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THE RABBIT PROPERDIN SYSTEM:

I. Identification of a New Factor and Purification of Rabbit Properdin[1]

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Pure preparations of rabbit properdin were obtained from rabbit serum by ion-exchange chromatography. These preparations functioned as properdin when they were measured with the zymosan assay or with a serum reagent selectively depleted of properdin by a specific immunoabsorbent. Properdin in these preparations was in its activated state.

A new serum factor was required to measure properdin activity when purified preparations of rabbit properdin were tested with the zymosan assay. This factor was designated as ZBP, or zymosan-binding protein. ZBP appeared to be distinct from known components of the alternative complement pathway and the classical complement system, and it did not appear to be an immunoglobulin.

The human properdin system was described by Pillemer and his associates (1) in 1954. This system has been studied extensively, and many investigators have shown that it is an alternative pathway of complement (C) activation. Components of the human properdin system that have been identified and characterized are properdin (2–6), factor B (7–11), factor D (11–13) and C3. The guinea pig properdin system has been partially characterized, and guinea pig properdin (14), factor B (15, 16), and factor D (17) have been purified.

The rabbit properdin system has not been investigated. We began our studies of this system by developing a method to purify rabbit properdin, using the zymosan assay (1, 18, 19) to monitor properdin activity. Under these conditions, purification of properdin was associated with a striking decrease in properdin activity. Further experiments demonstrated that an additional factor was required to measure purified preparations of rabbit properdin by the zymosan assay. This factor has been tentatively designated as ZBP,[3] or zymosan-binding protein, and it appears to be distinct from known components of the properdin system.

This paper will describe some initial work on the purification and identification of ZBP and experiments performed to purify rabbit properdin.

MATERIALS AND METHODS

Rabbit serum. Pooled fresh rabbit serum was obtained from Pel-Freez Biologicals, Inc., Rogers, Ark., and kept at −70°C.

Human serum. Human blood was collected from professional donors, allowed to clot at room temperature for 1 hr, and stored at 4°C overnight for maximal clot retraction. The serum was separated by centrifugation at 1°C. Serum pools from 10 to 20 donors were stored at −70°C.

Antiserum. Antisera to human properdin were obtained from Kent Laboratories Ltd., Vancouver, B. C., and from Dr. J. T. Boyer, Tucson, Arizona. Goat anti-rabbit IgM (heavy chain specific) was obtained from Cappel Laboratories, Inc., Cochranville, Pa., and Miles Laboratories, Inc., Elkhart, Ind. Goat anti-rabbit IgG (heavy chain specific) and goat anti-rabbit C3 were obtained from Cappel Laboratories, Inc. Goat anti-rabbit IgA was purchased from Miles Laboratories, Inc. Goat anti-human GBG (factor B) was obtained from Atlantic Antibodies, Westbrook, Maine.

Resins and gels. DEAE-cellulose, high capacity, type 20, exchange capacity 1.05 mEq/g, was obtained from Carl Scheicher and Schull Co., Keene, N. H. DEAE-cellulose was prepared and packed in 4 x 50 cm chromatography tubes as described previously (20). CM-Sephadex, C-50, medium, capacity 4.5 mEq/g, QAE-Sephadex, A-50, capacity 3.0 mEq/g, SP-Sephadex, C-50, capacity 2.3 mEq/g, and CNBr-activated Seph-

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[3] Abbreviations used in this paper: TBS, triethanolamine-buffered saline; EDTA, ethylenediamine tetraacetic acid; RP, a human serum made deficient in properdin by absorption with zymosan; RP-A, human serum rendered deficient in properdin by absorption with Sepharose 4B-coupled anti-human properdin; ZBP, zymosan-binding protein; ZBP-Z complexes, a solid phase complex consisting of ZBP bound to zymosan; EA, a sheep erythrocyte (E) sensitized with specific antibody (A); EAC4 and EAC14, intermediate complexes with EA bearing the hemolytically active fragment of the fourth (C4) component of complement (C) and EA bearing hemolytically active first (C1) and fourth (C4) components of C; DEAE-1-P, pool of properdin-containing fractions eluted from DEAE-cellulose columns in TBS at pH 7.4; DEAE-2-P, pool of properdin-containing fractions eluted from DEAE-cellulose columns in TBS at pH 8.0; CM-P, pool of properdin-containing fractions eluted from CM-Sephadex columns with a linear salt gradient in phosphate buffer at pH 5.9; QAE-P, pool of properdin-containing fractions eluted from QAE-Sephadex columns in glycine-sodium hydroxide buffer at pH 8.6; SP-P, properdin-containing fractions eluted from SP-Sephadex columns by NaCl in β-alanine-acetic acid buffer at pH 4.7.

arose 4B were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Bio-Gel A-15 m agarose beads were purchased from Bio-Rad Laboratories, Richmond, Calif.

