Structural and functional analysis of the complement component Factor H with the use of different enzymes and monoclonal antibodies to Factor H

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The action of six different enzymes on the function and structure of Factor H was investigated by use of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, haemagglutination, two enzyme-linked immunosorbent assay systems and an assay for Factor I cofactor activity. Six monoclonal antibodies directed against the 38 kDa tryptic fragment of Factor H [which contains the binding site for C3b (a 180 kDa fragment of the third component of complement) and the cofactor activity] were also used to detect cleavage products derived from the same fragment. Elastase, chymotrypsin A4 or trypsin first cleaved Factor H to 36–38 kDa fragments carrying all six monoclonal anti-(Factor H)-binding sites. In parallel, the interaction of Factor H with surface-bound C3b was lost, whereas the cofactor function was preserved. Further cleavage of the 36–38 kDa fragments into two 13–19 kDa fragments (one carrying the MAH4 and MRC OX 24 epitopes, the other the MAH1, MAH2, MAH3 and MRC OX 23 epitopes) destroyed cofactor activity. Pepsin, bromelain or papain rapidly split off a 13–15 kDa fragment of Factor H carrying the MAH1, MAH2, MAH3 and MRC OX 23 epitopes and destroyed all tested functions of Factor H. Ficin cleaved Factor H into disulphide-linked fragments smaller than 25 kDa, but did not affect the functions of the Factor H molecule. The 38 kDa tryptic fragment of Factor H is the *N*-terminal end of the Factor H molecule, as determined by *N*-terminal sequence analysis. A model is presented of the substructure of Factor H.

INTRODUCTION

Factor H (formerly β 1H) was first described by Nilsson & Müller-Eberhard (1965) as a contaminant of C3 and C5 preparations. Its function as a control protein of the alternative complement pathway was recognized by Whaley & Ruddy (1976*a,b*). Factor H has several regulatory functions in the complement system. Binding of Factor H to C3b prevents the interaction of C3b with Factor B (Pangburn & Müller-Eberhard, 1978) and C5 (Isenmann *et al.*, 1980) and displaces the Bb fragment of Factor B from the active C3 convertase and C5 convertase of the alternative pathway (Weiler *et al.*, 1976). Factor H also acts as a cofactor for the serine proteinase Factor I, which cleaves C3b to iC3b, thus abrogating the action of C3b (Pangburn *et al.*, 1977). Other studies suggest that Factor H releases endogenous Factor I from lymphocytes (Lambris *et al.*, 1980) via a specific Factor H receptor (Lambris & Ross, 1982; Schulz *et al.*, 1984), triggers the proliferation of mouse spleen cells (Hammann *et al.*, 1981), initiates the oxidative metabolism in monocytes (Schopf *et al.*, 1982), blocks the differentiation but not the proliferation of B-cells (Tsokos *et al.*, 1984) and is probably involved in the recognition of activating and non-activating cell surfaces (Horstmann *et al.*, 1984).

Factor H is a single-chain plasma glycoprotein of about 150–160 kDa containing 9.3% carbohydrate (Sim & DiScipio, 1982). Its frictional ratio of 2.11 (Sim & DiScipio, 1982), its unusual c.d. spectrum (DiScipio & Hugli, 1982) and its appearance in electron microscopy (Smith *et al.*, 1983) suggest that Factor H is an elongated asymmetric molecule approx. 28 nm $\times$ 3 nm (280 Å $\times$ 30 Å), one end of which is larger and more globular than the other. Factor H contains about 33 disulphide bridges, which not only stabilize the secondary conformation of the molecule but are also functionally relevant, since their cleavage results in a total loss of all activities (DiScipio & Hugli, 1982).

Tryptic cleavage of Factor H generates 38 kDa and 142 kDa fragments linked together by disulphide bonds (Sim & DiScipio, 1982; Hong *et al.*, 1982; Alsenz *et al.*, 1984). The cleaved Factor H no longer interacts with surface-bound C3b, whereas the cofactor function for the cleavage of fluid-phase C3b by Factor I remains unaffected (Sim & DiScipio, 1982; Hong *et al.*, 1982; Alsenz *et al.*, 1984). We have shown that the C3b-binding site and the cofactor function of Factor H are localized in the 38 kDa tryptic fragment of Factor H (Alsenz *et al.*, 1984).

