Decreased opsonization for *Streptococcus pneumoniae* in sickle cell disease: Studies on selected complement components and immunoglobulins

Opsonic activity for *Streptococcus pneumoniae* in the sera of patients with sickle cell disease was reduced in comparison to the opsonic activity of sera from age-matched normal children. No difference in opsonic activity for *Escherichia coli* was observed in the sera from patients or normals. Total hemolytic complement, conversion of C3 by inulin and cobra venom factor, and levels of C3, factor B, properdin, C3b inactivator, and immunoglobulins G, A, and M were normal in patients' sera. The opsonic abnormality for *S. pneumoniae* was attributed to a deficiency of serum proteins rather than to an inhibitor of opsonic function. The data suggest that decreased opsonization was not associated with a deficiency of those complement components or immunoglobulins measured in this study.

Ann B. Bjornson, Ph.D., Marilyn H. Gaston, M.D., and Constance L. Zellner, B.S., Cincinnati, Ohio

A primary cause of morbidity and mortality in sickle cell disease is infection caused by *Streptococcus pneumoniae*; predisposition to pneumococcal bacteremia and meningitis is particularly common in children with SCD who are under the age of three. Several abnormalities of host defense have been demonstrated in patients with SCD including splenic hypofunction, neutrophil dysfunction, and reduction in serum opsonization. The relationship between these immunologic abnormalities and susceptibility to pneumococcal infection has not been clearly defined.

Deficiency of serum opsonic activity for *S. pneumoniae* in patients with SCD was originally documented by Winkelstein and Drachman. These investigators demonstrated that the sera from patients with SCD had reduced ability to promote phagocytosis of a type 25 test strain of *S. pneumoniae* by normal human peripheral leukocytes, in comparison to the phagocytosis-promoting activity of sera from age-matched normal children. Phagocytic tests using *Salmonella* were also performed with sera from the patients and controls, and no differences in opsonic activity of the sera were demonstrated. Total hemolytic complement was also found to be normal in the patients' sera. Serum agglutinins to the type 25 *S. pneumoniae* were rarely present in normal or patients' sera, suggesting that...
the deficiency of pneumococcal opsonizing activity was unrelated to antibody.

Johnston and associates subsequently tested the ability of sera from patients with SCD and normal controls to promote phagocytosis of a type 2 strain of *S. pneumoniae* by normal leukocytes in the presence of varying amounts of type specific antibody. When the pneumococci were maximally sensitized with antibody, no difference in the extent of phagocytosis was observed in the presence of normal or patients' sera. However, when the amount of antibody fixed to the pneumococci was reduced, the normal sera opsonized efficiently, whereas many of the patients' sera did not. The investigators suggested that a possible cause for decreased opsonization was an abnormality in the alternative complement pathway.

The present report provides evidence that decreased opsonization for a type 10 strain of *S. pneumoniae* in sera of patients with SCD is unassociated with a deficiency of certain components of the alternative or classical pathways of complement activation or of immunoglobulins.

**MATERIALS AND METHODS**

**Patients and normal volunteers.** Fifty-one patients with SCD (41 SS, 7 SC, 1 SD, and 2 S thalassemia) were studied. The mean age of the patients was 7.5 years with a range of nine months to 19 years. The patients were neither infected nor in crisis at the time of blood collection. Twenty-one black children of healthy laboratory personnel or normal siblings of patients with SCD constituted the control group. The mean age of the normal children was eight years with a range of two months to 21 years. With the exception of one child who had sickle trait, all of the other control children had AA hemoglobin. Informed written consent for the study was obtained from the parents of both the patients and the normal volunteers.

**Collection of blood.** Blood specimens were collected from patients and normal donors in glass tubes and were allowed to clot for one hour at room temperature and for one to four hours at 4°C. The tubes were centrifuged at 5,000 × g for 10 minutes, and the sera were removed. All sera were dialyzed for 18 hours against phosphate-buffered saline containing 1.0 × 10⁻³ M MgCl₂ and 1.5 × 10⁻³ M CaCl₂. Dialysis of the sera was performed to standardize the concentration of Mg²⁺ and Ca²⁺ in the sera. The dialysis procedure was not found to reduce complement levels or activities in normal sera.

