Antithymocyte Globulin Reacts With Many Normal Human Cell Types

By Barbara Greco, Leonard Bielory, David Stephany, Su Ming Hsu, Pedro Gascon, Arthur Nienhuis, and Neal Young

Antithymocyte globulin (ATG) is frequently effective therapy for aplastic anemia. Its mechanism of action is often assumed to be upon a lymphocyte inhibitor of hematopoiesis. However, specificity for T lymphocytes would not be anticipated from consideration of the method of preparing ATG. In fact, using flow microfluorometry and fluorescence immunohistochemistry, we have found that ATG binds to virtually all circulating lymphocytes, granulocytes, and platelets, as well as to bone marrow cells. Extensive absorption of ATG with either granulocytes or lymphocytes does not eliminate reactivity with the opposite cells, indicating that ATG recognizes some distinct antigens on each cell type. Treatment of cells with ATG blocks the binding of monoclonal antibodies directed against either lymphocyte differentiation or histocompatibility antigens. ATG also binds to visceral tissues, including thymus and testis cell membranes and the nuclear and cytoplasmic components of tonsil, kidney, liver, breast, lung, and intestine. In vitro cytotoxicity of ATG was demonstrated for both T and non-T lymphocytes and platelets. Despite its name, ATG is not specific for a particular cell type, and it would be premature to conclude that its effect is mediated through a specific lymphocyte population.

A significant number of patients with aplastic anemia show a hematologic response on treatment with antithymocyte globulin (ATG).1,2 A potent immunosuppressive agent, ATG has been used successfully in the prevention and treatment of allograft rejection.3 Because of laboratory evidence that suppressor lymphocytes may inhibit hematopoiesis in some patients with aplastic anemia,4–6 the action of ATG in bone marrow failure is often assumed to be upon a lymphocyte target. However, the method of preparation of commercially available ATG is such that this agent is unlikely to be specific for a single human cell type.7 To prepare ATG, horses are immunized with homogenized thymus tissue fragments obtained from normal organs during pediatric surgery. Homogenization leads to disruption of cells, and the immunogen is actually a suspension of cell membrane, nucleic, and cytoplasmic components. Horse serum is absorbed with human erythrocyte stroma to reduce the hemagglutinin titer and with human plasma to remove antibodies to circulating proteins. An IgG fraction is made by passage of serum through an ion-exchange resin to remove beta-globulins.

As thymocytes express antigens common to many human cell types, some of which are highly immunogenic, many of the antibodies in the “ATG” resulting from this method would be expected to recognize cells other than thymocytes or T lymphocytes. ATG has been reported to bind to all normal blood mononuclear cells, T and B cells, and a variety of lymphoid tumor cells.8 In humans treated with ATG, almost all lymphocytes disappear from the circulation9 and marked decreases in platelet, and to a more viable degree, granulocyte number are observed.10,11

To determine the specificity of ATG, we studied the binding characteristics of several lots of this immunoglobulin preparation now in use for patient treatment. ATG binding to cell membranes of peripheral blood cells, including lymphocytes, granulocytes, and platelets, as well as to normal bone marrow cells was analyzed by immunofluorescence and flow microfluorometry (FMF); binding to visceral tissues was studied by immunohistochemical staining. The relevance of antibody binding to the potential mechanism for cell destruction was investigated using cytotoxicity assays.

MATERIALS AND METHODS

Reagents

ATG lots 17908, 17919, 17924, 842JX, 844JX, 731KM, and 953KS were provided by the Upjohn Company (Kalamazoo, MI) in solution at a protein concentration of 50 mg/ml and were heat inactivated by exposure to 56°C for 30 min. For some experiments, ATG was absorbed to normal cells by repeated reaction of 20 μl of a 1:50 dilution to 2–4 x 106 cells for 40 sec at 4°C, in order to reduce binding to that specific cell type to a constant minimal amount. Fluorescein-conjugated, affinity-purified F(ab’)2 goat anti-horse Ig was purchased from Cappel Laboratories (Cochranville, PA). Fluorescein-conjugated monoclonal antibodies specific for T cells (OKT3), the helper/inducer (OKT4), and suppressor/cytotoxic (OKT8) subsets of lymphocytes were purchased from Ortho Pharmaceuticals (Raritan, NJ), and anti-HLA-DR antibody was obtained from Becton Dickinson (Sunnyvale, CA). For tissue studies, a biotin-labeled rabbit anti-horse IgG was obtained from Vector Laboratories (Burlingame, CA) and was absorbed with human IgG and spleen cells prior to use. Chromatographically purified horse IgG was obtained from Amersham (Arlington Heights, IL) at a specific activity of 1.85 mCi/mg Cr.

