Ultra Low Dose Interleukin-2 Therapy Promotes a Type 1 Cytokine Profile In Vivo in Patients with AIDS and AIDS-associated Malignancies

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Abstract

This study was undertaken to determine if prolonged daily subcutaneous administration of ultra low dose IL-2 could influence the constitutive endogenous production of a type 1 (IFN-γ) cytokine in patients with AIDS or AIDS-associated malignancies. Using a quantitative reverse transcription PCR assay, we demonstrate that daily administration of one type 1 cytokine, IL-2, for 3 mo increases significantly the constitutive endogenous gene expression of another type 1 cytokine, IFN-γ, in vivo. The predominant source of IFN-γ appears to be IL-2–expanded natural killer cells and CD8+ T cells. Moreover, PBMC obtained from these patients during IL-2 therapy showed normalization of a profound deficit in IFN-γ protein production after stimulation with extracts from infectious agents in vitro. Our data suggest that prolonged exogenous administration of a type 1 cytokine in a nontoxic fashion to patients with AIDS and AIDS-associated malignancies can enhance significantly the endogenous type 1 cytokine profile in vivo. Consequently, ultra low dose IL-2 therapy has the potential to improve the immunodeficient hosts’ immune response to infectious pathogens that require IFN-γ for clearance. (J. Clin. Invest. 1998. 101: 1373–1378.) Key words: interleukin-2 • interferon-γ • AIDS • immunotherapy • natural immunity

Introduction

The clinical progression of HIV infection is thought to result in part from dysregulation of cytokine production. The gradual decline of the type 1 cytokine profile (i.e., IL-2 and IFN-γ) has been attributed to the impaired production of IL-2 and IFN-γ by HIV-infected CD4+ T cells and impaired IL-12 production by monocytes (1–4). IFN-γ is critical for effective control and clearance of many obligate intracellular pathogens that infect HIV-positive patients, such as Mycobacterium species and toxoplasma gondii (5–8). We and others have shown that IL-2 can greatly potentiate IFN-γ production in vitro (9, 10). Therefore, exogenous administration of a type 1 cytokine to HIV-infected patients has been proposed to potentially improve the type 1 cytokine deficiency in vivo, and to restore some degree of immune competence which may in turn impede the increased incidence of opportunistic infections and virally associated malignancies in these patients (11–13). Indeed, in a phase I study of prolonged ultra low dose IL-2 therapy (0.45–1.2 × 10^6 IU/m^2/d) given to patients with AIDS-associated malignancies, no opportunistic infections were observed during 50 mo of cumulative therapy, despite 70% of patients having CD4+ counts < 100/mm^3 (14). A phase II study administering a daily subcutaneous injection of ultra low dose IL-2 (1.2 × 10^6 IU/m^2/d) to similar patients with AIDS or AIDS-associated malignancies for 90 consecutive days was completed recently, again, without any new opportunistic infections during > 50 mo of cumulative therapy (15).

Therefore, we hypothesized that ultra low dose IL-2 therapy may be enhancing IFN-γ production in vivo. We evaluated type 1 and 2 cytokine gene expression in unfractionated PBMC, and in enriched PBMC subsets taken directly from patients’ blood before, during, and after receiving a 3-mo course of daily IL-2 in the phase II study (15). We also evaluated cytokine protein production in an in vitro assay using extracts from infectious agents. Our results showed that ultra low dose IL-2 therapy induces a significant increase in the constitutive endogenous IFN-γ gene expression without a significant change in IL-10 gene expression in vivo. Normalization of a profound deficit in IFN-γ protein production in response to infectious stimuli was also demonstrated in vitro.

