PROPERDIN: BINDING TO C3b AND STABILIZATION OF THE C3b-DEPENDENT C3 CONVERTASE*

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Activated properdin ($\bar{P}$),[1] a gamma globulin with an estimated mol wt of 223,000 daltons and an isoelectric point of greater than 9.5, has been purified free of other serum proteins and observed to consist of four apparently identical subunits of 45,000 daltons each (8, 9). $\bar{P}$ eluted from a zymosan-serum complex or purified from the euglobulin obtained from serum at pH 5.4 interacts with normal serum in the absence of zymosan, resulting in cleavage of C3 and B (9, 10). Müller-Eberhard and Götze (6) have showed that the hydrazine-sensitive factor (factor A) of the properdin system is C3 and that its major cleavage product, C3b, interacts with B and $\bar{D}$ to form a C3b-dependent C3 convertase. Formation of this convertase on the surface of an erythrocyte, EAC43, permitted stoichiometric hemolytic titrations of B and $\bar{D}$ to be performed (5, 11) and revealed that $\bar{P}$ profoundly augmented the number of effective convertase sites formed (12). This action, previously attributed to a capacity of $\bar{P}$ to convert D to $\bar{D}$, is shown now to be the result of binding of $\bar{P}$ to C3b with concomitant retardation in decay of the C3b-dependent convertase.

Materials and Methods

Functionally pure guinea pig C$\bar{1}$ and C2 were prepared as described (13). Human C3 (14, 15) and B (4) were purified to homogeneity as assessed by alkaline disc gel electrophoresis and quantitated by radial immunodiffusion against monospecific antisera. $\bar{D}$ was purified from citrated plasma which was adjusted to pH 7.8, diluted with 0.0035 M PO$_4$, 0.002 M EDTA, pH 7.8, to a conductivity of 5 mS at 4°C and applied directly to quaternary aminoethyl (QAE) Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) that had been equilibrated with the PO$_4$ buffer brought to 5 mS by addition of NaCl. $\bar{D}$ eluted at 7 mS and was further purified by gel filtration twice on Sephadex G-75 Superfine. The final preparation of $\bar{D}$ had no detectable trypsin-activable D

[1] In the nomenclature for the alternative pathway the components are designated by capital letters, with a bar over the letter indicating the active factor. B (1) is the properdin factor which also has been termed C3 proactivator (2) and glycine-rich beta glycoprotein (3); D (4,5) is also termed precursor of C3 proactivator convertase (6); factor A (1) is now known to be C3 (6); and P is the symbol for properdin (7).

Other abbreviations used in this paper: C-EDTA, rat serum diluted in GVB-EDTA; DFP, diisopropylphosphofluoridate; DGVB^{++}, GVB^{++} plus dextrose; GVB^{++}, gelatin veronal-buffered saline with Mg^{++} and Ca^{++}; QAE, quaternary aminoethyl; Z, average number of hemolytic sites per cell.

activity, and exhibited a single band on alkaline disc gel electrophoresis. $\bar{D}$ protein concentration was estimated by Folin analysis (16). $\bar{P}$ was isolated from the euglobulin fraction of citrated plasma at pH 6.0 by chromatography on QAE Sephadex, sulphopropyl Sephadex, and Sephadex G-200 (12). The final product exhibited a single band on acidic disc gel electrophoresis and induced cleavage of C3 and B when added to serum in the absence of zymosan. $\bar{P}$ was quantitated by radial immunodiffusion (12).

