In Vivo Cytokine Profiles in Patients with Kala-azar

Marked Elevation of Both Interleukin-10 and Interferon-gamma

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Abstract

The immunological mechanisms underlying the susceptibility to disseminated visceral parasitism of mononuclear phagocytes in patients with kala-azar remain undefined. Resistance and susceptibility are correlated with distinct patterns of cytokine production in murine models of disseminated leishmanial disease. To assess lesional cytokine profiles in patients with kala-azar, bone marrow aspirates were analyzed using a quantitative reverse transcriptase PCR technique to amplify specific mRNA sequences of multiple Th1-, Th2-, and/or macrophage-associated cytokines. Transcript levels of IL-10 as well as IFN-γ were significantly elevated in patients with active visceral leishmaniasis; IL-10 levels decreased markedly with resolution of disease. These findings suggest that IL-10, a potent, pleiotropic suppressor of all known microbicidal effector functions of macrophages, may contribute to the pathogenesis of kala-azar by inhibiting the cytokine-mediated activation of host macrophages that is necessary for the control of leishmanial infection. (*J. Clin. Invest.* 1993.91:1644–1648.) Key words: cytokines • leishmaniasis • polymerase chain reaction • human • macrophage

Introduction

Infection of humans with trypanosomatid protozoa of the genus *Leishmania* causes a wide spectrum of clinical manifestations ranging from inapparent infection or localized cutaneous disease to disseminated visceral disease. The full clinical expression of the latter, kala-azar, which is due to *L. donovani*–complex organisms, is presently epidemic in foci in India and Sudan. Population mortality rates have been estimated to be ~ 10% in the ongoing epidemic in southern Sudan (1). A critical immunological feature of kala-azar is a profound antigen-specific unresponsiveness in traditional assays of T cell function (2–5). Patients with kala-azar have been shown to have negative delayed-type hypersensitivity skin tests, absent lymphocyte blastogenesis, and decreased or absent IL-2 and IFN-γ production by PBMC stimulated in vitro with parasite antigens. Because IFN-γ has been shown to be critical in the activation of macrophages to kill leishmania (6), organisms which are obligate intracellular parasites of mononuclear phagocytes in their mammalian hosts, this lack of T cell responsiveness is likely to be of pathophysiological importance.

Despite such antigen-specific unresponsiveness, there is evidence that antigen-reactive T cells are present in patients with kala-azar. After successful chemotherapy, there is restoration of antigen-specific reactivity as well as the development of immunity against reinfection (7). Additionally, visceral leishmaniasis is accompanied by high titers of antileishmanial antibodies, likely to be T cell dependent. Finally, murine models of *L. donovani*–induced visceral disease have demonstrated that T cells are required for successful chemotherapy despite a lack of detectable antigen-specific proliferative activity or lymphokine secretion (8), observations which find their counterpart in the resistance to therapy of visceral leishmaniasis in patients with coexisting HIV infection (9).

Experimental infection of inbred strains of mice with *L. major* produces a spectrum of disease mirroring that of human infection with leishmania. In these murine models, the phenotype of disease expression is associated with distinct cytokine profiles. Susceptibility to progressive infection is associated with the expansion of a subset of CD4+ cells expressing a Th2 phenotype, marked by the production of IL-4 and IL-10. In contrast, resistance to infection is associated with the expansion of a subset of CD4+ cells expressing a Th1 phenotype, producing IL-2 and IFN-γ (10–13). The ability to render susceptible mice resistant by suppression of the Th2 response with anti-IL-4 mAb, and to render resistant mice susceptible with anti-IFN-γ mAb, suggests that these cytokine profiles are causally related to the phenotype of disease expression in these models (12). The existence of a similar polarity in cytokine responses in humans infected with leishmania remains to be demonstrated.

In the present study, we assessed in vivo cytokine patterns in patients with visceral leishmaniasis using a quantitative reverse transcriptase PCR method for the amplification of cytokine mRNA. We report here that levels of mRNA for both IL-10 and IFN-γ are markedly elevated in the bone marrow of patients with active kala-azar.