**Measurement of serum opsonic activity.** A minor modification of the method of Hirsch and Strauss was used for measuring serum opsonic activity. The test strain of *S. pneumoniae* (type 10) was a clinical isolate and was typed by the Quellung reaction. The pneumococci were maintained in intraperitoneally infected mice which, prior to death from infection, were killed by cervical dislocation and were frozen at −70°C. Prior to each experiment, a mouse was thawed, the spleen was plated on blood agar, and the plate was incubated at 37°C in 5% CO₂ overnight. A single colony was then transferred to a tube of brain-heart infusion broth which was incubated at 37°C in CO₂ overnight.

The strain of *Escherichia coli* 075 was maintained in frozen culture in brain-heart infusion broth. Prior to each experiment, a tube was thawed and inoculated into a tube of brain-heart infusion broth and incubated at 37°C overnight. These methods of culture were very efficient since virulence of the organisms was maintained from experiment to experiment, and the methods yielded reproducible numbers of viable bacteria.

To summarize the methodology, 1.0 × 10⁴ organisms and 5.0 × 10⁴ human peripheral polymorphonuclear leukocytes from normal donors were incubated with the test serum in a final volume of 1 ml of Hank balanced salt solution containing 0.1% gelatin (HBG). HBG was substituted for the leukocytes in the controls. Reaction mixture and control tubes were rotated end-over-end for one hour at 37°C. The number of bacteria surviving in the controls and reaction mixtures was determined by plating three tenfold dilutions of the samples in distilled water containing 0.1% gelatin on blood agar, and counting the colonies after overnight incubation. The percent of micro-organisms killed intracellularly was determined by the formula a-b/a × 100, where a was the number of bacteria surviving in the control lacking leukocytes and b was the number of bacteria surviving in the corresponding reaction mixture containing leukocytes.

Ten percent was the concentration of serum selected for the opsonic assays when *S. pneumoniae* was used as the test strain, since this was found to be the minimal concentration of pooled normal human serum which promoted maximal killing of this organism after one hour of incubation at 37°C. The concentration of serum used in the *E. coli* assays was 2%, which was the minimal concentration of serum required for maximal killing, and incubation was carried out at 37°C for three hours. No reduction in bacterial counts was observed in any of the controls containing patient or normal sera and either bacterial strain in the absence of the leukocytes or in the control containing leukocytes and bacteria in the absence of serum. When the total number of viable bacteria surviving in the reaction mixtures after lysis of the leukocytes was compared to the number of viable extra-
cellular bacteria surviving in supernatants of the reaction mixtures, similar counts were obtained. These findings indicated that phagocytosis of the test strains was rapidly followed by intracellular killing and that killing could therefore be used as a method for measuring opsonization.

**Purified proteins and antisera.** Human factor B was purified according to the method of Gotze and Muller-Eberhard. Properdin was isolated by the method of Pensky and associates except that a euglobulin fraction of human serum rather than serum reacted with zymosan was utilized as the starting material. By polyacrylamide gel electrophoresis, the final preparations of properdin and factor B were found to contain only trace amounts of contaminating gamma globulin. Human C3b inactivator and C3 were purchased from Cordis Laboratories, Miami, Fla.

Antibody was raised in goats by multiple subcutaneous injections of the respective partially purified protein in saline containing 0.01M EDTA, pH 7.0, incorporated in Freund complete adjuvant. Antiserum to B antigen of C3 was prepared by absorbing antiserum to C3 with the minimal amount of aged normal human serum which fully removed antibodies to the A and D antigens. The antiserum to factor B was absorbed with heated normal serum (50°C, 30 minute). The antiserum to properdin was absorbed with properdin depleted human serum, and the antiserum to C3b INA was absorbed with normal human serum heated at 56°C for two hours; the procedures removed antibodies to contaminating proteins. Each of the absorbed antisera in agarose yielded single lines against normal human serum in immunoelectrophoresis and in double immunodiffusion and complete identity in double immunodiffusion with reference goat antiserum to each respective protein. The reference antiserum to factor B, C3b INA, properdin, and B antigen of C3 were generously provided by Dr. Clark D. West, Children's Hospital Research Foundation, Cincinnati, Ohio. Rabbit antiserum to human IgG, IgM, and IgA were purchased from Behring Diagnostics, Somerville, N. J.

