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Immunology

Properdin: Initiation of alternative complement pathway

(properdin pathway/C3 convertases)

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ABSTRACT Activation of the classical complement (C) system involves conversion of C1 to its active state with subsequent cleavage of C4 and C2 so as to form the classical C3 convertase, $C\overline{42}$ (a bar indicates the activated form of a protein), which sequentially cleaves C3 and C5 to initiate the cytolytic event associated with the complete reaction. An alternative, or properdin-dependent, pathway to complement activation generates a C3 convertase, $C\overline{3B}$, that is formed by cleavage of B with $\bar{D}$ in the presence of C3b, the major cleavage fragment of C3. C3b is capable of binding activated properidin ($\bar{P}$) with resultant stabilization of $C\overline{3B}$, which otherwise rapidly decays by loss of $\bar{B}$ activity.

Initial cleavage of C3, a prerequisite for formation of $C\overline{3B}$, is demonstrated to occur through the interaction of native C3 and B in the presence of either $\bar{D}$ or $\bar{P}$ alone, or together. The effect of $\bar{P}$ on the interaction of $\bar{D}$, B, and C3 is attributed to stabilization of $C3\tilde{B}$ as has been shown for $C\overline{3B}$. Larger amounts of $\bar{P}$ and B with C3 in the absence of $\bar{D}$ form a C3 convertase that is designated $(\bar{P})C3\tilde{B}$ to indicate that demonstrable cleavage of B does not occur although the active site is available. The generation of this initial convertase, as assessed by C3 inactivation, is dose-related to $\bar{P}$ and B inputs. The presence of both $\bar{P}$ and $\bar{D}$ greatly augments initial cleavage of C3 with $\bar{D}$ fully uncovering the active site of B and $\bar{P}$ stabilizing that site.

Activation of the classical complement system by an antigen–antibody complex containing immunoglobulin of the appropriate class involves conversion of C1 from its precursor form to its active state, $C\bar{1}$ (8), with subsequent cleavage of C4 (9) and C2 so as to form the classical C3 convertase, $C\overline{42}$ (10). The latter sequentially cleaves C3 and C5, thereby initiating formation of the terminal $C\overline{56789}$ complex (11) which mediates the cytolytic effect of the complement reaction.

An alternative pathway to complement activation was described some 20 years ago as consisting of a group of serum factors which interacted with complex microbial polysaccharides to form a C3-cleaving enzyme distinct from $C\overline{42}$ (7). An alternative C3 convertase, $C\overline{3B}$, was subsequently characterized as being formed by the interaction of C3b, the major cleavage fragment of C3, with B and $\bar{D}$, two plasma proteins of the alternative pathway (6). $\bar{D}$, a 25,000 molecular weight protein (4) whose active site is of the serine esterase class (5), cleaves B in the presence of C3b to form the labile bimolecular complex, $C\overline{3B}$, which is similar to $C\overline{42}$ in its capacity to activate C3–C9 (12). $C\overline{3B}$ decays by loss of $\bar{B}$ activity, which limits its potential for cytolytic activity, but can be regenerated by the action of $\bar{D}$ on additional B (12). Activated properdin, $\bar{P}$, a gamma globulin of 223,000 daltons (13) with an isoelectric point of greater than 9.5, binds to C3b and retards decay of convertase function, thereby profoundly augmenting activation of C3–C9 by $C\overline{3B}$ (14). Formation of $C\overline{3B}$ requires the presence of C3b, indicating that an additional C3 convertase must be formed during initial activation of the alternative pathway. The finding that the introduction of $\bar{P}$ to a mixture of C3, B and $\bar{D}$ results in C3 cleavage (15) does not distinguish between a contribution to generation of the initial C3 convertase or a stabilizing effect on the $C\overline{3B}$ subsequently formed. Although interaction of C3, B, and $\bar{D}$ is sufficient to result in initial C3 cleavage, B or $\bar{D}$ can be limited to the extent that C3 cleavage is not sustained unless $\bar{P}$ is present. The further finding that the interaction of C3 with larger amounts of $\bar{P}$ and B in the absence of $\bar{D}$ leads to C3 cleavage without B cleavage establishes a role for $\bar{P}$ in the formation of an initial convertase of the alternative pathway distinct from $C\overline{3B}$.

