Erythrocyte Autoantibody Associated with Alpha-Methyldopa: Heterogeneity of Structure and Specificity

By Richard F. Bakemeier and John P. Leddy

The association of positive direct antiglobulin (Coombs) tests and hemolytic anemia with the antihypertensive drug, alpha-methyldopa, was first reported in 1966.2,3 This association has potential significance as a model for studies of the pathogenesis of idiopathic autoimmune hemolytic anemia. As part of a broader effort to gain insights into the nature of erythrocyte autoantibodies through systematic study of their structural characteristics,4 the red cell antibodies occurring in a patient during alpha-methyldopa therapy have been examined in detail. The results indicate that this antibody population is heterogeneous in structure and specificity.

Case Summary

F.A., a 60 year old white woman with essential hypertension and diabetes mellitus, had been treated with alpha-methyldopa (α-MD) since suffering a mild cerebral vascular accident in September 1965. Initially the dose was 0.75 Gm. daily, but on March 1, 1966, it was raised to 1 Gm. daily. As shown in Table 1, her hematocrit was initially normal. However, in mid-April 1966, the patient noticed increasing fatigue and in May was hospitalized with Coombs-positive hemolytic anemia. No evidence of underlying lymphoproliferative or connective tissue disease was found. The patient had taken no other drugs except insulin and an occasional aspirin. She had never been pregnant or received a blood transfusion. No therapy was given except to withdraw the α-MD. Recovery was prompt (Table 1). Clinical details of this case will be recorded elsewhere.5

Materials and Methods

Serologic reagents and procedures for detecting RBC antibodies and determining their light-chain types in hemagglutination reactions have been previously described.4,6 RBC anti-
Table 1.—Sequential Hematologic Data

<table>
<thead>
<tr>
<th>Date</th>
<th>α-Methyl-dopa (Gm./day)</th>
<th>Hct. (%)</th>
<th>Retics (%)</th>
<th>DCT (titer)</th>
<th>Coombs Consumption Test</th>
<th>Serum Antibody ICT (titer)</th>
<th>Trypsinized RBC</th>
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</thead>
<tbody>
<tr>
<td>9/15/65</td>
<td>None</td>
<td>55§</td>
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<tr>
<td>9/17/65</td>
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<td>0.75</td>
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</tr>
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<td>0.75</td>
<td>40</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3/1/66</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/30/66</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5/11/66</td>
<td>1.00</td>
<td>26</td>
<td>16.0</td>
<td>1280</td>
<td>2</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>5/27/66</td>
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<td>34</td>
<td>5.3</td>
<td>320</td>
<td>Trace</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/28/66</td>
<td>None</td>
<td>41</td>
<td>0.6</td>
<td>160</td>
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<tr>
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<td>42</td>
<td>2.0</td>
<td>320</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>9/16/66</td>
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<td>41</td>
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<td></td>
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<tr>
<td>11/1/66</td>
<td>None</td>
<td>41</td>
<td>1.8</td>
<td>0</td>
<td>Pos.</td>
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</tr>
<tr>
<td>12/12/66</td>
<td>None</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/27/67</td>
<td>None</td>
<td>45</td>
<td>1.3</td>
<td>0</td>
<td>Neg.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations: Hct. = hematocrit; retics = reticulocytes; DCT, ICT = direct and indirect Coombs tests, respectively.

†Highest dilution of rabbit antihuman γG globulin giving macroscopic agglutination.

This modified Coombs consumption tests, possessing greatly enhanced sensitivity, was developed and performed by Dr. B. C. Gilliland. A positive result indicates RBC coating with a quantity of γG globulin which is abnormal but below the threshold of the Coombs test.

§This hct. was obtained at time of admission for cerebrovascular accident and is thought to reflect dehydration.

Bodies were eluted in pH 3.0 glycine-HCl buffer from large volumes of stromata prepared by a modification of the method of Kochwa and Rosenfield from thoroughly washed RBC. Eluates were immediately dialyzed in the cold against a neutral saline buffer and concentrated by negative pressure ultrafiltration. Eluate F.A. (5/11/66) (cf. Table 4) was prepared from 26 ml. of washed, packed RBC, and the initial eluate was concentrated from 60 ml. to 2 ml. before testing for immunoglobulin content. In contrast, pooled eluate F.A. (6/28 to 8/4/66) was derived from 135 ml. of packed RBC and was concentrated to 1 ml. Tests of antibody specificity were carried out by the indirect antiglobulin reaction employing untreated red cells. All agglutination reactions shown in the tables are macroscopic readings.

