Interaction of $C3b_2$–IgG complexes with complement proteins properdin, factor B and factor H: implications for amplification

Emiliana JELEZAROVA, Anna VOGT[1] and Hans U. LUTZ[2]

Institute of Biochemistry, Swiss Federal Institute of Technology, ETH-Zentrum, CH 8092, Zurich, Switzerland

Nascent C3b can form ester bonds with various target molecules on the cell surface and in the fluid phase. Previously, we showed that $C3b_2$–IgG complexes represent the major covalent product of C3 activation in serum [Lutz, Stammler, Jelezarova, Nater and Späth (1996) Blood **88**, 184–193]. In the present report, binding of alternative pathway proteins to purified $C3b_2$–IgG complexes was studied in the fluid phase by using biotinylated IgG for $C3b_2$–IgG generation and avidin-coated plates to capture complexes. Up to seven moles of properdin 'monomer' bound per mole of $C3b_2$–IgG at physiological conditions in the absence of any other complement protein. At low properdin/$C3b_2$–IgG ratios bivalent binding was preferred. Neither factor H nor factor B affected properdin binding. On the other hand, properdin strongly stimulated factor B binding. Interactions of all three proteins with $C3b_2$–IgG exhibited pH optima. An ionic strength optimum was most pronounced for properdin, while factor B binding was largely independent of the salt concentration. $C3b_2$–IgG complexes were powerful precursors of the alternative pathway C3 convertase. In the presence of properdin, C3 convertase generated from $C3b_2$–IgG cleaved about sevenfold more C3 than the enzyme generated on C3b. $C3b_2$–IgG complexes could therefore maintain the amplification loop of complement longer than free C3b.

Key words: C3 convertase, immunoglobulins, C3.

INTRODUCTION

Proteolytic activation of the third component of human complement, C3, liberates the anaphylatoxic fragment C3a and generates C3b. The reactive thioester within C3b forms a very short-lived intermediate which reacts preferentially with hydroxyl groups on target molecules and forms ester bonds [1,2]. Much effort has been focused on the binding selectivity of C3 with respect to the molecular structures in the immediate environment of the hydroxyl group [3,4] as well as at higher structural levels [4,5].

The efficiency of covalent interaction of nascent C3b is low. Only approx. 10% of the activated C3b binds covalently to target molecules on cells in the near vicinity [1]. The majority of C3b molecules remain in the fluid phase, where they react with water or form covalent complexes with serum proteins such as C4 or IgG. IgG has a measurable affinity for C3 [6] and can serve as a target for covalent attachment of C3b. It has been demonstrated that C3b in complex with IgG [7] or C4b [8] is partially protected from factor I-dependent inactivation, and therefore has a longer half-life than free C3b (30–40 min in 2% serum [7] or 3–4 min in 20% serum [9]). These properties make C3b- and IgG-containing covalent complexes ($C3b_n$–IgG complexes) powerful activators of the amplification loop [7] and efficient opsonins that bind simultaneously to C3 and Fc receptors [10]. The same complexes may be involved in systemic, excessive complement activation which initiates inflammation and contributes to persisting injury. In many such cases intravenously applied IgG has beneficial effects. High-dose IgG diverts nascent C3b from immune complexes to fluid-phase IgG [11,12] by forming $C3b_n$–IgG complexes, and stimulates their factor I-dependent inactivation [9].

Though covalent complexes containing C3b and IgG appear to have important functional properties, their architecture and bonding is insufficiently understood. Evidence from several laboratories suggests that C3b is ester-bonded exclusively to IgG heavy chain [13–17], and within the heavy chain to the CH1 domain [15,17]. Some of these complexes contain not one, but two C3b molecules [9,16], most likely in the form of C3b–C3b–IgG complexes. The ester-linkage between the two C3b molecules is not arranged at random, but probably occurs between the 65 kDa fragments of α'C3 molecules. This type of bonding maintains the sequential arrangement during inactivation of the complexes. The dimeric organization of the inactivated C3b molecules may increase their affinity for binding to complement receptor, CR3. A detailed analysis of the properties of these complexes was hampered by the fact that complement activation generates a whole series of complexes containing C3b, C4b and IgG. The major $C3b_n$–IgG complex observed in serum, the $C3b_2$–IgG complex, can also be generated *in vitro* from C3b and IgG [16], and we have designed a new protocol to purify it. In the present paper, we address the functional properties of purified $C3b_2$–IgG complexes, and study their interaction with the proteins of the alternative pathway in order to establish their potential involvement in persistent complement amplification.

EXPERIMENTAL

Materials

As whole human IgG we used Sandoglobulin® (manufactured by the Central Laboratory, Blood Transfusion Service, Bern, Switzerland). Sephadex, $Na^{125}I$, and Bolton and Hunter Reagent {*N*-succinimidyl-3-(4-hydroxy, 5[^{125}I] iodophenyl) propionate} were from Amersham Pharmacia Biotech; factor B, properdin,

Abbreviations used: HABA, 2-(4′-hydroxybenzeneazo)benzoic acid; HSA, human serum albumin; IgG^B, biotinylated IgG; VBS, veronal-buffered saline; GVBS, VBS containing 0.1% gelatin.

[1] Present address: Karolinska Institutet, Mikrobiologiskt okt Tumörbiologiskt Centrum, Parasitologen, S-17177 Stockholm, Sweden.