Isotonic veronal-buffered saline containing 0.1% gelatin, 5×10^{-4} M Mg^{++}, and 1.5×10^{-4} M Ca^{++} (GVB^{++}); half-isotonic gelatin veronal buffer with dextrose ($DGVB^{++}$); and GVB in which cations were omitted and containing 0.04 M EDTA (GVB-EDTA) were used as diluents for hemolytic assays (13). Tube and microtiter plate hemolytic assays for B, $\bar{D}$, and trypsin-activatable D were performed (5, 11, 12, 17) with rat serum diluted 1:15 in GVB-EDTA (C-EDTA) as the source of C3-C9. Activity determinations obtained by tube titration are expressed as the average number of hemolytic sites per cell (Z) and microtiter plate titrations are expressed as CH50 units per milliliter. Düsopropylphosphofluoridate (DFP) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Results

Capacity of Properdin to Increase the Hemolytic Titer of $\bar{D}$ and B. To examine whether $\bar{P}$ and trypsin were acting upon the same factor, plasma obtained from blood collected into hexadimethrine and EDTA (5) was chromatographed on QAE Sephadex as for isolation of $\bar{D}$, and fractions were assayed for $\bar{D}$, trypsin-activatable D, and for the activity enhanced by $\bar{P}$. Trypsin-activatable D activity eluted at 5.8 mS while $\bar{P}$-inducible activity was found only in fractions containing $\bar{D}$ which eluted with peak activity at 6.8 mS. To assess further whether the expression of $\bar{P}$ was dependent on $\bar{D}$ activity, 5 μg $\bar{D}$, purified to give a single band on disc gel electrophoresis and free of trypsin-activatable D, was incubated with 5×10^{-3} M DFP or $DGVB^{++}$ for 30 min at 37°C, extensively dialyzed, and assayed for residual $\bar{D}$ activity, with and without 1 μg $\bar{P}/10^8$ EAC43. $\bar{D}$ incubated in $DGVB^{++}$ contained 0.08 U/ng alone and 1 U/ng in the presence of $\bar{P}$, while $\bar{D}$ treated with DFP exhibited 0.007 U/ng alone and 0.086 U/ng with $\bar{P}$. Thus, the $\bar{P}$-enhanceable activity, which is DFP-sensitive and separable from trypsin-activatable D, is $\bar{D}$.

To assess the capacity of $\bar{P}$ to increase the titer of a set, limited amount of B in the presence of excess $\bar{D}$ and to establish a standard assay for $\bar{P}$, 1×10^7 EAC43 with 10 ng $\bar{D}$, 0.16 ng B, and variable amounts of $\bar{P}$ in 0.2 ml $DGVB^{++}$ were incubated for 30 min at 30°C. After addition of 0.3 ml C-EDTA and further incubation for 60 min at 37°C, 1.5 ml saline was added to each reaction mixture, the extent of hemolysis was determined, and the Z was calculated. In the absence of $\bar{P}$, 0.16 Z were generated and the presence of increasing amounts of properdin was associated with a linear increase in the number of hemolytic sites per cell (Fig. 1). In the absence of B, no hemolytic sites were formed, so that this activity of $\bar{P}$ cannot be attributed to contamination with B. In subsequent hemolytic titrations, $\bar{P}$ activity is expressed as the increase in Z achieved over that obtained with EAC43, limited B and excess $\bar{D}$ alone; reaction mixtures containing B and $\bar{D}$ alone always generated less than 0.2 Z.

Binding of $\bar{P}$ to EAC43. 1×10^7 EAC43, 0.14 ng B, and 10 ng $\bar{D}$ were incubated with or without 10 ng $\bar{P}$ in 0.2 ml $DGVB^{++}$ for 30 min at 30°C, followed by addition of C-EDTA to bring the hemolytic reaction to completion; the activity of 10 ng $\bar{P}$ was 1.50 Z. An additional 5×10^7 EAC43 were incubated with 50 ng $\bar{P}$ in 0.5 ml $DGVB^{++}$ for 10 min at 15°C, after which the reaction mixture
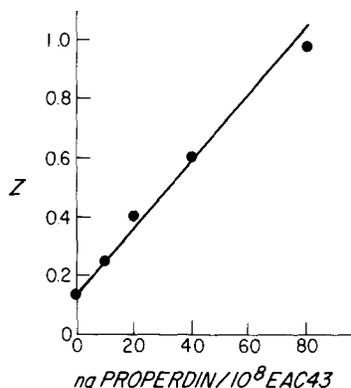
FIG. 1.   Hemolytic activity (Z) generated during incubation of EAC43, D̄, constant limited B, and variable amounts of P̄.

