Rapid, Sensitive Diagnosis of Hemolytic Anemia Using Antihemoglobin Antibody in Hypotonic Solution

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Abstract. We have developed a new and simple flow cytometric method to detect damaged red blood cells (RBCs) using anti-Hb in hypotonic solution. We studied a total of 200 patients, including 62 patients with schistocytosis, 8 postsplenectomy patients, and 108 healthy controls. Peripheral blood (2 µl) was stained with phycoerythrin-conjugated (PE) antihemoglobin antibody (anti-Hb) in 0.6% (w/v) NaCl solution, and analyzed by flow cytometry omitting the washing step. The proportion of RBCs stained by anti-Hb was 0.55% (SD ± 0.23%) in normal controls and was significantly higher in patients with schistocytosis (2.95 ± 2.95%, p <0.001). Six of 108 blood samples from normal controls and 60 of 62 samples from schistocytosis patients showed ≥1.01% stained RBCs (ie, values > mean+2SD of normal controls). The number of schistocytes counted by microscopic examination correlated with the proportion of RBCs stained by anti-Hb (r = 0.637, p <0.001). The proportions of stained RBCs in blood samples with malaria, spherocytosis, and elliptocytosis were also significantly higher than in normal controls. However, the results in postsplenectomy and iron-deficiency anemia (IDA) patients were not significantly different from the normal controls; the number of schistocytes in postsplenectomy patients was not related to the proportion of RBCs stained by anti-Hb. Based on these findings, flow cytometry of damaged RBCs using anti-Hb in hypotonic solution is a simple, sensitive, and accurate method to detect active hemolysis. (received 29 July 2001, accepted 27 September 2001)

Keywords: hemolytic anemia, antihemoglobin antibody, flow cytometry

Introduction

Damage to the red blood cell (RBC) membrane is a fundamental finding in the diagnosis of hemolytic anemias, both intravascular and extravascular. In patients with intravascular hemolysis, the presence of schistocytes in the peripheral blood is a cardinal sign of thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) [1]. Numerous TTP/HUS cases associated with drugs, cancer, and other diseases have been reported [1-9].

Schistocytosis is frequently found in patients after bone marrow transplantation (BMT) and some show significant hemolytic anemia [10-12]. Despite therapeutic developments, mortality from TTP is increasing [13] and the incidence of relapse of TTP is high [14,15]. The only available method to confirm and monitor the presence of schistocytes is the microscopic examination of blood smears after Romanovsky staining. This is labor-intensive and time-consuming process; it may be difficult to distinguish schistocytes from indented normal RBCs. In extravascular hemolysis, RBC membranes have defects, frequently showing poikilocytosis, spherocytosis, and elliptocytosis [15]. Although specific laboratory tests for individual diseases are available (eg, the antiglobulin test for autoimmune hemolytic anemia), early diagnosis of extravascular...
hemolysis is still difficult [17,18]. RBCs frequently show spherocytic morphology as an artifact. Therefore, an improved diagnostic test for hemolytic anemia is needed. We have developed a new, sensitive test for hemolytic anemia by flow cytometry using anti-Hb, which detects many types of membrane damaged RBCs.

Methods and Materials

Patients. We studied 92 patients with anemia, 62 patients with schistocytosis who did not have splenectomy, 8 patients with malaria, 8 patients with spherocytosis (including 3 patients with hereditary spherocytosis, 3 patients with autoimmune hemolytic anemia, and 2 patients with spherocytosis of unknown origin), 2 patients with elliptocytosis, 4 patients with iron deficiency anemia (IDA), and 108 normal controls (healthy adult platelet donors).

Fifty of 62 patients with schistocytosis had acute or chronic leukemia and 39 received BMT. Three of 8 post-splenectomy patients had splenectomy due to idiopathic thrombocytopenic purpura 1 mo to 5 yr prior to the study and 5 patients had splenectomy due to splenomegaly by chronic myelogenous leukemia 2 mo to 3 yr prior to the study. In all 3 hereditary spherocytosis patients, samples from parents and siblings were also studied. The osmotic fragility tests of the patients showed hemolysis beginning at 0.52% to 0.62% (w/v) sodium chloride (NaCl). All malaria patients were infected with Plasmodium vivax.

All of the patients gave informed consent to participate in this study. EDTA-anticoagulated blood samples for complete blood cell count were used within 4 hr post-collection. The samples were kept at room temperature (18 to 20°C) until analysis.

