Acquired Immune Hemolytic Anemia Associated With IgA Erythrocyte Coating: Investigation of Hemolytic Mechanisms

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We have investigated the hemolytic mechanisms in a patient with acquired immune hemolytic anemia whose red cells appeared to be coated with IgA alone. The clinical course was similar to that of patients with hemolytic anemia mediated by warm-reacting IgG antibody. Splenic sequestration of red cells was demonstrated, and marked reduction of hemolysis occurred after corticosteroid therapy. Antibody was eluted from the patient's red cells and used to sensitize normal red cells in vitro. These sensitized red cells were not lysed by fresh autologous serum, nor did they fix detectable amounts of C3. However, red cells sensitized by eluted antibody were lysed by normal human peripheral blood monocytes in a system designed to demonstrate antibody-dependent cell-mediated cytotoxicity. Monocyte-mediated hemolysis of sensitized red cells was inhibited by the addition of low concentrations of normal serum IgA to the system, but not by IgG. The ability of the eluate to induce monocyte-mediated hemolysis was abolished by its adsorption on Sepharose-bound anti-IgA, but not by preincubation with Sepharose-bound anti-IgG. In addition, normal human monocytes were demonstrated to ingest eluate-sensitized red cells. These data demonstrate an in vitro interaction of IgA-sensitized red cells with leukocytes and suggest a possible mechanism for the patient's hemolysis.

**Case Report**

The patient, a 71-year-old white man, was cared for at Vanderbilt University Medical Center (VUMC) for seven years and was diagnosed clinically as having chronic autoimmune thrombocytopenic purpura, which was treated with intermittent low doses of prednisone. In July 1980, he was maintaining a normal platelet count while taking no corticosteroids, when he was admitted to his local hospital because of sudden onset of anemia and hemoglobinuria. The only drugs he had been taking were ibuprofen and chlorthalidone. Physical examination was unremarkable. The urine sediment contained no red cells, although the urine was strongly positive for hemoglobin by dipstick. The WBC count was 15,500/µL; the platelet count was 155,000/µL; and the hematocrit was 23.5%, with 11.1% reticulocytes. The peripheral blood smear demonstrated marked spherocytosis. A chemistry survey revealed a bilirubin level of 5.5 mg/dL and a lactate dehydrogenase (LDH) level of 1,900 U/L. A liver-spleen scan demonstrated splenomegaly, and a bone marrow aspirate showed erythroid hyperplasia. Indirect antiglobulin testing was negative, but the direct antiglobulin test (DAT) was weakly positive with polyspecific and anti-C3 antisera and was negative with anti-IgG antisera. A diagnosis of acquired immune hemolytic anemia was made. Ibuprofen was discontinued. The patient was begun on prednisone at 80 mg/d and referred to VUMC.

On arrival of the patient at VUMC, his physical examination was unchanged, except that a spleen tip was noted at the left costal margin. The hematocrit had declined to 20.5%, with 43.7% reticulocytes, and the patient was transfused with 2 units of frozen red cells. Quantitative assays of serum immunoglobulins revealed: IgG 800 mg/dL, IgA 70 mg/dL, IgM 123 mg/dL, IgE 230 U/mL, and IgD undetectable. Hemolytic complement and total C3 were decreased: CH50 = 144, C3 = 90 mg/dL, and antinuclear antibody and latex fixation tests were negative. A sucrose hemolysis test was negative. An acidified serum hemolysis test was initially positive, but this was thought to be due to spherocytosis. This test became negative as his spherocytosis lessened. Red cell survival studies demonstrated a 11Cr half-life of 7.1 days, with evidence of splenic sequestration. Repeat
antiglobulin testing at VUMC was negative using polyspecific, anti-C3, and anti-IgG antisera. However, further direct antiglobulin testing at the Nashville regional American Red Cross reference laboratory was positive with polyspecific antisera and with antisera monospecific for IgA (3+), but not for IgG, IgM, C3, or C4. A red cell eluate coated all normal red cells with IgA, but no red cell antibody was detected in the serum, even when tested with anti-IgA.

The patient responded to prednisone with an increase in his hematocrit to 34% to 40%. He continued to have active, compensated hemolysis 20 months after diagnosis. Exacerbations of hemolysis were treated with increased doses of prednisone. Azathioprine was prescribed in order to reduce the prednisone requirement. Direct antiglobulin testing remained positive for IgA only. In December 1982, the patient underwent splenectomy incidental to an emergency cholecystectomy. Since then, his DAT has become negative, and he has maintained a normal hematocrit as his steroid dose has been tapered.

