Increased Circulating Neutrophils With Surface Receptor Activity for Immunoglobulin G in Polycythemia Vera and Myeloid Metaplasia

By Harriet S. Gilbert, Robert Goldberg, and Lawrence Ward

Reports of heterogeneity of IgG receptor activity of normal circulating neutrophils prompted measurements in myeloproliferative disease to determine if dysplasia of the hematogenous cell resulted in an abnormality of this membrane property. IgG receptors were assayed by rosette formation in suspension with human Rh-positive erythrocytes sensitized with high-titer Rh antiserum. IgG receptors were detected on 19 ± 1.6% (mean ± SEM) of neutrophils from 45 normal subjects. A significant increase in IgG-receptor-bearing neutrophils was found in polycythemia vera (PV) and myeloid metaplasia (MyM), with values of 70 ± 3.6% and 69.7 ± 4.3%, respectively. Normal values were observed in polycythemic states not due to myeloproliferative disease and in chronic myelocytic leukemia. Rosette-forming neutrophils were increased to 52.3 ± 3.7% in infection and inflammatory disease, but this value was significantly lower than those in PV and MyM. Increased IgG receptors in PV and MyM may be related to the activated state of the neutrophil and may result from an intrinsic cellular abnormality of the proliferating clone or from altered bone marrow release. Quantitation of neutrophil IgG receptors may be of value in the differential diagnosis of PV and MyM and may offer insights into the derangement of hematopoiesis that underlies these myeloproliferative disorders.

Surface receptors for immunoglobulin G have been demonstrated on human neutrophils by several investigators.1-3 Heterogeneity of circulating neutrophils for IgG receptor activity has been observed, and Fc-receptor-bearing neutrophils have been demonstrated to be more active in adherence to nylon wool, in phagocytosis, in bactericidal capacity, and in chemotaxis than neutrophils without such activity.4 The recognition of the heterogeneity of surface receptor activity of normal circulating neutrophils prompted this study of neutrophil IgG receptors in a group of disorders characterized by clonal proliferation of the pluripotential stem cell that may result in alterations in the normal heterogeneity of circulating hematocytic cells.5

Materials and Methods

Patient Population

The study population consisted of normal subjects and patients under treatment at the Mount Sinai Medical Center for hematologic and other disorders. Informed consent was obtained for blood collection, and the protocol for this study was approved by the Institutional Review Board. The...
Diagnostic criteria used for classification of patients with myeloproliferative disorders and polycythemic states have been described previously.6,7

Leukocyte Suspensions

Leukocytes were obtained from venous blood that was anticoagulated with preservative-free heparin at a final concentration of 8 U/mL. Neutrophil-nich leukocyte suspensions were prepared by sedimentation of blood for 15-30 min with an equal volume of 3% dextran 1500, followed by centrifugation at 150 g for 10 min. Residual erythrocytes were removed by hypotonic lysis. Leukocytes were washed twice with Hanks’ balanced salt solution (HBSS) and suspended at a final concentration of 4 x 10^6/mL. Total leukocyte counting was performed using a Coulter S and leukocyte differential counting by examination of Jenner-Giemsa stained smears. Cell suspensions contained more than 85% neutrophils, with the remaining cells being mainly lymphocytes. Cell viability was more than 98%, as judged by trypan blue dye exclusion. In some experiments highly purified neutrophil suspensions were prepared by Ficoll-Hypaque density centrifugation followed by dextran sedimentation and removal of residual erythrocytes.8 These suspensions contained more than 97% neutrophils.

