ABSTRACT  Antithymocyte globulin (ATG) therapy is an important treatment alternative for patients with acquired aplastic anemia. The mechanism by which it exerts its effects on hematopoiesis is unknown. In this report, we describe the ability of horse ATG to induce growth and differentiation of normal bone marrow. A single cell suspension of normal human bone marrow was cultured in methylcellulose medium and examined for the growth and maturation after incubation with ATG (10 μg/ml). After 3-4 days of culture, spherical colonies containing mature myeloid elements were found in cultures containing ATG but not in cultures containing medium or preimmunization horse IgG. The addition of 10% colony-stimulating factor increased growth by 40%. The number of spherical colonies is not dependent on the presence of macrophages or T lymphocytes. This property of ATG may be relevant to the mechanism behind the hematologic recovery in some patients with acquired aplastic anemia. We also describe the ability of ATG to induce terminal differentiation in the HL60 leukemic cell line. ATG binds to HL60 cells and at concentrations between 10 and 100 μg/ml, 50% of the cells become mature granulocytes, acquire the ability to reduce nitroblue tetrazolium, and lose their proliferative capacity in the clonogenic assay. These new observations of ATG-induced differentiation of normal marrow myeloid elements and terminal differentiation of the HL60 cell line point to different avenues for future search of differentiation-inducing agents.

Antithymocyte globulin (ATG) is a heteroantiserum prepared by immunizing animals with pooled human thymocytes. The only available commercial preparation in the United States, ATGAM, is a concentrate of equine IgG antibody. Other noncommercial preparations using thoracic duct lymphocytes as immunogen are also available in the United States and Europe. Biologic activity is determined by its ability to inhibit sheep erythrocyte rosette formation and delay animal skin graft rejection (1). The antilymphocyte immunosuppressive properties of ATG have led to its use in ameliorating kidney transplant rejection (2, 3) and in treatment of graft-vers-host disease in allogeneic bone marrow transplant recipients (4, 5).

The treatment with ATG or other similar preparations has emerged as an important therapy for acquired aplastic anemia since 1978. Autologous hematologic reconstitution occurs in 50-74% of patients receiving these preparations (6, 7). The mechanism for the hematologic recovery in these responding patients is unknown. Many investigators have postulated a reversal of immunosuppression of hematopoiesis as the probable cause of recovery (6, 8). This hypothesis is supported by some clinical and laboratory evidence. Other mechanisms probably exist, because many patients with severe aplastic anemia do not demonstrate immune-mediated marrow suppression. Also, successful marrow reconstitution has occurred in 11 of 22 patients with aplastic anemia who received a bone marrow transplant from a genetically identical twin in the absence of any form of immunosuppressive and marrow ablative conditioning regimen (9).

In an effort to explore other mechanisms for ATG-induced hematopoietic recovery, we examined the effect of ATG on hematopoietic precursor cells. In the present report, we describe the ability of ATG to induce morphological differentiation of human bone marrow cultured in semi-solid medium without mediation via T lymphocytes or macrophages. We also describe the ability of ATG to induce terminal differentiation in the HL60 cell line.

MATERIALS AND METHODS

Human Bone Marrow. After obtaining informed consent, we aspirated normal bone marrow from the posterior iliac crest of donors for allogeneic bone marrow transplantation into preservative-free heparinized syringes. The marrow was forced through a 22-gauge needle, diluted 1:2 in Hanks’ balanced salt solution without Ca2+ and Mg2+ (GIBCO), and layered on a Ficoll-diatrizoate gradient (density 1.077–1.080) (Litton Bionetics). Mononuclear cells with occasional mature elements were recovered from the interface and suspended in Iscove’s medium (GIBCO) containing 20% fetal calf serum. Duplicate cultures of 2 × 10⁵ cells in single cell suspension were plated in 1 ml of 0.8% methylcellulose in Iscove’s medium supplemented with 30% fetal calf serum, 100 units penicillin, 100 μg of streptomycin, and 2 mM L-glutamine in 35-mm tissue culture dishes. Five to 10% human placental conditioned medium prepared by the method of Schlunk and Schleyer (10) was added as a source of colony-stimulating factor (CSF). ATG at various concentrations (1–10 μg/ml) (gift of Upjohn) was added to test for effects on cell differentiation and colony formation. Culture plates were incubated in a 5% CO2/95% air humidified incubator at 37°C. To assess differentiation effects, cells were harvested after 3–4 days of culture by the addition of Hanks’ balanced salt solution with gentle swirling and rinsing into 50-ml centrifuge tubes. Cells were spun at 50 × g for 10 min and then resuspended in 1–2 ml of Hanks’ balanced solution containing 4% fetal calf serum. Cytocentrifuge preparations were made and stained with Wright’s staining reagent. The degree of maturity of the myeloid elements was scored by defined morphologic criteria (11).

