The binding of human complement proteins C5, factor B, β_1 H and properdin to complement fragment C3b on zymosan

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The covalent binding of complement fragment C3b to zymosan by the action of the alternative-pathway C3 convertase and the reversible binding of several complement proteins (component C5, factor B, β_1 H and properdin) to C3b on zymosan have been investigated. When C3b is deposited on zymosan after activation by a surface-bound C3 convertase, the C3b molecules are deposited in foci around the C3 convertase site, with an average of 30 C3b molecules per site. The association constants of C5, factor B, β_1 H, and properdin for C3b bound to zymosan have been determined. The association constants ranged from $6.5 \times 10^{-5} \text{ M}^{-1}$ for factor B to $2.9 \times 10^7 \text{ M}^{-1}$ for properdin. An approximate stoichiometry of 1:1 for C5, factor B, and properdin binding to C3b has been observed. Curvilinear Scatchard plots were observed for β_1 H binding to C3b, with the maximal extrapolated ratio of β_1 H to C3b of 0.32. Physiological amounts of properdin increase by 7-fold the affinity constant for factor B binding to C3b with no alteration in the stoichiometry. Similarly, physiological amounts of factor B increase the affinity constant of properdin to C3b about 4-fold with only a small measured difference in stoichiometry. Competition binding studies and protein modification suggest that C5, factor B, β_1 H, and properdin each bind to a distinct region on C3b.

Complement component C3 is a two-chain plasma glycoprotein of mol.wt. 187 000 and it is the central protein of the complement system (Tack & Prahl, 1976; Müller-Eberhard & Schreiber, 1980). When C3 is activated by C3 convertase, the nascent complement fragment C3b can apparently bind to surfaces (such as yeast cell walls, cell membranes and Sepharose beads) by a covalent bond that has been proposed to be an ester (Law & Levine, 1977; Law *et al.*, 1979b). Other lines of evidence have led to the suggestion that native C3 contains an intrachain thioester within its structure. During activation, this thioester becomes exposed and available for reaction with water or surface hydroxy groups (Sim *et al.*, 1981; Janatova *et al.*, 1980; Tack *et al.*, 1980).

When bound to a surface by the ester bond, C3b can form part of the complex proteinases of the classical-pathway C5 convertase, C4b2a2b3b (Müller-Eberhard, 1975; Cooper & Müller-Eberhard, 1970), the alternative-pathway C3 convertase,

C3bBbP , and the alternative-pathway C5 convertase, $\text{C3b}_n\text{BbP}$ (Müller-Eberhard & Götze, 1972; Medicus *et al.*, 1976; Vogt *et al.*, 1978). Furthermore, covalently bound C3b can interact with receptors on membrane of primate erythrocytes, polymorphonuclear leukocytes, B-lymphocytes, and monocytes (Dierich & Bokisch, 1977; Bianco *et al.*, 1970; Lay & Nussenzweig, 1968; Ross & Polley, 1975). Thus bound C3b is an essential component of the immune system and its activities depend on its ability to bind complement factors and cell-membrane receptors.

Vogt *et al.* (1978) observed that covalently bound C3b can bind component C5, and Isenman *et al.* (1980a) proposed that C5 may bind C3b in a multivalent fashion. Properdin, which is a stabilizer of the alternative-pathway C3/C5 convertase, was shown to bind to C3b (Medicus *et al.*, 1976); however, neither the affinity nor the stoichiometry of the association of properdin with C3b was established. Kazatchkine *et al.* (1979) have studied the binding of factor B and β_1 H to bound C3b, and they have found differences in the degree of binding of β_1 H for activators and non-activators of the alternative pathway. Vogt *et al.* (1977) observed a 1:1 stoichiometry of factor B binding to fluid-phase C3b.

Abbreviations used: IgG, immunoglobulin G; *K*, the equilibrium association constant; SDS, sodium dodecyl sulphate. The nomenclature of complement components is that recommended by the World Health Organisation (1968).

I have investigated further the covalent binding of component C3 to zymosan, and the reversible binding of component C5, factor B, $\beta_1\text{H}$, and properdin to zymosan-bound C3b. The stoichiometries and affinities of these various complement proteins for zymosan bound C3b were determined.

Materials and methods

Materials

Outdated human plasma was obtained from the National Blood Transfusion Service, John Radcliffe Hospital, Oxford, U.K. Lactoperoxidase, *p*-chloromercuribenzoate, 6-aminohexanoic acid, poly(ethylene glycol) 4000, BaCl_2 , and imidazole were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. Sepharose CL6B, DEAE-Sephadex A50, CM-Sephadex C50 were products of Pharmacia Fine Chemicals Ltd., Hounslow, Middx., U.K. Dithiothreitol was obtained from Calbiochem Corp., La Jolla, CA, U.S.A. Benzamidine was a product of Aldrich Chemical Co., Gillingham, Dorset, U.K. Lenzol was obtained from Hopkin and Williams Co., Chadwell Heath, Essex, U.K. Na^{125}I (100 mCi/ml) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Several of the human complement components were purified by adapting and modifying the C3/C5 purification method of Tack & Prahl (1976). The modifications of the C3/C5 preparation were: (a) treatment of 1 litre of outdated human plasma with 40 ml of 1 M- BaCl_2 in order to remove the vitamin K-dependent proteins (DiScipio *et al.*, 1977); (b) substitution of DEAE-Sephadex for DEAE-cellulose (the buffer that was used at this step was 25 mM-imidazole/ H_3PO_4 (pH 7.0)/75 mM-NaCl/20 mM-6-aminohexanoic acid/10 mM-EDTA/2 mM-benzamidine/0.01% NaN_3 /20% (v/v) glycerol); (c) substitution of hydroxyapatite column chromatography by affinity chromatography on C3b-Sepharose to separate C3 and C5; (d) fractionation of C3, C3u and C3b on sulphated Sepharose (Parkes *et al.*, 1981).