MATERIALS AND METHODS

Preparation of Alternative Pathway Factors and Classical Complement Components from Human Plasma. B (4) and $\bar{D}$ (5, 14) were purified to homogeneity so that each preparation presented a single band on alkaline disc gel electrophoresis corresponding to the position in a replicate, unstained gel from which activity was eluted. B was measured by radial immunodiffusion (4) and $\bar{D}$ by Folin analysis (16) with human serum albumin as the reference standard. $\bar{P}$, purified from the pH 6.0 euglobulin of fresh acid-citrate-dextrose (ACD) plasma by sequential chromatography on quaternary aminoethyl Sephadex (Pharmacia Fine Chemicals, Piscataway, N.J.), sulfopropyl Sephadex, and Sephadex G-200, yielded a single band on acid disc gel electrophoresis (17). On Ouchterlony analysis, $\bar{P}$ presented no precipitin

Abbreviations: ACD plasma, acid–citrate–dextrose plasma; VBS, isotonic Veronal-buffered saline; GVB^{++}, VBS containing 5×10^{-4} M magnesium, 1.5×10^{-4} M calcium, and 0.1% gelatin; $DGVB^{++}$, half-isotonic GVB^{++} with 2.5% dextrose; EDTA, ethylenediaminetetraacetate; GVB-EDTA, GVB^{++} from which cations were omitted and containing 0.04 M EDTA.

Nomenclature: The nomenclature for the classical complement (C) components conforms to that agreed upon under the auspices of the World Health Organization (1968) *Bull. W.H.O.* **39**, 395. The nomenclature for alternative pathway factors is that provisionally adopted at the Second International Congress of Immunology, 1974, Brighton, United Kingdom. B (1) is the symbol for a protein that has been termed C3 proactivator (2) and glycine-rich beta glycoprotein (GBG) (3); D (4, 5) is the symbol for the protein that has been termed precursor C3 proactivator convertase (6); and P represents properdin (7). A bar over a letter indicates the activated form of a protein. The symbol ~ over a letter is introduced to indicate activation of a protein without demonstrable cleavage. With respect to fragmentation, the larger fragment is designated by the suffix b and the lesser fragment by a. ^{hu} indicates human origin; ^{gp}, guinea pig; ^{oxy} indicates the oxidized form of C2. EA is a sheep erythrocyte sensitized with rabbit antibody.

arcs with anti-whole human serum and a single arc with monospecific rabbit anti-P. $\bar{P}$ was quantitated by radial immunodiffusion (17). C3 was isolated from the pH 5.4 euglobulin of fresh ACD plasma by chromatography on DE-52 (DEAE) cellulose (Whatman Biochemicals, Kent, England) and hydroxyapatite (Bio-Rad Laboratories, Rockville Centre, N.Y.) (18, 19), followed by repeat chromatography on DE-52 cellulose. The final product, with or without radiolabel, was homogeneous on alkaline disc gel electrophoresis and was free of C3b as analyzed by immunoelectrophoresis, alkaline disc gel electrophoresis and isoelectric focusing. C3b was prepared by incubating this C3 with $EAC1^{gp}4^{hu\ oxy}2$ (20), sedimenting the cells, and removing the supernatant; more than 99% of C3 was rendered hemolytically inactive as assessed by a classical C3 titration (19). C3 and C3b protein concentrations were measured by radial immunodiffusion. $C\bar{1}^{gp}$, $C2^{gp}$ (21), and $C2^{hu}$ (22) were functionally purified; and C5, C6, C7, C8, and C9 were obtained from Cordis Corp., Miami, Fla.

Assays. Isotonic Veronal-buffered saline (VBS), pH 7.5, containing 5×10^{-4} M magnesium, 1.5×10^{-4} M calcium and 0.1% gelatin (GVB^{++}), half-isotonic GVB^{++} with 2.5% dextrose ($DGVB^{++}$), and GVB^{++} from which cations were omitted and containing 0.04 M ethylenediaminetetraacetate (GVB-EDTA) were used as diluents in hemolytic assays (21). C3 was assayed with $EAC1^{gp}4^{hu\ oxy}2$ as described (19). Hemolytically active B was assessed with EAC43P, the cellular intermediate bearing $\bar{P}$ (14), to permit detection of smaller amounts of B than was possible with EAC43 (12). EAC43P, generated by incubation of 4.0 μg of $\bar{P}$ with 10^8 EAC43 for 10 min at 15°, were incubated at 1×10^7 with limiting dilutions of B and 10 ng of $\bar{D}$ in 0.2 ml of $DGVB^{++}$ for 30 min at 30° to form the intermediate bearing the $\bar{P}$-stabilized $\overline{C3B}$ convertase. The hemolytic sites were developed by addition of 0.3 ml of rat serum (Pel-Freez Biologicals, Rogers, Ark.) diluted 1:15 in GVB-EDTA and further incubation for 60 min at 37°. After addition of 1.5 ml of saline, the extent of hemolysis was determined, and the average number of hemolytic sites per cell (Z) was calculated, with a reaction mixture lacking B representing the reagent blank.

