Interferon (IFN)-γ is a major effector T cell–derived lymphokine in host defense against *Leishmania donovani*, an intracellular protozoan that causes visceral leishmaniasis (kala-azar). In vitro, recombinant IFN-γ activates macrophages to kill the parasite [1]. In the BALB/c mouse model, IFN-γ plays a central role in controlling the growth of *L. donovani*, since administration of a monoclonal antibody against IFN-γ impairs acquired resistance [2]. Recently, a BALB/c model of persistent visceral *L. donovani* infection was developed and showed an associated Th2 response dependent on interleukin (IL)-4 and IL-10 [3]. Administration of exogenous IFN-γ also induces antileishmanial activity in this model [2]. In humans, infusion of local IFN-γ into cutaneous leishmanial lesions has been shown to promote cell-mediated immunity and effect a cure of most lesions [4].

When used as adjunctive therapy with antimony, IFN-γ may be beneficial in disease that is highly resistant to standard therapy [5, 6]. To determine if this drug alone would be adequate to enhance the intracellular antimicrobial defense and lead to a cure, we used IFN-γ as monotherapy in patients with previously untreated primary disease [7]. Untreated patients who responded to and were cured by a standard course of pentavalent antimony and patients who were not cured by antimony but responded to and were cured by amphotericin B lipid complex (ABLC) were chosen for comparison of splenic cytokine levels during treatment [8].

Splenic aspirates were used for analysis of the cytokine response to therapy by an ex vivo method. Although the amount of material is limited, an adequate number of cells is obtained to evaluate several cytokines. The immune status of the patient was assayed before, during, and at the end of treatment by analysis of the mRNA for various cytokines present in the spleen. The amount of cytokine mRNA in serial splenic aspirate samples can be semiquantitatively determined by normalizing each sample to the amount of a constitutively expressed gene [9].

Materials and Methods

**Patients.** The IFN-γ monotherapy [7] and ABLC [8] studies were open-label trials conducted at the Institute of Medical Sciences, Banaras Hindu University in Varanasi, India. Concurrent, previously untreated patients receiving standard sodium antimony gluconate (SAG) therapy were used for comparison. Patients were diagnosed by examination of splenic aspirates and treated with IFN-γ (100 μg/m² daily for 20 days), SAG (20 mg/kg daily for 30 days), or ABLC (3 mg/kg every other day for a total of 5 doses) [7, 8]. Splenic aspirates that were obtained to monitor progression of disease were used as the source of clinical material for these ex vivo studies.

**Cytokine assays.** Splenic aspirate samples were treated, and specific cytokine mRNA was analyzed as described [9]. In brief, total RNA was extracted and reverse transcribed (SuperScript RNaseH⁻; Life Technologies, Gaithersburg, MD), and an aliquot was used for specific amplification with Taq DNA polymerase (Promega, Madison, WI). Optimization of the reactions for the number of amplification cycles was predetermined for each product: hypoxanthine-guanine phosphoribosyl transferase (HPRT; the constitutive housekeeping gene), 30 cycles; IFN-γ and IL-10, 32 cycles; and IL-4, 40 cycles. Polymerase chain reaction products
were electrophoresed, transferred to Hybond-N⁺ (Amersham International, Amersham, UK), probed with an internal gene-specific primer, and detected by an enhanced chemiluminescence system (Amersham). Intensity of the bands was measured by densitometry and normalized on the basis of HPRT expression. To control for the relative amount of mRNA in each sample, we diluted the reverse transcription product if the HPRT band was above the linear range. If the HPRT signal was lower than pretreatment levels in all but 1 patient (Figure 1), then the reactions were run with the IFN-γ treated patients and then rose again to the pretreatment level despite continued exogenous IFN-γ (Figure 1A). On the other hand, the level of message for IFN-γ decreased steadily throughout treatment in most patients in the SAG and ABLC groups. The mean decreasing levels in the SAG and ABLC groups. The mean level of IFN-γ message in these latter groups was significantly lower midway through treatment and remained disease free at 6 months without relapse (table 1).

### Statistical analysis.
A Wilcoxon signed rank analysis (JMP software; SAS Institute, Cary, NC) was done in all cases to determine the significance of paired results.