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Isolation of Lymphocytes, Granulocytes, and Platelets

Peripheral blood lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized whole blood obtained from normal volunteers. For fluorescent staining, the mononuclear cells were washed in RPMI 1640 medium (M.A. Bioproducts, Walkersville, MD) supplemented with 2% fetal calf serum, and resuspended in Hanks’ balanced salt solution (HBSS) containing 0.1% bovine serum albumin and 0.1% sodium azide. T cells were separated from non-T-lymphocytes by rosetting with sheep red blood cells. Granulocytes were isolated by osmotic lysis of the pellet obtained from Ficoll-Hypaque sedimentation with an erythrocyte lysing solution containing 0.15 M NH₄Cl, 0.05 M KHCO₃, and 0.1 mM EDTA. Intact cells were pelleted, resuspended in HBSS, and examined morphologically after staining to verify that more than 90% were granulocytes. Single-cell suspensions of lymphocytes were prepared from platelet-rich plasma by a Stractan (arabinose-galactose) gradient method. Platelets were washed 3 times with calcium-free Tyrodes buffer containing 10 mM EDTA and 0.1 g/ml prostaglandin-E₁ (Sigma) and resuspended in HBSS, containing 10 mM EDTA.

Bone Marrow Cells

Bone marrow aspirates were collected from the posterior iliac crest of normal healthy donors, using a Jamshidi needle, directly into syringes containing 1 ml of Iscove’s modification of Eagle’s medium (GIBCO, Grand Island, NY) and 50 U of preservative-free heparin (Jones, McNeal & Feldman, St. Louis, MO). The mononuclear cell fraction was obtained by Percoll (Pharmacia, Piscataway, NJ) sedimentation, according to the manufacturer’s direction, and resuspended in HBSS, 10 mM EDTA.

Immunofluorescence Staining

Antibodies were used at dilutions predetermined to be at saturation levels. For fluoresceinated antibodies, the following dilutions were employed: OKT3, 1:15, OKT4, 1:15, OKT8, 1:15, and anti-HLA-DR, 1:30; and 1:100 dilutions were used for all lots of ATG and the fluoresceinated anti-horse reagent. For blocking control cells, were incubated with 5 μl of a tenfold higher concentration of ATG (1:10) or unlabeled mouse monoclonal antibody (1:2-1:4 range) for 30 sec at 4°C with intermittent mixing, followed by addition of the second antibody without washing. Horse IgG was used at a concentration of protein equivalent to that of UPjohn ATG. Immunofluorescence staining of single-cell suspensions of lymphocytes, granulocytes, platelets, and bone marrow cells was performed as described previously. Briefly, aliquots of 1-6 x 10⁶ cells were incubated at 4°C for 30 min with ATG, horse IgG, or HBSS alone. Cells then were washed and incubated for 30 min with the fluoresceinated reagent. Following labeling, cells were washed and resuspended in the appropriate HBSS solution.

Flow Microfluorometry (FMF) Analysis

FMF analysis was performed as described previously, using a Becton Dickinson FACS II (Becton Dickinson FACS Systems, Sunnyvale, CA) interfaced to a PDP 11/34 computer (Digital Equipment Corporation, Maynard, MA). Fluorescein was excited by 200 mW of argon laser light at 488 nm, and fluorescence emission was collected at 90° to intersection by an S-11 response photomultiplier tube (EMI, 650 V) after passage through 520-nm longpass, 530-nm cut on (Ditic Optics, Marlboro, MA), and 510-550-nm bandpass (Pomphret Optics, Slamford, Connecticut) optical filters. All fluorescence data were collected on 2-5 x 10⁴ individual viable cells as determined by forward light scatter intensity. Data for quantitative comparison of fluorescence intensity were collected using linear electronic amplifications and were analyzed as immunofluorescence (IF) profiles in which increasing fluorescence intensity is on the x axis and number of cells is on the y axis.

Because the reactions analyzed resulted in a wide range of fluorescence intensities, higher amplification (gain) was needed to assess relatively dull cells, while lower amplification (gain) was required for relatively bright cells (which were off scale at high amplifications). In every case, controls for each cell type were always analyzed at every gain at which an experimental antibody was analyzed on that cell. Mean fluorescence channel numbers (Mn Ch #) were calculated by using the formula:

\[ \text{Mn Ch #} = \frac{\sum N(C) \times C}{\sum N(C)} \]

where \(N(C)\) was the number of cells in channel \(C\), and the summations were made over all channels of data collected. In order to compare data collected at different linear gains, Mn Ch #s were converted to fluorescence units (FU) at a single arbitrarily chosen gain (16) by the following formula:

\[ \text{Mn FU (at gain 16)} = \left( \frac{\text{Mn Ch #} - P}{16/Gain used} \right) \]

\(P\) is the value in channel numbers of an electronically imposed voltage that is independent of amplifier gain. Using these conditions, Mn FU are proportional to fluorescence intensity.

