Effect of complement-protein-C3b density on the binding of complement factor H to surface-bound C3b

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Various amounts of the activation fragment C3b of the complement (C) protein C3 were coupled to Sepharose 4B by catalysis with the C3 convertase of the alternative pathway of C. The binding of radioactively labelled C proteins B and H (= factor H) to the C3b-carrying particles was assayed. It was found that the relative binding of H, but not of B, fell rapidly with decreasing densities of solid-phase C3b, suggesting a sigmoidal relationship between C3b density and binding of H. To study the phenomenon in more detail, preformed C3b was coupled to activated thiopropyl-Sepharose 6B at various densities. By using this model system, it was shown that the binding of H/unit amount of C3b was positively correlated to C3b density up to a C3b concentration of about 0.5 mg/ml of gel, whereas binding of B was independent of C3b density. The results show that accumulation of high densities of C3b on a surface creates high-affinity binding sites for H. Because H has recently been shown to form dimers in solution, the interaction of dimeric H with neighbouring C3b molecules is a likely explanation for the phenomenon. The C3b density effect may be a regulatory mechanism keeping the activation of the alternative pathway of C on activating surface within reasonable limits.

INTRODUCTION

One of the initial events in the activation of the alternative pathway (AP) of C is the binding of the fragment C3b of the C protein C3 to the activator surface through an ester bond via a specific site in its α' -chain [1–3]. The complement protein B can associate with C3b in the presence of Mg^{2+} . The cleavage of C3b-bound B by factor D to fragments Ba and Bb leads to the formation of the AP C3 convertase, C3b,Bb, which can then catalyse further formation of C3b [4,5].

The plasma protein H is a regulatory factor that inhibits binding of B to C3b, dissociates the C3 convertase and functions as a cofactor for serine proteinase I in the cleavage of C3b to the iC3b form, which is inactive in AP activation [6]. The affinity of H for surface-bound C3b is a decisive factor determining whether extensive formation of the C3 convertase on the surface, i.e. activation of the AP, can occur, and this affinity has been found to depend on the quality of the surface, so that the average affinity is lower on activating than on non-activating surfaces. A characteristic feature of the interaction between H and surface-bound C3b is a heterogeneous affinity, and association constants ranging from about $10^5 M^{-1}$ up to about $10^9 M^{-1}$ have been reported [7–10]. In the fluid phase, C3b and H associate with a homogeneous affinity, the association constant being about $4 \times 10^6 M^{-1}$ at $+20^\circ C$ [11].

The molecular basis of the surface effect remains to be fully elucidated, although several clues exist. Thus sialic acid on the surface has been found to increase the affinity of H for C3b, thereby inhibiting activation, and is thought to be an important factor in the restriction of activation on homologous cells [7,12–16]. Also some non-sialic acid-containing polyanions have been found to increase the affinity between C3b and H [17,18]. The idea of restriction of activation by specific structures that increase the affinity of H for C3b implies that the affinity of H for surface-bound C3b is basically low. However, it has remained unexplained why high-affinity H-binding sites are also formed when C3b is bound to activators of the AP, such as zymosan,

although high-affinity H-binding sites are relatively more abundant on non-activators [7,9,10].

Evidence from two previous studies, both of which employed erythrocytes as the solid phase, suggests that a high density of C3b on a surface could promote high-affinity binding of H [19,20]. In the present study, the gel-filtration matrix Sepharose 4B was used to show that C3b density is indeed an independent factor in determining the affinity of H. The conclusion was confirmed, and the C3b density effect was characterized in more detail by using the pre-activated matrix thiopropyl-Sepharose 6B as a solid phase.

MATERIALS AND METHODS

Buffers, chemicals and proteins

Buffers used in ligand-binding assays were as follows: 0.01 M-sodium 5,5'-diethylbarbiturate / 0.15 M-NaCl / 0.02 % $NaNO_3$, pH 7.3 (Veronal-buffered saline, VBS); VBS/0.1 % gelatin/0.2 % BSA (BVB); BVB/1 mM- $NiCl_2$ (BVB-Ni); BVB/2 mM-EDTA (BVBE). Sepharose 4B and thiopropyl-Sepharose 6B (TPS) were purchased from Pharmacia, Uppsala, Sweden. $Na^{125}I$ was purchased from Amersham International. All chemicals were of reagent grade.

