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Alternative pathway	properdin
C3, C3b	serum
complement	sickle cell disease
inulin	total hemolytic activity (CH50)

Alternative Pathway of Complement in Sickle Cell Disease

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Summary

Thirty-one patients, 10 months to 20 years of age, were studied. A complement abnormality was not identified in sera from patients with sickle cell disease (SCD) by the methods employed in the present study. Concentrations of C3, factor B, total hemolytic activity (CH50), properdin, and C3b inactivator were similar in sera from patients and control subjects (Table 1 and Fig. 2). Although concentrations of C3b inactivator protein were below normal in a few patients, there was no evidence that these levels were low enough to alter the functions mediated by this protein.

Initiation of the complement sequence via the alternative pathway by reaction with inulin was equal in patient and control sera when assessed by the activation of factor B, cleavage of C3, and the consumption of hemolytic complement components (Table 1). Lysis of erythrocytes treated with reduced glutathione was similar in patient and control sera during alternative pathway activation (Fig. 3), indicating comparable formation of lytic complexes via this pathway.

An abnormality of the alternative pathway was not detected when the serum from patients with sickle cell disease was reacted with inulin. Thus, this polysaccharide, although commonly employed to assess alternative pathway function, is not satisfactory for studying serum from these patients. In addition, activation of the alternative pathway by cobra venom factor was comparable

with controls when assessed by the lysis of glutathione-treated erythrocytes.

Speculation

Defective function of the alternative pathway of complement activation has been described in the serum of patients with sickle cell anemia. The precise abnormality may be revealed by a quantitative and functional assessment of the components of this pathway.

Fulminant bacterial infections, particularly with *Streptococcus pneumoniae*, occur with increased frequency in children with SCD (1, 20), and several abnormalities of the body defense mechanisms have been reported to account for the propensity to infections in this disorder (7). Defects related to the opsonization, phagocytosis, and killing of bacteria are most important because these defenses are required to control pyogenic infections. An abnormality of pneumococcal opsonization in serum from patients with SCD has been attributed to the heat-labile opsonin system (23). The precise serum defect is as yet unidentified, but evidence suggests that it involves the alternative pathway of complement (8). In this report a quantitative and functional assessment was made of this pathway and related proteins

in serum from patients with SCD in an attempt to define this abnormality.

MATERIALS AND METHODS

EXPERIMENTAL SUBJECTS

Thirty-one patients, 10 months to 20 years of age, were studied. The hemoglobinopathies, as documented by electrophoresis and family studies, were 25 SS, 3 SC, 2 S thalassemia, and 1 SD. Results of experiments from the few patients who were not homozygous SS could not be distinguished from the total group, and all data were combined. A total of 59 blood samples were collected, and the patient was judged at the time of venipuncture to be well, infected, or in crisis. Well patients were free of illness for at least 2 weeks. The difficulty in accurately distinguishing infection from crisis is well known, but infection was considered to be present if suggestive symptoms, signs, or chest roentgenograms were accompanied by a temperature higher than 101°F. It is possible that some of these patients were suffering merely from crisis. The diagnosis of crisis was made when patients were ill, but were afebrile and exhibited no features of infection. Healthy laboratory personnel and 32 normal children (ages 10 months to 19 years) admitted for elective surgery served as control subjects. This study was approved by the committee for human investigation and informed written consent to participate in the study was obtained.

PREPARATION OF SERUM

Venous blood was clotted at room temperature. The serum was separated within 1 hr by centrifugation, divided into small aliquots, and frozen at -70°. In most instances each aliquot was thawed once and used in a single experiment.

QUANTITATION OF COMPLEMENT COMPONENTS AND RELATED PROTEINS

A simplified version of the complement sequence with components of the classic and the alternative pathways is illustrated in Figure 1. Serum concentrations of C3, factor B and C3b inactivator were determined by radial immunodiffusion in 2% glycine agarose containing 0.04 M ethylenediaminetetracetic acid (EDTA). Properdin was determined by similar methods using 1% agarose with 0.04 M EDTA.

The measurement of C3 employed antiserum specific for the B antigenic determinant (22). Antibody was prepared by immunizing goats with C3 isolated from fresh human serum. Antibodies against other antigens of C3 were cleared by absorption with

aged serum. The absorbed antibody, when studied by immunoelectrophoresis or by double diffusion, produced a single arc against fresh serum and purified C3 and no arcs with aged serum. The quantity of the B antigen in serum was arbitrarily expressed as B antigen units.

