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Depletion of Interleukin-4 in BALB/c Mice with Established *Leishmania major* Infections Increases the Efficacy of Antimony Therapy and Promotes Th1-Like Responses

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Whereas most inbred mouse strains mount a protective Th1 helper T-cell response following infection with *Leishmania major*, an ineffective Th2 response develops in BALB/c mice, leading to the development of disseminated, ultimately fatal disease. Interleukin-4 (IL-4) production is required for the initiation of the Th2 response, though little is known about the requirements for the long-term maintenance of this response. In order to investigate the role of the expanding parasite population on the Th2 response, mice infected for 2 weeks with *L. major*, which exhibited a Th2-like cytokine profile, were treated with a leishmanicidal agent (Pentostam) and/or various doses of anti-IL-4 antibody. Untreated mice, mice treated with Pentostam alone, or mice treated with 2.5 mg of anti-IL-4 antibody given at days 13 and 21 of infection developed progressive disease. However, in 8 of 10 mice treated with this dose of anti-IL-4 antibody plus Pentostam lesion development was arrested and lesions were either controlled or eventually healed. Healing was associated with the production of high levels of gamma interferon by spleen cells, and low levels of immunoglobulin E in serum compared with levels for control animals, indicating that a Th1-like response had developed in mice receiving both treatments. Thus, depletion of IL-4 only in combination with a reduction in the parasite burden allowed the expression of a Th1 response. When the dose of anti-IL-4 antibody was increased to 5 mg per injection, all mice treated with this dose of antibody, with or without Pentostam therapy, healed. However, combined therapy with Pentostam in mice treated with this dose of antibody had an additional protective effect. As expected, a Th1 response developed in mice treated with this dose of anti-IL-4 antibody with or without combined therapy with Pentostam, whereas a Th2 response developed in control mice. Thus, a significant effect on the course of disease is noted when mice with established *L. major* infections are treated with anti-IL-4 antibody in combination with Pentostam, suggesting that the combined effect of inhibiting IL-4 and reducing the parasite burden has a dramatic effect on the development of resistance to *L. major*.

The differential expansion of functionally distinct $CD4^+$ T-cell subsets in response to *Leishmania major* infection determines the outcome of infection in murine hosts (11). Resistance is known to be dependent on the production of gamma interferon (IFN-γ), which activates macrophages to kill intracellular amastigotes (14). Most strains of mice are capable of controlling experimental infections with *L. major*, as a result of their ability to preferentially expand IFN-γ-producing Th1 cells. A different type of response develops in infected BALB/c mice, however, in which interleukin-4 (IL-4)-secreting Th2 cells rather than cells secreting IFN-γ predominate (11). A Th2 response, characterized by humoral immunity and poor cell-meditated immune responsiveness, is not effective against *L. major*, and as a result, BALB/c mice develop a nonhealing, progressive disease which is ultimately fatal.

Despite the marked susceptibility of BALB/c mice to *L. major* infection, certain prophylactic treatments can induce these mice to progress towards healing and remain resistant to reinfection. For example, a single injection of anti-IL-4 antibody prior to or shortly after infection can promote the development of a protective Th1 response rather than a Th2 response and leads to healing of the lesion (4, 18, 19). Depletion of IL-4 at the time of infection is believed to induce healing because IL-4 preferentially induces undifferentiated T cells to develop into nonprotective IL-4-producing Th2 cells. Subsequent studies have shown that coadministration of anti-IL-4 and anti-IFN-γ antibody at the time of infection completely reverses the ability of anti-IL-4 antibody to induce healing (4), suggesting that IFN-γ, even in the absence of IL-4, is required for the development of the protective Th1 cells.

In contrast to the efficacy of treatments given prior to or at the time of infection in promoting healing in susceptible mice, it has been more difficult to modify infection patterns in mice after T-cell subset profiles have been established. For example, treatment of *L. major*-infected mice weekly with 10 mg of anti-IL-4 antibody beginning at 2 weeks postinfection or with a single 10-mg dose of anti-IL-4 antibody 2 weeks after infection could arrest lesion development, but did not promote healing of lesions (4). Similarly, treatment of mice with sublethal levels of irradiation (8), anti-CD4 antibody (23), or IL-12 (22) is effective in promoting healing if given near the time of infection, but not if administered later, supporting the concept that these treatments alter the induction of helper T-cell subsets but are less effective in modifying the in vivo balance of differentiated Th cells.