For studies on antibody specificity, eluates were absorbed with one-fifth to one-twentieth volume of washed, packed RBC of a given phenotype at 37 C. for 60 min. This step was repeated with fresh RBC until the absorbed eluate gave negative indirect antiglobulin reactions with the RBC phenotype used for absorption. The number of absorptions used varied with the potency of the eluate. In most experiments, three to five absorptions sufficed. With the most potent eluates, as many as nine to fifteen absorptions were required with certain RBC phenotypes. More than one example of the RBC phenotypes indicated in Table 2 were used for most absorptions and for all subsequent tests of absorbed eluates for residual antibody activity. For example, to demonstrate anti-e, aliquots of F.A. eluate were absorbed with four different DcE/DcE cells, and the respective supernatants were tested against a combined total of 6 DcE/DcE cells, 5 DcE/DcE cells, 2 ce/ce, 2 cE/ce, etc.

For the production of the 2* and 3* eluates depicted in Figure 1, the aliquots of RBC of a given phenotype which had been used for absorption, were pooled and thoroughly washed. Their stromata were eluted as above, elutions being repeated until antibody could no longer be detected in concentrated eluates. Active eluates were pooled for the indirect Coombs tests shown in Figure 1.

Antisera to human γG globulin and to κ or λ light chains were prepared and characterized...
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Fig. 1.—Schema of absorptions and elutions of F.A. autoantibodies. ICT = indirect Coombs test. SN = supernatant. The procedures used in absorptions and in production of 2° and 3° eluates are set forth in Methods.

as previously described. Antisera to the γG heavy-chain subclasses* γG1 (γ2b, We), γG2 (γ2a, Ne), and γG3 (γ2c, Vi) were prepared by immunization of monkeys or rabbits with purified myeloma proteins or isolated heavy chains of appropriate subclass. These proteins were originally typed by Dr. William Terry. Each antiserum was carefully absorbed with agammaglobulinemic serum, light chains, γA and γM globulins, and γG myeloma proteins of other subclasses. After the specificity of these antisera had been established in Ouchterlony reactions and radioprecipitin analysis against purified myeloma globulins of each subclass, aliquots of each antiserum were absorbed thoroughly with normal human RBC (groups A, B, and O) before use as antiglobulin reagents. The specificity of these antiglobulin reagents was then verified in hemagglutination studies employing RBC coated with myeloma proteins of known subclass by the bisdiazotized benzidine technic (cf. Ref. 4). Antiserum to the minor subclass, γG4 (γ2d, Ge), was not available at the time of this study.

A radioprecipitin inhibition (RPI) technic, modified from the methods of Weiler et al. and of Fahey and Lawrence, allowed quantitative measurement of light-chain types, heavy-chain subclasses, and total γG globulin in FA eluates. Isolated γG myeloma proteins of appropriate light- or heavy-chain type and normal γG globulin (chromatographically purified Cohn Fr. II) were trace-labeled with 125I by the method of Bale et al. An autoantibody eluate was then compared to a standard γG myeloma protein of known light or heavy chain type, or to normal γG globulin, in its ability to inhibit the precipitation of radiolabeled antigen by the corresponding antiserum. In order to avoid reactions based on "individual" antigenic determinants of myeloma proteins, the myeloma protein chosen as radiolabeled antigen or standard inhibitor was not the one used for immunization. In practice, the labeled

*The newly proposed nomenclature for immunoglobulin subclasses will be employed.
protein, the unlabeled inhibitor or eluate, and specific antiserum were mixed in a total volume of 1.5 ml and incubated at 37 C. for 30 min. and then overnight at 4 C. Radioactivity remaining in the supernatant fluid after centrifugation and in washed precipitates were each counted in a well-type gamma counter. Duplicate determinations agreed within 6 per cent, with a minimum activity of 20,000 cpm usually being present. Concentrations of \( \gamma \)G globulin as low as 0.1 to 1.0 \( \mu \)g./ml. could be measured accurately. The specificity of each system was verified by absence of significant inhibition of precipitation by 5 to 10 \( \mu \)g./ml. of the non-homologous proteins.

