Interferon is a mediator of hematopoietic suppression in aplastic anemia \textit{in vitro} and possibly \textit{in vivo}  

\textbf{(inhibitors/myelopoiesis/colony culture)}

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\textbf{ABSTRACT}  
We have investigated interferon as a mediator of hematopoietic suppression in bone marrow failure. Interferon production by stimulated peripheral blood mononuclear cells from patients with aplastic anemia was significantly higher than that observed in controls; spontaneous interferon production by these cells was also high for more than half of aplastic anemia patients. Circulating interferon, not detectable in normal individuals, was detected in 10 of 24 patients. Interferon is a potent inhibitor of hematopoietic cell proliferation and, therefore, may be the mediator of suppression in many \textit{in vitro} models employing patients’ cells and sera. The possible pathogenic importance of interferon in aplastic anemia was suggested by an increase in hematopoietic colony formation \textit{in vitro} after exposure of bone marrow cells to anti-interferon antisera (277 ± 71% increase for patients compared to 1.6 ± 1.6% for normal individuals). Interferon levels in the bone marrow sera of aplastic anemia patients were high (mean = 203 international units (IU)/ml, \(n = 8\)), even in comparison to circulating levels in the same patients. Normal bone marrow sera also contained measurable interferon but at lower levels (41 IU/ml, \(n = 16\)), indicating that interferon may be a normal bone marrow product. High concentration of bone marrow interferon, possibly due to abnormal immunologic activity or a reaction to virus infection of the bone marrow, may mediate hematopoietic suppression in aplastic anemia patients.

Several lines of evidence suggest that aplastic anemia may be mediated by cells of the immune system. Occasionally, patients recover autologous hematopoietic function after immunosuppressive therapy prior to unsuccessful bone marrow transplantation (1, 2). Conversely, some syngeneic twins require immunosuppression to effect bone marrow engraftment (3). Anti-thymocyte and anti-lymphocyte globulin treatments, which result in hematopoietic recovery in 40–50% of patients with aplasia, are presumed to function by their immunosuppressive action (4). Removal of T cells from aplastic bone marrow \textit{in vitro} can improve hematopoietic colony formation (5). Coculture of bone marrow or blood cells from aplastic anemia patients with histocompatible bone marrow from normal individuals results, in some cases, in suppression of colony formation by the normal cells (6). Hematopoietic suppressor activity also has been generated in mixed leukocyte cultures (7).

Lymphocytes that suppress hematopoiesis also may have a role in the normal regulation of blood cell production (8). T cells are required for erythroid colony formation from normal peripheral blood \textit{in vitro} (9) a helper effect enhanced by the prior removal of T cells bearing the HLA-DR antigen (10). Bacigalupo and his colleagues (11, 12) have generated suppressor cells from normal peripheral blood lymphocytes by exposing them to plant lectins; the resultant suppressor activity is similar to that spontaneously present in cultures of cells from patients with bone marrow failure (13).

We have shown that the hematopoietic suppressor activity in medium from lectin-stimulated T-cell cultures is \(\gamma\)-interferon (14). This observation led to the current investigation of the role of interferon in hematopoietic suppression in aplastic anemia.

\textbf{METHODS}

\textbf{Patients.} Twenty-three adult patients with severe aplastic anemia, as defined by peripheral blood cell counts and bone marrow biopsy (15), were studied before treatment with antithymocyte globulin or androgens. Two patients had histories of drug or chemical exposure. Three other patients who had achieved independence from transfusions after treatment are designated “post-recovery.” None of the patients studied showed clinical evidence of active viral infection, and their titers of antivirus antibodies were lower than those in multiply transfused patients. In the control population were patients with a variety of other hematologic diseases, including myelofibrosis, myelodysplasia, congenital pure red cell aplasia, and chronic refractory anemia; patients with thalassemia and sickle-cell disease who had received multiple transfusions; and normal, apparently healthy volunteers.

\textbf{Tissues.} Peripheral blood was collected in preservative-free heparin (O’Neill and Feldman, St. Louis, MO). Blood was allowed to clot for 1 hr before the serum was separated. T lymphocytes were separated by rosetting with sheep erythrocytes. For colony assays, bone marrow was aspirated into 1 ml of Iscove’s modification of Dulbecco’s medium containing 250 units of heparin; for interferon measurements, undiluted bone marrow samples were allowed to clot before centrifugation to separate bone marrow sera.

