Lymphokine Abnormalities in Aplastic Anemia: Implications for the Mechanism of Action of Antithymocyte Globulin

By Pedro Gascon, Nicholas C. Zoumbos, Giuseppe Scala, Julie Y. Djeu, Jeffrey G. Moore, and Neal S. Young

Anti-thymocyte globulin (ATG) provides effective therapy for many patients with aplastic anemia, and its mechanism of action has been presumed to be secondary to lymphocyctotoxicity. However, our studies of lymphocyte function in aplastic anemia show marked abnormalities of lymphokine production, which ATG may modulate. In 12 of 17 patients with aplastic anemia, interleukin 2 (IL2) production was markedly elevated in vitro (P < .01 by paired statistical analysis). Expression of the IL2 receptor, or Tac antigen, on peripheral lymphocytes assessed by flow microfluorometry was also increased above the normal range in 11 of 15 cases. Studies of ATG suggested that it might act to stimulate lymphocyte function. In vitro, ATG is a mitogen, as measured by incorporation of \(^{3}H\)-thymidine into blood mononuclear cells; the response of cells to ATG from patients with aplastic anemia was exaggerated in comparison with normals. Cell proliferation was accompanied by production of IL2 to levels that were, in some cases, similar to those obtained with lectin stimulation. Finally, supernatants from lymphocytes cultured in the presence of ATG were able to replace adherent cells in providing growth factors for the support of nonadherent cells in methylcellulose hematopoietic colony assays. These results provide a mechanism for an “immunostimulatory” action of ATG in effecting hematopoietic response in some patients with aplastic anemia.

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THE EFFECTIVENESS of antiserums directed to human lymphocytes in the treatment of aplastic anemia has been demonstrated in large clinical studies of anti-thoracic duct lymphocyte serum (ALG) in Europe and in more recent controlled trials of ALG and anti-thymocyte globulin (ATG) in the United States. Although the mechanism of action of ALG and ATG has been presumed to be lymphocyctotoxic, other forms of immunosuppression, including cytotoxic drugs, plasmapheresis, and lymphocyte depletion, and monoclonal pan-T cell antibodies have been much less effective than either ATG or ALG. Furthermore, there are no in vitro tests of immunosuppression that consistently correlate with response to ATG or ALG.

Our interest in a possible immunoregulatory role for ATG was initially suggested by observations of disorder in vitro lymphokine production, especially interleukin 2 (IL2), or T cell growth factor), in patients with aplastic anemia. IL2 is a soluble glycoprotein factor produced by T cells that is required for the in vitro proliferation and maturation of certain T lymphocytes, natural killer cells, and antibody production by B cells. Responsiveness of T cells to IL2 is at least partly modulated by the T cell growth factor receptor, measured by the monoclonal anti-Tac antibody. Because the production of growth factors such as IL2 and colony-stimulating factor (CSF) by lymphocytes in some biologic systems is coordinated, these results suggested that hematopoietin production may also be dysregulated in aplastic anemia. Antiserums raised against peripheral blood lymphocytes has been reported to be mitogenic for T lymphocytes. In the experiments we report here, ATG also was shown to have mitogeniclike effects on T cells, including stimulation of both IL2 and hematopoietin production. These data suggest a possible “immunostimulatory” role for ATG in some patients with aplastic anemia.

MATERIALS AND METHODS

Patients

Seventeen adult patients with severe aplastic anemia, as defined by peripheral blood counts and bone marrow biopsy, were studied. Twelve had acute disease (less than three months from the time of diagnosis to study) and five had chronic disease. Two of the chronic patients had received ATG treatment with no hematologic improvement. The 17 aplastic patients had received an average of 26 ± 33 U (x ± SD; range, zero to 100) units of packed red blood cells and 65 ± 136 U (range, zero to 520) units of platelet transfusions. Four patients had received no platelet transfusions, two patients had received no red blood cell transfusions, and six other patients had received less than five erythrocyte units at the time of study. None of the aplastic anemia patients showed clinical evidence of active fungal, bacterial, or viral infection, and titers of antivirus antibodies were lower than in multiply transfused patients. Six aplastic anemia patients were studied three times to confirm the reproducibility of the immunologic assays described below.

