Successful Therapy of Chronic, Nonhealing Murine Cutaneous Leishmaniasis with Sodium Stibogluconate and Gamma Interferon Depends on Continued Interleukin-12 Production

JIAN LI, SHAHEEN SUTTERWALA, AND JAY P. FARRELL*

Department of Pathobiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Treatment of nonhealing forms of human leishmaniasis with antimonial drugs in combination with gamma interferon (IFN- γ) may promote healing more effectively than conventional drug therapy. Although the natures of immune responses in patients prior to treatment are often unclear, it is generally assumed that such therapy also promotes a switch from a Th2-type response to a dominant Th1-type response. We have examined the efficacy of IFN- γ therapy, in combination with drug therapy, to promote healing and a Th2-to-Th1 switch in highly susceptible BALB/c mice infected with *Leishmania major*. Short-term treatment with the antileishmanial drug sodium stibogluconate failed to significantly alter the course of disease or the immune response when it was given during the third and fourth weeks of infection. IFN- γ therapy, administered over the same time period, also failed to induce cure or a Th1 dominant response. In contrast, mice treated with a combination of drug and IFN- γ therapy resolved their infections and developed Th1-type responses. However, administration of an antibody to interleukin 12 (IL-12) reversed the therapeutic effects of therapy with drug plus IFN- γ , suggesting that IFN- γ promotes cure through an IL-12-dependent mechanism. Analysis of mRNA levels within parasitized lesions suggests that drug treatment plus IFN- γ treatment, in addition to reducing parasite numbers, results in reduced levels of IL-4, IL-10, and transforming growth factor β transcripts but increased levels of transcripts of the p40 chain of IL-12 and inducible nitric oxide synthase, which catalyzes the production of nitric oxide. Together, these results suggest that such immunotherapy may promote the development of a protective Th1-type response in susceptible mice by a mechanism which involves both suppression of regulatory cytokines and enhancement of IL-12 and nitric oxide production.

Patients with leishmaniasis may exhibit a wide range of clinical disease manifestations depending upon the tissue tropism of the invading leishmanial species and the individual's immunological response to infection. In general, patients with mild or healing forms of cutaneous leishmaniasis exhibit positive delayed-type-hypersensitivity (DTH) responses and their cells produce gamma interferon (IFN- γ) in response to stimulation with parasite antigens while individuals with nonhealing diffuse cutaneous leishmaniasis or visceral leishmaniasis have negative DTH responses to leishmanial antigens and their cells may produce interleukin 4 (IL-4), in addition to IFN- γ , following in vitro stimulation (5–7, 9, 40). Successful cure of individuals with progressive forms of leishmaniasis is usually accompanied by expression of positive DTH responses, as well as other positive correlates of a protective cell-mediated immunological response (5, 16).

Experimental *Leishmania major* infections in mice have proven to be extremely valuable models for the study of immunity to cutaneous leishmaniasis. These studies have shown unequivocally that healing infections in resistant strains of mice are accompanied by the activation of Th1-type effector cells which produce the macrophage-activating cytokine IFN- γ while nonhealing infections in susceptible strains of mice such as BALB/c are accompanied by the preferential expansion of IL-4-producing Th2-type cells (13, 14, 28). Studies of murine leishmaniasis have also helped to establish the roles of IL-12 and IL-4 as the dominant cytokines which influence Th1 and

Th2 cell development, respectively, since treatment of BALB/c mice with either recombinant IL-12 or a neutralizing antibody to IL-4 will dramatically reverse the expected nonhealing-disease phenotype of an *L. major* infection (15, 27, 33). Importantly, these treatments must be administered at the beginning of infection in order to be effective, suggesting that IL-12 and IL-4 function early during an immune response to influence the differentiation of naive CD4⁺ cells into Th1 or Th2 effector cells (8, 33). IFN- γ also appears to play a role in Th1 cell development, since resistant strains of mice with disrupted IFN- γ genes (38) or mice treated with an anti-IFN- γ antibody develop progressive disease and Th2 dominant immune responses following infection with *L. major* (1, 27). However, IFN- γ treatment alone is not sufficient to promote a Th1 response and/or suppress in vivo activation of Th2 cells since even continued IFN- γ therapy has little effect on the eventual outcome of an *L. major* infection in BALB/c mice (27).

