

MODULATION OF THE ALTERNATIVE COMPLEMENT PATHWAY BY β 1H GLOBULIN*

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The biologic activities of the major cleavage product of C3 (C3b) are blocked by C3b inactivator, a plasma protein with a mol wt of approximately 100,000 daltons (1) which cleaves C3b into two smaller fragments, C3c and C3d. A second factor which participates in C3b inactivation was provisionally termed C3b inactivator accelerator (A·C3bINA)¹ (2), because it potentiated the activity of C3b inactivator. The activity of A·C3bINA resides in a plasma protein (3) previously named " β 1H globulin" because of its electrophoretic mobility (4). We now describe the purification and some physicochemical characteristics of β 1H. In addition, we show that β 1H not only potentiates the inactivation of C3b by C3b inactivator but also has two other important regulatory actions. Firstly, it possesses a direct C3b inhibitory activity of its own, distinct from C3b inactivator; and secondly, it directly inhibits the activity of the alternative pathway convertases $\overline{\text{C3bB}}$ and $\overline{\text{C3bBP}}$ formed by the interactions of C3b, properdin (P), and factor B (B) in the presence of factor $\overline{\text{D}}$.

Materials and Methods

Reagents. The following reagents were obtained from the sources shown: DEAE-cellulose (DE 52) and carboxymethyl cellulose (CM 52) (Reeve Angel, Clifton, N. J.); hydroxyl apatite (Bio-Gel HT), Bio Rex 70, precast polyacrylamide gels, dithiothreitol, and sodium dodecyl sulphate (SDS) (Bio-Rad Laboratories, Richmond, Calif.); quaternary aminoethyl Sephadex A-50 (QAE-Sephadex A-50), sulphopropyl Sephadex G-25 (SP-Sephadex G-25), Sephadex G-200 and G-75, aldolase, and ribonuclease (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.); agarose (Fisher Scientific Co., Pittsburgh, Pa.); trypsin, soybean trypsin inhibitor (SBTI), and five times recrystallized egg albumin (Worthington Biochemical Corp., Freehold, N. J.); zymosan, *N*-ethyl maleimide (NEMI), and epsilon amino caproic acid (EACA) (Sigma Chemical Co., St. Louis, Mo.); diisopropylfluorophosphate (DFP) (Aldrich Chemical Co., Inc., Milwaukee, Wis.); human or bovine serum albumin (Calbiochem, San Diego, Calif.); human factor XIII (American Red Cross, Bethesda, Md.).

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¹ *Abbreviations used in this paper:* A, rabbit antibody; A·C3bINA, C3b inactivator accelerator; B, factor B; DFP, diisopropylfluorophosphate; DGVB⁺⁺, mixture of equal volumes of GVB⁺⁺ and D5W⁺⁺; D5W⁺⁺, 5% dextrose in water; E, sheep erythrocytes; EACA, epsilon amino caproic acid; GVB⁺⁺, 0.1% gelatin veronal buffer; NEMI, *N*-ethyl maleimide; P, properdin; SPTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulphate; Z, average number of hemolytic sites formed per cell.

Buffers. Isotonic veronal-buffered saline containing 0.00015 M Ca^{++} , 0.0005 M Mg^{++} , and 0.1% gelatin veronal buffer (GVB⁺⁺); 5% dextrose in water containing the same concentrations of divalent cations (D5W⁺⁺), and a mixture of equal volumes of GVB⁺⁺ and D5W⁺⁺ (DGVB⁺⁺) were prepared (5). D5W⁻⁻ and GVB⁻⁻ were made as described above for D5W⁺⁺ and GVB⁺⁺, but the divalent cations were omitted. A stock solution of 0.086 M EDTA, pH 7.5, was diluted in GVB⁻⁻ to prepare 0.04 M EDTA GVB⁻⁻ and 0.01 M EDTA GVB⁻⁻. For assays of C3b inactivation, 0.01 M EDTA DGVB⁻⁻, ionic strength 0.0375 and pH 6.0, was used as previously described (6).

Hemolytic Assays. Guinea pig C1 (5), human C4 (7), C2 (7), C3 (4), C5 (4), B (8), D (8), P (9), and C3b inactivator (1) were purified by previously published techniques. Rat serum (Pel-Freez Biologicals, Inc., Rogers, Ark.) diluted 1:15 in 0.04 M EDTA GVB⁻⁻ served as a source of C3-C9.

Sheep erythrocytes (E) were sensitized with rabbit antibody (A) and used to produce EAC14 as previously described (7). EAC43 cells bearing limited C3b sites were prepared from EAC14 using purified C2 (50 effective molecules per cell) and limiting amounts of purified C3 followed by treatment in 0.04 M EDTA GVB⁻⁻ for 2 h at 37°C (6). EAC14 cells bearing limited numbers of C4b sites were prepared by incubating EAC1 (400 effective C1 molecules per cell) with a limiting concentration of C4 at 30°C for 15 min (7). EAC43 cells bearing the unstable alternative pathway convertase, C3bB, were prepared by exposing EAC43 cells generated with 1,000 effective molecules of C3 per cell at 30°C for 30 min and a concentration of 5×10^7 /ml DGVB⁺⁺ to 5 ng of B/ml and 50 U of D/ml. When required the convertase on these cells was stabilized (10) by treating them at 0° with 250 ng of P/ml in 0.01 M EDTA GVB⁻⁻.

To determine the average number of hemolytic C3b sites per erythrocyte, 0.1 ml of EAC43 (1×10^6 /ml DGVB⁺⁺) were incubated for 30 min at 30°C with an equal volume of DGVB⁺⁺ containing 1.0 μg of B and 50 U of factor D. The resulting EAC43bB (D) were then incubated for 60 min at 37°C with 0.3 ml of C3-C9. 1.5 ml of 0.15 M NaCl was added, the tubes centrifuged, and the supernatant hemoglobin measured spectrophotometrically at 414 nm. Calculation of the proportion of cells lysed (y), and the average number of hemolytic sites formed per cell (Z) were performed as previously described (11).

For the measurement of the inhibition of cell-bound C3b, equal volumes of EAC43 bearing limited C3b sites and test sample were incubated in 0.01 M EDTA DGVB⁻⁻ at 37°C for 1 h, the cells washed once in 0.01 M EDTA GVB⁻⁻, once in DGVB⁺⁺, and the residual average number of hemolytic C3b sites determined as described above. Inhibition of hemolysis, which reflects inactivation of C3b, was expressed either as percentage inhibition of hemolytic sites, or alternatively as Z' units where $Z' = -\ln(1 - \text{proportion of C3b lysis inhibited})$ (12).