Despite the dominance of the Th2 response which develops in BALB/c mice, *L. major*-reactive Th1 cells are present in BALB/c mice with chronic leishmaniasis, and low levels of IFN-γ can be detected in lymph node cell supernatants from these mice (5, 20). Adoptive transfer studies have recently shown that this underrepresented population of IFN-γ-producing cells can transfer resistance to C.B-17 *scid* mice (16).

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Thus IL-4, produced during an ongoing Th2 response, may actively be suppressing the further development of a Th1 response.

In the present study, we have attempted to address the requirements for the maintenance and expansion of the Th2 response in BALB/c mice with chronic cutaneous leishmaniasis. To investigate whether the expanding parasite population plays a role in the perpetuation of the Th2 response, and thus the continued suppression of Th1 cell function, we used the pentavalent antimony compound Pentostam (Burroughs Wellcome) to reduce the parasite burden in mice with established infections. Antimony compounds such as Pentostam have been used for the treatment of visceral and cutaneous leishmaniasis for decades (15). In susceptible mice experimentally infected with *L. major*, Pentostam has an effective parasiticidal effect, although lesion development almost invariably resumes with the cessation of drug treatment (2). We treated mice receiving Pentostam with anti-IL-4 antibody in order to investigate whether inhibition of IL-4 function coupled with a reduction in the parasite burden might allow the expansion of the otherwise suppressed Th1 response in these mice.

MATERIALS AND METHODS

Parasites and animals. BALB/cByJ (BALB/c) and C3HeB/FeJ (C3H) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) and were 5 to 7 weeks old at the time of infection. The clone of *L. major* (WHO MHOM/IL/80/Friedlin) used in this study was maintained in Grace's insect cell culture medium (GIBCO Laboratories, Grand Island, N.Y.) containing 20% fetal bovine serum (GIBCO), 2 mM L-glutamine, 100 mg of streptomycin sulfate per ml, and 100 U of penicillin G-potassium per ml. Soluble leishmanial antigen was prepared as previously described (21) and was used at a concentration of 50 μg/ml.

Infections and treatment protocol. Mice were inoculated in the right hind footpad with 1×10^5 metacyclic promastigotes selected from stationary-phase cultures, by using *Arachis hypogaea* agglutinin (Sigma Chemical Co., St. Louis, Mo.) as described previously (17). Lesion development was monitored with a dial caliper (L. S. Starrett Co., Athol, Mass.), and lesion size was expressed as the difference in thickness between the infected footpad and the contralateral footpad. The degree of infection in tissues was determined by limiting dilution analysis of homogenized tissue, as described elsewhere (1). Pentostam (sodium stibogluconate containing 100 mg of pentavalent antimony per ml; proprietary name of Burroughs Wellcome) was a gift from Max Grogl, Walter Reed Army Medical Center, Washington, D.C. Mice treated with Pentostam received 10 intramuscular injections of the drug (250 mg/kg of body weight per day) over a period of 2 weeks, beginning 14 days after infection. Antibody-treated mice received two intraperitoneal injections of either 2.5 or 5 mg of 11B11 monoclonal rat anti-mouse IL-4 or isotype control antibody (GL113 monoclonal rat anti-β-galactosidase) on days 13 and 21 postinfection. Antibody was purified from ascites fluid generated in pristane-primed BALB/c *nu/nu* mice by ammonium sulfate precipitation.

Production and measurement of cytokines. Spleen or popliteal lymph node cells were harvested from infected mice and ground in glass tissue homogenizers to single-cell suspensions in RPMI 1640 (GIBCO/BRL, Grand Island, N.Y.) containing 10% fetal bovine serum, 50 μg of gentamicin per ml, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (hereafter designated RPMI-10%). Following hypotonic lysis of erythrocytes (for spleens only), cells were cultured for 72 h in RPMI-10% at 5×10^6/ml in the presence of 50 μl of soluble leishmanial antigen, after which supernatants were harvested and analyzed for the presence of cytokines. IFN-γ (6) and IL-4 (4) concentrations were determined by enzyme-linked immunosorbent assays (ELISAs), and the standards used in cytokine ELISAs were recombinant murine IL-4 (a gift from R. Coffman, DNAX, Palo Alto, Calif.; 1 U = 45 pg) and recombinant murine IFN-γ (a gift from Genentech, San Francisco, Calif.).