In contrast to the efficacy of IL-12 and anti-IL-4 antibody treatments given at the time of an *L. major* infection to promote resistance in BALB/c mice, it has proven more difficult to induce healing following therapy in chronically infected mice with established Th2-type responses. For example, treatment of mice with IL-12 or anti-IL-4 antibody has been shown to be ineffective in mice when the antibody was administered after the second week of infection (8, 21, 34). However, we have shown that both of these treatments will induce healing and a switch from a Th2 to a Th1 dominant response in BALB/c mice if the cytokine or anticytokine therapy is combined with treatment with an antileishmanial drug (21, 22). These results suggest that reducing the in vivo level of infection enhances the efficacy of specific therapies designed to either promote Th1 cell activation or suppress Th2 cell activation.

* Corresponding author. Mailing address: Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St., Philadelphia, PA 19104. Phone: (215) 898-8561. Fax: (215) 573-7023.

Reports on cytokine therapy of human leishmaniasis are somewhat limited; however, it has been shown that combined therapy with IFN- γ plus the antiparasite drug sodium stibogluconate may be more efficacious than drug therapy alone in promoting cure of visceral disease (12, 30–32, 36). Since IFN- γ treatment alone will not induce cure or promote a Th1 dominant response in *L. major*-infected BALB/c mice, we have examined whether therapy with a combination of IFN- γ plus sodium stibogluconate will promote healing in similar mice with established Th2-type responses. Here, we show that therapy with IFN- γ and drug combined is an effective treatment. However, this combined therapy was totally ineffective in mice treated with an antibody to IL-12, suggesting that IFN- γ treatment promotes resistance through an IL-12-dependent mechanism.

MATERIALS AND METHODS

Animals and infections. BLAB/cByJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and were used at 6 to 8 weeks of age. Mice were inoculated in one hind footpad with metacyclic promastigotes of the Friedlin strain of *L. major* selected from stationary-phase cultures with *Arachis hypogaea* agglutinin (Sigma Chemical Co., St. Louis, Mo.) as previously described (26). Lesion size was measured with a dial caliper (L. S. Starrett Co., Athol, Mass.) and expressed as the difference in thickness between the infected footpad and the contralateral footpad. The degree of infection in parasitized footpad lesions was determined by limiting dilution analysis of homogenized tissue, as described elsewhere (8).

Antibody isotype assays. Levels of total immunoglobulin E (IgE) in serum as well as parasite-specific IgG1 and IgG2a were analyzed by enzyme-linked immunosorbent assay (ELISA) as previously described (25).

ELISA and ELISPOT assays. For ELISPOT assays, single-cell suspensions were prepared from lymph nodes and serial dilutions of these cells were cultured in duplicate in 96-well tissue culture plates coated with monoclonal antibody to IFN- γ (R46A2) starting at 10^6 cells per 100 μ l in RPMI 1640 (GIBCO) containing 5% fetal bovine serum, antibiotics, and 20 μ g of soluble leishmanial antigen (SLA). After overnight incubation at 37°C, plates were washed with phosphate-buffered saline and incubated for 1 h with rabbit antiserum against IFN- γ . After being washed with phosphate-buffered saline, plates were incubated for one additional hour with alkaline phosphatase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, Pa.). Color was developed with BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) in 0.1 M 2-amino-2-methyl-1-propanol buffer (Sigma) in 0.6% SeaPlaque agarose (FMC Bio-products). Following solidification of the agarose, individual spots indicative of cytokine-producing cells were counted with a dissecting microscope. For determination of total cytokine production, spleen cells were cultured at 5×10^6 cells/ml with SLA (50 μ g/ml) for 72 h. IFN- γ levels in supernates were determined by ELISA as previously described with recombinant murine IFN- γ (a gift of Genentech, San Francisco, Calif.) as a standard (10).