Flow cytometric detection of damaged RBCs. The anti-Hb (Dako A/S, Denmark) was conjugated with phycoerythrin (PE) and diluted in phosphate-buffered saline with 0.1% (w/v) gelatin and penta-chlorophenol as a preservative. The osmolality of the antibody base solution was 316 mOsm/kg H₂O (316 mmol/kg). Anti-Hb (5 µl) was mixed in 50 µl of each NaCl solution. Peripheral blood (2 µl) was added to the antibody-NaCl mixture and incubated in the dark for 15 min at room temperature. Next, 3 ml of saline was added without any washing step. Fluorescence was analyzed by flow cytometry (FACSCalibur, Beckton-Dickinson Co., San Jose, CA) using CELLQuest software. Quality control of the flow cytometer was carried out twice each week using CaliBRITE™ beads (Becton Dickinson) and monthly using Autocomp software. The instrumental settings of the forward- and side-scatter channels were log mode and the threshold level of forward-scatter channel was set at 52. Twenty thousand cells were analyzed.

Because some schistocytes were smaller than intact RBCs, a large gate was set that included all intact RBCs and platelets (Fig. 1). All samples were also stained with the isotypic control antibody (Becton-Dickinson) to set markers. Damaged RBCs were not stained by the anti-Hb in normal saline. To find the optimal concentration of NaCl in which hemoglobin in the damaged RBCs could be exposed to anti-Hb through damaged membranes, peripheral blood samples from 10 patients with schistocytosis and 10 normal controls were stained with anti-Hb in a serially diluted solution of NaCl from 0.2% to 2.0% (Fig. 2). To discriminate schistocytosis from normal samples, the best concentration was 0.6% NaCl.

To check the stability of reagents, 5 normal samples and 5 schistocytosis samples were stained with antibody immediately, and then daily for the next 7 da after mixing in 0.6% NaCl. The antibody was stable in 0.6% NaCl for 1 wk after preparation and the proportion of stained RBCs did not decrease. The osmolality of the PE conjugated anti-Hb-0.6% NaCl mixture was 213 mOsm/kg H₂O (213 mmol/kg). All of the other samples were analyzed with the PE-conjugated anti-Hb and 0.6% NaCl mixture.

Microscopic examination of RBC morphology. Blood smears from all of the samples were air-dried. The slides were stained with Wright’s stain and examined by two pathologists. The schistocytes were counted under the oil immersion fields (HPF); because the average number of RBCs in one HPF is 200, the number of schistocytes per HPF x 1/2 was used to express the schistocytes as a percentage.
Fig. 1 (at left). Bivariate distribution of the peripheral blood cells displayed in a plot of forward scatter (abscissa, log scale) versus side scatter (ordinate, log scale), showing a large gate set (rectangle) including intact RBCs and smaller fragmented RBCs.

Fig. 2 (below). In 0.6% NaCl solution, microangiopathic hemolytic anemia (MAHA) samples (right panel) show a significant proportion of RBCs stained with anti-Hb (bold line). Most RBCs of both normal controls (left panel) and MAHA samples are stained with anti-Hb in 0.2% NaCl (narrow line), and the proportions of stained RBCs decrease upon increase of NaCl concentration up to 0.6%. In 0.8% NaCl, none of the normal control and MAHA samples show stained RBCs (dashed line).

Table 1. The proportions of red blood cells (RBCs) stained by anti-Hb antibody in 0.6% NaCl solution in healthy controls and patients with various hematological disorders.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>N</th>
<th>Stained RBCs (%) mean±SD</th>
<th>p versus controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal controls</td>
<td>108</td>
<td>0.55±0.23</td>
<td>–</td>
</tr>
<tr>
<td>schistocytosis</td>
<td>62</td>
<td>2.95±2.95</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>malaria</td>
<td>8</td>
<td>1.87±0.72</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>spherocytosis</td>
<td>8</td>
<td>3.68±1.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>postsplenectomy</td>
<td>8</td>
<td>0.78±0.24</td>
<td>ns</td>
</tr>
<tr>
<td>iron deficiency anemia</td>
<td>4</td>
<td>0.59±0.11</td>
<td>ns</td>
</tr>
</tbody>
</table>

* ns, not significant (p > 0.05).
Statistics. All data was analyzed by the independent sample t-test. The Pearson correlation coefficient was used to measure the correlation between the percentage of RBCs labeled with anti-Hb and the number of schistocytes counted by microscopy. The statistical significance was evaluated with the Wilcoxon signed-ranks test using SPSS software.