\textbf{Interferon Measurements.} Interferon was measured by its ability to inhibit cytopathic effects (CPE) of vesicular stomatitis virus on human amnion WISH cells (16). The interferon titer is the reciprocal of the dilution that yielded 50% CPE and is expressed in international units (IU), standardized with National Institutes of Health reference human leukocyte interferon (G-023-901-527).

\textbf{Lymphocyte Cultures.} Peripheral blood mononuclear cells obtained by Percoll density gradient sedimentation were incubated for 1–7 days at 37°C in a humidified 5% CO\textsubscript{2} atmosphere at a concentration of 1–2 \(\times\) 10\textsuperscript{6} per ml in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence or absence of pokeweed mitogen or phytohemagglutinin at 10 \(\mu\)g/ml.

\textbf{Hematopoietic Colony Culture.} Mononuclear cells from bone marrow were separated by Percoll density gradient sedimentation and cultured in methylcellulose containing appropriate conditioned medium to support myeloid colony-forming unit-culture (CFU-C); i.e., granulocyte/macrophage

\begin{footnotesize}
\begin{itemize}
  \item Abbreviations: CFU-C, granulocyte/macrophage colony-forming cell; IU, international units.
\end{itemize}
\end{footnotesize}
colony-forming cell) or erythroid [burst-forming unit-erythropoietic (BFU-E); i.e., erythroid burst-colony-forming cell] colony formation (17). Interferon activity was neutralized with anti-α-interferon antiserum, prepared in sheep (Research Resources Branch, National Institute of Allergy and Infectious Diseases), anti-γ-interferon antiserum, prepared in rabbits (Interferon Sciences, New Brunswick, NJ), or a monoclonal antibody to γ-interferon (18) (kindly provided by B. Rubin). Controls included sheep and rabbit immune sera and monoclonal antibodies to various cell-surface determinants and normal human serum proteins.

RESULTS

Production of Interferon by Peripheral Blood Mononuclear Cells in Vitro. We have shown that the hematopoietic suppressor activity generated by normal peripheral blood T cells stimulated with lectins is γ-interferon (14). Fig. 1 shows the results of a representative experiment with peripheral blood mononuclear cells from a patient with severe aplastic anemia; the cells were exposed to phytohemagglutinin for 7 days, and the supernatant fluid was periodically sampled and tested for interferon by anti-viral assay and for hematopoietic suppressor activity in normal bone marrow colony culture. In comparison to normal cells (Fig. 1b), cells from the patient with aplastic anemia (Fig. 1a) showed a marked increase in total interferon produced at each of the time points assayed. Inhibitory activity in colony culture was more pronounced in the patient’s cell supernatant and correlated with the interferon concentrations in the individual supernatant samples (Fig. 1 Inset).

There were two important abnormalities in the behavior of mononuclear cells from the aplastic anemia patients. First, the levels of interferon produced were much higher than normal (Fig. 2); cells from 8 of 14 patients tested produced levels more than two standard deviations higher than the normal mean. Cells from patients with other hematologic diseases and from those receiving multiple blood transfusions did not show abnormal production of interferon. Nor were high levels in the aplastic anemia patients related to the numbers of platelet transfusions they had received; two untransfused patients showed marked elevations (data not shown). Second, the pattern of interferon production by the patients’ cells in vitro differed from normal: in contrast to the normal fall in interferon levels by day 7, in the cultures of the patients’ cells interferon levels remained high throughout the 7-day incubation period (Fig. 2).

Further evidence of the disordered regulation of interferon production in aplastic anemia was provided by observations on unstimulated peripheral blood mononuclear cells. In the absence of a lectin stimulant, normal cells and cells from patients who had received multiple blood transfusions did not produce detectable quantities of interferon (Table 1). In contrast, cells from 10 of 17 patients with aplastic anemia showed spontaneous in vitro production of interferon (16–3125 IU/ml).

The inhibition of hematopoietic colony formation by supernatants from lectin-stimulated cultures of patients’ cells was due to the presence of γ-interferon. Addition of sufficient anti-γ-interferon to neutralize 1000 IU of interferon to supernatants containing comparable quantities of interferon completely abrogated the inhibitory effect (data not shown). This result is identical to that reported for supernatants from lectin-stimulated normal cell cultures, in which addition of anti-γ-interferon antiserum or monoclonal antibody, and to a

![Fig. 1. Relationship of production of interferon to hematopoietic inhibitor activity in peripheral blood mononuclear cell cultures. Peripheral blood mononuclear cells from a patient with aplastic anemia (a) or a normal individual (b) were incubated at a concentration of 2 × 10^6 cells per ml with phytohemagglutinin at 10 μg/ml. Supernatants were collected on days 1, 3, 5, and 7 and assayed for interferon by anti-viral assay (line graphs) and for hematopoietic inhibitor activity when present at a concentration of 10% (vol/vol) in bone marrow tissue culture medium (bar graphs). The correlation (r = 0.97) between interferon levels and inhibition of hematopoiesis in this representative experiment is shown in the Inset.](image-url)

