Aplastic Anemia: Lack of Inhibitory Effect of Bone Marrow Lymphocytes on In Vitro Granulopoiesis

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Prompted by previous reports that in certain patients with aplastic anemia, cell-mediated autoimmune suppression of myeloid stem cell proliferation may be demonstrable in vitro, we studied the effects of bone marrow lymphocytes from 18 patients with aplastic anemia on the proliferation of committed granulocytic-monozytic progenitor cells (CFU-C). When assayed in soft agar cultures, marrow suspensions from 10 patients with aplastic anemia contained significantly fewer viable CFU-C than similar cell preparations from control subjects. To deplete marrow cell suspensions of lymphocytes, we employed rabbit anti-human thymocyte serum (ATS), which after multiple adsorptions exhibited marked cytotoxicity for human B and T lymphocytes but had negligible effect on normal CFU-C proliferation. Preincubation of marrow samples from 12 patients with ATS and complement resulted in no inhibition or enhancement of CFU-C growth. In further experiments, marrow cells from 8 patients were incubated with marrow from control subjects prior to CFU-C culture. No suppression of donor CFU-C proliferation was observed in any of these studies, and in 4 cocultures, mixture of the 2 marrow suspensions resulted in stimulation of CFU-C growth. Using these assays, we detected no evidence of cell-mediated inhibition of CFU-C proliferation in any of the 18 patients that we evaluated. Our data support the conclusion that in the majority of patients with aplastic anemia, an absolute deficiency of hemopoietic stem cells is present within the marrow that does not appear to be effected or sustained by suppressor lymphocytes. Whether the reduction of viable stem cells is the cause or the consequence of the process that leads to marrow failure remains unknown.

In the syndrome of acquired aplastic anemia, peripheral pancytopenia is directly due to hypoproliferation of all myeloid cellular lineages within the bone marrow. Although approximately half of the cases of aplastic anemia have no readily discernible etiology, apparent causes of marrow failure in man include exposure to ionizing irradiation, chemical agents, or toxic drugs; viral hepatitis; paroxysmal nocturnal hemoglobinuria; and antecedent autoimmune diseases. It is probable that the syndrome of marrow aplasia clinically represents a common expression of several distinct pathophysiologic insults to the pluripotential hemopoietic stem cell compartment, since etiologies recognized to predispose to this disorder are multiple and diverse.

In recent years, considerable interest has been aroused by the growing number of reports describing individual patients with aplastic anemia whose disease has remitted in close conjunction with immunosuppressive therapy.1-7 This observation has raised the possibility that in certain instances, marrow failure may result from direct autoimmune inhibition of hemopoietic stem cell proliferation. In pursuit of this hypothesis, several laboratories have employed techniques for the cloning of human committed myeloid progenitor cells in viscous medium8-10 to evaluate the potential role of immunologic suppression of myeloid precursor cell proliferation in patients with aplastic anemia. Some studies11-19 but not others,20-21 have provided evidence that suggests that lymphocyte-mediated suppression of in vitro myelopoiesis may exist in certain patients with marrow aplasia, and in some cases, inhibition by soluble antibody has been implicated.22-25 To date, the fraction of patients in whom evidence of immunologic inhibition of in vitro myelopoiesis has been reported remains small when compared to the total number of patients with aplastic anemia who have been evaluated using marrow culture techniques, and much of the literature has appeared in the form of individual case studies.

In 1976, we initiated a program designed to evaluate the role of lymphocyte-mediated inhibition of in vitro granulopoiesis in patients with bone marrow failure. We employed the techniques of cellular incubation with antithymocyte serum and complement for the depletion of T lymphocytes, coculture of aplastic and normal marrow suspensions to evaluate lymphocyte-mediated suppressor activity, and the soft agar culture of the committed granulocytic-monozytic progenitor cell (CFU-C) to quantify in vitro granulopoiesis. In this report, we review results of experiments...
based on a total of 18 patients with severe aplastic anemia and other disorders of myeloid hypoplasia.

