High-Level Secretion of Tumor Necrosis Factor-alpha Contributes to Hematopoietic Failure in Hairy Cell Leukemia

By A. Lindemann, W.-D. Ludwig, W. Oster, R. Mertelsmann, and F. Herrmann

Hairy cell leukemia (HCL) is frequently associated with severe pancytopenia. The authors detected high levels of tumor necrosis factor (TNF)-alpha in the bone marrow serum of patients with HCL and found anti–TNF-alpha neutralizing monoclonal antibodies (MoAbs) to be able to enhance hematopoiesis of HCL patients in vitro colony assays. As potent producers of TNF-alpha, hairy cells could be identified, thus implicating the malignant population in the pathogenesis of hematopoietic failure due to inappropriate secretion of this cytokine.

OVERSECRETION of certain soluble cell products may contribute to insufficient production of blood cells by exerting profound inhibitory effects on hematopoietic progenitor cells.1,2 Foremost, aplastic anemia has been shown to possibly result from excessive production of lymphokines inhibitory to hematopoiesis.3,4 Hairy cell leukemia (HCL) is also characterized by severe pancytopenia, which can sometimes be observed in spite of only marginal hairy cell infiltration of the bone marrow.5 The authors have observed high levels of tumor necrosis factor (TNF)-alpha in the bone marrow of patients with HCL. Since TNF-alpha has been implicated in the suppression of hematopoiesis in vitro,6,7 the following study was designed to investigate whether increased TNF-alpha levels in the bone marrow of patients with HCL would contribute to the pancytopenia seen in these patients and to further define the cellular source of TNF-alpha in HCL.

MATERIALS AND METHODS

Patients. Peripheral blood and bone marrow specimens of 21 individuals—five healthy donors (HD), five patients with HCL, three patients with chronic lymphocytic leukemia (CLL), one patient with prolymphocytic leukemia (PLL), two patients with Waldenström's macroglobulinemia (WM), two patients with follicular-center cell lymphoma (FCC), and three patients with acute lymphocytic leukemia (ALL)—were investigated for TNF-alpha production. The diagnosis was based on morphology, cytochemistry, surface marker studies including CD5, CD11c, CD19, CD20, CD22, kappa/lambda analysis, and immunoglobulin gene rearrangement studies to confirm clonality and B-cell origin. The hematologic characteristics of the patients investigated are summarized in Table 1. None of the patients with HCL had been splenectomized; three were in a leukemic state. None of the patients had received any therapy within 6 weeks prior to study. Informed consent was obtained from all patients and volunteers approved by the local institutional review board.

Cell lines. In addition to patients' peripheral blood or bone marrow specimens, some cell lines were also studied for TNF-alpha production. JOK-1, a HCL cell line was kindly provided by Dr T. Tedder, Dana Farber Cancer Institute, Boston.7 Three Burkitt's lymphoma cell lines, Daudi, Namalwa, Raji, and an Epstein-Barr virus (EBV)-transformed B-cell line, Cess, were obtained from the American Type Culture Collection (ATCC), Rockville, MD; another three EBV-transformed B-cell lines, Khamb-C, Khamb-V, Khamb-M, were generously provided by Dr O.A. Haas, Department of Pediatrics, University of Vienna, Austria.

Culture preparation and culture. Mononuclear cells (MNC) from peripheral blood and bone marrow aspirates or core biopsies (in all patients with HCL) were obtained by Ficoll-Hypaque (FH; density 1.077 g/mL) gradient centrifugation. Cells assayed for in vitro TNF-alpha secretion and messenger RNA (mRNA) analysis were derived from leukemic peripheral blood specimens (malignant cells >50%, or absolute number >1,000/mL), depleted of T cells and monocytes by two E-rosetting cycles and subsequent repeated adherence steps of the E-rosette negative fraction. Purity was >98% for neoplastic B cells in CLL, PLL, WM, and ALL; and >95% in HCL and FCC as defined by immunofluorescence staining with the appropriate monoclonal antibodies (MoAbs).

Culture supernatants to be assayed for TNF-alpha activity were generated by 24-hour incubation of neoplastic cells at 5 × 10^3/mL in standard culture medium (SCM): RPMI 1640 supplemented with 5% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 ng/mL streptomycin, and 1% sodium pyruvate (GIBCO, Grand Island, NY) in 24-well flat-bottom plates (Falcon, Oxnard, CA) at 37°C in a 5% CO2 atmosphere in air.