![Fig. 2. Interferon production after lectin stimulation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells from 4 patients with aplastic anemia and 4 hematologically normal individuals were treated as described in the legend to Fig. 1, and the culture supernatants were assayed periodically. Both the quantity of interferon produced and the sustained levels at day 7 were characteristic of cultures of cells from aplastic anemia patients. The production of interferon in IU/ml, given as the mean ± SEM in 3-day cultures was measured for a larger population of patients and controls: Aplastic anemia patients, 3862 ± 1088 (n = 14); normal controls, 490 ± 190 (n = 16). The difference in interferon production between the two populations was significant (P < 0.01).](image-url)

<table>
<thead>
<tr>
<th>Source of cells</th>
<th>Interferon, IU/ml</th>
<th>No. &gt; 10 IU/ml*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aplastic anemia patients</td>
<td>17 213 ± 182</td>
<td>10/17</td>
</tr>
<tr>
<td>Multiply transfused patients</td>
<td>10 0</td>
<td>0/10</td>
</tr>
<tr>
<td>Normal controls</td>
<td>15 0.3 ± 0.3</td>
<td>0/15</td>
</tr>
</tbody>
</table>

Individual values of interferon activity in culture fluid of cells from aplastic anemia patients were 3125, 150, 125, 50, 25, 25, 16, 16, 10, 5, 5, 0, 0, 0, and 0 (IU/ml). Only one normal person’s cells produced detectable interferon activity of 5 IU/ml. The levels for 14 other normals and for 10 multiply transfused patients were 0 IU/ml.

*P < 0.001 for the differences between the aplastic anemia group and each of the other two groups.
lesser degree anti-α-interferon antiserum, reversed the inhibitory effect (14).

**Serum Interferon Levels.** Circulating levels of interferon were measured in aplastic anemia patients, patients who had received multiple transfusions, and normal controls (Table 2). In both the normal and the multiply transfused control populations, interferon was not detectable in sera (5 IU/ml was the limit of sensitivity of the virus protection assay). However, in 42% of the patients with aplastic anemia, high levels of interferon were measured. There was no correlation between high serum levels of interferon and spontaneous production of interferon by peripheral blood mononuclear cells *in vitro*.

**Effect of Anti-Interferon on Bone Marrow Colony Formation in Vitro.** We tested the ability of bone marrow mononuclear cells to form hematopoietic colonies *in vitro* in the presence of anti-interferon antisera. In cultures of 10 normal bone marrow, there was no effect on colony formation in the presence of anti-γ-interferon sufficient to neutralize ~800 IU of interferon per 1-ml dish; in 4 of the normal cultures, anti-α-interferon at 5000 neutralizing units per dish was also without effect. In contrast, anti-γ-interferon antiserum significantly increased hematopoietic colony formation by CFU-C from 9 of 10 patients and returned colony number to the normal range in 4 cases (Fig. 3). Similar results were obtained for BFU-E (erythroid)-derived colonies (data not shown). CFU-GEMM (granulocyte/erythroid/macrophage/megakaryocyte colony-forming cell)-derived colonies were not observed in control or anti-interferon-treated dishes. In cultures from two patients tested, a monoclonal antibody to γ-interferon was as effective as the anti-γ-interferon antiserum in increasing colony formation (data not shown). Anti-α-interferon antiserum showed a similar but less dramatic effect on colony formation. Manipulations of culture conditions that served as controls for the antisera effects included incubation with a sheep antiserum against an interferon-deficient antigen and hyperimmune rabbit sera from multiple sources. An average 4-fold increase in colony formation with anti-γ-interferon was observed in the cultures from the aplastic patients (Table 3), although the interpretation of the increases must be tempered by the finding of very low colony number in untreated dishes in some cases.

**Interferon in Aplastic Bone Marrow.** Interferon was measured in bone marrow sera in the last patients studied in this series (Table 4). All 8 patients with aplastic anemia had interferon in their bone marrow sera at concentrations that result in significant inhibition of hematopoietic colony formation *in vitro* (14, 19). Interferon activity also was present in normal bone marrow sera but at significantly lower levels than that measured in bone marrow from aplastic anemia patients. Mononuclear bone marrow cells from all 6 patients tested showed significantly increased colony formation *in vitro* in the presence of anti-interferon antiserum. Bone marrow sera levels of interferon were higher than simultaneously obtained circulating sera levels in 6 patients tested, suggesting local production or concentration of interferon in the bone marrow.

