Sucrose density profile of ^{125}I-labelled aggregated IgG. Heat-aggregated IgG was centrifuged for 6 hr at 263,000 *g* on 10%–30% (w/v) sucrose density gradients. The arrows indicate the fractions where aggregates containing 20, 10 and 5 molecules IgG were calculated to be located. P = pellet.

RESULTS

Aggregates

Aggregation and ultracentrifugation of IgG, as described under Materials and Methods, resulted in the distribution profile shown in Fig. 1. About 60% of the IgG (fractions 22–30) appeared not to be aggregated. Prolonging the aggregation diminished this percentage, but this decrease was only reflected by an increase of the amount of IgG found in the pellet; the amount of the desired small aggregates found in fractions 5–20 did not change. The distribution profile of non-aggregated ^{125}I-IgG, centrifuged under the same conditions, showed that fractions 1–20 were free of monomeric IgG.

Precursor C1

The procedure to isolate precursor C1 was derived from the method of Gigli, Porter & Sim (1976). Because in our hands this method yielded preparations that contained several contaminants, we modified the procedure. Euglobulin was precipitated from serum by dialysis (at pH 7·4), and the dissolved precipitate was subjected to gel filtration on Biogel-A1·5 (Fig. 2). A third step consisted of immunoadsorption in order to remove the remaining contaminants, which by Ouchterlony analysis were found to be IgG, IgA, C3, C4 and β_1H. To this end, we prepared an immunoadsorbent of agarose-coupled rabbit $(Fab')_2$ fragments against these five proteins. Batch-wise treatment of the C1 containing fractions of the Biogel-column resulted in a preparation which, at a protein concentration of 0·75 mg/ml, did not react in Ouchter-

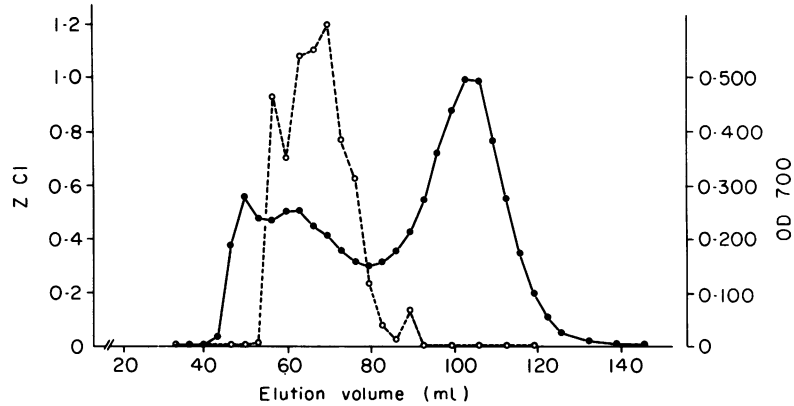

Figure 2. Gel filtration of an euglobulin preparation on Biogel-A1.5. The protein content (●——●) and C1 haemolytic activity (measured in a 1/20,000 dilution) of the fractions (O- - - -O) are indicated.

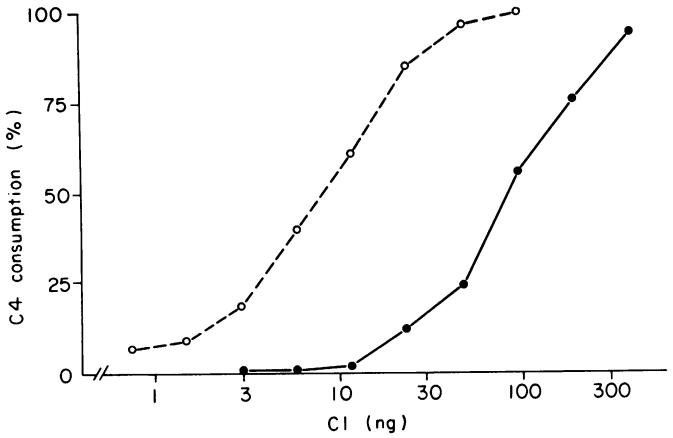

Figure 3. Determination of the percentage activated $C\bar{1}$ in the C1 preparation. Samples were incubated for 2 min at 37° with buffer or trypsin (2·5% w/v), after which their C4-consuming capacity was measured. From the difference between the two titration curves the degree of activation in the untreated sample was calculated.