Sodium stibogluconate, IFN- γ , and anti-IL-12 treatments. Sodium stibogluconate (Pentostam; Burroughs Wellcome, London, United Kingdom) containing 100 mg of pentovalent antimony per ml was a gift of Max Grogl, Walter Reed Army Institute of Research, Washington, D.C. Designated mice received a total of 10 daily intramuscular injections with 250 mg of drug per kg of body weight during weeks 3 and 4 of infection. Additional mice received a total of six intraperitoneal injections of recombinant murine IFN- γ (kindly provided by Genentech Inc.) during weeks 3 and 4 of infection at a dose of 1.5×10^4 U per injection. Mice treated with C17.8 monoclonal anti-IL-12 antibody (kindly provided by Giorgio Trinchieri, Wistar Institute, Philadelphia, Pa.) received 2 mg of antibody intraperitoneally on day 15 of infection and an additional 1 mg of antibody on day 22 of infection.

Reverse transcription-PCR. Infected mice were sacrificed at designated times after infection, and footpad tissues were collected. Footpad lesion tissues were homogenized in RNazol (Biotex Laboratories, Houston, Tex.) for preparation of total RNA. Analysis of mRNA levels in infected tissues was performed as previously described (25). Nucleotide sequences of primers and probes for IFN- γ , hypoxanthine phosphoribosyltransferase, and IL-12 p40 were as previously published (11, 21, 25, 39). Different numbers of PCR cycles were selected to give a positive signal for each product within lesion tissue and to fit on the linear portions of positive cDNA standard curves. PCR products were applied to Hybond-N⁺ membranes with a slot blot apparatus after denaturation in 0.4 M NaOH–25 mM EDTA and neutralization in 1 M Tris, pH 8.0. Membranes were then hybridized with oligonucleotide probes, and specific hybridization was detected by an enhanced-chemiluminescence system (Amersham, Arlington Heights, Ill.). Chemiluminescent signals were analyzed according to published procedures (39), and relative mRNA levels were compared between groups of infected mice.

FIG. 1. Course of *L. major* infection in BALB/c mice. Groups of mice were either untreated or treated with various combinations of sodium stibogluconate, IFN- γ , and anti-IL-12 antibody. Treated mice received 10 intramuscular injections of sodium stibogluconate (250 mg/kg) during weeks 3 and 4 of infection, either alone or in combination with 6 intraperitoneal injections of murine IFN- γ (1.5×10^4 U per injection). In addition, mice receiving both sodium stibogluconate and IFN- γ also received intraperitoneal injections of anti-IL-12 antibody on days 15 (2 mg) and 22 (1 mg) of infection. Values represent the means $\pm$ standard errors for at least five mice per group.

Statistical analysis. Data were analyzed for statistical significance by the unpaired Student's *t* test. Results were considered significant at a *P* of <0.05 .

RESULTS

Drug and/or IFN- γ treatment of infected mice. BALB/c mice were inoculated with 5×10^5 metacyclic *L. major* promastigotes into a hind footpad. Starting on day 15 of infection, groups of mice were treated for 2 weeks (weeks 3 and 4 of infection) with either sodium stibogluconate or recombinant murine IFN- γ or with a combination of the drug and IFN- γ . An additional group of mice which received both sodium stibogluconate and recombinant IFN- γ also received two injections of anti-IL-12 antibody on days 15 and 22 of infection. As can be seen in Fig. 1, the course of lesion development in mice treated with IFN- γ alone did not differ from that in control mice at any time during infection. As would be expected, sodium stibogluconate treatment suppressed lesion development in all groups of mice and lesions remained small through week 6 of infection. However, after week 6, lesion size increased rapidly in these mice, showing that short-term drug treatment did not effect a cure. In contrast to lesion size progression in the drug-treated group, lesion size in the group of mice treated with both sodium stibogluconate and IFN- γ did not progress. These mice maintained small lesions (average diameter, 0.4 mm) through at least the 12-week observation period used in this study. Anti-IL-12 treatment, however, totally reversed the positive effects of therapy with drug plus IFN- γ , and mice in the anti-IL-12-treated group developed lesions which were indistinguishable from those in the control group by week 12 of infection. Analysis of lesion parasite numbers at 12 weeks supports the course-of-infection data; lesion parasite numbers were approximately significantly lower in mice treated with both sodium stibogluconate and IFN- γ than in the other treatment and control groups, which all harbored similar numbers of parasites (Fig. 2).