Immunoelectrophoresis was performed in 1.5% agarose (Indubiose Agarose, Fisher Scientific Co., Pittsburgh, Pa.), 0.025 M Veronal, 0.002 M EDTA, pH 8.4, at 15 V/cm for 20 min. Precipitin arcs were developed with monospecific antisera to C3 and B which had been harvested from rabbits previously immunized with homogeneous preparations of C3 and B, respectively.

RESULTS

Capacity of $\bar{P}$ to sustain inactivation of C3 when $\bar{D}$ or B is limited

Variable amounts of $\bar{D}$ were incubated with B and C3 to define an amount of $\bar{D}$ insufficient to generate a $\overline{C3}$ convertase so that a contribution of $\bar{P}$ to initial C3 cleavage could be assessed. The utilization of B in the presence of C3b also was determined at variable inputs of $\bar{D}$. Four-hundredths micrograms of B, 0.4 μg of C3 or C3b, and variable amounts of $\bar{D}$ in 80 μl of $DGVB^{++}$ were incubated for 60 min at 30°, and residual B and C3 were hemolytically assessed. The activity of B or C3 incubated in $DGVB^{++}$ alone did not change during 60 min at 30° and the percent inactivation in the various reaction mixtures is expressed relative to these control activities. The dose-response effect of $\bar{D}$ on inactivation of B and C3 in the presence of C3 was sigmoidal, while inactivation of B in the presence of C3B WAS FIRST ORDER (Fig. 1).

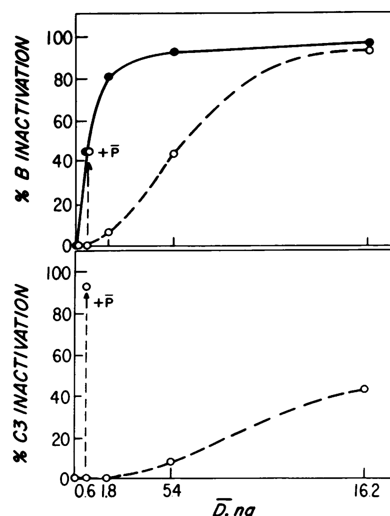

FIG. 1. Inactivation of B (O- -O, upper panel) and C3 (O- -O, lower panel) by their interaction with variable amounts of $\bar{D}$. The effects of $\bar{P}$ on the reaction with limited $\bar{D}$ and the result of substituting C3b for C3 on B inactivation (●—●) are depicted for comparison.

The reaction mixture containing C3, B, and 0.6 ng $\bar{D}$ exhibited neither B nor C3 inactivation, while addition of 0.1 μg of $\bar{P}$ resulted in 45% and 93% B and C3 inactivation, respectively; this amount of $\bar{P}$ had no effect in the absence of $\bar{D}$. The capacity of $\bar{P}$ to effect an interaction of C3, B, and limited $\bar{D}$ is consistent with a role for $\bar{P}$ in the formation of the initial C3 convertase of the alternative pathway.

The dose-response effect of $\bar{P}$ on the interaction of 0.04 μg of B, 0.4 μg of C3, and 1 ng of $\bar{D}$ in 80 μl of $DGVB^{++}$ was assessed after incubation for 60 min at 30° by hemolytic assay of residual B and C3. In the absence of $\bar{P}$, 8% of B and 2% of C3, respectively, were inactivated, while the presence of $\bar{P}$ effected a dose-related inactivation of both proteins (Fig. 2).