The complement proteins C3, B, H and D were isolated as described previously [21]. Proteins were labelled on ice with ^{125}I using the Iodogen method [22] to specific radioactivities of 1–3 $\mu Ci/\mu g$.

Coupling of C3b to Sepharose 4B and TPS

C3b was coupled to Sepharose 4B by catalysis with the AP C3 convertase at three stages of C3b deposition. All incubations were performed at $+30^\circ C$.

Stage 1. Into a 50–70 % (v/v) suspension of the particles in BVB-Ni, solutions of C3, B and D in VBS were added to approximate final concentrations of 1.0, 0.025 and 0.002 mg/ml respectively. The mixture was incubated for 30 min, and the particles were washed twice with BVB-Ni.

Abbreviations used: AP, alternative activation pathway of complement; C, complement; C3, B, D and H, complement proteins C3, B, D and H (= factor H); VBS, Veronal-buffered saline (for composition, see the text); BVB, VBS containing 0.1 % gelatin and 0.2 % BSA; BVB-Ni, BVB containing 1 mM- $NiCl_2$; BVBE, BVB containing 2 mM-EDTA; DTT, dithiothreitol; TPS, thiopropyl-Sepharose 6B.

Stage 2. B and D were added to the above concentrations and the mixture was incubated for 5 min, followed by quick washing with ice-cold BVB and addition of C3 in VBS (1 mg/ml final concn.). The mixture was incubated for 30 min, and the particles were washed with BVB-Ni.

Stage 3. The particles were incubated with B and D, followed by washing and incubation with C3, as at stage 2. Samples of the suspension were withdrawn after each coupling stage, washed with BVB and used for the assays as explained in the Results section.

The amount of C3b coupled to particles was quantified after each stage by including a small amount of ^{125}I -labelled C3 in the coupling mixture together with unlabelled C3, and measuring the radioactivity of the particles after washing with BVB. The radioactivity of the coupled C3b was subtracted when determining specific binding of ligands to the C3b-coupled gel (about 250 c.p.m./test maximally).

For coupling of C3b to TPS through the thiol group, C3 was first converted into C3b by incubating it in VBS at a concentration of 2 mg/ml in the presence of B (0.05 mg/ml), D (0.002 mg/ml) and NiCl_2 (1 mM) for 30 min at $+30^\circ\text{C}$. After adding 0.05 vol. of 0.2 M-disodium/0.2 M-tetrasodium EDTA, pH 7.3, the solution was mixed with preswollen TPS in VBS and incubated for 1 h at room temperature at a 40% (v/v) concentration of gel. After the coupling step, remaining active groups on the gel were blocked by the following treatment: The gel was suspended in 20 times its volume of 0.5 mM-2-mercaptoethanol in 0.1 M-sodium acetate/0.1 M-NaCl, pH 4.5, and incubated for 10 min at room temperature; the suspension was centrifuged, the supernatant was removed and the 2-mercaptoethanol treatment was repeated four more times. This treatment was found to lead to near-quantitative release of 2-thiopyridone from the gel, as assayed by measuring A_{343} of the supernatant. The bound C3b was not found to be adversely affected or released by this treatment, as assayed by binding of radiolabelled B and H to the 2-mercaptoethanol-treated as compared with the non-treated gel. Bound C3b was quantified by including a small amount of ^{125}I -labelled C3 in the C3 preparation used to make C3b and measuring the radioactivity of the gel after the 2-mercaptoethanol treatment. The efficiency of coupling was in the range $61 \pm 4\%$, irrespective of the amount of C3b offered.

Inactivated TPS, used as a control for determining background binding in ligand-binding experiments with TPS, was prepared by treating TPS with 2-mercaptoethanol as above without prior immobilization of protein.