Monospecific antibody to factor B was produced by immunizing goats with purified factor B isolated from human pseudoglobulin (5). The antiserum was absorbed with normal serum previously heated at 56° for 60 min. This absorbed antiserum, when studied by immunoelectrophoresis and double diffusion, produced a single arc against normal serum and factor B.

Monospecific antibody to C3b inactivator was produced by immunizing a goat with C3b inactivator (Cordis Laboratories, Miami, Fla.). The antiserum was absorbed with a C3b inactivator-free fraction of human pseudoglobulin. This fraction is the protein peak eluted from a BioRex 70 column before factor B, the preparation of which is detailed in the method for isolating factor B (5). The absorbed antiserum, which was studied by immunoelectrophoresis or by double diffusion, produced a single arc when reacted against normal serum and no arcs when reacted against factor B or serum from the patient with C3b inactivator deficiency (24). In addition, this antibody was used to absorb normal human serum rendered 0.01 M with respect to EDTA. When the absorbed serum was reconstituted with magnesium, extremely rapid breakdown of C3, assessed by decrease in B antigen concentration, occurred spontaneously. Reconstitution of unabsorbed EDTA serum with magnesium did not lead to C3 breakdown. Thus, the absorption specifically depleted the serum of the C3b inactivator, released its inhibition on the C3b feedback loop, and permitted spontaneous cleavage of C3 (16).

Monospecific antibody to properdin was prepared as follows. A goat was injected with crude properdin (18). The antiserum was rendered monospecific for properdin by absorption with human serum depleted of properdin by reaction with zymosan (17). This antibody was then coupled to Sepharose by cyanogen bromide and used to isolate properdin from normal human serum. The same goat was then boosted with the purified properdin, and the antiserum was again absorbed. When studied by immunoelectrophoresis or by double diffusion, the antiserum produced a single arc against normal serum and no arcs against several complement proteins, factor B, and properdin-depleted serum. Total hemolytic complement was measured as CH50 (9).

ACTIVATION OF ALTERNATIVE PATHWAY

The complement sequence can be initiated via either the classic or alternative pathway (Fig. 1). Inulin selectivity initiates the complement sequence via the alternative pathway, and this polysaccharide is frequently employed to assess the function of this pathway. If the alternative pathway is intact, reaction of serum with inulin will result in activation of factor B, cleavage of C3 and consumption of several complement components (5, 14). The method used was as follows. Serum was thawed and held in an ice bath. Particulate inulin was added (10 mg/ml serum); next, the mixture was vortexed and placed in a shaking water bath for 30 min at 37°. It was established in preliminary experiments that complement activation was complete within this period. After incubation, the reaction mixtures were cooled in an ice bath, the inulin was removed by centrifugation at 0-4°, and the supernatants were held briefly in an ice bath until studied.

Activation of factor B was assessed by the conversion of the electrophoretic mobility of this protein from β to γ (5). Immunoelectrophoresis to study factor B mobility was performed in 1.5% agarose for 25 min at 40 V/cm.

Cleavage of C3 was assessed by the decrease in concentration of the B antigenic determinant that normally accompanies cleavage of this protein (22). C3 is present in fresh serum as β -1-C-globulin. When serum ages or when the complement system is activated in fresh serum, C3 is converted to C3c (β -1-A-globulin) and C3d (α -2-D-globulin); at this point a portion of the molecule (the B antigenic determinant) can no longer be de-

Fig. 1. The components of the classic pathway are listed on the right and the alternative on the left. This simplified diagram is not intended to depict the sequence of reactions accurately.

tested by immunoelectrophoresis. The extent of reduction of B antigen concentration is a sensitive indicator of C3 cleavage. Activation of the complement system by inulin with the subsequent consumption of lytic components was assessed by the decrease of CH50 units.