Results

Flow cytometry in serial NaCl concentrations. In the normal controls and patients with schistocytosis, most RBCs were stained with anti-Hb in 0.2% NaCl solution, and the proportions of stained RBCs decreased when the NaCl concentration was increased to 0.6% (Fig. 2). At 0.6% NaCl, only the schistocytosis samples showed >1% stained RBCs, whereas all of the normal control samples showed <1% stained RBCs. Between 0.40% and 0.55% NaCl, a portion of RBCs in samples from normal controls and schistocytosis patients were stained with anti-Hb. At higher concentrations of NaCl (0.7% to 2.0%), none of the normal or schistocytosis samples showed stained RBCs.

Flow cytometry in 0.6% NaCl. The proportion of stained RBCs in each group is shown in Table 1. The mean proportion of stained RBCs in normal controls was 0.55 ±0.23%, and the cutoff value (ie, mean +2SD) was 1.01%. Only 6 of 108 normal controls showed >1.01% stained RBCs. The mean proportion of stained RBCs in patients with schistocytosis (2.95 ±2.95%) was significantly higher than the normal controls (p <0.001), and the proportion of stained RBCs in patients with malaria (1.87 ±0.72%) was significantly higher than normal controls (p=0.001). The only case that showed <1.01% stained RBCs revealed a single ring form of Plasmodium on the blood smears. The mean proportion of stained RBCs in patients with spherocytosis (3.68 ±1.05%) was significantly higher than the normal controls (p <0.001); all 8 samples revealed >1.01% stained RBCs. No significant difference was observed between the results in patients with hereditary spherocytosis versus autoimmune hemolytic anemia.

The results of flow cytometry were compared to the results of osmotic fragility tests. Two of the hereditary spherocytosis samples began hemolysis at 0.52% NaCl and did not show hemolysis at 0.6% NaCl by osmotic fragility test, whereas 5.06% and 2.24% of RBCs in these samples were stained with anti-Hb in 0.6% NaCl. The other hereditary spherocytosis sample began hemolysis at 0.62% and 3.40% of RBCs were stained with anti-Hb in 0.6% NaCl. A blood sample from this patient's father began hemolysis at 0.52% and did not show hemolysis at 0.6% NaCl by osmotic fragility test, while 4.04% of the RBCs were stained with anti-Hb in 0.6% NaCl.

Two cases showing marked elliptocytosis revealed 2.20% and 2.21% stained RBCs. The mean proportion of stained RBCs in patients who had prior splenectomy (0.78±0.24%) was similar to normal controls (p=0.07). Many poikilocytes, including target cells and spherocytes, and a few schistocytes (0 to 2.5%, mean 1.42%), were found in peripheral blood of the post-splenectomy patients, but the proportion of stained RBCs did not increase nor did the schistocytes counts correlate (p=0.85) with the proportion of RBCs stained by anti-Hb. All cases of iron-deficiency anemia (IDA) showed <1.01% stained RBCs (0.59±0.11%).

Discussion

How fragmented RBCs, ie, schistocytes, circulate in blood without losing their content of hemoglobin is unknown. Because a schistocyte loses a part of its cytoplasm, the hemoglobin inside the cell should be available through the damaged cytoplasmic membrane to the anti-Hb antibody outside the cell. However, schistocytes are not stained with anti-Hb in isotonic solution. This suggests that the damaged parts of the cytoplasm may be covered by temporary attachment or closure of both sides of the cytoplasmic membrane. Therefore, we attempted to expose the hemoglobin through the temporary cover of the damaged cytoplasm by incubating schistocytes
in hypotonic solution, 0.6% NaCl. Below this concentration, some normal RBCs were also stained with anti-Hb. Evidently, normal RBCs can be damaged by incubation in hypotonic solution that contains <0.6% NaCl, although they are incompletely hemolysed. Upon incubation in hypertonic solution up to 2% NaCl, the RBCs in normal and schistocytosis samples did not stain the RBCs with the anti-Hb. Deflated RBCs did not expose hemoglobin through the damaged cytoplasm.

The proportion of stained RBCs in normal controls was 0.55±0.23%, and it was significantly higher in patients with schistocytosis (2.95±2.95%, p <0.001). The number of schistocytes was correlated to the proportion of RBCs stained by anti-Hb (r=0.637, p <0.001). It appears, therefore, that staining RBCs with anti-Hb in 0.6% NaCl is an accurate method of estimating schistocytes. This flow cytometric method does not entail washing or lysing RBCs and the results are read within 20 min after blood collection.