The great sensitivity of this RPI method and the known tendency of gamma globulin to nonspecifically adhere to washed normal RBC\(^6\),\(^17\) made it mandatory to compare eluates from patient F.A. to eluates made from comparable volumes of RBC from several normal donors. Despite our practice of washing RBC twice with 25 volumes of neutral buffered saline and four times with similar volumes of 0.15 M NaCl, and then washing the RBC stroma five more times with 0.15 M NaCl, significant amounts of \( \gamma \)G globulin were recovered from the RBC of four normal donors. Such eluates from normal donors, even after concentration, failed to sensitize normal RBC for antiglobulin reactions. This eluted \( \gamma \)G globulin is therefore thought to have been nonspecifically bound to the RBC membranes in vivo. Preliminary data indicate the quantity of nonspecific \( \gamma \)G globulin recovered in eluates from normal RBC is proportionate to the volume of packed red cells eluted. Because of this problem, the results of RPI determinations on F.A. eluates (Table 4) have been compared to the quantities of \( \gamma \)G globulin, or of light- and heavy-chain components, which one could potentially elute from a comparable volume of normal, Coombs-negative RBC.

RBC of phenotypes O D--/D-- (L.H. and J.C.) and A\(_r-/-/-\) (\( \text{Rh}_{null} \)) (L.M.)\(^18\) were generously donated by Drs. F. H. Allen and Arthur Rowe. A second \( \text{Rh}_{null} \) blood (J.R.B.) (group A) was provided by Dr. R. E. Rosenfield.\(^18a\)

**RESULTS**

Direct antiglobulin reactions with the patient’s RBC were strongly positive with anti-\( \gamma \)G globulin and negative with antisera to \( \gamma \)A and \( \gamma \)M globulins and to complement components (C\(_4\) and C\(_3\)). A low titer (1:2) of free antibody was initially found in the patient’s serum by indirect antiglobulin tests employing untreated group O RBC. Serial tests of the patient’s RBC (Table 1) revealed the gradual disappearance of the positive direct antiglobulin reaction within four months after withdrawal of \( \alpha \)-MD. However, two months after the antiglobulin reaction had become negative, an abnormal quantity of \( \gamma \)G globulin could still be detected on the patient’s RBC by a very sensitive Coombs consumption technic\(^19\) (Table 1). A highly concentrated eluate prepared from a large volume of RBC on this same date gave positive indirect antiglobulin reactions with normal human RBC. Seven months later, no increase in \( \gamma \)G globulin above control levels could be detected on the patient’s RBC by the same Coombs consumption method.

**Antibody Specificity**

The patient’s RBC phenotype was confirmed after the direct Coombs reaction had become negative and is O R\(_{1r}\) (CcDee), MsNs, P\(_{1+}\), kk, Le (a–b+), Fy (a–), Jk (a+). Antibody which had been eluted from the patient’s RBC at the height of the hemolytic anemia reacted strongly with autologous Coombs-negative RBC obtained during remission. Eluted antibody and serum antibody reacted essentially equally with all human RBC in several standard test panels. Eluted antibody gave slightly weaker but clearly positive reactions with two examples of D--/D-- RBC. However no reaction was observed with two \( \text{Rh}_{null} \) (\( \text{---}/\text{---} \)) RBC, which lack all known Rh antigens as
well as the “D-like” LW antigen.\(^{18}\) The failure of F.A. eluate to produce detectable sensitization of the RBC of three different rhesus monkeys appears to rule against anti-LW specificity (cf. Ref. 20). The RBC of two *Macaca speciosa* (“stump tail”) monkeys also gave negative reactions.

Failure of erythrocyte autoantibodies to react with D--/D-- or Rh\(^{null}\) RBC, despite strong reactions with all other available human RBC phenotypes, has been considered to suggest that such autoantibodies may possess specificity for one or more determinants related to the Rh system.\(^{21,29}\) This seemed an attractive possibility in regard to the autoantibodies of F.A. particularly since Worlidge et al.\(^{3}\) had reported evidence for Rh-related specificity in some of their cases. The apparent nonspecificity of F.A. eluate in studies with standard cell panels could be explained if multiple Rh-related specificities were represented.