Antisera. Antisera to human C1q (13), C4, C3, and C5, and B (14), P (9), and C3b inactivator (15) were produced and used in radial immunodiffusion as previously described (14). Antisera to IgG, IgA, IgM, α_2 -macroglobulin, transferrin, β_2 -glycoproteins I and III, hemopexin, plasminogen, β -lipoprotein, and cholinesterase were purchased from Behring Diagnostics, American Hoechst Corp., Somerville, N. J. Specific F(ab')₂ fragments were prepared from monospecific antisera by the method of Lachmann (16).

Analytical Procedures. Immunoelectrophoresis (17) and SDS polyacrylamide gel electrophoresis (18) were performed by standard techniques.

Analytical ultracentrifugation was performed using a Beckman Model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The sedimentation coefficient of β 1H was determined during centrifugation runs at 60,000 rpm at 20°C in 0.15 M saline buffered with 0.05 M veronal at pH 7.5. To determine the molecular weight of β 1H, short-column equilibrium sedimentation studies were performed in an analytical ultracentrifuge at a speed of 9,900 rpm for 24 h at 20°C; a partial specific volume of 0.733 was assumed.

For sucrose density ultracentrifugation, linear gradients of 7.5–30% sucrose in DGVB⁺⁺ were prepared using a Buchler gradient mixer (Searle Analytic, Inc., Ft. Lee, N. J.). Centrifugation was performed for 16 h in a Beckman Model L ultracentrifuge at 40,000 rpm and 4°C using an SW50.1 rotor. 0.15-ml samples were collected from the bottom of the gradient and the positions of C1q, C4, C3, IgG, and B determined by radial immunodiffusion. The sedimentation characteristics of β 1H in a NaBr gradient were studied by mixing an equal volume of 5.2 M NaBr with β 1H at 4 $\mu\text{g}/\text{ml}$ veronal-buffered saline to increase the relative density to 1.21 (19), and centrifuging at 39,000 rpm for 48 h in a Beckman Model L ultracentrifuge using an SW39 rotor. The top 25% of the gradient (potentially lipoprotein rich) was removed using a Pasteur pipette, and the lower 75% (potentially lipoprotein depleted) was sampled through the bottom of the tube.

Protein concentrations of pools from column chromatography and purified preparations of β 1H were determined using the Folin method (20) with five times recrystallized bovine serum albumin as standard. Immunoabsorbents were prepared by linking the adsorbing material to Sepharose 4B by the cyanogen bromide technique (21).

The sensitivity of β 1H to various chemical and physical treatments was tested as follows: 0.1 ml of a solution of 4 μ g/ml was treated for 15 min at 37°C with the following reagents at the final concentrations shown: 10^{-3} M DFP, 100 μ g/ml SBTI, 10^{-2} M NEMI, 10^{-4} M I_2 , and 1.0 M EACA. To determine the sensitivity of β 1H to heat, 4 μ g/ml DGVB⁺⁺ were incubated at 56°C for 30 min. Sensitivity to trypsin was tested by treatment with trypsin, 100 μ g/ml for 5 min at 37°C followed by the addition of 200 μ g/ml of SBTI.

Results

Identification of A·C3bINA Activity in Normal Serum. Because it was repeatedly observed that purified C3b inactivator had less C3b-inactivating activity than the same concentration of C3b inactivator contained in whole serum, other substances in plasma, distinct from C3b inactivator, which could either inactivate C3b directly or potentiate the effect of C3b inactivator, were sought. 2.5 ml of normal human serum were chromatographed on a 2.5×90 cm column of Sephadex G-200 in 0.05 M veronal-buffered isotonic saline, pH 7.5, containing 0.01 M EDTA. Direct C3b-inactivating activity was measured by testing fractions with EAC43 bearing 0.8 C3b sites per cell as described in the Materials and Methods. A single peak of C3b-inactivating activity eluted synchronously with C3b inactivator protein detected by radial immunodiffusion at a volume corresponding to a mol wt of approximately 100,000 (Fig. 1). When fractions were tested for their ability to potentiate the C3b inactivation reaction by including 0.4 U of purified C3b inactivator/ml in the assay, a second peak of activity appeared between the 19S and 7S protein peaks in a position corresponding to an approximate mol wt of 300,000 daltons; this was not associated with C3b inactivator protein. The active principal in this early peak, which, under the conditions of the assay, possessed no direct C3b-inactivating activity of its own but potentiated the inactivation of C3b by the C3b inactivator, was tentatively named the C3b inactivator accelerator (A·C3bINA).

Purification of A·C3bINA. 5 U of fresh frozen plasma were thawed and recalcified; clotting was allowed to proceed at 4°C overnight. After separation from the fibrin clot, the serum was dialyzed against 0.008 M EDTA, pH 7.5. The precipitate was separated by centrifugation, and the pH of the supernate reduced to 6.5 by the addition of 1.0 N HCl. After 2 h at 4°C, the precipitate was separated by centrifugation, washed once, redissolved in 0.75 M sodium chloride buffered with 0.25 M veronal at pH 7.5, and dialyzed against 0.01 M Tris HCl containing 0.002 M EDTA, pH 7.5, with sufficient added NaCl to adjust the conductivity to 5.0 mS at 0°C. The dialyzed sample was applied to a QAE-Sephadex A-50 column (2.5×100 cm) equilibrated with the same buffer. The column was washed with one column volume of buffer, and then eluted with a linear NaCl gradient increasing to 0.7 M NaCl. As assessed by radial immunodiffusion, C3b inactivator eluted with the starting buffer, behind the initial peak of unadsorbed protein. Using the A·C3bINA assay, two major peaks of C3b inactivation were observed. The first corresponded to C3b inactivator protein, and the second eluted with the gradient at a conductivity of 11.0 mS (0°C) (Fig. 2a). This second peak (fractions 105–115) was concentrated to 15 ml by ultrafil-

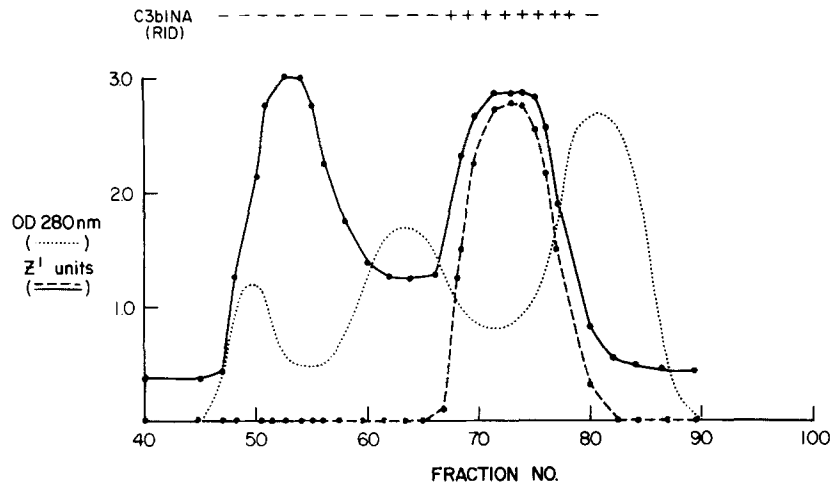


FIG. 1. Sephadex G-200 chromatography of normal human serum. 2.5 ml of serum were applied to a 2.5×90 cm column. Fractions were assayed for OD₂₈₀ (· · · ·), Z' units of direct C3b-inactivating activity (●---●), Z' units of C3b inactivator-potentiating activity (●—●), and presence of C3b inactivator protein (\pm at top). RID, radial immunodiffusion.