[2] To whom correspondence should be addressed (e-mail hlutz@bc.biol.ethz.ch).

and factor D, from Calbiochem–Novabiochem (San Diego, CA, U.S.A.). Complement C3 was isolated from fresh plasma as described elsewhere [9,18], and was deprived of inactivated C3 before use [19].

$C3b_2$–IgG^B complexes

Biotinylation of IgG was performed according to manufacturer's instructions. Briefly, 500 mg of whole human IgG in 50 mM bicarbonate buffer (pH 8.5) and 20 mg of sulfo-*N*-hydroxysuccinimidobiotin (Pierce, Rockford, IL, U.S.A.) were incubated at room temperature for 45 min with rotation. The reaction was stopped by dilution with cold bicarbonate buffer. Free sulfo-*N*-hydroxysuccinimidobiotin was removed by ultrafiltration in Vivaspin 15 (cut-off 100 kDa; Vivascience, Westford, MA, U.S.A.), and IgG^B was concentrated to approx. 80 mg/ml.

$C3b_2$–IgG^B complexes were generated from 6 mg of C3 and 400 mg of biotinylated IgG (IgG^B), using trypsin to activate C3 [7,16]. Free IgG^B was removed by ion-exchange chromatography on QAE Sephadex A-50. The sample was dialysed and loaded to a QAE Sephadex column (2.5 cm × 7 cm), equilibrated in 20 mM Tris, 140 mM NaCl, pH 8.3 (30 ml/h, 4 °C). After washing, the bound material was eluted with a stepwise gradient using 160 mM, 180 mM and 200 mM NaCl in starting buffer. $C3b_2$–IgG^B eluted at 180 mM NaCl, as judged by SDS/PAGE. Most of the free C3b was removed on a Sephacryl S 300 HR column (1.6 cm × 60 cm, FPLC) equilibrated with veronal-buffered saline (VBS), pH 7.4, containing 0.04 % sodium azide (18 ml/h, room temperature). Fractions corresponding to $C3b_2$–IgG^B complexes were dialysed and finally purified on MonoQ HR 5/5 (FPLC) using a stepwise NaCl gradient from 100 mM to 1 M NaCl in 20 mM Tris (pH 7.8). Purified $C3b_2$–IgG^B complexes were dialysed against VBS and stored at 4 °C.

The degree of biotinylation of IgG was determined by the 2-(4′-hydroxybenzeneazo)benzoic acid (HABA) method [20] using several different IgG^B concentrations and a mean value was taken. The average molecular mass of IgG was considered to be 160 kDa. The apparent degree of biotinylation of IgG was 4–5 moles of biotin per mole of IgG. Using this number, and the determined biotin concentration in the $C3b_2$–IgG^B preparation, the $C3b_2$–IgG^B concentration was calculated.

Protein iodination

Factor H, C3 and monoclonal antibody H206 were labelled by the chloramine-T procedure as previously described [21]. Properdin and factor B were labelled by the milder Bolton and Hunter method. Dimethyl formamide was evaporated from Bolton and Hunter Reagent under nitrogen. Protein, in 10 mM phosphate buffer (pH 8), 50 mM NaCl, was added and incubated for 30 min at room temperature with gentle shaking. The reaction was stopped by 0.1 M ethanolamine, and the labelled protein was purified on Sephadex G-25 in PBS (pH 7.4). Specific activity was 0.5×10^6 c.p.m./μg for properdin and 3.8×10^6 c.p.m./μg for factor B. Labelled proteins were stored in aliquots at −20 °C and used within two weeks. Prior to use, radioiodinated proteins were dialysed and centrifuged for 3 min at 14000 ***g*** to remove any aggregated material.

Radioimmune assay

Deglycosylated avidin (neutro-avidin; Fluka) or C3b were covalently coupled at 20 μg/ml to Chemobond® microtitre plates as described before [22]. Prior to use, the plates were blocked with 150 μl/well of blocking solution [20 mg/ml of IgG, 10 mg/ml of human serum albumin (HSA) in VBS, pH 7.4] for 1 h at room temperature, and washed with 300 μl/well of suitable buffer.

Purified $C3b_2$–IgG^B complexes in VBS (pH 7.4) were mixed with the ^{125}I-labelled protein in the presence of 10 mg/ml of HSA in a total volume of 180 μl. The samples were incubated for 2 h at 37 °C, and then loaded in triplicate on the neutro-avidin-coated plates (50 μl/well). The plates were incubated for 2 h at room temperature and washed three times with 300 μl/well of VBS (pH 7.4). Bound radioactivity in each well was determined.

Factor B binding was studied in the presence of 3 mM $MgCl_2$ and, where indicated, in the presence of 7.8 μg/ml of properdin.

Properdin molar concentration was calculated with respect to the monomer.

To study the ionic strength dependence of the binding, samples were prepared in different final NaCl concentrations in veronal buffer (pH 7.4), and corresponding buffers were used for all subsequent steps. The pH dependence of the binding was assayed in a buffer containing 1.25 mM glycine, 1.25 mM Mes, 1.25 mM Hepes and 1.25 mM Tris, which was brought to different pH values by the addition of HCl or NaOH [23]. This buffer mixture has a constant ionic strength over the whole pH interval and was supplemented with 150 mM NaCl. All incubations and washings were performed with buffer of the corresponding pH.

In the controls, $C3b_2$–IgG^B complexes were replaced by IgG^B at the same molar concentration. Radioactivity bound to those wells was subtracted from the corresponding data with complexes. It was 3–5 % of that in the equivalent samples and was dependent solely on the amount of radioactive protein.