**Human Subjects**

Informed written consent was obtained from the patient and from normal volunteers for all blood samples drawn for experimental study. The research plan was approved by the Vanderbilt Committee for the Protection of Human Subjects.

**Reagents/Immunglobulins**

IgG was prepared by ammonium sulfate precipitation\(^\text{10}\) and DEAE-cellulose (Schleicher and Schuell, Inc, Keene, NH) ion-exchange chromatography.\(^\text{11}\) Pure normal human serum IgA was purchased commercially (Cappel Laboratories, Inc, Cochranville, PA), and human IgM was the kind gift of Dr Larry B. Vogler of the Pediatric Immunology Division of Vanderbilt Medical Center.

**Antisera**

The monospecificity of antiglobulin reagents (anti-IgA and anti-IgM: Boeringer Mannheim GmbH, Mannheim, West Germany; anti-IgG: American Red Cross Blood Services, Washington, DC; anti-C3: Gamma Biologicals, Houston; anti-C4: Melloy Laboratories, Inc, Springfield, Va) was confirmed by a passive hemagglutination technique.\(^\text{12}\)

**Red Cell Eluate**

Eluates were prepared from the patient’s red cells at several times during his course by an acid digitonin method.\(^\text{13}\) The protein concentrations of the eluates were determined by the method of Lowry et al.\(^\text{14}\)

**Immunoabsorbsents**

Solid-phase immunoabsorbsents were removed to IgG or IgA selectively from the red cell eluate. Fifteen milligrams of goat heavy chain-specific anti-human IgG or anti-human IgA antiseraum containing 5 mg of antibody was coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, AB, Uppsala, Sweden).\(^\text{15}\) One volume of red cell eluate was incubated with 1 vol of settled anti-IgA or anti-IgG gel for 20 minutes at 20 °C. The gel was allowed to settle, and the supernatant was assayed for activity in promoting antibody-dependent cell-mediated cytototoxicity (ADCC). Eluate incubated with Sepharose with no bound antibody was a negative control.

**Measurement of Cytotoxicity/Target Cells**

Since the patient’s cells were spherocytic and too fragile to withstand extensive manipulation, all target cells were obtained from normal donors. Fifty microliters of packed, washed (three times), group O red cells were incubated with 20 μL of \(^{51}\)Cr as sodium chromate (ICN, Irvine, Calif) (2 mCi/mL) for 60 minutes. The radioactive cells were washed three times with 20 vol of saline and were then adjusted to a concentration of \(2 \times 10^8/\text{mL}\). The labeled cells were then sensitized by incubation with an equal volume of eluate at 37 °C for 60 to 120 minutes. Labeled red cells incubated with 6% bovine serum albumin (Ortho Diagnostics, Raritan, NJ) in saline, to provide an equivalent protein concentration, served as negative controls. Labeled Rh-positive red cells incubated with a 1:50 dilution of commercial, partially purified IgG anti-D (Cutter Biologicals, Calgary, Alberta, Canada) served as a positive control.

**Effectors Cells**

Leukocytes were prepared from 60 to 90 mL of normal heparinized blood by density centrifugation over Ficoll-Hypaque (Winthrop Laboratories, New York) (sp gr 1.075) by the method of Boyum.\(^\text{17}\) Mononuclear leukocytes from the plasma–Ficoll-Hypaque interface were washed three times with the alpha modification of Eagle’s minimum essential medium (α-MEM) and suspended (2 to 4 \(\times 10^8/\text{mL}\) in α-MEM containing 10% heat-inactivated human AB serum. Ten-milliliter aliquots of the suspension were incubated for 60 to 120 minutes at 37 °C in a 5% CO\(_2\) atmosphere in 100 x 15 mm plastic Petri dishes (Fisher Scientific Co, Pittsburgh).\(^\text{18}\) The adherent cells were then dislodged by gently scraping with a rubber policeman. Both cell fractions were washed twice with α-MEM and resuspended to \(1 \times 10^7/\text{mL}\) in RPMI 1640 ( Gibco, Grand Island, NY) buffered with 25 mmol/L HEPES (Calbiochem-Behring Corp, La Jolla, Calif), pH 7.3, and supplemented with 15% heat-inactivated fetal calf serum (Reheis Chemical, Phoenix). Microscopic inspection of Giemsa-stained films of the cell suspensions revealed that the adherent fraction consisted of 75% to 90% large mononuclear cells resembling monocytes; the principal contaminating cells were small lymphocytes, by morphological criteria, although 1% to 5% granulocytes were present in some preparations. The nonadherent, lymphocyte fraction consisted of 60% to 85% typical small lymphocytes and 15% to 40% larger mononuclear cells. Sixty to ninety percent of the adherent cells and 2% to 5% of the nonadherent cells prepared in this way were phagocytic, as judged by latex bead ingestion.\(^\text{19}\)