tration, dialyzed against 0.02 M phosphate buffer, pH 7.0, and applied to a Bio Rex 70 column (2.5×35 cm) equilibrated with the same buffer. After the appearance of the unadsorbed protein, a linear NaCl gradient of 0.7 M was applied. A·C3bINA eluted at 11.0 mS (0° C) with the bulk of the adsorbed protein. The A·C3bINA pool (fractions 73–77) was concentrated by ultrafiltration and chromatographed on a Sephadex G-200 column (2.5×100 cm) in veronal-buffered saline from which it eluted with an apparent mol wt of 300,000 daltons (Fig. 3). The final pool was concentrated by ultrafiltration to a protein concentration of 395 μ g/ml.

Properties of Purified A·C3bINA. In polyacrylamide gel electrophoresis, using 0.188 M Tris-glycine buffer, pH 8.9, purified A·C3bINA failed to enter 7.5% gels, and only partially entered 4% gels, forming a smear near the origin. Incubation of A·C3bINA with 2% SDS and 8.0 M urea at 37° C for 2 h followed by electrophoresis in 4% acrylamide and 0.2% SDS resulted in the presence of a single dense band after staining with Coomassie blue (Fig. 4). This band contained 93% of the stain as determined by densitometry. As judged by comparison with standards of human serum albumin and its polymers, aldolase, ovalbumin, and RNase, the position of the A·C3bINA band corresponded to an approximate mol wt of 150,000 daltons. The appearance of the band was not altered by prior reduction with 0.2 M 2-mercaptoethanol or 0.1 M dithiothreitol in 8.0 M urea followed by incubation with 0.2 M iodoacetamide, suggesting that A·C3bINA is a single polypeptide chain. The band stained heavily in the periodic acid-Schiff (PAS) reaction which indicated that it contained a significant quantity of carbohydrate.

In the analytical ultracentrifuge at a concentration of 367 μ g A·C3bINA/ml a single sharp peak was observed which had a sedimentation coefficient of 5.6. This value was unaltered when the protein concentration was reduced to 183.5 μ g/ml. By sucrose density gradient ultracentrifugation, the sedimentation coef-

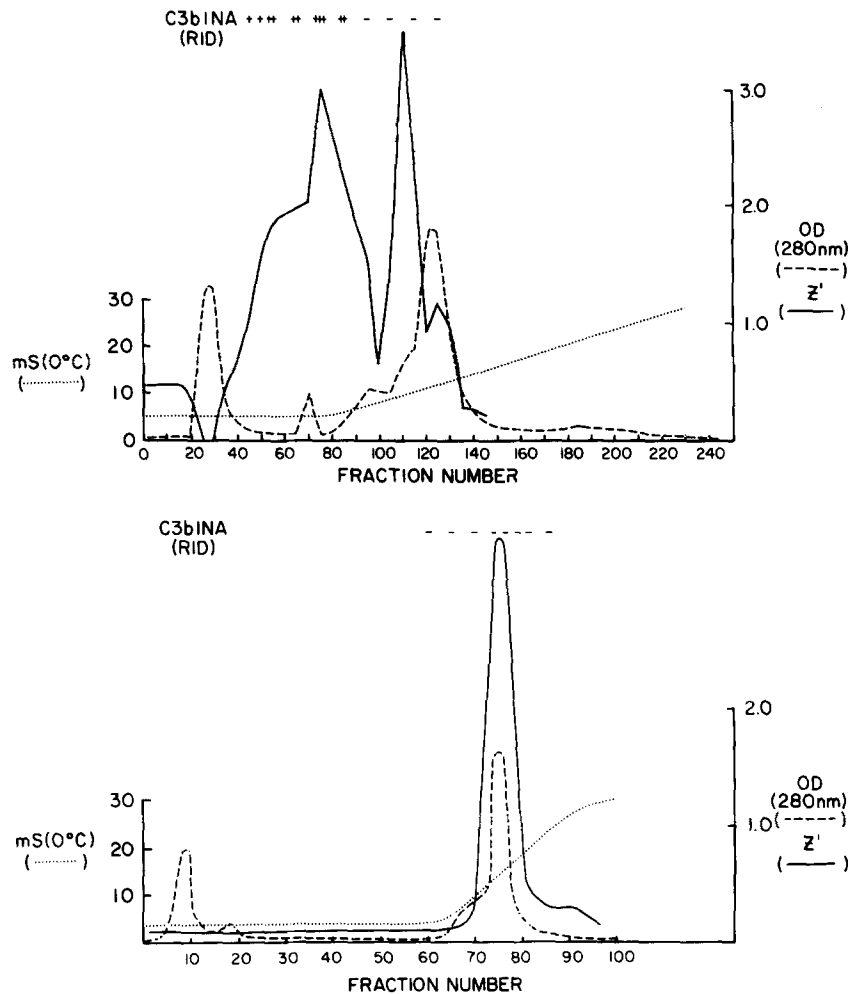


FIG. 2. Purification of A·C3bINA. (a), QAE-Sephadex A-50 separation of pH 6.5 euglobulin of human serum. Fractions were assayed for A·C3bINA activity as described in the text. Two major peaks of C3b inactivation were seen; the early peak, corresponding to C3b inactivator (C3bINA) protein, eluting with the starting buffer; and the second, presumed A·C3bINA, eluting at 11.0 mS at 0°C. RID, radial immunodiffusion. (b), A·C3bINA peak from QAE-Sephadex A-50 rechromatographed over Bio Rex 70. Fractions were assayed for A·C3bINA activity. The single peak of activity eluted at 11.0 mS (0°C) and did not correspond to C3b inactivator (C3bINA) protein. RID, radial immunodiffusion.

ficient of A·C3bINA was 6.4. In NaBr ultracentrifugation A·C3bINA did not float, indicating a density of >1.21 . By equilibrium sedimentation in the ultracentrifuge, a mol wt of 150,000 daltons was determined.

A·C3bINA activity was completely destroyed by trypsin. Heating to 56°C for 30 min resulted in approximately 30% loss of activity. DFP, SBTI, NEMI, or iodine were without effect. Because EACA directly inhibited C3b inactivator activity, it was impossible to assess its effect on A·C3bINA.