The biotin-binding capacity of the plate was determined by incubating with an excess of IgG^B under the same assay conditions. The biotin content of the solution before and after incubation was measured by the HABA method [20]. There were 109 ± 60 pM of biotin bound per well (mean from four independent measurements). The biotin content of $C3b_2$–IgG^B at the highest concentration used (12 μg/ml) was approx. 5.6 pM per well, i.e. 20-fold lower. Therefore it was assumed that most of the $C3b_2$–IgG^B complexes were trapped on the plate.

Detection of $C3b_2$–IgG^B or C3b on the plate

$C3b_2$–IgG^B was incubated at 0.05–0.6 μg/well (1.93–23.2 nM) in VBS, pH 7.0, on a neutro-avidin plate overnight at room temperature. C3b was covalently coupled to a Chemobond® plate at 0.035–0.42 μg/well (3.86–46.4 nM). The plates were blocked with 20 mg/ml of human IgG in VBS, containing 0.1 % gelatine, pH 7.0 (GVBS), and incubated with 0.03 μg/well (3.75 nM) of ^{125}I-labelled H206 for 2 h at room temperature in the same buffer. The plates were washed three times with GVBS containing 0.04 % Tween 20 and cut to determine bound radioactivity.

C3 convertase activity

$C3b_2$-IgG^B and C3b plates were prepared and blocked with human IgG as described above. C3 convertase generation was carried out in GVBS, pH 7.0, containing 3 mM $MgCl_2$ for 30 min at 37 °C (50 μl/well). Factor B was used at 50 μg/ml, factor D at 0.5 μg/ml, and properdin, where indicated, was added at a final concentration of 5 μg/ml. The plates were washed three times with GVBS containing 0.04 % Tween 20 and 20 ng/well of ^{125}I-labelled C3 (11×10^6 c.p.m./μg) was added for 30 min at 37 °C. In one series of wells C3 cleavage was performed in the presence of 0.5 M *N*-acetyl-L-tyrosine (Fluka) and aliquots from those samples were taken for SDS/PAGE prior to plate washing. The plates were washed three times with GVBS

containing 0.04% Tween 20, and bound radioactivity was determined. Control wells were treated in exactly the same way except that convertase generation was omitted. The deposition of ^{125}I-labelled C3 to those wells was subtracted from the corresponding data.

SDS/PAGE

SDS/PAGE was performed according to a modified Neville gel system using 5% or 7% separating gels [24]. Gels were stained with Coomassie Brilliant Blue R250 and scanned on a laser densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The amount of protein was quantified using the Image Quant program.

Protein concentration

Protein concentration was determined spectrophotometrically using the following $A^{1\%}_{280,\ 1cm}$: 9.7 for C3 and C3b, 14.1 for IgG, 17.8 for properdin, 12.7 for factor B, and 13.1 for factor H. Molecular masses were taken as follows: 190 kDa and 181 kDa for C3 and C3b, respectively; 53 kDa for properdin monomer; 150 kDa for factor H; 93 kDa for factor B; and 518 kDa for $C3b_2$–IgG.

RESULTS

Assay system for studying the binding of AP proteins to $C3b_2$–IgG complexes

An assay system based on the biotin–avidin interaction was developed for the purpose of this study. $C3b_2$–IgG complexes were generated from IgG^B and purified to yield a preparation containing only traces of C3b (Figure 1, lane 1). After reduction the $C3b_2$–IgG^B complex appeared on the gel as a characteristic pattern of several bands including α'-α'-heavy chain, β, heavy chain and light chain (Figure 1, lane 3). The preparation contained no detectable amount of IgG^B (Figure 1, lane 1), which at this acrylamide concentration is well separated from C3b (Figure 1, lanes 2 and 4). The presence of α' and α'-α' bands in the reduced complexes (Figure 1, lane 3) indicates the existence of free C3b and traces of C3b–C3b dimers in the preparation. Their amount was quantified from densitometric scans and was not more than 20% of the total protein.

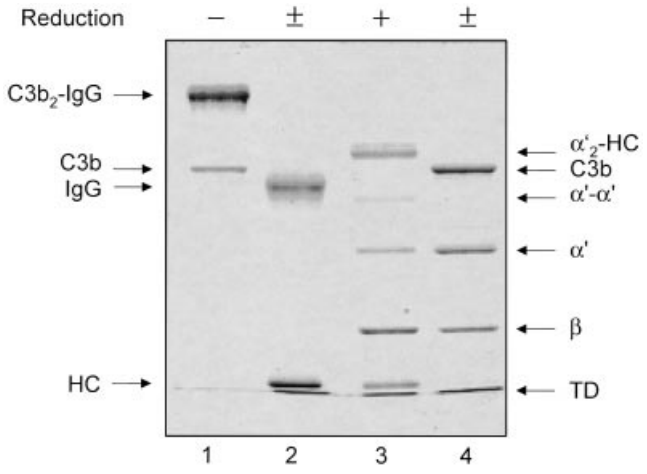

Figure 1 SDS/PAGE of purified $C3b_2$–IgG^B complexes

$C3b_2$–IgG^B complexes were boiled in sample buffer for 3 min, alkylated, and run on 5% gels (lanes 1 and 3). C3b (lane 4) and IgG^B (lane 2) were prepared reduced and non-reduced, and mixed after alkylation. Note that IgG light chain migrates together with the tracking dye (TD). HC, heavy chain.

