Defective Binding of the Third Component of Complement (C3) to \textit{Streptococcus pneumoniae} in Multiple Myeloma

By Bruce D. Cheson, Harold S. Walker, Margo E. Heath, R. Joseph Gobel, and Jarmila Janatova

Patients with multiple myeloma (MM) are at an increased risk for infections with bacteria that require opsonization with complement. Because \textit{Streptococcus pneumoniae} is the most frequently encountered pathogen in these patients, we investigated the ability of serum from patients with MM to mediate the binding of C3b, the major opsonin of the complement system, to \textit{S. pneumoniae}. \textit{S. pneumoniae} types 3, 14, and 25 were chosen for study, since \textit{S. pneumoniae} type 3 activates primarily the classical complement pathway (CCP), type 25 primarily the alternative complement pathway (ACP), and type 14 both pathways. \textit{S. pneumoniae} were treated with normal serum or serum from 17 patients with MM, and the bound C3b was quantified with fluorescein-conjugated anti-C3 in a spectrofluorometric assay. Despite normal or elevated serum concentrations of C3, total hemolytic complement, and C-reactive protein, patients with MM bound significantly less C3 to types 3 (32.7\% ± 8\% of normal), and in addition, serum from 15/17 patients bound decreased amounts of C3b to types 14 (39.6\% ± 8\%) and 25 (52.2\% ± 8\%). Mixing normal serum with MM serum restored MM C3b binding activity to all three \textit{S. pneumoniae} types, suggesting that the defect was related to a deficiency rather than an inhibitor of C3 activation. Although MM patients are unable to produce specific antibodies against invading bacteria, the addition of anti-\textit{S. pneumoniae} antibodies to MM serum did not enhance C3b binding to any of the \textit{S. pneumoniae} types. However, when \textit{S. pneumoniae} were opsonized in a mixture of MM serum and C3-depleted normal serum, C3b binding was restored to all three \textit{S. pneumoniae} types, demonstrating that MM C3 functions normally in the presence of other normal serum factors. In the present studies, the MM C3b binding defect appeared to correlate with the incidence of \textit{S. pneumoniae} infections. Serum from patients with a history of an \textit{S. pneumoniae} infection bound significantly less C3 (20.5\% ± 4\%) than those study patients without a history of an \textit{S. pneumoniae} infection (55.8\% ± 8\%) (\(p < 0.0025\)). Thus, MM serum has a defect in the activation of C3, and this may contribute to the increased susceptibility of MM patients to \textit{S. pneumoniae} infections.

Bacterial infections occur in up to 80\% of patients with multiple myeloma (MM) and, in half the cases, these are fatal.\textsuperscript{1,2} The infections tend to be refractory to therapy with conventional antibiotics, and neither gamma globulin injections nor antibiotics have been of any benefit when given prophylactically.\textsuperscript{3,4} Because \textit{Streptococcus pneumoniae} (\textit{S. pneumoniae}) is the most frequent pathogen in MM,\textsuperscript{1,3} several clinical trials have explored the use of polyvalent pneumococcal vaccine in preventing infections in MM. However, the results have not been encouraging.\textsuperscript{5,6}

Although there is a marked increase in susceptibility to bacterial infections in MM, the nature of the defect in host defense is not completely understood. Since most infections occur in the presence of a normal number of circulating neutrophils,\textsuperscript{2,3} which appear to function normally,\textsuperscript{7,10} further investigations were directed toward humoral, rather than cellular, abnormalities of opsonization and bacterial killing. Patients with MM produce large amounts of tumor-related monoclonal immunoglobulins and decreased amounts of normal, polyclonal immunoglobulins.\textsuperscript{1,11-14} Therefore, it was assumed that the inability of MM patients to provide specific antibodies against invading bacteria is responsible for their increased susceptibility to bacterial infections.\textsuperscript{1,12}

There is now evidence, however, supporting the hypothesis that the decrease in resistance to bacterial infections in MM is due primarily to a defect in the complement system, namely: (a) complement plays a protective role, especially during the first line of defense against infection;\textsuperscript{15} (b) the most common bacterial pathogens encountered in MM require opsonization with complement prior to interaction with neutrophils;\textsuperscript{16,22} (c) infections with the bacteria encountered in MM patients may lead to increased consumption of complement components, particularly those of the alternative complement pathway;\textsuperscript{16,26} which is independent of specific antibody for its activation;\textsuperscript{27} and (d) MM patients may already have reduced serum concentrations of complement components,\textsuperscript{9,10,28} which may increase mortality related to infection.

