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Immunochemical Analysis of Rabbit Antihuman Fibrinopeptide B Antibodies†

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ABSTRACT: The serologic immune response to human fibrinopeptide B (hFPB), a unique thrombin proteolytic product of the fibrinogen Bβ chain (Bβ1–14), was studied as a basis for developing immune probes for sequential regions in the fibrinogen molecule. Outbred rabbits hyperimmunized with hFPB analogue–bovine albumin conjugates produced antisera specific for native hFPB, as measured by radioimmunoassay using [^{125}I]Tyr-hFPB analogue as a tracer. By use of this assay system, these sera were found capable of distinguishing free hFPB from the peptide bound to its parent fibrinogen molecule. Immunoreactive sites seen by these sera were characterized in terms of displacement of bound radiolabeled tracer by synthetic homologues of the hFPB sequence. In four immune rabbit sera, displacement of binding comparable on a molar basis using hFPB as inhibitor was obtained with fragments Bβ3–14, Bβ5–14, and Bβ7–14 but not with fragment Bβ9–14. In an additional three sera, inhibition comparable to that of intact hFPB occurred only with fragments Bβ3–14 and Bβ5–14. In five of the seven sera tested, Arg^{14} seemed to contribute critically to hFPB antigenicity. Isoelectric focusing experiments showed that the immune response of each sera was limited to 8–10 discrete bands, indicating that these functional restrictions in site specificity were associated with a limited structural heterogeneity. Our data suggest that the region comprising the COOH-terminal 8–10 residues of hFPB is immunologically hindered by its attachment to the Bβ chain of its parent fibrinogen molecule. These findings are analogous to those of earlier studies on the immunochemistry of human fibrinopeptide A (hFPA) wherein immunogenic determinants in hFPA which were characteristic of the free peptide in solution could be localized to the COOH-terminal decapeptide sequence [Wilner, G. D., Nossel, H. L., Canfield, R. E., & Butler, V. P., Jr. (1976) *Biochemistry 15*, 1209]. Taken together, these results suggest that antisera which are specific for free fibrinopeptides in solution may be directed against conformationally ordered regions shown to be present in the COOH-terminal halves of these peptides [Huseby, R. M. (1973) *Physiol. Chem. Phys. 5*, 1]. It is concluded that antibodies of limited heterogeneity may be produced by immunization with peptide haptens possessing limited numbers of determinants and that antibodies so produced are potentially useful as sequence-specific immune probes.

The transformation of soluble fibrinogen into insoluble fibrin is preceded by the highly selective, limited proteolysis of two pairs of small activation peptides from the NH_2 terminal of the Aα and Bβ chains of fibrinogen (Blombäck, 1967; Blombäck et al., 1966). These activation peptides, termed fibrinopeptide A (hFPA)[1] and fibrinopeptide B (hFPB) after their respective chains of origin, consist of the NH_2-terminal 15 residues of the human Aα chain and the NH_2-terminal 14 residues from the human Bβ chain of fibrinogen (Blombäck, 1967). The primary structure of these peptides is shown in Figure 1. These activation peptides are thought to function as enzymatically labile protecting groups, covering specific polymerization sites located in the NH_2-terminal (E) region of the fibrinogen molecule (Blombäck et al., 1978). Our major interest in fibrinopeptides has been in their measurement as specific indices of thrombin action in vivo (Wilner, 1978). Sensitive and specific radioimmunoassays have been developed by ourselves and others for the quantitation of fibrinopeptides

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[1] Abbreviations used: hFPA, human fibrinopeptide A; hFPB, human fibrinopeptide B; Dnp, dinitrophenyl; Boc, N^{α}-*tert*-butoxycarbonyl.