Methods

Patients. After obtaining informed consent, bone marrow aspiration was performed for diagnostic reasons, or to assess response at the conclusion of therapy, on Sudanese patients with suspected kala-azar. Bone marrow samples were evaluated from (*a*) 10 patients with a parasitological diagnosis of active kala-azar, samples having being drawn before the initiation of therapy; (*b*) 6 patients just completing a successful 28-d course of pentavalent antimony for parasitologically proven kala-azar; and (*c*) 4 patients with other diagnoses in whom leishmaniasis had been excluded: schistosomiasis ($n = 1$), peritoneal tuberculosis ($n = 2$), and megaloblastic anemia ($n = 1$). Bone marrow samples were likewise evaluated from two normal volunteers in the United States.

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Table I. Demographic and Clinical Characteristics of Patients

	Active kala-azar	Treated kala-azar	Endemic control	U.S. control
Number of patients	10	6	4	2
Male	4	3	3	1
Female	6	3	1	1
Mean age (range), *yr*	22.1 (11–40)	25.8 (16–43)	22.8 (10–50)	33.5 (27–40)
Mean illness length before diagnosis (range), *wk*	13.2 (3.5–52)*	9.8 (4–26)	11 (4–18)	N/A
Number with marked weight loss before diagnosis	10	5‡	2	N/A

* Two patients presented with recurrences 5 and 11 mo, respectively, after initially successful therapy with pentavalent antimony for an earlier episode of kala-azar.

‡ All kala-azar patients with weight loss before diagnosis had reversal of same during treatment.

Demographic and clinical characteristics of patients are shown in Table I. All protocols were approved by the human subject review committees of the respective institutions involved.

Analysis of cytokine mRNA. Upon aspiration, 200 μl of bone marrow aspirate was placed directly into 5 ml of RNAzol B (Tel-Test, Friendswood, Texas) and stored at −75°C until use. RNA isolation was performed as described (14). Cytokine transcript levels were measured using a modification of a quantitative reverse transcriptase PCR technique previously described (15, 16). Briefly, 1 μg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD) in a 25-μl reaction. The reaction mixture was then diluted 1:8, and 10 μl of diluted product was used for specific amplification of cytokine mRNA using Taq DNA Polymerase (Promega Corp., Madison, WI). PCR product was separated on a 1% agarose gel and transferred to a Hybond N+ membrane (Amersham International, Amersham, UK) using standard blotting techniques. Southern transfers were subsequently probed with internal cytokine-specific oligonucleotides, and visualized using the enhanced chemiluminescence detection system (Amersham International). Autoradiographs were scanned with a 600 ZS scanner (Microtek, Torrance, CA) calibrated with a densitometric step tablet (Kodak, Rochester, NY).

PCR reaction conditions were strictly defined for each cytokine such that a log-linear relationship was obtained between the amount of specific cytokine mRNA and the signal density of the probed PCR product in the detection system, throughout the range of specific cytokine mRNA levels in the samples evaluated. The amount of cytokine-specific mRNA in a sample was determined by comparison of the signal density of the probed PCR product to that of a standard curve generated by simultaneous amplification and probing of step-wise dilutions of reverse transcriptase product from a patient sample with a high amount (as determined in initial experiments) of mRNA for the cytokine in question. The amount of cytokine-specific mRNA in a sample was thus expressed as a function (undiluted = 1, 1:2 dilution = 0.5, et cetera) of such a simultaneously generated standard curve. For reliable comparison of cytokine mRNA amounts, all samples were tested simultaneously for a given cytokine. To control for the relative amount of total mRNA transcribed in each reverse transcriptase reaction, the reverse transcription products used for the analysis of specific cytokine mRNA levels were also analyzed, by identical PCR and measurement techniques, to assess the amount of mRNA in each sample specific for the constitutively expressed housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT).[1] These data were used to derive an arithmetic correction factor, unique to each reverse transcriptase reaction, which was subsequently used to normalize the data on cytokine-specific mRNA levels for variation in amount of total mRNA transcribed in the reverse transcription step. Data on amounts of cytokine mRNA is thus expressed as a "normalized dilution." To minimize error, reverse transcription of samples was repeatedly performed with varying amounts of total RNA until the variation between samples in the amount of amplified HPRT product was minimal.