FIG. 2. Lesion parasite burdens at 12 weeks of infection of the mice whose lesion size progression is shown in Fig. 1. Values are the means $\pm$ the standard errors of the $\log_{10}$ dilutions of footpad tissues positive for parasites. Each group contained four to five mice. *, $P < 0.05$ compared to other groups of mice.

Cytokine and immunoglobulin production in treated mice.

The effects of the various therapies on the immune response to infection were assessed 3 days after the last day of treatment (4 weeks) and at 10 weeks of infection when healing versus nonhealing patterns of disease had been clearly established. ELISPOT assays were used at 4 and 10 weeks of infection to determine the frequencies of IFN- γ -producing cells within popliteal lymph nodes draining the cutaneous sites of infection, while reverse transcription-PCR analysis of infected lesion tissue was used to determine cytokine transcript levels. Figure 3 shows the ELISPOT data from control and treated mice at 4 and 10 weeks of infection. Lymph nodes from mice treated with IFN- γ alone contained a higher frequency of IFN- γ -producing cells than those from infected mice which received no treatment. The frequency of IFN- γ -producing cells was significantly reduced in all mice receiving sodium stibogluconate therapy, as might be expected since the reduction in parasite numbers which accompanies drug therapy probably reduces the antigenic stimulus necessary to fully promote T-cell activation. However, among the drug-treated groups, mice which received treatment with sodium stibogluconate plus IFN- γ had the highest frequency of IFN- γ -producing cells while mice treated with anti-IL-12 antibody had low numbers of IFN- γ -producing cells, similar to those noted for untreated mice, which is consistent with fact that anti-IL-12 antibody reversed the therapeutic effects of IFN- γ therapy in drug-treated mice.

Analysis of cytokine transcript levels in parasitized lesions at week 4 of infection provided some additional insight into the effects of therapy on the local immune response. We initially compared transcript levels in footpad tissues of 4-week-infected and noninfected mice to determine the relative increases in levels of cytokine mRNA within parasitized lesions. The fold increases in transcript levels in infected footpads were as follows: 2.9-fold for IFN- γ , 5.8-fold for IL-4, 2.5-fold for IL-12 p40, 4.5-fold for IL-10, 2.5-fold for transforming growth factor β (TGF- β), and 1.6-fold for inducible nitric oxide synthase (iNOS). The relatively small increase in transcript levels measured in these assays may in part be due to the fact that parasitized tissue comprised only a portion of the total footpad tissue processed for RNA isolation. We next analyzed the effects of therapy with drug and/or IFN- γ on transcript levels in footpad lesions and compared the levels to those in infected, nontreated mice, which were assigned a value of 100%. The results are shown in Fig. 4. Transcript levels for IFN- γ , IL-4, IL-12 p40, IL-10, and TGF- β , as well as for the *L. major* small ribosomal subunit, were uniformly lower in footpad lesions of drug-treated mice than in those of control mice. In contrast, only TGF- β mRNA levels were reduced in lesions of IFN- γ -treated mice, although TGF- β mRNA levels must be inter-