Production of Antiserum to A·C3bINA. New Zealand white rabbits were immunized subcutaneously with 100 μ g of purified A·C3bINA in Freund's

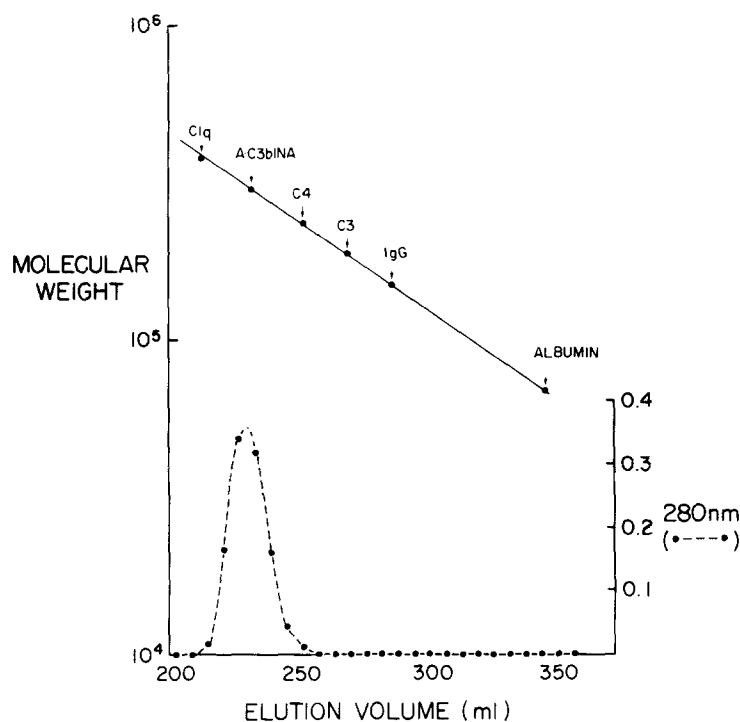


FIG. 3. Sephadex G-200 gel filtration of purified A·C3bINA. OD₂₈₀ peak (●---●) corresponded with A·C3bINA activity. The elution volumes of proteins of known molecular weight are shown above. A·C3bINA eluted with an apparent mol wt of 300,000 daltons.

complete adjuvant. 6 wk later, the animals were reimmunized intravenously with 100 μ g of A·C3bINA, and they were bled 7, 10, and 14 days later. On immunoelectrophoresis against whole human plasma, the antiserum produced a dense arc in the β -region and a faint arc in the α -region. Analysis of various fractions of serum indicated that the protein with α -mobility was present in high concentration in the pH 5.4 pseudoglobulin, and that the protein with β -mobility was virtually absent from this fraction. Since preliminary experiments had shown that A·C3bINA activity had a β -mobility in agarose at pH 8.3, the antiserum was adsorbed with insolubilized pH 5.4 pseudoglobulin to render it specific for the protein of β -mobility. In immunoelectrophoresis, this antiserum reacted both with normal human plasma and purified A·C3bINA to yield a single arc in the β -region (Fig. 5) which trailed toward the origin. When agarose strips were cut from the same electrophoresis plate, eluted with DGVB⁺⁺, and the eluates tested for A·C3bINA activity, the position of this activity corresponded to the precipitin arc.

In double diffusion against normal human serum in agarose gel, the antiserum to A·C3bINA gave reactions of nonidentity with antisera to C1q, C4, C3, B, P, C5, C6, C7, C8, C1 inhibitor, C3b inactivator, β_2 -glycoproteins I and III, β -lipoprotein, hemopexin, cholinesterase, α_2 -macroglobulin, plasminogen, transferrin, IgG, IgA, or IgM. No reaction between the antiserum and purified factor XIII was observed. A reaction of identity with antibody to β 1H globulin was

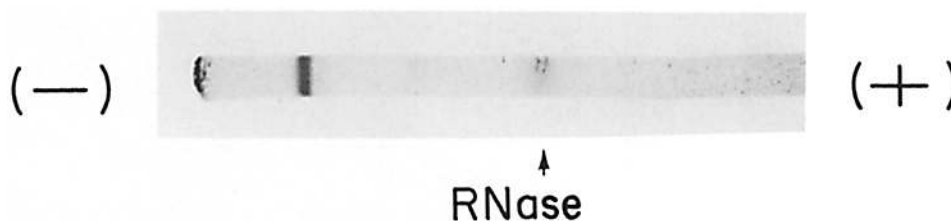


FIG. 4. SDS polyacrylamide gel electrophoresis of purified A·C3bINA; stain, Coomassie blue. 93% of the stain was in the A·C3bINA band as determined by densitometry. A·C3bINA band corresponded to an approximate mol wt of 150,000 daltons. The position of a RNase marker is shown.

initially found by Dr. Chester Alper, Center for Blood Research, Boston, Mass., and subsequently confirmed by Dr. Ulf Nilsson, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pa. β 1H was first described as a prominent contaminant of both C3 and C5 preparations (4). A purified sample of β 1H from Dr. Nilsson's laboratory had the same electrophoretic, ultracentrifugal, functional, and antigenic properties as our preparations. For the sake of both simplicity and historical accuracy, therefore, the name C3b inactivator accelerator has been discarded and β 1H used in its place.

Using monospecific antiserum to β 1H we were able to measure the degrees of purification and yields of β 1H at various stages during the preparative procedures (Table I). From 1,120 ml of plasma, containing 133 μ g of β 1H/ml, 2.20 mg of purified β 1H were obtained, which is a yield of 1.48%. Since by densitometry only 93% of the Coomassie blue stain was in the β 1H band on SDS polyacrylamide gel electrophoresis, we have assumed that the preparation is 93% homogeneous, and that the β 1H was purified by a factor of 376.5.

Activity of β 1H. The ability of β 1H to potentiate the inactivation of C3b by purified C3b inactivator was assessed in the following experiment. Two sets of EAC43 bearing 0.83 hemolytic C3b sites per cell were incubated at 5×10^7 /ml 0.01 M EDTA DGVB⁻ in the presence of varying concentrations of β 1H, ranging from 0 to 5 μ g/ml. One set of cells was incubated with β 1H alone, and the other with 0.2 Z' U/ml of C3b inactivator in the presence of β 1H. At the end of 60 min incubation at 37°C, the extent of C3b inhibition was determined as described in the Materials and Methods. In the absence of C3b inactivator, β 1H produced some dose-dependent inhibition of C3b (Fig. 6). C3b inactivator alone reduced the total number of hemolytic sites from 0.83 to 0.69. The extent of inhibition of C3b produced by C3b inactivator and β 1H together greatly exceeded the sum of those achieved independently, demonstrating a synergistic effect between these proteins in the inactivation of C3b.