**Radial immunodiffusion assays.** Single radial immunodiffusion was performed by a minor modification of the method of Mancini and associates. For assaying concentration of B antigen of C3, factor B, and C3b INA, agarose (1%) dissolved in veronal buffer (μ = 0.05, pH = 8.6) containing 0.04M EDTA was used. For assaying concentration of properdin, agarose (0.6%) dissolved in phosphate-buffered saline, pH 7.0, containing 0.01M EDTA was used. For assays of Ig levels, agarose (1%) dissolved in phosphate-buffered saline, pH 7.0, was used. An appropriate dilution of antiserum in agarose was poured into plastic plates which were stored at 4°C for up to two days. For assays of B antigen of C3, factor B and Ig levels, thirty 2 mm diameter wells were made on each plate, and 3 μl of serum were delivered into each well. For assays of C3b INA and properdin concentration, 4 mm diameter wells were made on each plate, and 10 μl of serum were delivered into each well. The unknowns and serial dilutions of the standard serum for the protein being measured were placed in the wells, and diffusion was carried out at room temperature for 18 hours (C3, factor B, C3b INA, and Ig) or at 37°C for 72 hours (properdin). All determinations were performed in duplicate. Normal reference serum for B antigen of C3 was generously provided by Dr. Clark D. West. Normal reference sera for Ig and factor B levels were obtained from Behring Diagnostics (lot 173B), and for properdin and C3b INA levels, the normal reference was a pool of 24 normal human sera.

**Measurement of C3 conversion and hemolytic complement.** Cobra venom factor (Cordis Laboratories) diluted 1:5 in saline or inulin (100 mg/ml) was added to patient or normal serum in the proportion of 10 μl of activating substance to 100 μl of serum, and the mixture was incubated for one hour at 37°C. Concentration of B antigen of C3 was determined by radial immunodiffusion before and after incubation with the activating substance, and the difference in concentration was taken as a measure of C3 conversion. Conversion of C3 in normal or patients’ sera was not found to occur during a one-hour incubation period in the absence of an activating substance.

Hemolytic complement was titrated by the method of Kabat and Mayer. **Analytical methods.** Immunoelectrophoresis was carried out in 1% agarose in veronal buffer (μ = 0.05, pH = 8.6) for 120 minutes at room temperature with a potential gradient of 5 v/cm. Double immunodiffusion was performed in 0.6% agarose in 0.01M phosphate-buffered saline, pH 7.0. Polyacrylamide disc gel electrophoresis was carried out using 6% vertical gels and tris glycine buffer.

**Statistical analysis.** The Wilcoxon rank sum test was used to compare the opsonic data from patients and controls. The Student’s t test was used to analyze the other data.

**RESULTS**

Opsonic activity of SCD sera for S. pneumoniae and E. coli. Opsonic activity for S. pneumoniae in the patients’ sera was significantly reduced (p < 0.01) in comparison to the opsonic activity of sera from the normal children or
Fig. 1. Opsonic activity of sera for *S. pneumoniae* from children with SCD, and from normal children and adults. The horizontal bars represent the mean values for each group of subjects.

Fig. 2. The temporal sequence of opsonic activity for *S. pneumoniae* in sera obtained from 20 SCD patients during the period from April, 1975, to April, 1976. The numbers refer to patient numbers.
Opsonization for *S. pneumoniae* in sickle cell disease

Opsonic activity of sera for *E. coli* from children with SCD, and from normal children and adults. The horizontal bars represent the mean values for each group of subjects.

Adults (Fig 1). Reduction in opsonic activity was not found to be related to the age of the patient and was demonstrated in patients with SC as well as SS disease. Reduction in opsonic activity was also demonstrated in patients with S thalassemia and SD disease, although too few of these patients were studied to draw a conclusion about them.

Serum opsonic activity for the test strain of *S. pneumoniae* was re-evaluated in 20 of the patients with SCD two to six times over a one-year period. If opsonic activity was below 2 SD from the normal mean (< 85%) in a patient's first serum sample, it remained low in his subsequent serum samples, and if opsonic activity was normal initially, it remained generally normal (Fig. 2). The opsonic activity of sera from Patients 11 and 23 increased to some extent with time, however, the rise was only transient. The opsonic activity of these patients' sera has been measured after February, 1976, and found to be below 80%. No difference in opsonic activity for *E. coli* was observed in sera from the patients or from the normal children or adults (Fig. 3).

Opsonic activity for *S. pneumoniae* in sera from ten of the patients was restored to normal by addition of a small amount of pooled normal human serum (2%); this concentration of normal serum was not found to be opsonic alone (Table I). These findings indicated that the opsonic abnormality was caused by a deficiency of critical normal serum proteins required for opsonization rather than to a serum inhibitor of opsonic function.