MATERIALS AND METHODS

Patients and Controls
All patients with aplastic anemia and other marrow hypoplastic syndromes included in this study were evaluated in consultation or managed directly by the hematology services of the Peter Bent Brigham Hospital, Children's Hospital Medical Center, or the Sidney Farber Cancer Institute. Control marrow specimens were obtained from healthy volunteers, marrow transplant donors, and patients undergoing marrow aspirations to determine the clinical stage of lymphoma or for the evaluation of anemia. In all cases, control marrow samples were determined to be free of morphological evidence of malignancy.

Collection of Marrow Samples
With the exception of samples obtained from normal volunteers, marrow specimens were collected only when bone marrow aspirations were carried out for diagnostic or therapeutic purposes. All specimens were obtained following guidelines approved by the Harvard University Institutional Review Committee for Human Experimentation. After the withdrawal of approximately 1.0 ml of marrow for histologic study, 5-8 ml were collected separately in dilute preservative-free heparin and allowed to sediment at unit gravity for 2 hr at room temperature. The nucleated cell-rich supernatant plasma was then separated and adjusted to the appropriate cell concentration with Hanks' balanced salts solution (HBSS) containing 10% fetal calf serum (FCS), 50 U/ml penicillin-G, and 50 µg/ml streptomycin (HBSS 10% FCS).

CFU-C Cultures
Bone marrow CFU-C were cloned in bilayer agar cultures in 35-mm Petri dishes using a modification of the technique of Pike and Robinson. A double-strength supplemented E-2020 tissue culture medium, prepared as previously described,6 was used in place of McCoy's 5-A Medium. Supplemented E-2020 medium diluted with an equal volume of triple-distilled water (E-1010) was used interchangeably with HBSS 10% FCS for cell washings and dilutions. Peripheral blood leukocytes from one of three healthy donors, suspended in underlayers at a concentration of 10^6 cells/ml, were used as a source of colony-stimulating activity (CSA) in all experiments. Nucleated marrow cells to be assayed for the presence of viable CFU-C were added to overlayers in a concentration of 0.82-3.10 x 10^3/ml. Cultures were maintained in a humid incubator at 37°C in the presence of 5% CO₂. On days 12-14, individual plates were scored with a dissecting microscope. Aggregates within the overlayers of more than 40 cells were counted as "colonies."

Preparation of Antithymocyte Serum (ATS)
Rabbit anti-human thymocyte serum was prepared by a modification of the technique previously described by Levey et al.17 Briefly, intact thymocytes separated from tissue obtained from children undergoing cardiopulmonary bypass surgery were injected into a panel of New Zealand white rabbits. Antiserum was harvested, heat-inactivated, and adsorbed sequentially with pooled human erythrocytes and cells from an Epstein-Barr virus-transformed B-lymphoblastoid tissue culture cell line (LAZ 156). The undiluted adsorbed ATS was shown to be cytotoxic to human T lymphocytes at a dilution of 1:10,000 and to B lymphocytes at a dilution of 1:1000 by the complement-dependent microcytotoxicity assay of Terasaki et al.18 In our studies, ATS was diluted 1:150 with HBSS prior to use (dilute ATS). To serve as a control, pooled serum from un.injected New Zealand white rabbits was heat-inactivated, adsorbed against pooled human erythrocytes, and diluted 1:150 with HBSS (dilute control serum). Antiserums were stored at 4°C.

Complement
Undiluted fresh pooled serum from New Zealand white rabbits (fresh rabbit serum) served as a source of hemolytic complement in the ATS incubation experiments described below. Aliquots of the same pooled serum were heated at 56°C for 30 min (heat-inactivated rabbit serum) to provide appropriate controls. Undiluted rabbit sera were stored at -20°C.