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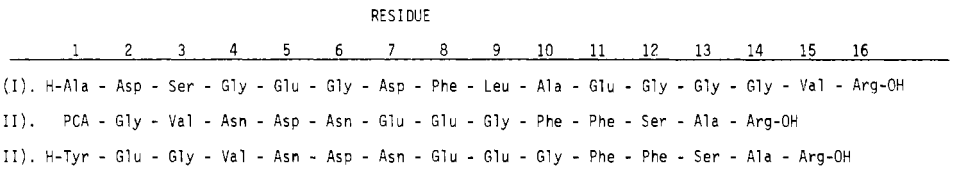

FIGURE 1: Primary structures of hFPA (I) and hFPB (II) are shown, as elucidated by Blombäck et al. (1966) and Blombäck (1967). The Tyr-hFPB (III) analogue was radiolabeled as described under Materials and Methods and used as a tracer in inhibition and immune-binding studies as described in the text.

in plasma samples of both man and experimental laboratory animals as tools for studying and defining the pathophysiology of both clinical and experimental thrombosis (Wilner, 1978).

Previous studies on the immunochemistry of hFPA showed that, while the reactivities of different sera with free hFPA in solution were all quite similar, marked differences were noted in the reactivities of these sera with larger fragments of the fibrinogen molecule containing fibrinopeptides (Canfield et al., 1976; Nossel et al., 1976). It was further demonstrated that these differences in the reactivity of sera with the free peptides as opposed to that of the peptide bound to its parent molecule could be correlated with differences in the localization of the antigenic immunoreactive site as determined by reactivity of the antisera with sequential homologues of the hFPA structure (Wilner et al., 1976). One implication from these studies was that conformational differences existed in the region of attachment of the peptide to the parent fibrinogen α chain, such that the COOH-terminal portion of hFPA is made unavailable for reactivity with those antisera as a result of this attachment. More recently, studies by Butler et al. (1978) have shown that certain rabbit antisera against hFPB also show differences in reactivity toward fragments of fibrinogen-containing hFPB as compared with that of free peptide in solution. Since spectral studies (Huseby, 1973) indicate that both fibrinopeptides A and B possess a similar ordered conformation in the COOH-terminal halves of the molecule, the question arises as to whether, as was previously noted with antibodies to FPA, the determinants seen by anti-hFPB antisera might be likewise localized to this conformationally ordered COOH-terminal region of the peptides. Therefore, studies were carried out by using synthetic peptide homologues to define the region of hFPB which binds to these rabbit antisera.

Materials and Methods

Reagents. All chemicals were of reagent grade. N^{α}-*tert*-Butoxycarbonyl (Boc) amino acids were purchased from Bachem, Inc., Torrance, CA. Boc amino acids with protected side chains were γ-benzylglutamic acid, β-benzylaspartic acid, *O*-benzylserine, and N^{g}-tosylarginine. 2-Pyrrolidone-5-carboxylic acid was introduced as the *N*-Boc derivative, and asparagine was introduced as the active ester. All Boc amino acids were of the L configuration with the exception of glycine. Purity of the Boc amino acids was assessed by melting points (uncorrected) and thin-layer chromatography. Sephadex G-25F and DEAE-Sephadex A-25 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Bio-Gel P-2, 100–200 mesh acrylamide, and methylenebis(acrylamide) were purchased from Bio-Rad Laboratories, Rockville Centre, NY. Carrier ampholytes were purchased from LKB, Inc., Rockville, MD. Styrene–divinylbenzene beads, 1% cross-linked, 200–400 mesh, chloromethylated (1.16 mequiv of Cl^- per g), were purchased from Lab Systems, Inc., San Mateo, CA, and were extracted with warm (60 °C) dimethylformamide for 18 h prior to use. Human fibrinogen solutions, native hFPB, and [^{125}I]Tyr-hFPB analogue were prepared as described previously (Bilezekian et al., 1975). Synthetic hFPB was purchased from Bachem, Inc., Torrance, CA. The preparation of anti-hFPB antisera has been described (Bilezekian et al., 1975). Protein-coated charcoal suspensions and buffered saline–ovalbumin solutions were prepared as previously described (Wilner et al., 1976). Solutions containing inhibitor or tracer were prepared in Tris-buffered saline, pH 8.5, containing 1% ovalbumin. ^{125}I-Labeled tracer dilutions were adjusted to 10 000 cpm and contained about 9.0×10^{-13} μCi of ^{125}I per 50 μL. Specific activity of the tracer varied from 40 to 100 μCi/μg.

