The binding of complement component C3 to antibody–antigen aggregates after activation of the alternative pathway in human serum

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Preformed immune aggregates, containing antigen and either IgG (immunoglobulin G) or F(ab')₂ rabbit antibody, were incubated with normal human serum under conditions allowing activation of only the alternative pathway of complement. Both the IgG and F(ab')₂ immune aggregates bound C3b, the activated form of the complement component C3, in a similar manner, 2–3% of the C3 available in the serum being bound to the aggregates as C3b, and the rest remaining in the fluid phase as inactive C3b or uncleaved C3. It was found that C3b was probably covalently bound to the IgG in the aggregates, since C3b–IgG complexes could be demonstrated on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, after repeated washing with buffers containing high salt or boiling under denaturing conditions. Incubation of the C3b–antibody–antigen aggregates in buffers known to destroy ester linkages had little effect on the C3b–IgG complexes, which suggested that C3b and IgG might be linked by an amide bond. Two main types of C3b–IgG complexes were found that had apparent mol.wts. of 360 000 and 580 000, corresponding to either one or two C3b molecules respectively bound to one molecule of antibody. On reduction of the C3b–IgG complexes it was found that the β -chain, but not the α' -chain, of C3b was released along with all the light chain of IgG but only about half or less of the heavy chain of IgG. These results indicate that, during activation of the alternative pathway of complement by immune aggregates containing IgG antibody, the α' -chain of C3b may become covalently bound at one or two sites in the Fd portion of the heavy chain of IgG.

Component C3, a glycoprotein of 186 000 mol.wt., is the most abundant complement component in serum (approx. 1.2 g/litre) and it plays a central role during the activation of both the classical and alternative pathways. During its activation, C3 is split, by the C3 convertase of either pathway, at one bond located near the *N*-terminal of its α -chain, to yield C3a (a polypeptide of 77 amino acid residues that displays anaphylatoxin activity) and C3b (a large fragment of approx. 178 000 mol.wt. composed of the α' -chain of 108 000 mol.wt. disulphide-linked to the entire β -

chain of 70 000 mol.wt.). The C3b fragment displays a short-lived activity, which allows it to bind to suitable surfaces such as cell membranes, polysaccharides and immune complexes (Müller-Eberhard & Biro, 1963; Müller-Eberhard *et al.*, 1966; Law & Levine, 1977; Capel *et al.*, 1978; Takahashi *et al.*, 1980; Mann *et al.*, 1980; Sim *et al.*, 1981). The same phenomenon, i.e. binding to surface acceptor sites, has also been described for complement component C4 immediately after its activation by bound subcomponent C1s to yield C4a and C4b (Dalmasso & Müller-Eberhard, 1964; Müller-Eberhard & Lepow, 1965; Cooper & Müller-Eberhard, 1968; Goers & Porter, 1978; Campbell *et al.*, 1980). The acceptor binding activity of freshly activated C3b and C4b is lost very rapidly in solution, and a half-life of approx. 25 μ s can be calculated for the C3b reactive intermediate generated from C3 by Sepharose–trypsin (Sim *et al.*, 1981).

The interaction between freshly activated C3b and

Abbreviations used: the nomenclature of the complement components and subcomponents is that recommended by the World Health Organisation (1968); IgG, immunoglobulin G; F(ab')₂, *N*-terminal half of each of the heavy chains plus the two light chains from peptic digestion of IgG; Fd, *N*-terminal half of the heavy chain of IgG; Fc, *C*-terminal half of the heavy-chain dimer; SDS, sodium dodecyl sulphate.

an acceptor surface was initially considered to be entirely hydrophobic in nature (Dalmasso & Müller-Eberhard, 1967; Müller-Eberhard, 1975; Capel *et al.*, 1978). However, recent evidence has suggested that C3b and C4b may be covalently linked to the surface of an activating particle by an ester or an amide bond, the carbonyl group of which is provided by the complement fragment (Law & Levine, 1977; Law *et al.*, 1979a,b; Campbell *et al.*, 1980; Sim *et al.*, 1981; Howard, 1980). Campbell *et al.* (1980) used partially purified components of the classical pathway to show that there was probably a covalent bond formed between the α' -chain of freshly activated C4b and the heavy chain of IgG during classical-pathway activation by immune aggregates. In the present study we have examined the binding of C3b to immune aggregates by using whole serum as a source of C3, under conditions allowing only activation of the alternative pathway to take place. Therefore the possibility of C4b binding is excluded, which allows a clearer interpretation of the results involving C3. It was found that C3b, in common with C4b, could be bound, probably covalently, via its α' -chain to the Fd portion of the heavy chain of IgG, but that certain aspects of C3b binding differed from that found with C4b, i.e. in terms of sensitivity of the binding to amines and the ability of the activated component to form dimers.

