Immunologic Abnormalities in Myelofibrosis With Activation of the Complement System

By Bruce R. Gordon, Morton Coleman, Parirokhe Kohen, and Noorbibi K. Day

Eighteen patients with agnogenic myeloid metaplasia with myelofibrosis were studied for clinical and laboratory evidence of immunologic dysfunction. Clinical findings included the presence of arthritis, vasculitis, and erythema nodosum. Laboratory abnormalities included the presence of circulating immune complexes, antinuclear antibodies, positive direct Coombs tests, elevated latex fixations, and a circulating lupus type anticoagulant. Total hemolytic complement was markedly depressed in four patients. Analysis of complement (C) components C1–C9 and factor B demonstrated significant reduction of only C3 and factor B. By crossed-immunoelectrophoresis, both C3 and factor B, but not C4, were cleaved, indicating that C activation was occurring predominantly via the alternative pathway. The control proteins βH and C3b inactivator were decreased in three of four patients with hypocomplementemia. These three patients subsequently developed systemic infections that contributed to their cause of death. These data suggest that immunologic mechanisms associated with activation of the complement system play an important role in the disease process of some patients with agnogenic myeloid metaplasia with myelofibrosis.

Patients were studied when they were considered to be free of infection.

Serum Samples

Blood was allowed to clot at room temperature for 1 hr. The serum was removed after centrifugation at 4°C, aliquoted, and stored at −70°C until used. Each aliquot was used only once. Samples subjected to immunoelectrophoresis were stored at 0.02 M EDTA.

Reagents for Complement Assays

Gelatin veronal buffers with and without Ca** and Mg** and glucose gelatin veronal buffer with Ca** and Mg** were used as previously described.1 The disodium salt of ethylene diamine tetra-acetic acid (EDTA) was titrated to pH 7.4 at a stock concentration of 0.15 M. Sheep erythrocyte intermediates coated with rabbit hemolysin and complement (C) were prepared according to the methods of Borsos and Rapp.8

Assays of Total Hemolytic Complement (TCH50), Component Assays C1–C9, Factor B, βH, and C3b Inactivator

TCH50 was titrated as described previously.9 Functionally pure C components for the assay of C1–C9 were obtained from Cordis Laboratories (Miami, Fla.). The assays were carried out according to the method described by Nelson et al.10 Factor B was determined by the method of Götzé and Müller-Eberhard11 using glutathionetreated human type O Rh-negative erythrocytes and serum incubated with isolated cobra venom factor.12 Immunochemical assay of C3 and C1q were carried out by the Mancini technique13 using monospecific antisera to isolated C3 and C1q raised in rabbits.14,15 βH and C3b inactivator were kindly measured by Dr. Hans Müller-Eberhard (Scripps Clinic, La Jolla, Calif.).

Assay for C3 Nephritic Factor

A modified method of Border et al. was used.14 Equal volumes of test serum and pooled normal human serum were incubated with 0.025 M EGTA or 0.02 M EDTA (37°C, 30 min). The reaction is stopped with 1 ml of ice-cold veronal buffer in 0.01 M EDTA. Serial dilutions of the reaction mixture were titered for C3 hemolytic activity as described previously. The percent consumption of C3 was calculated from the total C3 available determined from the reaction mixture containing EDTA. Normal serum incubated with MgEGTA or EDTA was used as a control.

MYELOFIBROSIS with myeloid metaplasia is a disease of unknown etiology characterized by splenomegaly, a leukoerythroblastic peripheral blood picture, and fibrosis on bone marrow biopsy. Bacterial infections are a frequent cause of death in myelofibrosis occurring in up to 41% of patients in one large series.1 While the asplenic state is often a contributing factor, adequate explanation for the increased susceptibility to infection is not always evident, suggesting additional immunologic impairment. Several reports have described the presence of positive antinuclear antibodies,3,4 elevated latex fixations,3,4 positive direct Coombs tests,5 and a lupus-type circulating anticoagulant. The relationship of these findings to the underlying disease process is unclear.

Our observations in several patients with myelofibrosis of complement activation via the alternative pathway prompted further analysis of 18 patients with myelofibrosis for immunologic abnormalities.