In the present study Factor H was treated with different enzymes, and the resulting products were tested for C3b binding, cofactor activity, and the binding of six different

Abbreviations used: C3b, 180 kDa fragment of the third component of complement; Factor H, a controlling protein of the complement system; MAH, monoclonal antibody to Factor H; Factor I, the C3b inactivator; SDS, sodium dodecyl sulphate; e.l.i.s.a., enzyme-linked immunosorbent assay.

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monoclonal antibodies to Factor H (MAH1, MAH2, MAH3, MAH4, MRC OX 23 and MRC OX 24). In addition, we determined the sequence of the *N*-terminal ten amino acids of the 38 kDa tryptic fragment.

MATERIALS AND METHODS

Materials

Bromelain (6 units/mg), chymotrypsin A4 (90 units/mg), ficin (3 units/mg), papain (30 units/mg) and pepsin (2500 units/mg) were purchased from Boehringer, Mannheim, Germany. Elastase (type 3; 70 units/mg) and trypsin [1-chloro-4-phenyl-3-tosylamidobutan-2-one-(‘TPCK’)-treated; 400 units/mg] were from Sigma Chemical Co., St. Louis, MO, U.S.A., and Serva, Heidelberg, Germany, respectively. All other reagents were of analytical grade and obtained from Merck, Darmstadt, Germany, or Sigma Chemical Co.

Complement components

Human C3, C3b, Factor H and Factor I were purified from fresh human plasma as previously described (Alsenz *et al.*, 1984; Lambris *et al.*, 1980). 125 I-C3b was prepared by the iodogen method (Pierce Chemical Co., Rockford, IL, U.S.A.) (Fraker & Speck, 1978) to a specific radioactivity of 5×10^6 – 5.5×10^6 c.p.m./ μ g of C3b.

Enzymic fragmentation of Factor H

Enzymic cleavage of Factor H was carried out in 150 mM-NaCl/10 mM-sodium phosphate buffer, at pH 7.3 (trypsin, ficin and chymotrypsin A4), at pH 8.5 (elastase), at pH 6.2 (bromelain and papain) or at pH 2.0 (pepsin). The enzymic reaction with trypsin, chymotrypsin A4 or elastase was stopped with 1 mM-di-isopropyl phosphorofluoridate. The cleavage of Factor H with ficin, bromelain or papain was stopped with the same volume of 100 mM-iodoacetamide (in 0.1 M-Tris/HCl buffer, pH 10) and incubated for 15 min at 37 °C. The pH was then adjusted to pH 7.0 with 0.1 M-Tris/HCl buffer, pH 2.0. The reaction with pepsin was terminated by adjusting the pH to 8.0 with 0.01 M-NaOH and allowing 10 min to inactivate the pepsin. Before use, bromelain and papain were activated by incubating 5 μ l of each (5 mg/ml) with 15 μ l of 150 mM-NaCl/10 mM-sodium phosphate/10 mM-L-cysteine/HCl/2 mM-EDTA buffer, pH 6.2, for 5 min at 22 °C.

Antibodies

The affinity-purified monoclonal antibodies against human Factor H, namely MAH1, MAH2, MAH3, MAH4, MRC OX 23 and MRC OX 24, were prepared as previously described (Alsenz *et al.*, 1984; Schulz *et al.*, 1984; Sim *et al.*, 1983).

Polyclonal goat antibodies to Factor H and C3 were prepared as described by Schulz *et al.* (1984). Peroxidase-conjugated rabbit anti-(goat γ -globulin) antibodies and peroxidase-conjugated rabbit antibodies to mouse immunoglobulins were purchased from E-Y Laboratories, San Marcos, CA, U.S.A., and Dako, Copenhagen, Denmark.

Affinity chromatography

The monoclonal antibodies MAH1 and MAH4 were coupled to CNBr-activated Sepharose 4B (Pharmacia, Freiburg, Germany). Purification of pepsin-generated

Factor H fragments was carried out in 150 mM-NaCl/10 mM-sodium phosphate buffer, pH 7.3, and bound fragments were eluted with 150 mM-NaCl/10 mM-sodium phosphate/3 M-KSCN buffer, pH 7.3 (MAH1), or 150 mM-NaCl/10 mM-sodium phosphate buffer, pH 4.0 (MAH4).