**ADCC**

The cytotoxicity assay was performed according to the method of Kurlander et al.\(^\text{16}\) Ten microliters of a saline suspension containing \(1 \times 10^7/\text{mL}\) sensitized, labeled red cells was added to triplicate or quadruplicate samples of 100 μL of leukocytes (1 \(\times 10^7/\text{mL}\) suspended in RPMI 1640 with 15% heat-inactivated fetal calf serum in round-bottom microtiter plates (Linbro Chemical Division of Flow Laboratories, Hamden, Conn). These were incubated at 37 °C in a 5% CO\(_2\) atmosphere on a rocking platform for 3% hours. Following incubation, the plates were centrifuged at 1,000 g for ten minutes, and the radioactivity in 50 μL of each supernatant was determined in an automatic gamma counter. The results are expressed as released index (RI):

\[
RI = \frac{\text{Radioactivity of the supernatant} \times 100}{\text{Total radioactivity}}
\]

Standard error of the mean of replicate determinations was calculated, and the differences were analyzed for significance using the Student’s t test for unpaired data.\(^\text{20}\) Positive and negative controls (anti-D sensitized and unsensitized red cells, respectively) were included in each experiment.
Inhibition of ADCC

In one set of experiments, various concentrations of normal IgG or IgA were added to the standard ADCC mixture to determine if either exerted an inhibitory effect. Results of these experiments are expressed as:

\[
\text{Percentage of inhibition} = 100 \times \left(1 - \frac{\text{Specific hemolysis with immunoglobulin}}{\text{Specific hemolysis without immunoglobulin}}\right),
\]

where specific hemolysis is the difference between leukocyte-mediated and baseline release index.

Complement-Induced Hemolysis

A micromodification of a standard hemolytic complement test was employed to determine whether the patient's antibody could initiate complement-dependent cytolysis in vitro. Group O red cells were labeled and sensitized as in the ADCC experiments. A suspension of 2 x 10⁶ red cells in 0.5 mL of barbital-buffered saline containing 0.25 mmol/L MgCl₂ and 1.8 mmol/L CaCl₂ (Barbitone; Inotrex Corp, Glenwood, Ill) was mixed with 2.0 mL of fresh or heat-inactivated autologous serum diluted 1:4 in barbital-buffered saline. The mixture was incubated for one hour at 37 °C and centrifuged at 1,000 g for ten minutes. Radioactivity of the supernatant fluid was determined in 1-mL aliquots. Complete hemolysis was produced by incubating the red cell suspension with 4 vol of 3% acetic acid, and results were expressed as percent positive control; red cells incubated with 6% bovine serum albumin in saline served as a negative control.

Fixation of Sublytic Complement

A 2% saline suspension of normal group O red cells was incubated with an equal volume of eluate at 37 °C for 30 minutes and then washed three times with saline before being resuspended at 4% in phosphate-buffered saline containing 100 mmol/L MgCl₂ and 30 mmol/L CaCl₂. These cells were then tested for agglutination with monospecific antiglobulin reagents before and after incubation for 60 minutes at 37 °C with an equal volume of fresh autologous serum.

Phagocytosis

With the remaining, somewhat less active, eluate, we investigated more directly the ability of mononuclear phagocytes to interact with eluate-sensitized red cells by means of a phagocytosis assay. Mononuclear leukocytes were prepared from normal whole blood as described above and suspended (1 x 10⁶/mL) in α-MEM supplemented with 10% heat-inactivated fetal calf serum that was previously absorbed with human red cells. One milliliter of this suspension was layered over a 25 x 25 mm coverslip in a 26 x 33 mm well coverslip were then washed vigorously with phosphate-buffered saline. The suspension was overlaid with red cells to be tested, which were suspended (1.7 x 10⁶/mL) in α-MEM with 10% heat-inactivated fetal calf serum. They were incubated a further 2% hours at 37 °C in 5% CO₂ atmosphere. The coverslips were then washed vigorously with phosphate-buffered saline (PBS), pH 7.2. The proportion of adherent leukocytes prepared in this manner was composed of phagocytes was > 90%, as judged by latex bead ingestion. The coverslips were overlaid with red cells to be tested, which were suspended (1.7 x 10⁶/mL) in α-MEM with 10% heat-inactivated fetal calf serum. They were incubated a further 2% hours at 37 °C in 5% CO₂. The coverslips were then washed gently with PBS, fixed with methanol, and stained with Wright's stain. The percentage of mononuclear cells ingesting red cells was determined in a count of 400 cells. Red cells were sensitized by incubating thrice-washed normal packed red cells in 10 vol of eluate for 120 to 180 minutes at 37 °C. Red cells sensitized with commercial anti-D served as positive controls, and red cells incubated in PBS in parallel with sensitized cells served as negative controls. To determine whether phagocytosis was specifically mediated by IgA autoantibody, normal IgA, or IgG (100 µg/mL) was added to some final incubations.