The affinity column of C3b-Sepharose was made as follows: to 1 litre of BaCl_2 -treated human plasma containing 5 mM-EGTA and 5 mM- MgCl_2 was added 100 ml of packed Sepharose CL6B. After stirring at 37°C for 20 min, the Sepharose was filtered and rapidly washed with 2 litres of 10 mM-imidazole/HCl (pH 7.4)/0.15 M-NaCl/0.01 M-EDTA at 4°C. Then 500 mg of pure native C3 was added to this Sepharose bearing C3 convertase. The reaction was allowed to proceed for 2 h at 37°C, and the Sepharose was washed with 4 litres of 10 mM-imidazole/HCl (pH 7.4)/0.75 M-NaCl and 2 litres of 10 mM-imidazole/HCl (pH 7.4)/0.15 M-NaCl. By

allowing ^{125}I -labelled C3 to react with a small portion of the activated Sepharose, it was estimated that about 6% of the C3b bound to the Sepharose by an apparent covalent bond (Law & Levine, 1977). As component C5 can bind to covalently bound C3b (Vogt *et al.*, 1978), this C3b-Sepharose absorbent can be used in the affinity purification of component C5.

The C3b-Sepharose column was used as follows. After the gel-filtration step described in the purification method of Tack & Prahl (1976), the mixture of proteins, containing predominantly C3, C5 and IgG (about 350 mg from 1 litre of starting plasma) was dialysed against 15 mM-imidazole/HCl (pH 7.8)/0.03 M-NaCl/2 mM-benzamidine/0.01% NaN_3 , and the protein mixture was passed over the column (2.5 cm $\times$ 14 cm) of C3b-Sepharose. The column was washed with 200 ml of the buffer, and a linear gradient of 150 ml each of 0–0.8 M-NaCl in the same buffer was applied to the column. Only component C5 has been observed to bind to this affinity column, and this protein was eluted off the column by the salt gradient (0.25–0.35 M-NaCl) without loss of haemolytic activity. The yield has been generally about 30%, resulting in about 20 mg of C5. The column can be regenerated by equilibration in the starting buffer.

$\beta_1\text{H}$ and C3bINA were purified as described by Crossley & Porter (1980). Properdin was purified by using as starting material the 5%-poly(ethylene glycol) precipitates of the BaCl_2 -treated human plasma (Tack & Prahl, 1976). This precipitate was resuspended in 25 mM-Tris/ H_3PO_4 (pH 6.6)/20 mM-6-aminohexanoic acid/10 mM-EDTA/0.01% NaN_3 /2 mM-benzamidine/10% (v/v) glycerol. The protein was applied to a column (3.4 cm $\times$ 30 cm) of CM-Sephadex equilibrated in the same buffer. A linear gradient of 400 ml each of 0–0.4 M-NaCl in the same buffer was applied to the column, and the properdin fractions were pooled and dialysed twice against 2 litres of water. The euglobulin was centrifuged at 9500 *g* for 15 min, and the pellet was dissolved in 25 mM-Tris/HCl (pH 8.0)/70 mM-NaCl/2 mM-benzamidine/20 mM-6-aminohexanoic acid/10 mM-EDTA/0.01% NaN_3 . The protein mixture was applied to a column (1.5 cm $\times$ 15 cm) of DEAE-Sephadex that had been equilibrated in the same buffer. The properdin passed through this column, but contaminants were retained. The properdin was passed over a rabbit anti(human IgG) antibody-Sepharose column (2.5 cm $\times$ 20 cm) equilibrated in the same buffer. The properdin passed directly through this column, and it was found to be a pure component on SDS/polyacrylamide gels. The yield was about 15%.

Factor B was purified from the same preparation as components C3, C5 and properdin. The factor B-containing fractions from the DEAE-Sephadex

column were found predominantly in fractions that passed directly through this column. These factor B-containing fractions were diluted by the addition of an equal volume of water, and were applied to a (3.1 cm $\times$ 30 cm) of CM-Sephadex column equilibrated in the same buffer, and the protein was eluted with an 800 ml linear salt gradient (0–0.4 M-NaCl) in the same buffer. The factor B-containing fractions were dialysed against 15 mM-imidazole/HCl (pH 7.3)/0.03 M-NaCl/2 mM-benzamidine, and the protein was applied to a column (1.7 cm $\times$ 20 cm) of benzamidine-Sepharose prepared by the method of DiScipio *et al.* (1977). The factor B was eluted with a linear gradient consisting of 0–0.5 M-NaCl in the same buffer. The factor B fractions were pooled, and passed through a column (1.1 cm $\times$ 10 cm) of soyabean trypsin inhibitor-Sepharose equilibrated in 15 mM-imidazole/HCl (pH 7.3)/0.03 M-NaCl/2 mM-benzamidine. This protein was homogeneous by the criterion of SDS/polyacrylamide-gel electrophoresis. The yield was typically about 30 mg, reflecting a 15% recovery from 1 litre of starting plasma.

Factor D was purified from the same preparation as the other complement proteins. After removal of C3 by precipitation with 12% (w/v) poly(ethylene glycol), the plasma was adjusted to 17% (w/v) poly(ethylene glycol). After centrifugation the pellet, containing factor D, was dissolved in 25 mM-Tris/ H_3PO_4 (pH 6.6)/50 mM-NaCl/10 mM-EDTA/20 mM-6-aminohexanoic acid / 2 mM-benzamidine / 0.01% NaN_3 /10% glycerol and fractionated on a column (3.4 cm $\times$ 30 cm) of CM-Sephadex with a linear salt gradient (0–0.45 M-NaCl). Then the factor D fractions were passed through a column (1.7 cm $\times$ 18 cm) of benzamidine-Sepharose, and fractionated on a column (2.6 cm $\times$ 90 cm) of Sephadex G-75. The factor D showed only a single band on SDS/polyacrylamide gels, and the yield was about 10%.

Zymosan was purified from the commercial product by the method of Law *et al.* (1980).