LYSIS OF GLUTATHIONE-TREATED ERYTHROCYTES

Human erythrocytes that are treated with reduced glutathione at alkaline pH are sensitive to complement-mediated lysis and can serve as indicator cells for the generation of lytic complexes via the alternative pathway in a manner similar to erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (6). Human erythrocytes (type O, Rh negative) were incubated in 0.2 M reduced glutathione (Boehringer Mannheim, New York) at pH 9.0 for 30 min at 37°. The cells were extensively washed until the supernatant was colorless, suspended in Veronal buffer (pH 7.4) containing 1.5×10^{-4} M calcium and 1.0×10^{-3} M magnesium, and used within 24 hr. These cells were exposed to serum during the activation of the complement system with inulin or cobra venom factor, another substance known to activate the alternative pathway (5). The cobra venom factor was not purified after purchase (Cordis Laboratories, Miami, Fla.), but its anticomplementary activity was assessed by its ability to activate factor B and to cleave C3 in whole serum. The reaction mixtures were incubated at 37° for 45 min with inulin and 60 min with cobra venom factor. Incubation with inulin was shortened because complete lysis occurred within 60 min. The lytic reaction was stopped by adding iced 0.15 M NaCl. The unlysed cells were removed by centrifugation at 4°, the supernatants placed into clean tubes, and the amount of hemolysis determined spectrophotometrically at 541 nm. Three blanks were used with each experiment: (1) complete lysis blank with glutathione-treated cells plus distilled water; (2) color blank containing all components of the reaction mixture except the activating substance (inulin or cobra venom factor); (3) a complete reaction mixture containing serum previously heated at 50° for 30 min to assess the requirement for heat-labile serum factors. The optical density of the color blank was subtracted from that of the sample, and the amount of lysis produced by the complement activation was expressed as the percentage of that observed after complete lysis by water.

RESULTS

Quantitation of complement components and related proteins. Concentrations of C3, factor B, and total hemolytic activity were similar in serum from patients and control subjects before incubation with inulin (Table 1). As expected, the concentrations of complement components were generally increased in patients who were ill. Concentrations of properdin were measured in sera from five patients and were slightly elevated (Fig. 2). Concentrations of C3b inactivator were measured in

sera from 17 patients, and the mean value was significantly lower than that of the control subjects (Fig. 2). However, there was considerable overlap into the normal range, and only two values were 2 or more SD below the control mean.

ACTIVATION OF ALTERNATIVE PATHWAY

Patients and control sera responded almost identically to incubation with inulin (Table 1). Factor B was activated in every serum as indicated by a cathodal shift in electrophoretic mobility to the γ region. The fall in B antigen concentrations after reaction with inulin was similar in patients and control subjects, indicating comparable cleavage of C3. The decrease in CH50 units was nearly identical, suggesting that the hemolytic complement components were consumed to a similar extent.

LYSIS OF GLUTATHIONE-TREATED ERYTHROCYTES

Lysis was measured in serum from 10 patients during incubation with inulin and from 17 patients during incubation with cobra venom factor. Lysis was equal to or greater than that of control subjects (Fig. 3). Although the data are not shown in the table, it is important that neither patient nor control serum supported lysis if previously heated to 50° for 30 min, indicating a requirement for factor B (5), and possibly other heat-labile serum factors. In addition, lysis did not occur in the absence of inulin or cobra venom factor.

Fig. 2. Concentrations of properdin and C3b inactivator in serum. The mean value and 2 SD for 45 healthy adult control subjects are represented by the bars. The points are individual patient studies.

Table 1. Results of studies performed on 32 samples from 32 normal children and 59 samples from 31 patients

Study	Results ¹			
	Control subjects (32)	Well patients (26)	Infected patients (22)	Crisis patients (11)
Serum without inulin				
C3 (B antigen units)	33.4	29.5	28.9	34.7
Properdin factor B (mg/dl)	25.1	28.0	30.5	34.8
Total hemolytic activity (CH50)	55.6	55.7	65.5	79.6 ²
Serum plus inulin				
% sera with shift of factor B from β to γ	100	100	100	100
% decrease of original concentration of C3	56.0	59.3	60.7	55.3
% decrease of original CH50 titer	46.1	46.3	48.9	55.5

¹ Mean values.

² Mean differing significantly from control mean.

Fig. 3. Lysis of erythrocytes treated with reduced glutathione. The points represent individual studies of control subjects and patients.

