Methyldopa: Physicochemical Characterization of the Erythrocyte Autoantibody

By Barry Wenz and Parviz Lalezari

A modification of the Polybrene technique for red blood cell antibody characterization has been employed to differentiate the panhemagglutinins arising during methyldopa administration from those accompanying other disease states. Dissociation characteristics of methyldopa-associated antigen-antibody complexes were determined by temperature gradient dissociation technique. Data obtained by this technique for cell-bound antibody were found to distinguish this antibody from those accompanying systemic lupus erythematosus (SLE) and Pronestyl therapy. Graphic data derived from temperature gradient dissociation curves at varying antibody concentrations were obtained for the methyldopa-induced serum antibodies. Results obtained with samples from all six patients were found to be relatively uniform in relation to each other, and different from similarly derived results for red cell antibodies accompanying idiopathic autoimmune hemolytic anemia and Hodgkin's disease. By means of these procedures, as well as standard blood banking techniques, distinguishing features are described that permit in vitro segregation of these distinct groups of red cell autoantibodies.

The increasing use of new therapeutic agents has been associated with a corresponding increase in the incidence of iatrogenically induced red blood cell antibodies. Three distinct types of drug-mediated immune response involving red blood cells have thus far been described:

1. The hapten effect is exemplified by the immunologic stimulation that develops following penicillin administration. In this type of reaction, there is an obligate binding of the drug or a metabolite to the red cell membrane.

2. The "innocent bystander reaction" occurs when antigen-antibody complexes are adsorbed onto the cell membrane, as observed with quinine administration.

3. The autoimmune reaction occurs following long-term methyldopa administration. In these instances, antibodies are directed against "normal" antigenic components of the cell.

The nature of the red cell antigens involved in the immunologic reactions associated with methyldopa has not been fully determined. However, commonly applied serologic tests reveal many qualities in common with reactions found in the idiopathic autoimmune hemolytic anemias. In the present study, the Polybrene technique, a newly developed method for detection and characterization of red cell antibodies, was employed to derive characteristic parameters that distinguish methyldopa-associated antibodies from those of other autoimmune diseases.
Subjects

Ten patients included in this study had confirmed essential hypertension of at least 2-yr duration and had been receiving methyldopa for periods of 6 mo or longer. The daily dose of the drug ranged from 0.5 to 2 g. In addition, the following criteria were required for patient selection: a positive direct antiglobulin test (AGT); a positive direct and indirect Polybrene test; negative antinuclear antibody (ANA), Lupus erythematosus preparation (LE prep), and latex fixation tests; and other than methyldopa, the patients had not been given any medications known to produce a positive AGT. Hematologic data for these patients are summarized in Table 1.

W.L. and S.S. were the only patients in this study with manifestations of a hemolytic anemia. W.L., a 60-yr-old man, had been receiving methyldopa (1 g daily) for 8 mo. Two months after the termination of drug therapy, and despite the persistence of a positive antiglobulin reaction, the patient's hemogram returned to normal (hematocrit 38%, reticulocytes 0.2%), and criteria for hemolysis were no longer present. S.S., a 60-yr-old man, had received methyldopa (0.5 g daily) for a period of 6 mo prior to the onset of hemolytic anemia. One month following drug withdrawal, the patient's hematocrit had increased 5 Vol/100 ml, despite the persistence of a positive direct AGT, an elevated reticulocyte count, and a depressed serum haptoglobin value.

Additional Patients

Four patients with idiopathic autoimmune hemolytic anemia and another patient with Hodgkin's disease accompanied by red blood cell autoantibodies were included in this study as examples of autoimmunity not associated with drug therapy. All patients had strongly positive direct and indirect Polybrene tests but had never received methyldopa or other drugs known to induce positive AGT reactions.