Incubation of Marrow Cells With ATS and Complement
In certain experiments, marrow samples harvested as described above were adjusted to a cell concentration of 4-5 x 10^6/ml and incubated with ATS and complement prior to CFU-C culture. To each of 4.0 5.0-ml aliquots of the marrow cell suspension were added 0.1 ml volumes of dilute ATS, dilute control serum, or E-1010. The mixtures were then incubated at 37°C for 30 min. At the end of the first incubation period, 0.065 ml volumes of fresh rabbit serum, heat-inactivated rabbit serum, or E-1010 were added, and a second 30-min incubation was carried out. Cell suspensions were then washed twice with 5 ml volumes of E-1010, resuspended in 10 ml E-1010 in 0.3% agar, and plated immediately in CFU-C cultures.

1 Chromium Labeling of Mononuclear Cells
Peripheral blood mononuclear cells were obtained by centrifugation of heparinized blood on sodium metrizoate-Ficoll gradients.29 One-thousand microcuries of Na_2 ^{51}CrO_4 (New England Nuclear, Boston, Mass.) were added to 10^6 washed mononuclear cells suspended in 1.0 ml HBSS and allowed to incubate for 1 hr at 37°C. The cells were then washed twice in HBSS and diluted to a concentration of 10^5 cells/ml. Aliquots (0.5 ml) of ^{51}Cr-labeled cells were incubated with ATS and complement according to the protocol described above. An additional control sample was added in which maximal cell lysis was produced by freezing and thawing the cell suspension three times. At the end of the final incubation, the cell suspensions were centrifuged, and radioactivity of the supernatant was determined by gamma spectrometry. The percentage of cellular lysis in each sample was calculated according to the following formula:

\[
\% \text{ Cytolysis} = \frac{\text{Radioactivity of supernatant from test sample}}{-} \times 100
\]

\[
\text{Background radioactivity}
\]

\[
\text{Radioactivity of supernatant from maximally lysed cells}
\]

\[
\text{Background radioactivity}
\]

Coculture Experiments
In certain experiments, marrow cells from patients with bone marrow hypoplasia and marrow cells from control subjects were harvested on the same day. A quantity of 0.6-3.5 x 10^6 unwashed cells from the patient and 2.0-4.0 x 10^6 unwashed cells from the control were suspended in separate tubes in 5 ml volumes of HBSS 10% FCS. A mixture of the two cell populations in an identical volume of HBSS 10% FCS was placed in a third tube. Mixtures were incubated at 37°C for 48 hr. In some experiments, the mixture tube contained the same number of cells found in each of the single-cell suspensions. In other studies, half the number of cells in each of the single-cell suspensions was placed into
the mixture. The single and mixed cell suspensions were incubated at 37°C in 5% CO₂ and 100% humidity for 20–24 hr. Each cell suspension was then washed twice in HBSS 10% FCS or E-1010. After the final wash, the cell pellets were resuspended in 10 ml E-1010 in 0.3% agar and plated in agar cultures. At the end of the culture period, CFU-C numbers were determined for each separate cell population and for the mixture of the two. From the CFU-C concentrations of each of the single-cell populations, an "expected" CFU-C value for the mixture was determined. Data from these studies are expressed as a percent of expected CFU-C concentration, as calculated by the formula:

\[
\text{% of Expected CFU-C} = \frac{\text{Observed CFU-C concentration}}{\text{Expected CFU-C concentration}} \times 100
\]

**Statistical Methods**

The Student’s t test was used to assess statistical significance between comparable experimental groups.

**RESULTS**

A total of 18 subjects with aplastic anemia and other hypoplastic syndromes were evaluated as a part of this study between April 1976 and May 1978. Clinical data pertaining to these patients are summarized in Table 1. Thirteen persons with acquired aplastic anemia showed evidence of marked marrow aplasia at the time of study (patients 1–13). These patients were dependent on erythrocyte and/or platelet transfusions, and 11 of the 13 had been transfused with more than 10 U of blood cells prior to culture. Only one patient (1), who subsequently required frequent transfusions, underwent marrow culture studies before his first transfusion was administered.

