In Vitro Production of Granulocyte-Macrophage Colony-Stimulating Factor in Aplastic Anemia: Possible Mechanisms of Action of Antithymocyte Globulin

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Various abnormalities of lymphokine production have been described in patients with aplastic anemia. To determine if abnormal production of hematopoietic growth factors could contribute to the process of aplastic anemia we studied the in vitro production of human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) by phytohemagglutinin (PHA)- and antithymocyte globulin (ATG)-stimulated peripheral blood lymphocytes from 29 patients with aplastic anemia and 15 normal controls. GM-CSF production in response to 1% PHA was seen in nearly all samples (43 of 44) and similar amounts of GM-CSF were produced by patients with aplastic anemia and normal controls. Production of GM-CSF by ATG-stimulated lymphocytes was seen in 7 of 23 patients with aplastic anemia (30%); two of these patients also demonstrated low-level spontaneous production of GM-CSF. Production of GM-CSF in response to ATG was also seen in 2 of 11 normal controls (18%) and barely detectable spontaneous production of GM-CSF was seen in both. Biologically active IL-3 could also be detected in PHA- or ATG-stimulated peripheral blood mononuclear cells in several patients and normal controls. Our results indicate that lymphocytes from patients with aplastic anemia can be stimulated in vitro to produce normal quantities of GM-CSF, suggesting that impaired potential for production of T-cell derived hematopoietic growth factors is unlikely to account for the marrow hypoplasia seen. In several patients overproduction of GM-CSF was observed, consistent with the notion that some patients with aplastic anemia may have circulating activated T cells. We also demonstrate that ATG can stimulate the production of growth factors such as IL-3 and GM-CSF, supporting the role for ATG in stimulating hematopoiesis.

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Numerous mechanisms have been postulated regarding the pathogenesis of aplastic anemia, including absent or defective stem cells, an abnormal stromal microenvironment, immune-mediated suppression of hematopoiesis, abnormalities in regulatory or accessory cells, and abnormal growth factor production. Several investigators have demonstrated abnormalities in peripheral blood mononuclear cell populations, including increased numbers of activated suppressor T cells (defined by the presence of HLA-DR, Tac, and CD8 antigens). A cell line consisting of CD8+, DR+ cells capable of suppressing hematopoiesis has been established from the peripheral blood of a patient with aplastic anemia, and Thy lymphocytes capable of suppressing hematopoiesis have been found in aplastic anemia bone marrow. A variety of abnormalities of lymphokine expression have also been demonstrated. γ-Interferon (γ-IFN), tumor necrosis factor-α (TNF-α), and tumor necrosis factor-β (TNF-β), lymphotoxin have all been implicated as potential inhibitors of hematopoiesis in aplastic anemia. A subgroup of patients with aplastic anemia has elevated circulating levels of γ-IFN, and production of high levels of γ-IFN by peripheral blood mononuclear cells has been demonstrated in some, but not all, patients with aplastic anemia. Increased in vitro production of interleukin-2 (IL-2) and TNF-α by phytohemagglutinin (PHA)-stimulated mononuclear cells from aplastic anemia patients has also been demonstrated.

Although increased production of inhibitory molecules has been observed, the production of substances that stimulate hematopoiesis, such as the colony-stimulating factors (CSFs), has not been well characterized. Granulocyte-macrophage CSF (GM-CSF) is a glycoprotein growth factor that stimulates the proliferation of myeloid progenitor cells and enhances the functional activity of mature effector cells. Multiple clinical trials have demonstrated the ability of GM-CSF to stimulate hematopoiesis in humans. Like IL-2 and γ-IFN, GM-CSF and IL-3 are elaborated by activated but not resting T lymphocytes. In vitro T-cell activation can be achieved using PHA, so we used a radioimmunoassay for GM-CSF and in certain instances bioassays for GM-CSF and IL-3 to analyze growth factor production from PHA-stimulated peripheral blood mononuclear cells.

