Biological and Immunological Characterization of ATG and ALG

By Eric L. Raefsky, Pedro Gascon, Alois Gratwohl, Bruno Speck, and Neal S. Young

Antithymocyte globulin (ATG) and antilymphocyte globulin (ALG) are effective therapies in aplastic anemia: their mechanism of action is undefined. We assayed multiple properties of ATG and ALG to address the biological and immunological bases for differences between ATG and ALG and lot variation. In addition, we studied a lot reported to be inactive in an American clinical trial; however in retrospect, this lot appeared to be active in patients treated in Europe. Immunoprecipitation of thymocyte and lymphocyte membrane proteins with ATG and ALG showed between 14 and 18 major bands on SDS-PAGE, but the patterns for ATG and ALG were not identical. The ability of ATG and ALG to block binding of labeled monoclonal antibodies was assessed using flow cytometry and a radioimmunoassay. In general, there was more lot variation among ALGs than ATGs; however, all ALG lots were more potent blockers of binding of anti-HLA-DR and anti-Leu 1 antibodies than was ATG. Both ALG and ATG effectively blocked binding of anti-Leu 2a, anti-Leu 3a, anti-Leu 4, anti-Leu 5b, and anti-IL 2 receptor abs; neither blocked binding of anti-Leu 7. All preparations were capable of inducing T-cell blastogenesis, although there was considerable lot variation. All lots lysed 60% to 75% T cells in a rabbit complement-mediated cytotoxicity assay, with most having a plateau of activity at 5 to 10 ug/mL. Two lots of ALG, including the lot reported to be clinically inactive, showed less toxicity at suboptimal concentrations and did not plateau even at 80 ug/mL. In total, these results indicate important differences between ATG and ALG in general, more lot variation among ALGs than ATGs and only differences in cytotoxicity between an “inactive” lot of ALG and most, but not all, other active ATG and ALG preparations.

MATERIALS AND METHODS

ATG and ALG preparations. ATG lots 17924, 844, and 731 were provided by the Upjohn Company (Kalamazoo, Mich) and ALG lots 160, 162, 15343, 181 S, and 97412 by the Swiss Serum and Vaccine Institute (Berne, Switzerland) as sterile solutions at 50 mg/mL in saline. The protein concentration was confirmed spectrophotometrically and the preparations determined to be greater than 95% IgG by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining.

ATG was prepared by injecting horses with homogenized emulsions of normal thymocytes removed from children undergoing cardiac surgery. Antibodies to RBC stroma and human plasma proteins were removed by absorption before the gamma globulin was purified. All ATG lots represent pooled sera from four inoculated animals, obtained and processed at different times. ALG lot 17924 has been successfully employed in clinical trials with response rates to transfusion independence in severe disease of about 40% (and the NHLBI multicenter trial, Young N, unpublished data). ALG EC162 and 15343 were prepared using fresh whole lymph from an adult patient undergoing therapeutic thoracic duct drainage; plasma from 2 horses were pooled before purification. The preparation of one ALG lot differed in that thoracic duct mononuclear cells which had been cryopreserved for 3 years were purified by Ficoll-Hypaque sedimentation before being used as the immunogen; the same factory lot (170S) was split to provide ALG for European use (ALG 92125) and American studies (ALG 97412) (N. Charriette, Swiss Serum Institute, personal communication). ALG 181S resulted from the immunization of new horses with fresh lymph from a new source. The production and clinical utility of these ALG preparations are summarized in Table 1; details of the clinical trials from Basel have been published elsewhere.

Preparation of peripheral blood cells. Peripheral blood was drawn into syringes containing 50 u/mL preservative-free heparin (Jones, McNeal, and Feldman, St. Louis, Mo). Peripheral blood mononuclear cells (PBMNC) were obtained by Ficoll-Hypaque
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Density sedimentation, washed three times, and resuspended in appropriate media. T cells were fractionated by rosetting with neuraminidase treated sheep red blood cells overnight at 4°C. Phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PHA-PBMC) were obtained by incubating PBMC at an initial cell concentration of 1.5 x 10⁶ cells/mL with PHA (GIBCO, Grand Island, NY) at a final concentration of 10 µg/mL in RPMI 1640 supplemented with 2 mmol/L glutamine, 20% heat-inactivated fetal calf serum (HyClone, Logan, Utah), and antibiotics at 37°C in a 5% CO₂ humidified chamber. After 48 hours, the cells were washed, resuspended in the same media without PHA but supplemented with 10% partially purified T-cell growth factor (Cellular Products, Portland, ME), and incubated for an additional 48 to 72 hours before harvesting. Thymocytes were obtained from a child undergoing cardiac surgery, frozen in media containing 10% dimethylsulfoxide, and stored in a liquid nitrogen freezer until used.