Evidence for the presence of multiple antibody specificities in F.A. eluates was obtained by absorbing eluates with human RBC of a given Rh phenotype and testing for residual antibody activity against other Rh phenotypes (Table 2). The data in Table 2 are in summary form; i.e., the actual experiments employed several examples of each of the apparent RBC genotypes listed in the table. Absorption of eluates with DeE/DeE (R\(^2\)R\(^2\)) cells consistently left antibody which behaved serologically as anti-e (hr\(^n\)). (Table 2, Row 2). The positive reaction of unabsorbed eluate with D--/D-- RBC could not be ascribed to anti-D (Rh\(_D\)), since absorption with ce/ce (R\(_c\)) RBC left no detectable antibody against D (Rh\(_D\)) positive RBC (Table 2, Row 4). Absorption of eluates with D--/D-- RBC removed the antibody reactive with these cells but left potent antibody capable of reacting with RBC of all other apparent Rh genotypes tested (Row 1). Conversely, absorption of a native eluate with RBC of phenotypes DCe/DCe (R\(^1\)R\(^1\)) or DeE/DeE (R\(^2\)R\(^2\)) uniformly removed all antibody reactive with D--/D-- RBC (Table 2). Thus, it seems clear that the antigenic determinants on D--/D-- RBC with which a portion of the eluted autoantibodies were reactive are shared by “normal” Rh phenotypes (cf. Ref. 21). These observations, therefore, serve to identify an antibody specificity other than “anti-e.” As explained in the Discussion section, this second antibody population, reactive with two different D--/D-- RBC but unreactive with the Rh\(^{null}\) RBC, may be conceived as having specificity for undefined “Rh-related” antigen(s). For lack of a better notation and for ease of reference in this report, we will designate this serological specificity as “anti-Rh\(_{ref}\)1.”

After thorough absorption of an eluate with D--/D-- RBC, there was still reactivity with DeE/DeE RBC (Table 2, Row 1), indicating the presence of antibody specificities other than “anti-e” and “anti-Rh\(_{ref}\)1.” In efforts to characterize this additional antibody, no evidence was found for anti-E (hr\(^E\)). The patient, moreover, lacked E antigen on her own RBC, although this would not rule out recovery of a cross reacting “anti-E” in her eluates.\(^{22}\) When F.A. eluate was fully absorbed with DCe/DCe RBC (Row 3), slight residual activity against DeE/DeE cells was detected in some, although not all, experiments. This raised the possibility of a minor population of anti-c (hr\(^c\)). These same absorbed eluates invariably gave positive reactions with ce/ce RBC (Rows 3 and 6). Conceivably, there could be some anti-f (hr\(^f\)) in this eluate.
Table 2.—Summary of Autoantibody Specificities Revealed by Absorption Studies

<table>
<thead>
<tr>
<th>RBC Used to Absorb Eluate</th>
<th>D--/D--</th>
<th>DeE/DeE</th>
<th>DCe/DCe</th>
<th>Apparent RBC Genotype Tested Against Absorbed Eluate*</th>
<th>DcE/DeE</th>
<th>cE</th>
<th>ce</th>
<th>Ce/ce</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. D--/D--</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>++ + + + +</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. DeE/DeE</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>++ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3. DCe/DCe</td>
<td>0</td>
<td>0/trace</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td>4. cE/cE</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. DCe/DCe</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. D--/D-- +</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCe/DCe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Unabsorbed</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
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</table>

*Indirect antiglobulin reactions using optimal dilution of anti-γ G serum.
†Results in several experiments varied between negative and trace +.
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Anti-G seems unlikely because absorption with ce/ce RBC, which are G-negative, left no detectable antibody reactive with Dce/cDE RBC, which are G-positive. Additional evidence for the possible presence of antibodies with "anti-c" specificity was obtained as a dividend of efforts to produce sufficient "anti-e" for structural studies (see below). A large volume of concentrated F.A. eluate was absorbed fully with DcE/DeE RBC, leaving residual "anti-e" as expected (Fig. 1). Antibody absorbed by these DcE/DeE cells was eluted (2° eluate) and found to react strongly with both D-/-/D-- and various common Rh phenotypes (Fig. 1). From a small aliquot of this 2° eluate (aliquot a Fig. 1) antibody reactive with D--/D-- RBC ("anti-Rhrei-1") was absorbed out, leaving reactivity with RBC of other phenotypes. A larger portion of the 2° eluate (aliquot b) was absorbed with DcE/DcE RBC. The supernatant from this absorption showed selective reactivity with all RBC containing the c antigen. Stronger reactions were observed with RBC homozygous for c and weaker reactivity with DcE/DeE RBC, suggesting a possible dose effect (Fig. 1). No reaction was obtained with DcE/DeE or D-/-/D-- RBC. These data are interpreted as supporting earlier, less conclusive evidence for "anti-c" specificity among F.A. autoantibodies.