Materials and methods

Materials

Human serum was prepared from fresh plasma by the addition of CaCl_2 and stored at -70°C . Before use it was either made 10 mM with respect to EGTA and 5 mM with respect to MgCl_2 or dialysed against 100 mM-NaCl/10 mM-EGTA/5 mM- MgCl_2 /10 mM-Tris/HCl, pH 7.4.

The sources of the chemicals used are described by Campbell *et al.* (1980).

Preparation of antibody-antigen aggregates

Pooled rabbit anti-ovalbumin serum (Porter, 1955) was heated to 56°C for 60 min and the IgG fraction isolated by a modification of the method of Wilkinson (1969) by using DEAE-cellulose 32 chromatography. F(ab')_2 fragments of the anti-ovalbumin IgG were prepared by pepsin digestion (Reid, 1971). Ovalbumin-anti-ovalbumin aggregates were prepared at equivalence and stored in 100 mM-NaCl/10 mM-Tris/HCl, pH 7.4, in the presence of 10 mM-di-isopropyl phosphorofluoridate. Before use, the immune aggregates were washed extensively in 100 mM-NaCl/10 mM-EGTA/5 mM- MgCl_2 /10 mM-Tris/HCl, pH 7.4, and the protein measured by amino acid analysis.

Complement components

C3 was purified from outdated human plasma by the method of Tack & Prahl (1976) or by a modification of this method (Sim *et al.*, 1981). Guinea-pig C2 was prepared by modification of the method described for human C2 (Kerr, 1979).

Haemolytic assay of complement components

Complement assay buffers [glucose/saline (0.45% NaCl)/sodium barbitone/gelatin and saline (0.9% NaCl)/veronal/gelatin] were used (Nelson *et al.*, 1966). The haemolytic activity of C1 was determined by the method of Borsos & Rapp (1967), and of C3 as described by Kerr (1980).

Radioiodination procedures

Radioiodination of C3, anti-ovalbumin IgG and F(ab')_2 preparations with ^{125}I was performed with chloramine-T (Sim *et al.*, 1981; Campbell *et al.*, 1980). C3 was labelled to 0.04 g-atom of ^{125}I /mol of C3, IgG to 0.03 g-atom of ^{125}I /mol of IgG and F(ab')_2 to 0.02 g-atom of ^{125}I /mol of F(ab')_2 .

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-slab-gel and -disc-gel electrophoresis was performed by the same procedures as used by Campbell *et al.* (1980). To calibrate gels for molecular-weight estimations under reducing conditions the following protein markers were used: reduced C4b binding protein (mol.wt. 67 500); $\beta_1\text{H}$ (mol.wt. 150 000), C3 (mol.wt. 115 000 and 70 000 respectively); bovine serum albumin (mol.wt. 68 000); ovalbumin (mol.wt. 44 500); α_2 -macroglobulin (mol.wt. 185 000). Under non-reducing conditions the following markers were used: IgM (mol.wt. 900 000); C4b binding protein (mol.wt. 565 000); α_2 -macroglobulin (mol.wt. 370 000); C3 (mol.wt. 186 000); $\beta_1\text{H}$ (mol.wt. 150 000).

Results

Optimal conditions for the binding of C3b to immune complexes after the activation of the alternative pathway in human serum

Conditions allowing only activation of the alternative pathway of complement in human serum were created by the addition of EGTA and MgCl_2 to final concentrations of 10 mM and 5 mM respectively, or by dialysis into 100 mM-NaCl/10 mM-EGTA/5 mM- MgCl_2 /10 mM-Tris/HCl, pH 7.4, before the addition of the serum to the immune aggregates. The addition of EGTA and Mg^{2+} prevents the activation of the classical pathway of complement by blocking utilization of C1, but allows alternative-pathway activation to take place (Platts-Mills & Ishizaka, 1974; Gadd & Reid, 1981). Ovalbumin-anti-ovalbumin aggregates (0.2–0.5 nmol of antibody) formed with either IgG or F(ab')_2 rabbit antibody were incu-

Table 1. *Binding of C3b to immune aggregates after activation of the alternative pathway of complement in human serum*
Ovalbumin-antiovalbumin aggregates (0.2–0.5 nmol) formed with either IgG or F(ab')₂ rabbit antibody were washed twice with 100 mM-NaCl/10 mM-EGTA/5 mM-MgCl₂/10 mM-Tris/HCl, pH 7.4, and incubated with human serum (220–550 μ l) made 10 mM and 5 mM with respect to EGTA and MgCl₂ and containing ¹²⁵I-labelled C3 (10 μ g/ml) for 15 min at 37°C. The mixture was centrifuged and the aggregates washed as described in the text. The C3b bound was determined by counting ¹²⁵I radioactivity after transferring the samples to clean tubes. Abbreviation used: ND, not determined.