Zymosan. Preparations of zymosan were obtained from Fleischman Yeast Division, Standard Brands, Inc., Stamford, Conn., Schwarz/Mann, Orangeburg, N. Y., and Sigma Chemical Co., St. Louis, Mo.

λ-Phage. λ-phage particles used to calibrate the Bio-Gel A-15 m column were a gift from Dr. Lazarus Astrachan, Department of Microbiology, School of Medicine, Case Western Reserve University, Cleveland, Ohio.

Buffers. Triethanolamine-buffered saline (TBS) at pH 7.4, ionic strength 0.15, containing 0.15 mM Ca^{++} and 0.5 mM Mg^{++}, and sodium phosphate buffers were prepared as noted previously (20). TBS was used routinely in the zymosan assay. When this buffer was employed for column chromatography, however, the Ca^{++} concentration was increased to 1.0 mM. Glycine-sodium hydroxide buffer was prepared by mixing 3.75 g of glycine with 85 ml of 1.0 *M* NaCl, and the mixture was titrated to pH 8.6 with 1.0 N NaOH. The final volume was adjusted to 1.0 L. β-Alanine-acetic acid buffer at pH 4.7 was prepared by taking 31.2 g of β-alanine and 8.0 ml of glacial acetic acid up to a final volume of 1.0 L. The conductivity was increased to the desired concentration with NaCl at room temperature, using a Radiometer conductivity meter.

Sensitized sheep erythrocytes (EA). Sterile sheep erythrocytes were sensitized and standardized at a concentration of 2.5 × 10^8/ml as described before (20).

Measurement of C1 and C3. Functional C1 and C3 activities were measured as described by Rapp and Borsos (21) and as recommended by Cordis Corp., Miami, Fla. Functionally pure human C components and intermediate complexes were obtained from Cordis Corp.

Measurement of properdin. Properdin activity was measured by the zymosan assay (18, 19). RP, the serum reagent lacking properdin activity, was prepared by incubating pooled serum with zymosan at 17°C for 60 min (18, 19). With most zymosan preparations, a second incubation at 17°C for 30 min with fresh zymosan was required to prepare RP that lacked properdin activity. Residual traces of zymosan were removed after the second incubation by centrifugation at 5°C for 20 min at 20,000 × G. In an additional method for measuring properdin activity, properdin was selectively removed from human serum by immunoabsorption by using goat anti-human properdin antibody coupled to CNBr-activated Sepharose 4B according to the procedure recommended by Pharmacia. Normal human serum was incubated with Sepharose 4B-coupled anti-human properdin for 90 min at 1°C with frequent mixing, and the Sepharose was removed by centrifugation. This reagent was identified as RP-A.

Rabbit euglobulin preparation. One volume of rabbit serum at 1°C was added with constant stirring to 8 volumes of cold sodium acetate buffer, pH 5.5, ionic strength 0.02. The mixture was allowed to stand at 1°C overnight, and the supernatant fluid was withdrawn by siphon. The residual precipitate was collected by centrifugation at 1°C, washed with acetate buffer at pH 5.5, ionic strength 0.02, and suspended in 0.3 M NaCl containing 1.0 mM Ca^{++} to one-eighth the original volume of serum. This mixture was centrifuged at 105,000 × G for 60 min at 5°C. The supernatant fluid was poured through a filter of glass-wool to exclude lipid particles, and the filtrate was dialyzed at 5°C in 50 volumes of TBS. After 12 to 18 hr, the dialyzed fraction was centrifuged, and the resulting solution, concentrated 12- to 14-fold with respect to serum, was used as the starting material for column chromatography.

Polyacrylamide gel electrophoresis. Acid disc gels were prepared with 7% acrylamide in pH 4.3 buffer and electrophoresed in a Canalco Model 6 disc gel apparatus at a constant current of 4 m amps/gel for 2 hr. Standard gels were made with 7% acrylamide in pH 9.5 buffer and electrophoresed for 1.5 hr with a constant current of 4 m amps/gel. The gels were fixed in 12% trichloroacetic acid for 30 min and stained with Coomassie Brilliant Blue dye.

Protein determinations. Protein concentration was estimated by absorption at OD 280 nm, assuming that 1.0 OD 280 nm equals 1.0 mg protein/ml.

RESULTS

Zymosan binding protein (ZBP): Recognition of ZBP. ZBP was recognized by its requirement for measuring properdin in purified preparations by the zymosan assay. Indeed, ZBP was required to restore full properdin activity to impure properdin preparations obtained by DEAE-cellulose chromatography. In the initial step to purify rabbit properdin, euglobulin preparations were separated into two fractions on DEAE-cellulose columns equilibrated with TBS at pH 7.4, ionic strength 0.15 (Fig. 1). Properdin was not bound by DEAE-cellulose, and it was detected in the first protein fraction obtained from the column. The yield was poor, however, since less than 20% of properdin activity was recovered. This properdin preparation was identified as DEAE-1-P.