Methods

Patients. Laboratory studies were performed on PBMC and enriched subsets of PBMC obtained from five patients with AIDS and three patients with AIDS-associated malignancies (two with lymphoma and one with Kaposi’s sarcoma). Patients with AIDS or AIDS-associated malignancies were selected randomly for this study from a group of HIV+ patients receiving ultra low dose IL-2 therapy (see Table I). Antiretroviral medications were not changed before or during therapy with IL-2. Each patient received a single daily subcutaneous injection of IL-2 (specific activity 1.8 × 10^6 IU/mg; Chiron Corp., Emeryville, CA) at a dose of 1.2 × 10^6 U/m^2/d for 90 consecutive days (15). Before participation in this study, each patient signed an informed consent approved by the Institutional Review Board at the Roswell Park Cancer Institute. Normal donor PBMC were obtained from buffy coats prepared by the American Red Cross (Buffalo, NY).

Isolation of PBMC and enrichment for cellular subsets. PBMC were obtained from patients as fresh blood before the initiation of IL-2 therapy, during IL-2 therapy between weeks 4 and 12, and 1 wk after the discontinuation of therapy. For isolation of total RNA, fresh
PBMC were separated by Ficoll density gradient centrifugation, washed, placed into RNAzol (Tel-Test, Inc., Friendswood, TX), and frozen at -70°C until all samples on a patient were collected. Enrichment for monocytes was performed by placing fresh PBMC in tissue culture flasks coated with 2% gelatin (E. Merck Science, Gibbstown, NJ) for 30 min. Nonadherent cells were removed by washing the plates four times with prewarmed medium, and monocytes were subsequently removed by washing with cold 15 mM EDTA (Sigma Chemical Co., St. Louis, MO) for 10 min. Nonadherent cells were enriched for T cell (CD4+ or CD8+) and natural killer (NK) cell (CD56+) subsets with mAb-coated immunomagnetic beads (MACS; Miltenyi Biotech Inc., Auburn, CA) following the manufacturer's recommendations. After enrichment, the purity of each fraction was ≥ 90%, as determined by flow cytometric analysis.

Flow cytometric analysis of blood. Three-color phenotypic analysis of leukocytes using directly conjugated mAbs was performed as described (16). The directly conjugated mAbs used were CD3-FITC, CD4-PE, CD8-TRI; and CD16-FITC, CD56-PE, and CD3-TRI (Becton Dickinson, San Jose, CA).

Quantitative-competitive reverse transcription (QC-RT) PCR to assess cytokine gene transcripts. The QC-RT-PCR method used to quantitate type 1 and 2 cytokine gene transcripts has been described by Reiner et al. (17) and was optimized for our purposes. The basic principle lies in the use of multiple competitive (C) cDNA templates with a small spacer (~ 100 bp DNA) spliced into the competitive amplon to allow for size discrimination from the wild-type (WT) cDNA amplon by agarose gel electrophoresis. The cDNA construct contains templates for IFN-γ, IL-2, -4, -5, -10, and -12, TNF-α, TGF-β, IL-13, and the housekeeping gene HPRT within the pGEM1Zf(+) vector (17).

QC-RT-PCR used in this study provides a method to compare relative amounts of cytokine transcripts between two or more experimental conditions, e.g., before, during, and after IL-2 therapy. 6–10 serial tubes containing known but differing amounts of cDNA for a gene of interest are each mixed and then amplified with a fixed amount of patient cDNA obtained from a single time point, presumably containing the WTcDNA of interest. The PCR that amplifies an equal amount of cDNA and WTcDNA produces doublet bands of equal intensity on an agarose gel. This point of equivalence between the WTcDNA and a known amount of cDNA template allows quantitation of WTcDNA relative to another experimental sample.

Adjustments for differences in WTcDNA loading between experimental samples from different time points or cell types were made by performing serial QC-RT-PCR on identical samples for the housekeeping gene HPRT, as described (17).