CFU-GM assay. CFU-GM were assayed in a double-layer agar18 (Agar Noble, Difco Laboratories, Inc, Detroit). Underlayers (0.5 mL) were composed of 0.5% agar in Iscove's modified Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, penicillin-streptomycin, 20% FCS, and 50 ng/mL recombinant human (rh)GM-CSF + 50 ng/mL rHIL-3 (Immunex, Seattle; obtained through Dr D. Krumwied, Behring, Marburg, FRG). The overlayer (0.5 mL) was composed of 0.3% agar in the same medium and contained 2 × 10^3 low-density bone marrow cells. The cultures were set up in triplicate in 24-well plastic culture plates (Corning Glass Works, Corning Science Products, New York) and were incubated at 37°C in 5% CO2 in a humidified atmosphere. In selected experiments the assay was performed in the presence of a neutralizing anti-TNF MoAb (lot No. 3314; protein concentration 1.4 mg/mL; neutralizing capacity: 300 pg TNF-alpha/µg as determined in pilot experiments; generously provided by Dr G. Adol, Ernst Boehringer Institute, Vienna), added to the overlayer in decreasing dilutions (1:5000 to 1:50). To analyze the role of TNF-alpha–induced prostaglandin release, indomethacin (Sigma, Munich, FRG) was added to the medium at 10^-7 mol/L in another set of experiments. To determine colony (>40 cells) number, the dried
cultures were fixed in acetone-methanol fixative (60% acetone, 10% methanol, 30 mmol/L sodium citrate, pH 7.4) for one minute and stained with acidic hematoxylin (Sigma, Munich, FRG).

Northern blot analysis. Total cellular RNA of purified cell populations was isolated using the guanidium/cesium chloride method. Fifteen micrograms of glyoxylated RNA were fractioned on a 1.2% agarose gel and blotted onto synthetic membranes (Schleicher & Schuell, Dassel, FRG). Specific cDNA probes were obtained for TNF-alpha by Dr O. Adolf, Ernst Bochninger Institute, Vienna; for \( \nu \)-fms by Dr C.J. Sherr, Department of Tumor Cell Biology, St Jude Children’s Hospital, Memphis; for alpha-actin (expressing 70% sequence homology and hybridizing to nonmuscle beta-actin) by Dr R.J. Schwarz, Department of Cell Biology, Baylor College, Houston; and for the constant region of the T-cell receptor (TCR)-beta chain by Dr H.D. Royer, German Cancer Research Center, Heidelberg, FRG. Radiolabeling and hybridization, including 5% dextran sulphate (mol wt 500,000, Sigma Chemicals, St Louis), were performed as described.11 Blots were developed on Kodak X-Omat films using intensifying screens.

**TNF-assay.** A TNF-alpha–specific enzyme-linked immunosorbent assay (ELISA) was obtained from T Cell Sciences, Inc, Cambridge, MA. The sensitivity of the assay is 10 pg TNF-alpha/mL. It is unaffected by the presence of denatured TNF-alpha, lymphotoxin, interleukin-1 (IL-1), or IL-2. Mean TNF-alpha serum levels in healthy donors were <20 pg/mL; the upper limit of normal use was 67 pg/mL. One milligram of recombinant TNF-alpha used as a standard for this assay is equivalent to 2 x 10^6 units of activity as defined in an L929 cytotoxicity assay in the presence of actinomycin D.

### RESULTS

**TNF-alpha production by neoplastic B cells.** Purified neoplastic B cells from patients with different disorders were assessed for TNF-alpha release using a commercially available TNF-alpha–specific ELISA. After 24 hours of unin-duced in vitro culture, high levels of TNF-alpha >2,500 pg/mL were detectable in hairy cell-conditioned medium, while other lymphoma or leukemia cell populations investigated failed to display TNF-alpha activities exceeding the normal range (Table 2). TNF-alpha concentration in the peripheral blood serum was below the threshold of detection with the exception of serum from those patients presenting with >1,000 hairy cells/mL. However, when bone marrow (BM) serum samples from HCL patients (>30% hairy cell

<table>
<thead>
<tr>
<th>Diagnosis (Patient No.)</th>
<th>WBC/mL</th>
<th>NC (%)</th>
<th>Platelets/mL</th>
<th>Hkt (%)</th>
<th>BM Infiltration (%)</th>
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<tr>
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**Abbreviations:** NC, neoplastic cells; Hkt, hematocrit; BM Infiltration, bone marrow infiltration by neoplastic cells.