Antithymocyte globulin (ATG) can be an effective treatment for aplastic anemia, but the mechanism of action of ATG in aplastic anemia is unknown. ATG was originally considered to act via an immunosuppressive mechanism, but conclusive data supporting this hypothesis is lacking. ATG can act as a T-cell mitogen, stimulating H-thymidine uptake and release of IL-2 from T cells. The in vitro production of hematopoietins in response to ATG was also reported in 1985, but the molecules responsible for this effect could not be identified. Other investigators have performed similar experiments but have not found detectable colony-stimulating activity in response to in vitro stimulation by ATG. To further investigate this issue, we examined the ability of ATG to act as an immunostimulant and stimulate GM-CSF (and IL-3) production.

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MATERIALS AND METHODS

Patient characteristics. Twenty-nine adult patients with acquired moderate or severe aplastic anemia were studied. The disease was idiopathic in 19 patients, secondary to paroxysmal nocturnal hemoglobinuria in 2, associated with autoimmune disease in 1, secondary to hepatitis in 2, and possibly drug related in 5. Fourteen men and 15 women were studied. The median age of the patients was 43 years, and all patients were previously transfused. Five patients were studied before receiving ATG therapy, and five patients who never received ATG were studied just before bone marrow transplantation. Of the 19 patients studied following ATG therapy, 17 had active aplastic anemia and 2 patients had had a complete response to ATG and had normal leukocyte counts.

Two control populations were used. The first control group consisted of eight patients, two of each with the following hematologic disorders: acute myelogenous leukemia (AML) in remission, refractory anemia, lymphoproliferative disorders, and status post-bone marrow transplantation for acute leukemia. Additionally, 15 normal volunteer donors were studied for production of GM-CSF.

Isolation of peripheral blood mononuclear cells. With appropriate informed consent, blood was collected in preservative-free heparin, and mononuclear cells isolated by Ficoll-Hypaque gradient separation. The cells were washed twice and resuspended to 2 x 10^6 cells/mL in Iscove's modified Dulbecco's medium containing 20% fetal bovine serum. Initially, results obtained using total peripheral blood mononuclear cells were compared with those obtained after depletion of adherent cells for six samples, and no difference in GM-CSF production was found by Wilcoxon rank sum analysis (0.7±0.2 ng/mL with adherent cells present vs 1.0±0.7 ng/mL with adherent cells depleted) (P = .51). Subsequent experiments were performed without depleting adherent cells. Peripheral blood mononuclear cells were incubated with or without PHA (1%) or were stimulated with ATG (Upjohn Co, Kalamazoo, MI; lot no. 163BC) at 1, 5, or 10 µg/mL. Conditioned media was obtained 72 to 84 hours after PHA stimulation, and 5 and 8 days following ATG stimulation.

Assays for CSFs. The conditioned media was stored at -20°C and later concentrated 6- to 12-fold using a Centricon 10 microcentrator (Amicon, Danvers, MA). Radioimmunoassays were performed on concentrated conditioned media to determine the concentration of immunologically reactive GM-CSF. By concentrating samples approximately 10-fold, the lower limit of detectability of the radioimmunoassay was approximately 0.15 ng/mL (~7 pmol/L). A sensitive bioassay for GM-CSF and IL-3 was used to detect IL-3 and to determine whether the GM-CSF detected in the radioimmunoassay was biologically active. This assay uses the M-07e subclone of the human myeloid leukemia M-07 cell line. The subclone is absolutely dependent on the presence of either GM-CSF or IL-3, even in serum containing cultures. Thymidine incorporation stimulated by the presence of either GM-CSF or IL-3 in the sample tested was compared with the incorporation stimulated by known concentrations of recombinant human GM-CSF or IL-3. Six serial fivefold dilutions of each sample and the recombinant standards were tested in the presence and absence of anti-GM-CSF or anti-IL-3 polyclonal rabbit antisera. These antisera block proliferation of M-07e cells in response to their homologous cytokine, but have no effect on proliferation supported by the heterologous factors.21 The amount of GM-CSF produced by lymphocytes from aplastic anemia patients was calculated and compared with the amount produced by normal donor lymphocytes using the Mann-Whitney rank sum test because the values were not normally distributed.27 Chi-square and Fisher exact tests were performed as indicated in the text.