In previous studies from our laboratory, we have demonstrated impaired complement-dependent opsonic activity in MM serum.\textsuperscript{30} Additionally, MM immunoglobulins appear to be able to interact with...
normal complement to create a functional opsonizing system.\textsuperscript{31} As \textit{S. pneumoniae} is the most frequent pathogen in patients with MM,\textsuperscript{1,3} and C3b the most potent of the complement-related opsonins,\textsuperscript{32} the present studies were designed to compare the activation and binding of C3b by \textit{S. pneumoniae} after treatment with MM serum, normal serum, and normal serum depleted of C3.

### MATERIALS AND METHODS

#### Patients

Seventeen consecutive patients with MM or macroglobulinemia were studied. Paraprotein types included IgG in 12 patients, IgA and IgM in a single patient each, and light chains only in 3 patients. Sera from 14 of the patients were obtained during a period of active disease, whereas sera from 3 patients were obtained during a period of clinical remission. No serum was obtained within 3 wk of chemotherapy, corticosteroid administration, or radiation therapy, or during a period of active infection. Active infection was defined as documented sepsis, pneumonia, meningitis, or a urinary tract infection requiring hospitalization.

#### Preparation of Serum

Serum was obtained from venous blood from either patients with MM or normal volunteers and stored at –70°C until use. No serum was used if it had been previously thawed.

#### Preparation of Bacteria

\textit{S. pneumoniae} types 14 and 25 were supplied by American Type Culture Collection (Rockville, MD), whereas type 3 was a laboratory isolate at the University of Utah Medical Center. The bacteria were maintained on blood agar (Bruscwick Biological Laboratories, Cockeysville, MD), types 14 and 25 at 37°C and type 3 at 25°C. Persistence of capsule was confirmed by the Quellung test.

Prior to study, the bacteria were transferred to Bacto Brain Heart Infusion broth (DIFCO Laboratories, Detroit, MI) and allowed to grow overnight at 37°C. Bacterial suspensions were then spun at 2,100 g in a Sorval RC-3 centrifuge (Ivan Sorval Inc., Norwalk, CT) for 10 min at 4°C. The pellets were resuspended in 7.5 ml of phosphate-buffered saline (PBS) (pH 7.4) and washed 3 times in 7.5 ml of PBS. Bacteria were then resuspended to a known standard concentration, as determined by optical density (OD) at a wave-length of 640 nm, in a Beckman DU spectrophotometer (Beckman Instruments, Pasadena, CA). The following OD values corresponded to the bacterial concentrations used in these studies:

<table>
<thead>
<tr>
<th>\textit{S. pneumoniae} Type</th>
<th>OD</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.200</td>
<td>$2 \times 10^9$/ml</td>
</tr>
<tr>
<td>14</td>
<td>0.300</td>
<td>$6 \times 10^8$/ml</td>
</tr>
<tr>
<td>25</td>
<td>0.300</td>
<td>$6 \times 10^8$/ml</td>
</tr>
</tbody>
</table>

These values were confirmed both by direct colony counts and by hand counting using a Petroff-Hauser counting chamber (Arthur C. Thomas Co., Philadelphia, PA).

#### Preparation of C3-Depleted Serum by Affinity Chromatography

The immunoadsorbent was prepared by reacting 20 ml of the IgG fraction of goat anti-human C3 (Cappel Laboratories, Cochrannville, PA) with 10 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). Covalent coupling of IgG to the beads was carried out at pH 6.8 in 50 mM sodium phosphate, containing 150 mM NaCl. Any residual active sites on the immunoadsorbent were blocked with 0.2 M glycine at pH 8.2.