Preparation of Sensitized Erythrocytes

Human IgG-coated erythrocytes were used to assay neutrophil IgG receptor activity. Normal type-O, D-positive (Rh: I^a) erythrocytes were sensitized with hypenimmune serum from patient Heym kindly provided by Dr. Richard Rosenfield. Heym serum contains an antibody of the IgG class with anti-CD (Rhl,2) specificity and an indirect Coombs titer of 1:25,000.9 Donor erythrocytes were washed twice with 0.15-M NaCl containing 0.01-M ethylenediaminetetraacetic acid, pH 7.2 (saline EDTA), incubated at 37°C for 30 min in Heym serum diluted with saline EDTA, washed once with saline EDTA and twice with HBSS, and resuspended in HBSS at a final concentration of 2 x 10^8/mL. Optimal erythrocyte sensitization without agglutination was obtained using a serum dilution of 1:100 with two normal type-O D-positive (Rh: I^a) donors, and unless otherwise stated, this is the sensitizing dilution employed in these experiments. Coombs testing and comparison of rosette formation with erythrocytes from the two donors showed comparable degrees of erythrocyte sensitization, and these donors were used throughout the study. The resulting erythrocytes, sensitized without agglutination, were strongly Coombs-positive with anti-IgG and Coombs-negative with anti-C3 and anti-C4 monospecific antisera and with anti-lgM. In some experiments the amount of IgG used to coat erythrocytes was reduced by further antiserum dilution from patient Fred, with an IgG antibody directed against D (RhI), and an indirect Coombs titer of 1:128 was used at a dilution of 1:10 in some experiments to produce erythrocytes with a strongly positive anti-IgG Coombs reaction and a negative anti-C3 and anti-C4 Coombs reaction.

Control erythrocytes prepared by incubation of D-positive cells without antisera or by incubation of D-negative (Rh: -I) erythrocytes with antisera were Coombs-negative with all test antisera.

Assay for IgG Receptor Activity of Neutrophils

Rosette formation was assayed by incubating leukocytes with sensitized erythrocytes at a ratio of 1:50. This was accomplished by mixing 0.05 mL of leukocyte suspension (0.2 x 10^6 leukocytes) with 0.05 mL of erythrocyte suspension (1 x 10^7 erythrocytes) in disposable plastic tubes (Falcon, Los Angeles, Calif.) and gently agitating for 1 hr at room temperature. The mixture was centrifuged at 150 g for 3 min, then gently rocked to disrupt the cell pellet, and aliquots were transferred to wells on PTFE-coated multispot microscope slides (C. A. Hendley & Co., Essex, England). After settling, 100 neutrophils in each of two wells were examined by phase-contrast microscopy. Cells with segmented or band-shaped nuclei were counted as neutrophils. The numbers of neutrophil rosettes (neutrophils to which three or more erythrocytes were bound) were counted, and the results were expressed as percentages rosette-forming neutrophils. In some experiments smears were prepared from the incubated cell suspensions and examined after Jenner-Giemsa staining.
phosphate buffer (pH 7.4) and IgG myeloma proteins of four known subclasses (IgG 1, 2, 3, and 4) were kindly provided by Dr. Shaul Kochwa. The myeloma proteins were isolated by starch-block electrophoresis. Whenever immunoelectrophoresis against anti-whole-human-serum revealed contamination, an additional purification on DEAE-cellulose was performed. The final product gave a reaction with only one anti-light-chain reagent and was chemically typed to determine the subgroup.12

Statistical Analysis

Comparison between groups was performed using Student's t test and paired t tests.

RESULTS

Human neutrophils incubated in suspension with human IgG-sensitized erythrocytes formed rosettes that were readily detectable by phase microscopy. Examination of stained smears confirmed the presence of neutrophil rosettes. No rosette formation was observed following incubation of neutrophils from any of the populations studied with unsensitized D-positive erythrocytes or D-negative erythrocytes treated with Rh antiserum.