was centrifuged, the supernate was removed, and the erythrocytes were washed twice in ice-cold DGVB^{++}. Development of the hemolytic sites on 1×10^7 washed erythrocytes with 0.14 ng B and 10 ng D̄ resulted in generation of 1.14 Z above that obtained with cells not exposed to P̄, while hemolytic assay of 0.1 ml of the supernate yielded 0.13 Z of residual P̄ activity. Thus, of the total P̄ activity initially available, 76% bound to EAC43, creating EAC43P, with 20% recovered in the supernate.

The specificity of P̄ binding for an intermediate bearing C3b was examined with EAC4 and EAC43. 20 ng P̄ were incubated alone or with 2×10^7 EAC4 or EAC43, ranging from 1.25×10^6 to 2×10^7 in 0.2 ml DGVB^{++} for 10 min at 15°C, after which the reaction mixtures were centrifuged, and the supernates removed and assayed for P̄ as in the previous experiment. P̄ input was 0.64 Z and an identical amount was recovered in the supernate of the reaction mixture containing 2×10^7 EAC4 (Fig. 2). In contrast, incubation of P̄ with increasing numbers of EAC43 resulted in a dose-related removal of P̄ activity plateauing at 85% depletion by 1×10^7 EAC43.

The kinetics of binding of P̄ to EAC43 at 15°C was compared to that at 0°C because of the failure of P in serum to bind to zymosan at 0°C (7). Samples of 1×10^7 EAC43 in 0.1 ml DGVB^{++} at either 0°C or 15°C were added to 10 ng P̄ in 0.1 ml DGVB^{++} equilibrated at the appropriate temperature, and incubated at 0°C or 15°C for up to 20 min. The reactions were stopped by the addition of 5 ml ice-cold DGVB^{++}, immediate centrifugation at 0°C, and washing twice with DGVB^{++}. The erythrocyte intermediates, and EAC43 that had been incubated with DGVB^{++} alone, were each resuspended in 0.2 ml DGVB^{++} containing 0.14 ng B and 10 ng D̄ and then lysed with C-EDTA. Binding of P̄, as assessed by incremental lysis, occurred rapidly and was complete in 5 min at 15°C and in 10 min at 0°C. No significant difference in the amount of P̄ activity bound was apparent at these two temperatures and there was no decay in 20 min (Fig. 3).

Binding of P̄ was compared in GVB^{++} and GVB-EDTA to examine the cation requirement. Samples of 1×10^7 EAC43 were incubated for 10 min at 15°C with 10 ng P̄ in 0.2 ml of GVB^{++} or GVB-EDTA. The intermediates were washed once with their initial buffers and twice with ice-cold DGVB^{++}, and assayed for
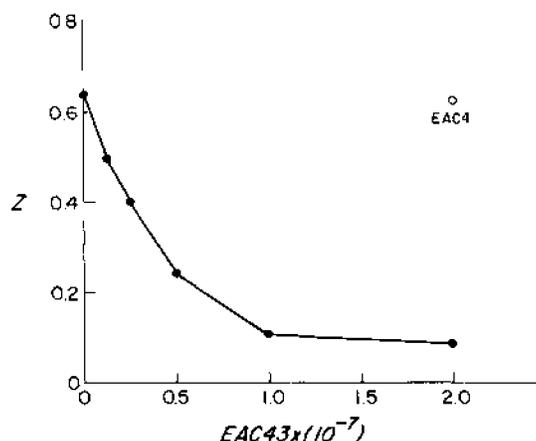
FIG. 2. Depletion of supernatant $\bar{P}$ by incremental concentrations of EAC43 (●) as compared to 2×10^7 EAC4 (O).
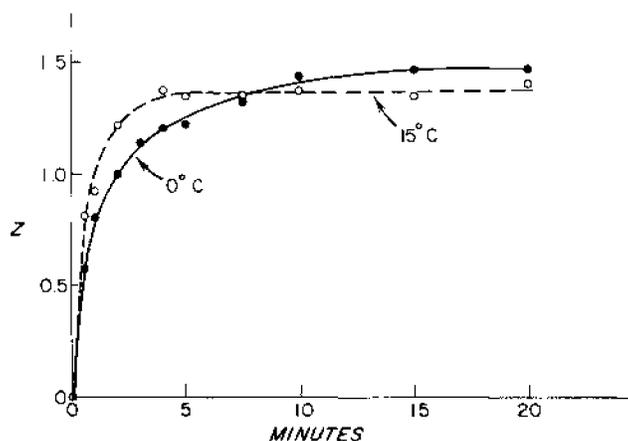

