Further Evidence for Lymphokine Overproduction in Severe Aplastic Anemia

By Wolfgang Hinterberger, Günther Adolf, Gerald Aichinger, Robert Dudczak, Klaus Geißler, Paul Höcker, Christoph Huber, Peter Kalhs, Walter Knapp, Ursula Köller, Klaus Lechner, and Beatrice Volc-Platzer

Interferon-γ (IFN-γ) and tumor necrosis factor (TNF) are lymphokines with a potent hematopoietic progenitor cell suppressive capacity. In untreated and immunosuppressed patients with severe aplastic anemia (SAA) and in control individuals we measured (a) serum levels of IFN-γ and TNF and its production by peripheral blood mononuclear cells (PBMCN); (b) serum levels of neopterin, a product that reflects endogenous IFN production; (c) resting and activated lymphocyte subpopulations; and (d) serum levels of soluble interleukin-2 receptor (IL-2R). Serum levels of IFN and TNF did not differ significantly in untreated and treated SAA patients and control individuals. Spontaneous and phytohemagglutinin-induced production of IFN and TNF by PBMCN, however, were highly increased in both untreated and treated SAA patients. Increased and decreased neopterin serum levels in untreated and treated SAA patients, respectively, suggest modulation of endogenous lymphokine release subsequent to immunosuppression. HLA-DR + antigen was mainly expressed by CD8 T cells. Circulating numbers of activated (CD4 and CD8) T cells and serum levels of IL-2R were not increased in both untreated and treated SAA patients. The proportion of HLA-DR + T cells in the PBMCN of untreated SAA patients correlated with the extent of lectin-induced IFN production. Although we were unable to confirm previous reports in SAA on (a) detectable IFN in blood and bone marrow serum, (b) improvement of stem cell growth upon neutralization of endogenous IFN, (c) absolutely increased numbers of circulating activated T cells, and (d) normalization of these abnormalities subsequent to successful immunosuppression, our data clearly support previous reports on abnormal lymphokine production in severe aplastic anemia. Our failure to relate this phenomenon to the severity of disease states, however, further raises doubts on the pathogenetic significance of lymphokine overproduction in SAA.

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who subsequent to immunosuppressive treatment achieved a state of transfusion independence but still had abnormal hematopoiesis.

**PATIENTS AND METHODS**

**Patients and control individuals.** Nineteen untreated SAA patients and 13 further patients (including four of the untreated) who had achieved transfusion independence subsequent to immunosuppressive therapy were studied (Table 1). The diagnosis was based on the morphological examination of a bone core biopsy specimen and blood and bone marrow smears and by exclusion of an underlying disease. One case was pregnancy associated; two cases were related to prolonged pyrazolone and chloramphenicol treatment, respectively. All other cases were considered idiopathic. Three patients had been treated with oxymetholone, without response. These patients had not been receiving oxymetholone for >9 months before study. Three of the untreated patients were untransfused, the remaining were pretransfused with two to 115 blood cell transfusions before study (median, 12). The untreated SAA patients were studied 0.5 to 32 months after diagnosis (median, 6). Immunosuppressive treatment consisted of a combination of ATG (Atgam, Upjohn Co, Kalamazoo, MI) and high-dose methylprednisolone (ATG- MP), which has been described by us in detail. The control groups consisted of (a) 20 healthy blood and eight bone marrow donors, (b) nine hematologically normal individuals who were given two to 14 blood transfusions 4 to 52 weeks before investigation during surgical procedures, and (c) ten patients with refractory anemia (myelodysplastic syndromes) who were pretreated with 18 to more than 100 blood transfusions. For at least six days before the study, patients and controls were afebrile and free of viral, bacterial, or fungal infection as judged by clinical, radiological, and laboratory examinations. Informed consent was obtained from all individuals studied.

**Laboratory investigations.** Blood samples drawn in the morning by venipuncture were divided and further processed by the laboratories involved in the study.