For graphics purposes, data were also collected with logarithmic electronic amplification using a 3-decade logarithmic amplifier constructed from an NIH-modified design of Rattiebert, LASL, Los Alamos, NM. Logarithmic amplification permitted visualization of all cell types, regardless of fluorescence intensity, on the same x-axis.

Data were also analyzed for numbers of positive cells by integration of IF profiles above arbitrarily chosen levels of fluorescence intensity. These levels were selected for each cell type as the point of intersection between curves for positive (ATG + FITC anti-horse Ig) and negative (media + FITC anti-horse Ig) controls. Values calculated at this level for horse IgG controls were subtracted to obtain a minimum percentage of cells positive for ATG binding.

Staining of Visceral Tissues

The following normal human tissues were obtained as either fresh surgical specimens or removed at autopsy within 2 hr postmortem: tonsil, thymus, kidney, liver, breast, colon, testis, and lung. Normal bone marrow aspirates were analyzed by FMF analysis, and cyt centrifuge preparations were made for histochemical staining. All tissues were fixed in acetone and stained by an avidin–biotin immunohistochemical technique described previously. Tissues were incubated with lot 17908 of ATG at 1:100 dilution, washed, and incubated with a 1:200 dilution of the biotin-labeled reagent that was previously determined to give minimal background staining. Controls were incubated with horse IgG rather than ATG.

Cytotoxicity Assays

Lymphocytes, granulocytes, and platelets were labeled with ⁵¹Cr, and the cytotoxicity assay was a modification of published methods. Briefly, radiolabeled cells (20,000 cpm in 90 μl) were placed in 96-well Cooke microtiter plates (Dynatech, Alexandria, VA), and 90 μl of ATG in varying dilutions in phosphate-buffered saline was added in quadruplicate. The optimal concentration of guinea pig complement (1:40, Cappel), which resulted in lysis of more than 90% of human T cells, was determined by titration using ATG. Equiva-
RESULTS

ATG Binding to Peripheral Blood Cells

Peripheral blood lymphocytes, granulocytes, and platelets were analyzed by FMF for ATG binding. Background fluorescence, as determined by immunofluorescence profiles of cells incubated with horse IgG, is depicted in Fig. 1D. Differences in background staining among the three peripheral blood elements were found to be related to differences in autofluorescence; immunofluorescence profiles of suspensions that had not been treated with the fluorescein-conjugated reagent correlated with background profiles.

Immunofluorescence profiles for each cell type incubated with lot 17908 of ATG, plotted on a logarithmic fluorescence intensity scale, are shown in Fig. 1A, and the calculated proportion of cells and mean fluorescence in a typical experiment are listed in Table 1. The minimal percentages of lymphocytes, granulocytes, and platelets positive for ATG binding were all very high, and the difference in percentages between platelets and other cells was not considered significant. Cell size analysis of platelet suspensions before and after immunofluorescence staining demonstrated broadening of the cell size distribution caused by osmotic swelling of cells. In addition, with time, there was an increase in the number of small platelet particles and debris. Since FMF analysis determines only the minimum number of cells that can be detected by immunofluorescence, results of a typical experiment comparing binding of a single lot of ATG to various cell types. Lymphocytes and granulocytes from three other donors also showed almost 100% staining by ATG; platelet binding in two other individuals ranged from 75% to 90%. Two other normal bone marrows tested showed ATG binding to >90% of cells. Although mean fluorescence is not readily comparable from one experiment to another because equilibrium conditions are not reached, mean fluorescence of these samples was 1.697 and 1.689, which were approximately those observed with granulocytes and less than T-cell fluorescence.

![Fig. 1](image)

**Table 1. Binding of ATG to Hematopoietic Cells**

<table>
<thead>
<tr>
<th>Percent Positive</th>
<th>Mean Fluorescence (FU)</th>
</tr>
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<tbody>
<tr>
<td>Lymphocytes</td>
<td>99</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>99</td>
</tr>
<tr>
<td>Platelets</td>
<td>88</td>
</tr>
<tr>
<td>Normal bone marrow</td>
<td>92</td>
</tr>
<tr>
<td>Erythroid bone marrow</td>
<td>94</td>
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</table>

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ATG REACTS WITH MANY TYPES OF CELLS

Results were obtained using autologous human serum or rabbit serum as complement sources. Following incubation with shaking at 37°C for 1 hr, the cells were centrifuged, and the supernatant fluid assayed by gamma counting.
fluorescence, there may be positive cells below the detection limits.