These experiments suggested that an additional factor might be required to measure properdin activity by the zymosan assay, and that this factor had been separated from properdin by chromatography on DEAE-cellulose. To investigate this possibility, aliquots from column fractions eluted by a linear saline gradient were tested for their ability to increase DEAE-1-P activity. Aliquots from fractions 84 through 164 (Fig. 1) did increase properdin activity. The active agent in these fractions was tentatively designated as ZBP, since further experiments have shown that it binds to zymosan. These experiments indicated that ZBP might be a component of the rabbit properdin system, and studies were undertaken to investigate the role of ZBP in the zymosan assay.

Function of ZBP in the zymosan assay for properdin. Ex-

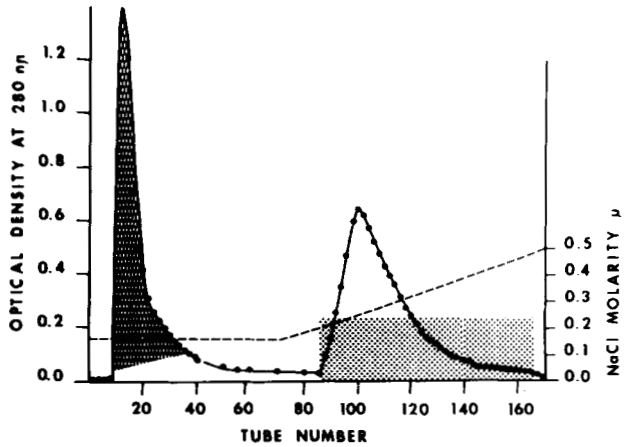

Figure 1. DEAE-cellulose chromatography of rabbit euglobulin preparations in TBS at pH 7.4: ●——●, protein eluted; -----, ionic strength of elutrient; cross-hatched area, properdin activity; stippled area, ZBP activity.

periments to assess the role of ZBP in this reaction were difficult, because the action of ZBP was measured indirectly through its effect on properdin activity. When aliquots of ZBP-containing fractions were added to mixtures containing zymosan, RP, and properdin, the assay was extremely erratic and marked variations were noted in properdin activity measured under apparently identical conditions. This problem was corrected when further experiments demonstrated that ZBP was bound by zymosan to form a solid phase complex (ZBP-Z complex).

In these experiments, serial dilutions (100 μl) of ZBP-containing fractions in TBS were incubated with 1.0 mg of zymosan suspended in 50 μl of TBS for 15 min at room temperature. After incubation, the mixtures were diluted with 2.0 ml of ice-cold TBS, centrifuged, the supernatant fluid was decanted, and the zymosan pellets were washed again with 2.0 ml of TBS. The zymosan residues were resuspended in 100 μl of RP and 100 μl of DEAE-1-P in TBS, and the mixtures were incubated for 45 min at 37°C. Five hundred microliters of EA (2.5×10^8/ml) were added to each tube; the mixtures were incubated for an additional 30 min at 37°C, centrifuged, and the extent of hemolysis was determined. Since constant amounts of properdin, zymosan, and RP were added to each mixture, changes in properdin activity were directly related to ZBP that had been bound by zymosan. In the original description of the zymosan assay of properdin (1, 18), one unit of properdin was defined as the smallest amount of test sample that would reduce the C3 titer of RP from 120 to 0 units during incubation with zymosan. For experiments described here, the definition of one unit of properdin has been changed to the smallest amount of test sample that would reduce hemolysis in the reaction mixture by 50%.

Data from a typical experiment are shown in Table I. In this experiment, ZBP-Z complexes were formed by incubating 1.0 mg of zymosan with ZBP at the concentrations shown in Table I. After incubation, the ZBP-Z complexes were washed and reincubated with RP and DEAE-1-P. To measure the properdin activity in a test preparation, decreasing amounts of the properdin preparation were incubated with a constant amount of ZBP-Z complexes and RP. Thus, the concentration of both ZBP and properdin were varied to measure properdin activity. To obtain the value of 80 units of properdin shown on the first line of Table I, for example, ZBP-Z complexes were prepared in a series of four tubes, with each tube containing zymosan that had been incubated with 18 μg of ZBP. One hundred microliters of RP were added to each tube, but the amount of properdin test sample added to each mixture was reduced, in sequence, by one-half. These mixtures were incubated at 37°C for 45 min; EA was added to each mixture, and the extent of hemolysis was determined after an additional incubation for 30 min at 37°C. This same procedure was carried out with ZBP-Z complexes formed in mixtures containing 9.0 and 4.5 μg of ZBP, so that the same amount of properdin test sample was incubated with variable amounts of ZBP.