The IgG receptor activity of neutrophils for IgG in the form of an anti-CD antibody on human erythrocytes was studied in 45 normal subjects (Fig. 1). The frequency of neutrophil rosettes was 19 ± 1.6% (mean ± SEM), with a range from 1% to 48%. Receiver activity of circulating neutrophils from 18 patients with active polycythemia vera (PV) was displayed by 70 ± 3.6% of neutrophils with a range from 40% to 96% (Fig. 1). These values were significantly greater than those observed in the normal group (p < 0.001). A comparable degree of elevation was observed in 10 patients with myeloid metaplasia (MyM), in whom rosetting was observed in 69.7 ± 4.3% of neutrophils (range 43%–93%, p < 0.001 compared with the normal group). Twelve patients with polycythemia not attributable to a myeloproliferative disorder were studied. This group consisted of 6 patients with secondary erythrocytosis (hypoxia of respiratory origin, 3; carboxyhemoglobinemia, 1; erythropoietin-secreting hypernephroma, 1; erythrocytosis of undetermined etiology in the absence of any stigmata of myeloproliferative disease, 1) and 6 patients with relative polycythemia. Rosetting activity in these patients was displayed by 15 ± 2.5% (range 3%–30%) of the circulating neutrophils, values not
significantly different from that of the normal group. IgG rosetting activity in 8 patients with active chronic myelocytic leukemia (CML) was $15.9 \pm 5.2\%$ (range $4\%-41\%$) and did not differ from that of the normal group. Since this study was concerned with IgG receptor activity of mature circulating neutrophils, less mature neutrophilic forms present in the circulation of patients with MyM and CML were not included in the scoring of receptor activity.

Since IgG receptor activity of neutrophils has been found to be dependent on the type of IgG used in the assay,2 additional studies were performed using erythrocytes sensitized with anti-D Fred antiserum (Table 1). In this test system normal neutrophils displayed significantly less IgG receptor activity than in assays using anti-CD antiserum. Patients with PV and MyM again showed significantly greater numbers of rosetting neutrophils, whereas patients with secondary and relative polycythemia had normal values. The increment in IgG receptor activity in the PV and MyM groups over that seen in the normal group was greater with anti-D-coated erythrocytes than with anti-CD-coated erythrocytes. These studies demonstrate that neutrophils in PV and MyM have increased receptor activity for more than one type of IgG antibody.

The relationship between rosette formation and concentration of IgG used to sensitize the test erythrocytes was studied. Decreasing concentrations of IgG (using anti-CD antiserum) resulted in decreasing rosette formation (Fig. 2). A significant reduction in rosetting was observed at antiserum dilutions of 1:500 or greater, as compared with lower dilutions ($p < 0.05$ by paired $t$ test). IgG dose dependence was observed in both normal subjects and patients with PV and MyM. However, a
significant increase in rosetting in PV and MyM was observed at all the dilutions of antibody studied, and the differences between normal controls and the PV and MyM groups were most marked when smaller amounts of antibody were used. The antibody dilutions at which rosetting ceased to occur also differed. Erythrocytes sensitized with antibody diluted beyond 1:2000 failed to form rosettes with normal neutrophils, whereas elimination of rosetting with PV and MyM neutrophils required dilutions of 1:4000 or more. Corresponding values for rosetting at a 1:2000 dilution of anti-CD antiserum were obtained using anti-D antiserum at a 1:100 dilution.

The specificity of neutrophil IgG receptors was studied by determining the degree of inhibition produced by pooled normal IgG and single subclasses of IgG purified from subjects with multiple myeloma. Table 2 summarizes the results of rosetting assays in 5 patients with PV or MyM using anti-CD antiserum for erythrocyte sensitization. Pooled IgG produced essentially complete inhibition of neutrophil receptor activity for the IgG in this antiserum. Subclasses of IgG were added at concentrations comparable to that contained in pooled IgG in order to determine subclass specificity. IgG 1 and 3 produced complete inhibition of the neutrophil receptor, whereas IgG 4 had no inhibitory effect. IgG 2 showed slight inhibition that was significant at the 0.05 level. These results corroborate the validity of the rosetting assay for testing IgG receptors on neutrophils, in that pooled IgG inhibited rosetting. The neutrophil receptor sites for the IgG on the anti-CD-coated erythrocytes used in this assay were specific for IgG subclasses 1 and 3.

The relationship between neutrophil IgG receptor activity and disease activity of PV and MyM was studied by serial assays in 8 patients receiving myelosuppressive therapy (chlorambucil, busulfan). Comparison of IgG receptor activities during periods of uncontrolled and controlled proliferation was performed by paired t testing and revealed no significant difference in rosetting activities during active and controlled disease. Increased IgG receptor activity was not correlated with the circulating neutrophil count, hematocrit, platelet count, or degree of splenomegaly.