RESULTS

GM-CSF production in response to PHA. Peripheral blood mononuclear cells from 15 normal donors produced detectable amounts of GM-CSF after 3 days of PHA (1%) stimulation. The quantity of GM-CSF produced ranged from 0.2 ng/mL to 2.0 ng/mL (mean ± SD; 0.8 ng/mL ± 0.6 ng/mL; median 0.7 ng/mL) (Fig 1). In 11 of the 15 normal controls, unstimulated peripheral blood mononuclear cells were assayed for GM-CSF production, and in four of these samples GM-CSF was intermittently barely detectable by our assay (~0.1 ng/mL in each sample).

Peripheral blood mononuclear cells from 28 of the 29 patients with aplastic anemia produced readily detectable amounts of GM-CSF after PHA stimulation. The amount of GM-CSF produced ranged from 0.15 ng/mL to 10.0 ng/mL (mean ± SD; 1.5 ng/mL ± 1.9 ng/mL; median 1.3 ng/mL). Two of the eight patients with aplastic anemia manifested spontaneous production of GM-CSF (0.3 ng/mL, 0.8 ng/mL). In six of eight control patients with other hematologic disorders, detectable levels of GM-CSF were produced after PHA stimulation (1.5 ng/mL ± 2.0 ng/mL [mean ± SD]; median 1.0 ng/mL; range 0.3 to 5.5 ng/mL). These results indicate that, using a sensitive radioimmunoassay, one can detect peripheral blood mononuclear cell populations in nearly all individuals that are capable of being activated by mitogens to produce lymphokines such as GM-CSF. The mean level of GM-CSF produced by patients with aplastic anemia was higher than that produced by the normal control donors, but this difference did not reach significance (P = .11; Mann-Whitney rank sum test). Nonetheless, GM-CSF production in several of the aplastic anemia samples was considerably higher than the normal controls (Fig 1).

The basal production of GM-CSF by unstimulated peripheral blood mononuclear cells from the aplastic anemia patients was not different than the normal controls. Addi-
tionally, the incremental increase in GM-CSF production in response to PHA stimulation was basically the same in the two patient populations (Fig 2). The levels of GM-CSF production by both the aplastic anemia patients and the patients with other hematologic disorders could not be correlated with their transfusion histories. The amount of GM-CSF produced in response to PHA in the 20 heavily transfused aplastic anemia patients (those who had received >10 U of red blood cells [RBCs]) was not statistically different than the nine aplastic anemia patients that received less than 10 U of RBCs (1.3 ng/mL ± 1.0 v. 2.2 ng/mL ± 3.1; P = .77 by the Mann-Whitney rank sum test). Comparing GM-CSF production by aplastic anemia patients receiving more than five platelet transfusions (24 patients) versus those receiving less than five platelet transfusions (five patients) also showed no real differences (1.6 ng/mL ± 2.0 v. 1.5 ng/mL ± 1.4; P = 1.0 using the Mann-Whitney rank sum test). Only one of the eight patients with a different hematologic disorder had received less than five platelet transfusions or less than 10 RBC transfusions, precluding any meaningful analysis.