lony analysis with antisera against C3, C4, $C\bar{1}$INH, β_1H, IgG, IgA and C4-binding protein, but did react with antisera against C1q, C1r and C1s. After immunoelectrophoresis in the presence of EDTA, only one precipitation line was found against anti-whole human serum, which represented C1q. SDS-PAGE after preincubation with EDTA, in the presence and absence of DTT, gave results, which were compatible with those found by Medicus & Chapuis (1980).

The amount of precursor C1 present in the preparation was determined by comparison of the C4-consuming activity of trypsin-treated and untreated samples of the preparation (Fig. 3). As can be seen, both samples consumed C4 in the same dose-dependent way, but their efficiency differed greatly: 91 ng untreated C1 was needed to consume 50% of the available C4, whereas the same effect was achieved by only 8·3 ng trypsin-treated C1.

This meant that 91% of the untreated C1 was still in its precursor state. This figure is a lower limit because it is based on the (unproven) assumption that the trypsin treatment resulted in 100% activation.

Binding of C1

The ability of IgG aggregates of different size to bind C1 was assessed by a consumption assay, essentially as described by Augener *et al.* (1971). Aggregates containing 20, 10 and 5 molecules and monomeric IgG, all isolated from the same sucrose gradient, were used

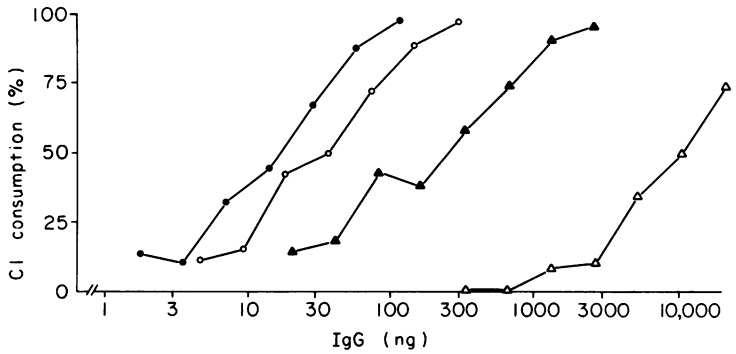

Figure 4. Binding of C1 to IgG aggregates of different size. The C1-binding capacities of aggregates containing 20 (●——●), 10 (○——○) and 5 (▲——▲) IgG molecules and of monomeric IgG (△——△) are shown.

(Fig. 1). In Fig. 4 the results of a representative experiment (out of 4) are shown. All four preparations consumed C1 in a dose-dependent way, but the efficiency was markedly different. The amount of IgG that consumed 50% C1, as calculated from the dose–response curves, appeared to diminish several orders of magnitude with increasing aggregate size, ranging from 9740 ng for monomeric IgG to 14·3 ng for $(IgG)_{20}$ (Table 1). Assuming mol. wt of 150,000 for IgG and 760,000 for C1, we calculated the corresponding IgG:C1 molar ratios (Table 1, second column) and the the number of C1 molecules bound per 10,000 aggregates at this ratio (Table 1, third column). The data clearly show that monomeric IgG binds C1 very poorly whereas aggregation strongly promotes the binding capacity, not only per aggregate, but also per IgG molecule.

Table 1. Binding of C1 by IgG aggregates of different size*

n†	IgG (ng) required for 50% C1 binding	IgG:C1 molar ratio	C1 molecules bound per 10^4 aggregates
Monomer	9740	5200	1
5	198	106	236
10	31	16·5	3030
20	14·3	7·6	12,660

* Purified C1 (9·5 ng) was incubated for 30 min at 30° with aggregates after which the percentage consumption of C1 haemolytic activity was determined.

† IgG molecules per aggregate.

