**CONCISE REPORT**

**Specific Antibody-Blocking Activities in Antilymphocyte Globulin as Correlates of Efficacy for the Treatment of Aplastic Anemia**

By Anajane G. Smith, Richard J. O'Reilly, John A. Hansen, and Paul J. Martin

Horse anti-human thoracic duct lymphocyte globulin (ATDLG) has been used successfully for the treatment of severe aplastic anemia, although not all lots have comparable efficacy. We have characterized the antibody specificities contained in one lot of Swiss ATDLG found to provide a response rate of 68% and another lot that provided only a 31% response rate. Antibody specificities were analyzed quantitatively by competitive inhibition assays with the use of a panel of fluorescein-conjugated murine monoclonal antibodies that recognize T cell antigens, common leukocyte antigens, and "Ia-like" antigens. Although there was wide variation in the amounts of individual antibody specificities within each lot, the effective lot of ATDLG contained an average of 2 1/2 times as much of each antibody specificity as the less effective lot. There were only two antibody specificities that differed remarkably from this pattern; and these deviations did not appear sufficient to account for the variation in ATDLG efficacy. It is possible that antibody specificities other than those tested were responsible for therapeutic efficacy. Alternatively, the data suggest that it might be possible to achieve improved results for the treatment of severe aplastic anemia with higher doses of less effective lots of ATDLG.

**MATERIALS AND METHODS**

Isolation of cells. Mononuclear cells were isolated from the blood of normal human volunteers by centrifugation over Ficoll-Hypaque (specific gravity, 1.077). T cells were enriched from peripheral blood mononuclear cells (PBMCs) by removal of the adherent non-T cell population on a nylon wool column. Granulocytes were prepared by dextran sedimentation of theuffy coat from the Ficoll-Hypaque bottom layer, followed by lysis of erythrocytes with Tris-buffered 0.83% NH₄Cl. Cells of the Burkitt's B cell line Daudi, positive for HLA class II ("Ia-like") antigens, were maintained under standard culture conditions.

Purification of monoclonal antibodies. Murine monoclonal IgG antibodies were purified from ascites fluid by affinity chromatography with the use of protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ).¹ Murine monoclonal IgM antibodies were purified by dialysis against water. The precipitate was washed once with water at pH 7.5 and then dissolved in phosphate-buffered saline (PBS), pH 7.2. Antibody concentration was determined by Bradford assay with the use of bovine IgG as a standard.² Fluorescein conjugation of antibodies. Purified monoclonal antibodies were diazoylated overnight against PBS, pH 7.2. Fluorescein isothiocyanate (FITC) (Molecular Probes, Cave Junction, Ore) was dissolved in 25 µL of dimethylsulfoxide and diluted to 1 mg/mL in water at pH 7.5.

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0.286 mol/L of carbonate/bicarbonate buffer, pH 9.4. FITC (0.25 mL) was mixed with purified antibody (1 to 5 mg in 1 mL of PBS) and incubated at 37°C. Aliquots of the mixture were removed at intervals up to 120 minutes and applied to a column of G25 Sephadex (Pharmacia) in order to separate conjugated antibody from unconjugated FITC.

**Competitive inhibition assays.** Cells (10⁶ in 0.1 mL of RPMI 1640 containing 5% fetal calf serum and 3 mmol/L of EDTA) were incubated for 30 minutes at 4°C with 25 μL of serially diluted ATDLG or purified monoclonal antibody in parallel assays. FITC-conjugated antibodies were added at optimal concentration (twice the amount required for saturation) without an intervening wash and the incubation was continued for another 30 minutes at 4°C. Cells were washed twice, resuspended in 0.5 mL of 0.85% saline containing 1% paraformaldehyde, and analyzed by flow microfluorimetry (FACS IV, Becton Dickinson, Mountain View, Calif) with logarithmic amplification of the fluorescence signal. The modal fluorescence intensity was determined according to a calibrated logarithmic scale and expressed as a percentage of the control fluorescence of cells stained in the absence of ATDLG with a correction for the background fluorescence of cells stained in the presence of a 50- to 100-fold excess of the unconjugated monoclonal antibody. The amount of ATDLG or monoclonal antibody required to block 50% of the binding of each fluorescein-conjugated monoclonal antibody was determined by semilog regression analysis and defined as 1 unit of blocking activity. Results were expressed as the milligram equivalent of monoclonal antibody contained in 1.0 g of ATDLG. For example, if 1 mg of ATDLG is determined to be the amount required to produce 50% blocking (1 unit), then 1 μg of ATDLG contains 1,000 units of blocking activity. If 1 μg of the monoclonal antibody also inhibits binding by 50% (1 unit), then 1 g of ATDLG contains blocking activity equivalent to 1 mg of the monoclonal activity.