TABLE 1. IgE levels in serum and cytokine production by lymph node cells from BALB/c and C3H mice infected with *L. major* for 2 weeks[a]

Mouse strain	IgE (μg/ml)[b]	Cytokine production by lymph node cells[c]	
		IFN-γ (ng/ml)	IL-4 (U/ml)
BALB/c	2.54 (1.7)	4.10 (0.6)	53.27 (16.5)
C3H	0.50 (0.2)	18.27 (11.9)	8.87 (2.6)

[a] BALB/c and C3H mice were infected in one hind footpad with 1×10^5 *L. major* metacyclic promastigotes and were sacrificed on day 14 postinfection.

[b] The concentration of IgE in serum was determined in an isotype-specific ELISA. Sera from normal BALB/c and C3H mice contained 0.30 and 0.29 μg of IgE per ml, respectively. The data shown represent means (± standard deviations) for five mice per group. The concentration of IgE shown for BALB/c mice is significantly greater than that for C3H mice ($P < 0.05$).

[c] Popliteal lymph node cells harvested from mice 2 weeks after infection were cultured in the presence of soluble leishmanial antigen for 48 h, after which supernatants were harvested and analyzed by ELISA. Data represent means (± standard deviations) for five animals per group. C3H lymph node cells produced significantly more IFN-γ and less IL-4 than those from BALB/c mice ($P < 0.05$).

Determination of IgE levels in serum. Levels of immunoglobulin E (IgE) in individual mouse serum samples were determined with an isotype-specific ELISA, using a monoclonal anti-mouse IgE (PharMingen, San Diego, Calif.) and a rat anti-mouse IgE-horseradish peroxidase conjugate (Southern Biotechnology Associates, Birmingham, Ala.) as a second antibody. The standard used in the ELISA was purified mouse IgE(κ) (PharMingen).

Statistical analysis. Data were analyzed for statistical significance by an unpaired Student *t* test. Data were considered significant at *P* of <0.05.

RESULTS

The nature of the anti-*L. major* response prior to treatment. A Th1 type immune response, characterized by the production of high levels of IFN-γ, is required for healing of *L. major* infections, while susceptibility is mediated by Th2 cells that produce IL-4 and stimulate IgE synthesis (11). Because we were interested in modifying ongoing Th2 responses in susceptible BALB/c mice infected with *L. major*, it was necessary to demonstrate that the cytokine response in these mice was heavily biased toward a Th2 type profile prior to the onset of treatment. To this end, we assessed the degrees of Th1 and Th2 activation in BALB/c mice and highly resistant C3H mice, 2 weeks after infection. The level of in vitro production of IFN-γ was used to determine the degree of Th1 activation, while Th2 activation was assessed by levels of IL-4 produced in vitro and levels of IgE in the serum of infected mice. Two weeks after infection, lymph node cells from BALB/c mice produced significantly less IFN-γ and more IL-4 than those from similarly infected C3H mice (Table 1). In addition, IgE levels in serum were significantly higher for BALB/c mice than for C3H mice at this time (Table 1). Thus, within 2 weeks of

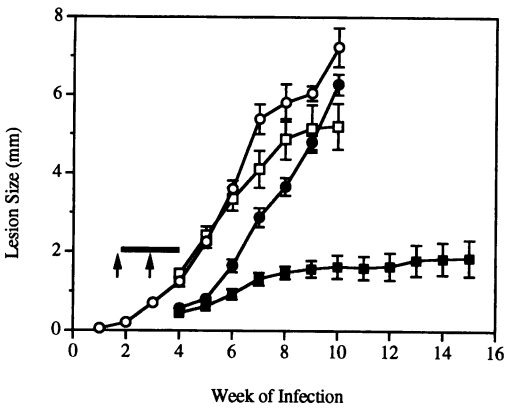

FIG. 1. Course of *L. major* infection in BALB/c mice. Groups of 10 mice either were untreated (○); were treated with Pentostam alone during the 3rd and 4th weeks of infection as indicated by the solid horizontal line (●) or with 2.5 mg of anti-IL-4 antibody alone on days 13 and 21 postinfection as indicated by the arrows (□); or received both agents (■). Anti-IL-4 antibody was administered intraperitoneally, and Pentostam was given intramuscularly at a dose of 250 mg/kg of body weight per day. Untreated mice and animals receiving anti-IL-4 antibody or Pentostam alone were sacrificed at week 10 postinfection because of the severity of the infection. The mean ± standard error is shown for each measurement.