Kinetics of Inactivation of C3b and C3b Inactivator and β 1H. Five sets of EAC43 bearing 1.1 C3b sites per cell (1×10^8 /ml in 0.01 M EDTA DGVB⁻) were incubated at 37°C with buffer alone, normal serum diluted 1:1,000, purified C3b inactivator containing the same concentration of C3b inactivator as present in the dilution of normal serum, β 1H (400 ng/ml), and β 1H with C3b inactivator together. Samples were removed from the reaction mixtures at timed intervals, diluted on ice-cold 0.01 M EDTA GVB⁻, and centrifuged. The cells were washed once in DGVB⁺, resuspended to 1×10^8 /ml DGVB⁺, and the residual C3b sites

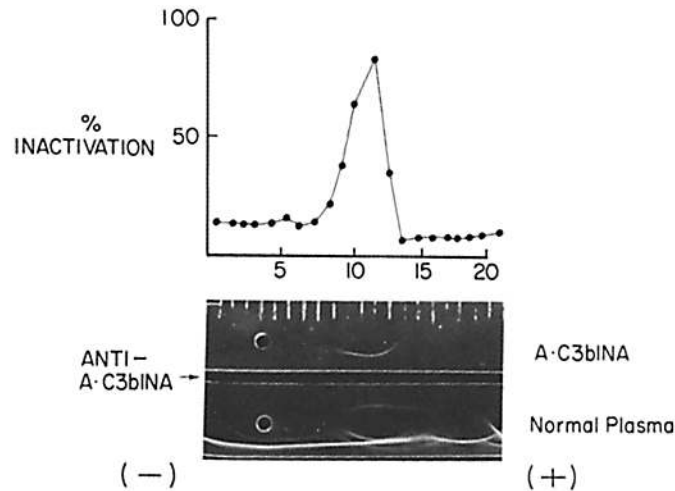


FIG. 5. Agarose gel electrophoresis of A·C3bINA. Upper well, purified A·C3bINA; lower well, normal plasma. A·C3bINA antiserum in upper trough and anti-normal human serum in lower trough. The antiserum produced a single arc in the β -region, which trailed into the origin. Slices were cut from the agarose, eluted, and the eluates tested for A·C3bINA activity as shown in the upper frame. A·C3bINA activity corresponded to immunoprecipitable protein.

TABLE I
Purification of β 1H

Pool	Volume	Total protein concentration	β 1H concentration	Yield at each stage	Purity β 1H concentration/protein concentration	Purification factor
	ml	μ g/ml	μ g/ml	%		
Plasma	1,120	53,800	133	100.0	2.47×10^{-3}	—
Euglobulin, pH 6.5	135	8,500	490	44.4	5.76×10^{-2}	23.3
QAE-Sephadex A-50	15	2,100	741	16.8	3.53×10^{-1}	142.9
Bio Rex 70	3	2,200	1,367	36.9	6.21×10^{-1}	251.4
Sephadex G200	6	395	367	57.8*	$9.30 \times 10^{-1}\ddagger$	376.5

* Total yield of β 1H: 2.20 mg, 1.48%.

‡ This factor is based on densitometric studies of purified β 1H on SDS acrylamide gel electrophoresis.

measured. The kinetics of inactivation of C3b (Fig. 7) by C3b inactivator alone, C3b inactivator and β 1H together, and normal serum were all first order, suggesting that the potentiation of C3b inactivator by β 1H is not a multistep process. β 1H alone produced a small amount of inactivation of C3b but the extent of this precluded firm conclusions as to the kinetics of this reaction.

Requirements for the Simultaneous Presence of β 1H and C3b Inactivator for the Potentiation of C3b Inactivation. Four identical aliquots of EAC43 bearing 1.1. C3b sites per cell (1×10^8 /ml in 0.01 M EDTA DGVB⁻) were incubated at 37°C for 1 h in the presence of either buffer alone, C3b inactivator alone (0.4 Z' U/ml, β 1H alone (400 ng/ml), or β 1H plus C3b inactivator. The tubes were then centrifuged, the cells washed in 0.01 M EDTA DGVB⁻, resuspended to their original volume, and reincubated for a second hour at 37°C. For the second hour of incubation, the sample initially exposed to C3b inactivator alone was divided into two portions, one of which was incubated in buffer and the second with β 1H

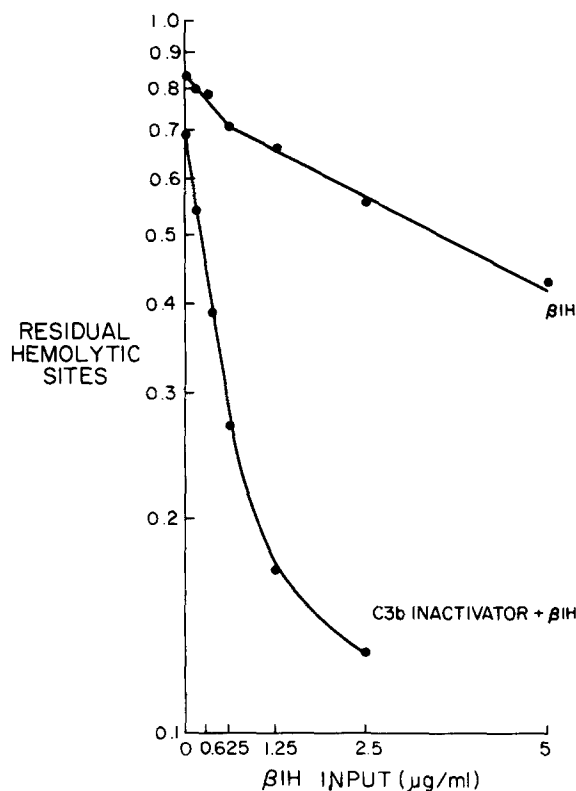


FIG. 6. Direct C3b inhibitory, and C3b inactivator-potentiating activities of β 1H. EAC43 were incubated with increasing concentrations of β 1H (0–5 μ g/ml), in the absence (upper line) or presence of a limited concentration of purified C3b inactivator (lower line). β 1H produced dose-dependent inhibition of C3b, and also potentiated the inactivation of C3b by C3b inactivator.

alone. Cells originally exposed to β 1H alone were likewise divided into two portions, one being incubated with buffer and the other with C3b inactivator alone. Those cells incubated with C3b inactivator and β 1H together during the first hour were incubated with buffer. Cells incubated with buffer during the first hour were reincubated with buffer for a second hour.

C3b inactivator alone produced 48% inactivation of C3b and β 1H alone produced 4% inactivation (Fig. 8). Either C3b inactivator followed by β 1H, or β 1H followed by C3b inactivator yielded inactivation similar to the sum of the values for C3b inactivator and β 1H, irrespective of the sequence in which these reagents were used. In contrast, when cells were exposed to β 1H and C3b inactivator simultaneously, 87% inactivation was achieved.