MATERIALS AND METHODS

Patient Population

In all 18 patients studied, the diagnosis of myelofibrosis with agnogenic myeloid metaplasia was based on the presence of splenomegaly, a leukoerythroblastic peripheral blood picture, and fibrosis on bone marrow biopsy. The sex distribution in this series was 10 males and 8 females. The average age was 62 yr (range 46–72 yr). The average patient age at time of initial diagnosis was 59 yr.

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Supported in part by Grant HL-07029 from the National Institute of Heart and Lung; NIH Grants CA-08748, CA-26287-Ol, and AI-11843; and American Cancer Society Grant AM-185.
Submitted January 21, 1981; accepted July 6, 1981.
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904 Blood, Vol. 58, No. 5 (November), 1981
Crossed-Immunoelectrophoresis for Determining Cleavage of C3, C4, and Factor B

Two-dimensional (crossed) immunoelectrophoresis according to the method of Laurell\(^{17}\) was used to determine whether activation of C3, C4, or factor B had occurred. C3 and factor B were isolated\(^{15}\) and used for the production of antisera in rabbits. Antibody to C4 containing circulating immune complexes (IgA CIC) were determined by a Raji cell assay as modified by Hall et al.\(^{176}\) and S. S. Witkin (manuscript in preparation). Levels of IgA CIC are considered elevated only when there are two standard deviations above the mean of 20 control serum samples.

Isolation of CIC Using Sucrose Density Gradient Ultracentrifugation

Two-hundred microliters of sera from six patients (3, 4, 5, 6, 7, and 9) were subjected to sucrose density gradient ultracentrifugation (10%-40% w/w) at 100,000 g for 15 hr (Beckman 65, Montgomery, N.J.). Radiolabeled \(^{125}\)I-IgG (7s), Clq (11s), and IgM (19s) were used as markers. Twenty fractions, each containing 15 drops, were collected. Every third fraction was tested for the presence of IgG by the Ouchterlony double immunodiffusion method (10%-40% w/w). Each sample was run with a control consisting of normal pooled serum as well as normal pooled serum that had been incubated with cobra venom factor (37\(^{\circ}\)C, 45 mm).

Results for Circulating Immune Complexes (CIC)

Samples were assayed for the presence of CIC using the Raji cell radioimmunoassay\(^{18}\) and by a solid phase Clq assay.\(^{19}\) Results for both assays were expressed as \(\mu g/ml\) equivalents of aggregated gammaglobulin (\(\mu g/ml\)). The Raji cell assay and solid phase Clq assay have sensitivities of 16 \(\mu g/ml\) and 25 \(\mu g/ml\), respectively. IgA containing circulating immune complexes (IgA CIC) were determined by a Raji cell assay as modified by Hall et al.\(^{20}\) and S. S. Witkin (manuscript in preparation). Levels of IgA CIC are considered elevated only when there are two standard deviations above the mean of 20 control serum samples.

Levels of CIC

Eleven of 18 patients studied had laboratory and/or clinical evidence for immunologic dysfunction (patients 1, 2, 3, 5, 6, 7, 8, 9, 11, 14, and 17) (Table 1). Clinical findings included arthritis in patients 1 and 3, skin rash with a medium vessel vasculitis in patient 8, fever of unknown origin in patient 11, and biopsy-proven erythema nodosum in patient 9. Five of 18 patients had a positive direct Coombs test without evidence for active hemolysis. Patient 5 had a lupus type circulating anticoagulant.\(^{21}\) Antinuclear antibodies were present in three patients (3, 7, and 11). A positive latex fixation was present in two patients (2 and 11).
Clq assay: <25 μg/mI. Normal (pg/mI). Patients with elevated levels of Clq activity disease, as manifest by either increasing C1q count was greater than 1,500/μl in each patient. Gram-negative sepsis in two patients and a disseminated fungal infection in one patient were directly related to the cause of death. Only one of these patients had been splenectomized. The total white cell count was greater than 100,000/μl, bone pain, or fever of unknown origin, was present in 7 of 9 patients with elevated CIC levels as compared to the remaining 14 patients. Factor D, Clq, and C3-C9 were normal in sera of all 18 patients studied. Normal values ± 1 standard deviation; for C2, C3, and factor B are expressed as a percentage of pooled normal sera. Normal values ± standard deviation: TCH50, 532; C4, 246,000 ± 144,000; C2, 1,350 ± 500; C3, 2,600 ± 532; C4, 246,000 ± 101,500; factor B, 80 ± 16.