Ratio of C3 molecules to antibody molecules present	No. of C3b molecules bound/1000 IgG molecules	No. of C3b molecules bound/1000 F(ab') ₂ molecules
0.5	35	14
1.0	85	38
2.0	156	168
5.0	265	185
7.5	ND	189
8.0	259	ND
10.0	160	50

Table 2. *Binding of C3b to immune aggregates under various incubation conditions*

Ovalbumin-antiovalbumin aggregates (0.2–0.5 nmol) formed with either IgG or F(ab')₂ rabbit antibody were washed twice with 100 mM-NaCl/10 mM-EGTA/5 mM-MgCl₂/10 mM-Tris/HCl buffer, pH 7.4, and then incubated with a 6-fold molar excess of C3, present in human serum containing ¹²⁵I-labelled C3 (10 μ g/ml) and EDTA or EGTA and MgCl₂, as described below. At the end of the incubations, ice-cold buffer was added and the samples centrifuged at 2000g for 10 min. After the aggregates had been washed three times, they were transferred to fresh tubes; the amount of C3b bound was determined by counting for ¹²⁵I radioactivity.

Incubation conditions	No. of C3b molecules bound/1000 IgG molecules	Aggregate solubilized (%)	No. of C3b molecules bound/1000 F(ab') ₂ molecules	Aggregate solubilized (%)
10 mM-EGTA/5 mM-MgCl ₂ ; 15 min at 37°C	183	15	150	9
10 mM-EGTA/5 mM-MgCl ₂ ; 18 h at 4°C	10	4	12	0
10 mM-EDTA; 15 min at 37°C	5	1	2	1

bated in EGTA-treated human serum (220–550 μ l) containing ¹²⁵I-labelled C₃ (10 μ g/ml) for 15 min at 37°C. After the incubation period the mixture was centrifuged and the immune aggregates washed three times with 100 mM-NaCl/10 mM-EGTA/5 mM-MgCl₂/10 mM-Tris/HCl, pH 7.4 (220–550 μ l) and the amount of C3b bound to them determined by counting ¹²⁵I radioactivity (Table 1). Immune aggregates containing either IgG or F(ab')₂ rabbit antibody were found to bind radiolabelled C3 (in the form of C3b as shown below) and form a stable complex after activation of the alternative pathway of human complement.

The relative amounts of C3 and antibody molecules present during each incubation were calculated by assuming that the serum concentration of C3 was 1.2 g/litre and that it had a mol.wt. of 186 000. Optimal C3b binding occurred when a ratio of five to eight C3 molecules to every antibody molecule present in the incubation mixture (Table 1) was used. Under optimal conditions, 2–3% of the C3 available became bound after the 15 min incubation

at 37°C. A ratio of six C3 molecules to one antibody molecule was used in all subsequent labelling experiments.

Negligible amounts of radiolabelled C3b were bound to the immune aggregates when the incubation was performed in human serum made 10 mM with respect to EDTA to prevent classical- and alternative-pathway activation (Table 2; Fig. 2a below). Activation of the alternative pathway is temperature-dependent, no activation occurring at 4°C (Reid, 1971). Immune aggregates formed by using either IgG or F(ab')₂ rabbit antibody were incubated in human serum, treated with EGTA and MgCl₂ to give final concentrations of 10 mM and 5 mM respectively for 18 h at 4°C. No significant binding of radiolabelled C3 took place under these conditions (Table 2). No losses of C1 haemolytic activity were detected when using human serum treated with EGTA and MgCl₂ or with human serum dialysed against buffer containing EGTA and MgCl₂.

Solubilization of the immune aggregates during

the incubation period was monitored by using aggregates prepared with ^{125}I -labelled IgG or F(ab')_2 antibody. Under the conditions routinely used, i.e. immune aggregates incubated with serum for 15 min at 37°C with a ratio of six C3 molecules to one antibody molecule present, the losses of immune aggregates due to their solubilization was low, 85–90% of the aggregates originally present being recovered after the incubation (Table 2).