Comparison of Effects on C3b and C4b. C4b is inactivated by a C4b inactivator, a plasma protein which has not been separated from C3b inactivator (22). The ability of purified C3b inactivator to inactivate cell-bound C3b and C4b, and for β 1H to potentiate these effects were compared in the following experiments. EAC43 and EAC14 bearing 1.1 and 1.0 hemolytic sites, respectively, were incubated with varying concentrations of purified C3b inactivator with or without a fixed input of β 1H (400 ng/ml). After incubation at 37°C for 1 h in 0.01 M

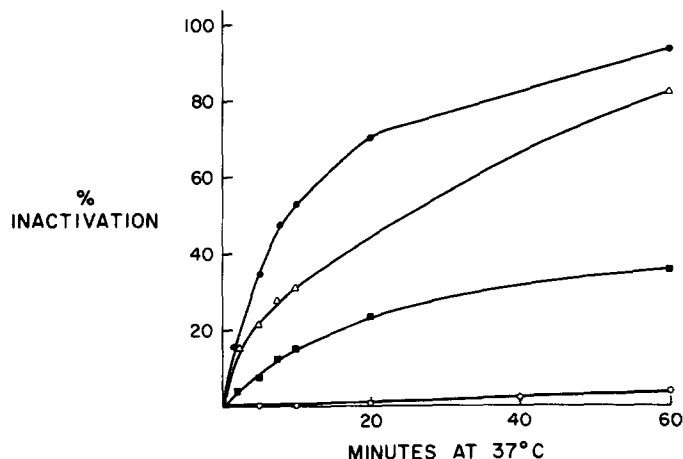


FIG. 7. Kinetics of inactivation of C3b by normal human serum diluted 1:1,000 (●—●), purified C3b inactivator at the same concentration as present in serum (■—■), β 1H alone 400 ng/ml (○—○), and C3b inactivator together with β 1H (Δ — Δ).

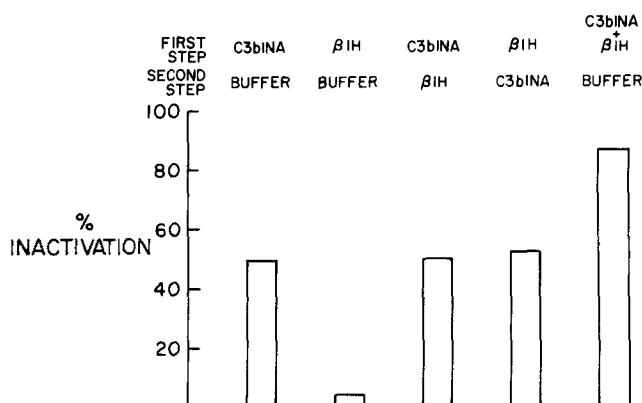


FIG. 8. Requirement for the simultaneous presence of C3b inactivator (C3bINA) and β 1H for the β 1H potentiation effect. EAC43 were incubated at 37°C for 1 h, with the reagents shown in the upper line. After washing, they were reincubated at 37°C with the reagents shown on the lower line. C3b inactivator alone, C3b inactivator followed by β 1H, and β 1H followed by C3b inactivator, all produced similar degrees of C3b inactivation. Significant potentiation was only seen when C3b inactivator and β 1H were incubated simultaneously.

EDTA DGVB⁻, the cells were washed twice in DGVB⁺ and resuspended to 1×10^8 /ml DGVB⁺. The residual C3b sites on EAC43 were developed as previously described. Residual C4b sites on EAC4 produced from the EAC14 by the exposure to EDTA were developed by incubation with C1 (200 U/ml) for 15 min at 30°C, C2 (50 U/ml) for 15 min at 30°C, and finally, in C3-C9 for 1 h at 37°C. C3b inactivator alone produced marked dose-dependent inactivation of C3b but had little effect on C4b (Fig. 9). As anticipated, β 1H produced considerable potentiation of C3b inactivation, but little enhancement of the small degree of C4b inactivation was observed.

In the converse experiment (Fig. 10), the input of β 1H was varied in the presence or absence of sufficient C3b inactivator to induce almost identical

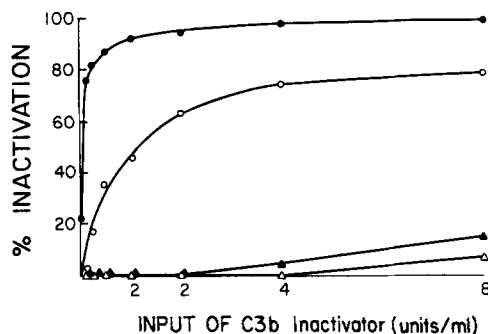


FIG. 9. Inactivation of C3b and C4b by varied inputs of C3b inactivator and a fixed input of β 1H (400 ng/ml). EAC43 showed significant dose-dependent inhibition of C3b by C3b inactivator alone (O—O); β 1H markedly enhanced this response (●—●). Little inactivation of C4b was achieved with high inputs of C3b inactivator (Δ — Δ) and β 1H did not significantly enhance this effect (\blacktriangle — \blacktriangle).

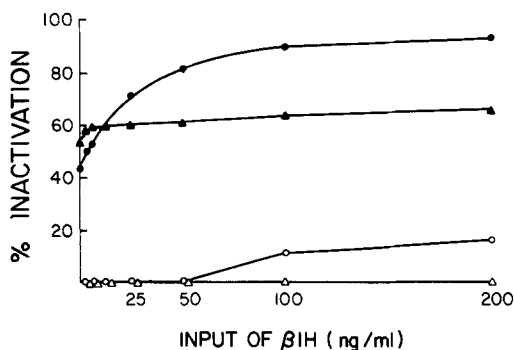


FIG. 10. Inactivation of C3b and C4b by a fixed input of C3b inactivator and varied inputs of β 1H. The inputs of C3b inactivator were adjusted to produce similar degrees of inactivation of C3b (0.4 Z' U/ml) and C4b (40 Z' U/ml). β 1H produced dose-dependent enhancement of C3b inactivation by C3b inactivator (●—●), and dose-dependent inhibition of C3b on its own (O—O). Little potentiation of the inactivation of C4b by C3b inactivator (\blacktriangle — \blacktriangle), and no direct C4b inhibition (Δ — Δ) were observed.

degrees of inactivation of C3b and C4b in the absence of β 1H. The input of C3b inactivator required to achieve 43% inactivation of C3b was 0.4 Z' U/ml, whereas that required to produce 52% inactivation of C4b was 40 Z' U/ml. Dose-dependent potentiation of C3b inactivation was observed when β 1H was added to the reaction mixtures, but the extent of inactivation of C4b by C3b inactivator was relatively independent of β 1H concentration. Significant inhibition of C3b by high concentrations of β 1H in the absence of C3b inactivator was observed.

