Restoration of Th1 Cytokine Synthesis by T Cells of Patients with Chronic Myelogenous Leukemia in Cytogenetic and Hematologic Remission with Interferon-α

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ABSTRACT
Chronic myelogenous leukemia (CML) is a disorder of the hematopoietic stem cell that results in malignant expansion of myeloid cells with a cytogenetic abnormality, the translocation between chromosomes 9 and 22 known as the Philadelphia chromosome. Treatment with IFN-α has proven to be an effective therapy, inducing cytogenetic remission in CML patients. However, it is unknown whether IFN-α can restore normal immune function for patients who achieve a complete cytogenetic remission. To address this question, we used a method of intracellular staining and flow cytometric analysis to ascribe the syntheses of Th1 or Th2 cytokines to T-cell subsets of patients in chronic, in accelerated, and in blast crisis phases as well as patients who had achieved a complete cytogenetic remission with IFN-α. We assessed the cytoplasmic synthesis of cytokine in phorbol ester (phorbol 12-myristate 13-acetate)-activated CD4+ and CD8+ T-cell subsets of 81 patients with various stages of CML and 21 normal controls. The percentages of CD4+ and CD8+ T cells from patients in chronic, in accelerated, and in blast crisis phases that synthesized Th1 cytokines interleukin (IL)-2, IFN-γ, and tumor necrosis factor-α were significantly lower than those of remission patients and normal controls. Conversely, the percentages of CD4+ and CD8+ T cells of patients in chronic, in accelerated, and in blast crisis phases of CML preferentially synthesized the Th2 cytokine IL-10. Patients who achieved a durable complete cytogenetic remission for >2 years without maintenance IFN-α therapy restored their preference for a Th1 cytokine profile that is necessary for efficient cytotoxic T-cell function.

INTRODUCTION
CML2 is a malignancy of the hematopoietic stem cells resulting in the clonal expansion of myeloid cells and their progenitors (reviewed in Refs. 1 and 2). At least 90% of the malignant cells in CML are characterized by a cytogenetic abnormality, known as the Philadelphia chromosome (Ph+; Ref. 3), the result of a reciprocal translocation between chromosomes 9(q34) and 22(q11) (Ref. 4). Patients with CML present in the Chronic phase characterized by a relatively indolent, benign stage with the differentiation of precursors into functional, mature hematopoietic cells. The disease can be either biphasic or triphasic, because some patients proceed into a less well-defined accelerated phase wherein the myeloid cells begin to stop maturing into granulocytes and instead become arrested at the level of the immature blast (5). Patients in ACC phase have 15–30% blasts and promyelocytes and ≈20% basophils in the blood or bone marrow. The natural course of CML is a transformation that closely resembles an acute leukemia, known as the BC phase, during which blasts of both lymphoid and myeloid origin make up at least 30% of the WBCs in bone marrow or blood (6). Transformation is often associated with karyotypic evolution (7). Patients become refractory to chemotherapy (8), and survival for patients in ACC/BC is on the order of only 3–12 months (6).

Conventional treatment of CML with chemotherapeutic agents normalizes the WBC count of patients but indiscriminately kills both the leukemia clone and normal cells (9); the chemotherapeutic agents are often considered palliative because they do not delay the onset of BC (10). Treatment with IFN-α, on the other hand, not only reduces the WBC count but also reduces the neoplastic, Ph+ cells (11, 12). Possible mechanisms of action by IFN-α include the reduction of tumor burden (11, 12), through the down-regulation of BCR/ABL mRNA and P210 (BCR/ABL) protein (13), regulation of tumor necrosis factor-related apoptosis-inducing ligand-mediated T-cell cytotoxicity (14), suppression of the chemotactic protein IL-8 (15), induction of the inflammatory factors IL-1 receptor antagonist (16) and soluble receptor p55 of tumor necrosis factor (17), and the regulation of the cytokine network by suppressing Th2-like cytokine responses (18) that are capable of suppressing normal T-cell proliferation (19).

Although IFN-α is known to inhibit the growth of bone marrow progenitors and to restore adherence properties to

Received 12/19/99; revised 2/11/00; accepted 2/11/00.

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2 The abbreviations used are: CML, chronic myelogenous leukemia; ACC, accelerated (phase of CML); BC, blast crisis; IL, interleukin; CCR, complete cytogenetic remission; Late-CCR, long-term remissions; Early-CCR, early remissions; PMA, phorbol 12-myristate 13-acetate; PE, phycoerythrin; TNF, tumor necrosis factor.

