The Erythrocyte-Coating Substance in Autoimmune Hemolytic Disease: Its Nature and Significance

By Hugh Fudenberg, Isabel Barry and William Dameshek

When thrice-washed red cells are agglutinated by rabbit anti-human-globulin sera (Coombs' reagent), they are said to be "coated" with an "incomplete" (blocking) antibody. Such coating occurs in vivo in erythroblastosis fetalis as a result of placental transfer of maternal isoantibody, and in acquired hemolytic anemia of the immunologic variety ("autoimmune" hemolytic disease) as a result of unknown mechanisms. It may also occur in vitro by exposure of red cell suspensions to antisera containing incomplete antibodies of the appropriate specificity.

Despite the sensitive serologic methods now available for the detection of the "incomplete" antibody bound to the erythrocyte surface in autoimmune hemolytic disease, study of the nature of this "erythrocyte-coating substance" has been greatly impeded by the difficulties inherent in obtaining significant amounts of this material free of contamination by serum proteins or red cell stromata. This report deals with studies of the coating substance eluted from the red cells of autoimmune hemolytic disease, with particular reference to possible differences in serologic properties of the "erythrocyte-coating substance" (ECS) in idiopathic and symptomatic autoimmune hemolytic disease (AIHD) and in cases with "warm" as contrasted with "cold" autoantibodies.

MATERIALS AND METHODS

1. Clinical material (tables 1 and 2)

Eluates were prepared from the erythrocytes of 14 patients with AIHD, 10 with "warm" autoantibodies and 4 with antibodies of the "cold" variety. In some cases, serial eluates were prepared before, during, and after steroid therapy. The red cells in all cases

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*Symptomatic "autoimmune" hemolytic disease is defined as autoimmune hemolytic disease occurring in association with such diseases as leukemia, lymphoma, neoplasm, and the "collagen" disturbances..."
displayed both a 4+ direct antiglobulin (Coombs') reaction at the time of eluate preparation and a decreased lifespan as measured by chromium$^{51}$ (Cr$^{51}$) red cell survival studies. Control eluates were also prepared from normal erythrocytes, from Rh+ erythrocytes coated in vitro with a potent anti-Rh$_{	ext{a}}$ antiserum, and from the erythrocytes of 8 patients with diverse hematologic malignancies, but with negative serologic tests for AIHD. In these latter 8 cases, anemia and decreased erythrocyte survival time, as demonstrable by Cr$^{51}$ red cell survival studies, were present.

2. Eluate preparation

Elution was carried out by a method of Kidd, so modified as to permit quantitative elution. The elution method was rigidly standardized in an attempt to obtain a relatively constant proportion of the total amount of coating substance in each case. After removal of the plasma from 500 ml. of citrated whole blood, the erythrocytes were divided into four equal aliquots and washed six times in 250 cc. centrifuge cups. In those cases with "warm" antibodies, washing was carried out at 4 C. with fivefold volumes of cold saline; in the cases with "cold" incomplete antibodies, two of the four aliquots were washed with cold saline and two with warm saline (37 C.). The supernatant obtained following the sixth washing was tested by the indirect globulin method to insure absence of traces of residual plasma antibody. The erythrocytes were then lysed in a tenfold volume of slightly acidified (1 x 10$^{-4}$ M HAc) distilled water, and lysis further insured by freezing and thawing. Stroma was precipitated by adjusting the pH of the mixture to 5.7 with 0.1 N HCl. The precipitate was removed by centrifugation and washed six times with cold M/15 phosphate buffer, pH 5.7, to facilitate removal of hemoglobin (and other hemochromogens). Two of the four preparations containing "cold" autoantibody were washed with buffer at 37 C. The final stromal preparation was white to slightly gray in color.

The packed residual stroma preparation, approximately 4 per cent of the original volume of packed erythrocytes, was centrifuged in graduated centrifuge tubes, the supernatant removed, and a 2.5-fold volume of citrate-HCl buffer, pH 3.3, added. After rapid mixing, the pH of the mixture was adjusted to 3.3 by the dropwise addition of 1 N HCl; only a few drops were necessary. The mixture was kept at 25 C. for 15 minutes. The stromata were then separated by centrifugation at 3000 r.p.m. for 6 minutes at room temperature; the supernatant was then removed and its pH rapidly adjusted to 7.2 by the addition of 5N NaOH (1.0 per cent of the volume of the supernatant, plus a few additional drops when needed). The stroma precipitate formed during neutralization was then removed by centrifugation at room temperature, and the colorless supernatant eluate stored in sealed screw-cap vials at −20 C. Sodium azide, 0.25 per cent, was used as a preservative.