Four patients with severe aplastic anemia (2, 3, 4, and 9) were treated with multiple courses of intravenous ATS injections. Of the four, one patient (3) underwent remission of aplasia during therapy and is currently well 3 yr after marrow recovery. In no other patient was a favorable response to ATS treatment noted. Six patients received marrow transplants from histoidentical sibling donors (4, 6, 7, 8, 12, and 13) of which two (6, 13) are alive 1–3 yr after transplantation with functioning marrow allografts.

Five patients had moderate marrow hypoplasia at the time that culture studies were performed (patients 14–18). All but one (patient 15) had previously received multiple transfusions. Two patients (16, 17)

Table 1. Clinical Profile of Patients With Marrow Aplasia

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Age (Yrs)</th>
<th>Sex</th>
<th>Etiology</th>
<th>Platelets (x 10^9/liter)</th>
<th>Hematocrit (%)</th>
<th>Reticulocytes (x 10^9/liter)</th>
<th>WBC (x 10^9/liter)</th>
<th>PMN (%)</th>
<th>Estimated Marrow Cellularity (%)</th>
<th>Months from Dx to Time of Study</th>
<th>Treatment and Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>M</td>
<td>Idiopathic</td>
<td>3,000</td>
<td>27</td>
<td>0.4</td>
<td>5,000</td>
<td>2.900</td>
<td>10</td>
<td>1</td>
<td>No response to corticosteroids; died</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>F</td>
<td>Chloramphenicol</td>
<td>4,000</td>
<td>25</td>
<td>1.0</td>
<td>2,400</td>
<td>600</td>
<td>10</td>
<td>6</td>
<td>No response to ATS; died</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>M</td>
<td>Idiopathic</td>
<td>65,000</td>
<td>20</td>
<td>0.9</td>
<td>2,300</td>
<td>600</td>
<td>10</td>
<td>4</td>
<td>Partial recovery after ATS; alive and well</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>F</td>
<td>Idiopathic</td>
<td>28,000</td>
<td>23</td>
<td>0.2</td>
<td>2,000</td>
<td>500</td>
<td>10</td>
<td>3</td>
<td>No response to ATS; transplanted; died 2' GVH</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>F</td>
<td>Insecticides</td>
<td>2,000</td>
<td>30</td>
<td>0.4</td>
<td>3,100</td>
<td>700</td>
<td>10</td>
<td>6</td>
<td>Developed AML after 36 mo of aplasia</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>M</td>
<td>Viral hepatitis</td>
<td>34,000</td>
<td>20</td>
<td>0.2</td>
<td>2,800</td>
<td>200</td>
<td>10</td>
<td>3</td>
<td>Transplanted; alive and well</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>F</td>
<td>Idiopathic</td>
<td>1,000</td>
<td>23</td>
<td>0.4</td>
<td>1,000</td>
<td>200</td>
<td>5</td>
<td>4</td>
<td>Transplanted; died 2' sepsis</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>F</td>
<td>Idiopathic</td>
<td>80,000</td>
<td>28</td>
<td>0.0</td>
<td>3,700</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>Transplanted; died 2' sepsis</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>M</td>
<td>Idiopathic</td>
<td>1,500</td>
<td>36</td>
<td>—</td>
<td>4,900</td>
<td>1,900</td>
<td>15</td>
<td>36</td>
<td>No response to ATS; died</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>M</td>
<td>Idiopathic</td>
<td>5,000</td>
<td>33</td>
<td>—</td>
<td>2,100</td>
<td>500</td>
<td>5</td>
<td>24</td>
<td>No response to androgens; died</td>
</tr>
<tr>
<td>11</td>
<td>57</td>
<td>F</td>
<td>Idiopathic</td>
<td>4,000</td>
<td>27</td>
<td>0.2</td>
<td>4,200</td>
<td>300</td>
<td>30</td>
<td>1</td>
<td>Spontaneously recovered; alive and well</td>
</tr>
<tr>
<td>12</td>
<td>19</td>
<td>M</td>
<td>Viral hepatitis</td>
<td>22,000</td>
<td>31</td>
<td>0.0</td>
<td>750</td>
<td>120</td>
<td>5</td>
<td>2</td>
<td>Transplanted; died 2' sepsis</td>
</tr>
<tr>
<td>13</td>
<td>18</td>
<td>M</td>
<td>Viral hepatitis</td>
<td>8,000</td>
<td>28</td>
<td>0.4</td>
<td>500</td>
<td>200</td>
<td>10</td>
<td>2</td>
<td>Transplanted; alive and well</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>M</td>
<td>Idiopathic</td>
<td>42,000</td>
<td>33</td>
<td>3.8</td>
<td>6,600</td>
<td>1,400</td>
<td>10</td>
<td>10</td>
<td>Transplanted; identical twin transplant; gradual but complete recovery</td>
</tr>
<tr>
<td>15</td>
<td>7</td>
<td>M</td>
<td>Hereditary hypoplasia</td>
<td>119,000</td>
<td>31</td>
<td>0.7</td>
<td>4,500</td>
<td>1,200</td>
<td>30</td>
<td>24</td>
<td>No therapy; has persistent hypoplasia</td>
</tr>
<tr>
<td>16</td>
<td>41</td>
<td>M</td>
<td>Benzene</td>
<td>153,000</td>
<td>29</td>
<td>3.4</td>
<td>2,300</td>
<td>900</td>
<td>30</td>
<td>12</td>
<td>Spontaneously recovered; alive and well</td>
</tr>
<tr>
<td>17</td>
<td>3</td>
<td>M</td>
<td>Idiopathic</td>
<td>170,000</td>
<td>34</td>
<td>3.2</td>
<td>6,900</td>
<td>2,800</td>
<td>60</td>
<td>12</td>
<td>Recovered while receiving androgens; alive and well</td>
</tr>
<tr>
<td>18</td>
<td>60</td>
<td>F</td>
<td>Systemic lupus erythematosus</td>
<td>8,000</td>
<td>24</td>
<td>2.4</td>
<td>7,100</td>
<td>3,400</td>
<td>30</td>
<td>6</td>
<td>Recovered while receiving corticosteroids; alive and well</td>
</tr>
</tbody>
</table>
had acquired aplastic anemia but spontaneously recovered before marrow samples were studied in culture. One additional patient (14) had formerly had severe aplastic anemia, and culture studies were carried out 6 mo after marrow transplantation from his identical twin resulted in incomplete engraftment. An elderly woman (18) had marrow hypoplasia in association with systemic lupus erythematosus. Finally, a 7-yr-old child (15) had a familial syndrome of myeloid hypoplasia.