Synthesis of Homologues of Human Fibrinopeptide B. Peptides Bβ3–14, Bβ5–14, Bβ7–14, Bβ9–14, and Bβ1–13 were synthesized manually by using Merrifield's solid-phase method as previously described (Wilner et al., 1976) but with the following modifications: (a) amino-group deprotection was accomplished with 50% trifluoroacetic acid in methylene chloride, (b) neutralization was carried out by using 5% diisopropylethylamine in methylene chloride, and (c) all residue couplings were repeated following repetition of the neutralization step. Following an initial desalting on a 2.6 × 46 cm Sephadex G-25F column eluted with 0.05 M NH_4HCO_3 buffer, the peptides were definitively purified on a 1.2 × 16 cm DEAE-Sephadex A-25 column equilibrated with 0.005 M sodium borate[2] buffer, pH 9.0, and eluted with a linear sodium chloride gradient varying to 0.5 M. Major peaks were pooled and rechromatographed on a 2.0 × 70 cm Bio-Gel P-2 column equilibrated with 0.05 M NH_4HCO_3. Eluate fractions were analyzed by absorbance at 225 nm by using a Gilford 240 spectrophotometer. Identity of the peptides was confirmed by amino acid analysis following acid hydrolysis, as described (Wilner et al., 1976). Peptides were stored freeze-dried in desiccators at −20 °C.

Inhibition Studies. The displacement of binding of antisera to [^{125}I]Tyr-hFPB analogue (Figure 1) by various peptides and by fibrinogen was performed by using the radioimmunoassay procedure previously described (Wilner et al., 1976), except that incubation was carried out for 18 h at 4 °C instead of 1 h. Peptide concentration in stock solutions of inhibitors was determined by amino acid analysis. Fibrinogen concentration was determined spectrophotometrically by using an extinction coefficient of 15.06 (Doolittle, 1975). For inhibition studies with fibrinogen, final dilutions of antisera were carried out in Tris–ovalbumin buffer solutions containing 1 unit of Trasylol (MOBAY, New York) and 0.01 unit of hirudin (Sigma Chemical Co., St. Louis, MO) per 100 μL of diluted serum to inhibit any plasmin or thrombin which might be present.

Equilibrium Dialysis Studies. These were performed as described by Eisen (1964) using [^{125}I]Tyr-hFPB analogue as the ligand (Figure 1).

Isoelectric Focusing Experiments. Isoelectric focusing of antibodies and detection by radioautography using radiolabeled antigen were carried out as described by Briles & Davie (1975), with later modifications (Nicolotti et al., 1979).

[2] Molarity refers to the initial concentration of sodium borate used in preparing the buffer solution.

Table I: Characteristics of Anti-hFPB Antisera

antisera	assay dilution[a]	B_{max}[b]	$K_a \times 10^{-9}$ (M)	hFPB, 50% (pmol)[c]
R22	1:2000	0.75	4.4	0.45
R24	1:3000	0.75	3.6	0.41
R30	1:2500	0.73	2.5	0.50
R28	1:5000	0.70	7.5	0.38
R31	1:4000	0.74	1.8	0.38
R29	1:1500	0.74	1.8	0.46
R23	1:1000	0.73	2.2	0.52

[a] Value represents initial antiserum dilution added to assay mixture. [b] Each value represents the ratio of bound to total added radiolabeled tracer dilution and 24-h incubation at 4 °C. [c] Each value represents the quantity (picomoles) of hFPB required for displacement of 50% of bound radiolabeled tracer at the given assay dilution. Radioimmunoassay was carried out as described under Materials and Methods.

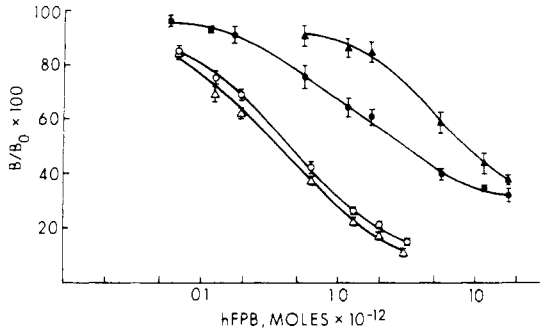

FIGURE 2: Relative abilities of different concentrations of hFPB and human fibrinogen to inhibit binding of the ^{125}I-labeled Tyr-hFPB analogue with antisera R22 and R28. R28 plus hFPB (△), R22 plus hFPB (○), R22 plus fibrinogen (●), and R28 plus fibrinogen (▲) are shown. Inhibition studies were carried out as detailed under Materials and Methods. Initial dilutions of R22 (1:2000) and R28 (1:5000) were carried out in Tris–ovalbumin buffer, pH 8.5, containing hirudin (0.01 unit) and Trasylol (1 unit).