Table 1. Laboratory Data on Patients Treated With Methyldopa

<table>
<thead>
<tr>
<th>Patient</th>
<th>Hematocrit (%)</th>
<th>Reticulocyte Count (%)</th>
<th>Serum Haptoglobin (mg/100 ml)</th>
<th>Red Cell Survival (days)</th>
<th>Bilirubin (indirect) (mg/100 ml)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELJ</td>
<td>39</td>
<td>1.7</td>
<td>230</td>
<td>30+</td>
<td>0.2</td>
<td>AGT results discovered and methyldopa discontinued 1 yr prior to the study</td>
</tr>
<tr>
<td>SB</td>
<td>42</td>
<td>0.6</td>
<td>—</td>
<td>29</td>
<td>—</td>
<td>On methyldopa and procaineamide</td>
</tr>
<tr>
<td>DG</td>
<td>40</td>
<td>1</td>
<td>273</td>
<td>—</td>
<td>0.1</td>
<td>Status postpancreas transplant on immunosuppressive therapy; anemia attributed to chronic renal disease</td>
</tr>
<tr>
<td>IJ</td>
<td>42</td>
<td>0.4</td>
<td>200</td>
<td>—</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>FB</td>
<td>19</td>
<td>0.7</td>
<td>110</td>
<td>—</td>
<td>0.3</td>
<td>—</td>
</tr>
<tr>
<td>WL</td>
<td>28</td>
<td>11.5</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>Overt hemolysis</td>
</tr>
<tr>
<td>RH</td>
<td>39</td>
<td>1</td>
<td>264</td>
<td>—</td>
<td>0.1</td>
<td>AGT results discovered and methyldopa discontinued 1 yr prior to this study</td>
</tr>
<tr>
<td>VB</td>
<td>40</td>
<td>1.2</td>
<td>—</td>
<td>—</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>SS</td>
<td>17</td>
<td>30</td>
<td>0</td>
<td>—</td>
<td>1.4</td>
<td>Overt hemolysis</td>
</tr>
<tr>
<td>MD</td>
<td>38</td>
<td>0.6</td>
<td>5*</td>
<td>—</td>
<td>0.6*</td>
<td>— Associated liver disease</td>
</tr>
</tbody>
</table>

*AGT, antiglobulin test.
† Not available.
Serologic Technique

Direct and indirect antiglobulin tests (AGT) and antibody identification procedures were carried out using a “broad spectrum” antiglobulin reagent (Ortho Diagnostics, Raritan, N.J.), the results being evaluated microscopically. Cells from two different cell panels (Spectra Biologicals [Tencell Panel], Oxnard, Calif. and Pfizer Diagnostics [Panocell 16], Groton, Conn.) were used according to standard blood banking techniques, for antibody identification. Reactivity was tested by both AGT and enzyme (Bromelase, Dade Co., Miami, Fla.) treatment of the test cells. Red cell antibody eluates were prepared by heating the washed sensitized cells at 56°C for 30 min.

Polybrene Technique

Polybrene (Aldrich Chemical Co., Milwaukee, Wis.), a positively charged synthetic polymer, has been used to facilitate the detection of both serum (indirect) and cell-bound antibodies (direct test). The test is performed in a continuous flow system, which provides automatic colorimetric recording of the results and also allows determination of thermal characteristics of the immunologic bonds. The latter is evaluated by subjecting the antibody-dependent red cell aggregates to progressively increasing temperatures. The thermal energy thus introduced results in gradual dissociation of the aggregates. This disaggregation depends on the specific thermal characteristics of the antigen-antibody reaction and the concentration of the antibodies being studied. Continuous colorimetric recording of the process produces a curve that has been termed the temperature gradient dissociation curve (TGDC), and the temperature at which one-half of the antibody-dependent aggregates dissociate has been defined as the “T 50°C.” It has been shown that erythrocyte autoantibodies so studied, produce T 50% values that permit the antigen-antibody reactions involved to be classified into three different groups: (1) cold-reacting antibodies with T 50% less than 30°C, such as anti-I and anti-i; (2) antibodies of intermediate thermal characteristics with T 50% values between 30°C-50°C, exemplified by the antibodies accompanying SLE, drug-induced lupus-like syndromes (e.g., caused by procainamide), and the majority of lymphosarcoma-induced red cell autoimmunities; and (3) warm antibodies, with T 50% values over 50°C, that have been shown to be associated with idiopathic autoimmune hemolytic anemia, certain additional cases of lymphosarcoma, Hodgkin’s disease, myelofibrosis, and several drug-associated antibodies, such as methyldopa and penicillin.

Since T 50% values are greatly influenced by antibody titer, a second analytical technique has been developed that permits discrimination of antibodies, independent of their serum concentration. This procedure is accomplished by obtaining T 50% values for different antibody concentrations and subsequently plotting the log of antibody concentration vs. the reciprocal of T 50%, in absolute temperature units. It has been established that such plots produce straight lines, the slope and intercepts of which are characteristic of various antigen-antibody systems. (The intercept may be considered as the intersection of the extrapolated data lines and the abscissa.) The results of such a study on blood group isoantibodies may be seen in Fig. 1 where distinctive slopes and intercepts are depicted.

Slope and intercept data were obtained by using serial dilutions of each of the test serums, run against an ABO compatible normal red cell, thereby obtaining discrete TGDC and T 50%, values for each dilution. These antibody dilutions were then tested against a second ABO compatible red cell suspension, and the respective T 50%, values for each cell were separately plotted against the log of antibody concentration. Accordingly, patients S.B. and W.C. were studied in duplicate analysis, employing different test cells, to confirm the reproducibility of this technique.

The units in terms of which antibody concentrations are expressed are equal to the activity in a dilution of the patient’s serum that produces a deflection of OD of 0.035 in the continuous flow system. All serum dilutions were made in ABO compatible serums, previously screened to exclude the possibility of interacting isoantibodies.