**Cytotoxicity assays.** T cells were labeled with 200 µL of ³¹Cr (1 mCi/mL solution, Amersham, Arlington Heights, Ill) per 1 x 10⁶ cells for one hour at 37°C, washed, and resuspended in RPMI plus 0.1% BSA at a concentration of 20,000 cpm/75 µL. Seventy-five µL of freshly prepared dilutions of heat inactivated ATG, ALG, or nonimmune horse IgG (HlgG, United States Biochemical Corp., Cleveland, OH) was added to 75 µL of labeled cells in triplicate and the cells were incubated for 30 minutes at room temperature. Rabbit complement (Cedarlane, Hornsby, Ontario) at an optimal final dilution of 1:6 or human anti-AB antibody negative serum at a final concentration of 1:24 was then added and the incubation was continued for an additional 60 minutes at 37°C. The cells were then centrifuged and cytotoxicity determined by assaying the supernatant with 10% bovine serum albumin (BSA) and 0.1% sodium azide (NaNO₂). 1 x 10⁶ cells were preincubated with 100 µL of HlgG, ATG, ALG, or media in varying concentrations for 30 minutes at 4°C, washed twice, and then incubated with 5 µL of commercially available monoclonal antibodies or control IgG directly conjugated to fluorescein (FITC-Ab, Becton Dickinson, Sunnyvale, Calif) for an additional 30 minutes at 4°C in the dark. The cells were washed twice, resuspended in HBSS plus 0.1% BSA and 0.1% NaNO₂, and analyzed by flow microfluorometry using an Epics V (Coulter, Hialeah, Fla). Fluorescence was measured using a logarithmic scale. Using the IMMUNO program of the EASY 88 Computer System (Coulter), negative cells in the control samples (ie, samples preincubated with media alone before labeled with FITC-Ab) were subtracted from the corresponding fluorescent channel from the samples preincubated with ATG or ALG. This program therefore effects a decrease in mean fluorescence proportional to the blocking of the cell surface protein by the preincubation preparation, but does not alter the proportion of cells scored as positive by comparison to control (nonimmune mouse monoclonal antibody) preparations. Extrapolation from these data allowed an estimation of the amount of the preincubation preparation needed for 50% blocking of each FITC-Ab. In most cases, preincubation with the highest concentration of HlgG resulted in less than 5% blocking; in the occasional case in which this nonspecific blocking was greater than 5%, the mean fluorescence of the sample preincubated with HlgG was used as the control.

**³²P blocking studies.** The ability of ATG, ALG, and HlgG to block a ³²P-monoclonal antibody directed against the interleukin-2 (IL-2) receptor (³²P-anti-Tac) from binding to HUT 102 cells (which contain approximately 300,000 IL-2 receptors per cell) was determined using a modification of a previously described assay.³² ¹⁰⁶ HUT 102 cells were washed in PBS and resuspended in binding media as previously described.³² Cells were incubated with varying dilutions of unlabeled anti-Tac, HlgG, ATG, or ALG for two hours at room temperature in duplicate. 5.5 ng of ³²P-anti-Tac (equivalent to 15,000 cpm, kindly supplied by Dr Thomas Waldmann) was added and the cells incubated for an additional 60 minutes at room temperature. Cell bound radioactivity was determined after the reaction mixture was centrifuged through a 1 mol/L sucrose cushion and the supernatant aspirated. Unbound ³²P-anti-Tac was determined by subtracting antibody bound from total antibody added.

