Monocyte-Erythrocyte Interaction In Vitro in Immune Hemolytic Anemias

By Neil E. Kay and Steven D. Douglas

Monocyte recognition of immunoprotein-coated erythrocytes in vitro was studied in 21 patients with immune hemolytic anemia. Simultaneous assessment of immunoglobulin and complement components on the patients' erythrocytes was performed. The patients were heterogeneous in relation to associated diseases, therapy, and duration of disease state; 15 were studied within 1 mo of diagnosis and 4 additional patients were studied in sequential fashion. Monocyte-erythrocyte interaction was assessed by both a morphologic and a radioactive assay; 12 of 17 patients had elevated ratios in the morphologic assay and 15 of 17 patients had elevated ratios in the radioactive assay. The majority of patients exhibited enhanced interaction between their monocytes and autologous immunoprotein-coated erythrocytes, as compared to normal monocytes exposed to the same immunoprotein-coated erythrocytes. These findings suggest that there is an enhanced capacity of monocytes from certain patients with immune hemolysis to interact with autologous immunoprotein-coated erythrocytes in vitro.

The diagnosis of immune hemolytic anemia (IHA) is based on the detection of immunoglobulins and/or complement by the direct antiglobulin test (DAT), which is positive in 85%-90% of patients. This widely used and imprecise documentation of immunoprotein-coated erythrocytes does not, however, provide information which directly correlates with the rates of hemolysis or responses to therapy. More precise methods other than agglutination which measure cell-bound antibody appear to show a relationship between immunoprotein coating and hemolytic rates. Nevertheless, it is evident that the pathophysiology of immune hemolysis involves a complex interaction between erythrocytes, antibody, the complement system, and phagocytic cells.

The mononuclear phagocyte (MNP) and the reticuloendothelial system may have a major role in the immunopathogenesis of this disease. MNP binding of sensitized erythrocytes has also been documented in drug-induced immune hemolysis. Plasma membrane receptor sites are present on blood, splenic, and hepatic MNP for the Fc portion of IgG and for complement components which are bound to the erythrocyte membrane. The interaction between receptor and antigen may result in partial or complete erythrocyte phagocytosis.

Since MNP interaction with immunoprotein-coated erythrocytes is most
likely the major source of removal of erythrocytes in IHA,7 we have investigated the MNP in vitro activity in patients with this disease. In order to examine the possible role of MNP in IHA, we have studied IgG and C3 receptor activity, and have compared the capacity of the patients' erythrocyte-bound autoantibody to interact with both the patients' monocytes and monocytes from normal donors.

**MATERIALS AND METHODS**

**MNP Source and Isolation**

Peripheral blood from normal donors and patients with positive DATs were obtained on the day of the assay. Normal donors who were ABO compatible with the patients studied were selected. MNP were isolated by dextran sedimentation of whole blood followed by flotation of leukocyte-rich plasma on 28% bovine serum albumin.8 After adherence to glass coverslips, these preparations contained 90%-95% MNP and 5%-10% lymphocytes. The adherent cells were determined to be monocytes by morphologic criteria using Wright-Giemsa stains and their ability to ingest latex.

**Patient Population**

Patients with positive DATs and prior or present well-documented anemia and reticulocytosis were included in this study. The patients varied with respect to associated diseases, hematologic parameters, and therapy. A summary of the clinical data is presented in Table 1. DATs were performed using either Ortho and/or Hyland broad-spectrum reagents.