Ligand-binding experiments

The medium used in ligand-binding experiments was either BVB or BVB-Ni when the ligand was either H or B respectively. Ni^{2+} was used instead of Mg^{2+} for studying binding of B to make the comparisons between H and B as accurate as possible, as it has been shown that a more stable complex between C3b and B is formed in the presence of Ni^{2+} than in the presence of Mg^{2+} , with functional properties similar to that formed in the presence of the latter [23]. To study binding of ligands to particles with or without coupled C3b, 60 μl of a 10% (v/v) suspension of particles was added to wells, prewetted with BVB, of a microtitre-format microfiltration plate (PVDF hydrophilic Durapore membrane; MultiScreen Assay System; Millipore, Bedford, MA, U.S.A.), followed by 60 μl of ligand solution. The plate was covered with a plastic sheet and incubated for 30 min at room temperature with agitation to keep the particles in suspension. The plate was transferred to a filtration chamber on which the bound and free ligand were separated under continuous vacuum by sucking off the liquid in the wells followed by three rinses with 150 μl of incubation medium. In practice, the rinsing

could be completed within 30 s. The rinsed particles were transferred into test tubes by flushing with buffer, and the radioactivity in the tubes was counted in a Clinigamma γ -radiation counter (Pharmacia-LKB, Uppsala, Sweden).

Non-specific binding to particles was estimated in each assay by testing the binding of the radiolabelled ligand to particles without coupled C3b. The non-specific binding, which was regularly less than 2% of total radioactivity, was subtracted from the radioactivity obtained on the C3b-carrying particles to obtain specific binding. All binding values in the text are expressed as specific binding.

When labelled B or H was incubated in the microfiltration wells without particles, but otherwise under the conditions of the assay, followed by aspiration of the liquid for counting, more than 95% of total radioactivity could be recovered, which shows that binding of ligands to the membrane does not occur to an appreciable extent.

All assays were done in triplicate.

Computer analysis of data

For curve-fitting and error estimation, the computer program InPlot, version 3.1 (GraphPAD Software, San Diego, CA, U.S.A.) was used.

RESULTS

Solid phases; nature and stability of the C3b-matrix bond

The solid phases used in the present study were the gel-filtration matrix Sepharose 4B and the pre-activated gel TPS, recently introduced by Edens *et al.* [24] for studying C-protein interactions. C3b can be coupled to TPS through the free thiol group generated from the thioester bond present in native C3 [25], which makes it feasible to couple preformed C3b to the gel in predetermined proportions.

The molecular-mass exclusion limit for globular proteins is 20×10^6 Da for Sepharose 4B, and 6×10^6 Da for TPS. Both matrices thus offer large reaction surfaces for the AP proteins.

The covalent binding of C3b to the gels was verified by elution experiments with ^{125}I -labelled C3b. Thus, when particles of

Fig. 1. Binding of B and H to Sepharose 4B carrying various concentrations of C3b

The binding of C3b to Sepharose 4B was catalysed by the AP C3 convertase, and the binding of ^{125}I -labelled B ($\circ$) and H ($\triangle$) to the gels was then studied as described in the Materials and methods section. The ordinate shows the concentrations of the bound ligands in the reaction mixtures, and the concentration of C3b/ml of packed gel (20 times the concentration in the reaction mixture) is indicated on the abscissa, the three points from left to right representing the gels from one, two or three cycles of C3b deposition. The total concentrations of radiolabelled ligand/well were 3.8 nM (H) and 2.3 nM (B). The error bars indicate the 95% confidence range (three replicates).

Fig. 2. Binding of ^{125}I -labelled B and H to TPS carrying various densities and concentrations, or a constant density but various concentrations, of C3b

The binding of constant amounts of ^{125}I -labelled B (a) or H (b) to TPS coupled with various densities of C3b (Δ), or to a gel carrying a high density (1.2 mg/ml) of C3b, diluted to various extents with inactivated TPS ($\circ$), was tested as described in the Materials and methods section. The total concentrations of the ligands in the reaction mixtures were 4.1 nM (H) and 1.6 nM (B). The concentration of C3b is indicated on the abscissa as the concentration in packed gel, which is 20 times the concentration in the incubation mixture. The error bars indicate the 95% confidence range (three replicates).

Sephacrose 4B coupled with ^{125}I -labelled C3b by catalysis with B and D as described in the Materials and methods section were treated for 1.5 h at $+45^\circ\text{C}$ either with 1% SDS in VBS or with 1% SDS/0.2 M-diethanolamine/0.1 M-NaCl, pH 11.5, less than 15% of the C3b was released with the former solution, whereas the latter released more than 95%. This shows that the great majority of the binding of C3b occurred through ester bonds [2]. Binding of C3b to TPS by S-S bonds was verified by showing that more than 90% of ^{125}I -labelled C3b, coupled to TPS by the method described in the Materials and methods section, was released by a 30 min treatment at room temperature with 0.05 M-DTT / 1% SDS / 0.05 M-Tris / HCl / 0.1 M-NaCl, pH 8.2, whereas less than 10% of the C3b was released without DTT under otherwise identical conditions.