Rh<sup>+</sup> cells were generously supplied by Dr. Arthur Rowe, Greater New York Blood Program, New York, from a frozen stock; normal frozen cells, similarly prepared by a droplet technique in a sucrose-glucose-NaCl solution, were used as control. Serums, which contained methyldopa-induced antibody, were separated from clotted blood samples and were kept at −20°C until use.
RESULTS

The results of the serologic tests performed by conventional manual techniques are summarized in Table 2. All ten patients had positive direct AGT, but only five had positive indirect tests. Eluted antibodies prepared from cells of seven of the ten patients demonstrated “panhemagglutinin” activity when reacted with enzyme-treated commercial panel cells. In contrast, AGT revealed panhemagglutinin activity in only five of these patients. In the remaining patients, although a definite specificity could not be determined, the eluted anti-

Table 2. Antiglobulin and Enzyme Reactions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Direct AGT</th>
<th>Indirect AGT</th>
<th>Cell Panel Studied by Bromelase</th>
<th>Cell Panel Studied by AGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELJ</td>
<td>3+</td>
<td>2+</td>
<td>PAN1</td>
<td>NS</td>
</tr>
<tr>
<td>SB</td>
<td>3+</td>
<td>Neg</td>
<td>NS (r’r-neg)</td>
<td>NS</td>
</tr>
<tr>
<td>DG</td>
<td>1+</td>
<td>Neg</td>
<td>NS (r’r, r-neg)</td>
<td>NS</td>
</tr>
<tr>
<td>IJ</td>
<td>2+</td>
<td>Neg</td>
<td>NS (r’r-neg)</td>
<td>NS</td>
</tr>
<tr>
<td>FB</td>
<td>1+</td>
<td>Neg</td>
<td>PAN</td>
<td>PAN</td>
</tr>
<tr>
<td>WL</td>
<td>3+</td>
<td>2+</td>
<td>PAN</td>
<td>PAN</td>
</tr>
<tr>
<td>RH</td>
<td>1+</td>
<td>Neg</td>
<td>PAN</td>
<td>NS</td>
</tr>
<tr>
<td>VB</td>
<td>3+</td>
<td>2+</td>
<td>PAN</td>
<td>PAN</td>
</tr>
<tr>
<td>SS</td>
<td>3+</td>
<td>2+</td>
<td>PAN</td>
<td>PAN</td>
</tr>
<tr>
<td>MD</td>
<td>3+</td>
<td>1+</td>
<td>PAN</td>
<td>PAN</td>
</tr>
</tbody>
</table>

* AGT, antiglobulin test.
† PAN, panhemagglutinin.
‡ NS, nonspecific (reactive with all cells except those with r’r genotype).
bodies were nonreactive, with one of the cells having the genotype r"r (dcE/dce). In addition, on eluted sample failed to react with a cell of the rr (dce/dce) genotype. This selective lack of reactivity was observed with enzyme-treated cells and with cells analyzed by the AGT. In contrast, sera from all ten patients were shown to have panhemagglutinin activity by the Polybrene techniques, but no quantitative studies were done.

Table 3 shows the results of the direct TGD curves obtained for red cell-bound antibodies expressed in terms of the $T_{50\%}$ and their corresponding $\Delta OD$ values. Nine of these ten patients displayed $T_{50\%}$ values greater than 60°C. Patient D.C., on whom the $T_{50\%}$ obtained was $57^\circ C$, was studied 1 yr after methyldopa had been withdrawn, presumably reflecting a decline in antibody concentration. Results of the indirect tests are expressed in optical density values alone (i.e., $\Delta OD$).

**Slope and Intercepts**

Seros subjected to this study must have a minimum initial titer of at least 1:20 in the Polybrene technique. This stipulation permits TGDCs to be generated on at least four different dilutions and the corresponding $T_{50\%}$ values to be utilized in the plot. Accordingly, four of the original ten patients whose sera had low antibody titers and produced a $\Delta OD$ less than 0.6 (Table 3) were not analyzed by this procedure. The data obtained for the remaining six patients studied are shown in Fig. 2. In Fig. 3, the data obtained for slope and intercept values of the methyldopa-induced red cell antibodies are schematically represented as a median line with intercept range values and are compared with data similarly obtained with anti-Rh (Ortho Diagnostics, Raritan, N.J.). In addition, this figure depicts slopes and intercepts obtained with panhemagglutinating sera from four patients with idiopathic autoimmune hemolytic anemia and from one patient with Hodgkin’s disease.