Results

The characteristics of the rabbit anti-hFPB antisera which were investigated are shown in Table I. The affinity constants, determined by equilibrium dialysis using [^{125}I]Tyr-hFPB analogue as a bindable tracer, were similar for all seven antisera and showed K_a values of (1.8–7.5) × 10^{-9} M. All sera were capable of maximal binding of at least 70% of added labeled tracer at dilutions of ≥1:50. The mean dose of hFPB required for displacing 50% of bound radiolabeled tracer was 0.44 ± 0.02 pmol.

To test whether the immunoreactive determinants of free hFPB are also present in the parent fibrinogen molecule, we compared the reactivities of antisera R22 and R28 with free hFPB to those with fibrinogen. Figure 2 illustrates the relative abilities of hFPB and intact human fibrinogen to displace [^{125}I]Tyr-hFPB analogue bound to antisera R22 and R28. While the reactivities of these two sera with free hFPB appear to be quite similar (Figure 2 and Table I), significant differences are noted in ability of fibrinogen to displace bound tracer as compared with that of hFPB (Figure 2). The molar ratios comparing 50% displacement of bound tracer for hFPB vs. that for fibrinogen are 1:6.9 for for antiserum R22 and 1:24 for antiserum R28. Similar differences in the reactivities of free hFPB vs. fibrinogen are evident in the remaining five anti-hFPB antisera as well.

The abilities of sequential synthetic homologues of hFPB to displace 50% of the bound radiolabeled Tyr-hFPB analogue from each of the seven antisera are shown in Table II. The dominant immunoreactive site for R22, R24, and R30 is within

Table II: Molar Reactivities of Anti-hFPB Antisera with Synthetic hFPB Homologues

peptide[a]	antisera R22	R24	R30	R28	R31	R29	R23
1 2 3 4 5 6 7 8 9 10 11 12 13 14							
PCA-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg-OH (Bβ1–14)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
H-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg-OH (Bβ3–14)	4.0	4.9	3.6	1.4	2.9	4.9	4.2
H-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg-OH (Bβ5–14)	3.0	2.7	2.6	1.1	2.4	3.1	6.7
H-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg-OH (Bβ7–14)	>2.8 × 10^4 (22%)	1.4 × 10^3	3.8 × 10^3	4.4	8.7	88	92
H-Gly-Phe-Phe-Ser-Ala-Arg-OH (Bβ9–14)	>5.4 × 10^4 (41%)	1.3 × 10^4	9 × 10^3	292	3.6 × 10^3	1.8 × 10^3	2.3 × 10^4
PCA-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-OH (Bβ1–13)	23	26	2.2	740	423	2.1	150

[a] The comparative abilities of hFPB homologues to displace 50% of bound [^{125}I]Tyr-hFPB analogue are shown, expressed as normalized molar ratios. Where 50% inhibition was not attained, the largest quantity of inhibitor used is listed, and percent inhibition achieved by that amount of inhibitor is shown in parentheses.