RESULTS

ADCC

A typical ADCC experiment is illustrated in Fig 1. An increased release index occurred only with red cells that were coated with the patient's red cell eluate or with IgG anti-D. Adherent mononuclear leukocytes were active effector cells, which were necessary for lysis in this system. In ten of 18 experiments where the activity of both adherent and nonadherent cell fractions was measured, the nonadherent fraction possessed significant activity, but less than that of the adherent cells. Further experiments were carried out with adherent leukocytes as effectors. A dose–response curve illustrating the dependence of cytotoxicity on the amount of eluate sensitizing the patient's red cells is shown in Fig 2.

Effect of Immunoabsorbents

When the eluate was treated with anti-IgA bound to Sepharose beads, its ability to promote ADCC was abolished (Fig 3). Similar treatment of the eluate with anti-IgG bound to Sepharose, or Sepharose that did not bind immunoglobulin, did not affect its ability to sensitize red cells for ADCC.

![Fig 1. Typical ADCC experiment. ⁶¹Cr-labeled red cells sensitized with patient's eluate were incubated with adherent and nonadherent mononuclear leukocytes. Unsensitized red cells served as negative controls, and Rh-positive cells coated with commercial anti-D served as positive controls in each experiment. Data are expressed as release indices ± SEM for quadruplicate determinations. Differences from baseline hemolysis were analyzed for significance by Student's t test: (a) not significant; (b) P < .06, (c) P < .01, (d) P < .001.](image-url)
not have antibody attached to it, did not destroy this activity.

**Inhibition of ADCC by Added Immunoglobulin**

The addition of normal human serum IgA to the ADCC system produced a marked inhibition of adherent leukocyte-dependent hemolysis at low concentrations. Addition of 70 μg/mL IgA decreased hemolysis by 50%, while addition of an equal amount of normal IgG produced one tenth of this degree of inhibition (Fig 4).

**Effects of Complement**

Red cells sensitized with patient eluate were not lysed by complement from fresh autologous serum, while group A red cells sensitized with group O serum showed about 100% lysis under the same conditions. Red cells sensitized with eluate also did not fix sublytic amounts of C3 detectable by direct antiglobulin testing.

**Phagocytosis**

Incubation of eluate-sensitized red cells on coverslips with adherent leukocytes resulted in red cell phagocytosis by 20.5% ± 1.0% (average ± SEM) of mononuclear cells in three separate experiments and no phagocytosis of unsensitized red cells (Table 1). Addition of normal IgA at a concentration of 100 μg/mL decreased the proportion of phagocytic cells by 70% (P < .01, Student’s t test for unpaired data), whereas addition of normal IgG at the same concentration resulted in no significant inhibition of phagocytosis.

**DISCUSSION**

There are three well-established mechanisms that lead to immune red cell destruction: (1) activation of the entire complement system, Cl through 9, producing direct red cell membrane damage; (2) adherence of C4b- and/or C3b-coated red cells to complement receptors on phagocytic cells; and (3) adherence of antibody-coated red cells to Fc receptors on phagocytes and possibly “killer” lymphocytes. It has not been clear which of these, if any, accounts for AIHA associated only with IgA, because the nature of the effector function of IgA is not well defined. Studies have failed to show that IgA can fix complement via the classical pathway, although it can utilize the alternate pathway. Receptors for Fc portions of human IgA have been recognized on human lymphocytes and granulocytes, but not on human monocytes. Some authors, therefore, are reluctant to assign any pathogenetic role to IgA in AIHA. However, recent work has demonstrated Fc receptors for

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**Table 1. Ingestion of Eluate-Sensitized Red Cells by Mononuclear Phagocytes**