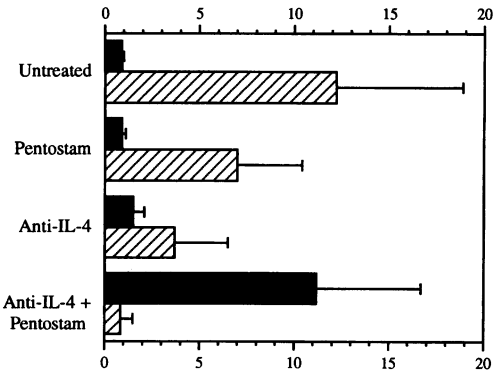

FIG. 2. IgE levels in serum (hatched bars; micrograms per milliliter) and in vitro production of IFN-γ by spleen cells (solid bars; nanograms per milliliter) from *L. major*-infected BALB/c mice. Mice were either untreated or treated as described in the legend for Fig. 1, and the means ± standard deviations for 10 mice per group are shown. Supernatants and serum samples from untreated mice and animals treated with anti-IL-4 antibody or Pentostam alone were prepared at week 10 postinfection, while samples from animals receiving both treatments were prepared at week 15 postinfection. IFN-γ and IgE levels were determined by ELISAs. Normal BALB/c serum contained 0.03 ± 0.02 μg of IgE per ml. The amounts of IFN-γ and IgE detected in supernatants and serum samples, respectively, from mice treated with Pentostam plus anti-IL-4 antibody were significantly different from amounts detected in samples generated from all other groups of mice.

infection, the anti-*L. major* response in BALB/c mice was heavily biased towards a Th2 profile, whereas in C3H mice, a Th1-like response had developed.

Parasite burdens and cytokine profiles shortly after treatment. To verify that Pentostam therapy effectively reduced the parasite burden during the treatment period, footpads from treated and untreated mice were analyzed for the presence of parasites 3 days after the cessation of treatment. Footpad tissue from treated mice had approximately 3,000-fold fewer parasites than tissue from untreated mice (minus log parasite titer of 2.8 ± 1.2 versus 6.3 ± 2.3, respectively), demonstrating the efficacy of this drug treatment regimen.

To determine whether Pentostam therapy altered cytokine profiles shortly after treatment, supernatants from 5×10^6 antigen-stimulated lymph node cells from untreated mice and Pentostam-treated mice 3 days after the termination of treatment (day 31 postinfection) were analyzed for the presence of IL-4 and IFN-γ 72 h after the initiation of culture. Although the levels of IL-4 produced by cells from treated and untreated mice did not differ, cells from Pentostam-treated mice produced approximately twofold more IFN-γ than those from untreated mice. Lymph node cells from untreated mice produced 3.87 ± 2.38 ng of IFN-γ per ml, while those from treated mice produced 7.36 ± 3.51 ng of IFN-γ per ml. Thus, Pentostam treatment significantly reduced parasite burdens during the treatment period and resulted in a marginal increase in IFN-γ production.

Pentostam therapy of mice receiving two 2.5-mg injections of anti-IL-4 antibody. *L. major*-infected BALB/c mice were treated with 2.5 mg of anti-IL-4 antibody on days 13 and 21 of infection, with or without combined therapy with Pentostam. Pentostam was administered during the 3rd and 4th weeks postinfection as described in Materials and Methods. Control animals receiving neither anti-IL-4 nor Pentostam developed progressive disease, as did mice which received only anti-IL-4 antibody (Fig. 1). Lesion development was significantly suppressed in mice treated only with Pentostam, although footpad lesions of all mice in this group rapidly increased in size soon after the cessation of treatment, indicating that the antiparasitic effect of the drug was only transient. However, in the group of mice that received Pentostam treatment in combination with anti-IL-4 antibody, footpad lesion development was arrested (Fig. 1). Only 2 of 10 mice in this group eventually developed progressive disease. Lesions of the remaining mice in the group either remained stable or were decreasing in size as evidenced by measurements of footpad swelling over time. Thus, this dose of anti-IL-4 antibody was effective only when the parasite burden was reduced during the antibody treatment period. Furthermore, these results indicate that combined therapy with both agents results in a synergistic antileishmanial effect.