Electrophoresis of samples of IgG, or F(ab')_2 , immune aggregates on SDS/polyacrylamide slab gels, after incubation in human serum containing

radiolabelled C3, EGTA and Mg^{2+} , showed that only small amounts of material could be seen in the expected positions of the C3 α' -chain or intact C3b in the reduced and non-reduced samples respectively after staining with Coomassie Blue (Fig. 1) or on radioautography (Fig. 2). However, in these samples, high-molecular-weight bands were present (bands 1, 2 and 1', 2' in Figs. 1 and 2) that were not seen in the control samples, i.e. in samples of immune aggregates that had been incubated with human serum containing radiolabelled C3 and 10 mM-EDTA (Fig. 2a, track iii). In the case of the

Fig. 1. SDS/polyacrylamide slab gels, stained with Coomassie Blue, showing the binding of high-molecular-weight material to immune aggregates after incubation with human serum

Immune aggregates containing ovalbumin and either IgG or F(ab')_2 rabbit antibody were incubated with human serum under conditions allowing only alternative-pathway activation, exactly as described in the text. After incubation, the washed aggregates were run under (a) non-reducing conditions, or (b) after reduction and alkylation, on SDS/7% (w/v) and 8.5%-polyacrylamide slab gels respectively. The gels were stained with Coomassie Blue. The samples run in the tracks shown in (a) and (b) were: (i) C3 + C3b marker proteins; (ii) control aggregates containing IgG; (iii) aggregates containing IgG after incubation with human serum in the presence of EGTA and Mg^{2+} ; (v) C3 + C3b marker proteins; (v) control aggregates containing F(ab')_2 ; (vi) aggregates containing F(ab')_2 after incubation with human serum in the presence of EGTA and Mg^{2+} .

Fig. 2. Radioautograph of SDS/polyacrylamide slab gels showing the binding of C3b to immune aggregates on incubation with human serum

Immune aggregates containing ovalbumin and either IgG or F(ab')₂ rabbit antibody were incubated with human serum containing ¹²⁵I-radiolabelled C3, under conditions allowing only activation of the alternative pathway, as described in the text. After incubation the washed aggregates were run under (a) non-reducing conditions, or (b) after reduction and alkylation, on SDS/7.5% (w/v)-polyacrylamide slab gels. The samples in the tracks shown in (a) are: (i) C3 marker; (ii) IgG immune aggregates after incubation with human serum containing EGTA and Mg²⁺; (iii) IgG immune aggregates after incubation with human serum containing EDTA; (iv) F(ab')₂ immune aggregates after incubation with human serum containing EGTA and magnesium. The samples in the tracks shown in (b) are: (i) C3 marker; (ii) IgG immune aggregates after incubation with human serum containing EGTA and Mg²⁺.

aggregates containing IgG, most of the radioactivity bound to these aggregates is located in material of higher molecular weight than the intact C3b, as judged from the non-reduced sample (Fig. 2a, track ii) and that although the β-chain of C3 is clearly seen in the reduced sample, the α'-chain is missing and two major bands of higher apparent molecular weight are present (Fig. 2b, track ii). Since these experiments were performed in whole serum it is probable that limited degradation of the α'-chain of bound C3b by the action of β₁H, C3b inactivator and other enzymes will take place. Such degradation would be expected to lead to the production of radiolabelled bands of lower molecular weights than the bands 1, 2, 1' and 2' seen in Figs. 1 and 2. Thus the material running near the C3 and C3b positions on the gels of non-reduced samples (Figs. 1a and 2a), of aggregates that had been incubated with human serum in the presence of EGTA and Mg²⁺, may not be intact C3 or C3b but a portion of degraded C3b (e.g. a portion of the α'-chain) bound to IgG. Results that would support this suggestion are: the material near

the C3 + C3b positions is of a slightly higher apparent molecular weight than are the C3 and C3b marker proteins (Fig. 2a, track ii); reduction and alkylation of this radiolabelled material, found near the C3 + C3b positions, does not yield significant amounts of the α- or α'-chain of C3 (Fig. 2b, track ii); the band in the high-molecular-weight band-1' position (Fig. 2b, track ii) is broad, indicating some heterogeneity in size; prolonged incubation periods (up to 60 min) result in a decrease in the amount of label found in the two principal high-molecular-weight bands and an increase in bands of lower molecular weight; incubation of the aggregates in serum containing EDTA rather than EGTA and Mg²⁺ results in no significant binding of radiolabelled C3 (Fig. 2b, track ii).

Generation of C3-cleaving activity on the surface of immune aggregates and its correlation with C3b binding

Fujita *et al.* (1977) reported that a C3-cleaving activity was formed on the surface of immune

aggregates containing ovalbumin and $F(ab')_2$ rabbit antibody after incubation in human serum. This observation has been confirmed and correlation made between C3b binding to the immune aggregates after alternative-pathway activation and the formation of a C3-cleaving activity on the aggregates.