Th1 Cytokine Synthesis by CML Patients in Remission

stoma in CML (20, 21), little is known of the effect of IFN-α therapy on the synthesis of Th1 and Th2 cytokines by T cells. Because Th1 and Th2 cytokines are important mediators of the host cellular immunity (22), the contribution of individual T-cell subsets with respect to cytokine synthesis warrants investigation. A shift from a Th1 to a Th2 cytokine profile is widely accepted as a prognosticator of disease progression in individuals with neoplasm (23, 24) or infection (25–28).

We expected to find a Th2 cytokine profile in CML patients in Chronic and ACC/BC phases of disease and a Th1 cytokine profile of patients in complete cytogenetic remission after treatment with IFN-α. Furthermore, we hypothesized that CCR patients would have a Th1 cytokine profile comparable with normal controls. To test this hypothesis, we used a method of flow cytometry that permitted the evaluation of Th1 and Th2 cytokine subsets with respect to cytokine synthesis warrants investigation. A shift from a Th1 to a Th2 cytokine profile is widely accepted as a prognosticator of disease progression in individuals with neoplasm (23, 24) or infection (25–28).

Table 1 WBC counts and percentages of lymphocytes of CML patients and normal controls

<table>
<thead>
<tr>
<th>Study group</th>
<th>No. per group</th>
<th>WBC × 10^3/μl</th>
<th>% lymphocytes</th>
<th>Lymphocytes count/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic</td>
<td>15</td>
<td>25.8±</td>
<td>10.6±</td>
<td>2434±</td>
</tr>
<tr>
<td>ACC/BC</td>
<td>11</td>
<td>7.1</td>
<td>11.3±</td>
<td>868±</td>
</tr>
<tr>
<td>Early-CCR</td>
<td>40</td>
<td>5.5±</td>
<td>27.4±</td>
<td>1380±</td>
</tr>
<tr>
<td>Late-CCR</td>
<td>15</td>
<td>6.8</td>
<td>29.4±</td>
<td>1795±</td>
</tr>
<tr>
<td>Controls</td>
<td>21</td>
<td>6.2</td>
<td>30.9±</td>
<td>1871±</td>
</tr>
</tbody>
</table>

Data are presented as median and ranges for each parameter. Statistical analyses were among groups and between groups: a Chronic versus ACC/BC, P <0.01; b Chronic, ACC/BC versus Early-CCR, Late-CCR, and Normal Control, P < 0.001; c Chronic versus other groups, P < 0.01; d ACC/BC versus Early-CCR, Late-CCR, and Normal Control, P < 0.01; e Early-CCR versus Normal Control, P = 0.02; f Early-CCR versus Late-CCR and Normal, P < 0.01.

Study Population

The study population consisted of 81 CML patients who were assigned to a disease stage based on cytogenetic analysis of bone marrow and clinical assessment (30). The clinical stages of the CML patients included 15 patients in the Chronic phase (Chronic), 11 in accelerated or blast crisis phases (ACC/BC), 40 in Early-CCR, and 15 in Late-CCR (Table 1).

Intracellular Cytokine Synthesis by T-Cell Subsets after Activation with PMA

Each subject provided 5 ml of blood in EDTA (Vacutainer; Becton Dickinson, San Jose, CA) for a complete blood count, leukocyte differential analysis, and determination of lymphocyte phenotypes by flow cytometry. Another 10 ml of blood in heparin was obtained for cytokine studies. Informed consent was obtained from each subject with approval for the study from the Human Experimentation Committee of the University of Texas M. D. Anderson Cancer Center.

Activation of T Cells by PMA. Blood samples from patients with a WBC count >15 × 10^3/μl were diluted to 15 × 10^3/μl to bring the WBC to within normal range. Intracellular cytokine synthesis was conducted in three stages as described previously (24). Briefly, 25 ng of PMA were added to 1 ml of whole blood, and the mixture was incubated for 4 h at 37°C in the presence of 1 μg of ionomycin and 10 μg of brefeldin-A [a nontoxic but potent inhibitor of intracellular transport (31)]. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Staining of the Activated T Cells. The PMA-activated cells were stained for the presence of cytoplasmic cytokines as described previously (24) using a panel of cytokine-specific monoclonal antibodies conjugated with PE to detect one of the following cytokines: IL-2, IL-10, IFN-γ, and TNF-α. Additionally, samples were reacted with anti-CD8 monoclonal antibody conjugated with FITC and anti-CD3 monoclonal antibody to delineate CD8+ and CD8– (or CD4+) T cells. The percentage of cells that expressed the early activation antigen, CD69 (32), was indicative of lymphocyte activation by PMA. All monoclonal antibody reagents except for IL-10 were purchased from Becton Dickinson Immunocytometry Systems, Inc. (Mountain View, CA); anti-IL-10 conjugated with PE was purchased from Caltag (Burlingame, CA). Stained cell preparations were fixed.
Determination of Cytokines in the Cytoplasm of Activated T Cells by Flow Cytometry