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**Table 1. Preparation and Clinical Results of ALG Lots**

<table>
<thead>
<tr>
<th>Lot</th>
<th>EC162</th>
<th>15343</th>
<th>92125 (97412)</th>
<th>181S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production date</td>
<td>5/78</td>
<td>2/80</td>
<td>8/82</td>
<td>1/84</td>
</tr>
<tr>
<td>Horses immunized</td>
<td>Vulcan III</td>
<td>Vulcan III</td>
<td>Vulcan III</td>
<td>Vulcan IV</td>
</tr>
<tr>
<td>Antigen</td>
<td>donor: VB concentrated lymph</td>
<td>donor: VB concentrated lymph</td>
<td>donor: VG Ficoll-separated lymphocytes cryopreserved 3 yr</td>
<td>donor: WG concentrated lymph</td>
</tr>
<tr>
<td>No. Patients treated</td>
<td>29*</td>
<td>28†</td>
<td>12†</td>
<td>17†</td>
</tr>
<tr>
<td>Age (median)</td>
<td>16</td>
<td>14</td>
<td>23</td>
<td>31</td>
</tr>
<tr>
<td>No. Severe disease‡</td>
<td>29‡</td>
<td>20</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>No. Acute disease§</td>
<td>28§</td>
<td>15§</td>
<td>8§</td>
<td>11§</td>
</tr>
<tr>
<td>No. Alive and transfusion independent (%)¶</td>
<td>20 (69%)</td>
<td>23 (82%)</td>
<td>9 (75%)</td>
<td>23 (71%)</td>
</tr>
</tbody>
</table>

*Clinical results of lot EC162 from reference 4.
†Clinical results of patients treated in Basel.
‡Treatment within 2 months of diagnosis.
§Improvement in blood counts so that patient's hematologic status no longer qualified as severe and blood product transfusion was not needed by 4 months after initiation of ALG therapy.

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Cell surface iodination, immunoprecipitation, and electrophoresis. 10^6 cells were washed and resuspended in Dulbecco's phosphate-buffered saline without calcium or magnesium (DPBS; GIBCO) and the cell surface proteins radiiodinated with 2 mCi of 125I-sodium iodide (New England Nuclear, Boston, Mass) via the lactoperoxidase-H_2O_2 method. Cells were washed 3 times in DPBS containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1 mmol/L diisopropylfluorophosphate (DFP, both from Sigma, St Louis, Mo) and the iodinated proteins solubilized by lysing the cells in 1 mL of DPBS containing 1 mmol/L PMSF, 1 mmol/L DFP, and 0.5% NP-40 for 30 minutes at 4°C followed by centrifugation to remove cellular debris. To immunoprecipitate cell surface proteins with ATG and ALG, 50 μL of solubilized iodinated lysate from PHA-PBMNC or thymocytes were precleared twice with 300 μL of other preparation: for example, ATG uniquely precipitated several major protein components, for example, ATG (Fig 1) and thymocytes (data not shown), without apparent major differences in results using the two cell types. Both ATG and ALG preparations consistently precipitated several major protein components, for example, proteins of molecular weight 182,000, 138,000, 112,000, 93,000, 43,000, and 35,000. However, other protein components were better or exclusively precipitated by one or the other preparation: for example, ATG uniquely precipitated bands of 295,000 and 30,000 and ALG a band of 166,000. Two lots of ATG (844 and 17924) had identical patterns of immunoprecipitation of thymocyte and PHA-PBMNC surface proteins. In contrast, there was variability in the number and intensity of proteins immunoprecipitated by different ALG preparations (lots EC162, 15343, and 97412).

Definition of antibody specificities. In order to define specific antibody specificities in horse sera, the binding of fluoresceinated monoclonal antibodies to T-cell antigens was quantitated after preincubation of cells with ATG or ALG. A typical experiment, illustrated in Fig 2a, shows that increasing concentrations of ATG 844 progressively inhibited binding of anti-Leu 4-FITC as demonstrated by a decrease in mean fluorescence. The blocking ability of different lots of ATG and ALG for anti-Leu 4-FITC varied (Fig 2b), presumably reflecting differences in the concentration of this antibody specificity within these preparations.