<table>
<thead>
<tr>
<th>Table 1. Patient Population Data: Therapeutic Status, Immunoprotein Coat on Erythrocytes, and Monocyte Assay Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient No.</strong></td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td><strong>IIHA</strong></td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>2</td>
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<td>3</td>
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<td>14</td>
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<td>16</td>
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<td>17</td>
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</tbody>
</table>

*IIHA, idiopathic immune hemolytic anemia; DIHA, disease-associated immune hemolytic anemia; CLL, chronic lymphocytic leukemia; Histo, fibrous histiocytoma; SLE, systemic lupus erythematosus.*

**Notes:**
1. N, no therapy; S, steroids; Sp, splenectomy.
2. Patients with immunoglobulin-coated erythrocytes; 2, patients with immunoglobulin and complement-coated erythrocytes.
MNP Receptor Assays

Isolated MNP were suspended at a concentration of $1 \times 10^6$/ml in medium 199 containing 15% fetal calf serum and incubated for 1 hr at 37°C in a 5% CO₂ incubator in order to allow adhesion to coverslips in Leighton tubes. Coverslips were washed three times with medium 199 in order to remove unbound mononuclear cells. Sheep erythrocytes were coated (dilution 1:100, 1:200, and 1:400) with 7S rabbit anti-sheep (Forssman) antibody (EA) purified by gel chromatography or 19S rabbit Forssman antibody (dilution 1:50, 1:100, and 1:200) in the presence of fresh human serum (dilution 1:100) as a complement source in order to prepare complement-coated erythrocytes (EAC).

Human erythrocyte-monocyte interaction was studied with normal, ABO-compatible erythrocytes and autologous immunoprotein-coated erythrocytes from the patients. The autologous erythrocytes were also radiolabeled by incubation with $^{51}$Cr for 30 min.9 Erythrocytes were suspended at a concentration of $10^7$/ml and 0.1 ml of the various erythrocyte preparations was incubated with the MNP for 1 hr at 37°C in 5% CO₂-air atmosphere. The coverslips were then washed three times with 1.5-mI aliquots of medium 199 in order to remove unbound erythrocytes. All assays were performed in duplicate.

In order to determine the number of erythrocytes bound, the coverslips were assayed for radioactivity in a Packard gamma counter (model 5120). The results were expressed as radioactivity per slide and compared between patient and control populations. The following formula was used to calculate the ratio:

$$\text{Radioactivity assay ratio} = \frac{\text{radioactivity detected in immunoprotein-coated}}{\text{radioactivity detected in immunoprotein-coated}} \cdot \frac{\text{erythrocyte-monocyte (patient) population}}{\text{erythrocyte-monocyte (control) preparation}}$$

The same MNP preparations were then fixed in methanol and stained with Wright-Giemsa. The morphologic activity of all slides was determined by microscopic examination for rosette formation (three or more erythrocytes bound to an MNP) and/or phagocytosis (at least one ingested erythrocyte). Two hundred cells were counted per slide and the results of the morphologic assay were compared between patient and control populations. The formula used to calculate the ratios was

$$\text{Morphologic assay ratio} = \frac{\text{total morphologic activity in the immunoprotein-coated}}{\text{total morphologic activity in the immunoprotein-coated}} \cdot \frac{\text{erythrocyte-monocyte (patient) preparation}}{\text{erythrocyte-monocyte (control) preparation}}$$

In order to define the specificity of MNP activity in both the patient and control populations, evaluation of radioactivity and morphologic parameters in relation to normal ABO compatible erythrocytes was also performed.

Immunoprotein Detection on Erythrocyte Membranes

Manual DATs were performed using broad-spectrum antiglobulin and monospecific antisera as described previously.10 The monospecific antisera were anti-IgG, IgM, IgA, IgD, IgE, C3, and C4 (Behring Diagnostics and Meloy Labs). In addition, polyvinylpyrrolidone instrumented antiglobulin tests utilizing the same monospecific antisera were performed for 11 patients.11 This test was utilized because of its greater sensitivity in the detection of erythrocyte-bound immunoprotein.12

Sequential Investigation

Four patients with immune hemolysis were reevaluated on 2-3 separate occasions during the course of their disease. Evaluations of immunoproteins bound to the erythrocytes and the monocyte assays were performed.
RESULTS

Patient Population

All 17 patients had positive DATs at the time of the study, although the strength of agglutination varied widely. Of the 17, 15 patients were studied within 1 mo and 2 patients within 6 mo of diagnosis. Eight patients were on therapy (7 on steroids, 1 postsplenectomy) and 9 were untreated at the time of initial evaluation.