To evaluate the possible contribution of varying percentages of T cells in the bone marrow samples to the described cytokine profiles, parallel analysis of reverse transcription products to assess the amount of mRNA in each sample specific for T cell receptor (TCR)-alpha, and normalization of the data for the same, was performed as described above for HPRT.

Oligonucleotide sequences. PCR primer pairs were chosen to span at least one intron. Nucleotide sequences for sense and antisense primers, and probes, respectively, were as follows: HPRT, CGAGATGTGATGAAGGAGATGG, GGATTATACTGCCTGACCAAGG, and GCTGACCTGCTGGATTACAT; TCR-α, GAACCCTGACCCTGCCGTGTACC, TCTCGACCAGCTTGACATCACAGG, and GGACTTCAAGAGCAACAGTG; IL-2, GCATTGCACTAAGTCTTGC, ATTGCTGATTAAGTCCCTGG, and GCCACAGAACTGAAACATCTT; IL-4, ACTTTGAACAGCCTCACAGAG, GATCGTCTTTAGCCTTTCC, and CATGAGAAGGACACTCGCT; IL-5, CCACAGAAATTCCCACAAGTG, ACTCTTGCAGGTAGTCTAGG, and CACCAACTGTGCACTGAA; IL-6, GATTCCAAAGATGTAGC, GATTTTCACCAGGCAAGTCTCC, and GGATGCTTCCAATCTGGA; IL-10, CTGAGAACCAAGACCCAGACATCAAG, CAATAAGGTTTCTCAAGGGGCTGGGTC, and GCCATGAGTGAGTTTGACATC; IFN-γ, GCAGGTCATTCAGATGTAG, GACAGTTCAGCCATCACTTGG, and TGCAGAGCCAAATTGTCTCC; TGF-β, CAGAAATACAGCAACAATT-CCTGG, TTGCAGTGTGTTATCCGTGCTGTC, and GGGCTACCATGCCAACTTCTG; TNF-α, AGGCAGTCAGATCATCTTCTCG, TCTTGATGGCAGAGAGGAGG, and GTGGAGCTGAGAGATAAC; and GM-CSF, ATGTGGCTGCAGAGCCTGCTGC, CTGGCTCCCAGCAGTCAAAGGG, and GCATGTGAATGCCATCCAGG.

Statistics. Results were analyzed using the Wilcoxon two-sample two-tailed test.

Results

Elevation of IL-10 and IFN-γ mRNA levels in the bone marrow of kala-azar patients. There was a highly significant elevation of IL-10 transcript levels in patients with active kala-azar compared with kala-azar patients after therapy ($P < 0.01$), as well as with both endemic ($P < 0.01$) and normal volunteer ($P < 0.05$) control groups (Fig. 1 *A*). In both patients in whom paired samples were available, there was a sharp drop in IL-10

1. *Abbreviations used in this paper:* HPRT, hypoxanthine-guanine phosphoribosyl transferase; TCR, T cell receptor.