preted with caution since message levels do not necessarily correlate with the production of bioactive TGF- β protein. In addition, IFN- γ treatment induced a variable increase in levels of iNOS transcripts within parasitized lesions. Administration of sodium stibogluconate and IFN- γ together, however, led to a significant increase in levels of iNOS transcripts as well as a small, but significant, increase in levels of IL-12 p40 mRNA, while IL-4, IL-10, and TGF- β transcripts were reduced to levels similar to those noted for mice which received only drug treatment. Since treatment with anti-IL-12 antibody reversed the therapeutic effects of treatment with IFN- γ plus sodium stibogluconate, we also examined how inoculation of anti-IL-12 antibody affected cytokine transcript levels within parasitized tissue. Not unexpectedly, mRNA levels for iNOS and IL-12 p40, which were increased following treatment with IFN- γ plus sodium stibogluconate, were markedly reduced following treatment with anti-IL-12. In addition, IFN- γ transcript levels within lesions were to only 20% of those noted in nontreated control mice. This observation supports the data in Fig. 3 which show that numbers of IFN-producing cells within draining lymph nodes are markedly reduced following anti-IL-12 treatment.

Analysis of responses of mice at 10 weeks of infection revealed clearer differences between healing and nonhealing groups. An increased frequency in IFN- γ -producing cells was noted only for the group of mice treated with a combination of drug and IFN- γ , which is consistent with the ability of this group of mice to control their infections (Fig. 3). The increase in IFN- γ -producing cells noted at 4 weeks of infection in mice treated with IFN- γ was not noted in this group at week 10 of infection, which is consistent with the failure of IFN- γ treatment alone to promote healing. We also examined IFN- γ production by antigen-stimulated spleen cells as well as levels of total IgE and parasite-specific IgG1 and IgG2a in serum at 12 weeks of infection. As can be seen in Fig. 5, spleen cells from both the IFN- γ -treated group and the group which received both IFN- γ and drug treatment produced significantly more IFN- γ than did cells from the nontreated control group. However, healing mice in the group treated with drug plus IFN- γ had significantly lower levels of the IL-4-dependent isotype, IgE, in serum than did mice which were treated with IFN- γ

FIG. 3. Numbers of IFN- γ -producing cells per 10^6 draining lymph node cells. Cells from representative mice described in the legend to Fig. 1 were harvested shortly following treatment (4 weeks) and at 10 weeks postinfection. ELISPOT assays were used to determine numbers of IFN- γ -producing cells. Values represent the means $\pm$ standard errors for three to five mice per group.

FIG. 4. Percent changes in amounts of mRNA within footpad lesions from mice described in the legend to Fig. 1. Gene expression was determined for three to four mice from each treatment group following termination of therapy (4 weeks). Values represent percent changes in levels of mRNA in treated mice $\pm$ standard errors relative to levels of mRNA in untreated controls, which were designated as 100%.

alone. The mice treated with both drug and IFN- γ also had low levels of parasite-specific IgG1, a Th2-associated isotype, but higher levels of parasite-specific IgG2a, an isotype dependent on IFN- γ production, suggesting a more pronounced Th1-type immune response (Fig. 6). When data on Ig isotype levels are considered with our data on IFN- γ production, it appears that only mice which healed following therapy with drug plus IFN- γ developed responses which tended toward Th1 dominance.

DISCUSSION

Using an experimental protocol similar to the one in this study, we have previously shown that treatment with sodium stibogluconate plus either recombinant IL-12 or anti-IL-4 antibody will induce a Th2-to-Th1 shift and cure in infected BALB/c mice (21, 22). In the current study, we show that treatment with IFN- γ plus sodium stibogluconate can also promote healing of an established *L. major* infection in highly susceptible BALB/c mice. As evidence for this conclusion, we note that mice receiving combined therapy had lesions which decreased in size and which contained fewer parasites than did those of control mice or mice which were treated with either the drug or IFN- γ alone. Importantly, healing mice had increased numbers of IFN- γ -producing cells in draining lymph nodes and increased production of IFN- γ by antigen-stimulated spleen cells as well as decreased levels of IgE in serum, suggesting that they developed characteristics of a protective Th1-type response. These results provide experimental support for previous observations which have shown that therapy with IFN- γ and drug may shorten the duration of treatment and lower the dose of antimony required to cure patients with nonhealing leishmanial infections as well as promote cure and a cell-mediated immune response in some patients who fail to respond to multiple courses of chemotherapy with pentavalent antimonial drugs (12, 30–32, 36).