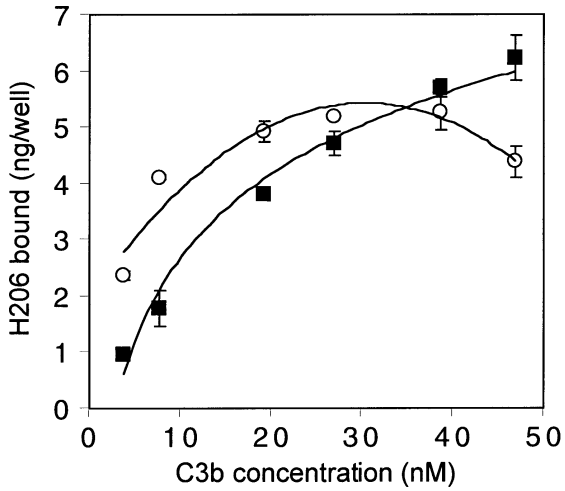

Figure 2 Immobilization of $C3b_2$–IgG^B or C3b

Increasing concentrations of $C3b_2$–IgG^B (1.93–23.2 nM, ■) or C3b (3.86–46.4 nM, ○) were immobilized on an avidin or a Chemobond® plate respectively. Note that the same number of C3b molecules per well was used for coating. The plates were incubated with ^{125}I-labelled monclonal antibody H206 for 2 h at 37 °C and the bound radioactivity was determined. $C3b_2$–IgG^B concentration is expressed as C3b residues. Data are given as means ± S.D. from triplicates.

The use of biotinylated complexes for binding studies has the advantage that protein–protein interactions occurred in solution rather than with immobilized ligand. Once equilibrium was reached, the $C3b_2$–IgG^B complexes were captured on the avidin plate together with any other protein that specifically interacted with them. We chose to label the IgG portion of the $C3b_2$–IgG complexes for several reasons. IgG has been successfully biotinylated without any loss of functional activity [25], while C3 has proved difficult in this respect [26]. The IgG portion of the complexes was not expected to take part in the interaction with the complement proteins, whilst the C3b moieties had to be in close-to-native state. In addition, the traces of C3b and C3b–C3b dimers in the $C3b_2$–IgG^B preparation could not bind to the plate, and thus interaction of the labelled ligand with them could not contribute to bound radioactivity.

A monoclonal anti-C3 antibody (H206) directed to the C-terminal 43-kDa portion of C3 [27] was used to verify capture of $C3b_2$–IgG^B complexes on the avidin plate and to determine the relative concentration of C3b moieties on the $C3b_2$–IgG^B/avidin and on the C3b plate. ^{125}I-labelled H206 binding increased with the $C3b_2$–IgG concentration (Figure 2). The same result was obtained when $C3b_2$–IgG^B complexes were incubated with the antibody in solution and then captured to the plate (results not shown). H206 binding to covalently immobilized C3b showed a different pattern, but revealed a similar number of C3b molecules per well at the corresponding concentrations.

Binding of properdin to $C3b_2$–IgG complexes

Binding of labelled properdin was indistinguishable from that of the unlabelled one at 0.25–10 μg/ml (results not shown). Properdin bound to $C3b_2$–IgG complexes in the absence of any other complement protein. The degree of binding was dependent on

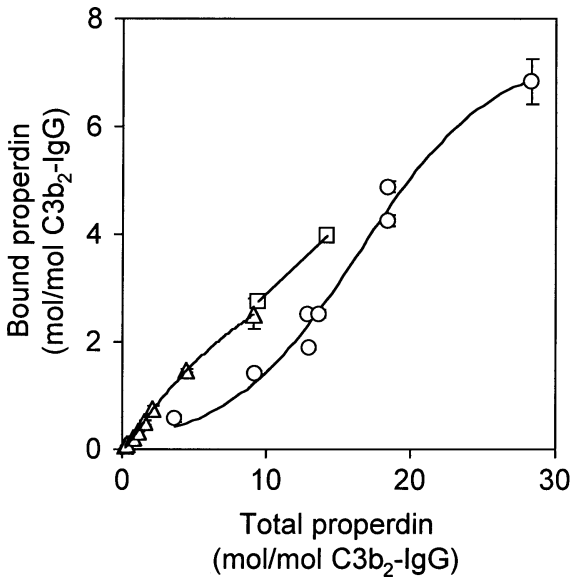

Figure 3 Concentration dependence of properdin binding to $C3b_2$–IgG^B complexes

^{125}I-labelled properdin was incubated with $C3b_2$–IgG^B complexes at increasing molar excess as described in the Experimental section. The molar concentration of properdin was calculated for monomer. Binding for 9.65 nM (○), 19.3 nM (□) and 23.2 nM (△) $C3b_2$–IgG^B is shown as means ± S.D. from at least triplicates of several independent experiments.