CFU-C assays were carried out on untreated marrow samples from 10 patients with severe aplastic anemia and 9 controls. The results of these experiments are shown in Fig. 1. The percentage of nucleated cells from the marrows of patients with aplastic anemia that were capable of forming colonies in agar cultures maximally stimulated by an exogenous source of CSA was severely reduced when compared to control values ($p < 0.001$).

Incubation of marrow specimens with ATS and complement prior to CFU-C cultures involved the exposure of nucleated marrow cells to four separate rabbit serum preparations. Studies were performed to determine whether any of these components exerted an intrinsic stimulatory or inhibitory effect on normal human CFU-C proliferation. At concentrations 3–4-fold greater than those used in the experiments cited below, none of the individual additives significantly modulated normal human CFU-C proliferation when tested separately (data not shown).

Experiments were undertaken to confirm that pretreatment of hemopoietic cell suspensions with ATS and complement in the concentrations used in these experiments resulted in a substantial depletion of viable lymphocytes. Peripheral blood mononuclear cell fractions were labeled with $^{51}$Cr and incubated with ATS and complement according to the protocol detailed above. Eighty percent cytolysis resulted when the cell suspensions were exposed to both ATS and complement, whereas treatment with ATS alone produced no measurable lysis of the labeled mononuclear cells (data not shown).