**Numbers of circulating and bone marrow infiltrating cells were assessed by immunofluorescence analysis using appropriate MoAbs.**
infiltration in the BM) were analyzed, all of them contained high levels of TNF-alpha, in contrast to BM samples obtained from healthy donors and patients with other B-cell disorders (Table 1). Analysis at the mRNA level by Northern blotting revealed a marked accumulation of TNF-alpha-specific mRNA in purified uninduced hairy cells. With the exception of the leukemic cells of one patient with PLL expressing a weak TNF-alpha-specific message, none of the remaining patients’ specimens investigated was found to transcribe the TNF gene (Fig 1 and Table 2). Any significant contamination by monocytes or T cells was ruled out by reprobing the filters with c-fms and TCR-beta-specific probes, respectively (Fig 1).

The analysis of various B-cell lines (JOK-1, Raji, Daudi, Namalwa, Cess, Kamb-C, Kamb-V, and Kamb-M) demonstrated accumulation of TNF-alpha-specific mRNA only in JOK-1 cells, which, however, failed to release detectable amounts of TNF-alpha into their culture supernatants (Table 1).

**Anti-TNF MoAb enhances in vitro colony growth of HCL patients.** To investigate a possible role of TNF-alpha in suppression of CFU-GM colony growth in HCL patients, low-density mononuclear BM cells were assayed for colony growth in a double-layer agar system in the presence or absence of anti-TNF MoAbs. While the colony number of BM samples drawn from a patient with PLL, two CLL patients, and healthy donors was unchanged in the presence of increasing concentrations of a neutralizing anti-TNF MoAb, a dose-dependent increase of colony numbers (day 14 CFU-GM) was observed in BM samples of HCL patients (Fig 2).

**DISCUSSION**

Hematopoiesis has recently been shown to be controlled by various humoral mediators. While proliferative effects of colony-stimulating factors are well established in vitro and in vivo, little is known about the in vivo role of inhibitory cytokines as possible mediators of hematopoietic failure. In vitro studies by several authors, however, have demonstrated inhibitory effects of interferon (IFN)-gamma and TNF-alpha on normal and malignant hematopoiesis. Aside from aplastic anemia (AA) in which oversecretion of inhibitory cytokines has been linked to BM failure, similar mechanisms may also be operative in other pancytopenic states in which cytopenia cannot be explained merely by displacement of normal hematopoiesis by neoplastic cells. The authors’ findings suggest a pathogenic role of TNF-alpha in HCL, since high levels of TNF-alpha were found in the bone marrow of HCL patients. However, only those patients presenting with a high count of hairy cells in the peripheral blood were found to exhibit measurable TNF-alpha levels in venous blood serum. The lack of detection of TNF-alpha in hairy cell-conditioned medium reported by another group differs from the authors’ results and may be due to the lower cell densities in the cultures used in this study. Since TNF-alpha was suggested to induce TNF-alpha secretion in an autocline loop, a threshold level dependent on cellular density may be crucial.

With regard to the high TNF-alpha levels detected in the bone marrow of HCL patients, interaction of hairy cells with their environment (eg, stroma and endothelial cells [EC]) may be important. Thus, TNF-alpha has been shown to induce EC to produce IL-1 and PDGF. Induction of IL-1 secretion by EC may in turn perpetuate hairy cell secretion of TNF-alpha (manuscript in preparation). Increased PDGF release, on the other hand, has been linked to marrow fibrosis, frequently observed in HCL.

Levels of TNF-alpha of up to 4 ng/mL, as measured in the authors’ samples, can inhibit hematopoiesis by nearly 50% in vitro. The doubling of colony numbers of HCL patients in vitro cultures, performed in the presence of high concentra-
Fig 2. Analysis of colony growth in a CFU-GM assay (day 14 CFU-GM). Low-density BM cells (2 x 10^5/mL) from patients with hairy cell leukemia (HCL Nos 1 through 3), CLL (Nos 1 and 2), PLL (No 1), and from HD were assayed as described in "Materials and Methods." The colony number grown in medium alone was defined as 100%, and was for HD, 141 ± 37 (n = 8); HCL No 1, 78 ± 8; HCL No 2, 83 ± 10; HCL No 3, 41 ± 3; CLL No 1, 46 ± 4; CLL No 2, 58 ± 5; PLL No 1, 60 ± 7. The number of colonies grown in the presence of decreasing dilutions of an anti-TNF-alpha MoAb is indicated in percent relative to cultures that had been performed in the absence of an anti-TNF-alpha MoAb.

REFERENCES

15. Cordinley FT, Hoffbrand AV, Heslop HE, Turner M,


High-level secretion of tumor necrosis factor-alpha contributes to hematopoietic failure in hairy cell leukemia [see comments]

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