**GM-CSF production in response to ATG**. Based on previously published studies, we initially studied the response of peripheral blood mononuclear cells from three aplastic anemia patients to 5 or 10 μg/mL of ATG. In two patients neither 5 μg/mL nor 10 μg/mL stimulated GM-CSF production, and in one patient both 5 μg/mL and 10 μg/mL of ATG stimulated equivalent amounts of GM-CSF (1.4 ng/mL v. 1.5 ng/mL). Because 8 days of ATG exposure was more effective than 5 days in stimulating GM-CSF production (data not shown), subsequent tests were run using 10 μg/mL of ATG and 8 days of stimulation. Samples from 23 of the 29 patients with aplastic anemia were studied for GM-CSF production in response to ATG. Seven of these patients produced GM-CSF (30.4%), whereas 2 of 13 normal control donors (15%) and one of the eight patients with hematologic malignancies (12%) produced GM-CSF in response to ATG (the difference between aplastic anemia patients and normal controls was not significant; chi-square = 1.65, P = .44). The amount of GM-CSF produced in response to ATG ranged from 0.2 to 2.1 ng/mL in the aplastic anemia patients (median 0.8 ng/mL); the two normal controls produced 0.5 and 0.7 ng/mL and one patient with Hodgkin's disease produced 0.2 ng/mL. Like the production of GM-CSF in response to PHA, production of GM-CSF in response to ATG also did not correlate with the number of platelet or RBC transfusions (Table 1). Two of the seven “GM-CSF producers” had received less than five platelet transfusions, whereas five of the seven “GM-CSF producers” received more than five platelet transfusions. Only 1 of the 17 “GM-CSF nonproducers” received less than five platelet transfusions. These sample sizes are small, but using the Fisher exact test one-tailed P values of .59 and .19 were obtained when the variables of greater than or less than 10 RBC transfusions and greater than or less than 5 platelet transfusions were analyzed, respectively.

We compared the absolute number of circulating lymphocytes and the percent of circulating lymphocytes in the peripheral blood (calculated from the complete blood count [CBC] and differential count) of the patients who produced GM-CSF in response to ATG to those that did not. The mean absolute number of circulating lymphocytes (1,087/μL) and the percentage (44.7%) found in the seven “GM-CSF producing” patients (44.7%) were virtually identical to the mean absolute number of lymphocytes (1,219/μL or the percentage (44.2%) found in the peripheral blood of the 14 aplastic anemia patients who did not demonstrate GM-CSF production in response to ATG. (CBC and differential counts were available on the day the peripheral blood mononuclear cells were obtained for only 14 of the 16 patients who did not produce GM-CSF in response to ATG).

Of the seven patients who produced GM-CSF in response to ATG, four had no clinical response to ATG, two had a partial response, and one patient had a complete response. Twenty-four of the aplastic anemia patients studied received treatment with ATG (20 mg/kg/d × 8 days). Eleven patients had a clinical response (45.8%); the clinical response rate in patients in whom ATG elicited the in vitro production of GM-CSF (42.9%) did not appear to be different than the clinical response rate in those patients whose lymphocytes did not produce GM-CSF after ATG stimulation (47.1%) (Table 2).

**GM-CSF and IL-3 bioassay**. Bioassays for GM-CSF and IL-3 were performed using the factor-dependent M-07e cell line and neutralizing antibodies against GM-CSF or IL-3. Because the amount of GM-CSF detected in several san-
bioassays for GM-CSF on 21 samples to confirm the presence of specific GM-CSF activity. The bioassay results were generally confirmatory of the results of the radioimmunoassay (one aplastic anemia [AA] sample had −0.2 ng/mL GM-CSF by bioassay but <0.1 ng/mL by radioimmunoassay). Of 15 positives by radioimmunoassay, all but one contained biologically active GM-CSF (one AA sample contained no GM-CSF). In eight of the samples (four PHA-stimulated T cells, three ATG-stimulated T cells, one unstimulated T cell) colony-stimulating activity not attributable to GM-CSF (ie, present after the addition of anti–GM-CSF antibody) was detected. In four of these samples, this activity was totally neutralized by specific anti-IL-3 antibodies, confirming the presence of both anti-GM-CSF and anti-IL-3 antibodies.

**DISCUSSION**

There is strong evidence for immune suppression of hematopoiesis as a pathogenetic mechanism in at least a subset of patients with aplastic anemia. Abnormal peripheral blood and bone marrow mononuclear cell populations have been identified that are capable of suppressing hematopoiesis in vitro, and in most cases these cells are T lymphocytes. Abnormalities in cytokine production have also been observed in patients with aplastic anemia, and increased production of IL-2, TNF-α, and γ-IFN has been observed in a proportion of patients with aplastic anemia. These abnormalities have been thought to reflect the presence of abnormal populations of T cells, which may be circulating in an activated state. These findings have not been universally observed, so it is unclear how common these findings are in patients with aplastic anemia.