As shown in Table I, properdin activity varied as a function of ZBP concentration. In the absence of ZBP, 4 units of properdin activity were detected, but when ZBP was employed, activity was increased 20-fold to 80 units/ml. The mixture containing ZBP-Z complexes and RP, but no added properdin, did not have detectable properdin activity. It should be noted that the preparations of ZBP tested were not pure and ZBP-Z complexes prepared with high concentrations of ZBP inactivated C3 when incubated with RP in the absence of added properdin. In other experiments with highly purified preparations of ZBP, however, ZBP-Z complexes prepared with large concentrations of ZBP did not inactivate C3 in the absence of added properdin.

TABLE I

Effect of ZBP on properdin activity

ZBP[a]	Zymosan	Properdin (DEAE-1-P)	Properdin Activity
μg/ml			*units/ml*
18.0	(+)	(+)	80
9.0	(+)	(+)	20
4.5	(+)	(+)	8
(−)	(+)	(+)	4
18.0	(−)	(−)	0

[a] μg of ZBP incubated with zymosan to form ZBP-Z complexes.
(−), Not present in the reaction mixture.
(+), Present in the reaction mixture.

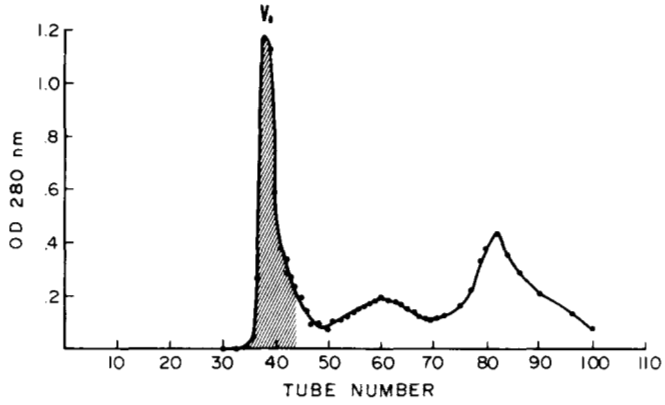

Figure 2. Bio-Gel A-15 m gel filtration of the pooled, concentrated ZBP-containing fractions from DEAE-cellulose: ●—●, protein eluted; stippled area, ZBP activity. The column was calibrated with lambda phage particles to obtain the void volume (V_0); the flow rate was 10 to 12 ml/hr and 4.4 ml fractions were collected.

Identification of ZBP. Highly purified preparations of ZBP were obtained by gel filtration. ZBP-containing fractions eluted from DEAE-cellulose (Fig. 1) were pooled, concentrated, and placed on a column (2.6 x 100 cm) containing Bio-Gel A-15 m agarose beads in TBS. ZBP activity was associated with a protein peak that eluted in the void volume (176 ml) as shown in Figure 2. In most experiments, two smaller protein peaks were detected as well (Fig. 2).

Column fractions containing ZBP activity were pooled, concentrated, and examined by polyacrylamide disc gel electrophoresis at pH 9.5. In every case, only one or two bands were detectable at the top of the separating gel after staining with Coomassie Brilliant Blue dye. Preparations of ZBP were analyzed by immunodiffusion with antisera to rabbit IgM, IgA, IgG, and C3, and no precipitin lines were detected. IgM was detected, however, in fractions from the second protein peak eluted from the Bio-Gel A-15 m column, and IgG and C3 were detected in fractions from the third protein peak (Fig. 2).

Additional experiments demonstrated that ZBP preparations obtained by gel filtration on Bio-Gel A-15 m columns would bind to zymosan to form ZBP-Z complexes, and ZBP activity was measured as described above. ZBP activity was destroyed by incubation at 56°C for 30 min, and it was particularly susceptible to inactivation by EDTA.[3] Preparations of ZBP were completely inactivated after incubation for 30 min at 37°C in TBS containing 1.0 mM EDTA. Indeed, ZBP activity could

not be detected when euglobulin preparations were suspended and chromatographed in buffers containing EDTA.

The inactivation of ZBP by EDTA suggested that ZBP might be rabbit C1. To investigate this possibility, ZBP-containing fractions and properdin-containing fractions eluted from DEAE-cellulose (Fig. 1) were tested for their ability to form the complex EAC14 when incubated with the complex EAC4. C1 activity was detected in fractions from both parts of the chromatogram, but C1 activity was not detectable in ZBP-containing fractions obtained from Bio-Gel A-15 m columns (Table II).

These experiments indicate that ZBP is a unique serum protein that is distinct from known components of the classic and alternative C systems and serum immunoglobulins. Additional work on the purification and characterization of ZBP that supports this conclusion will be reported separately.