A 3° eluate prepared from the absorbing DcE/DcE RBC (Fig. 1) reacted strongly with all Rh phenotypes tested, including D-/-/D--. The antibody to D-/-/D-- ("anti-Rhrei-1") was readily absorbed out, leaving potent reactivity against DcE/DcE, DcE/DeE, and ce/ce RBC. The latter reactions may represent a second kind of specificity for undefined, possibly Rh-related antigen(s) common to many human RBC. This reactivity will be designated "anti-Rhrei-2." Other interpretations of this latter reactivity are possible, however.

Two variables which must be acknowledged in the foregoing studies utilizing serial absorptions are the effects of dilution and non-specific loss of antibody with each addition of packed RBC. However, the differential decrease of certain "specificities" in comparison to others during these absorptions suggests that a general, nonspecific decrease in antibody activity is not a major factor in these results. For example, in parallel studies of one eluate, three absorptions with one-fifth volume of DcE/DeE cells left potent antibody reactive only with RBC containing the e antigen; similar absorptions with ce/ce RBC removed all detectable antibody.

Antibody Structure

Direct antiglobulin tests were performed on the patient's RBC with rabbit antiserum to the κ and λ light chain types of human immunoglobulins. Agglutination was observed only with anti-κ. However, this reaction was relatively weak and the presence of type L molecules could not be excluded.*

*Direct antiglobulin tests with antiserum to the γG subclass determinants were not carried out. It has been our experience that when RBC are not sufficiently sensitized to give strong reactions with anti-light-chain sera their reactivity with anti-subclass sera is also too weak to be convincing.
Table 3.—Detection of Light and Heavy Chain Types by Agglutination of Normal RBC Sensitized by Unabsorbed F.A. Eluate

<table>
<thead>
<tr>
<th>Antiserum Employed</th>
<th>DcE/ce</th>
<th>Antiglobulin Reaction with RBC of Apparent Genotype*</th>
<th>DcE/DcE</th>
<th>ce/ce</th>
<th>D-./-D-.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-γG</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
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<td>+ + + +</td>
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<tr>
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<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Anti-λ</td>
<td>+ + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Anti-γG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>n.d.†</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>n.d.</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Anti-γG&lt;sub&gt;2&lt;/sub&gt;</td>
<td>n.d.</td>
<td>+ + + +</td>
<td>+ + + +</td>
<td>n.d.</td>
<td>+ + +</td>
</tr>
<tr>
<td>Anti-γG&lt;sub&gt;3&lt;/sub&gt;</td>
<td>+ + +</td>
<td>trace +</td>
<td>+ + + +</td>
<td>n.d.</td>
<td>trace +</td>
</tr>
</tbody>
</table>

*Negative control tests with normal monkey serum pool and normal rabbit serum pool are not shown.
†n.d. = not determined.

Table 4.—Measurement of Light and Heavy Chain Types of RBC Eluates by Radioprecipitin Inhibition

<table>
<thead>
<tr>
<th>Eluate</th>
<th>Type K</th>
<th>Type L</th>
<th>μg./ml. Eluted Protein γG&lt;sub&gt;1&lt;/sub&gt;</th>
<th>γG&lt;sub&gt;2&lt;/sub&gt;</th>
<th>γG&lt;sub&gt;3&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>5.5±3.6</td>
<td>6.0±3.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. F. A. (6/28 + 8/4/66 pool)</td>
<td>162</td>
<td>96</td>
<td>60</td>
<td>41</td>
<td>45</td>
</tr>
<tr>
<td>Control*</td>
<td>28±19</td>
<td>31±17</td>
<td>13±5</td>
<td>17±19</td>
<td>34±34</td>
</tr>
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</table>

*μg. protein of each specific type which might be recovered in eluates from comparable volume of normal RBC (mean of six normal donors ± standard deviation).
†n.d. = Insufficient quantity of eluate available for these determinations.