Cell preparations of PMA-activated cells were analyzed with the FACSCalibur flow cytometer (Becton Dickinson) using the CellQuest software. Briefly, 10,000 events were collected based on on scatter and anti-CD3 reactivity. Immunoglobulin isotype controls were used to verify the staining specificity of the anti-cytokine reagents and to set markers delineating positive and negative populations. Cytokine synthesis by the two T-cell subsets (CD3+ and CD3-) were measured based on the reactivity of the lymphocytes with anti-CD8. Anti-CD69-PE was used to identify activated T cells after exposure to PMA.

Statistical Analysis

The mean ± SE percentages of T-cell subsets synthesizing the cytokines of interest were obtained for each sample. Data are presented as histograms of the mean ± SE percentages of CD8+ and CD4+ T cells/µl synthesizing a cytokine. Statistical differences between study groups with respect to the percentages of T cells synthesizing cytokines were determined by the Mann-Whitney test.

RESULTS

Intracellular Cytokine Synthesis by T-Cell Subsets. Eighty-one CML patients and 21 normal controls were recruited for study. The breakdown of the medians and ranges of the WBC count, percentage of lymphocytes, and absolute count of lymphocytes of each of the groups are shown in Table 1. Negligible percentages of resting T cells from all groups synthesized cytokines, and significantly higher percentages of T cells were found to synthesize cytokines after activation with PMA.

Intracellular Synthesis of IL-2 by PMA-activated T Cells. Mean ± SE percentage of CD8+ T cells synthesizing IL-2 in response to stimulation with PMA is shown in Fig. 1a. The mean percentages (± SE) of CD8+ T cells producing IL-2 from normal controls (13.7% ± 1.5%) and CML patients in Early-CCR (11.7% ± 1.5%) or Late-CCR (14.0% ± 2.5%) were similar. However, there was a significant difference in the mean percentages of CD8+ T cells synthesizing IL-2 from normal controls and those of patients in Chronic (3.6% ± 1.3%) and in ACC/BC (4.7% ± 2.3%) phases (P < 0.001). Moreover, the mean percentages of IL-2-producing CD8+ T cells of patients in Chronic and in ACC/BC phases were significantly lower than those of Early-CCR and Late-CCR patients (P < 0.01).

Although patients in Late-CCR (33.2% ± 5.5%) and normal controls (47.1% ± 3.4%) had similar percentages of CD4+ T cells that produced IL-2, they were significantly higher than those of patients in Chronic (9.1% ± 3.3%, P < 0.002) and in ACC/BC phases (11.3% ± 4.8%, P < 0.005), respectively (Fig. 1b). There were no significant differences in the percentages of CD4+ T cells that produced IL-2 between Early-CCR and Late-CCR patients; however, both groups of remission patients had a greater percentage of CD4+ T cells that synthesized IL-2 than patients in Chronic and in ACC/BC phases (P < 0.005).

Intracellular Synthesis of IFN-γ by PMA-activated T Cells. The mean percentage ± SE of CD8+ T cells that synthesized IFN-γ was greatest in normal controls (44.7% ± 3.5%) and followed in descending order by patients in Late-CCR (30.5% ± 5.6%), in Early-CCR (25.0% ± 3.3%), in ACC/BC (9.6% ± 2.9%), and in Chronic phases (9.3% ± 2.9%), respectively (Fig. 2a). The percentages of CD8+ T cells that synthesized IFN-γ were significantly higher in normal controls than in remission patients (P < 0.01). However, patients in ACC/BC and in Chronic phase had significantly lower percentages of CD8+ T cells that synthesized IFN-γ compared with normal controls and both remission groups (P < 0.01).
A similar trend in IFN-γ production was seen in CD4+ T cells as with CD8+ T cells. Normal controls had the greatest mean percentage of CD4+ T cells (25.8% ± 2.2%) that synthesized IFN-γ, followed in decreasing order by the patients in Late-CCR (24.7% ± 5.2%), Early-CCR (16.2% ± 2.0%), Chronic (8.6% ± 3.3%), and ACC/BC (9.2% ± 2.6%), respectively (Fig. 2b). The percentages of CD4+ T cells from normal controls and Late-CCR patients that produced IFN-γ were similar. On the other hand, patients in Early-CCR, in ACC/BC, and in Chronic phases had significantly lower percentages of CD4+ T cells that produced IFN-γ than normal controls (P < 0.001). There was a direct correlation between IFN-γ synthesis and disease progression because patients in Chronic and in ACC/BC phases who had significantly lower percentages of CD4+ T cells that synthesized IFN-γ than patients in Early-CCR and Late-CCR (P < 0.05).