Cytokine responses of mice receiving Pentostam and two 2.5-mg injections of anti-IL-4 antibody. We next investigated whether the nature of the antiparasite immune responses differed between the groups of mice described above. Little IFN-γ was produced in vitro by lymph node cells from untreated mice and from mice treated with Pentostam or anti-IL-4 antibody alone (Fig. 2). However, lymph node cells from the group of mice treated with anti-IL-4 antibody in combination with Pentostam produced significantly more IFN-γ than those from all other groups in the study. Levels of IgE in serum were highest in untreated mice and were not significantly different from the levels observed in animals treated with Pentostam alone (Fig. 2). Mice treated only with anti-IL-4 antibody had levels of IgE in their sera significantly lower than those of untreated mice. More importantly, however, treatment with Pentostam and anti-IL-4 antibody resulted in levels of IgE in serum significantly lower than those of untreated mice and mice treated with either anti-IL-4 antibody or Pentostam alone (Fig. 2). Together, these data indicate that the most pronounced Th1-like response existed in the group of mice that received combined treatment with Pentostam and anti-IL-4 antibody, the only treatment protocol which arrested

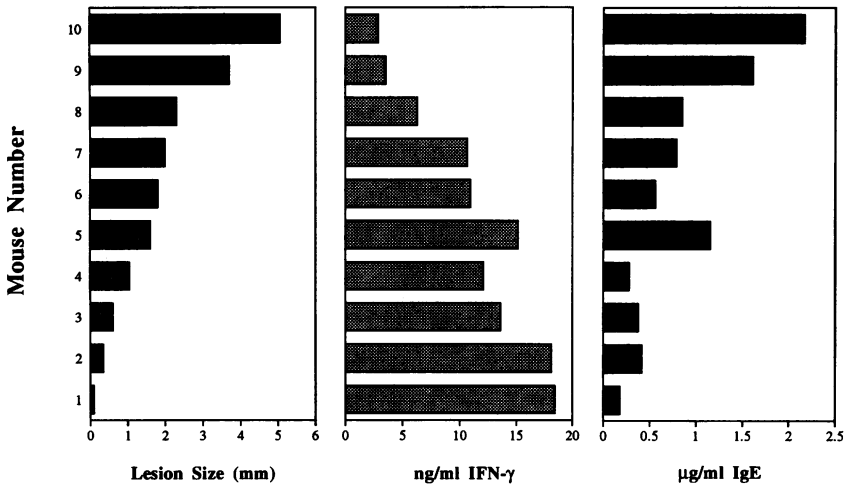

FIG. 3. IgE levels in serum and in vitro production of IFN-γ by spleen cells from 10 individual *L. major*-infected mice after treatment with Pentostam and anti-IL-4 antibody. Mice were infected in one hind footpad with 1×10^5 metacyclic promastigotes and after 2 weeks began receiving treatments as described in the legend for Fig. 1. Lesion size, IgE levels in serum, and the concentrations of IFN-γ in cytokine supernatants were determined at week 15 postinfection.

lesion development. The T-cell response was skewed towards Th2 cell predominance in untreated mice and in mice receiving only anti-IL-4 antibody or Pentostam treatments, an observation that is consistent with the fact that the infection was not controlled in these groups of mice.

Although lesion development was suppressed in all mice treated with anti-IL-4 antibody and Pentostam, not all mice in this group responded equally to treatment. We therefore assessed the relative degrees of Th1 and Th2 activation among the individual mice in this group. The sizes of the footpad lesions at 15 weeks after infection, the amounts of IFN-γ produced by spleen cells in vitro, and IgE levels in the serum of individual mice were examined (Fig. 3). A very strong positive correlation was found between the size of the footpad lesion and the concentration of IgE in the serum of individual mice in this group ($r = 0.948$), indicating that a high degree of Th2 activation was present in mice with large lesions. In contrast, a strong negative correlation was observed between lesion sizes from individual mice and levels of IFN-γ produced in vitro ($r = -0.922$). A negative correlation also existed between the level of IFN-γ produced in vitro and the concentration of IgE in serum of individual mice treated with anti-IL-4 antibody and Pentostam ($r = -0.786$). Thus, control of lesion development as a result of treatment with both agents was associated with low levels of IgE in serum and the production of high levels of IFN-γ in vitro and therefore correlated with the ability of mice to generate a Th1 response.