Total cellular RNA was isolated from ~ 106 cells, and WTcDNA was generated by RT of 0.5–2 µg of cellular RNA in the presence of random hexamer primers (Clontech, Palo Alto, CA) following the manufacturer's recommendations. Primers specific for the gene of interest (i.e., HPRT, IFN-γ, IL-12, -10, or -13) were added (20 µM), followed by 5 µl of 10× PCR buffer (15 mM MgCl2, 100 mM Tris, pH 9.0, 500 mM KCl, 1% Triton X-100; Perkin-Elmer Corp., Foster City, CA), dNTPs (20 mM; Pharmacia Biotech AB, Uppsala, Sweden), and 0.5 µl of Tag polymerase (Perkin-Elmer Corp.). Total mixtures of 50 µl were placed in a thermocycler (Perkin-Elmer Corp.) and allowed to amplify for 35 cycles. Cycling conditions and primer sequences used have been published (17). After amplification, products were electrophoresed on a 3% agarose gel. The gel images were then analyzed and quantified for equivalence as described above using a computerized densitometer (BioMax: Eastman Kodak Co., New Haven, CT).

For measurement of IFN-γ and IL-10 gene expression in PBMC, four time points were analyzed in seven patients (i.e., pre–IL-2 therapy, day +60, day +90, and 1 wk post–IL-2 therapy), while one patient (UPN [unique patient number] 4) had only three evaluable time points (i.e., no day +60 sample). Enriched CD3+CD4+ T cells, CD3+CD8+ T cells, CD3+CD56+ NK cells, and monocytes obtained at day +60 of IL-2 therapy were analyzed for IFN-γ and IL-10 in three patients. Enriched monocytes from five of the patients were also analyzed for IL-12 gene expression at day +60 and again at day +90 of IL-2 therapy. Pre- and post–IL-2 therapy samples were not available for enrichment studies.

Quality control of QC-RT-PCR reaction. A single large-scale plasmid preparation of the polycotester construct was performed. The CcDNAs within the polycotester were confirmed to be of correct size, and were cloned randomly and sequenced for verification. Before analyzing patient samples, positive controls for cytokine gene expression (IFN-γ, IL-10, -12, and -13) were added (20 µg of plasmid DNA/l reaction) to ensure that the CcDNA polycotester would reliably quantitate changes in gene transcripts. For analysis of IFN-γ gene expression, we used the NK-92 cell line (18, 19). For analysis of IL-10, a mixture of cells containing an increasing number of EBV+ lymphoblastoid cells was used. For analysis of IL-13 gene expression, resting and activated T cells were used, and for analysis of IL-12 gene expression, resting and LPS-activated monocytes were used.

In vitro assessment of IFN-γ protein production by stimulated PBMC. Normal donor or frozen PBMC obtained before and during IL-2 therapy were thawed simultaneously (viability ≥ 95%) at 37°C, plated at 107/ml in culture medium (RPMI 1640) supplemented with

**Table I. Clinical Profile of Study Patients**

<table>
<thead>
<tr>
<th>Patients</th>
<th>CD4 count</th>
<th>Absolute NK count</th>
<th>Viral load</th>
<th>Antiviral drugs</th>
<th>HIV-related symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells/mm³</td>
<td>cells/mm³</td>
<td>copies/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UPN 1</td>
<td>593.6</td>
<td>106.3</td>
<td>25</td>
<td>Zidovudine, Lamivudine, Saquinavir</td>
<td>Fatigue, nasal congestion, myalgia, arthralgia</td>
</tr>
<tr>
<td>UPN 2</td>
<td>19.6</td>
<td>126.7</td>
<td>5000</td>
<td>Stavudine</td>
<td>Fatigue, folliculitis</td>
</tr>
<tr>
<td>UPN 3</td>
<td>163</td>
<td>186.4</td>
<td>500</td>
<td>Zidovudine, Didanosine</td>
<td>Arthralgia, myalgia</td>
</tr>
<tr>
<td>UPN 4</td>
<td>57</td>
<td>97.1</td>
<td>500000</td>
<td>Zidovudine, Stavudine</td>
<td>Fatigue, arthralgia, memory loss</td>
</tr>
<tr>
<td>UPN 5</td>
<td>183</td>
<td>382.7</td>
<td>25</td>
<td>Zidovudine, Zalcitabine, Delavirdine</td>
<td>Myalgias</td>
</tr>
<tr>
<td>UPN 6</td>
<td>85.3</td>
<td>88.7</td>
<td>500000</td>
<td>Zidovudine, Zalcitabine</td>
<td>Fatigue, myalgia</td>
</tr>
<tr>
<td>UPN 7</td>
<td>448.2</td>
<td>122.7</td>
<td>5000</td>
<td>Stavudine</td>
<td>Nasal congestion</td>
</tr>
<tr>
<td>UPN 8</td>
<td>343.2</td>
<td>77.6</td>
<td>500</td>
<td>Zidovudine, Lamivudine, Saquinavir</td>
<td>Fatigue</td>
</tr>
</tbody>
</table>