RESULTS

Immunoprecipitation of cell surface membrane proteins. To determine which cell surface proteins were immunoprecipitated by ATG and ALG, cells similar to the immunogens used for the preparation of ATG and ALG were employed. Both ATG and ALG precipitated a discrete number of major protein bands from membranes of both PHA-PBMNC (Fig 1) and thymocytes (data not shown), without apparent major differences in results using the two cell types. Both ATG and ALG preparations consistently precipitated several major protein components, for example, proteins of molecular weight 182,000, 138,000, 112,000, 93,000, 43,000, and 35,000. However, other protein components were better or exclusively precipitated by one or the other preparation: for example, ATG uniquely precipitated bands of 295,000 and 30,000 and ALG a band of 166,000. Two lots of ATG (844 and 17924) had identical patterns of immunoprecipitation of thymocyte and PHA-PBMNC surface proteins. In contrast, there was variability in the number and intensity of proteins immunoprecipitated by different ALG preparations (lots EC162, 15343, and 97412).

Extrapolation from these data allowed an estimation of the concentration of ATG, ALG, or unlabeled monoclonal antibody needed for a 50% decrease in mean fluorescence (Table 2). Comparison of the relative blocking ability for the various antibodies is complicated by differences in antigen quantity on the cell surface, unknown antibody affinities for antigens, and the likelihood of significant interactions among antibodies of differing specificity. However, these results do allow a quantitative comparison of different preparations of ATG and ALG for each monoclonal antibody. Comparison of an ATG lot (844) and an ALG lot (EC162) showed similar amounts of blocking ability for antigens recognized by monoclonal antibodies to Leu 2a, Leu 3a, Leu 4, and Leu 5b. However, ALG EC162 was over 30 times more potent than ATG 844 in blocking anti-Leu 1 (50% inhibition concentration: 5.8 mg/mL vs 0.16 mg/mL) and more than 20 times more potent in blocking anti-HLA-DR binding (50% inhibition concentration: 104 mg/mL vs 4.7 mg/mL).

These differences in the blocking ability of ATG and ALG were not due to lot variation. The 50% blocking concentrations for ATG lots 17924 and 844 were similar, with less than twofold variation for all monoclonal antibodies tested (data not shown). Parallel to the immunoprecipitation data, however, the differences among different ALG lots were more pronounced. ALG EC162 was more potent than ALG 97412 in blocking antibody binding to Leu 1 (4 x), Leu 2a (3 x), Leu 4 (4 x), and Leu 5b (3 x); these preparations were similar in their ability to block binding of anti-Leu 3a and anti-HLA-DR. In general, lot 15343 was more similar to 97412 than to EC162. However, even the least potent ALG lot was still 10 times more potent than ATG in preventing the binding of anti-Leu 1 and anti-HLA-DR antibodies.

This analysis was not able to demonstrate antibodies in ATG or ALG able to block the binding of monoclonal
antibodies to Leu 7 (a marker for large granular lymphocytes) or the interleukin 2 receptor (Table 2). However, as both thymocytes and thoracic duct lymphocytes include cells that express the interleukin-2 receptor, a more sensitive assay evaluating the ability of these horse immunoglobulin preparations to block the binding of a tritiated monoclonal antibody reactive with the interleukin-2 receptor (called anti-Tac) to HUT 102 cells was performed (Fig 3). Under these conditions, both ATG and ALG were able to significantly block the binding of 3H-anti-Tac with equal potency. The potency of ATGs and ALGs compared to the specific anti-Tac monoclonal antibody (about 1000 x) was similar to the relative potency of the antisera compared to other monoclonal antibodies (Table 2). The explanation for the discrepancy between the behavior of ATGs and ALGs in the radioimmunoassay and the flow cytometry blocking experiments is unclear, but may reflect the greater density of Tac antigen on the surface of the malignant cell line HUT 102 compared to PHA-PBMNC and the use of different monoclonal antibodies directed against the interleukin-2 receptor which were employed in the two assays.