Pentostam therapy of mice treated with two 5-mg injections anti-IL-4 antibody. In a second series of similar experiments, the dose of anti-IL-4 antibody was increased to 5 mg per injection and BALB/c mice were treated with Pentostam as before. Unexpectedly, the lesions of animals receiving 5-mg injections of anti-IL-4 antibody on days 13 and 21 postinfection eventually healed (Fig. 4), an effect that was not noted in a previous study (4). However, lesion development was even more dramatically suppressed when mice were treated with this dose of anti-IL-4 antibody plus Pentostam (Fig. 4). The mean lesion size in animals treated with anti-IL-4 antibody peaked at the 6th week postinfection (1.08 ± 0.32 mm), at which time lesions were significantly larger than in mice treated with Pentostam and anti-IL-4 antibody (0.33 ± 0.13 mm; $P < 0.005$). Lesions remained significantly larger in the group of mice treated only with anti-IL-4 antibody than in the group treated with Pentostam and anti-IL-4 antibody through the 9th week of infection, after which the lesions of all mice in this group eventually healed. The observation that the lesions of animals treated with anti-IL-4 antibody with or without combined therapy with Pentostam were indeed healing was confirmed by using a limiting dilution assay for the presence of parasites in footpad tissues. Significantly fewer parasites were

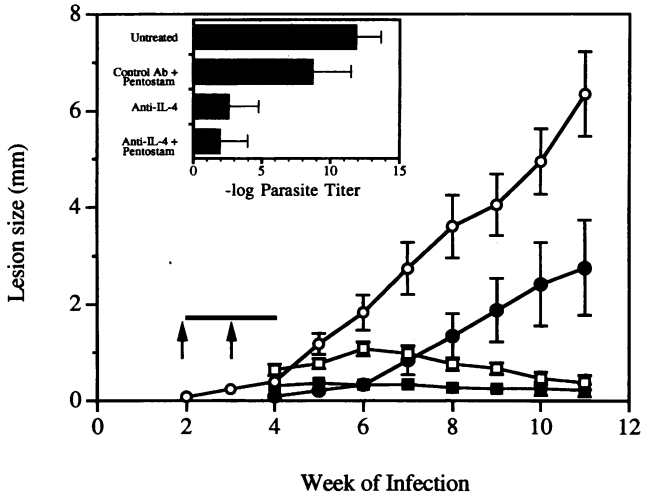

FIG. 4. Course of *L. major* infection in untreated mice (○) and in mice treated with either Pentostam plus control antibody as described in the Fig. 1 legend (●), 5 mg of anti-IL-4 antibody alone on days 13 and 21 of infection as indicated by the arrows (□), or anti-IL-4 antibody plus Pentostam (■). The mean ± standard error for five mice per group is shown for each footpad measurement. A limiting dilution assay was performed on footpads from mice at 11 weeks postinfection, and these data are shown in the inset. Footpads were homogenized in a fixed volume, after which the homogenate was serially diluted and 1 week later inspected for the presence of parasites. The data represent the means ± standard deviations for five mice per group. Ab, antibody.

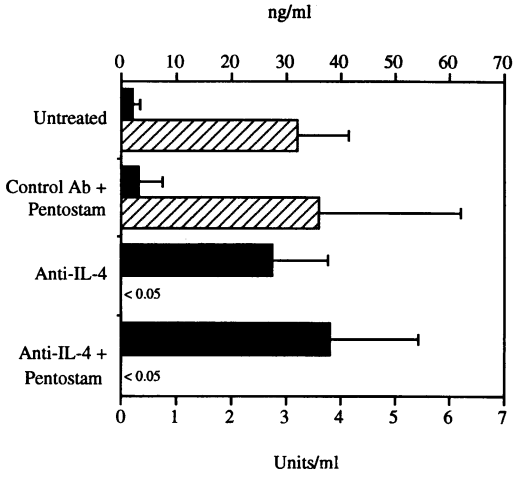

FIG. 5. IL-4 (hatched bars; units per milliliter) and IFN-γ (solid bars; nanograms per milliliter) production by spleen cells from the untreated and treated mice described in the Fig. 4 legend. At week 11 postinfection, spleens were ground to single-cell suspensions and 5×10^6 cells were incubated for 72 h in vitro in the presence of soluble leishmanial antigen, after which supernatants were analyzed for cytokine content. Each value represents the mean ± standard deviation for five individual mice per group.