As we have noted previously, a two-week course of sodium stibogluconate therapy alone did not induce cure of an established *L. major* infection, even though treatment did suppress lesion development and dramatically reduce levels of parasites within infected lesions (23). In this study, numbers of IFN- γ -

producing cells were significantly reduced at week 4 of infection in mice receiving drug treatment, probably as a result of a reduced level of antigenic stimulation, which accompanied the drug-mediated decrease in numbers of parasites. However, by week 10 of infection, no differences were noted between the numbers of IFN- γ -producing cells in drug-treated mice versus nontreated control mice. In addition, both drug-treated and nontreated groups had similar high levels of IgE and parasite-specific IgG1, suggesting that drug treatment had little long-term effect on the immune response.

IFN- γ treatment alone also had little effect on the course of disease or the overall phenotype of immune responses in infected mice. Treated mice did have increased numbers of IFN- γ -producing cells within draining lymph nodes immediately after therapy, and IFN- γ production by antigen-stimulated spleen cells was elevated in these mice at week 12 of infection compared to that in drug-treated or nontreated control mice.

FIG. 5. Serum IgE levels and IFN- γ production by spleen cells from treated and control mice at 12 weeks of infection. Spleen cells from the mice described in the legend to Fig. 1 were cultured in the presence of SLA for 72 h, after which culture supernates were collected and IFN- γ levels were determined by ELISA. Values represent the means $\pm$ standard errors for four to five mice. Serum IgE levels were determined for these same mice. *, $P < 0.05$ compared to untreated control mice.

FIG. 6. *Leishmania*-specific IgG1 and IgG2a levels in the sera of treated and control mice at week 12 of infection. Values are the means $\pm$ standard errors for four to five mice per group. *, $P < 0.05$ compared to untreated control mice. Optical density (OD) values for normal serum were less than 0.15 at a 1:150 dilution.

However, IFN- γ treatment failed to alter the rate of lesion development during the early weeks of infection and by weeks 10 to 12 of infection, treated mice had numbers of parasites in lesions as well as levels of parasite-specific IgG1 and IgG2a and total IgE similar to those of control mice, suggesting that IFN- γ treatment did not effectively induce a protective Th1-type response.

Although IFN- γ therapy clearly promotes healing in mice given concurrent drug therapy, the mechanism by which exogenously administered IFN- γ functions with sodium stibogluconate to induce cure is currently unclear. IFN- γ can directly activate macrophages to kill leishmanial parasites (20); however, it is unlikely that IFN- γ therapy functions simply to enhance in vivo macrophage microbicidal activity since lesion transcript levels for parasite rRNA were not dramatically altered by such treatment in this study. In addition, a previous study has shown that treatment of T-cell-deficient mice with IFN- γ does not enhance the destruction of *Leishmania donovani* in infected liver tissue (19). However, IFN- γ treatment will enhance the killing of *L. donovani* amastigotes in the livers of euthymic mice or nude mice reconstituted with either CD4⁺ or CD8⁺ T cells, suggesting that IFN- γ may alter in vivo T-cell function rather than directly induce macrophage activation (19). Our results also suggest that IFN- γ treatment functions in vivo to alter the phenotype of the T-cell response and further suggest that the effects of IFN- γ are dependent upon the activity of IL-12, since treatment of infected mice with an antibody to IL-12 inhibits the ability of treatment with IFN- γ plus drug to develop a Th1-type response and resolve their infections.

Multiple cytokines are known to have regulatory effects on both T-cell and macrophage function and may potentially interact to either enhance or suppress the response to immunotherapy. For example, IFN- γ can activate macrophages to produce increased levels of NO and can also prime macrophages for IL-12 p40 production (18, 37). IL-4 promotes CD4⁺ cells to differentiate into Th2-type effector cells and can also suppress NO and IL-12 production by macrophages exposed to IFN- γ (2, 3, 35). TGF- β and IL-10 can also suppress NO production as well as IL-12 production by macrophages (2, 35), and a previous study has suggested that TGF- β production may down-regulate iNOS production in nonhealing lesions as well