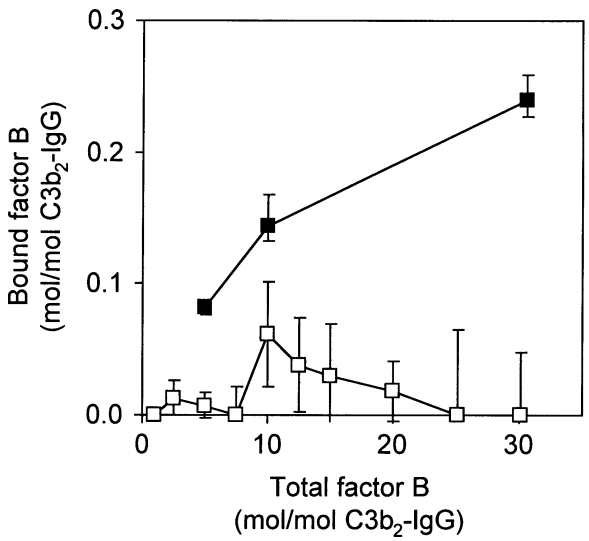

Figure 4 Binding of factor B to $C3b_2$–IgG^B complexes

^{125}I-labelled factor B (10–290 nM) was incubated with 1.93 nM $C3b_2$–IgG^B complexes without properdin (□) or in the presence of 147 nM properdin (■) in VBS (pH 7.4), containing 3 mM Mg^{2+}. Results are from triplicates and are given as means ± S.D.

the concentration of both reacting species. Up to seven moles of properdin 'monomer' bound per mole of $C3b_2$–IgG complex, provided that a large enough excess of properdin was available (Figure 3). This extent of binding corresponds to one tetramer and one trimer bound per $C3b_2$–IgG complex. On the other hand, at lower properdin/$C3b_2$–IgG ratios, 2–4 moles of properdin were bound per complex, suggesting that properdin oligomers reacted monovalently or bivalently depending on the availability of binding sites. In addition, the binding curves at higher concentrations of complexes showed higher stoichiometry of interaction at the same molar ratio of reacting species. At 10-fold molar excess of properdin, the number of bound properdin monomers increased from 1.4 to 2.7 per mole of $C3b_2$–IgG (see different curves in Figure 3). Although such behaviour is in accordance with the general predictions of the mass law, it is possible that preferential binding of a particular properdin oligomer contributes to the effect. Factor B (83 μg/ml) and factor H (117 μg/ml) had no effect on properdin binding, the differences in both cases being within one S.D.

Binding of factors B and H

The same assay system was utilized to investigate the interaction between $C3b_2$–IgG^B complexes and factors B and H. Binding of factor B to $C3b_2$–IgG^B complexes in the absence of properdin was not detectable within the concentration range tested (up to 290 nM; Figure 4). Addition of 147 nM properdin resulted in binding of up to 0.25 M of factor B per mole of $C3b_2$–IgG complex. The amount of bound factor B was low, but specific, since addition of factor D during incubation diminished bound radioactivity by approx. 40 %, which roughly corresponds to the portion of the released Ba fragment (results not shown).

Factor H binding was also relatively low and a high excess of it was required to obtain significant binding. Therefore concentration dependence was not performed. However, it is worth mentioning that of all three proteins, factor B showed the lowest binding in the absence of a stabilizer such as properdin.

Ionic strength and pH dependence

To further characterize the functional properties of $C3b_2$–IgG complexes, the ionic strength and pH dependence of their interaction with properdin, factor B and factor H was investigated under conditions giving optimal binding for each individual protein. Interaction with properdin was performed at concentrations corresponding to the steep part of the binding curve (Figure 3). For factors B and H, 1.93 nM $C3b_2$–IgG complexes were used in order to obtain high molar excess of the ligands. Binding of properdin and factor H exhibited a clear maximum at 110 mM and 60 mM NaCl respectively (Figure 5A). Factor B binding did not show a clear peak, but maximal values were approx. 120 mM NaCl and were within one S.D. in the range between 60 and 170 mM NaCl.

As the pronounced optimum of properdin and factor H binding to $C3b_2$–IgG complexes appeared rather unexpected, when compared to their binding to C3b [28], care was taken to exclude potential errors. Substitution of IgG^B in controls (see the Experimental section) with biotinylated HSA had no effect on the type of the curves. Another potential source of error was poor solubility and/or aggregation of properdin, factor B and factor H during incubation at low ionic strength. To address this issue, the proteins were incubated for 30 min at 37 °C at the corresponding salt concentration in the absence of $C3b_2$–IgG complexes and centrifuged for 30 min at 14000 ***g*** to separate any pelletable material. The radioactivity in the pellet was negligible except for properdin at low ionic strength. A comparison of bound/total to bound/soluble properdin showed that the impact on the binding curve was minimal. The decreased binding was therefore not due to poor solubility (Figure 5B).

The pH dependence of the interactions was studied at physiological salt concentration in the range between pH 4.5 and pH 8.5. A pH optimum of 5.5, 6 and 6.5 was observed for factor B, properdin and factor H respectively (Figure 6).