Preparation of zymosan with covalently bound C3b. Zymosan (9 mg) was washed several times with 10 mM-imidazole/HCl (pH 7.3)/0.15 M-NaCl. C3b (9 ml of a solution of concentration 1 mg/ml) in the same buffer was mixed with the zymosan. The suspension was freeze-dried. Subsequently the material was washed five times with 10 mM-imidazole/HCl (pH 7.3)/0.15 M-NaCl. A small portion, about 1%, of the C3b remained absorbed to the zymosan. Although this non-covalently absorbed C3b is non-specifically associated with the zymosan, a small portion of it is available for factor B binding. To zymosan bearing non-covalently absorbed C3b was added 5.3 μg of properdin, 45 μg of factor B, and 0.3 μg of factor D in a final volume of 270 μl in 10 mM-imidazole/HCl (pH 7.3)/0.15 M-

NaCl/5 mM-MgCl₂. After incubation at 37°C for 20 min, 50 μl of 0.2 M-EDTA was added, the activated zymosan was centrifuged at 9980 g for 1 min and the supernatant was discarded. The pellet was washed once in 300 μl of 10 mM-imidazole/HCl (pH 7.3)/0.075 M-NaCl, transferred to a new tube, and centrifuged. Then 900 μg of ^{125}I -labelled C3 (580 000 c.p.m.) was added to the activated zymosan in a final volume of 800 μl in 10 mM-imidazole/HCl (pH 7.3)/0.15 M-NaCl. At various times, portions (100 μl) were removed and centrifuged at 9980 g for 1 min. The supernatant was assayed for C3 haemolytic activity, and the pellet was quantitatively transferred to a new tube and washed eight times with 20 mM-Tris/ H_3PO_4 (pH 7.0)/6 M-urea/0.5% SDS. Controls were performed by employing the same procedure as above, except that zymosan devoid of C3b was used. The pellets were counted for radioactivity in an LKB Rackgamma counter. The amount of radiolabelled C3b in the activated zymosan was estimated after subtraction of the control values.

The entire procedure of studying a zymosan-associated C3 convertase was repeated for a second stage, except that non-radiolabelled C3 was used in the first stage for the priming of the zymosan with covalent C3b. A C3 convertase was constructed on zymosan (9 mg) bearing 72 pmol of covalently bound C3b, and the C3-cleaving activity and the ^{125}I -labelled C3b covalent binding were determined as previously described.

Estimates were made of the C3 convertase activity by measuring the amount of ^{125}I -labelled Bb (the active-site subunit) associated with the zymosan pellet. Samples of the zymosan–C3b, or zymosan with non-covalent C3b or untreated zymosan were treated as described above, except that ^{125}I -labelled factor B (100 000 c.p.m.) was used, and after the 20 min preincubation, a 50 μl portion of each sample was layered over 250 μl of oil (Lenzol), and material bound to the solid phase was separated from the aqueous phase by centrifugation at 9980 g for 1 min (Sim & Sim, 1981). The bottom of the tube was cut off and counted for radioactivity.

Binding studies. Zymosan bearing covalent C3b was prepared as described above except that native C3 was made to react with the first-stage C3 convertase for 2 h at 37°C, and the zymosan–C3b was washed five times with 10 mM-imidazole/HCl (pH 7.3)/0.65 M-NaCl/0.3% gelatin and then five times with 10 mM-imidazole/HCl (pH 7.3)/0.15 M-NaCl. By monitoring the reaction with radiolabelled C3, and by counting the zymosan particles microscopically with a haemocytometer, it was found that 1 ml of zymosan–C3b contained between 1.5×10^9 – 2.0×10^9 particles, with each particle having between 90 000–120 000 molecules of covalently bound C3b. The bound C3b was considered to have

been covalently bound, as further incubation and washing with 0.5% SDS removed only negligible amounts of the radiolabelled C3b, whereas treatment for 2 h with 1 M-hydroxylamine and 0.1% SDS in 25 mM-carbonate, pH 10.5, removed about 70% of the labelled C3b.

The binding of radioiodinated proteins (0.4–0.7 μ g) to 1.5 mg of zymosan–C3b was measured in 10 mM-imidazole/HCl (pH 7.3)/0.3% gelatin with either 0.15 M-NaCl or 0.075 M-NaCl, along with various amounts of non-radiolabelled competitor protein in a final volume of 70 μ l. For the factor B determinations the buffers contained 3 mM-MgCl₂. After incubation at 37°C, 50 μ l of the sample was layered over 250 μ l of oil (Lenzol) in a plastic tube, and the sample was centrifuged at 9980 g for 1 min in an Eppendorf 5412 microcentrifuge. The bottom of the tube was cut off and both the pellet and a portion of the supernatant were counted for radioactivity in an LKB 1270 Rackgamma counter. Controls were performed that involved the same procedure but utilized zymosan that was devoid of any covalently bound C3b. Control values were subtracted from the experimental results. The results were presented as Scatchard (1949) plots or as dose–response binding graphs.

Effects of p-chloromercuribenzoate treatment on zymosan–C3b on the binding of ¹²⁵I-labelled component C5, ¹²⁵I-labelled factor B, ¹²⁵I-labelled β_1 H and ¹²⁵I-labelled properdin. A mixture of zymosan–C3b (30 mg) containing 200 pmol of covalently bound C3b, 8 mM-p-chloromercuribenzoate in 10 mM-imidazole/HCl (pH 7.3)/0.075 M-NaCl in a final volume of 1 ml was incubated at 23°C for 15 h. A control incubation of zymosan with p-chloromercuribenzoate was treated identically. After incubation the zymosan–C3b and zymosan were washed five times with 10 mM-imidazole/HCl (pH 7.3)/0.65 M-NaCl/0.3% gelatin, and five times with 10 mM-imidazole/HCl (pH 7.3)/0.15 M-NaCl. Binding studies were performed as above.

Effect of dithiothreitol on zymosan–C3b on the binding of ¹²⁵I-component C5, ¹²⁵I-labelled factor B, ¹²⁵I-labelled β_1 H and ¹²⁵I-labelled properdin. Samples of zymosan–C3b (6 mg) were incubated with various amounts of dithiothreitol for 1 h at 37°C in 10 mM-imidazole/HCl (pH 7.3)/0.15 M-NaCl. Then the samples were washed eight times with the incubation buffer. Binding studies were performed as above.