Activation of C1

The same preparations of IgG aggregates that were used in the C1-binding experiments were also tested for their capacity to activate precursor C1. The appearance of $C\bar{1}$ after 30 min incubation at 30° of C1 with diluted samples of the aggregates was assessed as the increased C4-consuming capability of the reaction mixtures. This was performed by incubating portions of 136 ng purified human C4 with 100 μl of a six-fold dilution of the reaction mixtures (containing 39 ng C1) for 30 min at 30°, after which the residual C4 activity was measured. Under these conditions untreated C1 consumed 20% of the available C4, whereas incubation with trypsin-treated C1 resulted in 94% consumption (Fig. 3). The results were expressed as the percentage C4 consumption; the C4 activity after incubation with untreated C1 served as the control. Just as for C1 binding, a dose-dependent activation of C1 was found for all four preparations tested, and the aggregate size had a strong influence on the efficiency of the activation (Fig. 5). Table 2 shows that amounts of IgG that resulted in 50% C4 consumption. Since the same level of C4 consumption was achieved with 12 ng of C1 that was fully activated by trypsin treatment (Fig. 3), approximately 31% (12/39) of the C1 was in the activated form after incubation with these amounts of IgG aggregates. Because 9% of the untreated C1 preparation was already activated, it follows that 50% C4 consumption in the activation experiments corresponds to approximately 22% C1 activation. The second and third column of Table 2 show the IgG:C1 molar ratios at which this 22% activation occurred and the number of C1 molecules activated per 10^4 aggregates at these ratios. Comparison of

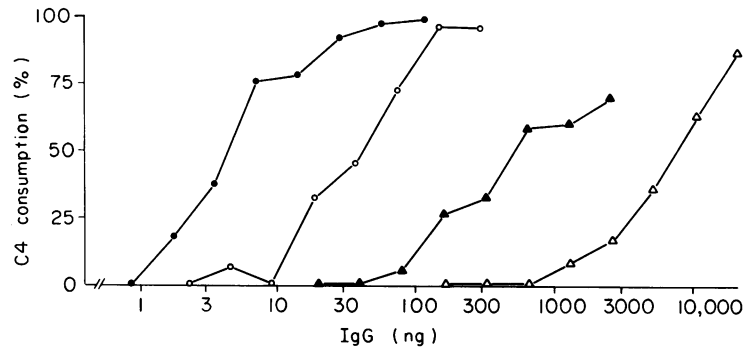

Figure 5. Activation of C1 by IgG aggregates of different size. The C4 consumption by C1 samples that were incubated with various amounts of aggregates containing 20 (●——●), 10 (○——○) and 5 (▲——▲) IgG molecules and of monomeric IgG (△——△) is shown.

Table 2. Activation of C1 by IgG aggregates of different size*

n†	IgG (ng) required for 22% C1 activation	IgG:C1 molar ratio	C1 molecules activated by 10^4 aggregates
Monomer	7240	158	40
5	702	15·3	720
10	35	0·76	29,000
20	5	0·11	400,000

* Purified C1 (233 ng) was incubated for 30 min at 30° with aggregates, after which the increased capacity to consume C4 was determined (see text).

† IgG molecules per aggregate.

Tables 1 and 2 reveals a striking difference between the efficiencies of the binding and the activation for all four preparations. For instance, the aggregates containing 20 IgG molecules bound approximately one C1 molecule per aggregate, whereas one aggregate was able to activate forty C1 molecules. These data clearly show that a substantial activation of C1 was achieved with amounts of IgG aggregates that exhibited only a very limited amount of C1 binding.