Prior to application of serum to the column, the immunoadsorbent was washed thoroughly with buffer of pH 5.0 (0.1 M sodium acetate) alternating with that of pH 8.3 (0.1 M sodium bicarbonate), both containing 250 mM NaCl. These were followed by 3 M NaCl, 50% ethylene glycol (both in the equilibrating buffer), 1 M acetic acid (pH 2.4), and finally with the equilibrating buffer (pH 7.4) alone, containing 50 mM sodium phosphate, 250 mM NaCl, and 5 mM disodium salt of ethylene diamine tetraacetic acid (Na$_2$EDTA) (Sigma Chemical Co., St Louis, MO).

Depleted human sera were prepared from freshly drawn, clotted, and centrifuged blood from normal healthy volunteers. Typically, 10 ml of normal serum was passed through 6 ml of immunoadsorbent at flow rates ≤10 ml/sq cm/hr. The eluate (0.5 ml/tube) was monitored for protein content by measuring the absorbance at 280 nm ($A_{280}$) on 1:100 diluted aliquots. Those protein fractions from the plate that did not exhibit C3 hemolytic activity were pooled to a total volume of 8 ml. Excess salts and Na$_2$EDTA were removed from 4.45 ml aliquots of postimmunoadsorbent serum by centrifugation\textsuperscript{33} through Sephadex G-25 (Pharmacia Fine Chemicals), pre-equilibrated with PBS. Immediately prior to snap-freezing, the depleted serum was supplemented with 1.5 mM Mg$^{2+}$ and 2.5 mM Ca$^{2+}$ (final concentrations).

Following application of the serum to the column, the flow rate was increased threefold and washing with the equilibrating buffer was continued until $A_{280}$ values of undiluted eluate were below 0.03. The regeneration was carried out in the direction opposite to that of sample application. Elution with the equilibrating buffer, containing $3.25$ M NaCl, removed practically all of the nonspecifically bound albumin, whereas the C3 antigen remained attached to the immunoadsorbent. Efficient dissociation of the C3 antigen was achieved by using 1 M acetic acid (pH 2.4).

#### Characterization of C3-Depleted Serum

Total antigenic levels of C3 in both the normal serum used as starting material and the depleted serum were quantified by rate nephelometry. While 1:36 dilutions of intact normal serum were used, undiluted samples of depleted sera were tested to allow for the detection of small amounts of C3. The level of reduction in the serum concentration of other serum proteins (e.g., C4) served as a measure of the degree of dilution of total proteins in the depleted serum. On the average, immunoadsorbent treatment of normal human serum, followed by centrifugation through Sephadex G-25, resulted in a 15% reduction of total protein concentration. This dilution was confirmed refractometrically and by $A_{280}$ determinations on 1:100 diluted aliquots. When the depleted serum was analyzed by quantitative agarose electrophoresis at pH 8.6, the ratio of all the protein fractions appeared to be the same as in the starting serum, except for the $\beta$-globulin fraction, which was reduced due to depletion of C3.

The depleted serum had less than 1% of the starting concentration of C3 as determined by rate nephelometry and hemolytic titrations.\textsuperscript{34} Hemolytic activity of this serum was fully restored after the addition of 0.8–1.2 mg/dl of highly purified and thoroughly characterized human C3.\textsuperscript{34,35} Immunoadsorbent-specific (i.e., C3) and nonspecific (i.e., albumin) protein removal from the depleted serum was monitored by SDS-polyacrylamide gel electrophoresis.\textsuperscript{36} No conversion of C3 into C3a or C3b chain was detected, and no other proteins were removed, except small amounts of albumin. Analysis of C1 by immunodiffusion\textsuperscript{37} demonstrated that C1q, C1r, and C1s remained undissociated, suggesting that the complement system was not
activated during preparation of the depleted serum. In addition, there was no change in the electrophoretic mobility of C2, C4, C5, or factor B, as determined by immunoelectrophoresis of the depleted sera. Factors H (gH-globulin) and I (C3b inactivator) were present in normal amounts as determined by radial immunodiffusion.