**Determination of IFN and TNF levels.** IFN was determined by a bioassay measuring the reduction of the cytopathic effect of encephalomyocarditis virus on A549 human lung cells (American Type Culture Collection, Rockville, Md; CCL 185). This assay is equally sensitive for human IFN-α, IFN-β, and IFN-γ. The level of detection varied between 1 and 3 U/mL. Serum samples were assayed in parallel by means of a commercially available immunoreadiometric assay specific for biologically active IFN-γ (IRMA; Centocor, Inc, Malvern, PA). The sensitivity of this assay ranged between 0.5 and 1 U/mL. A recombinant IFN-γ preparation was included in all assays. This preparation had previously been standardized against the World Health Organization international standard Gg 23-901-530; all IFN levels are thus given in international units per milliliter.

TNF levels in culture supernatants were determined by a serum-free in vitro bioassay. Murine connective tissue cells L-M obtained from the American Type Culture Collection, a derivative of L 929 cells, served as targets. The quantitation of cytolytic activity is based on the ability of TNF to lyse L-M cells in the presence of actinomycin D, as measured by crystal violet dye uptake of residual viable cells. The assay is sensitive to <100 pg/mL TNF-α.

**Determination of neopterin levels.** Serum neopterin levels were evaluated by means of a commercially available radioimmunoassay (Henning, Berlin). In this laboratory, serum neopterin levels of normal individuals ranged between 0.8 and 2.4 ng/mL. The interassay variation coefficient (VC) was 13%, the intraassay VC was 7% (for high values, the intraassay VC was 10%, and the intraassay VC was 8%).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median</th>
<th>Range</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>33</td>
<td>8-78</td>
</tr>
<tr>
<td>Duration of disease before study (mo)</td>
<td>6</td>
<td>0.5-32</td>
</tr>
<tr>
<td>Blood cell transfusions before study</td>
<td>12</td>
<td>0-115</td>
</tr>
<tr>
<td>Blood cell counts on day of study (n x 10⁶/L)</td>
<td>18</td>
<td>0-58</td>
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<tr>
<td>Reticulocytes</td>
<td>0.4</td>
<td>0-2.1</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>1.2</td>
<td>0.2-2.3</td>
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<tr>
<td>Lymphocytes</td>
<td>14</td>
<td>3-50</td>
</tr>
<tr>
<td>Platelets</td>
<td>54</td>
<td>26-169</td>
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**Preparation and harvest of PBMNC culture supernatants.** After Ficoll-Hypaque separation (400 g, 40 minutes of centrifugation, room temperature), 2 x 10⁶ PBMNC were cultured in 1.0 mL RPMI medium (supplemented with 10% fetal calf serum) in the presence or absence of 2.0 μg/mL phytohemagglutinin (PHA, Difco, Detroit). Because our initial studies had shown peak levels of IFN after three days, supernatants were harvested after exactly 72 hours and stored at -20°C.

**Analysis of bone marrow colony growth.** A quantity of 10⁶ Ficoll-Hypaque-separated mononuclear bone marrow cells from eight normal bone marrow donors and ten untreated SAA patients was cultured in 1 mL methylcellulose-containing culture medium appropriate to support the growth of granulocyte-macrophage colony-forming cells (CFU-GM), burst-forming units (BFU-E), and mixed colony-forming cells (CFU-GEMM). The technique is described in detail by one of us. Endogenous IFN-γ and TNF activity were neutralized by preincubation (two hours) and subsequent coculture of the bone marrow cell suspension with either (a) monoclonal antibody (MoAb) directed against IFN-γ (no. 3800, Interferon Sciences, Inc, lot 4039.2, New Brunswick, NJ) sufficient to neutralize 530 IU IFN-γ/mL or (b) MoAb directed against TNF-α (lot 3314-16, Interferon Sciences) sufficient to neutralize 6 x 10⁶ IU TNF-α/mL. Colonies were scored after 14 days of culture by means of an inverted microscope.

**Analysis of lymphocyte subpopulations.** PBMNC were stained with either the CD4/T4 antibody VIT 4 or the CD8/T8 antibody VIT 8, both fluorescein isothiocyanate labeled and counterstained with the phycoerythrin-conjugated anti–HLA-DR antibody from Becton Dickinson (Sunnyvale, CA). Analysis of the two-color fluorescence experiments was done on a FACS 440 cell sorter (Becton Dickinson). A total of 20 x 10⁶ cells were analyzed in each experiment.