### Results

#### Clinical.
Of the 9 patients, 5 improved with IFN-γ monotherapy, with relief of symptoms and reduction of the splenic aspirate parasitologic score. All 9 patients, including the 1 who was apparently cured by IFN-γ and then relapsed, were subsequently treated with the standard regimen of antimony with good clinical results and long-term cures [7]. The SAG and ABLC [8] patients showed a prompt clinical response during treatment and remained disease free at 6 months without relapse (table 1).

### Splenic aspirate cytokine message for IFN-γ.
Before treatment was started, 18 (86%) of 21 patients tested from all 3 groups had measurable amounts of IFN-γ message in their splenic aspirate (figure 1). Paired pre- and posttreatment aspirate samples were available for 18 patients. By mid-treatment, the amount of message dropped significantly in the IFN-γ-treated patients and then rose again to the pretreatment level despite continued exogenous IFN-γ (figure 1A). On the other hand, the level of message for IFN-γ decreased steadily throughout treatment in most patients in the SAG and ABLC groups. By the end of treatment, the level of IFN-γ message was lower than pretreatment levels in all but 1 patient (figure 1B, C).

### Splenic aspirate cytokine message for IL-10.
All the available samples (21 total for the 3 groups) had measurable IL-10 message before treatment (figure 1D–F). IFN-γ–treated patients had persistent or increasing levels of IL-10 mRNA midway through and after treatment, compared with steadily decreasing levels in the SAG and ABLC groups. The mean level of IL-10 message in these latter groups was significantly lower midway through (P = .03 and .008) and after (P = .008).
Figure 1. Cytokine message in serial splenic aspirates. Relative amounts of interferon (IFN-γ) mRNA signal in serial splenic aspirates during treatment with IFN-γ monotherapy (A), pentavalent antimony (B), and amphotericin B lipid complex (ABLC; C), expressed as function of dilutional standard curve and relative to mid-range positive sample. Symbols represent individual patients. Heavy line is mean (bars, ±SE). * P < .05 compared with initial value by Wilcoxon signed rank test. Relative signals for interleukin (IL)-10 (D–F) and IL-4 (G–I) are represented similarly. Nos. indicate no. of samples available for testing for each time point. SAG = sodium antimony gluconate.

and .004) treatment compared with levels in the pretreatment samples.

Splenic aspirate cytokine message for IL-4. Message for IL-4, which was detected at baseline in 10 (48%) of 21 patients, did not change significantly during the course of treatment with IFN-γ or during treatment with SAG or ABLC (figure 1G–I). Others have also found IL-4 to be unpredictable as an indicator of response to treatment [9, 10].

Discussion

Cytokine message from lesional tissue has been shown to be an indicator of the immune status in patients with kala-azar. Active disease was associated with increased levels of both IL-10 and IFN-γ mRNA in bone marrow [9] and lymphoid tissues [10] that were lower in posttreatment samples from patients treated with standard regimens. Paired pre- and posttreatment samples were available from only a few patients in prior studies, so it has been difficult to fully interpret treatment responses in vivo. The current study extends previous studies with an analysis of splenic aspirates, tissue that is most intimately involved in the immune response to this parasitic infection. This series is also the largest to date to evaluate kinetic data of lesional cytokines and provides observations in patients treated by different approaches.

Successful treatment of kala-azar is likely to depend, in part, on a change in the host immune response to the parasite. Peripheral blood mononuclear cells from patients with active disease will proliferate in response to leishmanial antigens if IL-10 is
neutralized by monoclonal antibody or if exogenous IL-12 is added during incubation [11], yet neither is available for patient use. In an attempt to augment the effector arm of the immune response in a clinical setting, we chose to treat previously untreated patients by directly stimulating macrophages in vivo with exogenous IFN-γ. The effect on the immune response was compared with standard SAG treatment in untreated patients and with ABLC in unresponsive patients.

In this trial, exogenous IFN-γ alone was not adequate to enhance intracellular antimicrobial defenses and lead to a cure in untreated kala-azar. While a partial response was seen in 5 of 9 patients, infection persisted in all. Splenic production of IFN-γ message may have been down-regulated early in the course of treatment due to the administration of the exogenous IFN-γ. The rise in IFN-γ message after continued treatment is likely to reflect the lack of resolution of disease and a return to the “untreated” immune response and to an increased response to the persistent IL-10 by a counter-regulatory mechanism. This scenario is similar to the finding that human T cell lines (cultured in the presence of IL-12 and anti-IL-4 monoclonal antibody) can simultaneously secrete both IL-10 and IFN-γ in response to recall antigen stimulation [12]. In SAG- and ABLC-treated patients, the steady decline in IFN-γ message may be an indicator of clinical improvement during curative treatment and a decrease in the overall level of inflammation.