Standard incubation conditions were used, 0.7 nmol of antibody being present in each sample of aggregates. Immune aggregates were washed with 100 mM-NaCl/10 mM-EGTA/5 mM-MgCl₂/10 mM-Tris/HCl, pH 7.4, and incubated for different lengths of time with human serum (790 μ l) containing EGTA and MgCl₂ at final concentrations of 10 mM and 5 mM respectively and ¹²⁵I-labelled C3 (10 μ g/ml). The incubations were stopped by the addition of ice-cold buffer and the aggregates were washed twice, before being transferred to a clean tube and counted for ¹²⁵I radioactivity to determine the amount of C3b bound. Purified C3 (50 μ g) in iso-osmotic 5 mM-veronal buffer, pH 7.5 (100 μ l), containing 1 mM-MgCl₂ and 0.3 mM-CaCl₂, was then added to each sample, and the incubation continued for 30 min at 30°C. The samples were then centrifuged at 2000 *g* for 10 min and a portion of the supernatant taken for SDS/7.5% (w/v)-polyacrylamide-slab-gel electrophoresis run under reducing conditions. After staining with Coomassie Blue the percentage of C3 which had been inactivated was determined by gel scanning. Loss of C3 haemolytic activity was also determined and very similar results were obtained by both methods (Table 3). Control experiments in which immune aggregates were incubated in human serum containing 10 mM-EDTA, or an equivalent volume of buffer, followed by incubation with C3, showed no inactivation of the C3 added.

The C3-cleaving activity formed by the alternative-pathway components bound to the surface of the immune aggregates showed a correlation with

C3b binding, although other components of the alternative pathway are also involved. The C3-cleaving activity was lost after prolonged incubation of the aggregates in human serum (Table 3) or by repeated washing of the serum-treated aggregates with 100 mM-NaCl/10 mM-EGTA/5 mM-MgCl₂/10 mM-Tris/HCl, pH 7.4, although the latter treatment had a negligible effect on the C3b bound to them. Preliminary results indicate that the loss of activity is due to the decay of factor B from the surface of the aggregates, since addition of purified factor B, followed by washing with buffer, allowed the regeneration of C3-cleaving activity on the immune aggregates.

Nature of the attachment of C3b to the antibody-antigen aggregates

The results presented above indicate that the interaction between C3b and the immune aggregates is very strong. The interaction is not disrupted by repeated washing ten times with 100 mM-NaCl/10 mM-EGTA/5 mM-MgCl₂/10 mM-Tris/HCl, pH 7.4, only 3–4% of the C3b which initially bound being removed by this treatment (this represents an average of five to eight C3b molecules removed/1000 antibody molecules present in the aggregates). Washing the aggregates containing bound C3b with high-salt buffer (1 M-NaCl/10 mM-EDTA/10 mM-Tris/HCl, pH 7.4), high (0.7 M-NaCl/20 mM-EDTA/50 mM-Tris/HCl, pH 10) and low (1 M-NaCl/20 mM-EDTA/50 mM-sodium acetate, pH 4.5) pH buffers, or with the non-ionic detergent Triton X-100 (1%, v/v), had a negligible effect on the C3b-antibody interaction (less than 4% of the bound C3b being removed). When aggregates containing ¹²⁵I-labelled antibody were used as controls, it was found that the conditions described above caused no disruption of antibody-antigen interactions.

The C3b-antibody interaction was also largely unaffected by boiling in 8 M-urea/2% (w/v) SDS/

Table 3. *Binding of C3b and formation of an alternative-pathway C3-cleaving activity on the surface of antibody-antigen aggregates*

Ovalbumin-anti-ovalbumin immune aggregates, containing either IgG or $F(ab')_2$ rabbit antibody (0.7 nmol), were incubated with human serum (790 μ l) containing 10 mM-EGTA, 5 mM-MgCl₂ and ¹²⁵I-labelled C3 (10 μ g/ml) for various time periods. The binding of C3b and formation of C3-cleaving activity were determined as described in the text.

Time (min)	No. of C3b molecules bound/ 1000 IgG molecules	C3 inactivated (%)	No. of C3b molecules bound/ 1000 $F(ab')_2$ molecules	C3 inactivated (%)
0	0	0	0	0
1	2	0	5	0
2	48	15	53	18
5	117	60	55	36
10	204	67	78	50
15	210	80	152	90
30	196	35	151	82
60	189	5	128	36

0.2M-Tris/HCl, pH 8.0, in the presence of dithiothreitol or iodoacetamide, as judged by the appearance of high-molecular-weight complexes after SDS/7.5% (w/v)-polyacrylamide-gel electrophoresis and radioautography of C3b-aggregate samples (Fig. 2). These conditions were used to prepare the samples for SDS/polyacrylamide-gel electrophoresis in the experiments described below.