The effect of $\bar{P}$ on the capacity of B to inactivate C3 in the presence of $\bar{D}$ was assessed over a 1000-fold range of B input. Four-tenths micrograms of C3, 100 ng of $\bar{D}$, and variable amounts of B in the presence and absence of 0.5 μg of $\bar{P}$ in 60 μl of $DGVB^{++}$ were incubated for 60 min at 30°, and residual C3 was hemolytically assayed. Although inactiva-

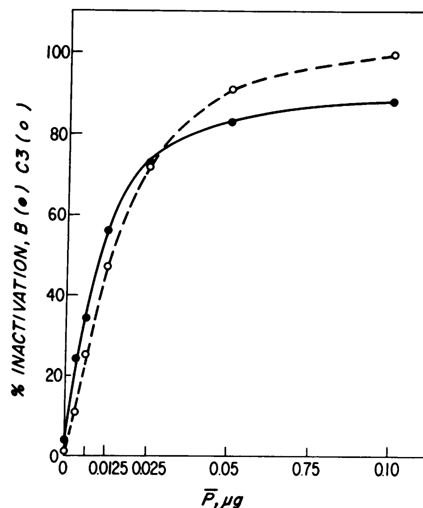

FIG. 2. Inactivation of B (●—●) and C3 (O- -O) in the presence of limited $\bar{D}$ and variable amounts of $\bar{P}$.

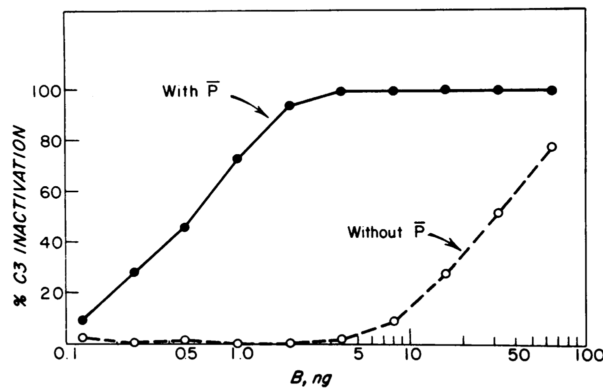

FIG. 3. Inactivation of C3 by interaction with $\bar{D}$ and variable amounts of B in the presence (●—●) and absence (○- -○) of $\bar{P}$.

tion of C3 was dose-related to B input without $\bar{P}$, the presence of $\bar{P}$ reduced the B requirement by 50-fold (Fig. 3). In the absence of $\bar{P}$, B was completely inactivated at all doses whether or not C3 was inactivated, indicating that B had been activated by $\bar{D}$ to form an initial C3 convertase which presumably decayed at lower doses of B before achieving detectable C3 inactivation. The capacity of $\bar{P}$ to sustain C3 inactivation in these reaction mixtures is attributed to retarding decay of this initial convertase.

Capacity of $\bar{P}$ to sustain cleavage of C3 with B in the absence of $\bar{D}$

Four-tenths micrograms of C3, 0.2 μg of B, 0.1 μg of $\bar{D}$, and 1 μg of $\bar{P}$, a larger amount than used previously, were interacted in various combinations in 60 μl of $DGVB^{++}$ for 60 min at 30°; and residual C3 and B were hemolytically assessed. Inactivation of C3 was complete with B and $\bar{P}$ with or without $\bar{D}$, while no other combinations were active (Table 1). In contrast, B inactivation was negligible in the mixture of C3 and $\bar{P}$ while the presence of $\bar{D}$ was accompanied by 82% inactivation of B. Thus, $\bar{P}$ interacted with C3 and B to yield a C3 convertase whose formation was not associated with B inactivation.

To determine if hemolytic inactivation of C3 by B and $\bar{P}$ reflected cleavage of C3, and if B had indeed functioned in its uncleaved state, the following reaction mixtures were incubated for 60 min at 30° in 60 μl of $DGVB^{++}$: 50 μg of C3 alone; 20 μg of B alone; C3 and B; C3, B and 2 μg of $\bar{P}$; and C3, B, $\bar{P}$, and 0.1 μg of $\bar{D}$. Each reaction mixture was subjected to immunoelectrophoretic analysis for the antigenic positions of C3 and B and assessed for residual C3 and B hemolytic activities. C3 incubated with B was in its native electrophoretic position, while C3 was partially converted to C3b, the major cleavage fragment of more anodal mobility, by incubation with $\bar{P}$ and B, and was completely converted

Table 1. Inactivation of C3 and B in various reaction mixtures

Reaction mixture	% Inactivation C3	% Inactivation B
$\bar{P}$ + C3	0	—
$\bar{D}$ + C3	1	—
B + C3	3	1
$\bar{P}$ + $\bar{D}$ + C3	2	—
$\bar{P}$ + B + C3	100	2
$\bar{P}$ + B + $\bar{D}$ + C3	100	82