### Table I. Restoration of serum opsonic activity for *S. pneumoniae* in patients with SCD by pooled normal human serum

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Intracellular killing (%)</th>
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<tbody>
<tr>
<td></td>
<td>SCD serum</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>3</td>
<td>43</td>
</tr>
<tr>
<td>11</td>
<td>57</td>
</tr>
<tr>
<td>12</td>
<td>39</td>
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<tr>
<td>110</td>
<td>22</td>
</tr>
<tr>
<td>119</td>
<td>78</td>
</tr>
<tr>
<td>203</td>
<td>61</td>
</tr>
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| SC = Sickle cell disease; NHS = normal human serum. |

Classical and alternative complement pathway activation and levels of immunoglobulins in the SCD sera. Levels of factor B, C3b INA, and properdin in the patients' sera were found to be equivalent to the levels of these components in the normal sera (Table II). The level of native C3 in the patients' sera was significantly reduced; however, no correlation between reduction in C3 and decreased opsonization for *S. pneumoniae* in individual sera could be demonstrated. Total hemolytic complement was normal, and C3 conversion by inulin and CoVF was
Table II. Levels and activities of the classical and alternative pathways of complement activation in the sera from children with SCD or from normal children*

<table>
<thead>
<tr>
<th>Source of serum</th>
<th>Native C3 (units/ml)</th>
<th>C3 conversion</th>
<th>Insulin (%)</th>
<th>CoVF (%)</th>
<th>Factor B (mg/dl)</th>
<th>Properdin (% of normal)</th>
<th>KAF (% of normal)</th>
<th>CHs0 (units/ml)</th>
</tr>
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<tbody>
<tr>
<td>Children with SCD (51)†</td>
<td>25.33 ± 5.80‡</td>
<td>78.04 ± 19.04‡</td>
<td>75.38 ± 19.13‡</td>
<td>23.17 ± 5.72</td>
<td>111.29 ± 36.54</td>
<td>95.98 ± 35.66</td>
<td>22.10 ± 6.0</td>
<td></td>
</tr>
<tr>
<td>Normal children (18)†</td>
<td>35.68 ± 8.0</td>
<td>65.30 ± 12.16</td>
<td>60.01 ± 16.60</td>
<td>25.11 ± 12.27</td>
<td>91.50 ± 22.18</td>
<td>82.67 ± 17.57</td>
<td>21.72 ± 4.34</td>
<td></td>
</tr>
</tbody>
</table>

*Mean values ± 1 SD are represented.
†Values in parentheses indicate the number of children who were studied.
‡p < 0.01 was considered to be one statistically significant. Significance between values for patients and controls was determined by the Student's t test.

Table III. Levels of immunoglobulins in the sera from children with SCD or from normal children*

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal†</td>
<td>SCD†</td>
<td>Normal†</td>
</tr>
<tr>
<td>0-4</td>
<td>1,131 ± 874 (7)‡</td>
<td>1,246 ± 620 (21)</td>
<td>88 ± 39 (7)</td>
</tr>
<tr>
<td>5-10</td>
<td>1,308 ± 1,051 (6)</td>
<td>1,541 ± 1,005 (16)</td>
<td>129 ± 73 (6)</td>
</tr>
<tr>
<td>11-22</td>
<td>1,520 ± 1,093 (5)</td>
<td>2,099 ± 1,130 (14)</td>
<td>220 ± 175 (5)</td>
</tr>
</tbody>
</table>

*Mean values ± 1 SD are represented.
†Source of serum.
‡Values in parentheses indicate the number of children who were studied.

significantly elevated in the patients' sera. Serum levels of IgG, IgA, and IgM in the patients were equivalent to the Ig levels in the normal children (Table III).

DISCUSSION

In the present study the previously reported observation that patients with SCD have reduced serum opsonic activity for S. pneumoniae was confirmed. In addition, the data show that the opsonic abnormality was found in patients with SC as well as SS disease and persisted during a one-year period, suggesting that it was not a transient phenomenon. Sera with reduced pneumococcal opsonizing activity had normal opsonizing activity for E. coli, a finding which may in part explain the lack of susceptibility of patients with SCD to E. coli infections. The opsonic abnormality for S. pneumoniae was found to be caused by a deficiency of critical normal serum proteins required for opsonization rather than to an inhibitor of opsonic function. Preliminary evidence was also provided to suggest that decreased opsonization was unassociated with a deficiency of the alternative or classical pathways of complement activation.