Eleven of the patients had idiopathic immune hemolytic anemia (IIHA) and 6 had diseases associated with IHA (DIHA)—chronic lymphocytic leukemia (2 patients), drug (1 patient), fibrous histiocytoma (1 patient), and systemic lupus erythematous (2 patients). Rates of hemolysis assessed by evaluation of the hemoglobin and reticulocyte levels were widely variable in the DIHA and IIHA population.

The patient population was divided into two groups (Table 1). Group 1 included patients with single or multiple membrane-bound immunoglobulins. The additional presence of C3 or C4 on the erythrocyte membrane led to designation as group 2.

Monocyte–Erythrocyte Interaction

The monocyte receptor activity ratios for both the morphologic and radioactive assays were analyzed in relation to the patient population (Fig. 1). A ratio of 1.0 would indicate that there was an equal avidity for the patients' immunoprotein-coated erythrocytes for both the patient and control monocyte preparations. Control monocyte preparations were evaluated concurrently with each patient and a total of 20 controls were studied. The duplicates in each assay had less than 5% variation.

The median ratio in both the morphologic and radioactive assays for the entire patient group was 1.28 and 1.55, respectively (Fig. 1). This finding suggests that the IHA patients had excessive monocyte activity toward the immunoglobulin-and/or complement-coated erythrocytes. Subdivision of the patients into DIHA and IIHA groups still showed elevated ratios for both groups,
**Table 2. IgG and C3 Monocyte Receptor Activity**

<table>
<thead>
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<th></th>
<th>N</th>
<th>IgG (%)</th>
<th>C3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>92 ± 3.6</td>
<td>73 ± 8.5</td>
</tr>
<tr>
<td>Patients</td>
<td>11</td>
<td>89 ± 6.1</td>
<td>72.2 ± 5.6</td>
</tr>
<tr>
<td>Idiopathic</td>
<td>8</td>
<td>92.6 ± 6.4</td>
<td>73 ± 5.6</td>
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<tr>
<td>Disease-associated</td>
<td>3</td>
<td>83 ± 7.8</td>
<td>75 ± 4.2</td>
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</tbody>
</table>

Values indicate percent of cells (mean ± SE) with rosette and/or phagocytosis. EA and EAC dilutions represented here are for EA 1:400 and EA 1:200, C 1:100.

suggesting there was no marked heterogeneity in monocyte reactivity to the immunoprotein-coated erythrocytes (Fig. 1).

Within the patient group, 5 patients had ratios of 1.0 in the morphologic assay (3 idiopathic, 2 disease associated) and 2 had 1.0 ratios in the radioactivity assay. Therefore, there was a subpopulation of patients without demonstrable increase in monocyte activity in comparison to the control group.*

The patient population was analyzed in relation to the presence or absence of complement and therapy status. All patient groups had elevated mean ratio levels. The mean ratio levels for the radioactivity assay were relatively consistent in all groups. The mean ratios for the morphologic assay were highest in the patient groups with complement bound to erythrocytes (4.52) and in patients who had been splenectomized or were on steroids (4.03). There were, however, no statistically significant differences between these groups.

Monocytes from the patients were incubated with ABO-compatible erythrocytes that had no detectable bound immunoglobulin or complement. The patients’ monocytes had no significant morphologic interaction or increased radioactivity per slide and were indistinguishable from monocytes from control donors. Using these erythrocytes, the radioactivity was close to background and morphologic activity varied from 0 to 5 rosettes and/or phagocytosis per 200 counted cells.

Control and patient monocyte populations (11 subjects each) were incubated with varying dilutions of EA and EAC preparations. Monocytes from 8 patients with IHA and 3 patients with DIHA were studied (Table 2). There was no statistical difference (Student’s t test) between control and patient monocyte populations in EA or EAC binding at the dilutions tested—EA 1:100, 1:200, and 1:400; and EA 1:50, 1:100, 1:200 with complement EAC (serum 1:100).