Impaired production of hematopoietic growth factors could contribute to the pathogenesis of aplastic anemia. Both GM-CSF and IL-3 are produced by activated but not resting T cells, so the amount of GM-CSF produced by PHA-stimulated peripheral blood mononuclear cells may reflect the state of circulating T cells and may identify specific defects in growth factor production in aplastic anemia. Using a sensitive radioimmunoassay (and a confirmatory bioassay) we have determined that GM-CSF production in PHA-stimulated peripheral blood mononuclear cells is preserved in patients with aplastic anemia. Although the mean amount of GM-CSF produced was not significantly higher for aplastic anemia patients than for normal controls (P = .11), in several patients the amount of GM-CSF produced was much greater than in normal controls (Fig 1). It has been suggested that there may be populations of T cells present in patients with aplastic anemia that are capable of heightened lymphokine production in response to PHA. Although overproduction of inhibitory molecules may be a common feature of aplastic anemia, our data suggest that overproduction of stimulatory molecules, such as GM-CSF, may be seen less commonly in aplastic anemia patients.

We observed spontaneous production of GM-CSF in only two of the aplastic anemia patients studied and barely detectable levels of GM-CSF in unstimulated peripheral blood mononuclear cell conditioned media in two normal controls. Although GM-CSF is not constitutively produced by T lymphocytes, GM-CSF mRNA can occasionally be detected in peripheral blood mononuclear cells. It is likely that this is due to circulating activated T cells because resting T cells and peripheral blood monocytes have undetectable levels of GM-CSF mRNA. The presence of circulating activated T cells has been described in aplastic anemia patients and these cells may be present in variable numbers in normal controls. The low level of GM-CSF detected could also be the result of cell-cell communications between monocytes in the peripheral blood mononuclear fraction and the T-cell populations capable of producing GM-CSF. In our initial studies of six patients and normal controls, no difference could be seen in the amount of GM-CSF produced in response to PHA when monocytes were removed by adherence to plastic, thus monocytes were not routinely depleted. However, it is possible that in some instances sufficient communication occurs between monocytes and T cells to result in in vitro T-cell activation.

The mechanism of action of ATG as a treatment for aplastic anemia remains to be determined. Both immuno-suppressive and immunostimulatory effects have been observed. ATG has been shown to be mitogenic for T cells, increasing thymidine incorporation and leading to IL-2 production in patients with aplastic anemia. Although early reports suggested that ATG had direct effects on hematopoietic progenitor cells and could induce differentiation of some myeloid cell lines in vitro, it appears that this effect is due to the antimicrobial preservative thimerosal that is present in the ATG preparations.

Our studies show that in a substantial proportion of patients with aplastic anemia (33%), ATG is capable of stimulating peripheral blood mononuclear cells to produce GM-CSF. We have also measured biologically active IL-3 in ATG-stimulated conditioned medium. Our findings con-