*Results expressed as particle-associated HIV-1 gag RNA copies per milliliter of plasma.

1. Abbreviations used in this paper: C, competitive; NK, natural killer; QC-RT, quantitative-competitive reverse transcription; STAg, soluble tachyzoite antigen; UPN, unique patient number; WT, wild-type.
10% human AB serum [C-Six Diagnostics, Mequon, WI] and antibiotics, and stimulated with either 5 μg/ml LPS purified from Escherichia coli (Sigma Chemical Co.), 5 μg/ml of a soluble tachyzoite antigen (STAg) preparation (20), or PBS (Sigma Chemical Co.) as a control. After a 48-h incubation at 37°C in a humidified 5% CO₂ atmosphere, cell-free culture supernatants were harvested and assayed for human IFN-γ protein production by ELISA (sensitivity ~ 5 pg/ml; Endogen, Inc., Woburn, MA).

Statistical analysis. The Wilcoxon rank sum test and the Spearman rank-order correlation coefficient (rₛ) were used to determine statistical significance.

Results

Cellular profile during IL-2 therapy. Table 1 provides the clinical profile of all eight patients included in this study. Flow cytometric lymphocyte analysis of the eight patients demonstrated a significant (P = 0.016, Wilcoxon rank sum test) expansion in the absolute number of CD3⁺CD56⁺ NK cells, which peaked at the end of the daily IL-2 therapy. Thereafter, the absolute number of NK cells decreased significantly (P = 0.008), compared with either day +60 or +90 during therapy (Fig. 1). There were no appreciable changes in the number of CD4⁺ or CD8⁺ T cells. The median CD4⁺ count for the eight patients immediately before initiation of IL-2 was 173/mm³.

Quantitative changes in IFN-γ and IL-10 gene expression in PBMC. Fresh PBMC were assessed by QC-RT-PCR for changes in type 1 (IFN-γ) and type 2 (IL-10) gene transcripts before, during, and after the 3-mo daily subcutaneous injection of ultra low dose IL-2. Representative analyses of IFN-γ and IL-10 gene expression, each with the housekeeping gene HPRT, are shown in Fig. 2. The results illustrate an increase in IFN-γ gene expression and a decrease in IL-10 gene expression during IL-2 therapy. After discontinuation of IL-2, IFN-γ gene expression decreased, while IL-10 gene expression remained unchanged. Changes in IFN-γ and IL-10 cytokine gene transcripts before, during, and after IL-2 therapy are summarized for the eight AIDS and AIDS-associated malignancy pa-
Overall, there was a significant increase in the mean IFN-γ gene expression from pretherapy to day +60 (\( P = 0.02 \)) that was maintained during therapy (\( PRE \) day +60), which is then maintained during therapy (\( POST \) \( P = 0.02 \)). There was a significant decline in IFN-γ expression after completion of IL-2 therapy (\( POST; P = 0.031 \)).

The mean IL-10 gene expression declines during and after IL-2 therapy, but this trend did not achieve statistical significance. Two patients who demonstrated an increase in IL-10 gene expression during IL-2 therapy showed decreases in another type 2 cytokine gene transcript (IL-13) during therapy (data not shown).