The eluates from the red cells coated with "warm" antibodies were tested for antibody activity prior to and subsequent to storage by exposing 2 per cent saline suspensions of thrice-washed pooled O Rh negative erythrocytes to equal volumes (usually 0.1 ml.) of serial dilution of each eluate. With eluates from cases with "cold" incomplete antibodies, tests were performed with both saline and fresh serum suspensions of washed, pooled O Rh negative erythrocytes.

3. Coombs' (antiglobulin) serum

Standard antiglobulin sera were prepared by the intramuscular injection of mixtures of normal human serum and Freund adjuvant* into rabbits weighing 4 Kg. Booster doses of human serum without adjuvant were given at 14 and 24 days, and the Coombs' sera harvested at 31 days. Antisera R18 and R19 were prepared against human sera obtained from normal donors. Antisera R21 to 28 were prepared against sera obtained from patients with malignant hematologic diseases (e.g., leukemia, lymphoma) without evidence of AIHD, despite diminished red cell survival time (as measured by Cr$^{51}$ erythrocyte half-life). Coombs' sera R$_{8,9,10}$, and 11 were prepared by intravenous injection of rabbits with eluates obtained from red cells of patients with AIHD, idiopathic in etiology.

*Obtained from Difco Laboratories, Detroit, as Bacto-Adjuvant.
ERYTHROCYTE-COATING SUBSTANCE IN AUTOIMMUNE HEMOLYTIC DISEASE

in 2 cases (R₀₈,9), and secondary to chronic lymphocytic leukemia and lymphosarcoma in R₀₁₀ and R₀₁₁, respectively. R₁₂ was prepared in a similar manner, using alum-precipitated human gamma globulin as the antigen. To avoid a disproportionate response to weaker, cross-reacting antigens, Freund adjuvant was not used in the preparation of R₀₈-₁₁. The rabbits were injected four times weekly for four weeks, using the immunization schedule of Kabat and Bezer. The rabbit antisera were harvested by intracardiac puncture. Aliquots of each antiserum were saved for precipitin tests. The remainder of each serum was diluted 1:10, heat inactivated at 56°C for 15 minutes, and absorbed with washed, pooled, normal A, B, A:B, and O cells. The antisera were diluted to comparable potency after parallel cross-titrations in block against Rh⁺ erythrocytes coated with serial dilutions of anti-Rh₀ antiserum.

4. Eluate total protein nitrogen
   This was measured by the biuret method.

5. Precipitin tests
   Precipitin tests were performed by adding 1.0 cc. of various antiglobulin sera to 4.0 cc. of eluate. This was followed by incubation for 1 hour at 37°C, refrigeration for 48 hours at 4°C, and centrifugation at 3000 r.p.m. for 1 hour at 4°C. Precipitates were washed three times in chilled saline. The eluate “antiglobulin reactive” ECS nitrogen concentrations were estimated by the Lowry method by comparison with standard curves previously calibrated by measurement of precipitates obtained after the addition of known amounts of gamma globulin to 1.00 cc. of antiglobulin serum. Supernatant fluids in all reaction tubes were tested for the presence of free antigen and free antibody.

6. Preparation of standard eluates
   Eluates 8, 9, 10, and 11 were titrated in parallel versus a 2 per cent saline suspension of pooled O cells by the indirect Coombs' method, using antiglobulin sera R₁₈ and 1₉. Titers obtained from eluates 8, 9, 10, and 11 were 1,280, 10,240, 2,560, and 1,280, respectively. Eluates 9 and 10 were therefore appropriately diluted to obtain “standard” titers of 1,280.

7. Red cell survival
   Apparent erythrocyte half-life was measured by the Cr⁵¹ method as modified by Donahue et al., using autologous red cells.

8. Electrophoretic analysis
   Electrophoretic analysis of the proteins of the sera of patients and of normal controls were carried out by the method of Bernfeld in a 2.5 ml. Tiselius cell of a Perkin-Elmer model 38 apparatus. The original protein buffer boundary was displaced 9 mm. into the limb of the cell and photographically recorded by means of the Longworth scanning method. The protein boundaries after exposure to an electric field of 10.5 volts/cm. (130 volt difference of potential between the two electrodes) for 70 minutes were photographed on the same film.