Immune aggregates containing bound C3b were incubated with buffers known to disrupt ester bonds linking C3b to other surfaces (Sim *et al.*, 1981), e.g. 1% (w/v) SDS/1M-hydroxylamine, pH 10, or 50mM-diethanolamine/100mM-NaCl, pH 11.5, for 60min at 37°C. The whole sample was then prepared for SDS/7.5% (w/v)-polyacrylamide-gel electrophoresis under reducing conditions. After staining with Coomassie Blue, the major high-molecular-weight species that were present were cut out, counted for C3-derived ^{125}I radioactivity and the results compared with those from a control sample incubated for 60min at 37°C with 1% (w/v) SDS and electrophoresed under identical conditions. Losses of 26 and 22% of ^{125}I radioactivity were found for C3b bound to IgG or F(ab')_2 immune aggregates incubated in the diethanolamine and hydroxylamine buffers respectively. If C3b were interacting with the aggregates by means of an ester bond, then a nearly 100% loss of C3 ^{125}I radioactivity would have been expected under these conditions.

Site of interaction of C3b with the antibody molecule

C3b bound to immune aggregates containing ^{125}I -labelled IgG was prepared by using the standard incubation conditions, washed three times with 100mM-NaCl/10mM-EGTA/5mM-MgCl₂/10mM-Tris/HCl, pH 7.4, and electrophoresed, without reduction, on an SDS/7.5% (w/v)-polyacrylamide slab gel. The two major high-molecular-weight bands containing radioactivity due to IgG, along with the IgG bands, were isolated and eluted from the gel as described previously (Campbell *et al.*, 1980; the present Fig. 3). The samples were then freeze-dried, re-run on an SDS/10% (w/v)-polyacrylamide slab gel, under reducing conditions, and the ratio of the distribution of radioactivity between the heavy and light chains was determined (Fig. 3). The results were compared with those obtained from a standard radiolabelled IgG sample also eluted from the SDS/7.5% (w/v)-polyacrylamide slab gel under reducing conditions and re-run on an SDS/10% (w/v)-polyacrylamide slab gel.

The ratio of radioactivity between the heavy and light chains of IgG was 1.90:1.00 (Fig. 3) in samples of IgG that had not been incubated with serum or in samples of IgG that migrated with the expected apparent mol.wt. of 150000 on SDS/polyacrylamide-gel electrophoresis after incubation

with human serum (i.e. contained no bound C3b) (Fig. 3). When the two C3b-IgG complexes were eluted and analysed, the ratios of radioactivity between the heavy and light chains were found to be 0.96:1.00 and 0.61:1.00 respectively (Fig. 3) and radioactivity, which could account for the amount lost from the heavy chains, was found in bands of higher molecular weight than that of the heavy chain (Fig. 3). These results, taken with the previous observations, suggest that the C3b in the C3b-IgG complexes is bound to the Fd region of the heavy chain of the antibody molecule through the α' -chain of C3b.

Molecular-weight estimations of the unreduced and reduced and alkylated C3b-IgG and C3b-F(ab')₂ complexes

The two main high-molecular-weight species (Figs. 1 and 3) were found to have apparent mol.wts., on SDS/polyacrylamide-gel electrophoresis, of 360000 and 580000 for the C3b-IgG complexes and 310000 and 470000 for the C3b-F(ab')₂ complexes when unreduced. Reduced samples were calculated to have apparent mol.wts. of 150000 and 250000 for the high-molecular-weight complexes from IgG aggregates (Fig. 3) and 135000 and 190000 for the high-molecular-weight complexes from F(ab')₂ aggregates.

The estimates obtained indicated that the two species, in both cases, were composed of one or two C3b molecules respectively, in association with a single antibody IgG or F(ab')₂ molecule. The expected molecular weights for complexes of the compositions C3b-IgG, (C3b)₂-IgG, C3b-F(ab')₂, (C3b)₂-F(ab')₂ were approx. 330000, 510000, 270000 and 450000 respectively for non-reduced samples. For reduced samples, complexes of the compositions α' -H-chain, (α')₂-H-chain, α' -Fd, (α')₂-Fd would be expected to have mol.wts. of 160000, 270000, 135000 and 245000 respectively.

Inhibition of C3b binding to immune complexes by the addition of inhibitors to serum

Of the amines tested, only putrescine and cadaverine were found to show significant inhibition of C3b binding to immune aggregates. Methylamine, ethylenediamine, propylamine and butylamine showed no more than 10% inhibition under the conditions used, at concentrations of up to 100mM. Increasing concentrations (0-100mM) of putrescine and cadaverine were added to human serum (790 μl) containing 10mM-EGTA/5mM-MgCl₂ and ^{125}I -radiolabelled C3 (10 $\mu\text{g/ml}$), before the addition of immune aggregates (containing 0.7nmol of IgG antibody). The incubation and washing procedure was performed as described above, and the amount of C3b bound determined by counting the washed aggregates for ^{125}I radioactivity. By comparison with