Two populations were used for controls. The first control group comprised 36 patients with the following hematologic diseases: sickle cell disease (eight cases), \(\beta\)-thalassemia (seven), refractory anemia (six), myelodysplasia (three), pancytopenia with normocellular bone marrow (three), bone marrow depression secondary to chemother-
apy (five), pure red cell aplasia (two), pure white cell aplasia (one), and idiopathic thrombocytopenic purpura (one). Almost all the patients with anemia were participants in red blood cell transfusion programs (n = 23; x ± SD = 155 ± 96; range, 30 to 700 U); patients with myelodysplasia, pancytopenia with cellular marrow, bone marrow depression owing to chemotherapy, and idiopathic thrombocytopenia had received variable quantities of platelets (n = 13; x ± SD = 33 ± 52; range, 0 to 200 U). In addition, 20 apparently healthy volunteers served as a second control population.

Peripheral Blood Lymphocytes

Fresh blood was drawn into syringes containing 20 U/mL preservative-free heparin (O’Neill and Feldman, St Louis), and mononuclear cells were separated on a Ficoll-Hypaque gradient (LSM, Litton Bionetics, Kensington, Md). Cells were washed three times and resuspended in RPMI 1640 medium (MA Bioproducts, Walkersville, Md), supplemented with 2 mmol/L glucose, 100 U/mL penicillin/streptomycin (MA Bioproducts), and 10% heat-inactivated fetal calf serum (FCS; HyClone, lot 100378, Sterile Systems, Inc Logan, Utah). Nonadherent cells were obtained after incubating mononuclear cells in tissue-culture dishes (Falcon 3003, Becton Dickinson, Oxnard, Calif) for one hour at 37 °C in a 5% CO2 humidified chamber. T lymphocytes were separated by rosetting nonadherent cells with neuraminidase-treated sheep red blood cells at 4 °C for one hour.

Interleukin 2 Assay

To measure IL2 production, 2 × 10⁶ peripheral blood mononuclear cells suspended in 5% FCS were incubated with or without phytohemagglutinin (PHA; Wellcome Research Laboratories, Beckenham, England) at 2.5 μg/mL for two days. To assay for IL2 activity, the supernatants of cultured lymphocytes were tested for their ability to support the growth of the murine IL2-dependent CT-6 cell line, using a 3H-thymidine proliferation test. Every assay was compared with a standard IL2 supernatant (256 U/mL) obtained from human mononuclear cells stimulated with 10 μg/mL Concanavalin A (ConA) and 10 μg/mL phorbol-12-myristate-13-acetate (PMA). In order to determine IL2 units, the standard and samples were tested at serial twofold dilutions starting at 1:4 or 1:6. The results generated in this microassay were evaluated by logarithmically plotting the counts per minute of 3H-TdR uptake against the logarithmic dilution of the standard sample. The IL2 titer in test samples was calculated by reciprocal titer of the test samples, which gave 50% of maximal counts per minute of standard.

Interferon Assay

Interferon was measured by its ability to inhibit cytopathic effects of vesicular stomatitis virus on human amnion WISH cells.

Blastogenesis Assay

DNA synthesis was assayed indirectly by measuring methyl-3H-thymidine incorporation (5 Ci/mmol, Amersham Corp, Arlington Heights, Ill). Then, 1 × 10⁶ peripheral blood mononuclear cells in 5% FCS were dispensed into 96-well microtiter plates in triplicate (Costar, Cambridge, Mass) and stimulated with different concentrations of various lots of ATG: 731, L88KS, 17908, 17924, 844, 953 (Upjohn Laboratories, Kalamazoo, Mich). Cells were incubated for 72 hours in a 5% CO2 humidified chamber. Sixteen hours before harvesting, 1 μCi methyl-3H-thymidine was added to each well. Cells were harvested, and the methyl-3H-thymidine incorporation was determined in a liquid scintillation counter.