Leakage of C3b from all the gels studied was less than 0.5%/h when suspensions in BVB were stored at 4°C .

Binding of radiolabelled B and H to Sepharose 4B coupled with different amounts of C3b

When C3b was coupled to Sepharose 4B at 1, 2 or 3 stages by catalysis with the AP C3 convertase as described in the Materials and methods section, increasing amounts of C3b were bound, the relative increase being highest between stages 1 and 2. The binding of radiolabelled B and H to the particles from the three C3b coupling stages was then analysed. A striking feature of the

Fig. 3. Binding of ^{125}I -labelled B and H to a constant amount of C3b on TPS as a function of C3b density

(a) Binding of a constant amount of ^{125}I -labelled B ($\circ$) and H (Δ) to TPS, coupled with various densities of C3b and diluted with inactivated TPS to the same C3b concentration (0.082 mg/ml of packed gel), was studied as described in the Materials and methods section. The total concentrations of ligands in the reaction mixtures were 4.1 nM (H) and 1.6 nM (B). The C3b concentration in each original undiluted gel is indicated on the abscissa. The error bars indicate the 95% confidence range (three replicates). (b) The curve shows the binding of H (same as in a) plotted as a function of the average distance from centre to centre of neighbouring C3b molecules. The average distance was calculated assuming that the three-dimensional distribution of C3b molecules within gel particles is random, that the void volume is 33% of packed gel volume, and that 4% of the particle volume is occupied by the matrix itself and thus not accessible to proteins.

results (Fig. 1) is the fact that, although the binding of B seemed to follow a normal binding isotherm, the binding of H fell rapidly to negligible levels with a decreasing C3b concentration in the gel, the relation between C3b concentration and the binding of H apparently being sigmoidal. This suggests that C3b density was a major factor in creating the high-affinity H-binding sites.

Analysis of the effect of C3b density on binding of H and B to C3b coupled to TPS

To analyse the effect of C3b density on the binding of the two ligands more closely, the matrix TPS was used next as a solid phase for the following reasons: First, by using preformed C3b it is possible to study whether the effect of C3b concentration on the binding of H, observed in the previous experiment, could be due to increased formation of dimeric C3b on successive coupling steps. Secondly, the random deposition of C3b on this matrix makes it possible to calculate average distances of neighbouring C3b molecules.

The binding of radiolabelled B and H to TPS-coupled C3b was analysed as follows. C3b was coupled to the particles at various densities, or particles carrying a high density of C3b were serially diluted with particles not carrying C3b. Thus two series of suspensions were obtained, either carrying various densities and concentrations, or a fixed density but various concentrations, of C3b. All of the suspensions were used for the ligand-binding experiments at a 10% (v/v) particle concentration.

As can be seen from Fig. 2(a), B bound to the same extent to the particles irrespective of C3b density, and an equation for a binding isotherm gave a good fit to the points in both cases. In contrast, the binding of H (Fig. 2b) was distinctly affected by C3b density: a sigmoidal binding curve was generated when the binding of H to gels with various densities of C3b was tested, whereas an equation for a binding isotherm gave a good fit to the points obtained by testing dilutions of the gel with a high density of C3b. The difference between the two curves obtained for H by the two methods (varying C3b density as against dilution only) is evident below a solid-phase C3b concentration of about 0.5 mg/ml, and the difference is maximally about 3-fold in the measurable range.

As further proof of the phenomenon, the following experiment was performed. All the gels carrying higher densities of C3b than the second lowest density tested in the previous experiment were diluted to this same C3b concentration (0.082 mg/ml), a series of gels with different densities, but a constant concentration, of C3b thus being obtained. The binding of ^{125}I -labelled H and B to the gels was then tested. The results (Fig. 3a) agree well with those of the previous experiment. Here too the relative binding of H decreased rapidly below a C3b density of about 0.5 mg/ml of gel, whereas C3b density did not affect binding of B.