Direct Effect of β 1H on C3b. The observations (Figs. 6-10) that high concentrations of β 1H inhibited C3b in the absence of C3b inactivator suggested that either β 1H had a direct C3b inhibitory activity or the preparation of β 1H was contaminated with a small amount of C3b inactivator. To discriminate between these possibilities, the following experiment was performed. Four Z' U of C3b inactivator and 20 μ g of β 1H were incubated for 15 min at 37°C with an equal volume of F(ab')₂ fragments prepared from 5 ml of anti- β 1H or anti-C3b inactivator or with buffer. The mixture was then diluted and the residual C3b

inhibitory activity was determined by the inhibition of lysis using the standard assay in which EAC43 were exposed to the mixtures, washed, and developed with B, \bar{D} , and C3-C9. Anti- β 1H removed virtually all of the direct C3b inhibitory activity of β 1H, whereas anti-C3b inactivator had little effect (Table II). Conversely, the anti-C3b inactivator removed almost all C3b inactivator activity; but anti- β 1H yielded insignificant inhibition. Controls indicated that neither F(ab')₂ fragments had any effects on the assay system. In other experiments, anti- β 1H removed the C3b inactivator-potentiating activity of β 1H. Since the direct C3b inhibitory and C3b inactivator-potentiating activities of β 1H were neutralized by specific anti- β 1H but not by anti-C3b inactivator, both of these activities reside in the same molecule, β 1H.

Effect of β 1H on the Alternative Pathway Convertases, $\overline{C3bB}$ and $\overline{C3bBP}$. The capacity of β 1H to inhibit a preformed alternative pathway convertase was assessed. EAC43 prepared with 1,000 effective molecules of C3 per cell were incubated at 1×10^8 /ml DGVB⁺⁺ for 30 min at 30°C in the presence of 5 ng/ml B and 50 U/ml of \bar{D} to allow the formation of EAC43 \overline{bB} . The cells were then centrifuged, washed once in ice-cold DGVB⁺⁺, resuspended to 1×10^8 /ml in 0.01 M EDTA GVB⁻⁻, and exposed to 250 ng of P/ml at 0°C to form EAC43 \overline{bBP} . After 3 min at 0°C to allow for binding of P to the EAC43 \overline{bB} , the cells were divided into two portions, one of which received 80 ng/ml of β 1H and the other buffer. Both mixtures were then incubated at 30°C and at time intervals 0.1-ml portions were sampled into 0.4 ml of C3-C9 for a further 60-min incubation at 37°C. The half-life of the EAC43 \overline{bBP} suspended in buffer was 17 min, whereas EAC43 \overline{bBP} suspended in β 1H exhibited a marked increase in the rate of loss of hemolytic sites (Fig. 11). When EAC43 cells were pretreated with the same concentration of β 1H before exposure to B, \bar{D} , and P for the formation of EAC43 \overline{bBP} , fewer hemolytic sites were observed at time zero, but no increased rate of decay resulted. In other experiments, EAC43 \overline{bB} bearing the labile convertase also exhibited an increased rate of decay upon exposure to β 1H. For both convertases, increase in rate of decay was a function of the concentration of β 1H.

By contrast, C3b inactivator had no effect on the preformed alternative pathway convertase, as demonstrated in the following experiment. EAC43 \overline{bBP} prepared with 1.5 C3b sites per cell, 10 μ g B, 50 U \bar{D} , and 250 ng P/ml were decayed at 30°C as described in the preceding experiment except that 80 Z' U C3b inactivator were substituted for the β 1H. No difference was observed in the rate of decay of EAC43 \overline{bBP} in the presence or absence of C3b inactivator (Fig. 12). As expected, treatment of the EAC43b with C3b inactivator before the formation of the convertase resulted in a decrease of total hemolytic sites at time zero, but no increase in the rate of decay of the EAC43 \overline{bBP} formed on subsequent exposure to B, \bar{D} , and P was observed.

Discussion

In this paper we have presented evidence that in addition to C3b inactivator there exists in human plasma a second protein which modulates the alternative complement pathway (Fig. 1). On the basis of its ability to potentiate the inactivation of C3b by the C3b inactivator, we originally named this second

TABLE II
Inhibition of C3b Inhibitory Activities of β 1H and C3b Inactivator by Specific F(ab')₂ Fragments

Reaction mixture	Z' units observed		
	Buffer	F(ab') ₂ anti-C3b inactivator	F(ab') ₂ anti- β 1H
Buffer	0	0	0
β 1H	0.70	0.66	0.07
C3b Inactivator	0.72	0.05	0.66

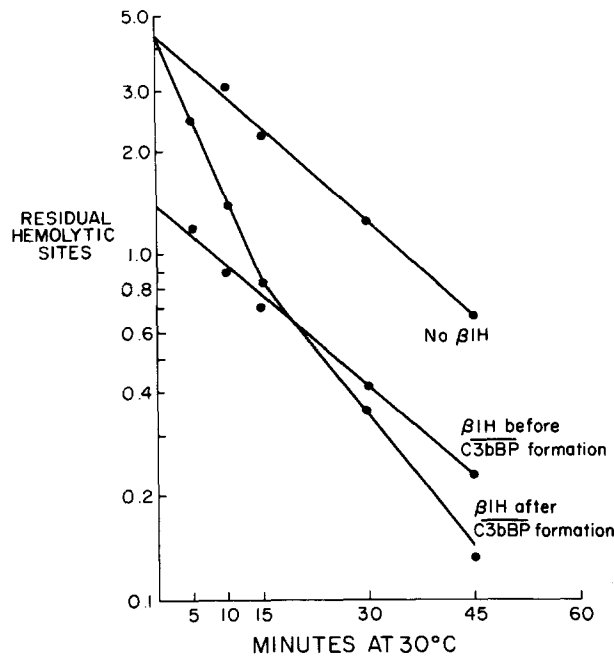


FIG. 11. Effect of β 1H on the rate of loss of hemolytic sites from EAC $\overline{3bBP}$. EAC $\overline{43bBP}$ were incubated at 30°C in the presence or absence of β 1H (80 ng/ml). β 1H markedly increased the rate of loss of hemolytic sites. In contrast when EAC43 were treated with β 1H before the formation of EAC $\overline{3bBP}$, fewer hemolytic sites were found, but the rate of loss of sites was the same as found with the untreated cells.

protein the C3b inactivator accelerator (A·C3bINA). An antiserum to purified A·C3bINA gave a reaction of identity with anti- β 1H; β 1H being a plasma protein without known function, named on the basis of its electrophoretic mobility (4). For the remainder of the discussion we will use β 1H in preference to A·C3bINA.