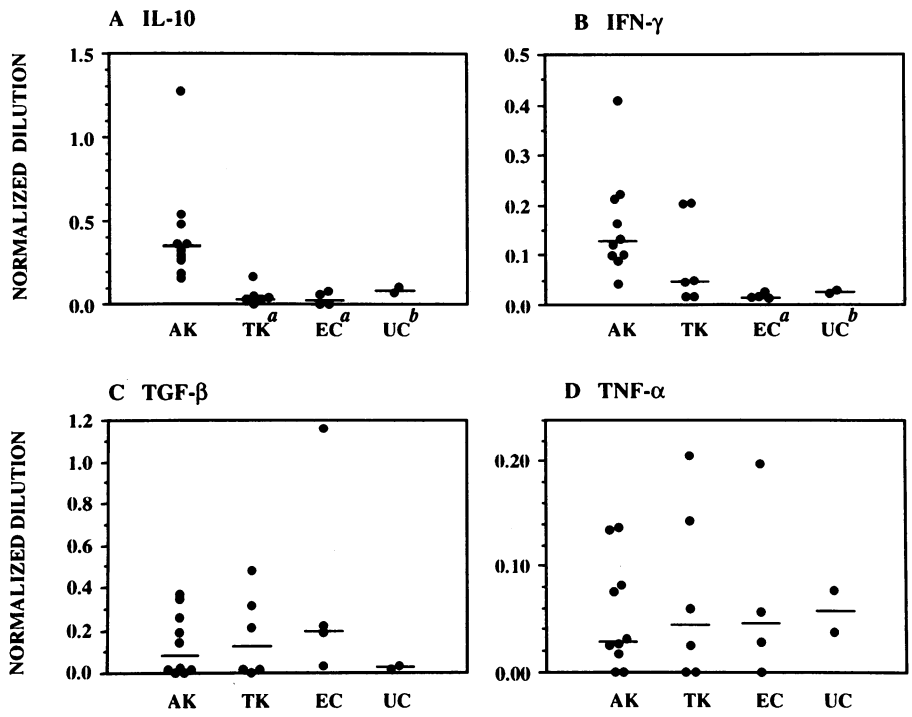

Figure 1. Levels of specific cytokine mRNA in bone marrow aspirates. Samples are from Sudanese patients with active kala-azar (*AK*), treated kala-azar (*TK*), or other endemic diseases (*EC*), and normal U. S. controls (*UC*). Each point represents the mean of at least two independent determinations, and is expressed as a function of the dilutional standard curve for a given cytokine, normalized for HPRT transcript levels. Means as indicated. (*A*) $P < 0.01$ in comparison with AK; (*B*) $P < 0.05$ in comparison with AK.

transcript levels over the course of treatment (Fig. 2 *A*). Surprisingly, IFN-γ mRNA levels were also significantly elevated in patients with active disease compared with endemic ($P < 0.01$) and U. S. ($P < 0.05$) control groups (Fig. 1 *B*). While there was a trend downwards in IFN-γ levels at the end of therapy, this did not reach significance. This downward trend was also observed in individual patients (Fig. 2 *B*).

Bone marrow mRNA levels of other Th1- and Th2-associated and macrophage-derived cytokines. No significant differences were seen between patient groups in levels of TGF-β or TNF-α mRNA (Fig. 1, *C* and *D*), although extensive posttranscriptional regulation (17, 18) renders the analysis of RNA transcripts an insensitive measure of differences in functional, secreted levels of these cytokines. There were similarly no significant differences in levels of IL-4 transcripts in the few samples in which IL-4 mRNA was demonstrable (3 out of 10 patients with active kala-azar, 2 out of 6 treated patients, 1 out of 4 endemic controls, and both U. S. controls). Levels of transcripts specific for IL-2, IL-5, IL-6, and GM-CSF were so low in all of the samples in which such transcripts were detected that standard curves could not reliably be generated, although such standard curves were readily generated using RNA isolated from in vitro stimulated human PBMC (data not shown). There were no significant differences between sample groups in the detection of transcripts for these cytokines (data not shown). Controlling for relative numbers of T cells in samples by normalizing results for the amount of mRNA in each sample specific for TCR-α, instead of HPRT, did not cause a significant change in the data for any cytokine evaluated.