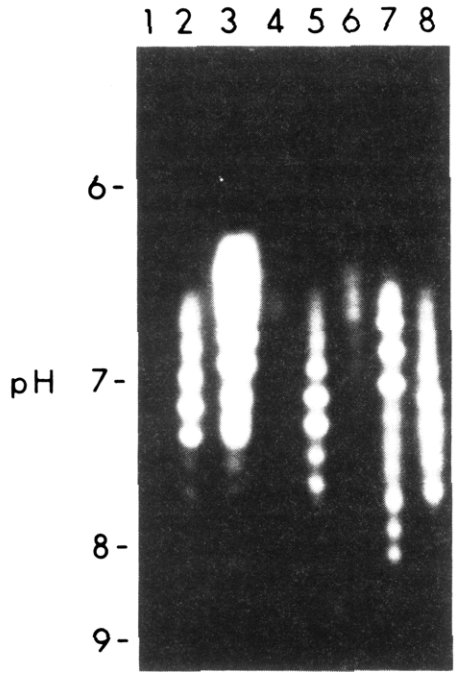

FIGURE 3: Radioautograph (48-h exposure) of isoelectrically focused rabbit anti-hFPB antisera carried out as described under Materials and Methods. Track 1, 10 μL of nonimmune rabbit serum; track 2, 1 μL of R31; track 3, 0.5 μL of R28; track 4, 3 μL of R23; track 5, 1 μL of R30; track 6, 3 μL of R24; track 7, 1 μL of R29; track 8, 1 μL of R22.

the COOH-terminal decapeptide (Bβ5–14). In antisera R23, R28, R29, and R31, the principal immunoreactive site appears shifted even further toward the COOH-terminal end of hFPB and is located within the COOH-terminal eight residues (Bβ7–14) of the hFPB sequence. All antisera appear sensitive to variable degrees to removal of COOH-terminal arginine, suggesting that Arg^{14} is also involved as an immunoreactive determinant.

Since the immunoreactive sites for all these sera were 8–10 residues in size, it was theorized that the antibodies were responding at most to two sets of determinants. If this were so, then the immune sera might show only limited heterogeneity. Isoelectric focusing studies (Figure 3) showed 8–10 discrete bands per sera, confirming the limited heterogeneity of the immune response. No binding response was obtained with isoelectrically focused nonimmune rabbit serum.

Discussion

As shown in Table II, the dominant immunoreactive site for all seven sera seems to reside within the COOH-terminal 10-residue segment of the peptide. With antisera R22, R24, and R30, Asp^5 and Asn^6 are critical determinants of immunoreactivity, since deletion of these residues results in at least a 500-fold decrease in the ability to displace bound counts. With the remaining four sera, Glu^7 and Glu^8 appear to be critical, and the immunoreactive site seems localized to the COOH-terminal eight-residue segment of the peptide. In addition, Arg^{14} appears to be a critical determinant in all seven sera tested. These functional restrictions in immunoreactive site specificity also correlated with restricted antibody heterogeneity, as shown in Figure 3. In most instances, these hFPB-specific antibodies are the product of two to three B lymphocyte clones, assuming that three bands represent one clone product (Askonas et al., 1970; Awdeh et al., 1970). Our findings suggest that immunization of outbred rabbits with hFPB–albumin produces a restricted immune response, localized to two overlapping immunoreactive sites within sequence Bβ5–14, the COOH-terminal decapeptide portion of hFPB. It is hypothesized that Asp^5 and Glu^7 represent the NH_2-terminal boundaries of these overlapping sites. With the exception of antisera R30 and R29, the COOH-terminal boundary of the sites extends to include Arg^{14}.

The differences seen in reactivity between fibrinogen and fibrinopeptide B with anti-hFPB antisera demonstrate that the COOH-terminal region of hFPB is immunologically hindered by its attachment to the Bβ chain in fibrinogen. These data are analogous to earlier findings with antisera to hFPA localizing antigenic determinants characteristic of free peptide in solution within the COOH-terminal 10-residue sequence (Wilner et al., 1976). Since it has been suggested on the basis of circular dichroic spectral and statistical analyses of a variety of mammalian fibrinopeptides in solution that an ordered conformation exists within the COOH-terminal halves of both fibrinopeptides A and B despite differences in sequence (Huseby, 1973), it is likely that antibodies specific for free fibrinopeptides are directed against these distinct, ordered regions. On the basis that regions of attachment of both hFPA and hFPB are accessible to proteolytic enzymes such as thrombin (Blombäck, 1967), which releases both hFPA and hFPB, or copperhead snake venom, which selectively cleaves hFPB (Shainoff & Dardik, 1979), we theorize that differences in immunoreactivities between free and attached fibrinopeptides reflect conformational differences associated with attachment rather than simple steric occlusion by other parts of the fibrinogen molecule. In this regard, our findings are analogous to studies by Maron et al. (1967), Sachs et al. (1972a–c), and Furie et al. (1974, 1979), demonstrating that conformational alterations in limited regions of proteins are detectable by restricted antibody probes, using radioimmunoassay systems as the means of detection. Our studies are unique, since, in this instance, restricted immunochemical probes for detecting conformationally distinct regions in proteins were prepared by restricting the size of the immunogen rather than by selection of a particular subpopulation of antibodies from a heterogeneous antibody repertoire.