When a highly concentrated eluate was used to sensitize a small number of normal RBC in vitro, agglutination was consistently obtained with both anti-κ and anti-λ sera and with antisera to heavy chain subclasses γG<sub>1</sub>, γG<sub>2</sub>, and γG<sub>3</sub> (Table 3). This held true when a variety of Rh phenotypes were represented in the test red cell. Reactions with anti-κ serum and anti- γG<sub>1</sub> were somewhat stronger than reactions with the other antisera.

Radioprecipitin inhibition (RPI) assays confirmed the presence of both light-chain types in two eluates prepared on different dates (Table 4). Molecules of the γG<sub>1</sub> heavy-chain subclass predominated. Although the γG<sub>2</sub> and γG<sub>3</sub> heavy-chain subclasses were readily detected by the RPI technic, their respective concentrations were not clearly above those which might be found in eluates from comparable volumes of unsensitized, normal RBC (Table 4) (cf. Materials and Methods). Therefore, the extent to which the measured quantities of γG<sub>2</sub> and γG<sub>3</sub> molecules in F.A. eluate represent autoantibodies is uncertain. The hemagglutination data (see above, Table 3), however, indicate the inclusion of γG<sub>2</sub> and γG<sub>3</sub> molecules in the autoantibody population, because it was consistently found that eluted "nonspecific" γG globulin...
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does not sensitize test RBC for antiglobulin reactions. The control values for K or L molecules recoverable from normal RBC (Table 4) are proportionately much higher for the 6/28 to 8/4 eluate than for the 5/11 eluate, for two reasons. First, a larger packed cell volume was used to make the later eluate (cf. Methods). Second, the in vivo sensitization of F.A. cells by autoantibody was weaker on the later date (Table 1). Thus, the eluted γG globulin, which presumably comprises both autoantibody and nonspecific γG globulin, might well include a relatively smaller proportion of autoantibody molecules in the later eluate. For this reason, we feel that the more meaningful K:L ratio is that of the earlier eluate, i.e., 1.7:1 or, subtracting the control values, 1.8:1.

The foregoing studies were carried out on whole, unabsorbed eluates. Because this antibody population behaved as a mixture of antibodies with differing specificity, efforts were made to study the structural characteristics of the subpopulations separable by absorption. The “anti-Rhrei-1” subpopulation has so far been studied only by means of antiglobulin reactions with heavily sensitized D-/D- RBC. Such RBC were clearly agglutinated by anti-κ, anti-λ, anti-γG1, and anti-γG2 (Table 3). Anti-γG3, however, gave only a trace reaction. γG3 molecules were similarly difficult to detect among the antibodies sensitizing DCE/DCE RBC (Table 3); antibodies capable of sensitizing DCE/DCE RBC could include our postulated “anti-e”, “anti-Rhrei-1” and “anti-Rhrei-2.”

Extensive efforts were made to determine whether the “anti-e” subpopulation remaining after exhaustive absorption of an eluate with DeE/DeE RBC was more structurally homogeneous than the parent eluate. RPI studies were inconclusive due to the relatively small quantity of “anti-e” antibody which remained after such absorption and because of the problem of interpreting RPI results against the “background” of elutable nonspecific γG globulin. When DCE/DCE RBC were sensitized by this “anti-e” antibody for antiglobulin tests, only anti-κ and anti-γG1 produced agglutination. These reactions