E.l.i.s.a.

The binding of monoclonal antibodies to Factor H, cleaved Factor H or isolated Factor H fragments was assayed as described by Schulz *et al.* (1984). The binding of fluid-phase C3b to micro-titre plates coated with Factor H or fragmented Factor H was detected by polyclonal goat anti-C3 antibodies and a peroxidase-conjugated anti-(goat IgG) antibody (Alsenz *et al.*, 1984). There was no difference in the binding capacity of the micro-titre plates for Factor H and cleaved forms of Factor H, as tested with a polyclonal anti-(Factor H) antibody. The interaction of Factor H or cleaved Factor H with C3b-coated micro-titre plates was described by Alsenz *et al.* (1984).

Factor H haemagglutination assay

The binding of serially diluted Factor H or enzyme-treated Factor H with cell-bound C3b was tested in a haemagglutination assay (Schmitt *et al.*, 1981).

Assay for the Factor H cofactor activity

The cofactor activity of Factor H for the cleavage of C3b by Factor I was assayed by the method of Alsenz *et al.* (1984).

SDS/polyacrylamide-gel electrophoresis and Western blotting

SDS/5–20% polyacrylamide-gel electrophoresis (Laemmli, 1970), Western blot analysis (Towbin *et al.*, 1979), estimation of molecular masses with reference proteins (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and other conditions have been described previously (Alsenz *et al.*, 1984).

Protein sequencing analysis

Edman degradation was performed in a Beckman model 890D sequencer, with the methods described by Brauer *et al.* (1975) and Zimmermann & Pisonzo (1977).

RESULTS

Cleavage of Factor H with elastase and chymotrypsin A4

Factor H was treated with elastase at an enzyme/substrate ratio of 1:10 (w/w) and the fragments were analysed by SDS/polyacrylamide-gel electrophoresis. In the non-reduced gel, the 148 kDa band of intact Factor H was degraded to a 32 kDa fragment via several intermediates (128 kDa, 106 kDa and 83 kDa) (Fig. 1a). Western blotting analysis demonstrated that the fragments reacted with each of the six monoclonal antibodies (results not shown). Under reduced conditions the 160 kDa band of the intact Factor H was first cleaved by elastase within 2 min into a 124 kDa and a 36 kDa fragment (Fig. 1b), the latter carrying all antigenic sites for the binding of the six monoclonal antibodies (Fig. 1c). The 124 kDa fragment was then stepwise degraded to 92 kDa, 85 kDa and 79 kDa pieces. In parallel with the first cleavage, the ability of Factor H to interact with surface-attached C3b was lost (Fig. 2a). In comparison,

Fig. 1. Cleavage of Factor H with elastase

Factor H was treated with elastase (E) (enzyme/substrate ratio 1:10) for different periods of time. The samples were analysed by SDS/5–20%-polyacrylamide-gel electrophoresis (30 μ g of Factor H/track) under non-reducing (a) and reducing (b) conditions. In addition, a mixture (1+1) of 1 min and 45 min elastase-cleaved Factor H was analysed by the immunoblotting technique on SDS/5–20%-polyacrylamide gels under reducing conditions (c).

the binding of fluid-phase C3b to surface-attached elastase-cleaved Factor H (Fig. 2b) as well as the cofactor activity (Fig. 2c) were not decreased to the same extent as was the binding of Factor H to surface-bound C3b.

The 36 kDa fragment was further cleaved (probably via a 30 kDa fragment) into a 13 kDa fragment carrying the MAH1, MAH2, MAH3 and MRC OX 23 epitopes and a 16 kDa fragment carrying the MAH4 and MRC OX 24 epitopes. Concomitant with the disappearance of the 36 kDa fragment, the binding of fluid-phase C3b to surface-bound and cleaved Factor H disappeared, and the cofactor activity was decreased (Figs. 2b and 2c, samples 5 and 6). The action of chymotrypsin A4 at an

enzyme/substrate ratio of 1:100 (w/w) was similar to that of elastase.