**Sequential Studies**

Four patients with IHA were followed by serial evaluation of monocyte function and immunoprotein erythrocyte patterns (Table 3).

For patient 1, the first study (day zero) at the time of initial diagnosis showed elevated monocyte assay ratios and multiple membrane-bound immunoglobu-

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*Two adult patients with a hemolytic uremic syndrome, (i.e., falling hemoglobin, reticulocytosis, and multiple schistocytes on peripheral smear) were studied with both the morphologic and radioactive assays. The ratios were 1.02 and 0.95 in the morphologic assay and 0.97 and 1.05 in the radioactive assay. Thus, at least in these hemolytic episodes, no obvious difference in monocyte activity from control was noted.
Table 3. Sequential Studies

<table>
<thead>
<tr>
<th>Patient</th>
<th>Day 0</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 13</th>
<th>Day 17</th>
<th>Day 14</th>
<th>Day 21</th>
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<tbody>
<tr>
<td>Therapy*</td>
<td>None</td>
<td>SP &amp; T</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Antiglobulin test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Immunoprotein group†</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<tr>
<td>Hemoglobin (g/dL)</td>
<td>8.9</td>
<td>12.3</td>
<td>8.0</td>
<td>9.5</td>
<td>10.4</td>
<td>4.2</td>
<td>6.5</td>
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<tr>
<td>Reticulocytes (%)</td>
<td>11.5</td>
<td>0.9</td>
<td>15.0</td>
<td>15.0</td>
<td>8.0</td>
<td>22.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Morphologic assay ratio</td>
<td>3.6</td>
<td>6.0</td>
<td>2.3</td>
<td>10.0</td>
<td>6.2</td>
<td>6.7</td>
<td>15.6</td>
</tr>
<tr>
<td>Radioactive assay ratio</td>
<td>2.0</td>
<td>3.4</td>
<td>1.3</td>
<td>1.1</td>
<td>1.0</td>
<td>1.3</td>
<td>1.0</td>
</tr>
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*Sp&T, splenectomy and transfusion; S, steroid therapy.
†See definitions in Table 1.

lin. A subsequent study, 10 days later (3 days postsplenectomy and multiple transfusions), revealed elevated monocyte activity ratios despite a normal hemoglobin and reticulocyte level (presumably secondary to transfusion effects).

Patient 2 was followed over a 20-day period. Neither transfusion nor specific therapy for IHA was administered. While there was slight improvement in the hemoglobin and reticulocyte levels, there were no changes in DAT or specific immunoproteins detected. The elevated morphologic monocyte assay ratio found on day zero increased on day 10, but on day 20, the ratio was less than on day 10. The radioactivity ratio was slightly elevated on day zero and fell to unity on day 10 and day 20.

Patient 3 was studied initially before therapy, and 13 and 17 days after steroids. Over the 17-day period there was a gradual improvement in the hemoglobin and reticulocyte levels; there was no change in the immunoprotein pattern detected on the erythrocytes and DAT reactivity. The elevated morphologic assay ratio observed on day zero was further increased by day 13 and day 17. The radioactivity ratios fluctuated from a moderate elevation initially to unity, and then increased on day 17.

Patient 4 was studied over a 21-day period and was not responsive to steroids. The elevated ratios observed at the onset of the study were either unchanged (in the morphologic assay) or fluctuated at high levels (radioactive assay).

DISCUSSION

Studies of the possible factors involved in immune hemolysis have primarily considered the role of immunoglobulin and complement bound to the erythrocyte. While erythrocyte-bound immunoprotein is of direct significance in determining decreased erythrocyte life span, the MNP has a major function in the removal of both aged normal and immunoprotein-coated erythrocytes. This combined study of the serologic and cellular components in a group of patients with IHA has been carried out to define further the roles of these two mechanisms in erythrocyte destruction.