The finding of increased IgG receptor activity in neutrophils from inflammatory exudate4 prompted an examination of neutrophil receptor activity in patients with infection or inflammatory disease (bacterial infection, 3; active rheumatoid arthritis, 5). Table 3 summarizes these results and indicates that neutrophil rosetting was significantly increased above normal in this group. Comparison between the PV and MyM patients and those with infection or inflammation showed that the patients

<table>
<thead>
<tr>
<th>Table 2. Abilities of Immunoglobulins to Inhibit IgG Receptor Activity of Neutrophils Incubated With Anti-CD-Coated Erythrocytes</th>
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<tbody>
<tr>
<td>Immunoglobulin Added to Incubation (concentration)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Pooled normal IgG (1000 μg/ml)</td>
</tr>
<tr>
<td>IgG 1 (660 μg/ml)</td>
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<tr>
<td>IgG 2 (230 μg/ml)</td>
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<tr>
<td>IgG 3 (70 μg/ml)</td>
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<td>IgG 4 (40 μg/ml)</td>
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*p value of paired t test of control incubation versus test incubation.
with myeloproliferative disease had significantly greater rosetting activity. Neutrophils from 8 untreated patients with lymphoproliferative disorders (chronic lymphocytic leukemia, 5; lymphosarcoma, 3) showed a significant reduction of IgG receptor activity (Table 3). A group of patients with dysplasia of the bone marrow (sideroblastic anemia, 4; pure red cell aplasia, 1; paroxysmal nocturnal hemoglobinuria, 1) had normal IgG receptor activity, as did 4 patients with congestive splenomegaly due to portal hypertension.

**DISCUSSION**

These studies demonstrate an increase in the number of circulating neutrophils that display surface membrane receptor activity for IgG in patients with PV and MyM. This abnormality was not observed in patients with secondary or relative polycythemia or chronic myelocytic leukemia. Its presence in PV and MyM was unrelated to the degree of proliferative activity. In this study the assay system for IgG receptors employed erythrocytes coated with IgG from two hyperimmune Rh sera, one with high anti-CD titers, the other with high anti-D titers. Neutrophils from patients with PV and MyM showed increased receptor activity in both test systems. Messner and Jelinek² studied IgG receptors of normal neutrophils using erythrocytes sensitized with a different anti-CD antibody and also a different assay system consisting of a slide adherence test in which monolayers of granulocyte-rich leukocyte suspensions were incubated with sensitized erythrocytes. Since the degree of adherence of circulating neutrophils may not be uniform, especially in abnormal subjects, we selected a suspension method in order to obtain information about the entire neutrophil population, rather than biasing the studies in favor of more adherent neutrophils that might be retained selectively on the monolayer. Among other factors, differences in antibody properties, as well as assay technique, may explain the values of 19 ± 1.6% for normal neutrophil rosetting in our study, as compared with their values of 34.1 ± 4.9%.

Although the degree of rosette formation was dependent on the concentration of erythrocyte IgG and the type of IgG used for erythrocyte sensitization, the increase in IgG receptor activity of neutrophils in PV and MyM was observed even when erythrocytes were sensitized with suboptimal amounts of IgG from the anti-CD antiserum or with the less active anti-D antiserum.

The inhibition of rosette formation by IgG and the specificity of IgG receptors of neutrophils for IgG subclasses 1 and 3 are properties of both normal neutrophil and monocyte IgG receptors.²,³ The concentrations of IgG required for complete inhibition of binding of IgG by PV and MyM neutrophils in this study were similar

### Table 3. Circulating Neutrophil IgG Receptor Activity in Infection and Hematologic Disorders Using Erythrocytes Sensitized With Anti-CD Antiserum