**Intracellular Synthesis of TNF-α by PMA-activated T Cells.** Similar mean ± SE percentages of CD8+ T cells synthesizing TNF-α were found in normal controls (33.6% ± 9.3%) and patients in Late-CCR (31.9% ± 6.9%) and Early-CCR (25.3% ± 3.2%; Fig. 3a). Significantly lower percentages of CD8+ T cells from patients in Chronic phase (6.8% ± 2.6%) synthesized TNF-α compared with normal controls (P < 0.004) and patients in Late-CCR (P < 0.002) and Early-CCR (P < 0.001). Similarly, a significantly lower percentage of CD8+ T cells of patients in ACC/BC phase (11.0% ± 3.6%) synthesized TNF-α compared with normal controls (P < 0.02), Early-CCR (P < 0.01), and Late-CCR patients (P < 0.05). There was no
difference in TNF-α synthesis by CD8+ T cells from patients with Chronic and ACC/BC phases.

The greatest percentage of CD4+ T cells synthesizing TNF-α was seen in normal controls (Fig. 3b). There was no statistical difference between the responses of normal controls (46.9% ± 9.9%) and those of Early-CCR (37.4% ± 3.5%) and Late-CCR (41.1% ± 6.7%) patients. In contrast, both Chronic (14.0% ± 3.9%) and ACC/BC (17.1% ± 6.6%) patients had significantly lower percentages of CD4+ T cells that synthesized TNF-α than normal controls (P < 0.02), Early-CCR (P < 0.001), and Late-CCR (P < 0.02), respectively.

Synthesis of IL-10 by PMA-activated T Cells. The means ± SE percentages of IL-10-producing CD8+ T cells in Early-CCR (9.6% ± 1.5%), in Chronic (9.2% ± 4.0%), in ACC/BC (8.0% ± 2.4%), in Late-CCR (7.9% ± 2.5%) patients, and in normal controls (4.4% ± 1.5%) were similar (Fig. 4a). By contrast, a statistically greater percentage of CD4+ T cells from patients in Early-CCR (26.9% ± 3.4%; P < 0.003), Chronic (23.1% ± 5.1%; P < 0.02), and ACC/BC (25.3% ± 5.0%; P < 0.03) phases synthesized IL-10 than CD4+ T cells of normal controls (10.8% ± 5.1%; Fig. 4b). Late-CCR (12.9% ± 3.6%) patients and normal controls had similar percentages of CD4+ T cells that synthesized IL-10. Late-CCR patients also had a significantly smaller percentage of CD4+ T cells that synthesized IL-10 than Early-CCR (P < 0.002) and ACC/BC (P < 0.02) phase patients.

DISCUSSION

CML patients in Chronic and in ACC/BC phases had significantly lower percentages of peripheral lymphocytes and significantly higher percentages of myeloid cells compared with patients in Early-CCR and Late-CCR. Compared with normal donors, there were normalizations of the WBC count (6.2 × 10⁹/µl versus 6.2 × 10³/µl), percentage of lymphocytes (30.9% versus 29.4%), and lymphocyte counts (1871/µl versus 1795/µl) in Late-CCR patients treated with IFN-α. These observations are consistent with an earlier report from this institution (33). The low percentage of peripheral T cells in Chronic and in ACC/BC patients deterred earlier evaluation of Th1 and Th2 cytokine production by these cells. Recent technological advancements make it possible to measure cytokine synthesis on a single-cell basis in whole-blood cultures without the requisite of isolating mononuclear cells (34). This experimental approach permitted the measurement of Th1 and Th2 cytokine production by T-cell subsets of CML patients in Chronic, ACC/BC, and CCR phases with IFN-α therapy.