Quantitative changes in IFN-γ and IL-10 gene expression in subsets of PBMC. Despite significant increases in both NK cell number and PBMC IFN-γ gene expression, there was no significant correlation between these two parameters. Therefore, we enriched subsets of T cells, NK cells, and monocytes from three patients on day +60 of IL-2 therapy in order to determine the predominant cellular source of IFN-γ and IL-10 measured in the unfractionated PBMC. To determine if there were differences in the quantity of IFN-γ and IL-10 mRNA expressed by the T cell subsets, NK cells, and monocytes on a per cell basis, we first equilibrated the cDNA obtained from these subsets with HPRT quantitation. On a per cell basis, CD3⁺CD56⁺NK cells showed substantially more IFN-γ transcript relative to CD3⁺CD4⁺T cells (mean fold difference 47.2±20.9) and CD3⁺CD8⁺T cells (mean fold difference 19.5±9.8) in all three patients (Fig. 4 A). However, when factored for the patients’ absolute cell number within the NK and T cell subsets, NK cells proved to be the predominant source of IFN-γ in two patients, whereas the CD8⁺T cell subset was the predominant source in the third patient. The predominant source of the IL-10 gene transcript was found consistently within the monocyte subset of PBMC before and after correction for the absolute cell number within mononuclear subsets (Fig. 4 B). Overall, a 5.5-fold greater level of IL-10 transcript was detected in monocytes compared with either T cell subset. In two of the three patients, CD8⁺T cells were also found to...
Before IL-2 therapy, PBMC from each patient demonstrated a profound (i.e., > 90%) reduction in IFN-γ production in response to LPS, compared with normal healthy controls (data not shown). During IL-2 therapy, LPS-stimulated PBMC produced significantly greater IFN-γ (1.165 ± 0.63 pg/ml) compared with PBMC obtained from patients not receiving IL-2 (1.714 ± 0.416 pg/ml for LPS-stimulated PBMC from normal donors; see reference 20 for STAg-stimulated PBMC).

**Discussion**

The progression of HIV infection to AIDS is characterized by immune dysregulation, emergence of opportunistic infections, and an increased incidence of certain malignancies. Impaired production of immunoregulatory cytokines such as IFN-γ, IL-2, and IL-12, which normally participate in the cell-mediated immune response, has been implicated in this enhanced susceptibility to infection and cancer (22, 23). Here, we demonstrate that exogenous administration of a type 1 cytokine, IL-2, increases the constitutive endogenous gene expression of IFN-γ in PBMC of AIDS or AIDS-associated malignancy patients with a median CD4 count of 173/mm³. The significant decline in IFN-γ gene expression shortly after the discontinuation of IL-2 supports our contention that the measured changes were indeed mediated by the prolonged ultra low dose IL-2 administration. Our direct assessment of cytokine gene expression in whole PBMC before and during in vivo cytokine therapy may provide a more complete picture of autocrine, paracrine, and endocrine immune regulation that occurs in vivo, compared with in vitro analyses of isolated T cell clones, as suggested by others (1).

The mechanism whereby the daily ultra low dose exogenous administration of IL-2 is able to increase the constitutive endogenous IFN-γ gene expression in PBMC is unknown. A limited analysis in a subset of patients suggests that the NK cell is one source of IFN-γ, but remaining CD8⁺ T cells also appear to contribute. Daily ultra low dose IL-2 therapy increases significantly the absolute number of NK cells in vivo, and IL-2 can increase IL-12 receptor expression on NK cells (24). The latter effect increases the cells’ sensitivity to IL-12, which in turn can induce NK cell IFN-γ production. Further, IL-12–mediated IFN-γ gene expression in NK cells is increased drastically with low amounts of exogenous IL-2 (9). IFN-γ in turn can increase monocyte/macrophage IL-12 gene expression (25, 26) and decrease IL-10 expression (27). Consistent with this, we observed an increase in IL-12 gene expression within enriched monocytes during IL-2 therapy (data not shown). However, we did not have pre- and posttherapy samples available to be certain the effect resulted from therapy. While this mechanism for the IL-2–induced in vivo increase in IFN-γ gene expression is possible, a direct effect of ultra low dose IL-2 on monocyte/macrophage gene expression, or participation by other factors and cells, cannot be excluded.