β 1H was purified from the pH 6.5 euglobulin of normal human serum by sequential chromatography on QAE-Sephadex A-50, Bio Rex 70, and Sephadex G-200 (Figs. 2 and 3). The final yield was 1.48% and the purification 376.5-fold (Table I). Three criteria for purity were: a single band on SDS polyacrylamide gel electrophoresis (Fig. 4), a homogeneous peak in the analytical ultracentrifuge, and the ability of the purified protein to produce an antiserum with a high degree of specificity. After the removal by immunoadsorption of a trace contami-

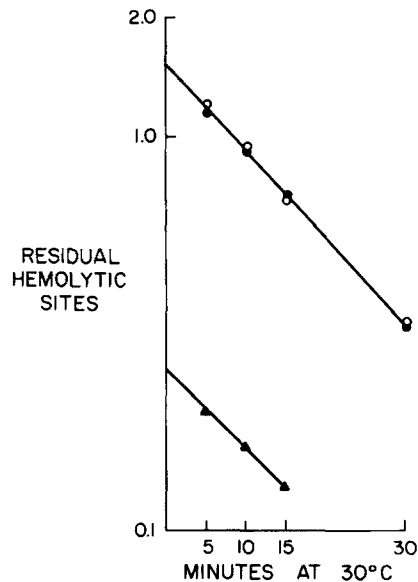


FIG. 12. Effect of C3b inactivator on the rate of loss of hemolytic sites from EAC43bBP. EAC43bBP were incubated at 30°C in the presence (○—○) or absence (●—●) of 80 Z' U/ml of purified C3b inactivator. C3b inactivator did not influence the rate of loss of hemolytic sites. When EAC43 were pretreated with C3b inactivator (▲—▲), fewer hemolytic sites were detected but the rate of loss of sites was unchanged.

nant in the α -region, the anti- β 1H was monospecific (Fig. 5). On agarose electrophoresis, β 1H activity was congruent with immunoprecipitable protein (Fig. 5), and F(ab')₂ fragments prepared from the anti- β 1H antiserum removed this activity (Table II).

On the basis of trypsin sensitivity, β 1H is a protein. Since reduction with 0.2 M 2-mercaptoethanol or 0.1 M dithiothreitol failed to alter the number of bands observed during SDS polyacrylamide gel electrophoresis, β 1H must be a single polypeptide chain. The PAS-positive nature of the band suggests that β 1H contains a significant quantity of carbohydrate. The mol wt of β 1H was determined to be approximately 150,000 daltons, both by SDS polyacrylamide gel electrophoresis and by equilibrium sedimentation in the analytical ultracentrifuge. Its elution pattern from Sephadex G-200 corresponds to an approximate mol wt of 300,000 daltons. This discrepancy suggests that the molecule is asymmetric. Since the same sedimentation coefficient of 5.6S was observed during ultracentrifugation at two different β 1H concentrations, this asymmetry may be rigid rather than subject to random coil formation as, for example, is DNA. The sedimentation coefficients determined in the analytical ultracentrifuge, 5.6, and in sucrose density gradients, 6.4, are consistent with a mol wt of 150,000, assuming a partial specific volume of 0.733. Since the total carbohydrate content of β 1H is at present unknown, the correct mol wt may be significantly lower than 150,000 daltons.

β 1H appears to potentiate the inactivation of C3b by C3b inactivator (Figs. 1 and 6-10), to prevent the fruitful interaction of C3b with C5 in the classical and B in the alternative pathways (3), and to diminish the activity of the preformed alternative pathway convertases, C3bB and C3bBP, by increasing their rate of

decay (Fig. 11). That these activities belong to the same molecule is shown by the ability of immunochemically purified β 1H to produce them, and the reversal of effects on C3b and C3b inactivation by specific $F(ab')_2$ fragments of anti- β 1H (Table II). In its effects on C3b, β 1H resembles C3b inactivator by modulating both classical and alternative pathways. In contrast, however, β 1H is able to inhibit the preformed alternative pathway convertases $C3b\bar{B}$ and $C3b\bar{BP}$ (Fig. 11), whereas C3b inactivator is without effect once C3b has interacted with factors B, \bar{D} , and P (Fig. 12). Another point of differentiation is that C3b inactivator is able to block immune adherence (23), whereas preliminary studies indicate that β 1H does not possess this function.

The ability of β 1H to enhance the inactivation of C3b by C3b inactivator is reminiscent of the action of a "10S globulin" to induce cleavage of C4b by C3b inactivator (24). Further similarity is the necessity for the simultaneous presence of C3b inactivator with either β 1H (Fig. 8) or the 10S globulin for these effects. Although we have not studied the cleavage of C4b, β 1H only weakly potentiates the inactivation of C4b by C3b inactivator (Figs. 9 and 10). A further difference is the sedimentation coefficient of 6.4 for β 1H on sucrose density gradients. Evidence to date suggests that β 1H is not the 10S globulin although appropriate neutralization studies with specific antibodies need to be performed.

In a search for an inhibitor of the preformed alternative pathway convertase, Fearon et al. (unpublished data and personal communication) have identified a protein with this effect; an antiserum to this protein gives a reaction of identity with our anti- β 1H. A "C5-site generation inhibitor" present in guinea pig serum was described in 1969 by Okada et al. (25). This inhibitor could bind with EAC1423 to block C5 activation, but had no effect on immune adherence. These facts, together with a similar elution profile on Sephadex G-200, suggest that the C5-site generation inhibitor is the guinea pig analogue of β 1H.

The mechanism of action of β 1H is not yet understood, but the available evidence suggests that it binds with C3b. Kinetic studies in which both the initial rate and final extent of C3b inhibition are proportional to β 1H concentration suggest a stoichiometric binding rather than an enzymatic reaction. The failure of known enzyme inhibitors such as DFP, SBTI, NEMI, and I_2 to block β 1H activity is consistent with this interpretation. Furthermore, EAC43 incubated with β 1H and then washed become agglutinable by anti- β 1H (3). Studies are currently underway to investigate the mechanism of interaction between C3b and β 1H.

Summary

C3b inactivator accelerator (A·C3bINA) was isolated from human plasma. An antiserum produced against the purified protein gave a reaction of identity with β 1H, a well-documented contaminant of C3 preparations. β 1H appears to be composed of a single polypeptide chain containing a significant quantity of carbohydrate, and having a sedimentation coefficient of 5.6 on analytical, and 6.4 on sucrose density gradient ultracentrifugation. Its mol wt based on SDS polyacrylamide gel electrophoresis and equilibrium sedimentation is approximately 150,000, whereas it elutes from Sephadex G200 with an apparent mol wt of 300,000, suggesting that β 1H is an asymmetric molecule.

β 1H potentiates the inactivation of C3b by C3b inactivator, binds to EAC43 to

limit the formation of EAC43bB and EAC43bBP , and in contrast to C3b inactivator, it increases the rate of loss of hemolytic sites from EAC43bB and EAC43bBP . For the C3b inactivator-potentiating effect, $\beta 1\text{H}$ and C3b inactivator must necessarily be simultaneously present. The kinetics of inactivation of C3b by C3b inactivator and $\beta 1\text{H}$ are first order, suggesting that potentiation is not a multistep process. The mechanisms of binding to C3b and inhibition of the alternative pathway convertases C3bB and C3bBP are currently unknown.

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