**Isolation of CIC Using Sucrose Density Gradient Ultracentrifugation**

Sera from six patients with myelofibrosis were fractionated (patients 3, 4, 5, 6, 7, and 9) using sucrose density gradient ultracentrifugation at pH 7.5. Patients 3, 5, and 7 had elevated CIC levels by the Raji cell radioimmunoassay and by the solid phase C1q test. CIC in these patients were found to sediment at approximately 19S. IgG was present in all the fractions containing immune complexes as well as the 7S region. Patient 6 had an elevated CIC level only by the Raji cell radioimmunoassay. In this patient, CIC were found to sediment at 11S. In contrast, no heavy sedimenting complexes containing IgG were found in normal sera or sera from patients 4 and 9.

**Complement Assays**

Total hemolytic complement levels for all 18 patients studied are shown in Table 1. TCH50 was one standard deviation or lower in patients 2, 3, 5, and 7. Each of these patients had increased levels of CIC, although the absolute CIC level did not correlate with the degree of hypocomplementemia. Further studies revealed that these patients were hypocomplementemic predominantly due to activation of the alternative pathway (Table 3). C3 by hemolytic assay revealed marked depression in patients 2, 3, 5, and 7 with concomitant lowering of factor B. C3 was found to be cleaved in these four patients while factor B was cleaved in 3 of 4 patients. These findings were observed in both serum and EDTA plasma (see Figs. 1 and 2). Although low normal levels of C1, C2, or C4 were observed in sera of patients 2, 5, and 7, the extent of C3 and factor B depletion were out of proportion to the changes seen in the early classical pathway components. Further, no patient had cleavage of C4. Individual complement components were normal in the sera of the remaining 14 patients. Factor D, Clq, and C5-C9 were normal in sera of all 18 patients studied (data not shown). The C3 level performed by an immunochemical method was not a true indication of the degree of complement activation when compared to the Raji cell radioimmunoassay.

**Table 2. Circulating Immune Complexes: Comparison of the Raji Cell Radioimmunoassay and C1q Binding Test**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Raji Cell Assay*</th>
<th>Solid Phase C1q Assay*</th>
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<tbody>
<tr>
<td>1</td>
<td>800</td>
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</tr>
<tr>
<td>2</td>
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<td>864</td>
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<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>6,400</td>
<td>3,000</td>
</tr>
<tr>
<td>8</td>
<td>3,000</td>
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<td>&lt;25</td>
</tr>
<tr>
<td>18</td>
<td>&lt;16</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>

*Results expressed in μg/ml equivalents of aggregated gamma globulin (μg/ml). Normal for Raji assay: <16 μg/ml. Normal for solid phase C1q assay: <25 μg/ml.
and C3b inactivator were measured by Dr. Hans Müller-Eberhard and found to be low in three of the

immunochemically was 108%, 61%, 57%, and 37% of normal compared to hemolytic titers of 63%, 15%, 5%, and 23% of normal in patients 2, 3, 5, and 7, respectively.

The presence of a C3 nephritic factor was sought in patients 2, 3, 5, and 7 as a possible explanation for activation of the alternative pathway, but none was found.

βIH and C3b inactivator were measured by Dr. Hans Müller-Eberhard and found to be low in three of the
same four patients (2, 5, and 7) who had C3 and factor B cleavage products in their sera (Table 4).

TCH50, hemolytic C3, factor B, and CIC levels (measured by the Raji cell radioimmunoassay) were serially followed in patient 3 until his death 6 mo after initial study (Table 5). Further reduction in complement levels with increasing CIC levels occurred during the terminal phase of the illness.

DISCUSSION

Several immunologic abnormalities have been reported to occur during the course of myelofibrosis including positive direct Coombs tests, elevated latex fixations, immune complexes, circulating anticoagulants, and increased platelet or white cell Fc receptors. In addition, in the present study of 18 patients with myelofibrosis, we found 4 patients with marked hypocomplementemia due to activation of the alternative pathway. Since abnormalities of the complement system are known to predispose to systemic infections, it is not surprising that three of four patients with myelofibrosis and low C3 levels developed severe infections that were directly related to their cause of death. At the time of initial immunologic evaluation, none of these four patients had infections.