**RESULTS**

The two lots of ATDLG contained equivalent amounts of protein (67 mg/mL) as determined by Bradford assay, and electrophoretic analysis demonstrated that both lots contained only gamma globulins. The antibody specificities in each lot of ATDLG were analyzed quantitatively by competitive inhibition assays with the use of a panel of fluorescein-conjugated murine monoclonal antibodies that react with T cells (35.1, 64.1, 66.1, 10.2, 12.1, 51.1), granulocytes and monocytes (60.1, 60.3), or Ia-like antigens (p4.1, 7.2). For each lot of ATDLG, there was wide variation in the amounts of individual antibody specificities (Table 1). For example, 1 g of the effective ATDLG contained the equivalent of 48.7 mg of antibody 10.2 but only 0.1 mg of antibody 60.1. Although it was determined that each lot of ATDLG contained the same amount of protein, the effective lot contained an average of 2½ times as much of each antibody specificity as the less effective lot. Only two of the 11 tested antibodies deviated remarkably from this pattern. At one extreme, the effective lot contained 5½ times more CD5 activity than the less effective lot. On the other hand, both lots contained equivalent amounts of anti-T200 activity.

**DISCUSSION**

We have characterized some of the antibody specificities contained in two lots of ATDLG, one of which was effective for the treatment of severe aplastic anemia and the other of which was less effective. Both lots contained antibody specificities that may have immunosuppressive effects. With the exception of anti-CD5 and anti-T200, the two lots of ATDLG had similar antibody specificity profiles. The interpretation of these data is necessarily equivocal because of the unexpected finding that, for most specificities, the effective lot of ATDLG contained more than twice as much binding activity as the less effective ATDLG. In this context, the amount of anti-CD5 activity in the effective ATDLG was only twice what otherwise might be expected and the amount of anti-T200 activity was approximately 40% of the expected value. It seems doubtful that single differences of this magnitude can account for the variation in efficacy between the two lots of ATDLG. It is possible that one or more antibody specificities other than those tested were responsible for the efficacy of ATDLG in the treatment of severe aplastic anemia. Alternatively, the data could suggest that if antibody-binding activity is responsible for the efficacy of ATDLG and assuming that certain other antibodies do not exert inhibitory effects, then it might be possible to achieve improved results with higher doses of less effective lots.

**Table 1. Milligram Equivalents of Monoclonal Antibodies in One Gram of Antithoracic Duct Lymphocyte Globulin**

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Antigen Mol Wt (kD)</th>
<th>Cells Tested</th>
<th>Effective ATDLG</th>
<th>Ineffective ATDLG</th>
<th>Ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.1</td>
<td>CD2</td>
<td>50</td>
<td>T cells</td>
<td>0.52</td>
<td>0.19</td>
</tr>
<tr>
<td>64.1</td>
<td>CD3</td>
<td>19</td>
<td>T cells</td>
<td>0.72</td>
<td>0.38</td>
</tr>
<tr>
<td>66.1</td>
<td>CD4</td>
<td>55</td>
<td>T cells</td>
<td>0.84</td>
<td>0.58</td>
</tr>
<tr>
<td>10.2</td>
<td>CD5</td>
<td>67</td>
<td>T cells</td>
<td>4.9</td>
<td>8.8</td>
</tr>
<tr>
<td>12.1</td>
<td>CD6</td>
<td>120</td>
<td>T cells</td>
<td>27</td>
<td>9.4</td>
</tr>
<tr>
<td>51.1</td>
<td>CD8</td>
<td>32</td>
<td>Granulocytes</td>
<td>0.30</td>
<td>0.15</td>
</tr>
<tr>
<td>60.1</td>
<td>CD11</td>
<td>95/150</td>
<td>Granulocytes</td>
<td>0.10</td>
<td>0.03</td>
</tr>
<tr>
<td>60.3</td>
<td>CDw18</td>
<td>95/130/150</td>
<td>Granulocytes</td>
<td>0.85</td>
<td>0.40</td>
</tr>
<tr>
<td>9.4</td>
<td>T-200</td>
<td>200</td>
<td>T cells</td>
<td>0.42</td>
<td>0.43</td>
</tr>
<tr>
<td>p4.1</td>
<td>DR</td>
<td>29/34</td>
<td>Daudi</td>
<td>1.5</td>
<td>0.72</td>
</tr>
<tr>
<td>7.2</td>
<td>DR + DO</td>
<td>29/34</td>
<td>Daudi</td>
<td>4.4</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Cluster designations were defined in the First and Second International Workshops on Human Leucocyte Differentiation Antigens. See references 11 through 13 for descriptions of individual monoclonal antibodies and definitions of antigen specificities.

†Effective/ineffective ATDLG ratio.
toxicity of ATDLG, however, may preclude testing of this hypothesis.

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REFERENCES

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