Blastogenesis. The mechanism of action of ATG and ALG in the treatment of aplastic anemia is unknown; investigators have shown both immunostimulatory and cytotoxic properties of these preparations. The ability of several ATG and ALG lots to induce the blastogenesis of PBMNC was measured as a general indicator of T-cell stimulatory potential (Table 3). Although all preparations were able to induce blastogenesis, there was variation among different ATG and ALG lots. Neither ATGs nor ALGs were uniformly superior as a class of sera. There was no correlation between activity in the blastogenesis assay and antibody specificity directed to Leu 4, an antigen required for mitogen activation of lymphocytes. Using nonadherent bone marrow cells as target populations, both ATGs and ALGs were also able to stimulate hematopoietin release from PBMNC to approximately the same degree (data not shown). At higher horse sera concentrations (greater than 10 ug/mL), blastogenesis was dose-responsive but below 100 ug/mL, blastogenesis was less than 50%.

![Fig 2. Flow microfluorometry blocking study of anti-Leu 4 ab. (A) Binding of anti-Leu 4-FITC to PBMCN after preincubation with media alone (control, upper panel) or increasing concentrations of ATG 844 was analyzed by flow microfluorometry in this representative example. Only the cells that were "positive" by the IMMUNO program are shown. (B) Binding of anti-Leu 4-FITC to PBMCN after preincubation with media alone (control, upper panel) or various lots of ATG or ALG at the same concentration. The decrease in mean fluorescence (MF) is proportional to the relative blocking ability of the different preparations.

### Table 2. Comparison of Antibody Specificities in ATG and ALG

<table>
<thead>
<tr>
<th>FITC-Ab</th>
<th>WHO &quot;CD&quot; Classification</th>
<th>50% Blocking Concentration (mg/mL)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unlabeled Antibody</td>
<td>ATG 844</td>
</tr>
<tr>
<td>Anti-Leu 1 (pan-T cell antigen)</td>
<td>CD5</td>
<td>0.0001</td>
</tr>
<tr>
<td>Anti-Leu 2a (suppressor/cytotoxic T cells)</td>
<td>CD8</td>
<td>0.0020</td>
</tr>
<tr>
<td>Anti-Leu 3a (helper T Cells)</td>
<td>CD4</td>
<td>0.0001</td>
</tr>
<tr>
<td>Anti-Leu 4 (T-cell receptor-associated antigen)</td>
<td>CD3</td>
<td>0.0010</td>
</tr>
<tr>
<td>Anti-Leu 5b (E-rosette receptor)</td>
<td>CD2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Anti-leu 7 (LGL, NK cells)</td>
<td>—</td>
<td>0.0400</td>
</tr>
<tr>
<td>Anti-HLA-DR (activated T cells)</td>
<td>DR</td>
<td>0.0100</td>
</tr>
<tr>
<td>Anti-IL 2 Receptor</td>
<td>CD25</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

WHO, World Health Organization; LGL, large granular lymphocytes; NK, natural killer; NT, not tested.

*Less than 10% blocking with the highest dilution (50 mg/mL) of ATG/ALG.
†Values represent the mean ± SD of the extrapolated concentration of ATG, ALG, or unlabeled antibody in 2 to 6 determinations that resulted in a 50% decrease in FITC-Ab mean fluorescence.
‡Cluster designation of antigens as defined by the First and Second International Workshop on Human Leucocyte Differentiation Antigens (reference 24 and 25).
genesis was often inhibited, even though cells were still viable. This effect may have been due to the blocking of the interleukin-2 receptor at these concentrations.

**Cytotoxicity.** All preparations of ATG and ALG tested were effective in a complement-dependent cytotoxicity assay using T cells as targets and rabbit serum as a source of complement (Fig 4). All lots of ATG and ALG had a peak cytotoxicity of 60% to 75%, with most having a plateau concentration between 5.0 to 10.0 ug/mL; significant lysis was not demonstrable at concentrations below 0.5 ug/mL. Although ALGs 97412 and 181S had peak cytotoxicity values comparable to other lots tested, they differed in that a plateau was never reached over the range of concentrations tested. At suboptimal concentrations (2.5 to 10 ug/mL), these same lots were significantly less cytotoxic than the other ATG and ALG preparations tested. Similar results were obtained using human serum as a source of complement, except 10 to 15 times higher concentrations of horse sera were needed for similar degrees of cytotoxicity (data not shown).