ATG-Conditioned Media

Peripheral blood mononuclear cells, 2 × 10⁶ cells/mL, in 5% FCS were incubated with or without ATG lot 731 (5, 2.5, 1, and 0.5 μg/mL) for seven days, and supernatants were harvested. Horse IgG (HIGG; United States Biochemical Corp, Cleveland)- and OKT3-conditioned media (Ortho Diagnostic Systems, Inc, Raritan, NJ) were obtained in the same way and used as controls. Aliquots of the supernatants were obtained on days 2 and 3 to measure IL2 and interferon, respectively.

Hematopoietic Colony Cultures

Bone marrow was obtained from patients and normal volunteers who had given informed consent. Bone marrow cells aspirated from the posterior iliac crest were drawn into Iscove’s modification of Dulbecco’s medium (IMDM; Flow Laboratories, McLean, Va) with 50 U/mL preservative-free heparin. Mononuclear cells were separated by Percoll sedimentation (Pharmacia Fine Chemicals, Piscataway, NJ). Cells were washed twice in IMDM-2% FCS and dispensed in a plastic flask (Corning 25100, Corning Glass Works, Corning, NY) for one hour at 37 °C in a 5% CO2 humidified chamber. Nonadherent bone marrow cells were then plated in 35 × 10 suspension culture dishes (Flow Laboratories) at final concentrations of 1 × 10⁷ cells per milliliter. Culture medium contained 0.8% methylocellulose (Dow Chemical Co, Midland, Mich), 30% FCS, 1% bovine serum albumin (BSA: Sigma, St Louis), 100 U/mL penicillin/streptomycin, and 10% volume supernatant of either ATG-conditioned medium, HIGG-conditioned medium, or OKT3-conditioned medium. Cultures were incubated at 37 °C in a 5% CO2 humidified chamber. Colonies were enumerated by their characteristic morphology: myeloid colonies [colony-forming unit cell (CFU-C)-derived] on day 10 and erythroid colonies [burst-forming cell (BFU-E)-derived] on day 18. Unfractionated bone marrow mononuclear cells grown under optimal conditions in medium containing 2.5 U/mL of erythropoietin (Epo; Connaught Laboratories, Willowdale, Ontario) and 10% phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) for erythroid colony formation or 10% human placental-conditioned medium for myeloid colony formation served as controls.

Tac Antigen Determination

Tac expression was quantitated by indirect immunofluorescence using anti-Tac monoclonal antibody (kindly provided by Dr Thomas A Waldmann) and a fluorescein-conjugated sheep anti-mouse IgG as previously described. T lymphocytes were suspended in Hanks’ balanced salt solution (MA Bioproducts) without phenol red, containing 0.1% sodium azide, 0.1% BSA. After centrifugation, 1 × 10⁶ cells were incubated with 10 μL of anti-Tac monoclonal antibody 1:5,000 dilution (MA Bioproducts) without phenol red, containing 0.1% sodium azide, 0.1% BSA. After centrifugation, 1 × 10⁶ cells were incubated with 10 μL of anti-Tac monoclonal antibody 1:5,000 dilution at 4 °C for 30 minutes. After two washings, a fluorescence-conjugated sheep anti-mouse IgG (Cappel 15649, Cappel Laboratories, Cochranville, Pa) was added in a 1:64 dilution, and the cells were incubated at 4 °C for 30 minutes. Mouse ascites IgG was used as control. Flow microfluorometry was performed using a fluorescence-activated cell sorter (FACS II; Becton Dickinson, FACS System, Sunnyvale, Calif). Data were selected, stored, and analyzed using a PDP 11/34 computer (Digital Equipment Corp, Marlboro, Mass).