The serologic evaluation at the time of diagnosis or within 1 mo of diagnosis has confirmed earlier reports which indicated a poor correlation between the degree of positivity of the DAT and hemoglobin or reticulocyte levels. In addition, the type of immunoproteins detected on the erythrocyte was not
specific in predicting apparent rates of hemolysis, as determined by hemoglobin and reticulocyte levels. It was somewhat surprising that the occurrence of multiple immunoglobulins and/or complement was not always associated with a low hemoglobin and high reticulocyte level. This finding is perhaps related to the fact that only receptors for IgG and C3 have been demonstrated on the MNP. Additionally, the known suppressive effect of steroids on both humoral and cellular immune mechanisms may modulate factors involved in the rate of hemolysis without significantly affecting the membrane-bound immunoprotein. Steroids have also been demonstrated to interfere with monocyte receptors.

MNP receptor activity, as defined by the radioactive and morphologic assay systems, suggested an increase in activity in some patients. This increase in activity was not uniform, in that 5 of 17 patients in the morphologic assay and 2 of 17 patients in the radioactive assay had normal ratios. There were no apparent clinical or hematologic differences for the patient group with ratios near unity. Sequential studies for 4 patients showed no change in immunoprotein characterization by both broad-spectrum and monospecific antisera. In contrast, the simultaneous sequential assessment of monocyte-erythrocyte interaction demonstrated increased activity which also showed fluctuation. These monocyte assays may make possible a more complete, kinetic appraisal of immune components affecting hemolysis.

The interaction of the patients’ MNP and immunoprotein-coated erythrocytes suggested a selectivity of these monocytes for the autologous protein-coated erythrocytes. There was no increase in activity of patients’ monocytes toward ABO-compatible or -incompatible human erythrocytes. In addition, there was no detectable difference in Fc and C3 receptor activity between the normal MNP and the patients’ MNP at the antibody dilutions studied. The possibility exists, however, that the IgG or C3 receptor activity of patients’ monocytes would be increased in comparison to controls if studied at higher antibody dilutions. Therefore, based on the available data, the interaction between the patients’ MNP and the immunoprotein-coated erythrocyte could not be explained on the basis of increased receptor activity or an increase in nonspecific ingestion. The most consistent elevation in MNP activity occurred for cells from patients with a single or multiple immunoglobulin(s) and complement on the erythrocyte. Therefore, the presence of complement may play a prominent role in modulating MNP activity. This finding tends to support observations that the presence of complement on erythrocytes may predict a more serious hemolytic process. In addition, a recent study has emphasized the increased binding of complement-coated erythrocytes to “activated” MNP.

These findings demonstrate that MNP from some patients with immune hemolysis had an increased capacity to bind autologous immunoprotein-coated erythrocytes in comparison to MNP obtained from a control population. The binding of rabbit (Forssman) IgG to patients’ MNP, however, was not distinguishable from control MNP at the dilutions of Forssman antibody studied. The latter observation differs strikingly from our previous findings of increased IgG receptor activity for patients with sarcoidosis, Crohn disease, and tuberculosis.

There are several possible interpretations of this observation that the pa-
patients' MNP had a greater capacity to bind autologous immunoprotein-coated erythrocytes than normal MNP. The first possibility is that there is a subpopulation of MNP which selectively bind the coated autologous erythrocyte. A subpopulation of patients' MNP could perhaps have more avid adherence to glass and a greater receptor activity. A second possibility is that the patients' MNP have either an increased affinity for autologous immunoprotein or an increase in the number of Fc receptor sites which have developed a restricted specificity. In this context, it is tempting to speculate further that the antigen-antibody complex which results when erythrocytes combine with antibody in immune hemolysis effects a structural modification in the Fc portion of the immunoglobulin molecule. This binding could result in a unique conformation of that portion of the Fc which binds to the MNP. A mechanism of this type could be of major immunopathogenic significance in immune hemolysis.

Further investigation of MNP binding sites and specific immunoproteins involved in immune hemolysis should make possible the precise elucidation of the interaction between MNP, immunoprotein, and erythrocytes. The development of more precise and specific quantitative assays of MNP receptors is essential to the characterization of these mechanisms.

ACKNOWLEDGMENT

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