The results of the marrow CFU-C assays obtained after incubation with ATS and complement are summarized in Table 2. The marrows of nine patients with severe aplastic anemia and three patients with myeloid hypoplasia were cultured, and in none was a statistically significant increase in cloning efficiency of CFU-C observed after ATS and complement pretreatment when compared with either untreated or serum-treated control groups. The marrows of four

Table 2. Results of Individual Experiments in Which Marrow Cell Suspensions Were Preincubated With ATS and Complement (C') Prior to CFU-C Culture

<table>
<thead>
<tr>
<th>Patient</th>
<th>Group I Untreated Control</th>
<th>Group II Serum-Treated Control</th>
<th>Group III ATS</th>
<th>Group IV ATS + C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.9 ± 3.1</td>
<td>11.8 ± 1.7</td>
<td>6.6 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>28.7 ± 5.1</td>
<td>23.2 ± 2.3</td>
<td>21.4 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.2 ± 0.2</td>
<td>0.6 ± 0.4</td>
<td>0.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.0 ± 1.2</td>
<td>5.0 ± 1.6</td>
<td>5.8 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>29.6 ± 3.5</td>
<td>30.0 ± 2.3</td>
<td>29.6 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.2 ± 0.2</td>
<td>0.5 ± 0.5</td>
<td>0.6 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>6.7 ± 1.1</td>
<td>5.8 ± 0.5</td>
<td>9.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.0 ± 0.0</td>
<td>1.8 ± 0.6</td>
<td>2.7 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>18.1 ± 1.8</td>
<td>12.7 ± 0.9</td>
<td>8.1 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.2 ± 1.5</td>
<td>24.5 ± 1.3</td>
<td>18.0 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.7 ± 0.7</td>
<td>1.5 ± 0.5</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>45.4 ± 3.3</td>
<td>32.4 ± 3.6</td>
<td>29.0 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.8 ± 0.0</td>
<td>26.6 ± 4.1</td>
<td>13.1 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>14.7 ± 2.4</td>
<td>22.8 ± 4.3</td>
<td>34.7 ± 3.6</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>173.8 ± 7.7</td>
<td>182.4 ± 10.3</td>
<td>146.2 ± 4.9</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents mean ± SEM of 1–5 individual culture plates stimulated by peripheral leukocyte feeder layers. The concentration of marrow cells plated in these experiments ranged from 0.8–3.0 x 10^5/ml. Values shown above are normalized to represent CFU-C per 2 x 10^5 cells plated.
control subjects were cultured in similar experiments. In two (ii and iii), CFU-C numbers were increased after ATS and complement pretreatment when compared with respective serum control groups but not when compared to untreated controls.

The results of individual coculture experiments are shown in Fig. 2. In these studies, marrow cells from patients with aplastic anemia or myeloid hypoplasia were admixed with marrow cells from control subjects prior to CFU-C assays. Similar coculture experiments were carried out between paired unrelated control subjects as shown. A total of 8 patients with aplastic anemia or myeloid hypoplasia were studied in 10 experiments. In one experiment (14), marrow cells from the patient were cocultured with cells from his hematologically normal identical twin. In all other cases, cocultured marrow samples were randomly paired and were assumed to be histoincompatible. In no case was suppression of CFU-C proliferation observed that exceeded that seen in the control cocultures. In three experiments (13, 14, and 15), a moderate increase in CFU-C proliferation was noted when compared to expected values, and in one instance (8) an extreme degree of enhanced CFU-C growth was evident. As noted above, cocultures were carried out at two separate cell concentrations. In order to determine whether cell density affected CFU-C proliferation in these mixtures, one pair of normal marrows (control A) and two aplastic-normal pairs (7 and 11) were simultaneously cocultured at both cell levels. In all three cases, the expected number of CFU-C recovered from the mixtures was reduced by a similar degree when the cells were cocultured at the higher concentration.