Purification of rabbit properdin: Chromatography on DEAE-cellulose. Rabbit properdin was purified from serum euglobulin preparations by ion-exchange chromatography on DEAE-cellulose, CM-Sephadex, QAE-Sephadex, and SP-Sephadex. The initial purification step on DEAE-cellulose was described above and shown in Figure 1. A second fractionation was performed on DEAE-cellulose in TBS at pH 8.0, ionic strength 0.1. When DEAE-1-P was rechromatographed on DEAE-cellulose under these conditions, properdin activity was again associated with proteins that were not bound, and a second large optical density peak was eluted by a linear sodium chloride gradient (Fig. 3). Column fractions containing properdin activity were combined (DEAE-2-P) and concentrated.

TABLE II

C1 Activity

Preparation	C1 Activity[a]/OD Unit
DEAE-1-P[b]	120,000
DEAE-ZBP[c]	245,000
Bio-Gel A-15 m-ZBP[d]	Undetectable

[a] C1 activity expressed as CH_{50} units/optical density unit of each preparation.

[b] DEAE-1-P, pool of fractions containing properdin activity from DEAE-cellulose column.

[c] DEAE-ZBP, pool of fractions containing ZBP activity from DEAE-cellulose.

[d] Bio-Gel A-15 m-ZBP, pool of fractions containing ZBP activity from Bio-Gel A-15 m column.

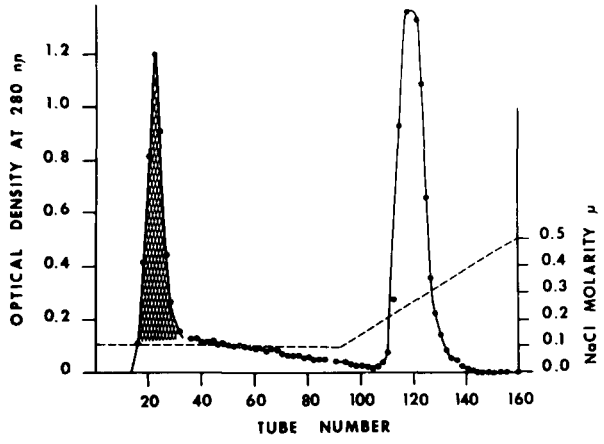

Figure 3. DEAE-cellulose chromatography of the pooled, concentrated properdin-containing fractions from DEAE-cellulose in TBS at pH 8.0: ●——●, protein eluted; -----, ionic strength of elutrient; cross-hatched area, properdin-activity.

Properdin activity and recovery are shown in Table III.

Chromatography on CM-Sephadex. Preparations of DEAE-2-P were dialyzed in phosphate buffer at pH 5.9, ionic strength 0.15, and placed on a column (2.5 x 30 cm) containing CM-Sephadex that had been equilibrated with the same buffer. Equilibration buffer was allowed to flow through the column, and a large protein fraction that did not contain properdin activity was always noted (Fig. 4). Properdin activity was usually associated with a well-defined protein peak eluted by increasing the sodium chloride concentration of the eluting buffer, as shown in Figure 4. On some occasions, three discrete protein peaks were found rather than the shoulders shown in Figure 4, and rarely the properdin activity was associated with proteins contained in the third or last protein peak. Column fractions containing properdin activity were pooled and concentrated (CM-P). The yield and activity in the presence and absence of ZBP are given in Table III.

Chromatography on QAE-Sephadex. Preparations of CM-P were dialyzed in glycine-sodium hydroxide buffer at pH 8.6, ionic strength 0.1, and placed on a column (1.5 x 25 cm) containing QAE-Sephadex equilibrated with the same buffer. As shown in Figure 5, two protein fractions were obtained. Properdin activity was associated with the first protein fraction, which was not bound by QAE-Sephadex, whereas no properdin activity was detected in the fraction eluted by a linearly increasing NaCl gradient in glycine-sodium hydroxide buffer. The yield and activity in the presence and absence of ZBP are shown in Table III. This preparation was designated QAE-P.

Chromatography on SP-Sephadex. Preparations of QAE-P were dialyzed in β-alanine-acetic acid buffer at pH 4.7, conductance of 10 m mho/cm, and placed on a column (1.5 x 25 cm) containing SP-Sephadex that had been equilibrated with this buffer. Properdin was bound by SP-Sephadex under these conditions, and the column was eluted in successive steps with 100 ml of β-alanine-acetic acid buffer at a conductance of 20, 30, 40, and 50 m mho/cm. Column fractions were immediately dialyzed in TBS, concentrated, and tested for properdin activity. These preparations were designated as SP-P.

For reasons that are not clear at this time, the activity and extent of purification of properdin preparations obtained in this step have been variable. In some preparations, properdin eluted as a single activity peak associated with column fractions obtained with either 30 or 40 m mho buffer, whereas in other preparations, properdin activity was detected in column fractions eluted with 30, 40, and 50 m mho buffer. In every case, however, properdin activity was not detected in the absence of ZBP, and the total properdin activity recovered did not vary by more than 2-fold. The results from a typical SP-P preparation are given in Table III.