Data also were analyzed using linear electronic amplification and revealed net FU per cell for lymphocytes, granulocytes, and platelets; the results of one of three typical experiments are summarized in Table 1. Lymphocytes have a higher FI than either granulocytes or platelets. The absolute numbers calculated for net FU indicate a much lower FI for platelets. Mean fluorescence is determined by cell size, density of reacting membrane antigens, and the accuracy of the instrument at lower fluorescence intensities. It was not possible to determine which factor was primarily responsible for the low platelet FI.

Antibodies present in ATG recognize multiple specificities on human cell surfaces. ATG that had been repetitively absorbed to T cells so as to reduce its binding to T cells to 0.4% of mean fluorescence units of unabsorbed ATG continued to show significant binding to granulocytes (6.3% of unabsorbed ATG). Similarly, ATG absorbed with granulocytes, so as to reduce its binding to granulocytes to 2.4%, showed residual activity against T cells (19.4% of unabsorbed ATG). In addition, experiments were performed to determine if some ATG specificities were directed against known lymphocyte antigens. Mouse monoclonal antibodies were not effective in blocking binding of ATG to lymphocytes. However, preincubation of T cells with ATG blocked binding of fluorescein-conjugated mouse monoclonal antibodies (results are expressed as percentage inhibition of ATG compared to H1g control): anti-OKT3, 68%; anti-OKT8, 72%; anti-OKT4, 53%. ATG also blocked the binding of anti-HLA-DR antibody to non-T-lymphocytes (59%). These results suggested that some antibodies present in ATG bound to, or bound close to, differentiation and histocompatibility antigens on lymphocyte surfaces.

Several lots of ATG were studied to determine potential differences in binding specificity that might have implications for therapeutic efficacy and adverse reactions to treatment. FMF analysis revealed no significant differences in IF profiles among three lots of ATG for all three cell types studied (Fig. 1, A–D). Because severe thrombocytopenia during ATG therapy has been observed in some individuals with normal platelet counts, binding of six lots to platelets was analyzed (Fig. 2, A–H). All lots tested showed binding to virtually 100% of platelets. Small differences in mean fluorescence were not reproducible from experiment to experiment.

Binding of ATG to Visceral Tissues

Immunohistochemical analysis demonstrated a relative specificity of ATG for thymocyte membranes. A comparison of positive membrane binding to minimal background staining with the biotin-labeled anti-horse antibody is shown for thymus in Fig. 3 (A and B). The field from thymus treated with ATG demonstrates binding to thymocyte membranes with a relatively lower intensity staining of cytoplasm and nucleus. In all other tissues studied for ATG binding, cytoplasm and nuclear staining were relatively more intense and masked cell membrane staining. Smooth muscle cells showed marked nuclear and cytoplasmic staining, including distinct fibrillae staining, as shown in a field from normal colon (Fig. 3C). A section of normal testis (Fig. 3E) illustrates cytoplasmic and nuclear staining of interstitial Leydig cells, endothelial cells, and tubular cells.

Binding of ATG to Bone Marrow Cells

To determine whether ATG showed a relative specificity for membranes of hematopoietic cells, single-cell suspensions of bone marrow aspirates from three normal individuals and a patient with hemolytic anemia were analyzed by FMF. The erythroid sample and one normal bone marrow were also examined by immunohistochemical staining (Table 1). Two hundred cell differential counts of the cytocentrifuge preparations showed a 2:1 myeloid-to-erythroid ratio of the normal bone marrows and a 1:5 ratio for erythroid marrow. By FMF analysis, almost all the cells of all bone marrows tested were positive for ATG binding; the net mean fluorescence of bone marrow cells was intermediate between granulocytes and lymphocytes. Immunohistochemical staining showed distinct nuclear membrane staining of apparently all bone marrow cells, with weak cytoplasmic and cell membrane staining as compared to background staining (Fig. 3, G and H). The lower intensity membrane staining using the biotin-labeled reagent may be an artifact of fixation of bone marrow. Data from the two methods suggest that both erythroid and myeloid bone marrow precursor cells, as well as mature granulocytes and platelets, bind ATG in vitro.