**DISCUSSION**

Both ATG and ALG precipitated a limited number of antigens from the cell surface membranes of thymocytes and lymphocytes. These bands almost certainly do not reflect all the antigens recognized by the horse sera, as the presence of certain major protein components will obscure minor bands during exposure of the autoradiograph. In addition, some of the densest bands precipitated most likely represent common cell membrane proteins, as ATG has been shown to recognize many normal human cell types and their molecular weights do not correspond to those for known T-cell-specific proteins. Nonetheless, while the patterns of immunoprecipitation were roughly similar within ATG and ALG lots, differences in proteins precipitated by ATG compared to ALG were evident. These differences were also recognized in quantitative blocking experiments with monoclonal antibodies to T-cell antigens. Remarkable in these experiments was the much greater ability of ALG to block binding of at least two antigens, the pan-T cell antigen Leu 1 and HLA-DR. While there was greater variability among lots of ALG than among lots of ATG in this assay, even the least potent lot of ALG was 10 times more active in blocking binding to these

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**Table 3. Summary of ATG- and ALG-Induced Blastogenesis of Peripheral Blood Mononuclear Cells from Patients with Aplastic Anemia**

<table>
<thead>
<tr>
<th>ATG Lot</th>
<th>Number (n)</th>
<th>Relative Blastogenesis</th>
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</thead>
<tbody>
<tr>
<td>731</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>844</td>
<td>4</td>
<td>58 ± 9†</td>
</tr>
<tr>
<td>17924</td>
<td>3</td>
<td>64 ± 18</td>
</tr>
<tr>
<td>ALG Lot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>162</td>
<td>3</td>
<td>74 ± 23</td>
</tr>
<tr>
<td>15343</td>
<td>3</td>
<td>41 ± 15</td>
</tr>
<tr>
<td>181S</td>
<td>3</td>
<td>50 ± 22</td>
</tr>
<tr>
<td>97412</td>
<td>7</td>
<td>74 ± 21</td>
</tr>
<tr>
<td>HlgG</td>
<td>3</td>
<td>18 ± 3</td>
</tr>
</tbody>
</table>

n, number of experiments.

*PBMNC were used instead of E-rosetted purified T cells because previous studies have shown that E rosetting can stimulate T cells from aplastic anemia patients (reference 9) and normals (reference 37). In two experiments, cells surface phenotyping before and after stimulation showed that the percentage of T cells (Leu 4+) increased from 79% to 86%, activated T cells (Leu 4+, IL 2R+) from 4% to 13%, while B cells (Leu 12+) decreased from 9% to 7%.

†Mean ± SEM.

Relative blastogenesis values were obtained by normalizing the maximal SI of each ATG and ALG lot for each experiment with the SI of ATG lot 731 which was given an arbitrary value of 100. ATG lot 731 was included in each experiment. The stimulation index was 9.0 ± 2.4. for ATG lot 731 and 1.6 ± 0.3 for HlgG. The cpm of unstimulated cells and ATG lot 731-stimulated cells were 188 ± 48 and 2029 ± 365, respectively.
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antigens. That the blocking ability of ATG and ALG was due to direct binding rather than steric hindrance could be demonstrated in a case in which we were technically able to immunoprecipitate with monoclonal antibodies; in this instance, preclearing of lysates with ATG and ALG but not H1G removed HLA-DR antigens (data not shown).

All the ATG and ALG preparations tested showed cytotoxic activity for T cells. Despite their poorer performance in some antibody competitions, all the ATG lots were equivalent to the best ALG lots in cytotoxicity. The ability to effect lysis must depend not only on the specific antigens recognized, but on factors such as the physical arrangement of antigens on the cell surface and the ability of antibodies to fix complement. There were no relative differences in cytotoxicity using rabbit or human serum as a source of complement. Furthermore, the concentrations of horse antisera needed for maximal cytotoxicity with human sera (160 to 320 ug/mL) were lower than the reported mean peak plasma levels of equine IgG after ATG infusion (727 ± 310 ug/mL).20,31 It may be difficult to relate in vitro cytotoxicity experiments to clinical performance as immunosuppressive agents, as the correspondence might be, for example, to suboptimal as opposed to plateau concentrations measured in vitro or dependent on the regimen for administration of sera to patients. The in vitro data suggests that the in vivo properties of ATG and ALG may be concentration related; T cells may be stimulated at low concentrations, whereas at higher concentrations, complement-mediated cytotoxicity and inhibition of T-cell proliferation by blocking important cell surface proteins such as the interleukin-2 receptor (CD25),22,23 the E-rosette receptor (CD2),24 or the T-cell receptor-associated antigen (CD3)25,26 may predominate.