Counting schistocytes in a blood smear is tedious and time consuming. Using this simple, rapid, flow cytometric method, the proportion of schistocytes can be estimated accurately and objectively. Furthermore, as in postsplenectomy cases, many poikilocytes (including target cells and acanthocytes) are sometimes found by microscopy without any clinical evidence of hemolysis [19]. In such cases, it is impossible to determine the significance of poikilocytosis, including the schistocytosis that develops in postsplenectomy cases, by use of microscopy alone. Using the flow cytometric method, poikilocytosis, including schistocytes in postsplenectomy patients, was not detected.

Since damaged cells are mainly eradicated from circulation by the spleen, it seems possible that schistocytes in postsplenectomy patients survive and circulate in the blood for sufficient time to repair the damaged cytoplasm. The flow cytometric method reported in this study could be the method of choice to diagnose and monitor microangiopathic hemolytic anemia in postsplenectomy patients.

The proportion of stained RBCs in patients with malaria (1.87±0.72%) was significantly higher than in normal controls (p=0.001). The RBCs are lysed during the course of malaria and cause hemolytic anemia [20]. Using the flow cytometric method, we found that some damaged RBCs circulate in the blood of malaria patients. How the RBCs of malaria patients develop cytoplasmic damage is poorly understood. However, the surface of red cells of children with severe Plasmodium falciparum anemia is modified by the deposition of IgG and by altered levels of complement regulatory proteins [20]. It is possible that such RBCs with modified surface membranes were stained with anti-Hb. In an endemic area, routine screening of febrile patients by this method might help to detect malaria patients and malaria-associated anemia.

The proportion of stained RBCs in patients with spherocytosis (3.02±1.12%) was significantly higher than in normal controls (p <0.001). There was no significant difference between hereditary spherocytosis and autoimmune hemolytic anemia in the proportion of stained RBCs. The mechanism of spherocytic change has previously been studied and defects in the structure of the cytoplasmic membrane have been reported [16,21]. Such defects seem large enough that anti-Hb can enter the cells. The laboratory tests currently available to diagnose hereditary spherocytosis are the osmotic fragility test and microscopic examination of peripheral blood. In a family with hereditary spherocytosis, the patient's parents and 2 siblings showed normal osmotic fragility and normal RBC morphology, but the patient's father showed an increased proportion of RBCs stained by the anti-Hb antibody using this flow cytometric method. This suggests that the flow cytometric method is more sensitive than the osmotic fragility test in detecting damaged RBCs.

Autoimmune diseases are a leading cause of death among young and middle-aged women in the United States [22]. Autoimmune hemolytic anemia occurs in about 10% of patients with systemic lupus erythematosus, but a mild degree of hemolysis may occur more frequently [1,23]. Hemolysis may be the sole presenting sign of autoimmune hemolytic anemia and may predate the appearance of other disease manifestations by several years [24]. However, little progress has been achieved in the laboratory diagnosis of autoimmune hemolytic anemia [25]. The direct antiglobulin test is the sole laboratory test for autoimmune hemolytic anemia.
and is insufficiently sensitive to diagnose this disease[17,18]. Early detection of mild hemolysis before the appearance of other disease manifestations may be feasible using the flow cytometric method. Elliptocytosis is a heterogeneous disease[16] and dysfunction or deficiency of membrane proteins has been reported [26,27]. Both of the elliptocytosis cases in this study showed increased RBC staining by anti-Hb using this flow cytometric method. Although spherocytosis and elliptocytosis are associated mainly with extravascular hemolysis, the results of this study demonstrate that some of these poikilocytes have large enough membrane defects to admit the anti-Hb in a hypotonic solution. None of the iron-deficiency anemia (IDA) cases or cases with tear-drop cells showed an increase in RBCs stained by anti-Hb. This suggests that these poikilocytes do not have sufficient membrane defects to expose the hemoglobin.

In conclusion, flow cytometric detection of damaged RBCs using anti-Hb in a hypotonic solution is a simple, sensitive, and accurate method for detection of both intravascular and extravascular hemolysis. This technique will be helpful in the early diagnosis of hemolytic anemia and will help to differentiate clinically significant damage of RBCs from clinically insignificant poikilocytosis.

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References

17. Gilliland BC, Baxter E, Evans RS. Red cell antibodies in acquired hemolytic anemia with negative