Statistics

A paired, or two-sample, comparison t test was applied to determine the significance (P value) of differences between the results of cohort experiments. The Omnibus test was used to assess ranking of summarized experimental data.
LYMPHOKINES IN APLASTIC ANEMIA

Table 1. Assay of Hematopoietic Growth Factors in ATG-Conditioned Medium: Representative Experiment

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>BFU-E (per 10^6 mononuclear cells)</th>
<th>CFU-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonadherent BM</td>
<td>ATG-CM</td>
<td>102 ± 10</td>
</tr>
<tr>
<td>HlgG-CM</td>
<td>32</td>
<td>21</td>
</tr>
<tr>
<td>OKT3-CM</td>
<td>36</td>
<td>31</td>
</tr>
<tr>
<td>Medium</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>Unfractionated BM</td>
<td>PHA-CM</td>
<td>85</td>
</tr>
<tr>
<td>Patient 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonadherent BM</td>
<td>ATG-CM</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>HlgG-CM</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>OKT3-CM</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Medium</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Unfractionated BM</td>
<td>PHA-CM</td>
<td>47 ± 8</td>
</tr>
<tr>
<td>Normal Control</td>
<td>ATG-CM</td>
<td>13</td>
</tr>
<tr>
<td>HlgG-CM</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>OKT3-CM</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Medium</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Unfractionated BM</td>
<td>PHA-CM</td>
<td>47 ± 8</td>
</tr>
</tbody>
</table>

BFU-E, erythroid burst-forming cell; CFU-C, colony-forming myeloid cell; BM, bone marrow; CM, conditioned medium; HlgG, horse IgG.

RESULTS

Interleukin 2 Production In Vitro

IL2 production by peripheral blood mononuclear cells that had been stimulated with PHA was measured using an IL2-dependent cell line. IL2 production was markedly increased in this population of patients with severe aplastic anemia, both acute and chronic, in comparison with cells from normal individuals (Fig 1). IL2 production was abnormally elevated in seven patients who had received little or no blood product transfusions. In patients receiving multiple red blood cell transfusions for other hematologic diseases, IL2 production was below normal. Because of the variability of the biologic assay, samples from patients were always tested in parallel with normal cells. Figure 1a shows the percentage of increase or decrease of IL2 production of each individual compared with his control. Figure 1b shows a compound result of the IL2 production, expressed in U/mL, in aplastic anemia patients (x ± SE = 77 ± 15), in normal individuals (x ± SE = 34 ± 6), and in patients with other hematologic diseases (x ± SE = 17 ± 3). In 12 of 17 cases, IL2 production by cells from patients was outside the normal range. Paired statistical analysis of the data showed significance at P < .01 with the normal controls (Table 1) and at P < .001 with the hematologic controls. The time course of IL2 production in vitro was similar for cells from both aplastic anemia patients and normal individuals (Fig 2).

Tac Antigen Expression

Anti-Tac monoclonal antibody recognizes the IL2 receptor. In normal individuals, Tac expression on circulating T cells is absent or low. In contrast, in the cells of 11 of 15 patients studied using flow microlimnetrometry, Tac expression was increased (x ± SE = 20.1 ± 3.4) compared with normals (7.6 ± 0.6) P < .005 (Fig 3). Tac expression was elevated in the seven patients with aplastic anemia who had received minimal amounts of blood products. The
two patients within the normal range were the only individuals in this series who had a history of drug or chemical exposure before development of aplastic anemia. Tac expression in 22 patients who had received multiple transfusions was within the normal range ($\pm$SE = 70 $\pm$ 0.6) $P < .005$ (Fig 3).

These results suggested dysregulation of IL2 production in patients with aplastic anemia. Lacking assays for the presence of IL2 in serum on bone marrow, it was not possible to extrapolate the in vitro findings to patients directly. However, serums raised against peripheral blood lymphocytes had been reported to induce lymphocyte blastogenesis, and we therefore examined the effects of ATG on T cell functions related to IL2 production.