Removal of T Lymphocytes and Macrophages. T lymphocytes were removed from the bone marrow cells by sequential treatment for 40 min on ice with OKT3 (Ortho Diagnostics) (1:50 dilution) and 3A1 (gift from Barton Haynes, Duke University Medical Center, Durham, NC) (1:75 dilution).

Abbreviations: CFU-GM granulocyte-macrophage progenitors; CSF, colony-stimulating factor; ATG, antithymocyte globulin; NBT, nitroblue tetrazolium.

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Cells were washed and then incubated at room temperature for 60 min with rabbit complement (Pel-Freez) at a 1:4 dilution. Macrophages were removed by treatment with Mo2 (Coulter) (1:50 dilution) and complement as described above. Cytofluorography was used to demonstrate effectiveness of depletion of T lymphocytes and macrophages from the normal marrow.

HL60 Cell Line. The HL60 cell line was obtained from D. W. Sedwick (Duke University Medical Center), who acquired it from R. C. Gallo (National Cancer Institute). The experiments reported were performed on cells in logarithmic phase between passage 15 and 30. The cells were routinely maintained in RPMI1640 medium with Hepes buffer (GIBCO)-supplemented with 10% fetal calf serum/2 mM L-glutamine/100 units of penicillin per ml/100 μg of streptomycin per ml.

Cells were suspended at a concentration of $1 \times 10^6$ per ml and incubated with various concentrations of ATG for 6 days, at which time the control cultures began to deteriorate. Cells were harvested daily for morphologic examination and viability by using trypan blue exclusion. After 6 days of culture, cells were harvested for cytofluorograph analysis, nitroblue tetrazolium reduction (NBT), and were assayed for clonogenic potential.

Antithymocyte Globulin. ATGAM (Upjohn) lot 17,924 was used in all experiments. Preimmunization horse IgG was also tested for its activity on human bone marrow and HL60 cells. In selected experiments, ATGAM was extensively absorbed with peripheral blood lymphocytes (five absorptions with $10^7$ cells each at 4°C for 1 hr).

NBT Reduction. Cells at a concentration of $2 \times 10^6$ per ml were incubated for 20 min in an equal volume of 0.4% NBTZ (Sigma) dissolved in Hanks' balanced salt solution containing 400 μg of freshly diluted phorbol 12-myristate 13-acetate (PMA) (PolyScience, Warrington, PA). PMA was stored at −70°C in 10 mM solutions of dimethyl sulfoxide. The reaction was stopped with cold Hanks' balanced salt solution and cytocentrifuge preparations were made. The slides were stained with 1% neutral red to contrast with the intracellular blue-black formazan deposits. A minimum of 200 cells were scored.

HL60 Clonogenic Assay. HL60 cells were first grown for 6 days in maintenance medium with and without the addition of ATG. The clonogenic activity of the cells was then determined by culturing $4 \times 10^4$ washed cells in 0.8% methylcellulose in Iscove's medium supplemented with 30% fetal calf serum, penicillin, streptomycin, and L-glutamine in 35-mm tissue culture dishes for 7 days at 37°C in a 5% CO₂/95% air humidified incubator. Colonies (>50 cells) were scored at the end of the incubation period.