RESULTS

1. Eluate reactivity
   Seventeen eluates obtained from the erythrocytes of the 10 patients with “warm” antibody AIHD gave positive results when tested by the indirect
Coombs’ method with antiglobulin sera R18 and R19. Tests with eluates obtained from the erythrocytes of four normal (control) donors, with eluates from the erythrocytes of four cases of AIHD with “cold” antibody and from the erythrocytes of 12 patients with various hematologic malignancies without AIHD (table 1), gave uniformly negative results.

Positive precipitin tests were obtained with six “warm” eluates but no “cold” eluates (table 2).

2. Effect of differential absorption of Coombs’ sera on reactivity

Aliquots of R18 and R19 were adsorbed with (a) normal human serum; (b) serum from a patient with congenital agammaglobulinemia*; (c) buffered solution of Fraction II, (99 per cent gamma globulin, by moving boundary electrophoresis).† Each aliquot was then used in precipitin tests with the six “warm” eluates with which positive precipitin tests were obtained with unabsorbed antiglobulin sera. Results of precipitin tests with native and absorbed antisera R18 are listed in table 3. Identical results were obtained with similarly absorbed aliquots of R19.

3. Relation of ECS concentration to disease severity

The survival time of the patient’s own erythrocytes, as measured by Cr⁵¹ red blood cell apparent half-life, appeared inversely related to the quantity of ECS present in the eluate of each patient with AIHD (table 2). Of the six eluates containing sufficient ECS to give visible precipitin reactions, only four formed enough precipitate to permit measurement of antiglobulin-reactive ECS nitrogen by the Lowry method. Even with these four eluates, the colorimetric readings obtained were at the extreme end of the curve, so that the estimated results are in error by perhaps 25 per cent. However, they

<table>
<thead>
<tr>
<th>Eluates</th>
<th>Condition</th>
<th>Antiglobulin (Coombs’) sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>warm autoimmune hemolytic disease</td>
<td>3+ to 4+</td>
</tr>
<tr>
<td>4</td>
<td>cold autoimmune hemolytic disease</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>chronic lymphocytic leukemia*</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>chronic myelocytic leukemia*</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>lymphosarcoma*</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Hodgkin’s disease*</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>myeloma (M type)*</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>myeloma (gamma type)*</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Rh⁰ + cells coated with anti-Rh⁰ (1:50)</td>
<td>3+</td>
</tr>
<tr>
<td>4</td>
<td>normal control</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>saline control</td>
<td>0</td>
</tr>
</tbody>
</table>

*Plus anemia, reticulocytosis, guaiac negative stools, shortened Cr⁵¹ erythrocyte half-life, and negative serologic tests for autoimmune hemolytic disease.
†Each of these antisera used in indirect Coombs’ test against 5 warm eluates, 2 cold eluates, and against the eluate used in the production of the given antiserum.

*Dr. David Gitlin, Children’s Medical Center, Boston, most generously supplied this.
†A gift from Dr. Robert B. Pennell, Protein Foundation, Inc., Boston.
### Table 2.—Autoimmune Hemolytic Disease: Relation of Degree of Hemolysis to the Quantity of Erythrocyte-Coating Substance