*Since submission of the manuscript, further experience with our antisera to the γG subclasses has uncovered several instances in which hemagglutination reactions appeared to be based on some specificity other than subclass. This occurred despite evidence for strict specificity by other criteria, including the sensitive radioprecipitin assay and hemagglutination reactions using RBC coated (by DDB) with subclass-specific γG myeloma globulins. We must admit, therefore, that some uncertainty exists about those hemagglutination reactions in Table 3 upon which we depended to implicate γG2 and γG3 molecules in F.A. eluate as autoantibodies rather than just nonspecifically elutable γG globulin (see controls, Table 4). Such problems have not been encountered in RPI assays with these antisera, and we tentatively conclude that the difficulties in hemagglutination studies may be due to exposure of a “new” antigenic structure(s) when the autoantibodies react with RBC. Moreover, similar problems have never been observed with the antisera to κ or λ light chains. That the F.A. autoantibody population comprises γG molecules with predominantly γG1 heavy chains and both κ and λ light chains is on firm ground. The only uncertainty is whether the measured γG2 and γG3 content of the eluate (Table 4) represents small amounts of autoantibody in these subclasses together with some nonspecific γG globulin or represents entirely nonspecific protein. A recent reexamination of F.A. eluate by immunoabsorption with antisera to each subclass suggests that γG2 and γG3 autoantibodies are no more than a minor part of the total autoantibody population. Furthermore, very recent RPI analysis indicates that F.A. eluate cannot continue more than very minor population of γG4 molecules.
were weak, however, and it is impossible to judge whether the other light- or heavy-chain types were absent or merely below the threshold of detection.

**Discussion**

The possible implication of alpha-methylldopa (α-MD) in the pathogenesis of erythrocyte autoantibodies depends upon quite striking epidemiologic evidence. Although in any single patient it is not possible to exclude a chance association, the onset and subsequent disappearance of the positive direct antiglobulin test and the hemolytic process in relation to drug therapy in the present case fit the pattern established in the larger surveys. Anemia appeared after approximately seven months of α-MD therapy (Table 1). Recovery occurred promptly with no treatment other than withdrawal of α-MD.

The strong reaction of the eluted F.A. antibodies with autologous (Coombs-negative) RBC obtained during remission indicates that transition from active disease to remission was not associated with a serologically recognizable alteration in the receptor (antigen) sites on the RBC membrane. This observation, together with the data on antibody specificity (Table 2, Fig. 1), supports the general impression that the antibodies in this syndrome do not require the presence of the drug or its derivatives on the RBC membrane to sensitize the red cells. Thus, the mechanism appears to differ from that found in penicillin-associated immune hemolytic anemia.

Worledge, et al. reported that the antibodies from their patients often showed apparent specificity for antigens of the Rh system. This conclusion was based on differences in titers against various RBC phenotypes. In the present case, the possibility of Rh-related specificity was first suggested by the selective failure of autoantibody eluates to react with Rhnull cells. The specificity of F.A. autoantibodies for known antigens of the Rh system was not apparent, however, until eluates had been absorbed and tested with RBC of differing Rh phenotypes (Table 2, Fig. 1). These studies suggested the presence of at least four serologically separable antibody activities or subpopulations. Two of these subpopulations behaved as “anti-e” and “anti-c,” respectively. In addition, it was possible to separate from the whole eluate antibodies reactive with two undefined antigens (or groups of antigens) which are absent from the Rhnull RBC and which, therefore, may be related to the Rh system. One of these undefined but possibly Rh-related antigens (“Rhrell”) is present on D--/D-- RBC; the other is absent from D--/D-- RBC but present on all phenotypically “normal” RBC tested (“Rhrec”). Our postulated “Rhrell” and “Rhrec” may well correspond to the RBC antigens reacting with the two “nonspecific” antibody components previously described in cases of idiopathic autoimmune hemolytic anemia by Dacie and Cutbush and with the “anti-pdi1” and “anti-nil1” specificities, respectively, of Weiner and Vos. It is quite plausible...

*Since only two examples of Rhnull RBC were available, we have not excluded specificity of F.A. autoantibodies for antigens outside the Rh system represented in both examples of D--/D-- RBC but not in the Rhnull cells of donors L.M. and J.R.B. It should be noted that both these Rhnull RBC also demonstrate anomalies of serologic reactivity involving the U and S antigens.*
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It is not possible at present to state whether these antibodies of differing specificity arose as discrete populations or whether they were elaborated as a more or less continuous spectrum of specificities or binding affinities, which were artificially segregated by our absorptions.

The antibody population eluted from F.A. red cells was also structurally heterogeneous (Tables 3 and 4). γG globulin molecules of both light-chain types were found in a ratio approximating that found in the γG globulin of normal human serum. Heterogeneity was further suggested by the detection in indirect antiglobulin tests of three heavy chain subclasses in the autoantibody population. Predominance of the γG1 subclass in F.A. eluates, determined by the RPI assay, recalls the predominance of this heavy-chain subclass in normal human serum.

