Lymphokine Overproduction in Severe Aplastic Anemia Is Not Related to Blood Transfusions

By Wolfgang Hinterberger, Günther Adolf, Peter Bettelheim, Klaus Geissler, Christoph Huber, Evelyn Ischick, Peter Kalhs, Ursula Köller, Klaus Lechner, Bernhard Meister, and Wolfgang Wolosczuk

The production of interferons (IFNs), IFN-γ, tumor necrosis factors (TNFs) and TNF-α (TNF-α) by peripheral blood mononuclear cells (PBMCs) of untransfused and transfused, but otherwise untreated patients with severe aplastic anemia (SAA) was determined using bioassays and immunoassays. In untransfused and pretransfused SAA patients, spontaneous and lectin-induced production of these cytokines by PBMCs was strongly enhanced. Cytokine production in untransfused SAA patients did not differ from that in pretransfused patients. Similar relative frequencies of activated (HLA-DR+) lymphocyte subpopulations present in the PBMCs demonstrated cytokine overproduction per cell. Cytokine production was studied in three SAA patients before and after blood cell transfusions. Spontaneous and lectin-induced production of these cytokines was abnormally high and unaffected by blood transfusions. In another patient exhibiting abnormal cytokine production, the hematopoietic response to cyclosporin-A was improved by normalization of cytokine production in vitro. We conclude that overproduction of IFN-γ and TNF-α by lectin-stimulated PBMCs is an intrinsic abnormality of SAA unrelated to blood transfusions. Normalization of production of IFN-γ and TNF-α accompanying a clinical response to cyclosporin-A may cautiously be taken as further evidence suggesting a pathogenetic role of cytokine overproduction in SAA.

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ture and diluted in 4 mL buffered (pH 7.4) EDTA. The probe was gently layered over Ficoll-Hypaque (specific weight 1.077) and centrifuged at 400 g for 40 minutes. The leukocyte-containing layer was removed with a Pasteur pipette and washed twice in phosphate-buffered saline. The cells were then adjusted to 2 x 10^6 cells/mL in RPMI 1640 medium (GIBCO, England) supplemented with 10% fetal calf serum (Seromed, FRG). PBMNCs were cultured with or without 2 μg/mL phytohemagglutinin (PHA, Difco). The dishes were incubated at 37°C in 5% CO2 in full humidity. Supernatants were harvested after 72 hours and stored at -20°C until assay.

**Measurement of IFN.** IFN was assayed (a) by a bioassay measuring reduction of the cytopathic effect of encephalomyocarditis virus on A-549 human lung carcinoma cells (American Type Culture Collection, ATCC CCL 185) and (b) using an immunoradiometric assay (IRMA) specific for biologically active IFN-γ (IRMA, Centocor, Malvern, PA). The sensitivity of the bioassay varied between 1 and 3 IU/mL. The sensitivity of the IRMA ranged between 0.5 and 1 IU/mL. All IFN levels are given in international units per milliliter in terms of the international reference preparation Gg 23-901-530 (National Institute of Allergy and Infectious Disease, Bethesda, MD).

**Measurement of TNF.** TNF levels in culture supernatants were determined by means of a bioassay sensitive to both TNF-α and TNF-β and an enzyme-linked immunosorbent assay (ELISA) specific for TNF-α. For the bioassay, murine connective tissue cells L-M served as targets (obtained from the ATCC). Quantitation of cytolytic activity was based on the ability to lyse the cells in the presence of actinomycin D, as measured by crystal violet dye uptake of residual viable cells. The assay is sensitive to less than 100 pg/mL TNF-α. The ELISA was performed in 96-well microtiter plates; it uses polyclonal rabbit antibodies to recombinant human TNF-α for coating and a monoclonal murine antibody to TNF-α coupled to hors eradish peroxidase. The assay is able to detect TNF-α in serum and supernatants with a sensitivity of 16 pg/mL.

**Analysis of lymphocyte subpopulations.** PBMNCs were stained with either the CD4/CD8 antibody VIT 4 or the CD4/CD8 antibody VIT 8 and counterstained with the anti–HLA-DR antibody from Becton Dickinson (Sunnyvale, CA), as previously described. The two-color experiments were analyzed on a FACS 440 cell sorter (Becton Dickinson). 2 x 10^6 cells were analyzed in each experiment.