Circulating HLA-DR + CD4 and CD8 lymphocytes were counted by multiplying circulating CD4 and CD8 cells by the proportion of respective T cells expressing HLA-DR antigen. Proportions of HLA-DR + T cells and monocytes (defined by light microscopy of a blood smear) in the PBMNC fractions were calculated according to

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</table>
the following formula: Percent HLA-DR+ T cells or percent monocytes equals (HLA-DR+ T lymphocytes or monocytes per
microliter blood x 100)/PBMNC per microliter blood.

Quantification of soluble low-affinity IL-2 receptor serum levels.
IL-2 receptor (IL-2R) serum levels were measured in ten SAA
patients before and 2 to 3 months after ATG/MP treatment. A
solid-phase enzyme-linked immunosorbent assay (ELISA) system,
described by Rubin et al.,28 was used. It was based upon two
non-cross-reactive anti-IL-2R MoAbs, kindly provided by D. Nel-
son, National Institutes of Health, Bethesda, MD. One of them,
anti-Tac, at a concentration of 4 μg/mL, was coated overnight at
4°C onto 96-well ELISA plates. The other, biotinylated MoAb
7G7/B6, at a dilution of 1:4,000, served for the detection of soluble
IL-2R fixed to the solid-phase anti-TAC. Streptavidin-peroxidase
complex (Amersham Corp. Buckinghamshire, UK) was used as a
detection system. The plates were read at 410 nm by a Dynatec
ELISA reader (Dynatech Laboratories, Inc. Alexandria, VA), and
results were expressed as optical densities (OD) corrected for OD of
phosphate-buffered saline control. Normal sera and serial dilutions
of concanavalin A supernatants of PBMNC were tested in addition
and used as negative or positive control, respectively.

Statistical analysis. The Mann-Whitney U test was applied to
compare nonparametrically distributed data from two groups. Fish-
ner's exact test was performed to compare the frequencies of positive
serum levels and spontaneous production by PBMNC of IFN
between two groups. The Spearman rank test was used to search for
possible correlations of parameters within groups.

RESULTS

Spontaneous and lectin-induced production of IFN and
TNF by PBMNC. Spontaneous IFN production by
PBMNC, as measured after 72 hours, could not be detected
in 18 normal controls, but was observed in 12 of 19 untreated
and seven of 13 treated SAA patients (P < .05 and P < .05,
respectively) (Fig 1 and Table 2). Upon PHA stimulation,
PBMNC of SAA patients produced approximately a tenfold
amount of IFN, irrespective of whether they were untreated
(P < .01) or had responded to ATG/MP treatment
(P < .01).

PBMNC from four of 19 untreated patients exhibited no
spontaneous release, and PHA-induced production was in
the normal range. These four patients did not differ from the
15 others with respect to blood cell counts, duration of
disease, number of blood transfusions given, and levels of
circulating resting or activated T cells.