We postulated that PBMC with increased IFN-γ gene expression and increased numbers of NK cells, as found in our patients’ PBMC during IL-2 therapy, might show some restoration of IFN-γ protein production in response to infectious insult. Indeed, in the absence of any exogenous cytokines, PBMC obtained from these patients during IL-2 therapy showed significant enhancement of IFN-γ protein production after stimulation with either LPS or STAg, compared with the

![Figure 5](image)

*Figure 5.* IFN-γ protein production after in vitro LPS and STAg stimulation of patient PBMC obtained before and during IL-2 therapy. (A) The amount of LPS-stimulated IFN-γ protein produced by patient PBMC obtained during IL-2 therapy was significantly greater than pretherapy levels in the seven patients evaluated (P = 0.016). (B) The amount of STAg-stimulated IFN-γ protein produced by patient PBMC obtained during IL-2 therapy was significantly greater than pretherapy levels in the six patients evaluated (P = 0.03). Results represent the mean of duplicate measurements (pg/ml) for each patient sample ± SD. No cytokines were added to the in vitro cultures.
same patients’ PBMC before IL-2 therapy. Further, the in vitro restoration of IFN-γ production in these patients’ PBMC in response to the bacterial and protozoan extracts was comparable to that seen in PBMC from healthy controls.

Whereas the reservoir of IFN-γ released in response to infectious challenge was increased, spontaneous production of IFN-γ protein during prolonged in vivo therapy with ultra low dose IL-2 was not measurable in serum (data not shown). This tightly regulated production of IFN-γ is likely to be responsible, at least in part, for limited clinical toxicity of the IL-2 regimen itself. In theory, the advantage of administering IL-2 alone is that IFN-γ would be released from T cells and, perhaps more importantly, the expanded NK cell population only after infectious insult. This would eliminate the need for constant exogenous provision of IFN-γ, a relatively toxic proinflammatory cytokine. T lymphocytes and NK cells have overlapping and often compensatory roles in host resistance. Mice lacking functional CD8+ T cells resist infection with T. gondii by a compensatory increase in NK cells, which produce large amounts of IFN-γ (28). In HIV infection, where the declining CD4+ increase in NK cells, which produce large amounts of IFN-γ tightly regulated production of IFN-γ-dose IL-2 was not measurable in serum (data not shown). This blunted exogenous provision of IFN-γ, a relatively toxic proinflammatory cytokine.

Acknowledgments

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References

19. Gong, J.H., G. Maki, and H.G. Kлинегман. 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia (Basingstoke). 8:562–569. Interferon-γ is likely to be responsible, at least in part, for limited clinical toxicity of the IL-2 regimen itself. In theory, the advantage of administering IL-2 alone is that IFN-γ would be released from T cells and, perhaps more importantly, the expanded NK cell population only after infectious insult. This would eliminate the need for constant exogenous provision of IFN-γ, a relatively toxic proinflammatory cytokine. T lymphocytes and NK cells have overlapping and often compensatory roles in host resistance. Mice lacking functional CD8+ T cells resist infection with T. gondii by a compensatory increase in NK cells, which produce large amounts of IFN-γ (28). In HIV infection, where the declining CD4+ population exhibits anergy and significant restriction of T cell receptor Vβ repertoire (29, 30), as well as activation-induced apoptosis (31, 32), NK cells may have the potential to function as an important T cell-independent alternative for the hosts’ antimicrobial resistance through their capacity to provide IFN-γ. IFN-γ confers protection against a variety of nonviral opportunistic pathogens that regularly infect patients with AIDS (33). Our data suggest that daily subcutaneous administration of a type 1 cytokine in a nontoxic fashion for several months to patients with AIDS can restore in part the type 1 cytokine profile, and may therefore provide a targeted approach to improve the hosts’ response to infectious insult.