**DISCUSSION**

At least three pathophysiologic processes have been considered to account for the development of spontaneous marrow aplasia in man. The frequency with which bone marrow failure closely follows exposure to toxic chemicals or ionizing irradiation and the high rate of successful engraftment seen with allogeneic marrow transplantation are compatible with the concept that an absolute deficiency or intrinsic defectiveness of the pluripotential hemopoietic stem cell (HSC) compartment may be a frequent mechanism of aplasia. Rarely, an inability of the hemopoietic inductive microenvironment (HIM) to sustain adequate proliferation or differentiation of myeloid precursor cells has been postulated. Finally, in recent years, the increasing number of reports of patients with bone marrow failure in whom prompt remission of aplasia has been observed after immunosuppressive therapy has lent support to the notion that in certain instances aplastic anemia may result from direct autoimmune suppression of HSC proliferation.

Although recent evidence suggests that the human pluripotential hemopoietic stem cell may proliferate in semisolid tissue culture, there is currently no convenient assay for the HSC in man. However, techniques have been standardized that permit quantitation of both committed granulocytic (CFU-C) and committed erythroid progenitor cells in human marrow suspensions. Several investigators, including ourselves, have employed these assays for the study of immunologically mediated suppression of human myelopoesis under the assumption that committed myeloid progenitor cells cloned in tissue culture represent close progeny of the HSC.

In 1976, Ascensao et al. and Kagan et al. reported in vitro studies carried out on the bone marrow cells of a young woman with severe aplastic anemia. They found that removal of lymphocytes from marrow cell suspensions by preincubation with ATS and complement or by velocity sedimentation produced an increase in the cloning efficiency of the
patient's CFU-C, and that coculture\textsuperscript{12} of the patient's marrow cells with marrow from normal donors resulted in a suppression of normal CFU-C growth. They concluded that suppressor lymphocytes in the population of marrow cells tested actively inhibited myeloid progenitor cell replication in vitro and suggested that cell-mediated immune suppression of HSC proliferation may have accounted for marrow hypoplasia in their patient. Following these initial observations, several laboratories submitted similar reports that suggested that lymphocyte-mediated suppression of CFU-C\textsuperscript{11,15,18} or of committed erythroid progenitor cells\textsuperscript{13,16,17} may be demonstrable in vitro in occasional patients with aplastic anemia. Others, however, have been unable to detect evidence of cell-mediated inhibition of in vitro myelopoiesis in such patients,\textsuperscript{31} and Singer et al.\textsuperscript{20} and Torok-Storb et al.\textsuperscript{34} have proposed that lymphocytic suppression of in vitro proliferation of granulocytic or erythroid progenitor cells in marrow cultures from patients with aplastic anemia may simply be a function of previous transfusion with histoincompatible blood components.

Our studies were designed to assess the frequency with which lymphocyte-mediated inhibition of granulopoiesis may occur in a large number of patients with marrow failure. Thirteen of the subjects that we studied comprised a homogeneous group with severe aplasia (Table 1), and all but one had received multiple transfusions prior to culture. Five patients had a moderate degree of marrow hypocellularity at the time of study or had partially recovered from severe aplasia.

When CFU-C assays were carried out on untreated marrow cells from 10 patients with severe aplasia (Fig. 1), we were not surprised to observe that the cloning efficiency was significantly lower than that of control marrows (\(p < 0.001\)). These data are in accord with previously reported studies in which the concentration of marrow CFU-C from patients with aplastic anemia has been quantified.\textsuperscript{19,35} When considered alone, these results are consistent with either an absolute deficiency of viable myeloid progenitor cells or with an immunologically mediated inhibition of stem cell proliferation operative in vitro, since the cultures were performed using unfractionated bone marrow cell suspensions in the presence of the patient's serum.

As shown in Table 2, we assayed CFU-C from washed bone marrow cell suspensions of nine patients with severe aplastic anemia, three patients with myeloid hypoplasia, and four controls after incubation with ATS and complement. Experiments using \(^{51}\text{Cr}\)-labeled peripheral blood mononuclear cells showed that the incubation protocol we utilized was capable of producing a substantial depletion of viable lymphocytes. Nonetheless, in none of the patients or controls was a statistically significant increase or decrease in CFU-C cloning efficiency observed after ATS and complement pretreatment (\(p > 0.05\)).