Fig. 3. Radioautograph of C3b- 125 I-labelled IgG aggregates

The method of Campbell *et al.* (1980) was used to determine the ratio of 125 I-labelling in each of the species shown above. Samples of 125 I-labelled-antibody-antigen aggregates with C3b bound were run in duplicate under non-reducing conditions on an SDS/7.5% (w/v) polyacrylamide slab gel. One duplicate was stained for protein, dried down and radioautographed; a line drawing of the radioautograph is shown. The other duplicate was cut into 2 mm strips and counted for radioactivity. Slices containing the C3b-IgG complexes and IgG were fragmented and were eluted with 0.5 ml of 20 mM-dithiothreitol at room temperature for 20 h. The extract and two washes with 0.25 ml of the same solution were combined, freeze-dried, and then electrophoresed under reducing conditions on an SDS/10% (w/v)-polyacrylamide slab gel. The gel was stained for protein, dried down and radioautographed; a line drawing of the radioautograph is shown. The bands containing 125 I-labelled material were cut out and counted for radioactivity. The radioactivity measured in each band ('c.p.m.') is shown and the ratio ('Ratio') of the counts in each band was calculated by assuming a light-chain value of 1.00.

control samples in which the immune aggregates had been incubated with an identical concentration of putrescine or cadaverine, washed three times and then incubated with normal human serum, containing 10 mM-EGTA/5 mM-MgCl₂ and 125 I-radio-labelled C3, it was found that 50% inhibition of C3b binding was observed at concentrations of 55 mM-putrescine and 30 mM-cadaverine. These concentrations are very high compared with the value of approx. 4 mM found to inhibit C4b binding to immune aggregates under the same conditions (Campbell *et al.*, 1980). Recent work has shown that, in the procedure used in the present study, putrescine and cadaverine at concentrations of approx. 40 mM cause inhibition of the cleavage of factor B by factor D in the presence of C3 and Mg²⁺ (K. J. Gadd, unpublished work). Thus the inhibition of C3b binding observed may be primarily due to inhibition of factor D activity rather than directly to interference with the binding of freshly activated C3b to IgG.

As expected from the results obtained by Sim *et al.* (1981), who studied the inhibition of binding freshly

activated C3 to Sepharose-trypsin, it was found that salicylhydroxamic acid is a more potent inhibitor of C3b binding than is putrescine or cadaverine. By using the experimental conditions described above, 50% inhibition of C3b binding to IgG immune aggregates was observed at a concentration of 0.8 mM-salicylhydroxamic acid.

Although the results obtained above showed that putrescine had little effect on the binding of C3b to immune aggregates, to confirm that this reaction was not a result of transglutaminase activity (Chung *et al.*, 1974; Folk & Finlayson, 1977), 20 mM-iodoacetamide was tested for inhibition with the experimental conditions described above. No inhibition of C3b binding was observed with iodoacetamide, indicating that a transglutaminase-catalysed reaction is probably not involved in the binding of C3b to immune aggregates.

Discussion

It is well established that, on activation of the complement system by immune aggregates, several

components, C1, C4 and C3, become tightly bound to the aggregates (Heidelberger, 1941; Müller-Eberhard & Biro, 1963; Müller-Eberhard & Lepow, 1965; Takahashi *et al.*, 1977; Goers & Porter, 1978; Campbell *et al.*, 1980). The C1 subcomponents C1r and C1s are readily removed by treatment with neutral iso-osmotic buffers, containing EDTA, and are considered to be linked to the aggregates primarily via the bound C1q (Dodds *et al.*, 1978; Arlaud *et al.*, 1979). Subcomponent C1q is more strongly bound, since buffers of low or high pH and containing a high salt concentration are required to bring about its complete removal from the aggregates (Arlaud *et al.*, 1979). Subcomponent C1q is bound non-covalently to a site in the C_H2 domain of the Fc region of the IgG molecule (for review, see Burton *et al.*, 1980), whereas activated C4 is considered to be bound via its α' -chain, probably covalently, to a site in the Fab region of the IgG molecule (Chan & Cebra, 1968; Goers & Porter, 1978; Campbell *et al.*, 1980). Before the present study, little was known about the nature and location of the binding of activated C3 to IgG in immune aggregates, although it is well established that freshly activated C3 can bind via a labile site on its α' -chain, probably by means of an ester bond, to the surface of various cells or particles (Law & Levine, 1977; Law *et al.*, 1979a,b; Sim *et al.*, 1981).

It has been shown that immune aggregates containing IgG or F(ab')₂ antibody caused exactly the same extent of utilization of alternative-pathway components, as judged by haemolytic assays, when incubated with human serum under conditions allowing only alternative-pathway activation (Gadd & Reid, 1981). These observations show that there is no involvement of the Fc region of IgG in the activation of complement, as judged by functional studies, when only alternative-pathway activation is permitted. Both IgG- and F(ab')₂-containing aggregates showed similar characteristics of C3b binding and ability to form a surface-bound C3-cleaving activity after incubation in human serum containing EGTA and Mg²⁺ (Tables 1 and 3). These results indicate that the site involved in C3b binding is probably located in the Fab region of the IgG molecule. Approx. 2–3% of the C3 available during the incubation was bound to this region of the molecule under the optimal conditions established (Table 1), and during this binding there was no classical-pathway activation and only a low amount of solubilization of the immune aggregates (Table 2).