The duration of disease before the study (more or less than

Table 2. Production of IFN and TNF by PBMNC. Percentage of HLA-DR+ T Cells and Monocytes in PBMNC, and Serum Levels of
IFN and Neopterin in SAA Patients, Normal Individuals, and Control Patients (Median, Range)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SAA Untreated</th>
<th>SAA Treated</th>
<th>Normal Individuals</th>
<th>Pretransfused Normal Individuals</th>
<th>Refractory Anemia</th>
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</thead>
<tbody>
<tr>
<td>IFN production (IU/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>2 (bdl-200)</td>
<td>3 (bdl-82)</td>
<td>bdl (bdl-bdl)</td>
<td>bdl (bdl-bdl)</td>
<td>bdl (bdl-39)</td>
</tr>
<tr>
<td>PMA-induced</td>
<td>800 (bdl-5,700)</td>
<td>1,300 (230-3,700)</td>
<td>67 (bdl-230)</td>
<td>7 (bdl-255)</td>
<td>66 (bdl-270)</td>
</tr>
<tr>
<td>TNF production (IU/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>0.2 (bdl-1.8)</td>
<td>bdl (bdl-0.3)</td>
<td>1 (1-4)</td>
<td>1 (bdl-2)</td>
<td>1 (bdl-6)</td>
</tr>
<tr>
<td>PMA-induced</td>
<td>263 (158-628)</td>
<td>164 (bdl-382)</td>
<td>22 (1-336)</td>
<td>18 (1-188)</td>
<td>43 (bdl-212)</td>
</tr>
<tr>
<td>Percentage of cells in PBMNC</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HLA-DR+ T cells</td>
<td>3.9 (0.5-12.3)</td>
<td>3.2 (1.5-10.5)</td>
<td>2.9 (0.6-4.3)</td>
<td>3.1 (0.7-4.3)</td>
<td>2.6 (0.7-1.1)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>3.7 (0.1-15.9)</td>
<td>10.5 (5.5-15.2)</td>
<td>14.5 (4.2-42)</td>
<td>19.3 (7-35.1)</td>
<td>3.7 (0.31-2)</td>
</tr>
<tr>
<td>IFN (IU/mL, serum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus inhibition assay</td>
<td>bdl (bdl-4)</td>
<td>bdl (bdl-bdl)</td>
<td>bdl (bdl-bdl)</td>
<td>bdl (bdl-bdl)</td>
<td>bdl (bdl-bdl)</td>
</tr>
<tr>
<td>Centocor+</td>
<td>bdl (bdl-4)</td>
<td>bdl (bdl-bdl)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Neopterin (ng/mL serum)</td>
<td>3.2 (1.0-25.1)</td>
<td>1.1 (0.7-2.3)</td>
<td>1.8 (0.8-2.4)</td>
<td>1.9 (0.6-2.6)</td>
<td>1.8 (0.6-5.2)</td>
</tr>
</tbody>
</table>

Abbreviations: bdl, below detection level; nd, not determined.
6 months) did not influence the release of IFN by PBMC (spontaneous IFN, \( r = -0.1 \); PHA-induced IFN, \( r = -0.4 \), not significant). The number of blood transfusions given before the study (more or less than 12) had likewise no influence on IFN production by PBMC (spontaneous IFN, \( u = 21.5 \); PHA-induced IFN, \( u = 26.0 \), not significant).

In untreated SAA patients, the proportion of HLA-DR+ T cells (CD4 plus CD8) in the PBMC correlated with the extent of lectin-induced IFN production (\( r = 0.54, P < 0.05 \)).

Also investigated were spontaneous and PHA-induced production of TNF by PBMC from SAA patients and controls: spontaneous release from PBMC was minimal and did not differ between controls and SAA patients. PHA-induced TNF production by PBMC from SAA patients was approximately ten times higher than that in normal individuals (\( P < 0.05 \) for untreated and \( P < 0.05 \) for treated SAA patients, respectively). There was no difference in spontaneous and lectin-induced production of IFN and TNF by PBMC of untreated and treated SAA patients. In the latter group of patients, a positive correlation was observed between spontaneous TNF production by PBMC and the proportion of HLA-DR+ T cells in PBMC fractions (\( r = 0.52, P < 0.05 \)).

In normal individuals, pretransfused normal persons, and patients with refractory anemia, IFN and TNF were not detectable in the serum samples. The proportions of HLA-DR+ T cells and monocytes in the PBMC suspensions were identical or diminished, respectively. The production of IFN and TNF by PBMC was in the normal range.

**Cellular composition of PBMC fractions.** The percentages of HLA-DR+ T cells in the PBMC fractions of untreated and treated SAA patients and in controls were not significantly different (Table 2). The percentages of monocytes, however, were much lower in untreated and slightly lower in treated SAA patients than in normal controls (\( P < 0.01 \) and \( P < 0.05 \), respectively).

**Serum and bone marrow serum levels of IFN and TNF.** By using a biologic assay sensitive to all types of human IFN as well as an immunoassay specific for human IFN-\( \gamma \), we were unable to detect IFN activity in the serum of normal individuals or treated SAA patients and in about 16 of 19 untreated SAA patients (not significant) (Table 2). Less than 4 IU/mL IFN was found in two cases by bioassay and in one other case by IRMA. Only four of 94 serum samples obtained during ATG/MP therapy showed IFN activity slightly above detectable levels (data not shown).