Absolute numbers of circulating HLA-DR⁺ CD4 and CD8 cells, respectively, were determined as previously described. The relative frequencies of activated (HLA-DR⁺) CD4 and CD8 cells in the PBMNC fractions studied for cytokine release were calculated according to the formula: Percentage of HLA-DR⁺ CD4 or CD8 cells equals (HLA-DR⁺ CD4 cells or CD8 cells per microliter of blood x 100)/PBMNCs/μL blood.

**Statistical analysis.** The Mann-Whitney U test was used to compare nonparametrically distributed data between different groups.

**RESULTS**

Spontaneous and PHA-induced production of IFN (bioassay) and IFN-γ (IRMA) and TNF (bioassay) and TNF-α (ELISA) by unseparated PBNMCs is shown in Fig 1. Spontaneous IFN production was higher in pretransfused and untransfused SAA patients than in normal controls, but these differences did not reach statistical significance. Spontaneous IFN-γ production was significantly higher in pretransfused (P < .01) and untransfused (P < .01) SAA patients than in normal controls, respectively. PHA-induced production of IFN and IFN-γ by PBMNCs was significantly higher in pretransfused (P < .01) and untransfused (P < .01) SAA patients, respectively, than in normal individuals.

Spontaneous production of TNF by PBMNCs did not differ within the three groups studied. Spontaneous production of TNF-α, however, was clearly enhanced in both pretransfused (P < .01) and untransfused (P < .05) SAA patients, respectively. PHA-induced production of TNF and TNF-α was significantly higher in pretransfused (P < .01 and P < .01, respectively) and untransfused (P < .01 and P < .01, respectively) SAA patients than in normal controls.

Analysis of relative frequencies of activated (HLA-DR⁺) CD4 and CD8 cells in the PBMNC fractions are shown in Table 1. There was a higher percentage of HLA-DR⁺ CD8 cells in PBMNCs of pretransfused SAA patients as compared with normal controls (P < .05), but two of the three untransfused SAA patients studied had percentages of HLA-DR⁺ CD8 cells lower than the median value of normal individuals and pretransfused SAA patients. Percentages of HLA-DR⁺ CD4 cells in the PBMNCs did not differ between normal individuals and pretransfused SAA patients; in untransfused SAA patients, however, percentages of HLA-
Cytokine overproduction in SAA

Table 1. Relative Frequencies of HLA-DR* T-Cell Subpopulations in PBMC Fractions Assayed for Cytokine Production

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Normal Individuals, Median (Range)</th>
<th>Pretransfused, Median (Range)</th>
<th>Untransfused, Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 14)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>HLA-DR* CD4</td>
<td>1.8 (0.6-3.2)</td>
<td>1.8 (0.8-7.2)</td>
<td>3.0 (2.5-7.2)</td>
</tr>
<tr>
<td>HLA-DR* CD8</td>
<td>0.9 (0.2-1.4)</td>
<td>2.0 (0.3-9.6)</td>
<td>0.5 (0.2-2.4)</td>
</tr>
</tbody>
</table>

Table 2. Resting and Activated T-Cell Subpopulations (Cells × 10^9/L Blood) and Their Respective Ratios in Blood of Normal Individuals and SAA Patients

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Normal Individuals, Median (Range)</th>
<th>SAA, Pretransfused, Median (Range)</th>
<th>SAA, Untransfused, Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 14)</td>
<td>(n = 3)</td>
</tr>
<tr>
<td>CD4</td>
<td>928 (497-1,302)</td>
<td>476 (5-2,662)</td>
<td>1,081 (371-1,753)</td>
</tr>
<tr>
<td>CD8</td>
<td>342 (168-618)</td>
<td>211 (7-961)</td>
<td>432 (119-465)</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>2.6 (1.4-7.8)</td>
<td>2.3 (0.7-6.4)</td>
<td>3.1 (2.4-3.8)</td>
</tr>
<tr>
<td>HLA-DR* CD4</td>
<td>35 (10-71)</td>
<td>16 (2-108)</td>
<td>74 (64-87)</td>
</tr>
<tr>
<td>HLA-DR* CD8</td>
<td>13 (6-27)</td>
<td>12 (8-68)</td>
<td>14 (4-21)</td>
</tr>
<tr>
<td>HLA-DR* CD4/CD8</td>
<td>1.8 (0.9-6.5)</td>
<td>1.1 (0.1-2.8)</td>
<td>6.2 (3.5-16)</td>
</tr>
</tbody>
</table>