DISCUSSION

The binding and activation of C1 by IgG-coated erythrocytes requires the presence of pairs of adjacent IgG molecules (doublets) on the cell surface (Borsos & Rapp, 1965; Ishizaka, Tada & Ishizaka, 1968; Linscott, 1970). This finding has led to the hypothesis that the activation of C1 by an immune complex requires the simultaneous binding of at least two of the six globular heads of the C1q molecule. However, it is not certain if this hypothesis rules out the activation of C1 by small immune complexes containing only one or a few IgG molecules. Although early reports on this subject suggested a minimal size of at least two (Cohen, 1968) or even four (Hyslop *et al.*, 1970) IgG molecules, it has been shown that complexes containing only one IgG molecule, or even non-complexed, monomeric IgG are able not only to bind $C\bar{1}$ (Augener *et al.*, 1971), but also to activate C1 (Goers *et al.*, 1977; Tschopp, Schultess, Engel & Jaton, 1980). We have investigated the binding and activation of purified C1 by small stabilized aggregates of human IgG, separated according to their size by sucrose density ultracentrifugation (Kauffmann *et al.*, 1979). The results described here confirm the findings of others, that C1 binding and activation can be achieved with very small IgG aggregates or immune complexes. Soluble aggregates containing an average of 20, 10 or 5 IgG molecules, but also the 7S fraction were all found to be able to bind and to activate C1. However, the efficiency of both processes was strongly dependent on the degree of polymerization of the IgG molecules. This was found earlier with several other models of small immune complexes (e.g. Hyslop *et al.*, 1970; Wright *et al.*, 1980) and is presumably due to the multivalency of the C1q molecule. Recently, Tschopp *et al.* (1980) have shown the C1 activating ability of chemically cross-linked oligomers of rabbit IgG. From their data it can be derived that 20% C1 activation occurred at IgG:C1 ratios of approximately 57:1, 4:1 and 5:6 for monomeric, dimeric and trimeric IgG, respectively. However, their trimeric IgG was sixty times more efficient than monomeric IgG, whereas in our system pentameric IgG was only ten times more efficient than monomeric IgG. This difference may be due to the source and the different methods used for polymerization of IgG. Possibly, some steric hindrance diminishes the apparent efficiency of heat-aggregated IgG for C1 binding and activation. Such a steric hindrance was suggested by earlier experiments with heat-aggregated guinea-pig IgG2 (Kijlstra, van Es & Daha, 1979) in which a decreased $C\bar{1}$ binding efficiency was found when the aggregates contained more than forty IgG molecules.

On the other hand, we could not confirm the conclusion of Tschopp *et al.* (1980) that C1 binding and activation are directly correlated. However, their conclusion was partially based on the assumption that the consumption of total haemolytic activity in serum by their IgG oligomers (Wright *et al.*, 1980) was merely due to the fixation of C1. In our system we found a substantial activation of C1 by small amounts of IgG aggregates that showed no detectable fixation of C1. The finding that one aggregate containing twenty IgG molecules was able to activate approximately forty C1 molecules (Table 2) confirms the suggestion of Füst *et al.* (1978), that C1 may be activated by an immune complex during a short, transient binding, after which it is released as $C\bar{1}$. Their suggestion was based on experiments in which the binding of $C\bar{1}$ was studied with partially purified $C\bar{1}$ according to Augener *et al.* (1971), whereas the consumption of C4 in whole serum was used as a parameter for C1 activation. The use of these two different systems prevents a quantifiable

comparison of the binding and activation capacities at a molecular level and therefore cannot be meaningfully compared with the results obtained in the present study.

The IgG:C1 ratio in normal human serum is approximately 375:1, assuming concentrations of 10 mg/ml for IgG and 135 μg/ml for C1 (Ziccardi & Cooper, 1977). We have found appreciable amounts of C1 activation at this IgG:C1 ratio with different batches of monomeric IgG. This phenomenon was also observed when the IgG preparations were subjected to centrifugation for 20 min at 25 000 *g*. On the other hand our results were obtained in a diluted system, in the absence of other serum proteins such as $C\bar{1}$ inhibitor which recently has been shown to be very effective in preventing full activation of the classical pathway (Ziccardi, 1981), and which may also have some influence on the activation of C1 (Folkerd *et al.*, 1980). An alternative explanation may be that not only the molar ratio but also the absolute concentration of IgG and C1 determine whether activation will occur. Experiments are now in progress to discriminate between these possibilities.

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