Effect of C3bINA/ β_1 H treatment on zymosan–C3b binding of ¹²⁵I-labelled component C5, ¹²⁵I-labelled factor B, ¹²⁵I-labelled β_1 H and ¹²⁵I-labelled properdin. To 30 mg of zymosan–C3b bearing 200 pmol of C3b, there was added 70 μ g of β_1 H and 8.5 μ g of C3bINA in a final volume of 1 ml in 10 mM-Mops(4-morpholine-ethanesulphonic acid)/Tris (pH 6.4)/0.075 M-NaCl. After incubation at

37°C for 10 h, the zymosan–C3b was washed five times with 10 mM-imidazole/HCl (pH 7.3)/0.65 M-NaCl/0.3% gelatin and five times with 10 mM-imidazole/HCl (pH 7.3)/0.15 M-NaCl. Binding studies were performed as described above.

Absorption coefficients and molecular weights that were employed for the present studies are: component C5: $A_{1\text{cm},280}^{1\%}$ 10, mol.wt. 200 000 (Tack *et al.*, 1979); factor B: $A_{1\text{cm},280}^{1\%}$ 12.7, mol.wt. 90 000 (Curman *et al.*, 1977); β_1 H: $A_{1\text{cm},280}^{1\%}$ 9.0, mol.wt. 150 000 (Nagaki *et al.*, 1978; Whaley & Ruddy, 1976); properdin: $A_{1\text{cm},280}^{1\%}$ 12.0, mol.wt. 184 000 (K. B. M. Reid, personal communication) (Minta & Lepow, 1974); component C3: $A_{1\text{cm},280}^{1\%}$ 9.7, mol.wt. 187 000 (Tack & Prahl, 1976).

Radioiodinations were performed by using the solid-phase lactoperoxidase method of David (1972). The specific radioactivities after radioiodinations were: component C5: 100 000–150 000 c.p.m./ μ g; factor B: 60 000–360 000 c.p.m./ μ g; β_1 H: 90 000–220 000 c.p.m./ μ g; properdin: 40 000–80 000 c.p.m./ μ g; component C3: 100 000–150 000 c.p.m./ μ g. The haemolytic activities of component C5, factor B, and properdin were retained after radioiodination.

Results

Covalent binding of C3b to zymosan

The activation of component C3 by a zymosan-bound C3 convertase and the covalent association of C3b with the zymosan was studied as a function of time. The experiment was performed in two stages. The first stage involved a low-grade C3 convertase constructed from C3b that had been non-covalently absorbed to the zymosan. The second stage involved a stronger C3 convertase generated from C3b that was, for the most part, covalently bound to the zymosan. The results are shown in Fig. 1. The first stage C3 convertase (Fig. 1a) has less effective C3-cleaving activity than the second-stage C3 convertase (Fig. 1b), and it is also observed that the covalent binding of ¹²⁵I-labelled C3b in stage I is attenuated before the C3 convertase activity has decayed. The threshold at stage I for covalent binding of ¹²⁵I-labelled C3b is reached when only 20% of the C3 is cleaved, under the conditions employed. A reasonable interpretation of these results is that because of the short half-life of nascent C3b, this newly activated protein can bind only in a locus around the original active centre of the convertase.

Parallel experiments were performed under exactly the same conditions but using ¹²⁵I-labelled factor B to estimate the amount of C3 convertase initially present. Determinations of ¹²⁵I-labelled Bb associated with the stage-I and stage-II C3 convertase at zero time were: (stage I) 0.2 pmol of Bb was asso-

Fig. 1. Activation of component C3 by zymosan-C3 convertase and the binding of ^{125}I -labelled C3b to zymosan. The C3 activity (O) and the ^{125}I -labelled C3b deposition (Δ) were studied in two stages. (a) Stage I: zymosan (9 mg) bearing non-covalently absorbed C3b, was mixed with properdin, factor B and factor D. After incubation at 37°C for 20 min, EDTA was added, and the zymosan-C3 convertase was centrifuged. Then ^{125}I -labelled C3 was added, and at various times portions were removed and centrifuged. The supernatant was assayed for C3 haemolytic activity, and the pellet was washed eight times with 20 mM-Tris/ H_3PO_4 (pH 7.0)/6 M-urea/0.5% SDS, and counted for radioactivity. Control values were subtracted. (b) Stage II: the same procedure was employed except that the zymosan (9 mg) had 72 pmol of covalently bound C3b. Details of the procedure are given under 'Methods'.

ciated with $750\mu\text{g}$ of zymosan bearing non-covalent C3b; (stage II) 2 pmol of Bb was associated with $750\mu\text{g}$ of zymosan bearing 6 pmol of covalently bound C3b. In these experiments, virtually all the radioactivity that was determined on the zymosan pellet was associated with the Bb subunit. Inspection of autoradiograms indicated that the Ba subunit was released from the zymosan after activation, and virtually all the factor B was converted into Bb and Ba after the 20 min pre-incubation period. The determinations indicated that 6 pmol of C3b can covalently bind to $750\mu\text{g}$ of zymosan originally bearing 0.2 pmol of Bb at zero time. Calculations from the amount of C3b that can bind per original Bb active centre for the stage-I C3 convertase, indicated an average of 30 C3b molecules per original Bb centre. Thus the C3b molecules deposited covalently on the zymosan are located in clusters on an average of about 30 molecules around the original convertase centres

Binding of component C5, factor B and properdin to zymosan-C3b

As C3b is known to bind several other complement proteins, determinations were made of the affinities and stoichiometries of the binding of several complement proteins (component C5, factor B, $\beta_1\text{H}$, and properdin) to zymosan-C3b. Scatchard-plot analyses of the binding of component C5 (Fig. 2a), factor B (Fig. 2b), $\beta_1\text{H}$ (Fig. 2c), and properdin (Fig. 2d) to zymosan-C3b are shown.

It is observed that component C5 binds to zymosan-C3b with $K = 1.1 (\pm 0.1) \times 10^7 \text{ M}^{-1}$ and $\text{C5/C3b} = 1.1$ (at 0.075 M-NaCl). In 0.15 M-NaCl , $K = 5.7 (\pm 0.5) \times 10^6 \text{ M}^{-1}$ and $\text{C5/C3b} = 0.7$ was observed.