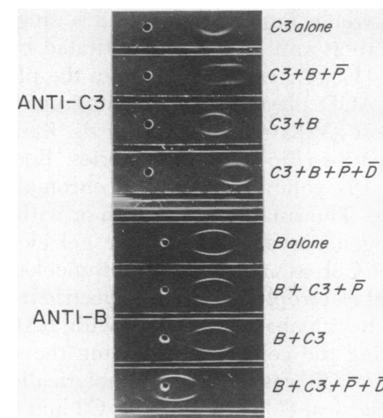

FIG. 4. Immunoelectrophoretic analysis of cleavage of C3 (upper four samples) and B (lower four samples) in reaction mixtures containing C3, B, $\bar{D}$, and $\bar{P}$ together and in various combinations.

in the presence of $\bar{D}$ (Fig. 4). There was no loss of C3 hemolytic activity in the presence of B alone, 50% inactivation with B and $\bar{P}$, and complete inactivation after incubation with B, $\bar{P}$, and $\bar{D}$. B presented in its native electrophoretic position after interaction with C3 or C3 and $\bar{P}$, and exhibited no loss of hemolytic activity in these reaction mixtures. The introduction of $\bar{D}$ to the mixture of B, C3 and $\bar{P}$ was associated with complete conversion of B to its Bb fragment of gamma mobility and complete loss of hemolytic activity.

To assess the quantitative requirements of $\bar{P}$ for inactivation of C3 in the absence of $\bar{D}$, 0.4 μg of C3 and 0.4 μg of B were incubated alone and with variable amounts of $\bar{P}$ in 60 μl of $DGVB^{++}$ for 60 min at 30°. Hemolytic assay of residual C3 and B revealed a sigmoidal relationship between the input of $\bar{P}$ and the percent of C3 inactivated. There was no loss of B hemolytic activity, even at the dose of $\bar{P}$ permitting 92% C3 inactivation (Fig. 5).

The dose-response effects of B on inactivation in the presence of $\bar{P}$ were examined by incubating 0.4 μg of C3 and 1.2 μg of $\bar{P}$ alone and with variable amounts of B in 60 μl of $DGVB^{++}$ for 60 min at 30°. Hemolytic assay of residual C3 revealed a sigmoidal dose response of B for C3 inactivation (Fig. 6).

DISCUSSION

Pillemer and associates initially defined properdin as a serum factor that bound to zymosan, a yeast cell wall polysaccharide, during incubation at 17°, thereby rendering the serum unable to generate C3-cleaving activity upon subse-

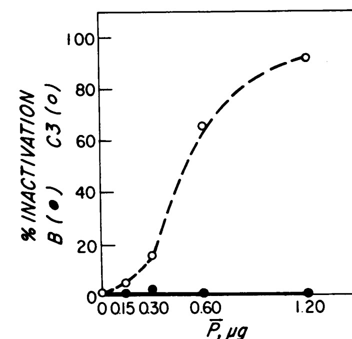

FIG. 5. Inactivation of C3 (○- -○) and B (●—●) in the absence of $\bar{D}$ by incubation with variable amounts of $\bar{P}$.

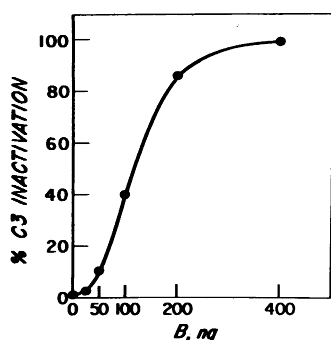

FIG. 6. Inactivation of C3 by incubation with P̄ and variable amounts of B in the absence of D̄.

quent incubation with fresh zymosan at 37° (7). The eluate of zymosan which had been interacted with serum at 17° restored C3-cleaving activity to the adsorbed serum (7). The active principle, P̄, subsequently was purified to homogeneity and shown to be functionally and antigenically distinct from immunoglobulin (13). Incubation of zymosan with serum at 0° did not remove properdin (7) and purified P̄ did not directly adhere to zymosan (23), indicating that binding is mediated by a temperature-dependent interaction of other serum proteins. The recent demonstration that P̄ binds at 0° to erythrocytes bearing C3b (14) suggests that the temperature-dependent steps permitting binding of P in serum to zymosan involve cleavage of C3 with adherence of C3b to zymosan. The additional finding that P̄ stabilizes the otherwise labile $\overline{\text{C3B}}$ convertase in a dose-related manner indicates a role for P̄ in augmenting activation of C3–C9 by the convertase formed after initial cleavage of C3 (14).