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Disease Process</th>
<th>Warm or Cold</th>
<th>Steroid Rx (Months)</th>
<th>Serum Globulin Protein</th>
<th>Eluate</th>
<th>RBC destruction</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RBC destruction (days)*</td>
<td>leukemia half-life (days).</td>
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<tr>
<td>1a</td>
<td>idiopathic</td>
<td>W pre-Rx</td>
<td>increased</td>
<td>++</td>
<td>0.325</td>
<td>1.08</td>
</tr>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>idiopathic</td>
<td>W 2</td>
<td>gamma</td>
<td>+</td>
<td>0.214</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1c</td>
<td>idiopathic</td>
<td>W 6</td>
<td>0</td>
<td>0.019</td>
<td>-Δ</td>
<td>20</td>
</tr>
<tr>
<td>2a</td>
<td>idiopathic</td>
<td>W pre-Rx</td>
<td>normal</td>
<td>++</td>
<td>0.302</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>idiopathic</td>
<td>W 2</td>
<td>0</td>
<td>0.069</td>
<td>-Δ</td>
<td>16</td>
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<tr>
<td>2c</td>
<td>idiopathic</td>
<td>W 4</td>
<td>0</td>
<td>0.049</td>
<td>-Δ</td>
<td>20</td>
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<td>W pre-Rx</td>
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<td>++</td>
<td>0.611</td>
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<td>W 6</td>
<td>gamma</td>
<td>++</td>
<td>0.165</td>
<td>-Δ</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>idiopathic</td>
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<td>alphaγ</td>
<td>0</td>
<td>0.078</td>
<td>-Δ</td>
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<td></td>
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<tr>
<td>5a</td>
<td>lymphosarcoma</td>
<td>W 6</td>
<td>decreased</td>
<td>0</td>
<td>0.038</td>
<td>-Δ</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>gamma</td>
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<td></td>
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<tr>
<td>5b</td>
<td>lymphosarcoma</td>
<td>W 12</td>
<td>gamma</td>
<td>0</td>
<td>0.019</td>
<td>-Δ</td>
</tr>
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<tr>
<td>6</td>
<td>lymphosarcoma</td>
<td>W 12</td>
<td>normal</td>
<td>0</td>
<td>0.030</td>
<td>-Δ</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>gamma</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>chr. lymph.</td>
<td>W 6</td>
<td>markedly increased</td>
<td>0</td>
<td>0.051</td>
<td>-Δ</td>
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<td></td>
<td>leukemia</td>
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<td>gamma</td>
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<tr>
<td>8</td>
<td>chr. lymph.</td>
<td>W 9</td>
<td>normal</td>
<td>0</td>
<td>0.037</td>
<td>-Δ</td>
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<tr>
<td></td>
<td>leukemia</td>
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<td>gamma</td>
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<td></td>
<td></td>
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<tr>
<td>9</td>
<td>L. E.</td>
<td>W 3</td>
<td>increased</td>
<td>+</td>
<td>0.138</td>
<td>-Δ</td>
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<td>gamma</td>
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<td>10</td>
<td>L. E.</td>
<td>W 6</td>
<td>increased</td>
<td>0</td>
<td>0.091</td>
<td>-Δ</td>
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<td>gamma</td>
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<tr>
<td>11</td>
<td>idiopathic</td>
<td>C pre-Rx</td>
<td>double</td>
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<tr>
<td>12</td>
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<td>C 3</td>
<td>normal</td>
<td>0</td>
<td>0.069</td>
<td>-Δ</td>
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<tr>
<td>13</td>
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<td>0</td>
<td>0.037</td>
<td>-Δ</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gamma</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>? postviral</td>
<td>C pre-Rx</td>
<td>normal</td>
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<td>0.058</td>
<td>-Δ</td>
</tr>
<tr>
<td></td>
<td>pneumonia</td>
<td></td>
<td>gamma</td>
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</tbody>
</table>

*Chromium⁵1 erythrocyte half-life (days).

†Donahue, D.M., et al.⁵¹

Δ No measurable reaction obtainable.

### Table 3.—Precipitin Tests with Antihuman Globulin Serum R18

<table>
<thead>
<tr>
<th>Eluate</th>
<th>R18 (non-absorbed)</th>
<th>R18γ (absorbed with normal serum)</th>
<th>R18β (absorbed with gamma-globulinemic serum)</th>
<th>R18γ (absorbed with gamma globulin)</th>
<th>Control rabbit serum</th>
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<tr>
<td>1a warm</td>
<td>++</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1b warm</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2a warm</td>
<td>++</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3a warm</td>
<td>+++</td>
<td>0</td>
<td>+++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3b warm</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9 warm</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11 cold</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>12 cold</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>anti-Rhγ</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>saline</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

do indicate the order of magnitude of “antibody” nitrogen in the eluate. Moreover, the ratio of antibody nitrogen to total nitrogen in these four instances is approximately the same (0.3 per cent), suggesting that relatively
constant proportions of the total amount of ECS were removed in each case by the standardized elution method used (table 4).

4. **Similarity of specificity in the ECS of idiopathic and symptomatic hemolytic anemia**

Electrophoretic analysis disclosed unusual serum protein electrophoretic patterns (double gamma, alpha\(^x\) and hypogammaglobulinemia patterns) in several of the idiopathic cases (figs. 1 and 2). Such patterns were not observed in the symptomatic cases (fig. 3). Attempts to demonstrate preferential specificity of antisera produced using idiopathic AIHD erythrocyte-coating substance (e.g., Re, 8,9) or symptomatic AIHD erythrocyte-coating substance (Re, 10,11) were unsuccessful. Tests in parallel with antiserum Re,8 against erythrocytes coated with standard eluates 8, 9, 10, and 11 gave identical end-points. Similar results were observed with anti-sera Re,9, Re,10, and Re,11 (table 5).