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>Percentage Neutrophil Rosettes</th>
<th>Mean ± SEM</th>
<th>Range</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>45</td>
<td>19.0 ± 1.6</td>
<td>3-48</td>
<td>&lt;0.001</td>
<td></td>
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<tr>
<td>Infection, inflammation</td>
<td>8</td>
<td>52.3 ± 3.7</td>
<td>37-64</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Lymphoproliferative disease</td>
<td>8</td>
<td>5.6 ± 1.8</td>
<td>2-15</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Bone marrow dysplasia</td>
<td>6</td>
<td>25.8 ± 6.5</td>
<td>2-40</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Congestive splenomegaly</td>
<td>4</td>
<td>27.5 ± 8.1</td>
<td>15-49</td>
<td>&gt;0.05</td>
<td></td>
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*p value of comparison with normal group.
to those reported by others. This suggests that the characteristics of the IgG receptors on PV and MyM neutrophils resemble those of normal neutrophils and monocyte receptors and that the increased numbers of rosetting neutrophils observed in PV and MyM are due to an increase in the number of IgG-receptor-bearing neutrophils present in circulating blood rather than to enhanced affinity of each IgG receptor present on the neutrophil surface.

Our studies of neutrophil receptor activity in small numbers of patients with conditions other than myeloproliferative disorders revealed an increase in IgG receptor activity in patients with infection or active inflammation. Levels were significantly elevated in comparison with those in normal subjects, but did not equal those seen in PV and MyM. Metabolic “activation” of neutrophils in infection and inflammation has been documented, and the observation of increased IgG receptor activity in patients likely to have a population of “activated” neutrophils is consistent with the finding of Klempner and Gallin, who demonstrated two neutrophil subpopulations as regards receptor activity. Those neutrophils with IgG receptors were functionally more active than those without receptors. These workers were unable to determine the origin of the two neutrophil subpopulations or to relate age to IgG receptor activity.

Neutrophil IgG receptors in some hematologic disorders were studied by Zipursky and Brown, who found varying degrees of increased binding and erythrophagocytosis of anti-D-sensitized erythrocytes in patients with myeloblastic, myelomonocytic, and lymphoblastic leukemia, in contrast to the absence of binding by normal neutrophils. They concluded that neutrophils formed as a result of abnormal myelopoiesis may have erythrocyte-binding characteristics similar to those of the monocyte. Characterization of surface receptor activity in PV and MyM has not been performed previously, although Munn and Chaplin noted in their studies of the effects of biologic variables of leukocyte monolayers on leukocyte rosette-forming capacity that the monolayer from a splenectomized patient with PV-myelofibrosis, composed primarily of neutrophilic granulocytes, had extraordinary binding of IgG-sensitized erythrocytes to levels 168 times the mean for normal donors. However, their second patient with PV-myelofibrosis had normal rosetting activity.

There are several possible explanations for the increased IgG receptor activity of neutrophils in PV and MyM. PV neutrophils have been shown to be in a metabolically activated state by other criteria, and IgG receptor activity may be another manifestation of this altered metabolic state. The presence of cells with IgG receptor activity may be the result of an abnormality of neutrophil release in PV and MyM and may indicate an age shift of the circulating neutrophils.

Alternatively, the presence of increased membrane IgG receptor activity in PV and MyM could be an intrinsic abnormality of the neutrophil that arises from the clone of hematic precursor cells responsible for the autonomous myeloproliferative activity that characterizes these syndromes and could reflect some maturational defect inherent in the dysplastic clone. Whatever the mechanism responsible for the observed phenomenon, the contrast between the markedly elevated values for IgG neutrophil receptor activity in PV and MyM and the normal values in secondary and relative polycythemia and CML suggests that this assay may be of value in the differential diagnosis of polycythemia and in the classification of patients with various myeloproliferative disorders.
The pathophysiologic consequences of an increased circulating population of IgG-receptor-bearing neutrophils remain to be elucidated. Of some relevance may be the recent report by Lewis and Pegrum\textsuperscript{19} that peripheral blood from patients with myelofibrosis has a high proportion of phagocytic cells containing immunoglobulin complexes. The sera of these patients also contain immune complexes. Those authors interpreted these findings to indicate an autoimmune basis for bone marrow fibrosis. Although they stated that they did not find the abnormalities in patients with polycythemia vera without myelofibrosis, it is notable that 2 of their 14 patients with immune complexes and myelofibrosis did have active PV, as evidenced by increased red cell mass. An increase in the number of phagocytes with intracellular immunoglobulin may relate to the presence of increased numbers of circulating neutrophils capable of binding IgG.

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