Factor B in the presence of 3 mM-MgCl_2 and 0.15 M-NaCl has $K = 6.5 (\pm 0.5) \times 10^5 \text{ M}^{-1}$ and $\text{B/C3b} = 1.0$. In the presence of $20\mu\text{g}$ of properdin/ml the factor B has $K = 4.3 (\pm 0.5) \times 10^6 \text{ M}^{-1}$ and $\text{B/C3b} = 1.0$. Thus properdin increases the affinity of factor B for C3b or the C3b-properdin complex, but does not influence the maximal amount of factor B that can bind to the zymosan-C3b.

The $\beta_1\text{H}$ Scatchard plot exhibits curvature, which lends itself to several interpretations. The high-affinity sites (in 0.15 M-NaCl) by linear extrapolation show $K = 2.1 (\pm 0.2) \times 10^7 \text{ M}^{-1}$ and $\beta_1\text{H/C3b} = 0.18$. The maximal extrapolated $\beta_1\text{H/C3b} = 0.32$. The $\beta_1\text{H}$ binding was not influenced by 5 mM-MgCl_2 or 5 mM-CaCl_2 .

Properdin binds to zymosan-C3b (in 0.15 M-NaCl) with $K = 2.9 (\pm 0.4) \times 10^7 \text{ M}^{-1}$ and $\text{P/C3b} = 1.0$. In the presence of $180\mu\text{g/ml}$ of factor B and 3 mM-MgCl_2 , the properdin (P) exhibited $K = 1.1 (\pm 0.1) \times 10^8 \text{ M}^{-1}$ with $\text{P/C3b} = 1.4$. Thus there is only a small change in the P/C3b ratio, with a 4-fold increase in the K value on the factor B binding.

It is noteworthy that neither ^{125}I -labelled C3 nor ^{125}I -labelled C3b bind to zymosan-C3b. As properdin has a multimeric structure, efforts were made to see if properdin were multivalent for C3 or C3b by adding various amounts of properdin ($\pm$

Fig. 2. *Scatchard plots of the binding of component C5, factor B, β_1H and properdin to zymosan-C3b*
 The binding to zymosan-C3b of the various complement proteins was analysed by Scatchard-plot analyses. The buffer was 10 mM-imidazole/HCl, pH 7.3, containing 0.15 M-NaCl for all the analyses except for the C5 determinations, where studies were performed in buffer containing 0.15 M-NaCl or 0.075 M-NaCl. The factor B analyses were performed in buffer containing 0.15 M-NaCl and 3 mM-MgCl₂. The identity of the graphs is as follows: (a) component C5 in 0.15 M-NaCl (Δ) and in 0.075 M-NaCl (O); (b) factor B without properdin (Δ) and with properdin (20 μ g/ml) (O); (c) β_1H (O); (d) properdin (P) without factor B (O) and with factor B (180 μ g/ml) (Δ).

factor B with 3 mM-MgCl₂) to zymosan-C3b with either ¹²⁵I-labelled C3 or ¹²⁵I-labelled C3b. If the properdin subunits were arranged in such a fashion that there could exist binding of both surface C3b and fluid-phase C3 or C3b, then one would predict some binding of ¹²⁵I-labelled C3 or ¹²⁵I-labelled C3b

to zymosan-C3b. The results were that there was no ¹²⁵I-labelled C3 or ¹²⁵I-labelled C3b bound to zymosan C3b in the presence or absence of properdin.

The binding of all the proteins that were tested exhibited ionic-strength-dependence (Fig. 3).

Fig. 3. Influence of ionic strength on the binding of ^{125}I -labelled component C5, factor B, $\beta_1\text{H}$ and properdin. The radiolabelled proteins C5 (0.2 μg) (●), factor B (0.7 μg) (○), $\beta_1\text{H}$ (0.3 μg) (△) and properdin (0.2 μg) (□) were mixed with 1.5 mg of zymosan-C3b in 70 μl of 10 mM-imidazole/HCl buffer, pH 7.3, at various concentrations of NaCl at 37°C for 15 min. The binding studies were performed as described under 'Methods'.

Competition binding studies

The influence of the various complement proteins (component C5, factor B, $\beta_1\text{H}$ and properdin) on the binding of each to zymosan-C3b was studied (Fig. 4). With ^{125}I -labelled component C5 (Fig. 4a), it is observed that factor B, $\beta_1\text{H}$ and properdin inhibit the binding of this protein, in confirmation of the results of Vogt *et al.* (1978) and Isenman *et al.* (1980a). In the case of ^{125}I -labelled factor B (Fig. 4b), properdin augments the binding as has been previously observed (Medicus *et al.*, 1976) and $\beta_1\text{H}$ inhibited the binding. It is noted that $\beta_1\text{H}$ can inhibit all the factor B association with zymosan-C3b, whereas the $\beta_1\text{H}$ can bind only to a maximum ratio ($\beta_1\text{H}/\text{C3b}$) of 0.32 (Fig. 2c). Component C5 is observed to inhibit factor B binding. The binding of ^{125}I -labelled $\beta_1\text{H}$ to zymosan-C3b (Fig. 4c) is inhibited by factor B, as previously noted (Pangburn & Müller-Eberhard, 1978; Nagaki *et al.*, 1978). Interestingly, there exists a portion of the ^{125}I -labelled $\beta_1\text{H}$ bound to zymosan-C3b that is resistant to very high amounts of competitor factor B. Properdin has no influence on ^{125}I -labelled $\beta_1\text{H}$ binding as previously observed (Pangburn & Müller-Eberhard, 1978). Conrad *et al.* (1978), in contrast,

Table 1. Effect of *p*-chloromercuribenzoate treatment of zymosan-C3b on the binding of properdin

Zymosan-C3b was made to react with *p*-chloromercuribenzoate. After washing, the material was employed for binding studies as described under 'Methods'. The tabulated values refer to the determinations after the control values were subtracted. 'Zymosan-C3b-*p*CMB' refers to the zymosan-C3b that was made to react with *p*-chloromercuribenzoate. The factor B study was performed in the presence and absence of properdin (20 $\mu\text{g}/\text{ml}$) and the properdin experiment was performed in the presence and absence of factor B (180 $\mu\text{g}/\text{ml}$).