The use of eluates 8, 9, 10, and 11 in antiglobulin inhibition tests gave

<table>
<thead>
<tr>
<th>Eluate</th>
<th>Total protein N (mg./ml.)</th>
<th>Antiglobulin reactive protein N gamma/ml.</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>0.325</td>
<td>1.08</td>
<td>3.32 x 10(^{-4})</td>
</tr>
<tr>
<td>1b</td>
<td>0.214</td>
<td>0.65</td>
<td>3.02 x 10(^{-4})</td>
</tr>
<tr>
<td>2a</td>
<td>0.302</td>
<td>0.93</td>
<td>2.82 x 10(^{-4})</td>
</tr>
<tr>
<td>3a</td>
<td>0.611</td>
<td>1.80</td>
<td>2.94 x 10(^{-4})</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>3.01 x 10(^{-4})</td>
</tr>
</tbody>
</table>

Fig. 1.—“Double gamma” serum electrophoretic pattern, case 11, idiopathic A.I.H.D. (autoimmune hemolytic disease).
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Fig. 2.—Alpha' globulin serum electrophoretic pattern, case 4, idiopathic A.I.H.D. (autoimmune hemolytic disease).

Fig. 3.—Normal serum electrophoretic pattern, case 6, symptomatic hemolytic anemia.

partial inhibition in dilutions 1:2, 1:4, and 1:8, and proved ineffective in greater dilution. The four eluates gave essentially identical results in such tests, regardless of the eluate used for coating or of the antiserum used.
5. Serologic investigations of hematologic malignancies

Eight antiglobulin sera (R 21–28) (table 6a) prepared using serum from patients with various hematologic malignancies (anti-H.M.-globulin sera) failed to produce positive reactions when tested by the indirect Coombs’ method against normal red cells coated with previously prepared eluates from the H.M. globulin donors. Uniformly negative results were also obtained when these antisera were used in direct “anti-H.M.-globulin tests” against the erythrocytes of numerous patients with each disease entity. All subjects (table 6b) displayed moderate to marked anemia (hemoglobin 6–10

### Table 5.—Titer of Standard Eluates* 8–11 Using Antisera R18-11

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Eluate: 8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>R18 8</td>
<td>1280</td>
<td>1280</td>
<td>1280</td>
<td>1280</td>
</tr>
<tr>
<td>R19 9</td>
<td>1280</td>
<td>1280</td>
<td>2560</td>
<td>1280</td>
</tr>
<tr>
<td>R11 10</td>
<td>1280</td>
<td>1280</td>
<td>1280</td>
<td>1280</td>
</tr>
<tr>
<td>R11 11</td>
<td>640</td>
<td>1280</td>
<td>1280</td>
<td>640</td>
</tr>
</tbody>
</table>

*Eluates diluted to provide identical end-points by indirect Coombs’ test with standard antiglobulin sera R18 and R19.

### Table 6a

<table>
<thead>
<tr>
<th>Anti-HM Globulin Serum</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 21</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>R 22</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>R 23</td>
<td>Chronic granulocytic leukemia</td>
</tr>
<tr>
<td>R 24</td>
<td>Chronic granulocytic leukemia</td>
</tr>
<tr>
<td>R 25</td>
<td>Lymphosarcoma</td>
</tr>
<tr>
<td>R 26</td>
<td>Hodgkin’s disease</td>
</tr>
<tr>
<td>R 27</td>
<td>Multiple myeloma (gamma type)</td>
</tr>
<tr>
<td>R 28</td>
<td>Multiple myeloma (M type)</td>
</tr>
</tbody>
</table>

### Table 6b.—Direct Antiglobulin Test

<table>
<thead>
<tr>
<th>Disease Entity</th>
<th>Patient Tested</th>
<th>Antiserum</th>
<th>Control Sera (Commercial)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without Autoimmune</td>
<td></td>
<td>R21</td>
<td>R22</td>
</tr>
<tr>
<td>Hemolytic Disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chr. lymph leukemia</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chr. gran. leukemia</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphosarcoma</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Giant follicle</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hodgkin’s myeloma</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>With Autoimmune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Idiopathic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Symptomatic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. disc. lupus erythematosus</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>2. chr. lymph leukemia</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>3. lymphoma</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>4. Hodgkin’s disease</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Identical results with R19.

†Ortho Laboratories, Raritan, N.J.; Blood Grouping Laboratory, Boston, Mass.; Knickerbocker Bio-Sales, Inc., N.Y., N.Y.
ERYTHROCYTE-COATING SUBSTANCE IN AUTOIMMUNE HEMOLYTIC DISEASE

Gm. per cent), reticulocytosis (3–8 per cent) and negative Coombs' tests using the usual type of antiglobulin sera. However, the anti-H.M. sera gave positive results in each instance in which positive Coombs’ tests were demonstrated using Coombs’ sera prepared from normal donors (tables 1 and 6b).