Cleavage of Factor H by pepsin, bromelain and papain

After enzymic digestion of Factor H with pepsin at an enzyme/substrate ratio of 1:1000 (w/w) followed by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions, Factor H was degraded very fast into several main bands (134 kDa, 73 kDa, 68 kDa, 62 kDa, 53 kDa and 15 kDa) and numerous minor bands (Fig. 3a). Immunoblotting showed that MAH1, MAH2, MAH3 and MRC OX 23 reacted with a 15 kDa fragment, whereas MAH4 and MRC OX 24 bound to the

Fig. 2. Functional activities of elastase-cleaved Factor H

Factor H was incubated with elastase (enzyme/substrate ratio 1:10) for 0 (sample 1), 1 (sample 2), 5 (sample 3), 15 (sample 4), 45 (sample 5), and 120 min (sample 6) at 37 °C. The samples were tested by incubation with C3b-coated sheep erythrocytes in a haemagglutination test (a) or attached to micro-titre plates and tested in an e.l.i.s.a. for the binding of C3b (b). In the cofactor assay (c) the samples (1–6) were incubated with ^{125}I -C3b and Factor I for 2 h at 37 °C, subjected to SDS/9–20% -polyacrylamide-gel electrophoresis under reducing conditions and the gels exposed to a Kodak X-ray film for 24 h. ^{125}I -C3b incubated with buffer (a), factor I (b), Factor H (c) or diisopropyl phosphorofluoridate-pretreated elastase (d) served as controls.

134 kDa fragment and to a variety of fragments with molecular masses between 134 kDa and 35 kDa (results not shown). Under reducing conditions, none of the fragments had a molecular mass of more than 59 kDa (Fig. 3b). The antibodies MAH1, MAH2, MAH3 and MRC OX 23 bound to one or two smaller fragments (14–16 kDa), whereas MAH4 and MRC OX 24 reacted only with higher-molecular-mass fragments (largest fragment: 48 kDa) (Fig. 3c).

Functional assays showed that treatment of Factor H with pepsin caused a complete loss of all activities (C3b-binding, cofactor activity) within 10 min (Figs. 4a–4c). In addition, Factor H was treated with pepsin for 3 min at 37 °C, and the resulting fragments were purified by affinity chromatography on MAH1–Sephacrose 4B and MAH4–Sephacrose 4B. SDS/polyacrylamide-gel electrophoresis under non-reducing conditions revealed that the breakthrough of the MAH1–Sephacrose 4B contained 134 kDa, 73 kDa and 62 kDa fragments, whereas the breakthrough of the MAH4–Sephacrose 4B contained

62 kDa and 15 kDa fragments and traces of a 73 kDa fragment. The eluates of MAH1–Sephacrose 4B and MAH4–Sephacrose 4B consisted of intact Factor H and a 15 kDa fragment and of intact Factor H and a 134 kDa fragment respectively. Both eluates reacted with all six monoclonal antibodies, whereas the breakthrough of the MAH1–Sephacrose 4B and MAH4–Sephacrose 4B bound only MAH4 and MRC OX 24 and MAH1, MAH2, MAH3 and MRC OX 23 respectively (results not shown). A comparison of the breakthroughs of the two columns on SDS/polyacrylamide-gel electrophoresis under reducing conditions revealed that both contained the 59 kDa, 32 kDa and 26 kDa fragments, indicating that none of these fragments carried epitopes responsible for MAH1 and MAH4 binding.

The C3b-binding and cofactor activities were only found in the eluates (results not shown). This was presumably due to the presence of uncleaved Factor H in the eluates.

Treatment of Factor H with bromelain or papain

Fig. 3. Breakdown of Factor H by pepsin

Factor H was incubated with pepsin for 1–120 min (enzyme/substrate ratio 1:1000). The resulting fragments were analysed by SDS/5–20% polyacrylamide-gel electrophoresis (30 μ g of Factor H/track) under non-reducing (a) and reducing (b) conditions. Under reducing conditions the immunoblotting technique was used to determine the binding of the different monoclonal antibodies to the fragments generated by pepsin treatment of 10 min (c).

(enzyme/substrate ratio 1:10, w/w) gave results similar to those of the cleavage with pepsin.