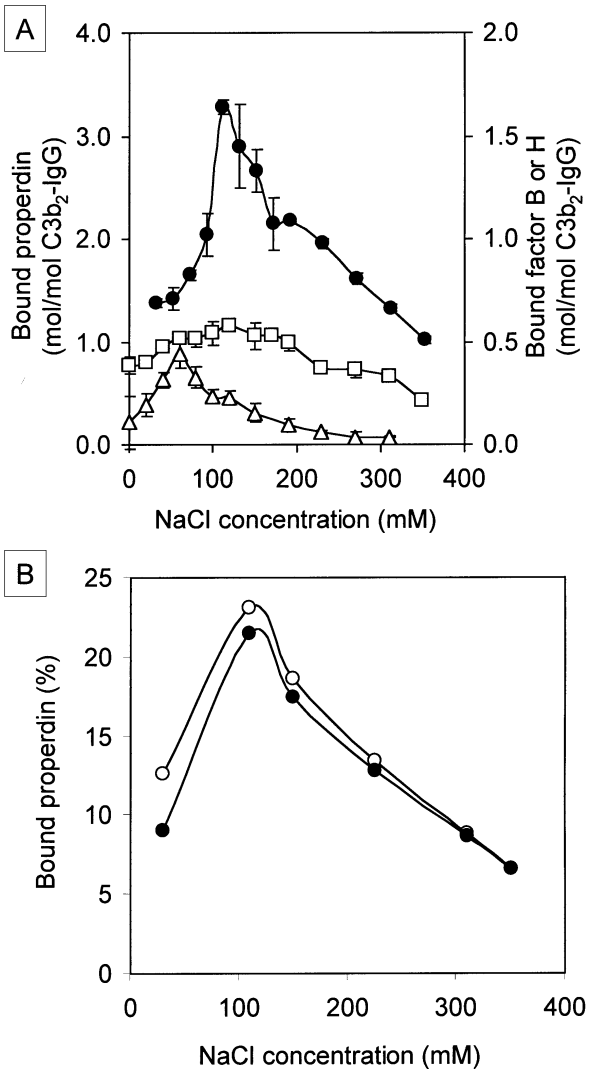

Figure 5 Effect of ionic strength on the interaction of $C3b_2$–IgG^B complexes with the alternative pathway proteins

(**A**) Ionic strength dependence. Binding of properdin (●) was studied at 9.65 nM $C3b_2$–IgG^B and 147 nM properdin. For the binding of factors B (□, 473 nM) and H (△, 147 nM) a concentration of 1.93 nM $C3b_2$–IgG^B was chosen. Binding of factor B was performed in the presence of 3 mM $MgCl_2$ and 147 nM properdin. Results are expressed as means ± S.D. from at least triplicates. (**B**) Impact of solubility on the binding of properdin. Bound properdin is expressed as percent of total (●) or as percent of soluble properdin (○).

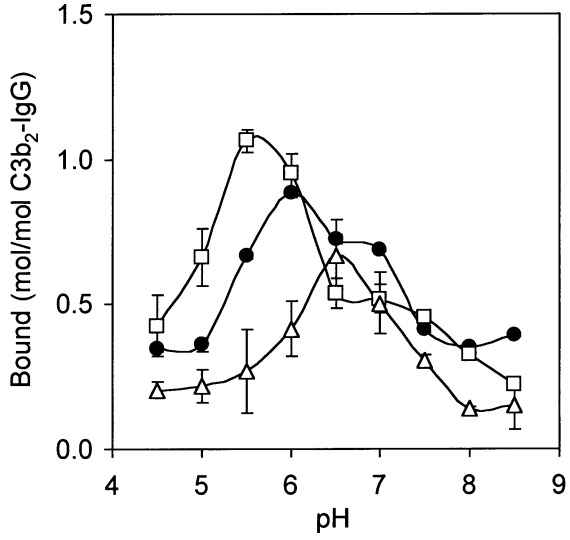

Figure 6 pH dependence of the interaction of $C3b_2$–IgG^B complexes with properdin and factors B and H

The concentration of $C3b_2$–IgG^B was 1.93 nM. Binding of factor B was performed in the presence of 3 mM $MgCl_2$ and 147 nM properdin. Labelled properdin (●), factor B (□) and factor H (△) were 29, 473 and 147 nM respectively. Results are given as means ± S.D. from at least triplicates.

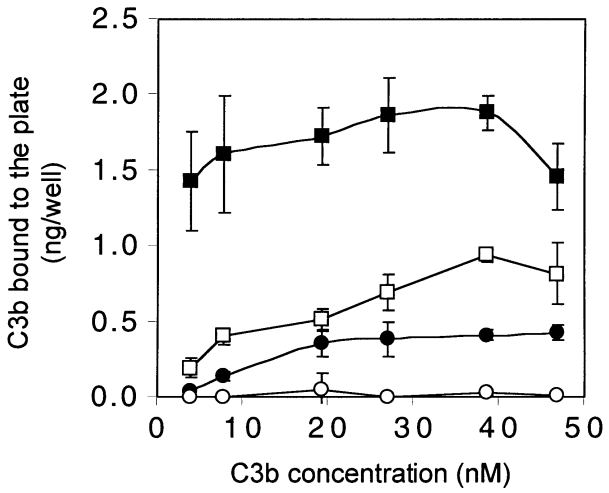

Figure 7 Comparison of C3 convertases generated from C3b or $C3b_2$–IgG^B

Complexes (squares) were immobilized on a neutro-avidin plate at 1.93 to 23.2 nM. C3b plate (circles) was prepared as described in the Experimental section using 3.86–46.4 nM C3b. Convertase was assembled by incubation with 538 nM factor B and 21 nM factor D in GVBS (pH 7.0), containing 3 mM Mg^{2+} (30 min at 37 °C). Properdin (94 nM) was added, where indicated (filled symbols). After washing, convertase activity was determined by the cleavage of ^{125}I-labelled C3 (2.1 nM, 30 min/room temperature). $C3b_2$–IgG^B molar concentration was multiplied by two in order to compare the same number of C3b molecules. Data are means ± S.D. from triplicates.