DISCUSSION

The nature of the “erythrocyte-coating substance” (ECS) responsible for the positive antiglobulin (Coombs') reaction in acquired hemolytic anemia remains obscure. Several laboratories have maintained that the substance is an autoantibody directed against the patient’s erythrocytes, and that this autoantibody is directly implicated in the pathogenesis of the hemolytic state. However, this concept has not been universally accepted.

Understanding of the ECS has been hampered by difficulty in obtaining sufficient quantities of the material. Attempts to correlate the severity of the hemolytic process with the quantity of “antibody” presumably present in a given case have been generally unsuccessful. Most attempts to measure concentration of erythrocyte-bound antibody have been based on results of titration of antiglobulin sera, the end-point being the greatest dilution of antiglobulin sera producing a positive direct antiglobulin (Coombs') reaction. Such methods, encompassing the nonlinear range of antigen-antibody interaction, and including the range of marked antigen excess, are based on obviously erroneous assumptions and are cited only to be condemned. Quantitative expression (1 to 4+) of the degree of positivity of the direct antiglobulin (Coombs') reaction is of little aid, since in most cases the direct Coombs’ test remains strongly positive for many months after marked clinical improvement has been produced by steroid administration. However, use of serial dilutions of ECS eluted from the erythrocyte stromata by semiquantitative technics, in combination with constant amount of Coombs’ antiglobulin sera (in effect anti-ECS sera) of sufficient strength to provide moderate antibody excess, provides an immunologically sound method of measuring relative amounts of the erythrocyte-bound coating substance. In such a reaction mixture, the ECS functions as the antigen and the antiglobulin serum as the antibody.

The finding that the antigen nitrogen/total protein nitrogen ratio was relatively constant in the four eluates in which the antigen (ECS) nitrogen was measurable affords support for the assumption that the rigidly standardized elution method employed did, indeed, remove a relatively constant amount of ECS in each instance.

Based upon the above assumption, the quantity of ECS present in each case was well correlated with the degree of hemolysis, as measured by diminution in Cr erythrocyte apparent half-life (table 2). Quantities of ECS sufficient to produce visible precipitin reactions were present only in those instances in which the erythrocyte survival time was most severely shortened. Furthermore, qualitative estimates of magnitude of precipitin reaction were in general inversely related to erythrocyte life span. Indeed, the four patients with the greatest diminution of erythrocyte life span were the only ones in whom the amount of ECS antigen nitrogen was sufficient to
warrant measurement by quantitative methods. It is thus evident that the severity of the hemolytic process was indeed closely related to the concentration of erythrocyte-bound coating substance. The findings of simultaneous progressive diminution in ECS concentration during the progressive increase in Cr^{3+} erythrocyte life span during steroid therapy in four cases of AIHD with "warm" antibodies (table 2) are further evidence of the interrelationship between the ECS concentration and the severity of the disease process. It is realized that a cause and effect relationship rather than an interrelationship does not necessarily follow, although the above data are strongly suggestive; demonstration of a cause and effect relationship would undoubtedly furnish strong support for the protagonists of the "autoimmune" etiology of acquired hemolytic anemia.

Since ACTH and the corticosteroids have been shown by many^{22-25} to decrease antibody production, the diminution in concentration of ECS following steroid administration, with the concomitant increase in erythrocyte life-span, suggests that the ECS is an antibody, as does the demonstration that the Coombs'-reactive ECS nitrogen is bound to the erythrocyte stroma and can be separated from the stroma by standard technics for antibody-antigen dissociation.^{6} The results of precipitin tests using antihuman-globulin sera absorbed with agammaglobulinemic and normal human sera suggest that the ECS in "warm" AIHD is a gamma globulin, or at least has cross-reactivity with gamma globulin. These findings confirm impressions gained by other investigators, using less reliable agglutination inhibition rather than precipitin inhibition methods.^{20,27} Attempts to characterize the ECS as to its immunologic similarity to, or difference from, the more usual variety of serum gamma globulins using agar diffusion methods^{28} are now in progress and will be reported later elsewhere. Demonstration of the gamma globulin character of the ECS is of more than academic interest in view of the similar nature of most antibodies. However, in contrast to other erythrocyte pan-antibodies, e.g., saline cold hemagglutinins, the "autoantibodies" of AIHD may display both species and antigen specificity. Although the saline cold hemagglutinin nonspecifically agglutinates the erythrocytes of all humans and of various mammalian species,^{29-31} the antibodies of "autoimmune" hemolytic anemia react only with human red cells, i.e., display species specificity.^{32,33} In addition, the use of panels of red cells varying antigenic composition has demonstrated that eluates obtained from the red cells of a patient with AIHD often possess preferential specificity for one or another of the blood group antigens present in the red cells of the donor patient,^{34-37} i.e., autospecificity. If the previously propounded evidence for the antibody nature of the ECS in AIHD is accepted, the findings of preferential specificity against blood group factors present in the patient's own red cells strongly suggest that the disease is autoimmune in nature, at least in the "warm" cases. Final proof of an "autoimmune" process awaits more precise delineation of the antigen or antigens implicated and the mechanism(s) whereby immunization is produced.