The capacity of P̄ to effect cleavage of C3 and B in serum without addition of zymosan or of isolated C3 and B in the presence of D̄ (15) could be due to stabilization of $\overline{\text{C3B}}$ formed by undetected amounts of C3b or to facilitation of an initial C3-cleaving enzyme. P̄ is not required for generation of the initial C3 convertase since interaction of C3, B, and D̄, each purified to homogeneity when analyzed by alkaline disc gel electrophoresis, results in cleavage of C3 (Fig. 1). The C3 used in this reaction was free of C3b as assessed by immunoelectrophoresis, disc gel electrophoresis, and isoelectric focusing. Further, pretreatment of C3 with the C3b inactivator (C3bINA) did not alter its subsequent capacity to permit inactivation of B by D̄ as compared to introducing C3bINA at the time of interaction of C3 with B and D̄ (24). Thus, a C3-dependent C3 convertase (C3B̄) could represent an initial convertase of the alternative pathway. These findings prompted an analysis of the effect of P̄ on the interaction of C3, B, and D̄ to determine whether P̄ supported initial cleavage of C3 by C3B̄, or merely augmented subsequent inactivation of C3 by stabilization of $\overline{\text{C3B}}$.

As shown in Fig. 1, when D̄ was limited so as not to sustain inactivation of B and C3, introduction of P̄ effected inactivation of these proteins. This capacity of P̄ to sustain inactivation of both C3 and B in the presence of limited amounts of D̄ was dose-related (Fig. 2). Further, P̄ effected a 50-fold reduction in the amounts of B required for 50% inactivation of C3 in the presence of excess D̄ (Fig. 3). Since B inactivation proceeded in the absence of P̄ in all reaction mixtures, regardless of C3 inactivation, it appears that in the presence of limited B, C3B̄ was generated but decayed without achieving inactivation of C3. The capacity of P̄ to sustain C3 inactivation in these reaction mixtures containing limited B is consistent with P̄ effecting stabilization of C3B̄ as has been shown for $\overline{\text{C3B}}$, and may represent a functional consequence of P̄ binding to C3 (25).

Larger amounts of P̄ and B sustained C3 cleavage and inactivation in the absence of D̄ (Table 1, Fig. 4). The magnitude of C3 inactivation was related to the dose of P̄ (Fig. 5) and B (Fig. 6), respectively. Formation of this convertase was not associated with conversion of B to its major fragment, Bb, or with its hemolytic inactivation (Figs. 4 and 5). The convertase is depicted as (P̄)C3B̄ to indicate that demonstrable B cleavage has not occurred and that the contribution of P̄ to formation of the complex may or may not involve its continued presence. In this regard it is noteworthy that interaction of cobra venom factor with B in the absence of D̄ is capable of generating some C3-cleaving activity (26). Furthermore, the serum from a patient with focal glomerulonephritis and glomerular deposition of C3, P, and IgA generated C3-cleaving activity during prolonged incubation at 0° that required B but was not associated with cleavage of B (27). Although an initial convertase can be formed without D̄, the presence of D̄ with its capacity to convert B to B̄ reduces the B requirement for C3 inactivation 200-fold, as illustrated by comparative dose responses of B (Figs. 3 and 6).

The first order dose response of D̄ for B inactivation in the presence of C3b is consistent with utilization of B in a single reaction, that is, formation of $\overline{\text{C3B}}$ (Fig. 1). The sigmoidal dose response of D̄ for C3 and B inactivation in the presence of C3 is attributed to the occurrence of two reactions: initial utilization of B in generation of C3B̄ which cleaves C3, thereby allowing subsequent formation of $\overline{\text{C3B}}$. The non-linearity of the dose responses of P̄ and B for inactivation of C3 in the absence of D̄ (Figs. 5 and 6) also is consistent with initial formation of (P̄)C3B̄, which, by cleavage of C3, permits subsequent generation of (P̄)$\overline{\text{C3B}}$.

Although effective interaction of native C3 and B to form an initial C3 convertase may be mediated by either D̄ or P̄, the presence of both factors greatly augments initial C3 cleavage because of the capacities of D̄ to reveal fully the active site of B and of P̄ to stabilize that site.

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