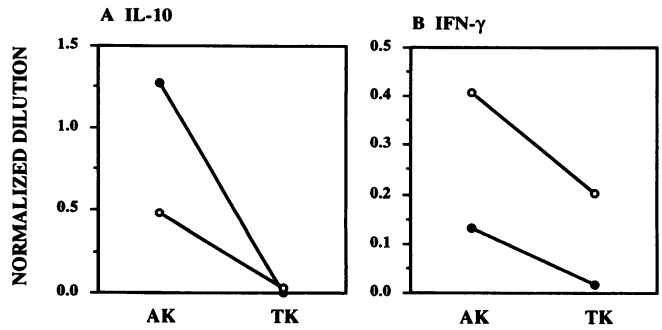

Figure 2. Cytokine transcript levels in individual patients before and after successful therapy with pentavalent antimony.

Discussion

These data clearly demonstrate the elevation of both IFN-γ and IL-10 mRNA levels in the lesional environment of the bone marrow in patients with kala-azar before therapy, findings which may be of importance in understanding how this organism is able to avoid immune-mediated destruction by its host macrophages.

IL-10 was initially characterized as a factor secreted by Th2 clones that inhibited the macrophage-dependent synthesis of cytokines by Th1 cells (19, 20). It has since been appreciated that it is produced by other cells including human macrophages (21, 22), and that it has pleiotropic effects on a wide variety of cells (23). Notably, it is a potent suppressor of all of the known microbicidal effector mechanisms of activated macrophages. IL-10 suppresses the ability of LPS- and/or IFN-γ-activated macrophages to produce inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, G-CSF, GM-CSF), reactive oxygen intermediates, and reactive nitrogen intermediates (16, 21, 24–29). Our finding of high levels of IL-10 in the face of high levels of IFN-γ in kala-azar is consistent with the hypothesis that IL-10 inhibits IFN-γ-induced activation of macrophages in this disease. Such a mechanism has previously been demonstrated in vivo in a murine model of *Trypanosoma cruzi* infec-

tion, in which a susceptible mouse strain was shown to express elevated levels of both IL-10 and IFN-γ in acute disease (30). The production of IL-10 has further been demonstrated in other murine models of parasitic disease (13, 31). To our knowledge, the present data provide the first in vivo evidence implicating IL-10 in the pathogenesis of an infectious disease in humans.

Previous failures to demonstrate elevations in IFN-γ levels in kala-azar are likely to have been due either to assay insensitivity or to the use of in vitro–stimulated PBMC for evaluation. The present study reinforces the recent demonstration of the utility of ex vivo analysis via PCR techniques of the cytokine responses within lesional tissue in human infections (32), especially using the more quantitative techniques employed in the present study.

TGF-β is another potent inhibitor of macrophage microbicidal activity (29, 33, 34) and, as such, has been shown to alter the course of murine infection with *T. cruzi* (35), *L. amazonensis,* and *L. braziliensis* (36). Whether TGF-β plays a role in kala-azar similar to the one postulated here for IL-10 is of great interest, and is not excluded by the present data as noted above. We find no evidence for a similar role for IL-4, also an inhibitor of macrophage effector mechanisms (29, 37), in the bone marrow of patients with visceral leishmaniasis. The fact that IL-10 is regulated independently of IL-4 and IL-5 in patients with active kala-azar suggests a source of this cytokine other than classically defined Th2 cells in this disease.

The lack of congruence between the cytokine profiles demonstrated here and those generated in experimental infections with *L. major* may provide further evidence of the lack of applicability of these models to disease with *L. donovani* (38). It should be noted, however, that patients with kala-azar present to medical attention weeks to months after infection, and that evaluation of cytokine patterns at this time is likely to miss the early immune-regulatory events in this disease. Furthermore a salient feature of the Th1/Th2 polarity in murine models of leishmaniasis, that disseminated disease is associated with a dysfunctional, not an absent, immune response, is replicated in the present findings. Kala-azar appears to be characterized not by the lack of cytokine production per se but by the production of counterregulatory and potentially counterprotective cytokines.

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