**ATG-Induced Blastogenesis**

ATG was tested for its ability to stimulate proliferation of peripheral blood mononuclear cells from both patients with aplastic anemia and normal individuals. The optimal ATG concentration for $^3$H-thymidine incorporation was found to be between 2.5 and 5.0 $\mu$g/mL; 10 $\mu$g/mL was toxic to the cells. ATG stimulated mononuclear cell proliferation; cells from patients with aplastic anemia showed greater responses than did cells from normal controls (Fig 4). The stimulatory index (SI), or ratio of ATG-induced to spontaneous isotope incorporation, was 22.5 $\pm$ 8.3 for cells from aplastic anemia patients and 2.5 $\pm$ 0.9 for normals ($P < .02$).

Different lots of ATG varied in their capacity for blastogenesis (Fig 5). Lot 17908 has been successfully used in a published controlled trial of ATG; lot 17924 is used in the current National Heart, Lung, and Blood Institute multicenter trial of ATG. The different blastogenic ability of the tested lots of ATG is in contrast to their identical binding specificities to lymphocytes and other human cell types. Lot 731, which gave the
Table 2. Production of Interleukin 2 by Peripheral Blood Mononuclear Cells From Aplastic Anemia Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Patient IL2 (U/mL)</th>
<th>Control IL2 (U/mL)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
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<td>75</td>
<td>62</td>
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<td>6</td>
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<td>7</td>
<td>71</td>
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<td>8</td>
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<td>15</td>
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<td>16</td>
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<td>60</td>
</tr>
<tr>
<td>17</td>
<td>17</td>
<td>32</td>
</tr>
</tbody>
</table>

The concentration of IL2 in supernatants from culture of lectin-stimulated peripheral blood mononuclear cells obtained from aplastic anemia patients and normal controls were assayed for their ability to support an IL2-dependent cell line. Variability in this biologic assay is high, and in general each patient sample was tested in a separated experiment with a normal sample. By pair statistical analysis, the difference between aplastic anemia and normal supernatants was significant at P < .01.

The highest blastogenic effect, has not been used in clinical trials.

ATG-Stimulated Lymphokine Production

Exposure of lymphocytes to lectins stimulates the production of lymphokines, including IL2 and interferon, as well as hematopoietic growth factors. ATG also promoted the production of IL2 by peripheral blood mononuclear cells in three of six patients and in two of eight controls tested. In patient No. 9 (Fig 6), IL2 production by ATG (0.5 µg/mL) was higher, 217 U/mL, than with PHA (2.5 µg/mL), 120 U/mL. However, in contrast to PHA, ATG did not stimulate measurable interferon production by cells from ten normal individuals. ATG did stimulate low levels of interferon production (20.5 ± 6.0 U/mL) in cells from four of ten aplastic anemia patients tested but to a much lower degree than when these cells were stimulated with PHA (2,060 ± 516 U/mL).

Hematopoietic Growth Factors in ATG-Conditioned Medium

The production of growth factors by lymphocytes may be coordinated. To assay for the production of hematopoietic growth factors by ATG-stimulated peripheral blood mononuclear cells, ATG-conditioned medium was added to normal nonadherent bone marrow cells, and hematopoietic colony formation in methylcellulose was determined. Hematopoietic colony formation by nonadherent cells in the absence of a source of growth factors, such as PHA-leucocyte-conditioned medium, is poor in comparison with unfracated bone marrow; this effect is more reproducibly observed for colonies derived from BFU-E than CFU-C. However, ATG-conditioned medium from cultures of lymphocytes of aplastic anemia patients increased colony formation by BFU-E and, to a lesser degree, CFU-C from normal bone marrow (Tables 2 and 3). The optimal concentration of ATG for hematopoietin pro-

DISCUSSION

Abnormalities of lymphokine production in vitro may characterize the circulating cells of many patients with aplastic anemia. IL2 production was equally abnormal in those aplastic anemia patients who had received no or minimal red blood cell or platelet transfusions as in heavily transfused aplastic anemia patients. Conversely, IL2 production was normal or lower than normal among the control population that had received large numbers of red blood cell or platelet transfusions. As in the case of IL2 production, aplastic patients who had received little or no blood products had high Tac antigen expression, and control patients who had received multiple erythrocyte or platelet transfusions had low Tac expression on peripheral blood mononuclear cells.