The observed heterogeneity of the autoantibodies produced by this patient must be taken into account by any hypothesis for the role of α-MD in the induction of their synthesis. There is now general agreement that a given, differentiated antibody-forming cell is nearly always committed to the synthesis of one type of light-chain, one type of heavy-chain, and antibodies of a single specificity. Whether this commitment is predetermined in the precursor (stem) cell from which the mature antibody-forming cell arises or occurs only at the time of differentiation from a pluripotential stem cell is a fundamental question which, to our knowledge, has not yet been answered by experimental evidence. In any case, the present observations would appear to require the emergence in our patient of differentiated cells, each producing one of two types of light chain, one of three kinds of heavy chain, and one of four or more antibody specificities. If each of the suggested specificities implies the presence of a distinct group of antibody-forming cells, which is probably an oversimplification, there would have to be at least four populations of differentiated cells producing at least three structurally distinguishable species of γG globulin. This type of immune response clearly resembles normal gamma-globulin synthesis and bears no resemblance to the type of monoclonal proliferation and homogeneous protein synthesis associated with myeloma. For these reasons we favor the hypothesis that, in this patient at least, an essentially normal lymphoid system is responding to several immunogenic determinants of an abnormal antigen or set of antigens. Such antigens could involve either an alteration of Rh sites on the red cell or some drug-derived hapten-carrier molecules which happens to resemble determinants of the Rh complex. A primary effect of α-MD on the lymphoid tissues to initiate autoantibody synthesis, possibly as a genetic derepressor, cannot be ruled out. However, this idea seems more difficult to reconcile with the multiple antibody specificities, or spectrum of specificities, observed in this case.

Because of the striking clinical resemblance of hemolytic anemia associated with α-MD to idiopathic autoimmune hemolytic anemia, the question arises of some common pathogenetic mechanism. Structural studies on erythrocyte autoantibodies from a series of patients with the idiopathic disease carried out...
in this laboratory have demonstrated that some autoantibody populations may show marked structural homogeneity while many other autoantibody populations show varying degrees of heterogeneity of structure approaching that of the autoantibodies of patient F.A. A more general comparison of the structural characteristics of the idiopathic autoantibodies and those associated with \( \alpha \)-MD must await study of further cases of the drug-associated disorder.

**SUMMARY**

A case of Coombs-positive hemolytic anemia associated with alpha-methyl-dopa therapy was studied with particular attention to the structure and specificity of the erythrocyte autoantibodies. Eluted autoantibodies were found to be heterogeneous in both respects. Serologic evidence was obtained for the presence of at least four antibody specificities, all possibly related to the Rh complex: “anti-e,” “anti-c,” and at least two additional specificities for undefined antigens which may be a part of the hypothetic Rh “core” or precursor substance. The autoantibody population was found to contain \( \gamma G \) globulins with both \( \kappa \) and \( \lambda \) light chains and three heavy-chain subclasses, in proportions approximating those found in normal human serum. These findings are judged to be most compatible with a response of basically normal lymphoid tissue to some antigenic alteration brought about by alpha-methyl-dopa or a derivative.

**SUMMARIO IN INTERLINGUA**

Un caso de anemia hemolytic Coombs-positive e associate con therapia a alpha-methyl-dopa esseva studiate con attention particular prestate al structura e al specificitate del autoanticorpo antierythrocytic. Eluite autoanticorpo esseva trovate heterogenee in ambe respectos. Evidentia serologic esseva obtenite pro le presentia de al minus quatro specificitates anticorpo, omnes possibilemente relationate al complexo de Rh: “anti-e,” “anti-c,” e al minus duo altere specificitates pro nondefinite antigenos que es possibilemente parte del hypothetic substantia “quintessential” o precursori de Rh. Le population de autoanticorpo contineva globulinas \( \gamma G \) con leve catenas \( \kappa \) e \( \lambda \) e tres subclasses de catena pesante, in proportiones approximativemente identic con illos trovate in normal sero human. Iste constatationes es reguardate como compatibile con le responsa de un fundamentalmente normal tissu lymphoide a un certe alteration antigenic effectuate per alpha-methyl-dopa o un derivato de illo.

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