Opsonization of Bacteria

Reaction mixtures for measuring bacterial opsonization contained 0.5 ml of bacterial suspension and either 1:4 dilutions of intact normal serum, C3-depleted normal serum, heated normal serum, or MM serum, or 1:8 dilutions of various combinations of two of these serum samples. The final volume of the suspension was adjusted with PBS to 2.0 ml. The samples were then vortexed for 10 sec and incubated at 37°C for 30 min in a Dubnoff Metabolic Shaker (Lab-line Instruments, Melrose, IL). Following the incubation, the samples were spun at 2,100 g for 10 min at 4°C. The pellet of bacteria in 0.5 ml of PBS was washed 3 times by repeated addition of 1.5 ml of PBS and, each time, 1.5 ml of supernatant was withdrawn.

Determination of the C3 Bound to S. pneumoniae

The amount of C3 bound to the bacteria was quantified using a modification of the spectrophotofluorometric assay of Gillis and Thompson. One-half milliliter of a 1:200 dilution of fluorescein-conjugated goat anti-human C3 (Cappel Laboratories) was added to each of the pellets of treated bacteria so that the final volume of the reaction mixture was 1.0 ml. The resulting suspensions were vortexed and incubated in the dark, at room temperature, for 15 min. They were then washed 3 times with 1.5 ml of PBS and resuspended in 2.0 ml of the same buffer. Next, the suspensions were placed into quartz cuvettes and analyzed in an Aminco Bowman spectrofluorimeter (American Instrument Co., Silver Spring, MD), using an excitation wavelength of 489 nm and an emission wavelength of 525 nm. Values from normal serum samples, after correcting for background intensity, were taken as being equivalent to 100% C3b binding activity. Serum from single donors was used for these studies. The C3b binding to bacteria treated with normal sera was 47.8% ± 10.8% (mean ± SD) for type 3, 75.2% ± 6.9% for type 14, and 62.1% ± 12.8% for type 25, as compared with fluorescein-conjugated anti-C3 diluted to 1:2,000 with PBS. These values were obtained from multiple replicate determinations performed on serum from 10 normal volunteers.

Serum Concentrations of Immunologic Factors

Serum concentrations of IgG, IgA, IgM, C3, C4, factor B, and C-reactive protein were quantified by rate nephelometry (Beckman Industries, Pasadena, CA). Total hemolytic activity of complement (CH$_{100}$) was assayed by radial immunodiffusion (Meloy Laboratories, Springfield, VA).

RESULTS

Serum Immunological Determinations

Serum concentrations of C3, C4, CH$_{100}$, factor B, and C-reactive protein for the 17 MM patients are listed in Table 1. All patients studied had normal or elevated serum concentrations of C3 and C-reactive protein, as well as CH$_{100}$ activity. Serum from one patient was deficient in factor B, and 3 sera were deficient in C4. Sera from controls had normal concentrations of all of these parameters.

Activation of the Different Complement Pathways by Various S. Pneumoniae Serotypes

The selection of S. pneumoniae types 3, 25, and 14 for the present study was based on data suggesting that type 3 activates the classical complement pathway (CCP), type 25 the alternative complement pathway (ACP), and that type 14 can activate both the CCP and the ACP. As the CCP can be selectively inhibited by chelating serum with ethylene glycol tetraacetic acid (EGTA) (Sigma Chemical Co.) in the presence of excess Mg$^{2+}$, we could estimate the contributions of the two complement pathways to C3 binding. Figure 1 demonstrates that, using MgEGTA-chelated normal serum in our spectrophotofluorometric assay, we obtained results that compared favorably with those previously reported using other assays. Inhibition of CCP in normal serum reduced C3 binding to S. pneumoniae type 3 to 8.7% ± 5% (mean ± SEM) of untreated serum, suggesting that type 3 activates complement primarily by the CCP. In contrast, inhibition of the CCP had no effect on C3 binding to type 25 (106.9% ± 15%), demonstrating that C3 can bind to type 25 as a result of activation of the ACP. When S. pneumoniae type 14 was treated with MgEGTA-chelated normal serum, C3 binding was 41.4% ± 3% of untreated serum, suggesting that it can activate both the CCP and ACP. Thus, we were able to compare the ability of S. pneumoniae to bind C3 from normal serum with that from MM serum via either complement pathway.