In preliminary studies, we were unable to detect constitutive production of Th1 and Th2 cytokines in the cytoplasm of resting T cells cultured for 16 h (data not shown). Upon short-term activation with PMA, the percentages of CD4+ and CD8+ T cells from Chronic and ACC/BC patients synthesizing the Th1 cytokines, IL-2 and IFN-γ, were significantly lower than the percentages of CD4+ and CD8+ T cells from patients in Early-CCR and in Late-CCR and normal controls. The lower percentage of Th1 cytokine-producing T cells of Chronic and ACC/BC patients was not unexpected because these CML patients had circulating leukaemia cells that are known to preferentially secrete the Th2 cytokine IL-10 (19). IL-10 can suppress the production of IL-2 and IFN-γ (35, 36) and can affect the quality and magnitude of the host immune response. Possible mechanisms by which IL-10 mediates suppression of the immune response include the inhibition of MHC class II-dependent antigen presentation (37), T-cell proliferation, and IL-2 production (36, 38). Among the lymphocytes evaluated, IL-10 was primarily produced by the CD4+ T cells of Chronic, ACC/BC, and Early-CCR patients, whereas the production of IL-10 in Late-CCR patients and normal controls by CD4+ T cells was negligible. These results strengthen our hypothesis that the increase in Th2-type cytokines and in particular IL-10 is a direct correlate of disease progression, and a shift from a Th2 to a Th1 cytokine profile is highly suggestive of improvement in clinical status.

Because patients achieved a CCR with IFN-α therapy and
sustained the CCR without maintenance therapy for >2 years, the percentages of T cells producing Th1 cytokines increased to levels characteristic of normal controls. The synthesis of Th1 by T cells in remission was accompanied by normalization in the percentage and number of T cells. Preferential syntheses of IL-2 by CD4+ T cells and IFN-γ by CD8+ T cells are desirable for leukemia-specific CTL responses in CML patients (39) because IL-2 is needed for the proliferation of the CTLs (40), and both IFN-γ and TNF-α are essential for cytotoxic activity (41, 42). The T cells of Late-CCR patients and normal donors were equally capable of Th1 cytokine synthesis, and these data suggested restoration of normal T-cell function in Late-CCR patients. Furthermore, IFN-α can increase the frequency of IFN-γ-producing human CD4+ T cells and ablate the suppressive effect of IL-4 on IFN-γ production (43).

In the current study, significantly higher percentages of CD4+ and CD8+ T cells from patients in Early-CCR and Late-CCR and normal controls synthesized TNF-α compared with the percentages of CD4+ and CD8+ T cells from CML patients in the Chronic and in ACC/BC phases of CML. TNF-α exerts both stimulatory and inhibitory control on hematopoiesis in vitro, depending on the conditions of the cell culture, making the role of TNF-α in CML ambivalent (44–49). Because high levels of TNF-α receptors were found in leukemic cells (50), it is tempting to suggest that the higher percentage of TNF-α-producing T cells in remission CML patients may contribute to the elimination of leukemic cells. Furthermore, others have identified CD8+ T cells with Th1 cytokines, IFN-γ and TNF-α, to be the classical CTLs (51) capable of producing perforin and granzyme, molecules needed for an effective cytotoxic response. Although we have shown that the synthesis of cytokines necessary for an effective cytotoxic response are restored in T cells of CML patients in CCR, further investigation is warranted to determine whether these T cells actually elicit effective cytotoxic activities.

To appreciate the inverse relationship between the syntheses of Th1 and Th2 cytokines by T cells from CML patients, the data can be examined as a ratio of T cells producing Th1 cytokine to that of T cells producing IL-10. In active CML disease, the number of lymphocytes were significantly outnumbered by leukemic myeloid cells that are known to produce Th2 cytokines (19), thereby suppressing Th1 responses and diminishing the ability of T cells to mount an effective host defense. When patients achieved a durable CCR of >2 years without maintenance IFN-α therapy, the ratio of Th1- to Th2-producing cells was comparable with that of normal controls. The switch from a Th2 to a Th1 cytokine profile is less apparent in Early-CCR patients; however, because these patients are more a heterogeneous group of individuals with clinical responses to IFN-α of varying lengths of time, there is a higher level of residual leukemic cells (52) and a higher incidence of relapse3 compared with Late-CCR. However, when patients achieved a Late-CCR with IFN-α, the imbalance in myeloid and lymphoid cells was reversed and resulted in a reduction in myeloid cells capable of producing Th2 cytokines. Upon antigenic stimulation of Th0 cells from CCR patients, the expansion of Th1 effector cells would be favored. Thus, IFN-α can be an effective therapeutic agent against CML by suppressing Th2 cytokine responses of leukemia cells and by restoring Th1 cytokine synthesis by T cells of patients in CCR. Whether the restoration of Th1 cytokine responses in CML patients achieving CCR is unique to IFN-α therapy or can be obtained with other forms of therapy that induce CCR in CML patients awaits further investigation.

ACKNOWLEDGMENTS

We are indebted to clinical and research nurses Shela Broussard and Pura Johnston, without whose perseverance it would have been impossible to recruit the patients necessary for this study. We also acknowledge Stephen Li and De-Yu Shen for their technical expertise in conducting the immunology assays.

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