DISCUSSION

It is clear that *S. pneumoniae* are opsonized inefficiently *in vitro* by the serum of some patients with SCD (8, 23). Winkelstein and Drachman (23) reported such a defect in 12 of 14 patients. They suggested a deficiency of heat-labile opsonins in SCD because: heating normal serum to 56° for 30 min destroyed its opsonizing activity and produced an opsonic defect resembling that demonstrated in the patient sera; the pneumococci were opsonized normally when preincubated in fresh normal serum, but not when preincubated in heated normal serum or fresh patient serum; and the concentration of pneumococcal antibodies (heat-stable opsonins) was similar in patients and control subjects. An inhibitor of opsonization was not identified in patient serum.

Johnston and associates (8) confirmed the opsonic defect. Pneumococci were coated with varying amounts of specific antibody and phagocytosis was studied in the presence of fresh serum from patients and control subjects. Antibody alone would not support phagocytosis and fresh serum was required for complete opsonization. Opsonization, as assessed by phagocytosis, was equal in patient and control serum when pneumococci were coated with maximal amounts of antibody. When the amount of antibody was decreased, the mean level of phagocytosis was significantly less in patient serum as compared with control serum. Further studies excluded a relationship of the phagocytic defect to abnormalities of pneumococcal antibodies or the classic complement pathway. An abnormality in the alternative pathway of complement was suggested because zymosan failed to cleave C3 in patient serum chelated with ethyleneglycol tetraacetic acid (EGTA); *i.e.*, there was no conversion of C3 into its more anodal form when studied by immunoelectrophoresis, and the zymosan particles were not opsonized with C3. A complement abnormality was not identified in sera from patients with SCD by the methods employed in the present study. Concentrations of C3, factor B, total hemolytic activity, properdin, and C3b inactivator were similar in sera from patients and control subjects. Although concentrations of C3b inactivator protein were below normal in a few patients, there was no evidence that these levels were low enough to alter the functions mediated by this protein.

C3b inactivator acts as an inhibitor of the C3b feedback loop. Continuous activation of the alternative pathway resulting in consumption of factor B and C3 has been demonstrated in a patient with C3b inactivator deficiency and hypercatabolism of C3 (24). Apparently, sufficient quantities of C3b inactivator to inhibit the loop were present in patient sera because concentrations of factor B and C3 were not decreased in sera with low

concentrations of C3b inactivator, nor was there spontaneous cleavage of C3 when these sera were incubated at 37° for 30 min. Initiation of the complement sequence via the alternative pathway by reaction with inulin was equal in patient and control sera when assessed by the activation of factor B, cleavage of C3, and the consumption of hemolytic complement components. Lysis of erythrocytes treated with reduced glutathione was similar in patient and control sera during alternative pathway activation, indicating comparable formation of lytic complexes via this pathway.

Our failure to identify an abnormality of alternative pathway function in SCD was unexpected in view of the report of Johnston and associates (8). The design of the two studies is dissimilar, and in some respects they are not comparable.

1. In the present report, specific complement components and functions were assessed in the sera of patients with sickle cell disease, but the opsonic ability of these sera was not determined. Thus, it is possible, although very unlikely because of the nearly universal finding of an opsonic defect by others (8, 23), that the serum of the patients in the present study possessed normal opsonizing ability.

2. Zymosan was used by Johnston *et al.* (8) to activate the alternative pathway, whereas insulin was selected in the present study for two reasons. The complement system is activated via both the alternative and classic pathways by zymosan (15, 21). It has been reported that both pathways participate in the consumption of terminal complement components by inulin (21), however, this substance is thought to *initiate* the complement sequence only through the alternative pathway (5). This polysaccharide has not been reported to fix C1, and presumably cannot activate the classic pathway by the usual mechanisms. In addition, the results of the reaction of inulin with serum from SCD patients are of interest because this polysaccharide is employed frequently to assess alternative pathway function.