FIG. 3. Kinetics of formation of EAC43P (Z) at 0°C and 15°C.

cell-bound $\bar{P}$ activity. 0.86 Z and 0.88 Z of $\bar{P}$ activity bound to EAC43 in GVB++ and GVB-EDTA, respectively.

Stabilization of the Hemolytically Active Site on EAC43B by $\bar{P}$. The capacity of $\bar{P}$ to bind to EAC43 and to increase by more than 10-fold the hemolytic efficiency of a set and limited amount of B prompted an analysis of the effect of $\bar{P}$ on the half-life ($t_{1/2}$) of the C3b-dependent C3 convertase. 4×10^8 EAC43, 80 ng B, and 0.4 μg $\bar{D}$ in 8 ml DGVB++ were incubated for 30 min at 30°C to generate EAC43B. The reaction mixture was divided into four equal portions which were centrifuged at 4°C, the supernates were removed, and the cells washed once in ice-cold DGVB++. The washed EAC43B were resuspended at 30°C in 2 ml GVB-EDTA, or GVB-EDTA containing either 500, 100, or 20 ng $\bar{P}$. Incubation was continued at 30°C during which 0.2-ml samples were removed from each reaction mixture at timed intervals and added to 0.3 ml C-EDTA to assess residual convertase sites. The hemolytically active site on EAC43B decayed with a $t_{1/2}$ of 4 min in the absence of $\bar{P}$, while the presence of $\bar{P}$ prolonged the $t_{1/2}$ up to 30 min
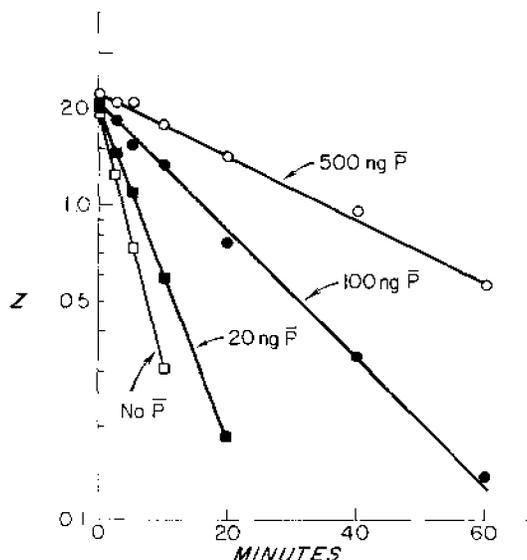
Fig. 4. Decay of EAC43B alone (□) and in the presence of 20 (■), 100 (●), and 500 ng P̄ (○), respectively.

in a dose-related manner (Fig. 4). There was no detectable lag in the stabilizing effect of P̂ so that decay was first order in each reaction mixture, and the presence of P̄ did not increase the number of active sites available at zero time. Washing the stabilized intermediate at 4°C in GVB-EDTA during the decay analysis did not alter the previously established decay rate upon subsequent resuspension and decay.