A clue to the mechanism of action of ATG and ALG might be provided by the comparative laboratory analysis of a lot (ALG 97412) described as being relatively ineffective in clinical trials in aplastic anemia patients.17 Unfortunately, clinical data from Europe and the United States are contradictory in regard to the “activity” of this particular lot. In an American study (reviewed in 38), this lot led to a 31% rate of transfusion independence in 35 patients as opposed to a 69% response rate in a previous study of 29 patients by the same investigators using ALG EC162.4 However, use of lot 97412 in Basel resulted in response of 9 of 12 patients with severe or moderate aplastic anemia, similar to excellent results obtained with a previous active lot (ALG 15343) and a subsequent lot (ALG 1815). While the reason for the discrepancy between the American and European experience with lot 97412 will probably never be determined, it is difficult to conclude that this ALG lot was clinically ineffective.

Our studies were undertaken on the report of clinical inactivity of lot 97412.17 The results of our blocking experiments are similar to a published comparison of this “inactive” ALG lot and an active ALG lot (EC162)36; in particular, the relative amounts of antibodies specific for known T-cell antigens were similar in the two studies. While Smith et al38 concluded that the overall 2- to 5-fold decrease in antibody blocking activity of the “inactive” lot, rather than the absence of a single antibody specificity, might account for clinical inactivity, we found the “inactive” lot very similar to another known active ALG lot (15343). The immunostimulatory capacity of this “inactive” lot, as determined by blastogenesis assays and hematopoietin production by T cells was equivalent to or superior to active lots of ALG and ATG. Although the “inactive” lot was less cytotoxic at suboptimal concentrations and failed to plateau even at concentrations 4- to 8-fold greater than the concentrations required for most other lots, this pattern was similar to that observed with an active lot, 1815. There was greater lot to lot variability among ALGs than among ATGs (which were remarkably homogenous in their properties as tested), but even when viewed independently of the clinical data, our laboratory studies failed to show a unique property of lot 97412 not shared by other ALG lots.

A question that has not yet been addressed in clinical trials is the relative effectiveness of ATG versus ALG. Response rates to ATG have averaged about 50%,4 whereas the first American trial of ALG resulted in a response rate of 68%,4 and more recent Swiss data have suggested response rates as high as 85% with ALG in combination with adjuvant therapies.3 If these response rates are truly different, they may reflect differences in patient selection, clinical management, the addition of high-dose corticosteroids and androgens, and the regimen for administration of the horse serum in addition to intrinsic differences in the preparations. Patients in Europe are often observed for several months for spontaneous recovery prior to therapy; the use of isolation and antibiotics is not uniform among centers; and androgens are employed with ALG more enthusiastically abroad7 than in the United States.19 In addition, there may be an advantage to administering horse serum in large doses over a short period of time (as in most ALG protocols), prior to the onset of serum sickness and more rapid clearance of horse IgG,19 rather than over 10 to 28 days (as in most ATG protocols). If intrinsic properties of ALG are responsible for higher response rates in clinical trials, our results would direct attention to proteins such as Leu 1 and HLA-DR, which are much better bound by ALGs than ATGs.

Monoclonal antibody therapy of aplastic anemia has not been successful.40,41 Another approach to more specific and less toxic immunotherapy of aplastic anemia would be the production of polyclonal antisera directed against a limited number of defined antigens present on target T cells. While the broad antibody specificities and multiple biologic properties of ATG and ALG have hindered the elucidation of their mechanism of action, comparison using these assays of ATGs and ALGs with more restricted antisera may ultimately be revealing.

ACKNOWLEDGMENT

We thank Dr Leonard Bielory for analyzing the protein concentration and purity of the various ATG and ALG lots, Dr N. Chariatte for providing details of the preparation of the different ALG lots, Mike Vail and James Wong for their assistance with the flow microfluorometry analysis, Dr Arthur Nienhuis for his helpful advice and critical reading of the manuscript, and Imogene Surrey for her help in preparation of this manuscript.
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