Our results appear to be at variance with those of Johnston and associates who related decreased opsonization for S. pneumoniae in sera from patients with SCD to an abnormality in C3 conversion via the alternative pathway. In our study, C3 in the patients' sera was found to convert normally by inulin or CoVF which are recognized activating substances of the alternative pathway. Levels of factor B, C3b INA, and properdin were also found to be normal, despite the inability of the patients' sera to support phagocytosis of the pneumococci by normal leukocytes. The level of native C3 was found to be slightly reduced in the patients' sera, although no correlation between reduced opsonization and C3 concentration was demonstrated. The slight reduction in native C3 might reflect partial activation; however, preliminary studies indicated that C3 conversion products were not present in the patients' sera.

The classical pathway, as assessed by measuring total hemolytic complement, was also found to be normal in the patients' sera. This observation is in agreement with other investigators who have provided evidence that total hemolytic complement was normal in sera from SCD patients. Since total hemolytic complement is a relatively gross measurement of C1 to C9 activity, further studies of the immunochemical and functional activities of individual classical components should be performed before any conclusions regarding the integrity of classical pathway are made.
The levels of factor B, properdin, and C3b INA in the sera of our SCD patients were found to be normal. Other alternative pathway components, however, which were not measured such as factor D or initiating factor may still be abnormal. It should also be emphasized that the measurements of alternative pathway components in our study were performed immunochemically, and the possibility exists that functional abnormalities of these components were missed. In this regard, Wilson and associates have recently reported that patients with SCD have decreased functional levels of factor B, and Koethe and associates have reported abnormal CoVF-induced alternative pathway activity in SCD. In our study, the C3 converting activity of the patients' sera by inulin or CoVF was found to be normal, suggesting that factors B and D were functionally active since conversion of C3 by the activating substances is dependent upon the functional integrity of these proteins.

Levels of IgG, IgA, and IgM in the sera of our SCD patients were also found to be normal. These findings confirm the observations of other investigators who have reported normal or elevated serum levels of immunoglobulin in black and white patients with SCD. Despite the normal immunoglobulin levels in SCD sera, a possible cause for decreased opsonization for the type 10 test strain by normal leukocytes. These results rise in heterophile titers in one normal child, one child with sickle trait, and two patients with sickle thalassemia. Antibody response in one of the patients with SCD to intramuscular injection of sheep erythrocytes was normal, suggesting that there was no generalized inability to respond to antigenic stimulation in this disease. Animal studies have confirmed that splenic hypofunction was responsible for the abnormality of antibody formation to intravenous stimulation. The splenectomized rat has been shown to be markedly reduced in patients with SCD, and this abnormality is postulated to be related to their splenic hypofunction. Schwartz and Pearson showed that five children with SCD had impaired response to intravenous injection of sheep erythrocytes in comparison to significant rises in heterophile titers in one normal child, one child with sickle trait, and two patients with sickle thalassemia. Antibody response in one of the patients with SCD to intramuscular injection of sheep erythrocytes was normal, suggesting that there was no generalized inability to respond to antigenic stimulation in this disease. Animal studies have confirmed that splenic hypofunction was responsible for the abnormality of antibody formation to intravenous stimulation. The splenectomized rat has a functionally active since conversion of C3 by the activating substances is dependent upon the functional integrity of these proteins.

Pneumococci are quite common in the upper respiratory tracts of children during the late winter and early spring. If a few pneumococci from the respiratory tract entered the circulation of a child with SCD, he might not be able to synthesize antibody rapidly enough or in sufficient quantity to effectively opsonize his infecting microorganism. Studies are in progress to define the nature of the opsonic abnormality and to determine the association between this humoral abnormality and susceptibility to pneumococcal infection.

Another possible cause of decreased opsonization for the type 10 test strain of S. pneumoniae may be a deficiency of the tetrapeptide tuftsin which is thought to stimulate phagocytosis nonspecifically at the leukocyte membrane. This possibility, however, is considered unlikely, since participation of tuftsin in opsonization of S. pneumoniae has not been previously demonstrated. Another hypothesis is that the factor causing decreased opsonization is an undefined serum protein which is neither a complement component or an immunoglobulin molecule.

Other strains of S. pneumoniae which we have isolated from infected children have been found to be more highly encapsulated than our type 10 test strain. The strains were isolated from blood cultures and were of the following types: type 19 (3), type 6 (2), type 3 (2), type 25 (1), and type 14 (2). None of the strains was killed by leukocytes in the presence of 10% pooled normal human serum, a concentration which promotes 90 to 99.9% killing of our type 10 test strain by normal leukocytes. These results emphasize the importance of future studies to determine if patients with SCD are capable of producing opsonizing activity to their infecting strain of S. pneumoniae during infection.

REFERENCES