IFN was also not detectable in bone marrow sera of six normal controls (bone marrow donors) and nine of ten untreated SAA patients. Only one patient showed 4 IU/mL IFN in the bone marrow serum as opposed to 2 IU/mL in serum.

TNF was detectable neither in serum nor in bone marrow serum of six untreated and two treated SAA patients (14 investigations, data not shown).

**Serum neopterin levels.** Serum neopterin levels (SNL) were higher in untreated SAA patients than in healthy controls (median, 3.2; \( P < 0.05 \)). In the 13 SAA patients who had previously been treated with ATG/MP, SNL were subnormal (median, 1.1 ng/mL; \( P < 0.05 \)). SNL, however, failed to correlate with serum levels of IFN and also with IFN production by PBMC. SNL were elevated in two of three untransfused, untreated SAA patients and declined after blood cell transfusions (data not shown).

SNL were slightly elevated in two of eight patients with refractory anemia although IFN production was normal.

**Bone marrow colony formation.** CFU-GM, BFU-E, and CFU-GEMM numbers were greatly diminished in SAA patients (Table 3). No increase in colony numbers was found on preincubation and coculture with MoAbs directed against IFN-\( \gamma \) or TNF. In additional experiments the following modifications were used: (a) antibodies directed against IFN-\( \gamma \) and TNF were applied simultaneously, (b) preincubation with MoAbs in suspension culture lasted 24 instead of two hours, and (c) a MoAb directed against lymphotoxin (LT) was used to neutralize endogenous LT.29 None of these measures led to an increase in colony growth of any of the progenitor cell classes studied (data not shown).

The capacity of MoAb 3,800 to neutralize IFN-\( \gamma \) in a methylcellulose colony assay was demonstrated as follows: normal bone marrow cells were induced to colony growth by the addition of PHA supernatants from SAA PBMCs. These supernatants were analyzed in parallel for their IFN-\( \gamma \) content. One aliquot was neutralized with an amount of MoAb 3,800 sufficient to neutralize 1,000 IU IFN-\( \gamma \)/mL, whereas the other was used as control. Differences in the CFU-GM frequencies obtained with neutralized/nonneutralized supernatants are shown in Fig 2 for ten different PHA supernatants, the differences in CFU-GM (\( \Delta \)) were clearly related to the concentration of IFN-\( \gamma \) in the respective supernatants.

**Resting and activated lymphocyte subpopulations in the blood.** Absolute numbers of circulating CD4+ lymphocytes were not statistically different in normal individuals and untreated and treated SAA patients (Table 4). CD8+ lymphocytes were lower in untreated than in treated SAA patients (\( P < 0.05 \) and \( P < 0.05 \), respectively). The ratios of resting CD4+/CD8+ T cells were not significantly different in the three groups. Absolute numbers of activated (HLA-DR+) CD4+ T lymphocytes were reduced in untreated in comparison to treated SAA patients (\( P < 0.05 \)) and normal individuals (\( P < 0.01 \)). HLA-DR+ CD8+ lymphocyte levels were normal in untreated SAA patients but elevated after ATG/MP.

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**Table 3. Number of Colonies/10^5 Mononuclear Bone Marrow Cells of Normal Individuals and Untreated SAA Patients (Median, Range). Cultures With and Without MoAbs Directed Against IFN-\( \gamma \) and TNF**