Table 1. Immunologic Determinations Performed on Serum From the 17 MM Patients

<table>
<thead>
<tr>
<th>Immunologic Parameter*</th>
<th>Mean (Range)</th>
<th>Normal</th>
<th>Increased</th>
<th>Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3 (80–150 mg/dl)</td>
<td>144.8 (94–196)</td>
<td>9</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>C4 (15–45 mg/dl)</td>
<td>34.1 (6–61)</td>
<td>8</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>CH$_{100}$ (40–80 U)</td>
<td>71.8 (48–98)</td>
<td>13</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Factor B (17–45 mg/dl)</td>
<td>34.7 (16–60)</td>
<td>12</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>C-reactive protein†</td>
<td>2.2 (0.2–4.7)</td>
<td>4</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate the normal range.
† Only 16 sera were available for determination of C-reactive protein.
During a period of clinical remission.

Indicate results from patients studied during a period of active MM mean of the samples from the 17 MM normal serum or 3.

S. compared (Figure 2). When serum was normal. suggesting that there was no reduction in C3b binding to type 25 (106.9% ± 7% the CCP reduces binding of C3b to type 14 to 41.4% ± 3% of normal. suggesting that it can activate both the CCP and the ACP.

Comparison of C3 Binding to S. pneumoniae From Normal Serum and MM Serum

S. pneumoniae types 3, 14, and 25 were treated with 1:4 dilutions of either intact normal serum or MM serum, and the relative amounts of C3 bound to the bacteria were compared (Figure 2). When S. pneumoniae type 3 was incubated with any of the 17 MM sera, considerably less C3 was bound to the bacteria than when normal serum was used, with a mean binding activity of 32.7% ± 6% of normal. Decreased amounts of C3 were also bound to S. pneumoniae types 14 and 25 from 15 of the 17 MM sera tested, the values being 39.6 ± 8% and 52.2 ± 8%, respectively, of normal C3 binding. The two sera that resulted in normal amounts of C3 bound to types 14 and 25 were from the same two patients who were in clinical remission. When the three patients in remission were excluded from the analysis, the C3 binding from MM sera to types 14 and 25 were 28.5% ± 6% and 43.4% ± 6% of values obtained with normal serum, respectively. The marked decrease in the level of C3 binding to all three S. pneumoniae types suggests that either there may be abnormalities present in both the CCP and the ACP in MM serum or there is a single defect involving a factors(s) influencing both pathways.

Investigations of the Mechanisms for Decreased C3 Binding From MM Serum

The above observations suggested several possible explanations for the decrease in C3 binding by MM serum-treated bacteria. First, there may be a deficiency of factors required for activation of C3 to its opsonically active form, C3b. Second, once activated, MM C3b may not be capable of binding to S. pneumoniae. The third possibility is that there is an inhibitor of C3 activation or binding in MM serum. To distinguish among these possibilities we performed a series of mixing experiments.

In the first experiments, we compared the amount of C3b bound to the bacteria following their incubation with either a 1:8 dilution of MM serum alone or mixtures of 1:8 dilutions of MM serum with various dilutions of intact normal serum (Fig. 3). As the amount of normal serum increased, C3b binding to all three S. pneumoniae types approached the level of a 1:4 dilution of normal serum (100% activity). In addition, using 1:8 dilutions of MM serum resulted in

**Fig. 1.** A comparison of activation of complement and binding of C3b by S. pneumoniae types 3, 14, and 25. Bacteria were treated with 1:4 dilutions of either intact normal serum or normal serum that had been chelated with 10 mM MgEGTA to inhibit CCP activity. Such chelation of normal serum reduces C3b binding to S. pneumoniae type 3 to 8.7% ± 5% (mean ± SEM) of normal, suggesting that type 3 primarily activates the CCP. In contrast, there was no reduction in C3b binding to type 25 (106.9% ± 15%), demonstrating that it primarily activates the ACP. Inactivation of the CCP reduces binding of C3b to type 14 to 41.4% ± 3% of normal. suggesting that it can activate both the CCP and the ACP.

**Fig. 2.** Determination of C3b binding to S. pneumoniae types 3, 14, and 25 following treatment with 1:4 dilutions of either normal serum or MM serum. The hatched lines represent the mean of the samples from the 17 MM patients. The solid dots indicate results from patients studied during a period of active disease, and the open dots indicate results from patients studied during a period of clinical remission.