When examined by acid disc gel electrophoresis, SP-P preparations (50 μg) that contained properdin activity only in column fractions eluted with either 30 or 40 m mho buffer generally showed one protein band about 8.0 mm from the top of the separating gel. On the other hand, when SP-P preparations that contained properdin activity in 30, 40, and 50 m mho column fractions were examined by acid disc gel electrophoresis, no bands were detected in gels containing 50 m mho fractions, whereas discs containing 30 or 40 m mho fractions showed one or two stained bands. The absence of a stainable band was not a function of concentration in gels that contained 50 m mho fractions, since the same result was obtained with samples ranging from 10 to 200 μg. No bands were detected after alkaline disc gel electrophoresis of preparations of SP-P that produced either a single or no band in acid disc gels.

TABLE III

Properdin activity at each purification step

Preparation	Zymosan Assay[a]		
	Standard[b]	Modified[c]	Yield[d]
	units/O.D. unit		*% of euglobulin*
Euglobulin	4	9	
DEAE-1-P	7	14	37
DEAE-2-P	3	57	31
CM-P	3	133	37
QAE-P	2	201	15
SP-P	0	435	2.5

[a] Properdin activity expressed as units/optical density unit of each properdin preparation.

[b] Standard zymosan assay that did not contain ZBP as a constituent of the mixture.

[c] Modified zymosan assay in which ZBP was incubated with zymosan to form ZBP-Z complexes. These complexes were then used to measure properdin activity and the assay was otherwise unchanged from the standard assay.

[d] Yield was expressed as % of euglobulin activity recovered at each step during purification when measured by the modified zymosan assay.

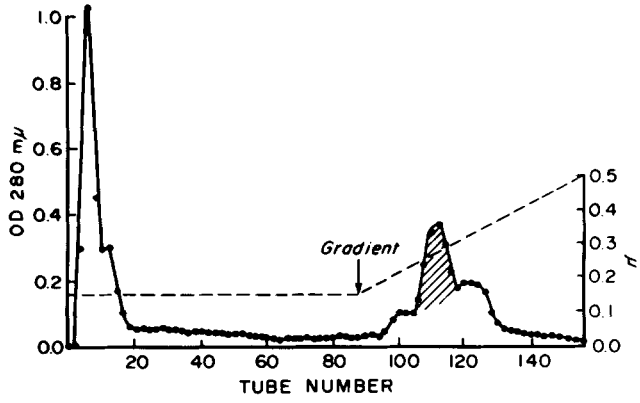

Figure 4. CM-Sephadex chromatography of the pooled, concentrated properdin-containing fractions from the second DEAE-cellulose column: ●——●, protein eluted; -----, ionic strength of elutrient; cross-hatched area, properdin activity.

Function of rabbit properdin. Human serum absorbed with Sepharose-coupled anti-human properdin (RP-A) was employed to study the function of rabbit properdin in an assay other than the traditional zymosan assay. In these experiments, 1.0 mg zymosan pellets were mixed with RP-A and with RP-A plus SP-P. After incubating for 30 min at 37°C, zymosan was removed from the reaction mixtures by centrifugation at 5°C, 3500 × G, and the supernatant fluids were tested for residual C3 activity. As shown in Figure 6, C3 was inactivated in a dose-dependent fashion when RP-A was reconstituted wilth SP-P. On the other hand, only minimal C3 inactivation was noted in mixtures containing RP-A and zymosan. It should be noted that properdin activity could be measured by this method without the addition of ZBP, since properdin was removed by a specific immunoabsorbent and ZBP remained in the serum reagent.

Other investigators have shown that purified preparations of properdin in its active state ($\bar{P}$) can initiate activation of the alternative C pathway in serum that does not contain zymosan or other activating factors (5, 6). Under these conditions, the immunoelectrophoretic patterns of C3 and factor B are changed and provide a method for identifying properdin. To determine whether properdin in SP-P preparations was in its precursor or active state, SP-P (60 μg) was incubated with human serum for 45 min at 37°C, and the immunoelectrophoretic behavior of factor B was determined. As shown in Figure 7, the immunoelectrophoretic mobility of factor B was altered in serum incubated with SP-P, indicating that properdin was in its active state.

DISCUSSION

Homogeneous protein preparations that functioned as properdin were separated from rabbit serum by ion-exchange chromatography. The chromatographic procedures with DEAE-cellulose, CM-Sephadex, and QAE-Sephadex were generally reproducible, but the final purification step with SP-Sephadex gave variable results. On some occasions, properdin activity was associated with column fractions that were eluted over a narrow range of ionic strength, and a single protein band was noted when these preparations were examined by acid disc gel electrophoresis. On other occasions, properdin activity was found in column fractions that were eluted over a broad range of ionic strength, and no protein bands were detectable in acid disc gels that contained aliquots of column fractions eluted at the highest ionic strength. The reason for the unusual staining characteristics exhibited by some properdin preparations is unknown.