$C3b_2$–IgG complexes as precursors of a C3 convertase

In view of the possible role of $C3b_2$–IgG complexes in maintaining the amplification loop, it was of interest to verify their ability to generate C3 convertase in comparison with C3b. C3b was covalently coupled to Chemobond® plates and $C3b_2$–IgG^B complexes were immobilized on neutro-avidin plates as described in the Experimental section. The coupling concentrations were chosen to be equal with respect to the C3b moieties. As revealed by monoclonal antibody H206 binding, there were similar numbers of C3b sites on both plates (Figure 2). Convertase activity was determined by the amount of ^{125}I-labelled nascent C3b which was deposited to the plate. The specificity of C3 cleavage was verified by measuring the release of ^{125}I-C3b to the fluid phase after incubation in the presence of *N*-acetyl-tyrosine (results not shown). In the absence of properdin, convertase activity on the C3b plate was measurable, but very low (Figure 7). Cleavage of ^{125}I-labelled C3b on the $C3b_2$–IgG plate was considerably higher and showed a clear dose response. Addition of 5 μg/ml of properdin resulted in a general stimulation of

convertase activity, but the absolute effect on ^{125}I-C3b cleavage was much higher in $C3b_2$–IgG plate. Thus convertase activity was 4–11-fold higher on the $C3b_2$–IgG plate over the entire concentration range tested.

DISCUSSION

In the physiological concentration range (2–15 μg/ml) properdin bound to $C3b_2$–IgG in a dose-dependent manner (Figure 3). The binding of seven properdin monomers to one molecule of $C3b_2$–IgG can be explained as the simultaneous binding of one trimer and one tetramer. Evidently, both C3b portions of the complex, each having one binding site for properdin [29], retained their binding capacity. Therefore the $C3b_2$–IgG complex as a whole can bind properdin bivalently. Since properdin oligomers are believed to possess significant flexibility [30], bivalent binding would be possible. Indeed, at low properdin/$C3b_2$–IgG ratios bivalency was favoured (Figure 4, sigmoidal curve). A bivalent contact would increase the avidity of interaction significantly, thus offering partial protection from inactivation already at low properdin concentration. It has been shown that properdin tetramers and trimers had higher affinities for C3b-coated particles than dimers [31], but the role of the different oligomers has yet to be clarified. Higher oligomers may geometrically better suit a bivalent interaction. While C3b clusters can be formed only at the cell surface, $C3b_2$–IgG complexes have the potential to bind properdin bivalently and support alternative pathway activation even in the fluid phase.

The effect of factor B and of factor H on properdin binding was studied at a properdin concentration corresponding to the steep part of the curve (Figure 4), where binding is most sensitive to modulation. Factor B and factor H did not affect properdin binding to $C3b_2$–IgG at all. In contrast, interaction of properdin with C3b bound to zymosan was augmented by factor B and inhibited in the presence of factor H [28]. This discrepancy may originate from different methods used to detect bound protein. Since we studied binding to the complexes in the fluid phase, no ligand could be entrapped by carrier material. More importantly, properdin strongly stimulated binding of factor B to $C3b_2$–IgG complexes (Figure 4). The same result was demonstrated for solid-phase immune complexes, which bound properdin in the absence of factor B [32]. Factor B is known to have a very low affinity even for zymosan-bound C3b, the K_a being in the order of 0.65 μM [28] to 2 μM [33]. These observations strongly question the sequence of events during alternative pathway convertase generation. Binding of factor B to C3b or $C3b_2$–IgG complexes is likely to be rather inefficient under physiological conditions and may require the presence of properdin. Moreover, properdin secretion by peripheral blood T cells, monocytes and neutrophils in response to inflammatory agents would result in elevated local levels of properdin, and would lead to transient amplification in sites of inflammation [34].

Interaction of properdin and factor H with $C3b_2$–IgG complexes in solution showed optima at 110 and 60 mM NaCl respectively. Binding of these proteins to C3b has been studied earlier using C3b bound to activating surfaces, like erythrocytes [35], zymosan [28], and microtitre plates [23]. Binding under those conditions did not show optima, but was favoured at low ionic strength with a sharp decrease towards physiological salt concentration, suggesting primarily ionic interactions. We think that this discrepancy originates from studying fluid-phase interactions as compared with interactions with solid supports, of which some were even highly charged. The lack of a well-defined ionic strength maximum of factor B binding to $C3b_2$–IgG is in good agreement with the proposed primarily hydrophobic nature of its interaction with C3b [36]. Maximal binding was found at pH 6 for properdin, 6.5 for factor H and 5.5 for factor B. In the interval between pH 6 and 7, where optimum of binding to $C3b_2$–IgG complexes was achieved, ionization of histidine residues is known to occur. Other groups have also indicated the importance of histidine residues for the interaction between factors H, I and C3b [23]. None of the proteins studied showed a pH optimum in the physiological range. Such a shift may contribute to the inherent regulation of the amplification loop. Whether the difference has structural reasons or originates from studying fluid-phase rather than solid-phase interactions, has to be further investigated.

$C3b_2$–IgG complexes served as precursors for C3 convertase generation and were considerably more efficient than C3b alone (Figure 7). This property can explain the correlation of complement activity in serum with the amount of $C3b_2$–IgG generated [9]. The presence of two C3b moieties within this complex did not perturb the substrate specificity of the subsequently generated convertase, although covalent C3b dimers were implicated in the C5 convertase [37]. On the other hand, recent work reported the ability of monomeric C3b to generate C5 convertase [38]. The specificity of the convertase, therefore, does not originate only from the structure of the enzyme, but has a more complex ground.

Our results altogether strengthen the conviction that properdin stabilizes $C3b_2$–IgG complexes before any other complement protein has bound to them and that, in general, the alternative pathway of convertase generation may depend on whether properdin is bound to its precursor, a C3b or a $C3b_2$–IgG complex. Since $C3b_2$–IgG complexes can bind properdin oligomers bivalently, they are far more potent activators of the alternative pathway than nascent C3b.