**Table 2. Clinical Versus In Vitro Response to ATG**

<table>
<thead>
<tr>
<th>Aplastic anemia patients</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. treated with ATG</td>
<td>24</td>
</tr>
<tr>
<td>Overall Clinical Outcome</td>
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<tr>
<td>Complete responses</td>
<td>3 (12.5%)</td>
</tr>
<tr>
<td>Partial responses</td>
<td>8 (33.3%)</td>
</tr>
<tr>
<td>No response</td>
<td>13 (54.2%)</td>
</tr>
<tr>
<td>GM-CSF Detected</td>
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<tr>
<td>(N = 7)</td>
<td></td>
</tr>
<tr>
<td>Complete responses</td>
<td>1 (14.3%)</td>
</tr>
<tr>
<td>Partial responses</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td>No response</td>
<td>4 (57.1%)</td>
</tr>
<tr>
<td>Not Detected</td>
<td></td>
</tr>
<tr>
<td>(N = 17)</td>
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</tr>
<tr>
<td>Complete responses</td>
<td>2 (11.8%)</td>
</tr>
<tr>
<td>Partial responses</td>
<td>6 (35.3%)</td>
</tr>
<tr>
<td>No response</td>
<td>9 (52.9%)</td>
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</tbody>
</table>
firm the early work of Gascon et al., who reported that ATG-stimulated peripheral blood mononuclear cell conditioned media could stimulate the in vitro growth of human bone marrow. Although stimulatory effects were seen on burst-forming unit-erythroid (BFU-E) and colony-forming unit in culture (CFU-C) formation, it was not possible to precisely identify which hematopoietic growth factors were present in that conditioned medium. Other studies, using antilymphocyte globulin (ALG) and ATG (ATGAM from Upjohn), failed to detect the presence of colony-stimulating activity in ATG-stimulated peripheral blood mononuclear cell conditioned medium, which may due to the presence of both stimulatory and inhibitory molecules in the conditioned medium. Using our radioimmunoassay and a confirmatory bioassay, we have identified several of the growth factors produced in response to ATG and showed that T cells responsive to the stimulatory effects of ATG are also present in normals. Cell sorting studies to determine which population of cells is stimulated by ATG to produce GM-CSF were not performed, but cells bearing CD4 or CD8 antigens on their surface have both been shown to produce lymphokines such as GM-CSF. We were unable to correlate the production of GM-CSF in response to ATG with absolute lymphocyte numbers. There was also no correlation between in vitro production of GM-CSF after stimulation with ATG and a clinical response to ATG therapy.

To evaluate any effect of transfusions on lymphokine production, we examined a heavily transfused group of patients with hematologic disorders other than aplastic anemia. Production of GM-CSF by these individuals was not significantly different than normals or patients with aplastic anemia. We also compared the amount of GM-CSF produced in the aplastic anemia samples as a function of the number of RBC and platelet transfusions and could detect no correlation (see Results and Table 1).

Hematopoiesis is probably regulated by a balance of stimulatory and inhibitory signals. Stimulatory signals include GM-CSF, IL-3, IL-6, and others, whereas IFNs, transforming growth factor-β, TNFs, and other factors act as inhibitors of hematopoiesis. Our study has demonstrated that ATG is capable of stimulating growth factor production in patients with aplastic anemia. It is possible that the occasional immediate improvement in hematopoiesis after ATG treatment is attributable to production of endogenous growth factors. The long-lasting effects of ATG on hematopoiesis may reflect a shift in the balance of positive and negative factors in the bone marrow microenvironment in favor of hematopoietic stimulation.

Patients with aplastic anemia have elevated serum levels of hematopoietic-stimulating activity, which has not yet been defined on a molecular or immunologic basis, but may represent M-CSF, or less likely G-CSF. M-CSF is detectable in serum and urine and G-CSF has recently been detected in the blood. We studied sera from six aplastic anemia patients and were unable to detect circulating levels of GM-CSF by radioimmunoassay (data not shown). Binding of GM-CSF to glycosaminoglycans in the bone marrow has been demonstrated; thus, GM-CSF probably acts locally to stimulate hematopoiesis. There appears to be little or no GM-CSF circulating in the blood; thus, GM-CSF contributes little to the increased amount of circulating colony-stimulating activity in the serum of aplastic anemia patients.

Our results do not show a significant difference in the amount of GM-CSF produced by PHA-stimulated T lymphocytes from aplastic anemia patients compared with normal controls. Thus, it is unlikely that impaired production of GM-CSF accounts for the marrow hypoplasia seen in patients with aplastic anemia. Although overproduction of GM-CSF was seen in several patients, the overproduction of lymphokines that are potential inhibitors of hematopoiesis may be a more consistent finding than overproduction of stimulatory lymphokines.

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In vitro production of granulocyte-macrophage colony-stimulating factor in aplastic anemia: possible mechanisms of action of antithymocyte globulin

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