Since the eluates from the cases of "cold" AIHD failed to give positive precipitin tests, failed to produce antiglobulin serum, and failed to react by
ERYTHROCYTE-COATING SUBSTANCE IN AUTOIMMUNE HEMOLYTIC DISEASE

indirect-Coombs' method with saline or fresh serum suspensions of pooled red cells, the assumption by previous workers that the "coating substance" in "cold" AIHD is an antibody merits re-evaluation. Our findings suggest that the "coating substance" in the "cold" AIHD cases is, instead, an adsorbed protein lacking antibody characteristics and not amenable to separation from the stroma by standard methods of antigen-antibody dissociation. These findings confirm objections to the "antibody" concept of the coating substance in "cold" AIHD raised recently by Dacie; this worker has provided evidence that the coating substance in such cases is complement adsorbed to the red cell surface rather than antibody.

As previously noted, only a small percentage of the protein nitrogen present in the eluates reacted in precipitin test with Coombs' (antiglobulin) serum. The nature of the non-Coombs' reactive protein nitrogen remains obscure, as does the nature of the nonprotein nitrogen present. Investigations of pooled lyophilized eluted material revealed a carbohydrate content equivalent (by weight) to 24 per cent of the total protein content. Hexosamine comprised less than 15 per cent of the total carbohydrates. Other amino sugars, pentoses, desoxypentose, and uronic acid could not be identified.

The large amount of non-Coombs reactive protein nitrogen may result from a considerable loss of immunologic reactivity of the ECS during the elution process, without degradation of a degree sufficient to alter the biuret reaction. It is also possible that the non-Coombs reactive protein is an enzyme derived from complement (C1), similar to the esterase eluted from antigen-antibody aggregates by Lepow et al., using pneumococcal specific soluble substance (SIII) and anti-SIII-rabbit sera. These latter investigators noted enhancement of C1 esterase activity upon elevation of the pH from 5.5 to 7.4. Studies on pooled lyophilized AIHD eluates revealed a marked decrease in titer following dialysis (at 4 C.) at slightly alkaline pH, without decrease in titer upon dialysis at pH ranges from 3.2 to 5.5, also suggesting the presence of proteolytic enzyme active at alkaline pH as a factor contributing to the non-Coombs-reactive eluate protein.

Others have speculated that positive antiglobulin tests in acquired hemolytic anemia are nonspecific manifestations not necessarily implicated in the disease process, implying that the hemolysis is secondary to the hematologic malignancy per se in the symptomatic cases and to unrecognized viral or chemical agents in the "idiopathic" cases. Such speculations would be strengthened if differential specificity could be demonstrated between the idiopathic and "symptomatic" cases. Observations of unusual serum globulin distribution patterns on moving boundary electrophoresis in several patients with idiopathic AIHD (figs. 1-2) in conjunction with relatively normal electrophoretic patterns in 8 patients with "symptomatic" AIHD (fig. 3) also suggested the possibility of differences in reactivity of the ECS in these two groups of cases. However, indirect Coombs' titration and inhibition tests using appropriate antisera against cells coated with ECS obtained from

*Performed by Dr. Peter Bernfeld, Tufts University School of Medicine, Massachusetts.
idiopathic and "symptomatic" cases of AIHD disclosed no preferential specificity, a finding in keeping with our clinical observations as to the identity of the clinical manifestations in these cases.