	Total radioactivity available for binding (c.p.m.)	Radioactivity bound to zymosan-C3b (c.p.m.)	Radioactivity bound to zymosan-C3b- <i>p</i> CMB (c.p.m.)
C5	58 100	17 300	14 300
B			
- Properdin	52 200	5 500	15 100
+ Properdin	52 500	15 700	40 500
$\beta_1\text{H}$	64 400	21 200	17 500
Properdin			
-B	35 000	17 200	12 600
+B	35 000	24 400	21 700

observed an increased uptake of ^{125}I -labelled $\beta_1\text{H}$ to EAC43 cells with the addition of properdin. Component C5 can inhibit ^{125}I -labelled $\beta_1\text{H}$ binding.

The binding of ^{125}I -labelled properdin (Fig. 4d) to zymosan-C3b is augmented in the presence of factor B as has been observed by Medicus *et al.* (1976), but is not affected by component C5 or $\beta_1\text{H}$.

Effects of *p*-chloromercuribenzoate treatment of zymosan-C3b on the binding of ^{125}I -labelled component C5, ^{125}I -labelled factor B, ^{125}I -labelled $\beta_1\text{H}$ and ^{125}I -labelled properdin

Treatment of zymosan-C3b with *p*-chloromercuribenzoate decreased slightly the binding of component C5, $\beta_1\text{H}$, and properdin, but resulted in a marked augmentation of factor B binding (Table 1).

The single free thiol group of C3b, when this protein is bound to zymosan, is available for reaction with *p*-chloromercuribenzoate. Factor B exhibited an 11-fold increase in its *K* value for C3b when the zymosan-C3b had been treated with *p*-chloromercuribenzoate, with a maximal ratio of factor B binding to C3b of 1.

Effect of dithiothreitol treatment of zymosan-C3b on the binding of ^{125}I -labelled component C5, ^{125}I -labelled factor B, ^{125}I -labelled $\beta_1\text{H}$ and ^{125}I -labelled properdin

Reduction of zymosan-C3b with up to 5 mM-dithiothreitol abrogates the binding of ^{125}I -labelled component C5, ^{125}I -labelled factor B and ^{125}I -

Fig. 4. Competition dose-response curves for the binding of ^{125}I -labelled component C5, factor B, $\beta_1\text{H}$ and properdin. The radioiodinated proteins ($0.6\text{ }\mu\text{g}$ of C5, $0.8\text{ }\mu\text{g}$ of factor B, $0.8\text{ }\mu\text{g}$ of $\beta_1\text{H}$, $0.5\text{ }\mu\text{g}$ of properdin) were mixed with 1.5 mg of zymosan-C3b in 10 mM -imidazole/HCl buffer, pH 7.3. The $\beta_1\text{H}$ and properdin studies included 0.15 M -NaCl in the buffer, the C5 studies were performed in buffer containing 0.075 M -NaCl, and the factor B studies included 0.15 M -NaCl and 3 mM -MgCl₂ in the buffer. The final volume of each sample was $70\text{ }\mu\text{l}$. The extent of binding of each radiolabelled protein was studied as a function of the amount of the other proteins after 15 min at 37°C . The identity of the graphs is as follows: (a) ^{125}I -labelled C5 binding as a function of factor B (Δ), $\beta_1\text{H}$ ($\square$) and properdin ($\circ$); (b) ^{125}I -labelled-factor B binding as a function of C5 (Δ), $\beta_1\text{H}$ ($\square$) and properdin ($\circ$); (c) ^{125}I -labelled $\beta_1\text{H}$ binding as a function of C5 ($\circ$), factor B ($\square$) and properdin (Δ); (d) ^{125}I -labelled properdin binding as a function of C5 ($\circ$), factor B ($\square$) and $\beta_1\text{H}$ (Δ).

labelled properdin, but not ^{125}I -labelled $\beta_1\text{H}$ (Fig. 5). The binding site for the $\beta_1\text{H}$ remains intact after dithiothreitol treatment of zymosan-C3b but the integrity of several disulphide bridges in the C3b appears to be necessary for component C5, factor B and properdin binding.

Effect of the treatment of zymosan-C3b with C3bINA/ $\beta_1\text{H}$

Treatment of bound C3b by C3bINA/ $\beta_1\text{H}$ is known to inactivate the C3b with regard to C3 and C5 convertase activities (Pangburn *et al.*, 1977;

Nagaki *et al.*, 1978; Law *et al.*, 1979a; Nagasawa & Stroud, 1977; Fujita *et al.*, 1978). The effect of the C3bINA/ $\beta_1\text{H}$ is to cleave a single internal polypeptide bond within the α' -chain of C3b (Crossley & Porter, 1980). It seemed worthwhile to study the effect of binding of the various components to zymosan-C3b after the latter was treated with C3bINA/ $\beta_1\text{H}$. The results are shown in Table 2. The binding of ^{125}I -labelled component C5, and ^{125}I -labelled factor B, is lost after zymosan-C3b is converted into zymosan-C3b' by the action of C3bINA/ $\beta_1\text{H}$. There is a marked decrease in the

Fig. 5. Effect of dithiothreitol treatment of zymosan–C3b on the binding of ¹²⁵I-labelled component C5, factor B, β_1 H and properdin

Zymosan–C3b was made to react with various amounts of dithiothreitol for 1 h at 37°C. After washing, the modified zymosan–C3b was employed for binding studies, as previously described under ‘Methods’, with the radioiodinated proteins: component C5 (Δ), factor B (●), β_1 H (□) and properdin (○).

Table 2. Effect of C3bINA/ β_1 H treatment of zymosan–C3b on the binding of ¹²⁵I-labelled complement proteins (component C5, factor B, β_1 H and properdin)

Zymosan–C3b was treated with C3bINA and β_1 H, washed, and used for binding studies as described under ‘Methods’. Zymosan–C3b’ refers to the C3bINA/ β_1 H-treated material. The tabulated values refer to the determinations after control values were subtracted.