**DISCUSSION**

Virtually every patient with aplastic anemia reported in this study showed abnormal interferon production *in vitro*. When exposed to lectins, the mononuclear cells from their periph-

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### Table 2. Circulating interferon levels

<table>
<thead>
<tr>
<th></th>
<th>Interferon,</th>
<th>No. &gt; 10</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>IU/ml</td>
</tr>
<tr>
<td>Aplastic anemia patients</td>
<td>24</td>
<td>87 ± 30</td>
</tr>
<tr>
<td>Normal persons</td>
<td>16</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Multiply transfused</td>
<td>18</td>
<td>&lt;10</td>
</tr>
<tr>
<td>patients with hereditary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>diseases</td>
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**Fig. 3.** Effect of anti-interferon antiserum on hematopoietic colony formation *in vitro* by bone marrow cells from patients with aplastic anemia. Bone marrow mononuclear cells from 10 untreated patients with severe aplastic anemia were cultured in methylcellulose at 1 x 10⁶ cells per ml and CFU-C-derived colonies were scored on day 10. Each group of bars represents the results obtained with cells from one of the patients. The culture medium contained no antiserum (bars a), control hyperimmune serum antiserum (bars b), control hyperimmune rabbit antiserum (bars c), sheep antiserum to human α-interferon (5000 neutralizing units/ml) (bars d), or rabbit antiserum to human γ-interferon (800 neutralizing units/ml) (bars e). Error bars represent standard deviations of replicate plates. Significant differences (by Student's t test) between plates containing anti-γ-interferon and control plates were observed for patients 1, 2, and 6 (P < 0.001); 3, 4, 5, 7, and 9 (P < 0.01); and 10 (P < 0.02). The number of bone marrow CFU-C per 10⁵ mononuclear cells in cultures from 10 normal persons was 91 ± 32 (mean ± SD) and was unaffected by anti-γ-interferon.
Table 3. The effect of anti-interferon antisera on hematopoietic colony formation (CFU-C)

<table>
<thead>
<tr>
<th>Source of bone marrow cells</th>
<th>Anti-α-interferon</th>
<th>Anti-γ-interferon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group^* Condition</td>
<td>n</td>
<td>± mean</td>
</tr>
<tr>
<td>A Aplastic anemia, prior to therapy</td>
<td>10</td>
<td>158 ± 37</td>
</tr>
<tr>
<td>B Aplastic anemia, post-recovery</td>
<td>3</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>C Other hematologic diseases</td>
<td>12</td>
<td>32 ± 31</td>
</tr>
<tr>
<td>D Normal</td>
<td>10</td>
<td>−0.5 ± 2</td>
</tr>
</tbody>
</table>

*Relative to control cultures from the same individuals. Given as mean ± SEM.

^Statistical analysis of differences in anti-γ-interferon effect: between A and D, P < 0.001; A and C, P < 0.001. Anti-α-interferon effect: between A and D, P < 0.02; A and C, P < 0.05.

eral blood showed very high levels of interferon production in comparison to cells from either normal persons or those receiving blood transfusions for other hematologic diseases. Furthermore, interferon production in vitro was sustained, with elevated amounts of interferon present in the cell supernatants 7–8 days after initiation of cultures, at which time normal cell culture interferon concentrations had returned to base-line values. Interferon is not normally measurable in cultures of lymphocytes unstimulated by lectins, but in cultures from about half of the aplastic anemia patients interferon was produced spontaneously, in some cases at very high concentrations. Interferon production by cells from these patients correlated with activity that suppressed normal hematopoietic colony formation in vitro, an effect completely reversed by the prior addition of antisera to γ-interferon.

The identification of a specific lymphokine as a hematopoietic inhibitor has implications for the interpretation of previously published experiments in which in vitro systems were used to demonstrate suppression in aplastic anemia. In retrospect, many experiments showing hematopoietic suppression in vitro for cells from patients with aplastic anemia likely were measuring the effects of interferon. Coculture of normal and aplastic anemia bone marrow or blood cells (6, 20), mixed leukocyte cultures (7), and lectin stimulation of mononuclear cells (11) are all conditions that promote interferon, particularly γ-interferon, production (21, 22). In other experiments, increased colony formation by aplastic bone marrow has been observed after removal of T lymphocytes (5). Interferon may also be implicated in these experiments, as T cells are the source of γ-interferon (23) and also may be required to mediate its effects upon target cells (24).