Cytofluorograph Analysis. HL60 cells ($1 \times 10^6$) were harvested after 45 min (with ATG at 100 μg/ml) or 6 days of continuous incubation (with ATG at 10 μg/ml) and washed three times with Hanks' balanced salt solution containing 2% fetal calf serum. To determine the actual binding of ATG, the cells were incubated with a fluorescein-conjugated goat anti-horse antibody (Cappel Laboratories, Cochranville, PA) for 45 min at 4°C and analyzed on an Ortho cytofluorograph model 50-H. The percent of positively stained cells was determined. A minimum of 5000 cells were counted. Cells incubated in the absence of ATG served as negative controls.

RESULTS

Morphologic Changes of Human Bone Marrow Induced by ATG. Single cell suspensions of human bone marrow cells were cultured in methylcellulose medium containing 5–10% CSF. In the presence of 10 μg of ATG per ml, tight spherical colonies developed from 1-, 2-, 4-, and 8-cell stages to 20–60 cells per colony after 3–4 days of culture. These colonies were not observed in cultures containing medium or preimmunization horse IgG (Fig. 1). In the presence of ATG, colony cells had mature myeloid characteristics (myelocytes, metamyelocytes, bands, neutrophils, and sometimes a large

Fig. 1. Spherical colonies grown from normal bone marrow after 3 days of exposure to ATG (10 μg/ml). These colonies were observed with or without exogenous CSF.
number of eosinophils (Figs. 2 and 3). Control cultures contained neither tight spherical colonies nor mature elements, although occasional small flat colonies characteristic of early colony forming units–granulocytes/macrophages (CFU-GM) were seen in all cultures.

To examine whether these differentiating tight colonies depended on T lymphocytes or macrophages for growth, T cells and macrophages were removed from normal marrow by treatment with monoclonal antibodies and complement. As seen in Table 1 from an average of 2 experiments, the number of spherical colonies was not affected by the removal of macrophages. However, colony size was larger (an aver-
age of 50–60 cells per colony), suggesting that prostaglandin E (produced by macrophages in culture) may be a negative regulator of cell proliferation of the spherical colony (12).

When lymphocytes were removed, the number of colonies decreased, although not significantly (Table 1). To investigate further the role of lymphocytes, we looked at spherical colonies induced by ATG that had been absorbed with lymphocytes. The number and size of colonies were increased over the controls (data not shown), suggesting that the ATG preparation may contain an inhibitor(s) of colony formation, removable by absorption with lymphocytes. The presence of lymphocytes in the crude marrow similarly may serve to reduce the concentration of the inhibitor, giving better colony growth. Alternatively, ATG may stimulate lymphocytes contained in the marrow aspirate to produce a growth-promoting factor, which in turn increases the number of colonies.

Spherical colony formation is dependent on the presence of ATG. Colonies were regularly seen with or without the addition of CSF, although CSF acts synergistically to promote spherical colony growth induced by ATG (40% increase) as seen in Fig. 4 (average of 2 experiments).

It is postulated that cells induced to differentiate in the presence of ATG are more mature than CFU-GM precursors, partly because of the brief time requirement for differentiated colonies to occur (3–4 days as compared to 6–7 days for CFU-GM) and partly because CFU-GM colonies simultaneously exist in culture and are readily recognizable by their appearance. Furthermore, CFU-GM colony growth approaches its peak at ~7 days of culture when spherical colonies are dying. Therefore, it was decided to study the effect of ATG on the HL60 leukemic cell line derived from a patient with acute promyelocytic leukemia.

Differentiation of HL60 Cells by ATG. After 45 min of incubation in ATG (100 μg/ml), 75–85% of HL60 cells demonstrated ATG binding, and 65–75% of the cells were still positive after 6 days of incubation. In tissue culture, only a small percentage of HL60 cells (<10%) spontaneously differentiate into more mature forms (myelocytes, metamyelocytes, bands, and neutrophils). HL60 cells grown in suspension in the presence of ATG undergo differentiation toward granulocytes. Similar to other differentiation inducers, effects of ATG are time and concentration dependent (Fig. 5).