Cytotoxicity Studies

Although a large proportion of lymphocytes, granulocytes, and platelets showed membrane bound ATG by fluorescent studies, there were marked differences in cytotoxicity of ATG for these different cell types (Table 2). With and without complement, lymphocytes and platelets were more susceptible than granulocytes to lysis by ATG, assessed by either the concentration of ATG required for maximal lysis or the quantity of total cell 51Cr released. However, it proved difficult to obtain granulocyte lysis using antibodies directed against HLA antigens and a variety of complement sources (data not shown). This relative
resistance of granulocytes to lysis by ATG may be a cellular characteristic. While there were statistically significant differences in the degree of cytotoxicity for T cells compared to non-T mononuclear cells, it was evident that ATG lysed both cell types. The concentration of ATG required and the amount of $^{51}$Cr release were similar to those reported previously by Fisher et al.

**DISCUSSION**

How specific for T lymphocytes is ATG? Using fluorescent methods, ATG binding can be demonstrated to the majority of circulating lymphocytes, granulocytes, and platelets. Antibodies in ATG preparations do not appear to be passively absorbed to these cells, as similar levels of binding are not seen with horse immunoglobulin controls. ATG binding detected by fluorescent methods does not correlate with in vitro measures of cytotoxicity, presumably due to differences among cells in antigen number, distribution, or susceptibility to complement-mediated lysis. The frequent decreases in platelet and granulocyte number observed following clinical administration of ATG may be the result of antibody binding to these cells and the subsequent removal of cells by tissue macrophages, rather than by a mechanism of intravascular complement-mediated cytotoxicity.

The present study confirms an earlier report that
Fig. 3. (a) Normal thymus treated with ATG, showing distinct membranous staining and weak cytoplasmic reaction. (b) Horse IgG control, normal thymus. (c) Smooth muscle cells in normal colon treated with ATG, showing cytoplasmic fibrillar reaction with intense nuclear staining. (d) Horse IgG control, normal smooth muscle. (e) Normal testis treated with ATG, showing nuclear and cytoplasmic reactions. (f) Horse IgG control, normal testis. (g) Normal bone marrow aspirate treated with ATG. (h) Horse IgG control, normal bone marrow aspirate.
ATG reacts with many types of cells achieved. After exposure of cells to water containing 0.1% Triton X-100, a degree of T-cell binding and cytotoxicity made it apparent that significant differences would be found between T-cell subsets. Indeed, ATG blocked the binding of antibodies directed against antigens on helper and suppressor T cells as well as B lymphocytes. In mice, low-dose antithymocyte serum administration has a more potent depleting effect on helper lymphocytes than on cytotoxic lymphocytes, but in man, peripheral blood T cell subsets, defined by monoclonal antibodies, are equally affected by ATG administration for renal allograft rejection and aplastic anemia. The responsiveness of patients with aplastic anemia to ATG therapy cannot be interpreted as evidence for the presence either of an abnormal suppressor type of lymphocyte or a functionally important inhibitor cell of hematopoiesis.

ATG binds to the majority of normal bone marrow cells. ATG incubation with bone marrow in tissue culture has been reported to stimulate erythroid colony formation and to enhance or inhibit granulocytic colony growth. Such experiments have been designed to detect ATG effects on inhibitory lymphocytes or to test for direct cytotoxicity of ATG for hematopoietic progenitors. However, binding of antibody to hematopoietic cell membranes may have important physiologic consequences that are now difficult to predict. For example, ATG binding has been reported to inhibit the response of lymphocytes to lectin stimulation, to block granulocyte chemotaxis, and to induce platelet aggregation and release.

All three lots of ATG tested showed identical reactivity with peripheral blood cells. We were unable to obtain samples of antilymphocyte globulin (ALG) for comparable testing. ALG is prepared by immunizing horses with human thoracic duct lymphocytes, and the resulting horse serum is absorbed with human placental cells. The use of intact cells for immunization and absorption with cells expressing fetal antigens may lead to immunoglobulin specificities in ALG quite different from those of ATG. Nonetheless, both ATG and ALG appear to be effective treatment for some patients with aplastic anemia.

The hematologic recovery of patients with bone marrow failure following ATG has important implications concerning the pathogenesis of aplastic anemia. It indicates that some proportion of patients with this disease have residual but functionally inapparent hematopoietic stem cells. ATG treatment can induce or recruit these cells to support near-normal hematopoiesis. Binding to another cell type involved in stem cell regulation, the stimulation of growth factor production by immune complex formation, or directly mediated alterations in the function of hematopoietic progenitors are other possible mechanisms of ATG action.

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