Antiglobulin (Coombs') sera prepared against the serum proteins of patients with various hematologic malignancies involving the antibody-forming tissue (spleen, lymph nodes, etc.) failed to give positive direct or indirect Coombs' tests in cases negative with tests using Coombs' sera prepared against normal, rather than abnormal, human sera. These antisera were produced to test the hypothesis that alteration of the reticuloendothelial system, i.e., the site of the body's antibody "factory," by proliferative disease might conceivably result in formation of abnormal serum proteins with resultant incorporation of abnormal protein into antibodies coating the erythrocytes. Conceivably, standard normal Coombs' antiglobulin serum, directed against normal human globulin, could fail to react with abnormal globulin coating the erythrocytes. However, the results obtained using Coombs' sera produced against serum obtained from patients with reticuloendothelial malignancies, anemia, and diminished erythrocyte survival differed in no way from the results obtained with the use of standard (antinormal human globulin) Coombs' serum. Use of the former sera failed to demonstrate erythrocyte-bound antibody in any instance where ECS was not detectable by standard (antinormal human globulin) Coombs' antiserum.

Conclusions

1. These observations indicate that the life span of the erythrocytes, measured in 14 instances of autoimmune hemolytic disease, was inversely proportional to the concentration of erythrocyte-coating substance.

2. Cortico-steroid therapy resulted in a decrease in the concentration of erythrocyte-coating substance and a concomitant increase in erythrocyte life span.

3. Indirect Coombs' inhibition tests, precipitin, and precipitin inhibition tests demonstrated that the erythrocyte-coating substance in "warm" autoimmune hemolytic disease was either a gamma globulin or a substance cross-reacting with gamma globulin. The lack of reactivity of the erythrocyte-coating substance of autoimmune hemolytic disease with "cold" antibody suggests that in this disease state the erythrocytes are coated by an adsorbed protein lacking antibody characteristics.

4. No difference in reactivity was observed in the erythrocyte-coating substance of cases of autoimmune hemolytic disease of the idiopathic and of the "symptomatic" varieties, despite unusual serum protein patterns obtained upon moving boundary electrophoresis in three cases of the idiopathic variety.

5. Antisera prepared against the serum proteins of patients with various hematologic malignancies, anemia and decreased erythrocyte survival and negative serologic tests for autoimmune hemolytic disease, failed to demonstrate erythrocyte-bound antibody in these cases.

6. These findings suggest the unitarian nature of all cases of autoimmune hemolytic disease of the "warm" variety and furnish evidence for the antibody nature of the erythrocyte-coating substance present in this disease.
ERYTHROCYTE-COATING SUBSTANCE IN AUTOIMMUNE HEMOLYTIC DISEASE

The hemolytic anemia of the hematologic malignancies with negative Coombs’ test is presumably nonimmune in origin.

Summary in Interlingua

1. Le duration del vita de erythrocytos, mesurate in 14 casos de autoimmun morbo hemolytic, esseva inversemente proportional al concentration del substantia revestiente le erythrocytos.

2. Cortico-stereide effectuava un reducite concentration del substantia de revestimento erythrocytic e concomitantemente un augmentate duration vital del erythrocytos.

3. Indirecte tests de inhibition de Coombs, tests de precipitina, e tests de inhibition de precipitina demonstrava que le substantia de revestimento erythrocytic in autoimmun morbo hemolytic a thermo-anti-corpore esseva un globulina gamma o un substantia capace de reaction cruciate con globulina gamma. Le absentia de reactivitate del parte del substantia de revestimento erythrocytic in autoimmun morbo hemolytic a cryo-anticorpore suggere que in iste condition le erythrocytos es revestite de un adsorbite proteina sin le caracteristicas de un anticorpore.

4. Esseva observate nulle differentia del reactivitate del substantia de revestimento erythrocytic inter casos de autoimmun morbo hemolytic del varietate idiopathic e casos del varietate symptomatic, in despecto del facto que inusual configurationes de proteina seral esseva obtenite per electrophorese a limite movente in tres casos del varietate idiopathic.

5. Antiseros preparate anti le proteinas seral de patientes con varie malignitates hematologic, con anemia, e con reducite superviventias erythrocytic e negative tests serologic pro autoimmun morbo hemolytic non demonstrava in iste casos le presentia de anticorpore ligate al erythrocytos.

6. Iste constatationes suggere le natura homogenee de omne casos de autoimmun morbo hemolytic del varietate a thermo-anticorpore. Illos supporta le conclusion del natura anticorporee del substantia de revestimento erythrocytic que es presente in iste morbo. Le anemia hemolytic de malignitates hematologic con negative tests de Coombs es probablemente de origine non-immun.

References


8. National Institutes of Health. Minimum requirements: Anti-human serum for the


b. ———: Unpublished data.


The Erythrocyte-Coating Substance in Autoimmune Hemolytic Disease: Its Nature and Significance

HUGH FUDENBERG, ISABEL BARRY and WILLIAM DAMESHEK