To interpret these data, it must be considered that the interaction between ATS and hemopoietic progenitor cells is complex from several standpoints. First, since unfractionated serum from animals injected with suspensions of intact human thymocytes contains a multiplicity of antibodies directed against many common cell surface antigens, and even after multiple adsorptions with other human cell types specific for T lymphocytes is usually incomplete,\textsuperscript{36} the potential for cross-reactive cytotoxicity to the CFU-C clearly exists.\textsuperscript{37} Second, human T lymphocytes have been shown to produce CSA,\textsuperscript{38} and their removal by preincubation with ATS and complement theoretically may spuriously diminish CFU-C proliferation by reducing the quantity of these stimulatory glycoproteins in individual cultures. Third, studies on the regulation of human erythropoiesis in vitro\textsuperscript{39} as well as experiments carried out in thymic-deficient mouse models\textsuperscript{40-42} suggest that T lymphocytes may normally serve to enhance proliferation of myeloid precursors more primitive than the CFU-C. We do not believe that the ATS preparation employed in these experiments produced measurable inhibition of CFU-C proliferation by any of these mechanisms, since in no individual experiment was a significant decrease in CFU-C growth noted after ATS and complement pretreatment. Furthermore, none of the separate rabbit serum preparations that we used enhanced or inhibited normal marrow CFU-C when tested individually.

We feel that the most plausible explanation for our findings that marrow CFU-C were diminished in these 12 patients with hypoplastic syndromes and failed to increase after preincubation with ATS and complement is that an absolute deficiency of myeloid progenitor cells was present in the marrows of each of these individuals at the time that culture studies were carried out. Our data do not permit us to speculate on whether a reduction in stem cell numbers was the primary etiology of aplasia in any of our patients, or merely the end result of the process that led to marrow failure. We cannot discount the possibility, based on these observations, that lymphocyte-mediated suppression of myeloid stem cell proliferation may have originally accounted for the evolution of aplasia in some of our patients, giving rise to an irreversible exhaustion of the stem cell mass. In such a case, it would not be expected that depletion of lymphocytes from marrow cell suspensions by preincubation with ATS and complement should result in an enhancement of the cloning efficiency of granulocytic progeni-
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We and others\(^{12,19}\) have performed crude assays of cellular interaction in which inhibition or stimulation of hemopoiesis may each occur by a number of mechanisms. Augmentation of CFU-C proliferation, as we observed in four of eight cocultures between normal and aplastic marrows, may be due to the production of CSA\(^{18}\) or other humoral stimulators of myeloipoiesis by activated lymphocytes, or to increased numbers of monocytes or macrophages within the culture. A decrease in CFU-C viability in this system may be caused by cytotoxicity mediated by suppressor lymphocytes, as others have reported;\(^{12,19}\) however, since our data suggest that CFU-C yield may diminish as the cell concentration within the coculture is increased, it is likely that CFU-C proliferation in these mixture experiments is dependent on many separate variables.

In summary, we have been unable to detect evidence of lymphocyte-mediated inhibition of in vitro granuloipoiesis in any one of 18 patients with marrow failure using the techniques of cellular preincubation with ATS and complement and marrow coculture. Although our data support the conclusion that an absolute deficiency of myeloid progenitor cells was present in the marrows of all of these patients at the time that our studies were carried out, we feel strongly that technical inadequacies of the assays that we have employed prevent us from excluding the possibility that suppressor lymphocytes capable of inhibiting myeloid progenitor cell proliferation may have been present within the marrows of some of our patients at the time of study. Although the likelihood that certain cases of aplastic anemia may be mediated by autoimmune suppression of myeloid stem cell proliferation is great, we believe that convincing evidence of this phenomenon has not yet been achieved in vitro because of significant limitations of the techniques currently available. We hope that in the near future, the development of specific antibodies to human lymphocyte subsets will provide new avenues for the study of cell-mediated autoimmune suppression of the HSC in the aplastic disorders. At present, the most compelling evidence that certain cases of aplastic anemia may be immunologically mediated is clinical.\(^{1,7}\)

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