Cleavage of Factor H with ficin

After Factor H was incubated with ficin at an enzyme/substrate ratio of 1:50 (w/w) for 120 min, SDS/polyacrylamide-gel electrophoresis under non-reducing conditions showed hardly any degradation fragments (Fig. 5). Some very faint bands were seen on the original gel after treatment for more than 120 min in

the molecular-mass range of 45–130 kDa. By immunoblotting (non-reducing conditions) all these cleavage products were shown to react with the six monoclonal antibodies. SDS/polyacrylamide-gel electrophoretic analysis under reducing conditions demonstrated that Factor H was cleaved by ficin within 3 min into a variety of fragments, none of which had a molecular mass greater than 25 kDa (Fig. 5b). Immunoblotting analysis under reducing conditions showed two types of fragments: one carrying the MAH1, MAH2, MAH3 and MRC OX 23

Fig. 5. Treatment of Factor H with ficin

Factor H was incubated with ficin for 3–120 min (enzyme/substrate ratio 1:50). The resulting fragments were analysed by SDS/5–20%-polyacrylamide-gel electrophoresis under non-reducing (a) and reducing (b) conditions. The binding of the monoclonal antibodies that react with Factor H to the fragments generated by ficin treatment (45 min, 37 °C, enzyme/substrate ratio 1:10) was determined by the Western blotting technique after SDS/15%-polyacrylamide-gel electrophoresis under reducing conditions (c).

Three types of enzymes were found that were different in their action on Factor H, as follows.

The first type (elastase/chymotrypsin A4) cleaved Factor H similarly to trypsin. Initially, Factor H was cleaved into a larger 124 kDa/125 kDa fragment and a smaller disulphide-linked 36 kDa/37 kDa fragment, the latter carrying the binding sites that reacted with the six monoclonal antibodies. Concomitant with that cleavage, Factor H no longer interacted with surface-bound C3b

(Fig. 2a), whereas it could still react with fluid-phase C3b (Fig. 2b) and had cofactor activity (Fig. 2c). Prolonged digestion resulted in the cleavage of the smaller fragment into a 13 kDa fragment, which reacted with the MAH1, MAH2, MAH3 and MRC OX 23 antibodies, and a 16 kDa/19 kDa fragment, which reacted with the MAH4 and MRC OX 24 antibodies. In parallel, Factor H lost the functional activities that were tested.

The second type of enzyme (pepsin, papain and bromelain) rapidly split off a 13–15 kDa fragment from the Factor H molecule that reacted with the MAH1, MAH2, MAH3 and MRC OX 23 antibodies (Fig. 3). This action destroyed all functional activities that were tested (Fig. 4). In addition, affinity-purified pepsin-induced fragments that carry the binding sites for either the antibodies MAH1, MAH2, MAH3 and MRC OX 23 or the antibodies MAH4 and MRC OX 24 were functionally inactive.

The third type of enzyme (ficin) cleaved Factor H into several fragments held together by disulphide bonds (Fig. 5). Under non-reducing conditions all the monoclonal antibodies bound to the same fragment (results not shown), whereas under reducing conditions the binding sites for MAH1, MAH2, MAH3 and MRC OX 23 and for MAH4 and MRC OX 24 were found on different fragments. Despite the fact that the molecule was cleaved by ficin at numerous sites, the functional activities of Factor H were not significantly affected (Fig. 6).

The 38 kDa tryptic fragment of Factor H containing the binding sites for all six monoclonal antibodies, the C3b-binding site and the cofactor activity was shown here to be the *N*-terminal end of the Factor H molecule. Since Smith *et al.* (1983) reported that C3b binds to the more-globular segment in the Factor H molecule, the globular portion must be the *N*-terminal end of the molecule, including the 38 kDa tryptic fragment.

Pepsin split off from the Factor H molecule a 15 kDa fragment carrying the MAH1, MAH2, MAH3 and MRC OX 23 epitopes. Since the pepsin-generated 48 kDa fragment (reduced conditions) carried the MAH4 and MRC OX 24 epitopes and was larger than the 38 kDa tryptic fragment, it must be the connecting part between the trypsin-induced 38 kDa and 142 kDa fragments. Therefore the 48 kDa fragment consists of the *C*-terminal end of the 38 kDa tryptic fragment and the *N*-terminal end of the 142 kDa tryptic fragment (Fig. 7). Therefore the 15 kDa fragment must be split off from the *N*-terminal end of the Factor H molecule (Fig. 7). In addition, the pepsin-generated 59 kDa, 32 kDa and 26 kDa fragments (reducing conditions), which were shown by affinity chromatography to carry no monoclonal-antibody-binding sites, must be derived from the 142 kDa tryptic fragment.