When cells are cultured at 1 × 10^6 cells per ml in the presence of ATG alone, 50–55% of the cells undergo morphological changes toward mature myeloid elements (myelocytes, metamyelocytes, bands, and neutrophils). These changes are more recognizable after 6 days of culture and at an ATG concentration of 10–100 μg/ml. Differentiation is seen in a small percentage of cells after 48 hr of culture. After 4 days of culture, the differentiated cells are predominantly myelocytes and metamyelocytes, and after 5–6 days the majority resemble segmented neutrophils. The morphological differentiation seen in these cells was further substantiated by studies with NBT dye reduction. NBT, a water soluble dye, is converted to insoluble intracellular blue formazan by phagocytizing neutrophils. After 6 days of culture at concentrations of ATG from 10 to 100 μg/ml, 35–40% of the HL60 cells reduced NBT as compared to 8% in the control cultures. Although the concentration of ATG for more apparent cell differentiation was found to be between 10 and 100 μg/ml, concentrations as low as 4 μg/ml also induced some differentiation.

To further evaluate ATG-induced terminal differentiation of HL60 cells, the clonogenic potential under the influence of ATG was measured after 6 days of incubation in liquid medium containing variable concentrations of ATG. After each experiment, the cells were washed, resuspended in fresh medium, and 2 × 10^5 cells were cultured in 0.8% methylcellulose with 10% CSF. Colonies were counted after 7 additional days of culture and the colony number was...
normalized according to the number of viable cells initially plated in semisolid medium. The results of these experiments are expressed as a percentage of the number of colonies from the ATG pretreated cells divided by the number of colonies in the control group times 100% (Fig. 6). Cells cultured with concentrations of ATG between 10 and 100 µg/ml expressed a 60–80% loss in clonogenic potential, which parallels the concentration range causing morphological differentiation.

The addition of ATG to HL60 cells in liquid culture also resulted in alterations in the rate of cell growth apparent after 48 hr of culture. The proliferative activity of HL60 cells in liquid cultures was reduced or eliminated with concentrations of ATG greater than 10 µg/ml (Fig. 7). Concentrations of 1000 µg/ml produced cell death. However, at concentrations between 10 and 100 µg/ml, cell proliferation ceases although cells remain viable over 6 days of culture. The range of ATG concentration associated with antiproliferative activity is again identical to that capable of inducing terminal differentiation.

DISCUSSION

ATG was found to induce tight spherical colony growth and myeloid differentiation of human bone marrow. This effect was seen early, after 3–4 days of culture, at an ATG concentration of 1–10 µg/ml and was not dependent on the presence of T lymphocytes or macrophages. Our studies also demonstrate that ATG is capable of inducing morphological and functional differentiation toward granulocytes in the human promyelocytic leukemia cell line, HL60. Fifty percent of the HL60 cells grown in suspension with ATG differentiated to mature myeloid elements and acquired the ability to reduce NBT dye. The optimal concentration of ATG for these effects to occur ranged between 10 and 100 µg/ml. At these concentrations, HL60 cell proliferation decreased and the clonogenic potential of cells incubated with ATG was reduced to 20–40% of controls. Other compounds capable of inducing terminal differentiation in this cell line, such as retinoic acid, dimethyl sulfoxide, or phorbol esters have been described (13–15). ATG as a differentiation inducer is unique: it is an IgG concentrate made against human T-lymphoid cells but it also binds to the membrane of normal marrow and leukemic HL60 cells. To our knowledge, this is the first report of an antibody preparation capable of inducing differentiation of normal marrow precursors as well as the HL60 cell line and it opens new possibilities for the development of a different species of differentiation inducers.

ATG is effective therapeutically in acquired idiopathic aplastic anemia. Our findings of ATG-induced marrow differentiation may be meaningful in explaining its efficacy in this disease. Higher rates of stem cell proliferation have been achieved in mice by depleting the committed progenitor pool (16). It is possible that increased differentiation of committed progenitors by ATG enhances the patients’ stem cell proliferation. If this hypothesis is true for human marrow, there could be considerable potential use for ATG in conditions involving failure of bone marrow differentiation.

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