The mechanism for activation of the alternative complement pathway may be related to decreased levels of \( \beta_{1H} \) and C3b inactivator. These proteins exert their regulatory influence at the level of the alternative pathway amplification C3 convertase C3bBb. The absence of either protein has been associated with spontaneous activation of the alternative pathway. Whaley et al. described 13 patients with systemic lupus erythematosus who had activation of the alternative complement pathway with decreased levels of C3b inactivator and \( \beta_{1H} \). The reason for decreased levels of these control proteins is unclear. Monocytes can synthesize \( \beta_{1H} \) and C3b inactivator. Since myelofibrosis appears to be a primary bone marrow disorder as evidenced by clonal hematopoietic cell proliferation, decreased synthesis of \( \beta_{1H} \) and C3b inactivator may be occurring. Metabolic studies would be required to differentiate between decreased synthesis and increased utilization of these proteins. A hereditary deficiency of these control proteins cannot be eliminated as a possibility. In the present study we were unable to carry out family studies.

Nine of 18 patients with myelofibrosis were also found to have increased CIC levels. The presence of elevated levels of CIC was associated with more active disease as manifest by increasing transfusion requirement, bone pain, or fever of unknown origin. Two independent methods were used to measure immune complexes in the sera of patients with myelofibrosis. The use of multiple techniques for measuring CIC has been recommended because of the limitations found in individual assays. In patients with myelofibrosis, a good correlation was found \( (r = 0.8) \) between the Raji cell radioimmunoassay and solid phase Clq assay. Patient 6 was found to have elevated CIC levels by the Raji cell radioimmunoassay but not by the solid phase

<table>
<thead>
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<th>Table 4. Alternative Pathway: Abnormalities in Control Proteins</th>
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<tbody>
<tr>
<td>Patient</td>
</tr>
<tr>
<td>TCH50*</td>
</tr>
<tr>
<td>C3</td>
</tr>
<tr>
<td>Factor B</td>
</tr>
<tr>
<td>( \beta_{1H} )+</td>
</tr>
<tr>
<td>C3bINA†</td>
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</table>

* TCH50, C3, factor B, \( \beta_{1H} \), and C3b inactivator (C3bINA) expressed as a percentage of normal sera.
† \( \beta_{1H} \) and C3bINA kindly measured by Dr. Hans Müller-Eberhard. Normal values: \( \beta_{1H} \) 475 \( \mu \)g/ml; C3bINA, 35 \( \mu \)g/ml.

<table>
<thead>
<tr>
<th>Table 5. Complement and CIC Levels in Patient 3</th>
</tr>
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<tbody>
<tr>
<td>6/79</td>
</tr>
<tr>
<td>CIC Level†</td>
</tr>
<tr>
<td>TCH50</td>
</tr>
<tr>
<td>C3</td>
</tr>
<tr>
<td>Factor B</td>
</tr>
</tbody>
</table>

* Studied at a time E. coli sepsis was present. Patient died 12/16/79.
† Measured by the Raji cell radioimmunoassay.

Normal values: CIC by Raji cell radioimmunoassay, \(< 16 \mu g/ml;\) TCH50, 80 \pm 15; C3, 2600 \pm 532; factor B, 80 \pm 16.
C1q assay. This was unlikely to be due to antilymphocyte antibodies as reported in some patients with systemic lupus erythematosus, since sucrose density gradient ultracentrifugation revealed the Raji cell binding activity to be in the 11S region.

There are several possible explanations for the presence of CIC in these patients. The association in some patients of hypergammaglobulinemia, Coombs positivity, circulating anticoagulants, and antigen-antibody complexes suggests the presence of an immunologic hyperresponsive state. CIC formed as a result of every day antigen exposure (e.g., bacteria in the gastrointestinal tract) may not be cleared normally in patients with myelofibrosis since the reticuloendothelial system may also be functionally impaired due to the presence of extramedullary hematopoiesis or prior splenectomy.

Immunologic dysfunction, including abnormalities of the complement system, may be an important part of the disease process in some patients with myelofibrosis. Activation of the alternative complement pathway leading to low levels of C3 may predispose these patients to serious systemic infections.

ACKNOWLEDGMENT

We would like to thank Regina O'Connor for providing excellent technical assistance.

REFERENCES

1. Ward HP, Block MH: The natural history of agnogenic myeloid metaplasia (AMM) and a critical evaluation of its relationship with the myeloproliferative syndrome. Medicine 50:357, 1971
32. Whaley K: Biosynthesis of the complement components and


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