Does interferon play a role in the suppression of bone marrow function in patients with aplastic anemia, or are the abnormalities described epiphenomena associated with bone marrow failure and its treatment? Many patients with aplasia showed evidence of circulating interferon. These observations were made in patients who, in addition to having little abnormal susceptibility to viral disease, showed no clinical evidence of active viral infection. Serum interferon levels, as well as in vitro production of interferon, were not abnormal in a control population of multiply transfused patients who had been more frequently exposed to the same transfusion-transmitted infections and who had higher levels of serum antibodies to hepatitis viruses, Epstein–Barr virus, and cyto-megalovirus (25). Some of the patients with aplastic anemia with marked abnormalities of interferon production had transfusion histories limited to only a few erythrocyte units. Our results are also consistent with the observation of bone marrow toxicity in cancer patients treated with intravenous infusions of recombinant interferon (19).

Levels of interferon were elevated in the bone marrow sera of patients with aplastic anemia, and the concentrations measured were in the range known to inhibit hematopoietic cell proliferation in vitro (14, 26). In addition, colony formation by aplastic bone marrow was improved by antisera to interferon, suggesting that hematopoietic suppression in the patients might be the result of the presence of interferon. Interferon activity was also present in sera from normal bone marrow, but at a significantly lower level than in aplastic bone marrow. Subtyping of the interferons present in aplastic bone marrow sera has shown the presence of both γ and α-interferon (unpublished observations). Synergy in the anti-proliferative effects of γ and α-interferon has been demonstrated with murine bone marrow (27) and leukemic cell lines (28). The higher levels of interferon in the bone marrow compared to the blood in aplastic anemia patients suggest that marrow is the origin of interferon.

Bone marrow interferon also was present, although at lower concentrations, in normal individuals, in whom interferon is not detectable in sera from circulating blood. Only α-interferon, and not γ-interferon, has been detected in subtyping of normal bone marrow sera (unpublished observations). That interferon might be a normal bone marrow product also has been suggested by the measurement of high levels of β-interferon in cultures of human marrow stromal cells (29). In contrast to its effect on aplastic bone marrow, addition of anti-interferon antisera to normal bone marrow cultures did not alter colony formation. The levels of interferon present in normal bone marrow or produced during normal marrow cell culture may not inhibit cell proliferation. Studies with mouse bone marrow cells suggest that preincubation with interferon (at low concentrations similar to those that we detected in normal human bone marrow) may enhance myeloid cell sensitivity to growth factors (30). Interferon first was defined by its ability to protect cells from viral infections (31); its roles in cell–cell interactions (32) and suppression of cell proliferation were recognized subsequently (33), but a possible regulatory function in hematopoiesis suggests that the description of interferon as an “immune mediator” may be too restrictive.

Table 4. Interferon activity in bone marrow serum and blood serum

<table>
<thead>
<tr>
<th>Group</th>
<th>Interferon, IU/ml</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Aplastic anemia patient</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>227</td>
</tr>
<tr>
<td>6</td>
<td>250</td>
</tr>
<tr>
<td>7</td>
<td>153</td>
</tr>
<tr>
<td>9</td>
<td>125</td>
</tr>
<tr>
<td>11</td>
<td>153</td>
</tr>
<tr>
<td>12</td>
<td>270</td>
</tr>
<tr>
<td>13</td>
<td>200</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>203 ± 54*</td>
</tr>
<tr>
<td>Normal individuals (n = 16)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>41 ± 23*</td>
</tr>
</tbody>
</table>

*P < 0.001.

Patient numbers 3–9 refer to Fig. 3. Patient 11 was partially recovered after anti-thymocyte globulin treatment. Patients 12 and 13 had been untreated.

Why is interferon production abnormal in aplastic anemia? Abnormalities of interferon production may be the consequence of the grossly disordered bone marrow that characterizes aplastic anemia; dysregulation of the production of other lymphokines, such as interleukins 1 and 2, is also common in aplastic anemia patients (34). γ-Interferon production may be the result of more specific inciting events. For exam-
ple, sensitized T cells produce γ-interferon after reexposure to virus (35), and the recent finding that a form of aplasia is caused by a parvovirus (36) raises the possibility that other forms of bone marrow failure might result from viral infection and perhaps be mediated by interferon's suppressive effects (37). Interferon-producing lymphocytes, whether a clone of abnormal suppressor cells or abnormally regulated normal cells, may be the targets of anti-thymocyte globulin and anti-lymphocyte globulin therapy (2).

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