as be responsible for the apparent hyporesponsiveness of BALB/c macrophages to activation with IFN- γ and lipopolysaccharide (29). Some insight as to how therapy with IFN- γ and drug influence the production of these T-cell and macrophage products was gained by examining lesion mRNA levels shortly after the last day of treatment. As was expected, drug treatment led to reduced levels of parasite RNA transcripts, indicating a reduction in the numbers of parasites in lesions. Transcript levels for IFN- γ , IL-4, IL-10, iNOS, IL-12 p40, and TGF- β were also lower in drug-treated mice and most likely reflect an overall reduction in the number of inflammatory macrophages and T cells within lesions in which parasite numbers were markedly reduced. IFN- γ therapy alone induced a decrease in levels of TGF- β transcripts and a slight increase in levels of iNOS transcripts but had less effect on mRNA levels for IFN- γ , IL-4, IL-10, and IL-12 p40. In contrast, transcript levels for *L. major*, IL-4, IL-10, and TGF- β in lesions were reduced while those for iNOS and IL-12 p40 were significantly elevated in mice treated with both IFN- γ and sodium stibogluconate. Since IL-4, IL-10, and TGF- β can suppress both NO production and IL-12 p40 production, it is possible that a reduction in the levels of these suppressive factors following drug treatment may facilitate the capacity of IFN- γ therapy to promote both macrophage NO and IL-12 production.

The capacity of treatment with anti-IL-12 to reverse the therapeutic effects of treatment with IFN- γ plus sodium stibogluconate illustrates the role of IL-12 as the predominant cytokine mediating the development of Th1-type responses. In addition to blocking healing, treatment with anti-IL-12 antibody also reversed the increases in transcript levels of iNOS and IL-12 p40 and the decreases in mRNA levels of IL-4 and TGF- β noted for mice treated with drug plus IFN- γ . In addition, numbers of IFN- γ -producing cells were significantly reduced in mice treated with anti-IL-12 antibody. These results suggest that a positive feedback loop between IL-12 and IFN- γ production may be important to the development of resistance following therapy in mice with established infections. Indeed, we did note a small, but significant, increase in levels of IL-12 p40 transcripts within lesions of mice treated with both IFN- γ and sodium stibogluconate. It has been previously noted that treatment with an antibody to IFN- γ will block the in vivo therapeutic effects of treatment with IL-12 in infected BALB/c mice (15). Since IFN- γ has been shown to prime phagocytic cells to produce IL-12 p40 (18), it is possible that the inoculation of exogenous IFN- γ , as was done in this study, acts as a positive stimulus for enhanced IL-12 production, which, in turn, may promote both higher IFN- γ production and Th1-cell development.

We have previously shown that a decrease in levels of IL-4 and TGF- β mRNAs in lesions also accompanies successful therapy of *L. major*-infected CB6F1 mice treated with IL-12 plus antibody to IL-4 (17). Thus, it is possible that reduced levels of cytokines such as IL-4, TGF- β , and IL-10 which occur following sodium stibogluconate therapy create an environment in which IFN- γ (or IL-12) can function to promote a more vigorous Th1-type response. Drug treatment may alter the local cytokine environment by inhibiting the overall inflammatory response within infected tissues or by reducing the numbers of infected macrophages, which may be potential sources of TGF- β and IL-10 production. Drug treatment, by reducing numbers of parasites, may also function to reduce the immunogenic stimulus driving the expansion of the Th2-type cell population, which predominates during an *L. major* infection in susceptible BALB/c mice. It is also possible that reduced levels of parasites are more conducive to the eventual development of a Th1-type response as has been shown for

BALB/c mice inoculated with low numbers of *L. major* promastigotes or for infected mice subjected to long-term drug therapy (4, 24). With respect to therapy of nonhealing forms of human leishmaniasis, these results suggest that reducing levels of infection with antileishmanial drugs, combined with IFN- γ immunotherapy, might be expected to promote cure, even in situations in which a patient exhibits a highly polarized Th2-type response.

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