**Reactivity of Methyldopa-induced Antibody With Cells of Differing Rh Genotypes**

Seros from these patients were tested against cells of the Rh genotypes $R_iR_i$, rr, and Rhnull, respectively. Reactivity was studied by the indirect AGT in all. The Polybrene technique was applied to the sera of four patients. The
Fig. 2. Slopes and intercepts produced by study of six examples of methyldopa-induced antibody. WL, WL', SB, and SB' represent duplication of results produced by the use of different red cells from two donors.

Fig. 3. Comparison of slopes and intercepts produced by anti-Rh₀, auto-antibodies associated with methyldopa, idiopathic autoimmune hemolytic anemia, and Hodgkin's disease. Shaded areas represent data range obtained by serial analysis of independent samples of anti-Rh₀ and methyldopa-induced antibodies.
results are shown in Table 4. It should be noted that although the indirect AGT gave negative results in five of the eight patients, the Polybrene reaction was consistently positive (Table 3).

**DISCUSSION**

In patients receiving methyldopa, an incidence of 20% positive direct antiglobulin reactions and 0.3% associated hemolytic anemias has been reported. This antibody has been shown to have properties similar to those of the Rh system. Using standard serologic techniques, Worlledge et al. and Bakemeier and Leddy were able to demonstrate specificity in some cases for the antigens hr’ (c) and hr” (e); most cases, however, possessed panagglutinins, and the antibodies were not distinguished from those arising in other types of autoimmune hemolytic anemias. Although the present study does not specifically define the antigenic “target” sites against which this antibody is active, it does provide distinctive physicochemical parameters that segregate the methyldopa-induced antibody from other panhemagglutinins.

1. It has been shown that in all examples studied, cell-bound antibodies were warm reacting, and with the exception of one sample, antigen-antibody complexes did not dissociate even at 60°C. This exception was attributed to diminishing antibody concentration, since treatment had been discontinued 1 yr prior to the study. This preliminary finding segregates methyldopa-induced antibody from other red cell autoantibodies, such as those accompanying SLE and Pronestyl therapy that, as previously stated, consistently produced T 50°C dissociation values in the 30°C–50°C range.

2. The second characteristic feature of this antibody is the similarity of slopes produced by plotting antibody concentration against the reciprocal of T 50°C values, in contrast to the different slopes obtained for the other autoimmune antibodies studied. Such differences were noted despite the fact that all antibodies studied by this technique possessed direct T 50°C values exceeding 60°C by TGDC. Differences were also noted between the slopes produced by methyldopa-related antibodies and the isoantibodies of the blood group systems displayed in Fig. 1. It is of interest that slope and intercept for methyldopa-induced antibody remained relatively uniform in either the presence or
absence of associated hemolysis. The determination of slopes and intercepts may thus allow in vitro discrimination of this antibody from other "warm" panagglutinins. In Figs. 1 and 3, data shown for anti-Rh, (D), anti-M, and methyldopa-associated antibody are indicated by range, demonstrating a degree of variation obtained for independent antibody samples of similar specificity. These variations are small and believed to represent the limitation of technique in employing identical antibody units for the various samples analyzed. The heterogeneity of the slopes and intercepts obtained for idiopathic autoimmune hemolytic anemia should be noted, a result that is suggestive of a diversity of the immunologic reactions involved.

(3) Finally, these antibodies have produced diverse reactions when tested with cells of differing Rh phenotypes. Of particular interest was the positive reaction between the methyldopa-induced antibody and the Rh<sup>null</sup> cells in the Polybrene test system, in contrast to the less sensitive AGT, which produced consistently negative reactions with the same cells. Although the strength of the reaction was decidedly weaker with Rh<sup>null</sup> cells, it was not absent. It is unlikely that this weak reaction was an artifact induced by the use of frozen and thawed cells, since the same cells demonstrated no reactivity when tested against ten different normal serums, nor did normal frozen and thawed cells produce false positive reactions. If this weak reaction is indeed valid, and assuming that methyldopa-related antibodies are directed against a factor or factors of the Rh system, it may then be considered that even the Rh<sup>null</sup> cell does not represent complete loss of Rh-related structure. The antibody could be directed against a still more primitive "ground substance" on which the remainder of the Rh system is built. These hypothetical considerations may possibly account for closeness of the slopes and intercepts between the methyldopa-induced antibody and representative members of the Rh system as shown in Fig. 3.

Thus, as conjecture, the theoretical Rh "ground substance" may be composed of several antigenic components, each capable of discrete interaction with a corresponding antibody, and accordingly, antibodies produced in association with different autoimmune diseases may possess reactivity with one, some, or all of these related antigenic sites. Alternatively, reactivity with the Rh<sup>null</sup> cells may represent an additional antigen-antibody reaction totally independent of the Rh system. The verification of these possibilities must await the availability of red cell antigens in pure and soluble form.

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Methyldopa: Physicochemical Characterization of the Erythrocyte Autoantibody

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