**Fig. 3.** The effect of adding increasing dilutions of normal serum (NS) to 1:8 dilutions of serum from 4 myeloma (MM) patients on C3b binding to S. pneumoniae types 3 (open dots), 14 (solid dots), and 25 (solid squares). There is a progressive increase in C3b binding to all three S. pneumoniae types as increasing amounts of NS are added.
less C3b binding to all three *S. pneumoniae* types (Table 2) as compared with the results obtained with 1:4 dilutions. These findings suggested that the C3b binding defect is not related to the presence of inhibitors in MM serum but, instead, that it is more likely related to a deficiency of a factor(s) necessary for the activation or function of MM C3.

As patients with MM are unable to produce specific antibodies in response to bacterial antigens, we next evaluated the possibility that the C3 binding defect represented a decrease in the activation of complement in MM serum due to a deficiency of anti-*S. pneumoniae* antibody. To test this hypothesis, we added a 1:8 dilution of heat-treated human serum containing anti-*S. pneumoniae* antibodies to 1:8 dilutions of serum from each of 14 MM patients and assayed the effect of these specific antibodies on C3 binding to *S. pneumoniae*. The source of anti-*S. pneumoniae* antibodies was serum from three normal volunteers who had received polyvalent pneumococcal vaccine (Pneumovax, Merck, Sharp, and Dohme, West Point, PA), which contains the capsular antigens of the three bacterial serotypes used in the present studies. These sera contained 577.7–819.5 ng antibody nitrogen (Ab N)/ml of specific anti-type 3 antibody, and 1,500–1,509 ng Ab N/ml of anti-type 25, as well as 79.7–139.6 ng Ab N/ml of anti-14.* By heating these sera at 56°C for 30 min, their complement was inactivated, as demonstrated by a complete loss of their C3 binding to the three *S. pneumoniae* types studied. As shown in Table 2, the addition of this source of anti-*S. pneumoniae* antibodies to MM serum did not significantly enhance C3 binding to any of the three *S. pneumoniae* types.

These observations suggested that the defect was either in the activation of C3 in MM serum or that the defect was in MM C3 itself. To distinguish between these possibilities, we next examined the binding of MM C3 to *S. pneumoniae* by MM serum alone or in the presence of C3-depleted normal serum. The C3 binding activity of the depleted sera was 3.6%, 2.6%, and 0% of normal for *S. pneumoniae* types 3, 14, and 25, respectively (at least 2 replicate determinations for each type). However, when 1.9 mg/ml (0.95 mg/ml native, hemolytically active C3) of highly purified and characterized C3 was added to the C3-depleted sera (final concentration 1.0 mg/ml of C3), C3 binding activity was restored to 104.6%, 117.8%, and 128.7% of normal for types 3, 14, and 25, respectively. Identical results were obtained when the studies were repeated in the presence of a threefold increase in the concentrations of Mg$^{2+}$ and Ca$^{2+}$ in the reaction mixture.

Table 2 presents the results of adding the C3-depleted sera to 8 MM sera. When 1:8 dilutions each of MM and C3-depleted serum were mixed and used to opsonize *S. pneumoniae*, the level of C3 binding to all three bacterial types was increased to the level observed with a 1:4 dilution of normal serum. This demonstrated that MM C3 can be activated and is capable of binding to *S. pneumoniae* in the presence of other serum factors. The function of MM C3 was also evaluated by means of hemolytic titrations. The hemolytic activity of serum from three MM patients was titrated against that of normal serum that had been depleted of C3 by the procedures described previously. In duplicate experiments performed on serum from each of these patients, the behavior of MM C3 was identical to that of normal C3.

**Clinical Correlations of the MM C3 Binding Defect**

We next examined the data to determine if any correlation existed between the MM C3 binding defect and clinical status. As shown in Fig. 2, serum from patients in remission could be activated so that normal amounts of C3 were bound to *S. pneumoniae* types 14 (91.3% ± 18%) and 25 (93.7% ± 19%), although they appeared to retain their defect for type 3 (31.6% ± 18%).