<table>
<thead>
<tr>
<th>Individuals</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-GEMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without MoAb</td>
<td>61 (21-312)</td>
<td>56 (14-174)</td>
<td>1.5 (0-19)</td>
</tr>
<tr>
<td>MoAb against IFN-( \gamma )</td>
<td>63 (30-416)</td>
<td>55 (14-194)</td>
<td>4.5 (1-15)</td>
</tr>
<tr>
<td>MoAb against TNF-( \alpha )</td>
<td>78 (30-386)</td>
<td>75 (18-302)</td>
<td>2.0 (0-18)</td>
</tr>
<tr>
<td>Untreated (n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without MoAb</td>
<td>3 (0-22)</td>
<td>1 (0-19)</td>
<td>0 (0-1)</td>
</tr>
<tr>
<td>MoAb against IFN-( \gamma )</td>
<td>4 (0-30)</td>
<td>1 (0-18)</td>
<td>0 (0-0.5)</td>
</tr>
<tr>
<td>MoAb against TNF-( \alpha )</td>
<td>4 (0-35)</td>
<td>2 (0-19)</td>
<td>0 (0-0.5)</td>
</tr>
</tbody>
</table>
therapy ($P < .05$). Consequently, the HLA-DR$^+$ CD4/CD8 T-cell ratios were lower in untreated ($P < .01$) and treated SAA patients ($P < .01$) than in normal controls.

**IL-2R serum levels.** Only one of ten untreated and two of ten treated SAA patients had IL-2R serum levels above the 95% confidence limit of normal individuals (not significant for untreated and treated SAA patients, respectively).

**DISCUSSION**

Our analysis on the impact of lymphokines in the pathogenesis of SAA was based on the parallel investigation of untreated and treated SAA patients. To exclude the interference of infections and/or blood transfusions with the parameters investigated, patients were selected who were free of clinically detectable infections and were either untransfused or not exposed to blood products for prolonged periods of time. Our data confirm and extend the view of lymphokine overproduction of SAA patients.

In this series, PBMC of many untreated and treated SAA patients spontaneously released detectable amounts of IFN-$\gamma$. When stimulated with $2 \mu g/mL$ PHA, PBMC of untreated and treated SAA patients produced on average approximately ten times more IFN-$\gamma$ and TNF than did PBMC of control individuals. It thus appears that the lymphokine overproduction of SAA patients is not restricted to full-blown disease states but is still present in patients who achieve a state of self-sustaining but still abnormal hematopoiesis subsequent to immunosuppressive therapy. These data partly contradict a recent investigation of Torok-Storb et al$^{77}$ stating that although spontaneous release was observed in 18 of 50 SAA patients, IFN-$\gamma$ production induced by $50 \mu g/mL$ of PHA did not clearly differ in each four SAA patients and controls studied. Our explanation of these discrepancies relates mainly to the fact that supramaximal stimulation was used in the study of Torok-Storb et al, whereas suboptimal concentrations of PHA were applied in our investigations.

Our measurement of circulating IFN levels, on the other hand, gained different results from the data of Zoumbos et al.$^{12}$ By using both a biologic IFN detection assay sensitive to all types of human IFNs as well as an IRMA specific for IFN-$\gamma$ we could detect low levels of circulating IFNs in only three of 19 untreated and in none of the treated SAA patients' sera. The maximum IFN levels detected in bone marrow and blood sera of SAA patients were always less than 4 IU/mL. In view of the parallel use of two different assays, we would conclude that measurable levels of IFN in SAA patients' sera are an exception rather than a characteristic feature of this disease.

We were also unable to observe normalized or even enhanced progenitor cell growth of SAA bone marrow cells by adding antibody to neutralize endogenous IFN-$\gamma$ and/or TNF. Possibly the effects of IFN or TNF are not direct or reversible and, therefore, not susceptible to blocking by antibody. At a concentration of 10,000 IU/mL IFN-$\gamma$, a 15-minute preincubation of adherent cells induced significant inhibition of subsequent BFU-E growth.$^{30}$ This observation may explain (a) an inability to reverse IFN-$\gamma$-induced stem cell suppression by neutralization of endogenous IFN-$\gamma$ and (b) reports on adherent cell–mediated stem cell inhibition in SAA.$^{31,32}$ Likewise, pulse exposure of normal bone marrow cells with TNF results in maximal or near-maximal colony growth inhibition when compared with cells left with TNF for the full culture period.$^{33}$ TNF exerts a potent suppressive effect on progenitor cell growth at concentrations of 50 to 100 IU TNF/mL.$^{34}$ The concentrations of TNF we measured in PBMC supernatants of SAA patients appear sufficiently high to mediate progenitor cell suppression.