The chromatographic behavior of rabbit and human properdin is similar. In both, properdin is not bound by DEAE-cellulose or QAE-Sephadex, but it is bound by CM-Sephadex and SP-Sephadex. Like human properdin, rabbit properdin is bound to negatively-charged resins, and like human properdin, it migrates into gels under acid but not alkaline conditions.

These experiments indicate that the rabbit serum protein isolated by this method is properdin, but additional experiments

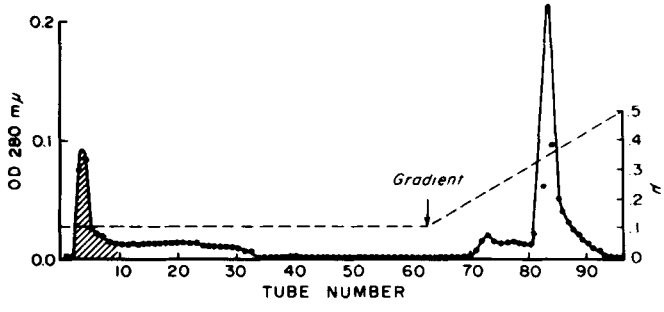

Figure 5. QAE-Sephadex chromatography of the pooled, concentrated properdin-containing fractions from CM-Sephadex: ●——●, protein eluted; -----, ionic strength of elutrient; cross-hatched area, properdin activity.

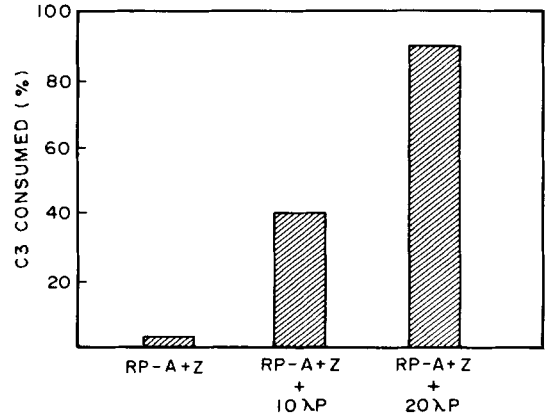

Figure 6. C3 inactivation by zymosan (*Z*) and properdin (*P*) in a human serum reagent (*RP-A*) made deficient in properdin activity with a specific immunoabsorbent.

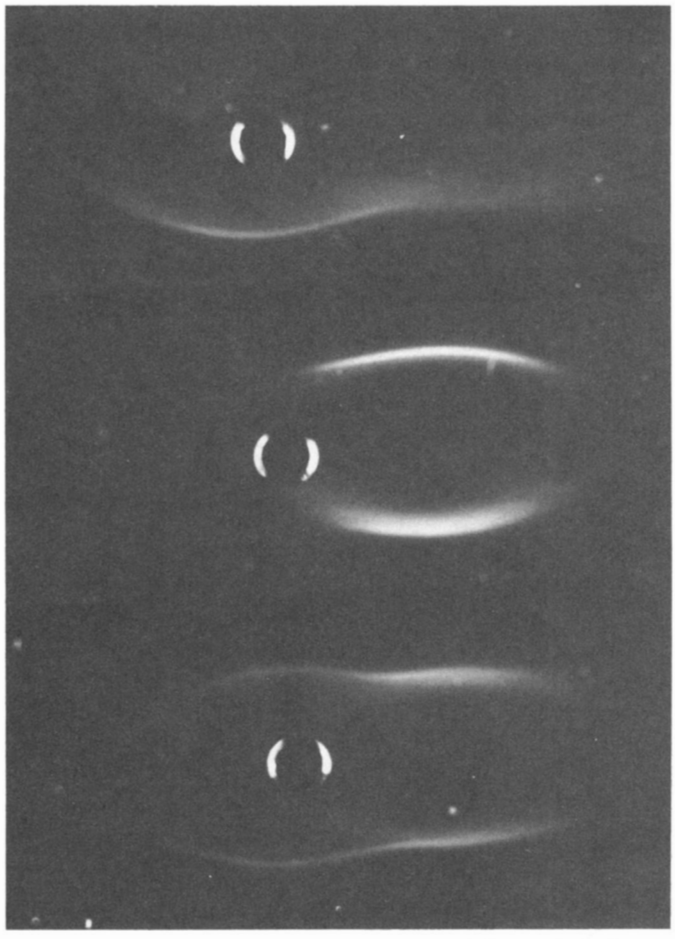

Figure 7. Immunoelectrophoretic behavior of factor B in human serum: *upper well*, 180 μl human serum incubated with 1.0 mg zymosan; *middle well*, 180 μl human serum incubated with buffer; *lower well*, 180 μl human serum incubated wiith 30 μg SP-P. Five microliters of each mixture was electrophoresed and developed with antihuman GBG (factor B).