Fig. 3(b) shows the H binding data of Fig. 3(a) replotted so that the binding of H is presented as a function of the average distance of neighbouring C3b molecules. As can be seen, there is a steep transition between high and low binding of H at a C3b density corresponding to an intermolecular distance of about 100 nm (1000 Å).

DISCUSSION

There are previous studies suggesting that the affinity of H for surface-bound C3b may depend on the density of C3b on the surface. Thus Parker *et al.* [19] observed that, when C3b was deposited on human erythrocytes at a low density with a random distribution, H bound to the C3b-coupled cells with a more homogeneous affinity than when the binding of C3b was catalysed with a surface-bound C3 convertase. They postulated that, in the latter case, high-affinity H-binding sites were due to a non-random distribution of C3b on the surface, i.e. H has a high affinity for clusters of C3b, which are formed around surface-bound C3 convertase complexes [2,9]. Pangburn *et al.* [20] observed that rabbit erythrocytes gradually became non-activators when incubated in the presence of the C proteins C3, B, D, H, I and P, and the relative binding of H to cell-bound C3b increased in the process.

However, the question of the possible link between C3b density and the affinity of H needs further clarification. Generalized conclusions based on experiments using cells as the solid phase are risky, because cell membranes are complex structures containing different proteins and chemical groups that may specifically affect the C3b-H interaction, thus obscuring interpretation of results.

The initial idea of this study was to look for model systems in which the interactions of H with surface-bound C3b could be studied in the absence of biospecific factors. Although an absolutely neutral surface exists only in theory, matrices such as

those used for gel filtration are an attractive alternative, because they are structurally simple and well characterized and contain very low densities of charged groups which would increase the affinity of H for C3b coupled to the matrices. That the latter pertains to Sepharose 4B, which was used as a solid phase in this study, is supported by the fact that it is a known activator of the AP [2].

The experiments with Sepharose 4B, to which C3b was coupled with the AP convertase (Fig. 1), demonstrated that a high concentration of C3b on the solid phase promotes high affinity binding of H. If the high affinity H binding sites had been induced by some chemical group on the solid phases, the binding of H should have followed a normal binding isotherm like that observed with B. In control experiments, results analogous to those obtained with Sepharose 4B were also obtained with Trisacryl GF 2000 (IBF Biotechnics, Villeneuve-la-Garenne, France), which is a non-carbohydrate polymer containing hydroxy groups as the prevalent surface group (results not shown).

An important question was whether the high-affinity H-binding sites were attributable to dimers (and higher oligomers) of C3b, which are known to accumulate on surfaces on repeated coupling of C3b [26]. However, the results obtained with TPS as the solid phase show that the spacing of C3b molecules, independently of the relative amount of C3b dimers, does affect the relative number of high-affinity H-binding sites, because in those experiments the proportion of dimers did not vary with C3b density. These results do not, however, rigorously rule out the possibility that dimers of C3b could also have a role in the phenomenon.

According to the results of the TPS experiments, the density effect was clearly manifest at relatively low densities of C3b, i.e. the phenomenon was saturated with increasing C3b densities. In this context it is most remarkable that H has recently been shown to form dimers in solution under physiological conditions [27]. Such dimers could be a natural explanation for the C3b density effect observed here, as the binding of bivalent H simultaneously to neighbouring C3b molecules would greatly increase the apparent affinity over that of the monomers. Starting from this hypothesis, the effective binding range of the H dimers can be approximated from the present results. According to Fig. 3(b), this would be about 100 nm (1000 Å), which corresponds to the middle of the steep portion of the curve describing the density effect. This would mean that if the dimers of H are formed by monomers of length 60 nm (600 Å) attached to each other at one end, the H molecules could form an angle wider than 90° when bound to solid phase C3b, that is, much more than the 5° given by Perkins *et al.* [27]. This apparent discrepancy may be explained by flexibility of the dimers.

From a physiological point of view, the C3b density effect could be seen as a means to limit consumption of C on activating surfaces when a considerable amount of C3b has accumulated, leading to the inactivation of the bound C3b and thereby facilitating the removal of the activating particles through adsorption to C receptors. It may also partially explain the generally observed heterogeneity of the affinity of H for surface-bound C3b.

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