Indirect support for the assumption of increased endogenous IFN-$\gamma$ production is derived from our analysis of a secondary IFN message: neopterin, a pterin that is produced

![Image](image-url)

**Table 4. Resting and Activated T-Cell Subpopulations ($\times 10^9$/L Blood, Median, Range) and Their Respective Ratios in Normal Controls and Untreated and Treated SAA Patients**

<table>
<thead>
<tr>
<th>Populations</th>
<th>Normal Controls ($n = 12$)</th>
<th>Untreated SAA ($n = 18$)</th>
<th>Treated SAA ($n = 13$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resting T cells</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD4</td>
<td>822 (388-1,710)</td>
<td>622 (6-2,662)</td>
<td>742 (226-1,172)</td>
</tr>
<tr>
<td>CD8</td>
<td>412 (198-819)</td>
<td>211 (7-961)</td>
<td>354 (123-761)</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>2 (1-4)</td>
<td>2 (1-6)</td>
<td>2 (1-4)</td>
</tr>
<tr>
<td><strong>Activated (HLA-DR$^+$) T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DR$^+$ CD4</td>
<td>34 (10-71)</td>
<td>12 (1-108)</td>
<td>27 (7-95)</td>
</tr>
<tr>
<td>HLA-DR$^+$ CD8</td>
<td>12 (12-27)</td>
<td>12 (8-28)</td>
<td>22 (4-69)</td>
</tr>
<tr>
<td>HLA-DR$^+$ CD4/CD8</td>
<td>2 (1-7)</td>
<td>1 (&lt;1-3)</td>
<td>1 (&lt;1-2)</td>
</tr>
</tbody>
</table>
in macrophages under control of IFNs, had elevated levels in untreated and subnormal ones in treated SAA patients. SNL are elevated in viral infections, autoimmune disorders, solid-organ graft rejection, and graft-v-host disease. Neopterin levels have not been studied in SAA; we would take elevated SNL in untreated SAA patients as further evidence suggesting an increased endogenous IFN production, although we were not able to directly confirm this by demonstration of circulating IFNs. Conversely, diminished neopterin levels in treated SAA patients may possibly indicate diminished IFN production in vivo, although lectin-induced lymphokine overproduction in vitro persists.

Zoumbos et al presented indirect evidence that HLA-DR+, Tac+, CD8+ cells are the cellular source of enhanced IFN production in SAA. Our observation of a significant correlation between the proportion of HLA-DR+ T cells in the PBMC fractions and the extent of lectin-induced IFN production in untreated SAA patients supports this assumption.

In contrast to the observation of Torok-Storb et al, no such correlation was found for monocytes in our study. Gascon et al described an increased proportion of lymphocytes bearing activation antigen (Tac+) in the blood of SAA patients and pretransfused normal controls. Zoumbos et al described normal numbers of activated (Tac+) CD4 cells but elevated numbers of activated CD8 T cells in SAA; although our data confirm HLA-DR+ expression mainly on the CD8 T-cell subset, we were unable to confirm absolutely increased numbers of circulating activated (HLA-DR+) CD8 T cells. Tac antigen expression may possibly more properly reflect lymphocyte activation in the particular disease studied; on the other hand, serum levels of soluble IL-2R, which reportedly appears to reflect activated T-cell concentrations (Rubin et al and own observation), were entirely in the normal range. Obviously, the number of circulating activated T cells may not necessarily reflect their local concentration, e.g., in the bone marrow, nor does the absolute number of circulating activated T cells necessarily relate to local production of lymphokines.

Our findings of abnormal IFN and TNF production in treated SAA patients are paralleled by persistently subnormal blood and stem cell numbers and also by the persistence of lymphocytes, which may inhibit stem cell growth. On the other hand, in this study neutralizing antibodies against IFN-γ and TNF did not normalize or even increase hematopoietic stem cell growth in SAA bone marrow samples. Control experiments clearly excluded the possibility that inappropriate neutralization in the colony culture system is responsible for this failure. Whether this finding indicates that cytokines with antiproliferative capacity other than IFN-γ or TNF are also involved or whether one in fact deals with an epiphenomenon remains to be investigated.

REFERENCES


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Further evidence for lymphokine overproduction in severe aplastic anemia [see comments]

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