3. Alternative pathway studies by Johnston and associates (8) were performed using serum chelated with EGTA without added magnesium in an attempt to block the classic pathway. When normal serum is rendered 0.01 M with respect to EGTA, the concentration of ionized calcium decreases from 10^{-3} M to approximately 10^{-12} M and the ionized magnesium from 10^{-3} to 10^{-6} M³. Although the magnesium concentration decreases by a factor of 1,000 with chelation, it has been demonstrated that such treatment selectively blocks the classic pathway while preserving certain alternative pathway functions (3). In a pilot study, sera from five of our patients with SCD and three control subjects were rendered 0.01 M with respect to EGTA (Sigma Chemical Co., St. Louis) and then reacted with inulin. Complement activation was inhibited by chelation in both patient and control sera. This inhibition was indicated by failure to activate factor B and to cleave C3. This inhibition did not occur when aliquots of these chelated sera were rendered 4×10^{-4} M with respect to magnesium before reacting with inulin (in some experiments magnesium was added to a concentration of 4×10^{-3} M). The pH did not fall below 7.2 during chelation, insuring that the calcium remained firmly bound and unavailable for classic pathway activity (19). There was no evidence that the magnesium activated the complement system before the addition of inulin, although this would not be of importance in implicating classic pathway activation since magnesium is known to activate only the alternative pathway (11). Fine and associates (3) emphasized that although their experiments provided quantitative information concerning the concentration of magnesium necessary to support alternative pathway functions, the optimal magnesium concentration would have to be determined separately for other experimental situations. Indeed, other investigators have found it necessary to add magnesium to sera chelated with EGTA to insure optimal alternative pathway function (2, 4, 12, 19). Thus, sera were not chelated with EGTA in this study and comparison of our data with that of Johnston and associates is difficult.

4. The methods for identifying C3 cleavage were different.

The decrease in B antigen concentration, as used in the present study, is a sensitive indicator that the C3 molecule has been altered. It is likely that the conformational changes in molecular structure that occur during the conversion of native C3 into its fragments (13) are detected with more precision by this method than by the studies of electrophoretic mobility and opsonization as employed by Johnston and associates (8).

5. Diluted serum was studied in the previous reports (8, 23), whereas whole serum was studied in the present one. Since undiluted plasma and inflammatory exudates provide the media in which these reactions occur within the body, it may be more analogous to the *in vivo* situation to study undiluted serum. The concentration of complement components in most exudates is unknown, and there is no proof that this assumption is valid. Winkelstein and Drachman (23) did observe improved opsonization when the concentration of patient serum was increased. Although opsonization was clearly different in patients as compared with control subjects when diluted serum was employed, data were not reported in either study (8, 22) to indicate whether a defect would be identified using undiluted serum.

An abnormality of the alternative pathway was not detected when the serum from patients with sickle cell disease was reacted with inulin. Thus, this polysaccharide, although commonly employed to assess alternative pathway function, is not satisfactory for studying serum from these patients. In addition, activation of the alternative pathway by cobra venom factor was also comparable with controls when assessed by the lysis of glutathione-treated erythrocytes.

Activation of the alternative pathway with cobra venom factor was recently reported to be defective in serum from sickle cell patients (10). It was suggested that the mechanism was reduced C3PA convertase (factor D) activity. There is no proven explanation for the different results. However, the hemolytic assays employed were different in the two studies. In addition, the cobra venom factor preparations were not purified further after purchase in either study, and it is possible that nonspecific enzymatic activity was present that could act independently of the cobra venom factor. It is to be emphasized that many substances, including pneumococcal polysaccharide, activate the alternative pathway. Several different mechanisms of activation have been described and each activating substance may require individual study to define the abnormal alternative pathway metabolism in SCD.

CONCLUSION

Defective opsonization of *S. pneumoniae* in serum from patients with SCD was previously reported to be caused by abnormal alternative pathway function. In the present study, several quantitative and functional studies of the alternative pathway were assessed in serum from children with this disorder in an attempt to define this abnormality. Concentrations of C3, factor B, CH50, properdin, and C3b inactivator were similar in serum from patients and control subjects. The alternative pathway was activated by inulin to the same extent in patient and control sera, when assessed by the activation of factor B, cleavage of C3, and the consumption of hemolytic complement components. Lysis of erythrocytes treated with reduced glutathione was similar in patient and control sera during activation of the alternative pathway with inulin and cobra venom factor. Thus, differences in the alternative pathway between patients with SCD and normal control subjects were not identified by the methods used in this study.

Note Added in Proof. Two relevant reports appeared after preparation of this manuscript. Grebner and associates (J. Lab. Clin. Med., 89: 153 (1977)) noted that zymosan was poorly opsonized in EGTA serum unless Mg was added. Provisor and associates ((Abstract) Pediat. Res., 10: 380 (1976)) found that *S. pneumoniae* was phagocytized equally well when opsonized

with SCD or control sera that had been chelated with EGTA and supplemented with Mg.

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