Ficin-cleaved Factor H contained under reducing conditions 13 kDa and 16 kDa fragments carrying the MAH1, MAH2, MAH3 and MRC OX 23 epitopes and a 16 kDa fragment bearing the binding sites for MAH4 and MRC OX 24. Under non-reducing conditions these epitopes were found on one fragment. Therefore the 38 kDa tryptic fragment must contain at least one disulphide bond.

Previously we showed that the monoclonal antibodies MAH1, MAH2, MAH4 and MRC OX 24 blocked the binding of Factor H to surface-bound C3b (Alsenz *et al.*, 1984; Sim *et al.*, 1983). In addition, the binding sites for MAH1, MAH2 and MAH4 are not available in

Fig. 6. Influence of ficin on the functional activities of Factor H

Factor H was treated with ficin (enzyme/substrate ratio 1:50) for 0 (sample 1), 5 (sample 2), 10 (sample 3), 30 (sample 4), 60 (sample 5) and 120 min (sample 6) at 37 °C. The binding of fluid-phase C3b to cleaved Factor H was tested in an e.l.i.s.a. (a), and a haemagglutination assay with sheep erythrocytes (b) was used to detect the binding of cleaved Factor H to surface-bound C3b (b). Buffer served as a control (sample 7). The cofactor activity (c) of Factor H was tested by incubating the samples 1–6 with ^{125}I -C3b and Factor I. ^{125}I -C3b treated with buffer (a), Factor H (b), Factor I (c), and iodoacetamide-pretreated ficin (d) served as controls.

preformed C3b–Factor H complexes (Schulz *et al.*, 1984). These antibodies therefore bind to the C3b-binding site, or close to it. In contrast, MAH3 and MRC OX 23 have no effect on C3b–Factor H interactions, and the binding site of MAH3 in preformed C3b–Factor H complexes is still detectable. In this study, all activities of Factor H were destroyed when the binding sites for MAH1 and MAH2 were separated from the MAH4 and MRC OX 24 epitopes. Therefore it is likely that the C3b-binding site within the 38 kDa tryptic fragment of Factor H is localized between the determinants where these two groups of monoclonal antibodies react, or is formed by two structures of the molecule that are separated by that cleavage (Fig. 7). As the model in Fig. 8 proposes, the cleavage of Factor H with trypsin, elastase or chymotrypsinogen A4, or the binding of the monoclonal antibody MRC OX 24 to Factor H (without cleavage of H), both

appear to modulate the C3b-binding site (Fig. 8, form B). This form (B) of Factor H has lost the ability to interact with surface-attached C3b, whereas the cofactor activity remains unaffected or is even increased (Hong *et al.*, 1982; Alsenz *et al.*, 1984; Sim *et al.*, 1983). Since the Factor H molecule was identical in both assays, the model suggests an additional difference between the conformation of surface-bound and fluid-phase C3b. Since MAH1, MAH2 and MAH4 inhibit both Factor H binding to C3b and cofactor activity, and because separation of the two groups of determinants destroys both activities, this suggests a single binding site for both fluid-phase and surface-bound C3b in Factor H. However, although it is likely that only one binding site exists, these data do not totally exclude the possibility that two close but distinct binding sites exist within the 38 kDa tryptic fragment of Factor H.

Fig. 7. Model of the substructure of Factor H

The size and shape of this model were selected from the data of Sim & DiScipio (1982), DiScipio & Hugli (1982) and Smith *et al.* (1983). The binding sites of the monoclonal antibodies to Factor H were assigned with respect to the data in the Discussion section. The cleavage sites (broken vertical lines) of the different enzymes, the orientation of the resulting Factor H fragments with regard to the N- and C-terminal ends and the possible positions of disulphide bonds are schematically shown below the model.

Fig. 8. Model of the interaction of both native and cleaved Factor H with surface-bound and fluid-phase C3b

Interactions of native Factor H (form A), trypsin-cleaved Factor H (form B) and pepsin-cleaved Factor H (form C) with surface-bound and fluid-phase C3b are shown.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 107 and A5) and from the Stiftung Volkswagenwerk. J.D.L. is the recipient of an E.M.B.O. grant (ALTF 121-1982).

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Received 26 February 1985/22 July 1985; accepted 27 August 1985