to study its function were required to confirm this thesis. Rabbit properdin was identified by the zymosan assay during its purification. This assay is based upon the observation that zymosan-induced activation of terminal C components does not occur in serum depleted of properdin activity. Although properdin activity can be selectively removed from human serum by this technique, properdin could not be selectively depleted from guinea pig serum by zymosan absorption (14). This also was the case with rabbit serum, since we were unable to remove properdin activity from rabbit serum by absorption with zymosan. It was necessary, therefore, to measure rabbit properdin activity in a heterologous system employing human serum depleted of properdin activity by zymosan absorption (RP). This assay provided a good method for monitoring properdin during purification, but it was complicated by a requirement for an additional factor (ZBP) in order to measure properdin activity in purified preparations. To document that the purified protein preparations identified as properdin by the zymosan assay were indeed properdin, these preparations were measured by an additional method that is specific for properdin activity (5, 14). In these experiments, properdin was selectively removed from human serum with a specific immunoabsorbent, and properdin activity was restored by the addition of purified preparations of rabbit properdin (Fig. 6).

Other investigators have shown that activated properdin ($\bar{P}$) will activate the alternative C pathway in the absence of zymosan or other activating factors (5, 6). The electrophoretic mobilities of C3 and factor B are altered under these conditions. We have shown that the electrophoretic mobility of factor B was altered when purified rabbit properdin preparations were incubated with human serum (Fig. 7), and that the mobility of C3 was altered in a similar manner when these preparations were incubated with rabbit serum (data not given). These experiments provide additional evidence that purified preparations of rabbit properdin do function as properdin, and that the properdin was present in its active state.

The purification of properdin was associated with a striking decrease in properdin activity when measured by the zymosan assay (Table III). Properdin activity was restored, however, when ZBP was included in the assay. Although ZBP has not been previously recognized as a component of the properdin system, it does not appear to be a unique requirement for measuring *rabbit* properdin; preliminary experiments in our laboratory have shown that it also is required to measure human properdin by the zymosan assay. Since these properdin preparations contained properdin in its activated state, the role of ZBP in reaction systems with precursor properdin remains to be established.

ZBP was identified in the protein fraction eluted from DEAE-cellulose columns by a salt gradient, and highly purified preparations of ZBP have been obtained by gel filtration. Indeed, ZBP activity was consistently associated with the protein peak eluted in the void volume of columns containing Bio-Gel A-15 m agarose beads. This observation indicated that ZBP might be an extremely large molecule, a complex of several different molecules, a polymer, or that it has an unusually asymetric shape.

The possibility that ZBP might be an aggregate of either IgM of IgA was considered. This postulate seems highly unlikely, however, since purified ZBP preparations did not contain these immunoglobulins or IgG when they were examined by immunodiffusion, using specific antisera to rabbit IgM, IgA or IgG. In addition, the susceptibility of ZBP to inactivation by either EDTA or incubation at low temperatures does not support the possibility that ZBP is an immunoglobulin aggregate.

ZBP also appears to be distinct from known components of the classical and alternative C systems. Studies with intermediate C complexes have shown that ZBP is not C1, and immunodiffusion experiments have demonstrated that purified ZBP preparations do not contain C3. The observation that ZBP in purified preparations will bind to zymosan indicates that it is not factor B or factor D, since these factors do not bind to zymosan unless C3 and factor B, or C3 and both factors B and D are present in the reaction mixture (22, 23). Furthermore, the detection of ZBP in the void volume of columns containing Bio-Gel A-15 m agarose beads clearly distinguishes it from known components of the classical or alternative C systems. These experiments indicate, therefore, that ZBP is a unique serum protein rather than an immunoglobulin aggregate or a complex composed of several different substances.

The function of ZBP has not been established by this work. We have shown that it binds to zymosan and that it is required to measure properdin activity in highly purified preparations of rabbit properdin by the zymosan assay, but the role of ZBP in the alternative C pathway remains to be determined. The direct binding of ZBP to zymosan suggests that this may initiate activation of the alternative pathway in a manner conceptually analogous to activation of the classical C pathway by the binding of C1 to immune complexes. On the other hand, ZBP

may function by stabilizing the alternative pathway C3 convertase, an action previously demonstrated for properdin (24), or by inhibiting the inactivation of alternative pathway C3 convertase by β1H (25, 26), an action previously documented for C3Nef (27, 28). Finally, ZBP may act to overcome the inhibitory effect of membrane-associated sialic acid (29, 30) on alternative pathway C3 convertase generation.

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