The capacity of P̄ introduced into the fluid phase to stabilize C3̄B raised the question of whether or not P̄ previously bound to C3b could transfer so as to achieve the same result. Donor cells were formed by interacting three batches of 1×10^7 EAC43 with 400 ng P̂, 200 ng P̄, or buffer for 10 min at 15°C, after which the intermediates were washed twice with ice-cold DGVB^{++} and resuspended in 1 ml GVB-EDTA at 30°C. Recipient EAC43B were generated by incubating 3×10^8 EAC43 with 30 ng B and 0.3 μg D̄ in 6 ml DGVB^{++} for 30 min at 30°C. The recipient cells were washed once in DGVB^{++}, resuspended in 3 ml GVB-EDTA at 30°C, and divided into three equal portions, each of which received 1 ml containing 1×10^7 EAC43P or EAC43. Incubation was continued at 30°C and 0.2-ml samples containing 1×10^7 recipient cells and 1×10^6 donor cells were withdrawn at timed intervals, added to 0.3 ml C-EDTA, and incubated for 60 min at 37°C to develop the recipient cell convertase sites. The $t_{1/2}$ of EAC43B interacted with EAC43 was 4.5 min, while the $t_{1/2}$ of EAC43B interacted with EAC43P, which had been formed with 200 and 400 ng P̄, was prolonged to 24 and 60 min, respectively (Fig. 5, left panel).

To confirm that stabilization of the recipient convertase was directly related to the quantity of P̄ made available by the donor cells, a fixed quantity of P̄ was interacted with two different concentrations of donor cells, which were then analyzed for their capacity to stabilize EAC43B. 4×10^7 EAC43 and 1×10^7
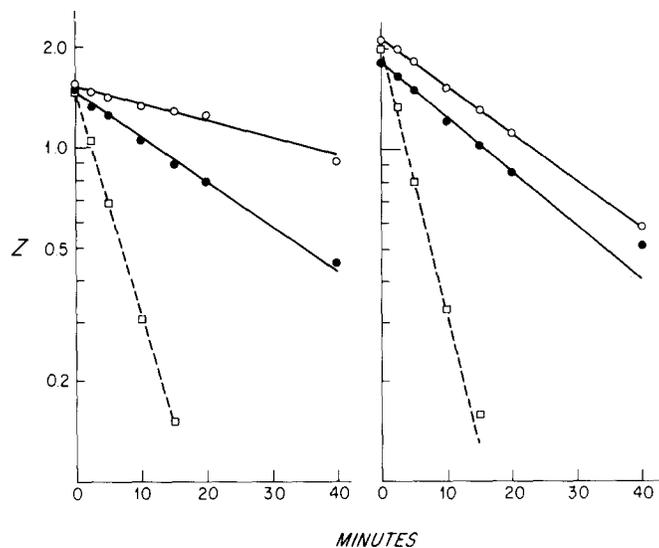
Fig. 5. (*Left panel*) Decay of EAC43B interacted with EAC43 (□) or EAC43P generated with 200 (●) and 400 ng P̄ (○), respectively. (*Right panel*) Decay of EAC43B interacted with 4×10^7 EAC43 (□), 1×10^7 EAC43P (●) generated with 200 ng P̄, or 4×10^7 EAC43P (○) generated with 200 ng P̄.

EAC43 were incubated with 200 ng P̄ in 1 ml DGVB^{++} for 10 min at 15°C, while 4×10^7 EAC43 were incubated with DGVB^{++} alone. After washing in ice-cold DGVB^{++} and resuspension in 1 ml GVB-EDTA at 30°C, the donor cells were added to 1×10^8 EAC43B in 1 ml GVB-EDTA, and transfer was assessed exactly as above. EAC43B incubated with EAC43 decayed with a $t_{1/2}$ of 4 min, while addition of EAC43P, either 1×10^7 or 4×10^7 formed with the same amount of P̄, resulted in a comparable degree of stabilization, extending the $t_{1/2}$ to 19 and 20 min, respectively (Fig. 5, right panel). Since stabilization of the convertase site on EAC43B varied with the amount of bound P̄ made available and was independent of the number of EAC43P, transfer of P̄ to EAC43B must have occurred.