Abnormalities of the lymphokine cascade are present in patients with aplastic anemia. As we report here, their peripheral blood mononuclear cells produce large amounts of IL2 in vitro, and their circulating T cells...
have high expression of Tac antigen, the IL2 receptor. Interferon production by peripheral blood mononuclear cells, both lectin-stimulated and spontaneous, is also markedly elevated in most aplastic anemia patients.\textsuperscript{24} In addition, production of the monokine, IL1, or lymphocyte-activating factor, by adherent cells from aplastic anemia patients is diminished (Gascon, Young, and Scala, manuscript in preparation). Lacking assays to measure circulating or bone marrow factors directly, in vitro abnormalities cannot be simply related to the biology of the aplastic anemia patient. Lymphokine abnormalities possibly may represent a physiologic response to bone marrow depression, although such abnormalities were not present in a control population consisting of patients with pancytopenia associated with other bone marrow morphology (myelodysplasia, cellular bone marrow) and in other clinical settings (secondary to chemotherapy). Our results suggest the possibility of dysregulation of the lymphokine network and possibly hypersensitivity of aplastic anemia cells to some biologic regulators.

Expression of Tac antigen is increased on the circulating mononuclear cells of patients with aplastic anemia. This result suggests that a subpopulation of the T lymphocytes of these patients are "preactivated," as Tac antigen is expressed on T cells that have been stimulated by antigens, mitogens, and lectins. Tac antigen expression may be a manifestation of an abnormal population of suppressor lymphocytes or of subtle, generalized immune dysregulation after bone marrow failure. High Tac expression is characteristic of only one other human disease—adult T cell leukemia,\textsuperscript{25} which is now closely linked to a human retrovirus. In vitro T cell lines transformed by human T cell leukemia virus also express IL2 receptor.\textsuperscript{26} Increased Tac expression in vitro may result from a priming effect of \(\gamma\)-interferon on lymphocytes;\textsuperscript{8} high Tac expression in patients with aplastic anemia may be related to abnormally high interferon production;\textsuperscript{24} both may point to a viral etiology of this disease.\textsuperscript{27}

Regardless of the underlying abnormalities of lymphokine expression and regulation in aplastic anemia, ATG, in addition to its known lymphocytotoxic properties, has been demonstrated to affect lymphocyte function. ATG behaves like a mitogen in stimulating lymphocyte proliferation and increases production of lymphokines like IL2. The ability of ATG to stimulate production of hematopoietins was directly demonstrated using peripheral blood mononuclear cells from patients with severe aplastic anemia. In these experiments, ATG-conditioned medium replaced lectin-conditioned medium in supporting colony formation by hematopoietic cells in the nonadherent fraction of normal bone marrow. Our experiments may explain, in part, the different results obtained in studies of the direct effects of ATG or ALG, in the presence or absence of added complement, on hematopoietic colony formation.\textsuperscript{28,29} Thus, although ATG is toxic both to lymphocytes elaborating growth factors in colony culture and to hematopoietic progenitors themselves, ATG at low concentrations, in the absence of complement, may stimulate production of growth factors by accessory cells within the bone marrow cultures and therefore enhance colony formation under suboptimal culture conditions.

In certain respects, the short-term methylcellulose culture replaces with T cells or their products the marrow stromal cells, represented by the adherent cells of long-term bone marrow culture.\textsuperscript{30} Although ATG’s effects have been readily measured using peripheral blood mononuclear cells and their defined products, its targets may be located within the bone marrow. ATG binds to many human cell types, including almost all the cells obtained by bone marrow aspiration.\textsuperscript{31} ATG may act on bone marrow cells that produce growth factors, the effects of which are mediated locally.

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LYMPHOKINES IN APLASTIC ANEMIA

Lymphokine abnormalities in aplastic anemia: implications for the mechanism of action of antithymocyte globulin

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