Miscellaneous studies. PBMCs of three pretransfused SAA patients were separated by adherence on Petri dishes into adherent and nonadherent cells and stimulated with PHA or LPS, respectively. There was no detectable cytokine production on stimulation of nonadherent cells with LPS as measured after 3, 24, and 72 hours. TNF production by adherent cells stimulated with LPS was not different in normal individuals, or in pretransfused or untransfused SAA patients (data not shown). There was no measurable production of IFN by adherent PBMCNs, regardless of whether PHA or LPS was used for stimulation. Likewise, there was no measurable production of TNF by adherent cells on stimulation with PHA as measured after 24 and 72 hours.

Discussion

Cytokines such as IFNs and TNFs (among their pleiotropic functions) are also myelosuppressive and were recently shown to represent a crucial pathogenetic principle in SAA. This view was mainly supported by findings of cytokine overproduction associated with SAA and of increased in vitro growth of CFU of SAA bone marrow after depletion from T cells or after neutralization of these factors with specific antibodies. We and other investigators showed that PBMCNs of pretransfused normal individuals and pancytopenic patients with preleukemic disorders produced IFN and TNF within the normal range.

SAA is a disease which at the time of clinical manifestation almost immediately requires blood cell transfusions. Antigens, in particular those associated with allogeneic

Fig 2. Spontaneous and PHA-induced production of IFN-γ and TNF by PBMCNs of three SAA patients in relation to blood transfusions.
leukocytes, profoundly influence in vitro and in vivo activation antigen expression and cytokine production.\textsuperscript{15,17} Our data confirm previous reports on enhanced HLA-DR\textsuperscript{+} antigen expression mainly by the CD8 lymphocyte subset in pretransfused SAA patients.\textsuperscript{7,11} Our observation of activation (HLA-DR\textsuperscript{+}) antigen expression mainly by CD4\textsuperscript{+} cells in untransfused SAA patients, however, should further stimulate studies on lymphocyte subpopulations in untransfused SAA patients. Considering the similar relative frequencies of HLA-DR\textsuperscript{+} expressing T cells in PBMCN fractions of SAA patients in relation to the approximately 10-fold increase in cytokine production, enhanced cytokine biosynthesis rather than overrepresentation of cytokine-producing cells accounts for the observed abnormality.

Recently, it was shown that intensive blood cell support might also adversely influence IFN-γ production by PBMCNs.\textsuperscript{22} Before accepting the view of unbalanced cytokine production as an intrinsic feature of SAA, it is mandatory to exclude that this represents only a consequence of allogeneic stimulation by repeated blood transfusions. Our data clearly exclude this possibility. We first showed that newly diagnosed and untransfused SAA patients carry the abnormality. Second, cytokine overproduction was demonstrated before and after blood transfusions in the same SAA patients. Third, we describe an SAA case with enhanced IFN-γ and TNF production before treatment with cyclosporin A.\textsuperscript{23} In this patient, cyclosporin A induced hematologic remission and normalized in vitro cytokine production at the same time. Although the reported case provides indirect evidence for a pathogenetic role of the cytokines studied, a larger number of patients must be monitored while receiving immunosuppressive treatment.

In this study, separation of PBMCNs by surface adherence procedures attributed cytokine overproduction to the nonadherent blood cells. Stimulation of adherent PBMCNs of SAA patients with LPS yielded TNF amounts in the range of normal individuals. We propose that the increased production of TNF observed in PHA-driven PBMCN cultures represents a consequence of induction of the cytokine cascade rather than a primary abnormality of adherent PBMCNs.\textsuperscript{24}

Our study demonstrates that enhanced biosynthesis of cytokines by PBMCNs of SAA patients is an intrinsic feature of the disease and is unrelated to blood transfusions. This further suggests, but does not prove, that overproduction of the cytokines IFN-γ and TNF which exhibit potent stem cell suppressive capacities are causally related to stem cell suppression in SAA.

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