SDS/polyacrylamide-gel electrophoresis of the reduced and alkylated C3b–IgG aggregates samples showed, on the Coomassie Blue-stained gel, that the β -chain of the C3b molecule was easily seen in its expected position, whereas only a minor band was present in the expected position of the α' -chain, and that two major high-molecular-weight bands (Fig. 1)

that contained ¹²⁵I-labelled material derived from C3 (Fig. 2) or IgG (Fig. 3) were present. Unidentified high-molecular-weight bands were also observed when C3b–IgG aggregate samples were electrophoresed under non-reducing conditions (Figs. 1–3). In the non-reduced samples, low-intensity bands were seen in the expected positions for C3b and for uncleaved C3 (Figs. 1a and 2a). The intensity of these bands could be decreased slightly by extensive washing with buffers containing high concentrations of salt, and it is possible, therefore, that these bands may be due to the non-specific binding of uncleaved C3 or C3b to the aggregates. However, it was observed that, in the presence of EDTA, there was a complete absence of C3 binding as judged from the radioautographs (Fig. 2, track iii), therefore it seems probable that these bands are produced by degradation of C3b bound to IgG, as was considered in the Results section.

By a comparison of the ratio of radioactivity distributed between the heavy and light chains derived from the reduced samples of ¹²⁵I-labelled IgG and the two major high-molecular-weight ¹²⁵I-labelled species (Fig. 3), it was established that in both species the α' -chain of the C3b molecule was bound to the heavy chain of IgG. An identical experiment could not be performed with immune aggregates containing F(ab')₂, owing to the poor separation of the Fd and light chain on SDS/polyacrylamide-slab-gel electrophoresis. However, it seems probable that C3b bound to these aggregates is also bound to the heavy chain of the antibody molecule, which indicates that the bond formed between C3b and IgG involves the α' -chain of C3b and the Fd region of the heavy chain of IgG.

The apparent molecular weights calculated for the two major high-molecular-weight species show that they are probably formed by the interaction of one or two C3b molecules with one antibody molecule. Other high-molecular-weight species were also found in lower amounts and were not studied, but they may have been formed by the degradation of the aggregate-bound C3b by the action of β_1 H, C3b inactivator and other enzymes. This was partially confirmed by the observation that these other high-molecular-weight species increased on prolonged (up to 60 min) incubation at 37°C, and some had lower apparent molecular weights than the C3b–IgG complex. The importance of a species thought to be composed of two C3b molecules bound to one antibody molecule is not clear, but Fujita *et al.* (1977) have demonstrated the presence of a C5-cleaving activity on the surface of F(ab')₂ immune aggregates after incubation in human serum, and C3b binding has been postulated to be essential in the generation of a C5 convertase enzyme from the C3 convertase (Vogt *et al.*, 1978; Janatova *et al.*, 1980b). The binding of C3b to the

Fd region of IgG antibody and its participation in the formation of a C3 convertase, may be of biological significance when considering those subclasses of IgG antibody that do not activate the classical pathway, for example the guinea-pig γ_1 IgG antibody (Sandberg *et al.*, 1971).

The interaction between C3b and the heavy chain of the antibody molecule would appear to be covalent, as judged by its stability (Table 4, Fig. 3). Law *et al.* (1979a) have suggested that activation of C3 results in the formation of a reactive acyl group in C3b, which can form an ester bond in the presence of a suitable activating surface, e.g. polysaccharides of the erythrocyte cell wall and zymosan. This has also been proposed by Sim *et al.* (1981) to account for the interaction between activated C3 and Sepharose-trypsin. The stability of the C3b-antibody interaction to treatment with high-pH buffers, diethanolamine and hydroxylamine as compared with the extreme lability of the C3b-polysaccharide interaction to these treatments (Sim *et al.*, 1981) implies that although the two mechanisms of interaction are likely to be very similar, the C3b-antibody interaction is unlikely to be due to the formation of an ester bond. The results presented here suggest that that active acyl group released from the thiol-ester bond present in native C3 (Tack *et al.*, 1980; Janatova *et al.*, 1980a) reacts with an amino acid in the Fd region of the antibody molecule and not with a carbohydrate moiety. However, this suggestion is not consistent with the finding that polysaccharides and glycopeptides isolated from rabbit IgG can inhibit the binding of C3b to Sepharose-trypsin (Capel *et al.*, 1978). Clearly further work is required to elucidate the precise nature of the C3b-antibody interaction.

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