Discussion

The capacity of P̄ to augment the hemolytic activity of D̄ was appreciated by three different approaches: chromatographic fractions of plasma that contained D̄ exhibited enhancement in their hemolytic titer when assayed in the presence of P̄, while fractions containing trypsin-activable D were not augmented; the P̄ enhancement of D̄ activity was fully apparent with D̄ that presented a single band on alkaline disc gel electrophoresis coinciding with the position from which D̄ activity was eluted in replicate unstained gels; and treatment of purified D̄ with DFP reduced both the D̄ titer and the net increment observed with P̄ by 92%. The previous conclusion that P̄ permits the hemolytic assay of D by conversion to D̄ (12) is not supported and is now attributed to the presence of less than 10% D̄ in the preparations of D. The capacity of P̄ to increase also the hemolytic activity of limited B (Fig. 1) is consistent with the view that the action

of $\bar{P}$ is not on an individual component but on the product of the interaction of several factors.

Although $\bar{P}$-dependent augmentation of the hemolytic titer of a set, limited amount of B in the presence of excess $\bar{D}$ was appreciated initially by introduction of $\bar{P}$ into the fluid phase (Fig. 1), the same result occurred when $\bar{P}$ was bound to EAC43 before interaction with B and $\bar{D}$. $\bar{P}$ did not bind to EAC4, and the extent of binding to EAC43 was related to the number of cells employed for adsorption (Fig. 2). Furthermore, the sum of the $\bar{P}$ activity bound to EAC43 and that remaining in the supernate was equal to the initial input. Formation of EAC43P was slightly more rapid at 15°C than at 0°C, but was quantitatively equal at these temperatures (Fig. 3), and did not require divalent cations. In contrast to these findings are the temperature and Mg^{++} requirements (7) for binding of P in serum to zymosan, which probably relate to the conditions necessary for initial cleavage of C3 and binding of C3b to zymosan.

The mechanism by which $\bar{P}$ augmented the hemolytic activity of either B or $\bar{D}$ was elucidated when $\bar{P}$ was shown to stabilize the otherwise labile convertase site on EAC43B, extending its $t_{1/2}$ up to 10-fold in a dose-response fashion (Fig. 4). The fact that each intermediate exhibited the same number of initial sites, irrespective of its subsequent rate of decay, indicates that the effect of $\bar{P}$ is to stabilize the cell-bound convertase rather than to uncover additional convertase sites. The additional observation that decay of convertase sites was first order at each dose of $\bar{P}$ suggests that a limited amount of $\bar{P}$ interacts equally among all sites. The reversible binding of $\bar{P}$ to C3b on EAC43 was appreciated by transfer from EAC43P to EAC43B with the stability of the resulting intermediate, EAC43PB, being dependent on the amount of cell-bound $\bar{P}$ available for transfer rather than the concentration of donor cells (Fig. 5). The capacity of $\bar{P}$ to bind to C3b and stabilize $\overline{C3B}$ contrasts with the inhibitory effect of the C3b inactivator on formation of this amplification convertase (15, 18).

Summary

A function of $\bar{P}$ in the alternative complement pathway is to prolong the first order decay of the hemolytic sites on EAC43B in a dose-dependent manner. As the number of initial convertase sites is not changed, even when activated properdin ($\bar{P}$) increases the $t_{1/2}$ 10-fold or more, $\bar{P}$ acts to stabilize rather than to uncover additional sites. $\bar{P}$ binds to EAC43 to generate EAC43P in a reaction that proceeds slightly more rapidly at 15°C than at 0°C, but reaches the same plateau and does not require divalent cations. The presence of $\bar{P}$ on EAC43P not only stabilizes the convertase subsequently formed on that cell, but, alternatively, permits transfer to convertase sites on other cells with the stability of the recipient intermediate being dependent on the $\bar{P}$ available for transfer. The capacity of $\bar{P}$ to bind to C3b and stabilize $\overline{C3B}$ contrasts with the inhibitory effect of the C3b inactivator on formation of this amplification convertase.

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