We next examined the data for a correlation between the level of C3 binding and the occurrence of clinical infections in these patients (Fig. 4). The patient population was divided into those who had not experienced an infection (7 patients), those who had a

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*The antibody levels were determined by radioimmunoassay through the kindness of Dr. Gerald Schiffman, Downstate Medical Center, Brooklyn, NY.*

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**Table 2. Effect of the Addition of 1:8 Dilutions of Intact Normal Serum (NS), Anti-Streptococcus pneumoniae Antibodies (Anti-SP), or C3-Depleted Normal Serum (C3DS) to 1:8 Dilutions of MM Serum on C3b Binding to *S. pneumoniae***

<table>
<thead>
<tr>
<th>SP Types</th>
<th>Percent Normal C3b Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MM (n = 17)</td>
</tr>
<tr>
<td>3</td>
<td>29.2% ± 4%</td>
</tr>
<tr>
<td>14</td>
<td>34.7% ± 8%</td>
</tr>
<tr>
<td>25</td>
<td>28.3% ± 6%</td>
</tr>
</tbody>
</table>

*The numbers represent the mean ± SEM.*
history of a bacterial infection, other than with *S. pneumoniae* (6 patients: *S. aureus*—2, *S. aureus* + *E. coli*—1, *H. influenzae*—2, *E. coli*—1), and those with a history of a *S. pneumoniae* infection (4 patients). Significantly less C3 was bound by *S. pneumoniae* when treated with serum from patients with a history of *S. pneumoniae* infections (20.5% ± 4%) than patients with a history of non-*S. pneumoniae* bacterial infections (40.9% ± 6%) (*p* < 0.01), patients without any history of a bacterial infection (55% ± 8%) (*p* < 0.0025), or the entire population of patients without a history of an *S. pneumoniae* infection (48.5% ± 5%) (*p* < 0.0025). Of the 10 patients with a history of a bacterial infection, only one who had *H. influenzae* pneumonia was neutropenic at the time of the infection.

**DISCUSSION**

In the present studies we have demonstrated that, in MM serum, the ability to mediate the binding of C3b to *S. pneumoniae* is markedly decreased. Although it had been previously assumed that a deficiency of specific antibody production was primarily responsible for the increased susceptibility to infections of these patients, the most frequent pathogens encountered in MM patients include *S. pneumoniae, S. aureus, H. influenzae,* and *E. coli* bacteria, which require opsonization with complement to facilitate interaction with granulocytes and activation of bactericidal mechanisms. Even those serotypes of *S. pneumoniae* that require specific antibody may also be dependent on complement for optimal opsonic activity.

These observations suggested that the lack of a functional complement system might be central to the increased susceptibility of myeloma patients to bacterial infections. Indeed, many of the in vitro bactericidal defects thus far described in myeloma appear to be mainly complement-mediated, with immunoglobulins playing only a secondary role. Spitzer et al. observed decreased adherence of polymorphonuclear leukocytes to nylon fiber columns in 16/26 myeloma patients studied, and this defect was more pronounced in the 14 patients with reduced serum concentrations of C4. A similar abnormality was reported by MacGregor et al. who demonstrated that the defect was related to the presence of a heat-labile factor in MM plasma that inhibited granulocyte adherence. In the latter article, however, there was no correlation between reduced serum C4 concentrations and defective granulocyte adherence. Since complement appears to be a major contributor to the opsonic activity required for killing the most common pathogens in myeloma patients, we performed studies to evaluate opsonic activity in MM serum. The results of these previous studies indicated a deficiency of complement-dependent opsonic activity in MM serum that was most pronounced in patients with a decreased serum concentration of C3, yet a decrease in C4 did not exert the same effect. These data have been confirmed by other investigators whose results also suggested depressed activity of the ACP in MM serum.

To further establish the importance of a complement defect in MM, we performed the present studies in which we compared the amount of C3b bound to *S. pneumoniae,* which were treated with either normal serum or MM serum. We observed that bacteria treated with MM serum bound significantly less C3b when compared with bacteria that had been treated with normal serum. The C3b binding defect appeared to be independent of a lack of anti-*S. pneumoniae* antibodies in MM serum, as there was no effect on C3b binding when heat-inactivated immune serum was added to MM serum. Previous studies from our laboratory using a neutrophil chemiluminescence assay have demonstrated that MM paraproteins can act as normal, nonspecific opsonins when in the presence of an intact complement system. In addition, the present experiments demonstrate that C3 functions as normal in the serum of patients with MM, as the hemolytic function of MM C3 was normal and MM C3 could be activated to bind to *S. pneumoniae* when in the presence of other normal serum factors. Therefore, the decrease in C3 binding to *S. pneumoniae* treated with MM serum reflects an abnormality in the activation of C3 in MM serum. Because the defect was apparent with three serotypes of *S. pneumoniae,* each of which...
activated complement by a different pathway or combination of pathways, the defect appears to influence both the CCP and the ACP.