	Total radioactivity available for binding (c.p.m.)	Radioactivity bound to zymosan–C3b (c.p.m.)	Radioactivity bound to zymosan–C3b’ (c.p.m.)
C5	52 600	16 700	0
B	58 000	5200	0
β_1 H	60 800	18 600	3100
Properdin	29 600	13 200	10 500

¹²⁵I-labelled β_1 H binding, but only a slight decrease in the binding of ¹²⁵I-labelled properdin to C3bINA/ β_1 H-treated zymosan–C3b.

Discussion

Law & Levine (1977) and Law *et al.* (1979b) have suggested that the complement fragment C3b

can bind surfaces by a covalent (probably ester) bond. It has been proposed that a thioester exists within component C3, and that this thioester is subjected to a nucleophilic attack by a surface hydroxy group or water when component C3 is activated to C3b (Janotova *et al.*, 1980; Tack *et al.*, 1980; Sim *et al.*, 1981; Howard, 1980). Component C4 and α_2 -macroglobulin appear to be similar in this respect (Campbell *et al.*, 1980; Law *et al.*, 1980; Salveson & Barrett, 1980; Swenson & Howard, 1979; Harpel & Hayes, 1979; Harpel *et al.*, 1979; Sottrup-Jensen *et al.*, 1980; Howard *et al.*, 1980).

When C3b is deposited on zymosan from a surface-associated alternative-pathway C3 convertase, the C3b molecules are deposited in foci with an average of 30 C3b molecules per site (Fig. 1). Mardiney *et al.* (1968), using electron microscopy, observed 10–40 C3b molecules per cluster on erythrocytes bearing C3b, and Sim *et al.* (1981), studying a trypsin–Sepharose system as a model system for a bound C3 convertase, observed that a maximum of 25–26 molecules of C3b were deposited around each native trypsin centre after activation of component C3. It is noteworthy that the alternative-pathway C3 convertase still functions when all the C3b acceptor sites are saturated by C3b (Fig. 1).

I have investigated the binding to zymosan–C3b of the human complement proteins component C5, factor B, β_1 H and properdin. One additional complement protein, C3bINA, which is known to bind C3b (Pangburn & Müller-Eberhard, 1978; Nagasawa *et al.*, 1980), was not investigated in the present studies.

When C3b is deposited on surfaces from a C3 convertase bound to that surface, the covalently bound C3b exhibits a retention of the B-antigenic determinant that is found on native C3 but not on fluid-phase C3b (Molenaar *et al.*, 1974). Vogt *et al.* (1978) first observed that component C5 has a binding capacity for surface-bound C3b. Isenman *et al.* (1980a) have proposed that the binding of component C5 for surface C3b could be multivalent. The results from Fig. 2 indicate that component C5 can bind to zymosan–C3b with a maximum ratio (C5/C3b) of 1.1 (at 0.075 M-NaCl); thus it is concluded that component C5 binds C3b as a 1:1 complex and not in a multivalent fashion. *K* values $1.1(\pm 0.1) \times 10^7 \text{ M}^{-1}$ in 0.075 M-NaCl and $5.7(\pm 0.5) \times 10^6 \text{ M}^{-1}$ in 0.15 M-NaCl have been calculated for the binding of C5 to zymosan–C3b (Fig. 2a). These evaluations of the association constants are 5–10-fold higher than the estimates of Isenman *et al.* (1980a), who used inhibition studies of fluid-phase C3b to inhibit the binding of C5 to cell-bound C3b to deduce their results. The fact that component C5 will bind reversibly and in an ionic-strength-dependent fashion to C3b that has

been bound to a surface through its labile binding site, has been exploited for the purification of the human component C5 (see under 'Methods'). An affinity column of C3b-Sepharose for the final step in the purification of this protein has been employed. A column of C3b-Sepharose, which was made by coupling C3b to Sepharose by a CNBr reaction (Cuatrecasas, 1970), was not successful for the purification of component C5.

Factor B has been observed to bind C3b in the fluid phase as a 1:1 complex (Vogt *et al.*, 1977). This has also been observed for the binding of factor B to zymosan-C3b at 3 mM-MgCl₂ (Fig. 2b). A K value of $6.5 (\pm 0.5) \times 10^5 \text{ M}^{-1}$ was observed, and this value is 3-fold lower than the results of Kazatchkine *et al.* (1979); however, their experiments employed 5 mM-MgCl₂ and they demonstrated that the efficiency of factor B with C3b was Mg²⁺-dependent. It has been well established that properdin will increase factor B binding to C3b (Medicus *et al.*, 1976). The present results indicate that in the presence of 20 $\mu\text{g}/\text{ml}$ of properdin there is no change in the maximal ratio of factor B bound per C3b, but there is a 7-fold augmentation in the association constant of factor B to C3b.

$\beta_1\text{H}$ has been shown to be a reversible inhibitor of factor B binding to C3b, and $\beta_1\text{H}$ is an accelerator of the decay of C3 convertase of the alternative pathway (Whaley & Ruddy, 1976; Pangburn & Müller-Eberhard, 1978). Conrad *et al.* (1978) have observed curvature for the Scatchard plot of $\beta_1\text{H}$ binding to erythrocytes bearing C3b, but the results of Kazatchkine *et al.* (1979) showed a linear Scatchard plot for the binding of $\beta_1\text{H}$ to zymosan-C3b; however, the latter group did not take sufficient data points near the abscissa and may have missed a possible curvature. The present results indicate a non-linear Scatchard plot (Fig. 2c). There are several interpretations for this non-linearity: (a) there may exist heterogeneity in the binding sites for $\beta_1\text{H}$; (b) there may exist negative co-operativity by the C3b clusters for the binding by $\beta_1\text{H}$; (c) the limitation on $\beta_1\text{H}$ binding to zymosan-C3b may be due to steric interference of the $\beta_1\text{H}$ molecules on the C3b clusters. $\beta_1\text{H}$ is known to be a rod-shaped molecule (Whaley & Ruddy, 1976). The present results indicate for the high-affinity $\beta_1\text{H}$ -binding sites on zymosan-C3b a K value of $2.1 (\pm 0.2) \times 10^7 \text{ M}^{-1}$, and this is about 3-fold lower than the results of Kazatchkine *et al.* (1979), but it is about 100-fold lower than that determined by Conrad *et al.* (1978) who were studying $\beta_1\text{H}$ binding to erythrocyte-bound C3b. Treatment of zymosan-C3b with up to 5 mM-dithiothreitol (Fig. 5) inhibits the binding of component C5, factor B, and properdin, but not $\beta_1\text{H}$. Zemel *et al.* (1980), and Isenman *et al.* (1980b) observed that reduction of component C3 with 1–2 mM-dithiothreitol resulted in an abrogation of