This work was supported by grants to H.U.L. from the Swiss Union Bank on behalf of a client and from the Swiss National Science Foundation (grant 3200-045844). The monoclonal anti-C3 antibody (H206) was kindly provided by Professor R. Burger, Robert Koch Institute, Berlin, Germany. The excellent technical assistance of Mrs Pia Stammler is gratefully acknowledged.

REFERENCES

1 Law, S. K. and Dodds, A. W. (1997) Protein Sci. **6**, 263–274
2 Law, S. K., Lichtenberg, N. A. and Levine, R. P. (1979) J. Immunol. **123**, 1388–1394
3 Sahu, A., Kozel, T. R. and Pangburn, M. K. (1994) Biochem. J. **302**, 429–436
4 Pangburn, M. K. (1989) J. Immunol. **142**, 2759–2765
5 Pangburn, M. K. (1989) J. Immunol. **142**, 2766–2770
6 Kulics, J., Rajnavolgyi, E., Fust, G. and Gergely, J. (1983) Mol. Immunol. **20**, 805–810
7 Fries, L. F., Gaither, T. A., Hammer, C. H. and Frank, M. M. (1984) J. Exp. Med. **160**, 1640–1655
8 Meri, S. and Pangburn, M. K. (1990) Eur. J. Immunol. **20**, 2555–2561
9 Lutz, H. U., Stammler, P., Jelezarova, E., Nater, M. and Spath, P. J. (1996) Blood **88**, 184–193
10 Fries, L. F., Siwik, S. A., Malbran, A. and Frank, M. M. (1987) Immunology **62**, 45–51
11 Basta, M., Kirshbom, P., Frank, M. M. and Fries, L. F. (1989) J. Clin. Invest. **84**, 1974–1981
12 Basta, M., Langlois, P. F., Marques, M., Frank, M. M. and Fries, L. F. (1989) Blood **74**, 326–333
13 Gadd, K. J. and Reid, K. B. (1981) Biochem. J. **195**, 471–480
14 Takata, Y., Tamura, N. and Fujita, T. (1984) J. Immunol. **132**, 2531–2537
15 Shohet, J. M., Bergamaschini, L., Davis, A. E. and Carroll, M. C. (1991) J. Biol. Chem. **266**, 18520–18524
16 Lutz, H. U., Stammler, P. and Fasler, S. (1993) J. Biol. Chem. **268**, 17418–17426
17 Sahu, A. and Pangburn, M. K. (1994) J. Biol. Chem. **269**, 28997–29002
18 Hammer, C. H., Wirtz, G. H., Renfer, L., Gresham, H. D. and Tack, B. F. (1981) J. Biol. Chem. **256**, 3995–4006
19 Janatova, J., Lorenz, P. E., Schechter, A. N., Prahl, J. W. and Tack, B. F. (1980) Biochemistry **19**, 4471–4478
20 Green, N. M. (1965) Biochem. J. **94**, 23–24

21 Lutz, H. U., Nater, M. and Stammler, P. (1993) Immunology **80**, 191–196
22 Lutz, H. U., Stammler, P. and Fischer, E. A. (1990) J. Immunol. Methods **129**, 211–220
23 Soames, C. J. and Sim, R. B. (1997) Biochem. J. **326**, 553–561
24 Fasler, S., Skvaril, F. and Lutz, H. U. (1988) Anal. Biochem. **174**, 593–600
25 Gretch, D. R., Suter, M. and Stinski, M. F. (1987) Anal. Biochem. **163**, 270–277
26 Berger, M., Gaither, T. A., Cole, R. M., Chused, T. M., Hammer, C. H. and Frank, M. M. (1982) Mol. Immunol. **19**, 857–864
27 Burger, R., Bader, A., Kirschfink, M., Rother, U., Schrod, L., Worner, I. and Zilow, G. (1987) Clin. Exp. Immunol. **68**, 703–711
28 DiScipio, R. G. (1981) Biochem. J. **199**, 485–486
29 Daoudaki, M. E., Becherer, J. D. and Lambris, J. D. (1988) J. Immunol. **140**, 1577–1580
30 Higgins, J. M., Wiedemann, H., Timpl, R. and Reid, K. B. (1995) J. Immunol. **155**, 5777–5785
31 Pangburn, M. K. (1989) J. Immunol. **142**, 202–207
32 Junker, A., Baatrup, G., Svehag, S. E., Wang, P., Holmstrom, E., Sturfelt, G. and Sjoholm, A. G. (1998) Scand. J. Immunol. **47**, 481–486
33 Kazatchkine, M. D., Fearon, D. T. and Austen, K. F. (1979) J. Immunol. **122**, 75–81
34 Schwaeble, W. J. and Reid, K. B. (1999) Immunol. Today **20**, 17–21
35 Fishelson, Z., Horstmann, R. D. and Muller-Eberhard, H. J. (1987) J. Immunol. **138**, 3392–3395
36 Pryzdial, E. L. and Isenman, D. E. (1988) J. Biol. Chem. **263**, 1733–1738
37 Kinoshita, T., Takata, Y., Kozono, H., Takeda, J., Hong, K. S. and Inoue, K. (1988) J. Immunol. **141**, 3895–3901
38 Rawal, N. and Pangburn, M. K. (1998) J. Biol. Chem. **273**, 16828–16835

Received 2 November 1999/24 March 2000; accepted 18 April 2000