<table>
<thead>
<tr>
<th>Red Cell Treatment</th>
<th>Percentage of Mononuclear Cells Ingesting Red Cells*</th>
</tr>
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<tbody>
<tr>
<td>Saline</td>
<td>0</td>
</tr>
<tr>
<td>Anti-D</td>
<td>66 ± 8</td>
</tr>
<tr>
<td>Patient eluate</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>Patient eluate + soluble IgA</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Patient eluate + soluble IgG</td>
<td>17 ± 5</td>
</tr>
</tbody>
</table>

* A suspension of red cells sensitized as indicated was layered over coverslips coated by adherent blood mononuclear leukocytes. The percent of mononuclear leukocytes ingesting red cells after a 2½-hour incubation was determined. Soluble immunoglobulin (100 mg/mL) was added to some incubations. Results are expressed as average ± SEM of three experiments.
rabbit IgA on subpopulations of human monocytes, and rabbit secretory IgA can synergize with IgG to promote ADCC by human monocytes, lymphocytes, and granulocytes. In addition, human IgA antibody against group C meningococci can promote human monocyte-mediated antimeningococci activity. These observations suggest that IgA might indeed produce immune hemolysis. The data reported here further support the notion that IgA (at least in some instances) is capable of producing hemolysis by directly mediating an immune adherence of sensitized red cells to phagocytes and possibly "killer" lymphocytes. First, although IgA was the only immunoglobulin detectable on the patient's red cells, he pursued a clinical course (spherocytosis, splenic sequestration of red cells, response to corticosteroids, and splenectomy) very similar to that of individuals with pure IgG warm antibody AIHA, which suggests that the mechanism of his hemolysis may be similar to that occurring in IgG AIHA. Second, the patient's eluate produced ADCC toward sensitized red cells that appeared to be IgA dependent, ie, the ADCC-promoting effect of the eluate was blocked by a solid-phase anti-IgA immunoadsorbent and by fluid-phase normal IgA. Third, the effect was dependent on the dose of IgA eluate used to sensitize red cells. Fourth, the patient's eluate promoted phagocytosis of sensitized red cells by normal mononuclear phagocytes. Fifth, both ADCC and phagocytosis promoted by the patient's eluate were complement independent. Sixth, the patient's eluate failed to produce complement-mediated hemolysis, and complement components could not be detected on sensitized red cells in vitro or in vivo. Taken together, these observations suggest a possible mechanism for what appears, clinically, in this patient to be a warm antibody AIHA mediated by IgA, ie, a complement-independent interaction of IgA-coated red cells with the patient's mononuclear leukocytes.

In the only reported study of the hemolytic mechanism of IgA AIHA, Suzuki et al described a patient whose red cells were coated with IgA and C3. These investigators presented evidence that their patient's IgA antibody fixed complement and that red cells so sensitized interacted with blood monocytes, at least in part, via complement receptors. Our findings suggest that a different mechanism may operate in our patient, but they are not necessarily in conflict with the data of Suzuki et al, since in neither case is the IgA subclass, its antigenic specificity, its degree of aggregation, or the degree of red cell sensitization in vivo known. Any of these factors might influence the effector function of IgA.

In the ADCC experiments performed with our patient's eluate, the adherent leukocyte fraction consistently exhibited effector activity. In some of these experiments, the nonadherent cell fraction produced 51Cr release as well, and some investigators believe that lymphocytes may produce significant immune hemolysis. The contamination of our nonadherent cell fraction with phagocytic cells makes it difficult for us to draw conclusions regarding the role of lymphocytes as effectors. However, it is clear from the phagocytosis experiments that mononuclear phagocytes can interact with eluate-sensitized red cells. Inhibition of eluate-promoted ADCC by IgA was not complete over the range of concentration tested, but the linearity of the inhibition curve suggests that higher concentrations of IgA would abolish ADCC altogether. Since these concentrations are in the physiologic range, our model does not entirely reproduce the in vivo situation if this patient's hemolysis was indeed mediated by IgA. In models of IgG AIHA, interaction of sensitized red cells with leukocytes is inhibited by similar concentrations of IgG. It has been shown that this inhibition occurs because these models do not approximate the conditions of hemoconcentration that obtain in the spleen, where most warm antibody hemolysis occurs.

Our findings indicate that treatment with corticosteroids and possibly cytotoxic drugs is rational therapy for some cases of IgA AIHA and show that splenectomy may be useful in patients with this disorder when it is resistant to drug therapy.

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