In conclusion, our present study supports the earlier contention of Haber et al. (1967) that antibodies of limited heterogeneity may be prepared by restricting antigenic heterogeneity. In the case of small aromatic haptens such as Dnp, studies by Keck et al. (1973) and later by Civin et al. (1976) show that the size and nature of the carrier protein may play a critical role in determining the heterogeneity of the immune response. More recently, Kipps et al. (1978) have presented data suggesting that restricted response to complex antigens containing Dnp as the haptenic group may involve regulation by suppressor T cell mechanisms. However, the nature of the immune response to peptide haptens may be different from the immune response to Dnp. We propose that, in the case of peptide haptens, restriction of the hapten-specific response is primarily a function of the number of immunoreactive sites present within the peptide antigen and is largely independent of the size or nature of the carrier protein.

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Human Factor $\bar{D}$ of the Alternative Complement Pathway. Physicochemical Characteristics and N-Terminal Amino Acid Sequence†

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ABSTRACT: Factor $\bar{D}$ is a plasma serine protease which is required for normal complement activation via the alternative pathway. Factor $\bar{D}$ was isolated from human plasma by sequential CM-Sephadex C-50, Sephadex G-75, and heparin–Sepharose chromatography. Isolated factor $\bar{D}$ (20 μg) showed a single protein band (molecular weight 25000) in the presence of 2-mercaptoethanol, when stained with either Coomassie Brilliant Blue or periodic acid–Schiff's reagent. Isoelectric focusing of purified factor $\bar{D}$ demonstrated two protein bands with isoelectric points at pH 7.0 and pH 6.6. Both bands displayed factor $\bar{D}$ hemolytic activity and both were also detected in normal human serum. On agarose gel electrophoresis, factor $\bar{D}$ (both purified and in serum) was of γ electrophoretic mobility in the presence of 1.8 mM calcium. In the presence of EDTA (5 mM), isolated factor $\bar{D}$ was of β mobility, while factor $\bar{D}$ in serum was of α_1-α_2 mobility. Eleven synthetic ester and nitroanilide substrates were evaluated for hydrolysis by factor $\bar{D}$. Among these, only the factor X_a substrate *N*-benzoylisoleucylglutamylarginine-*p*-nitroanilide hydrochloride was hydrolyzed by factor $\bar{D}$. Minimum molecular weight was 22000; no unusual characteristics in amino acid composition were revealed. Amino-terminal amino acid sequence was determined by manual Edman degradation. The amino-terminal sequence of factor $\bar{D}$ is Ile-Leu-Gly-Gly-Arg-Glx-Ala-Glx-Ala–. Factor $\bar{D}$, therefore, is distinct from, but homologous with, other plasma serine proteases, including thrombin, $C1\bar{r}$, $C1\bar{s}$, coagulation factors X_a and $X1_a$, and plasmin.

Factor $\bar{D}$ is a plasma protease which catalyzes the proteolysis of factor B and is required for normal complement activation via the alternative pathway (Alper & Rosen, 1971; Götze & Müller-Eberhard, 1971; Müller-Eberhard & Götze, 1972; Hunsicker et al., 1973). It consists of a single polypeptide chain with a molecular weight of 25000 (Fearon et al., 1974; Götze, 1976; Dieminger et al., 1976; Brade et al., 1974a,b; Volanakis et al., 1977; Lesavre & Müller-Eberhard, 1978; Götze, 1975). The results of various studies differ regarding the electrical charge of factor $\bar{D}$, whether determined by electrophoresis or isoelectric focusing (Fearon & Austen, 1975; Müller-Eberhard & Götze, 1972; Hunsicker et al., 1973; Lesavre & Müller-Eberhard, 1978; Konno et al., 1978; Martin et al., 1976; Götze & Müller-Eberhard, 1977; Davis et al., 1978). Factor B and C3b, in the presence of magnesium ions, form a reversible complex; this results in a conformational alteration of factor B, which allows its cleavage by factor $\bar{D}$

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