We next performed experiments designed to characterize the defect in MM serum leading to the decrease in C3b binding to S. pneumoniae. As the addition of normal serum to MM serum enhanced C3b binding to all three S. pneumoniae types, our data suggest that the defect is a deficiency of C3 activation and is not related to the presence of inhibitors in MM serum. These results are in agreement with our previous studies. Although data from MacGregor et al. suggested that decreased granulocyte adherence was related to the presence of inhibitory factors in MM plasma, those factors were not apparent in MM serum. In contrast to the present studies, MacGregor et al. were not able to correct the MM defect by adding normal serum. However, whereas in the present studies we mixed equal volumes of MM and normal human serum, those authors added 20% guinea pig serum to MM serum, which may explain some of the differences between these studies. Those factors that are responsive for the MM C3b binding defect would have to be heat-labile, as they are present in untreated normal serum but not in heated normal serum. In addition, they must be able to exert an influence on the activation of both the CCP and ACP. Because serum concentrations of C3, C4, factor B, and C-reactive protein were normal or elevated in almost all patients studied, a quantitative deficiency of one of these factors could not explain the defect observed. Guckian et al. have recently described a heat-labile factor that apparently is not complement, yet which may be required for killing S. pneumoniae type 25. The relationship between this activity and the MM defect remains to be determined. Of interest was the observation that, although the MM sera tested had a profound opsonic defect, they had normal hemolytic activity. This suggests either that fewer molecules of C3 must be activated for hemolysis or that activation of C3 in MM serum leads to the generation of a molecule that cannot bind to bacteria, but can activate the terminal components of the complement system.

Also of interest in the present studies was that serum from 12 of the 16 patients evaluated contained elevated serum concentrations of C-reactive protein. We assayed for this acute phase reactant based on reports by Mold and colleagues and Kaplan and Volanakis that suggested that C-reactive protein may enhance opsonization of S. pneumoniae. More recent data from Mold et al. suggest that, although C-reactive protein bound to S. pneumoniae may facilitate activation of the CCP, the ACP may be inhibited. In the present series, we have been unable to determine a correlation between serum levels of C-reactive protein and either remission status, C3 binding capacity, history of an infection with S. pneumoniae, or paraprotein concentration. Other proteins that may influence the activation of the complement system, such as β2 microglobulin, are often present in increased concentrations in MM serum. The relationship between these proteins and the MM C3 binding defect remains to be explored.

The present data demonstrated that the C3 binding defect in MM serum may have clinical relevance. First, when patients were separated by infection history, we observed a correlation between the severity of the C3b binding defect and the incidence of S. pneumoniae infections. Such a correlation was not noted in previous studies, although Penny and Galton and MacGregor et al. reported that the in vitro defect that they were investigating appeared to correlate with clinical status. Secondly, the MM C3b binding defect was not affected by the addition of anti-S. pneumoniae antibodies. Ingram reported a similar finding in which the addition of immune serum to MM plasma did not improve a defect in phagocytosis. Although immunoglobulins may facilitate efficient killing of bacteria by neutrophils, the present studies suggest that, in the absence of a functional complement system, the potential use of gamma globulin injections or pneumococcal vaccines may be of limited value in MM patients. Not only is it unlikely that they will respond to vaccines with a protective antibody titer, but an influence on complement activation would not be predicted from the present data. In fact, two patients in the present study developed repeated S. pneumoniae infections despite the administration of pneumococcal vaccine. Instead, the administration of an exogenous source of factors necessary for complement activation, such as plasma, might provide a useful adjunct to conventional antimicrobial therapy in infected MM patients.

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BD Cheson, HS Walker, ME Heath, RJ Gobel and J Janatova