Message for IL-10, a cytokine thought to play a major role in kala-azar [9, 10], increased despite IFN-γ treatment. This may be what blocked development of a dominant Th1 response and subsequent cure in these patients. Absence of disease resolution is therefore not due to a lack of IFN-γ but to an inhibition at some level of the effect of IFN-γ on the activation of macrophages. IL-10 has been shown to directly inhibit macrophage activation in other in vitro systems [13]. Message for IL-10 dropped quickly in both the SAG- and ABLC-treated patients, groups that eventually fully responded to therapy. Increased levels of IL-10 may thus be a central cause of persistent disease in kala-azar patients.

To our knowledge, this is the first time a large number of patients have been followed during the course of curative treatment to study lesional cytokine responses. While we were not able to determine the cell types responsible for these cytokines using this semiquantitative methodology, it is clear that the spleen represents an infected site with highly active transcription of cytokines from both arms of the T helper cell response that diminishes during treatment. Activated (HLA-DR+) T cells have been shown to be elevated in splenic aspirates from patients with active kala-azar despite antigen-specific unresponsiveness in culture [14]. Detectable serum levels of IFN-γ, IL-4, and IL-10 protein were found in most patients with active kala-azar [15]. This suggests an increase in cytokine production in response to the increased levels of transcription we have shown here, although it remains unclear whether biologically relevant levels of each of these cytokines exist at the cell surface in vivo.

The human immune response during active kala-azar must be incapable of down-regulating Th2-related cytokines, given the typical unrelenting progression of disease. A comparison of data from before and after standard curative therapy shows that kala-azar can be characterized by a generalized activation of the immune response. Pretreatment cytokine message levels showed a mixed picture from the standpoint of the T helper cell response, with no clear expression of Th2 predominance in the lesional tissue sampled. Message for IL-10 increased with IFN-γ treatment, compared to a steady decrease during curative therapy with SAG and ABLC. The increased amount of IL-10 may have blocked the differentiation and proliferation of active Th1 cells needed to overcome macrophage inactivation at the cellular level and inhibited a curative response. Further work is needed to delineate the complex interactions for these cytokines that lead to the persistence of disease in kala-azar.

References

Little empirical evidence from field-based studies exists on the relative magnitude or duration of clinical protection from *Plasmodium falciparum* malaria in infancy. A prospective study was undertaken to examine the age distribution of hospital admissions in four geographically and demographically well-defined areas with differing intensities of *P. falciparum* transmission. Where transmission was perennial, significant clinical protection from severe morbidity was observed up to the third month of life; in the seasonal transmission area, disease rates rose after the sixth month of life. Infants exposed to the highest rates of *P. falciparum* exposure demonstrated significant declines in the risks of severe malaria from 6 months of age. These data provide direct evidence for the very early acquisition of clinical immunity and for the existence of a period of clinical protection, which together may explain why, in these communities, the cumulative risk of malarial disease throughout childhood appears to decline with increasing transmission intensity.

It was widely accepted that the clinical consequences of *Plasmodium falciparum* infection are ameliorated during early infancy. However, this view derives from studies that are either small [1] or largely anecdotal [2] or that used parasitologic rather than clinical criteria [3]. Reduced parasite densities during a child’s first encounters with the parasite early in life have been repeatedly demonstrated across a wide range of endemicities in Africa [3]. There are, however, very few epidemiologic descriptions of the clinical consequences of these infections during infancy. After a recent series of prospective studies on the clinical presentation and epidemiology of severe childhood malaria in Africa, we have highlighted the potential importance of passively acquired or innate resistance to clinical disease among infants [4]. Here we reexamine the data collected during this study to define the extent of clinical protection and disease risk among infants aged 1–11 months living in communities with differing risks of parasite exposure from birth.

**Materials and Methods**

Prospective clinical surveillance data were available from four hospital settings in The Gambia and Kenya, covering 3 and 5 complete years. These surveillance protocols have been described in detail elsewhere [4]. Each pediatric ward surveillance protocol included the identification of usual residence of every post-neonatal patient admitted, age at admission, and details of the clinical presentation of the illness. Children were examined by study physicians and clinical officers who maintained clinical cover 24 h daily, 7 days a week. Malaria as the primary cause of admission was defined after the detection of *P. falciparum* infec-