C5 convertase, but left the immune adherence response largely intact. It is plausible that the erythrocyte and monocyte receptor is recognizing the $\beta_1\text{H}$ binding site on C3b.

Properdin is a plasma glycoprotein of mol.wt. 184 000, and it has been proposed that this protein exists as a tetramer of four apparently identical subunits (Minta & Lepow, 1974). The present work indicates an affinity of properdin (P) for zymosan-C3b of $K = 2.9 (\pm 0.4) \times 10^7 \text{ M}^{-1}$ with a maximum ratio (P/C3b) of 1.0 (Fig. 2d). In the presence of factor B (180 $\mu\text{g}/\text{ml}$) and 3 mM-MgCl₂ the properdin affinity for zymosan-C3b is $K = 1.1 (\pm 0.1) \times 10^8 \text{ M}^{-1}$ and P/C3b = 1.4. Because of its multimeric structure it was an attractive notion that properdin could be multivalent for C3b, but none of the data suggests that this is the case. Properdin can exist in at least two states, referred to as 'native' and 'active' (Medicus *et al.*, 1976; Gotze *et al.*, 1977; Medicus *et al.*, 1980). The two forms of properdin do not differ in covalent structure. Studies of properdin described here were performed with properdin that corresponds to the native form on the basis of the following observation: (a) when the properdin was added to normal human plasma up to a concentration of 10 $\mu\text{g}/\text{ml}$ for 30 min at 37°C in the presence of 0.5 mM-MgCl₂, there was only negligible consumption of the C3 in the plasma; (b) the properdin exhibited saturation binding to zymosan-C3b; (c) the properdin could be eluted off zymosan-C3b with 0.7 M-NaCl, and the stimulatory effect by factor B (with 3 mM-MgCl₂) for the binding of properdin to zymosan-C3b could be reversed by 20 mM-EDTA.

It remains an open question as to whether properdin has weak binding sites for factor B. This is plausible, as physiological amounts of properdin increase the binding affinity for factor B binding to zymosan-C3b about 7-fold, and physiological amounts of factor B increase the properdin binding affinity to zymosan-C3b about 4-fold.

It is suggested that the binding regions on the C3b molecule for component C5, factor B, $\beta_1\text{H}$, and properdin are distinct, although it is possible that in some cases the binding sites could overlap. The evidence for this is summarized as follows.

Component C5

Evidence of other workers suggest that optimal binding of component C5 for C3b occurs when C3b is bound by its apparent intrinsic ester bond to surfaces (Vogt *et al.*, 1979); this is not necessarily true for factor B and $\beta_1\text{H}$ binding to C3b. The simplest interpretation of the fact that properdin augments factor B binding but diminishes C5 binding to zymosan-C3b (Figs. 4a and 4b) would be that there exist topographically distinct sites on C3b for component C5 and factor B. In addition,

treatment of zymosan-C3b with *p*-chloromercuribenzoate diminished component C5 binding but augmented factor B binding (Table 1); thus the factor B site and component C5 site on C3b are probably different. It is unlikely that the binding site of component C5 is the same as the β_1 H binding site, since treatment with up to 5 mM-dithiothreitol of zymosan-C3b diminished component C5 binding but had little influence on the β_1 H binding (Fig. 5). The properdin site must be separate from the component C5 site, since component C5 cannot competitively inhibit properdin binding to zymosan-C3b (Fig. 4c).

Factor B

Factor B appears to have a distinct binding site from component C5 (see above). Factor B is unlikely to have the same site as β_1 H as the fact that properdin augments factor B binding but does not influence β_1 H binding would lend itself to the simplest interpretation that the binding sites are separate (Fig. 4). Furthermore, treatment of zymosan-C3b with up to 5 mM-dithiothreitol markedly diminishes factor B binding but leaves the β_1 H binding intact (Fig. 5). The properdin binding site is separate from that of factor B as properdin augments factor B binding and factor B augments properdin binding (Fig. 4). Furthermore, *p*-chloromercuribenzoate treatment of zymosan-C3b resulted in a higher affinity for factor B binding but slightly diminished the affinity for the other components.

β_1 H

The β_1 H binding site appears to differ from that of component C5 and factor B (see above). The β_1 H-binding site cannot be the same as that of properdin, since β_1 H has no influence on properdin binding and properdin has no influence on β_1 H binding (Fig. 4).

Properdin

Properdin evidently engages a unique site on C3b distinct from that of component C5, factor B, and β_1 H (see above). Furthermore, treatment of zymosan-C3b with C3bINA/ β_1 H (Table 2) resulted in decreases in the binding of component C5, factor B, and β_1 H but not properdin.

The fact that each of these components influences the binding of some or all of the others for zymosan-C3b can be attributed to (a) a conformational alteration in the C3b molecule; (b) steric interference for binding; (c) in the special case of properdin and factor B, the properdin could have a weak binding site for the factor B.

As all the components exhibited an ionic-strength-dependence for the binding affinity to zymosan-C3b (Fig. 3), it is suggested that salt bridges are an

important mechanism for associations of these components with C3b.

In conclusion, C3b that is deposited on zymosan by a surface alternative-pathway C3 convertase is distributed in clusters of an average of 30 C3b molecules per site. There exist on zymosan-bound C3b four distinct binding sites for component C5, factor B, β_1 H, and properdin.

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