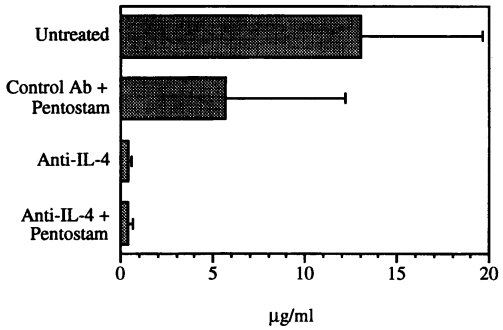

FIG. 6. IgE levels in serum of *L. major*-infected mice after treatment with Pentostam plus control antibody, two 5-mg injections of anti-IL-4 antibody, or a combination of both agents and of untreated mice. The treatment schedule is described in the Fig. 4 legend. Mice were bled 11 weeks after infection, and IgE levels were determined by an isotype-specific ELISA. The means ± standard deviations for five mice per group are shown. Ab, antibody.

detected in footpads of mice receiving anti-IL-4 antibody or anti-IL-4 antibody plus Pentostam than in footpads of untreated mice or mice treated with Pentostam plus control antibody (Fig. 4, inset). Although all mice treated with this dose of anti-IL-4 antibody eventually progressed towards healing, combined therapy with Pentostam and anti-IL-4 antibody was more effective than treatment with anti-IL-4 antibody alone. These results support those shown in Fig. 1, in which a synergistic antileishmanial effect was observed when animals were treated with Pentostam in combination with the low dose of anti-IL-4 antibody.

Cytokine responses of mice receiving Pentostam and two 5-mg injections of anti-IL-4 antibody. To determine the degree of Th1 and Th2 activation in the mice from the experiment in which the high dose of anti-IL-4 antibody was used, we measured in vitro production of IFN-γ and IL-4 by spleen cells, as well as IgE levels in serum at 11 weeks postinfection. As expected, spleen cells from untreated mice and mice treated with Pentostam plus control antibody produced significantly less IFN-γ and more IL-4 in vitro than those from mice treated with anti-IL-4 or anti-IL-4 and Pentostam whose lesions healed (Fig. 5). In addition, IgE levels in serum were significantly higher in untreated mice and mice treated with Pentostam plus control antibody than those in mice treated with anti-IL-4, with or without Pentostam (Fig. 6). Together, these data demonstrate that treatment with this dose of anti-IL-4 antibody can result in the eventual resolution of the lesion and that healing was associated with the development of a Th1 response. Additionally, a Th1 response was predominant in mice treated with this dose of anti-IL-4 antibody and Pentostam, a protocol which resulted in significantly less cutaneous pathology than treatment with anti-IL-4 antibody alone.

DISCUSSION

It has been recognized for over a decade that otherwise highly susceptible BALB/c mice can be manipulated to recover from an *L. major* infection and develop resistance to reinfection. Generally, such manipulations, which include prophylactic sublethal irradiation (8) or treatment with anti-CD4 (23) or anti-IL-4 (4, 18, 19) antibody, must be given prior to or near the time of parasite inoculation, and they are significantly less effective in controlling the rate at which the primary lesion expands than when administered later in infection. These results suggest that the effects of early intervention with such treatments, which ultimately lead to the preferential development of a Th1 response, are on the induction rather than the maintenance phase of the antiparasite immune response.

In the present study, we considered the possibility that the failure of immunomodulatory treatments to promote healing in BALB/c mice with chronic leishmaniasis may be due, in part, to the effect of the continued immunological stimulation caused by an expanding parasite population. In addition, since studies indicated that the antigen dose can determine whether cell-mediated or humoral immune responses predominate (3), we hypothesized that anti-IL-4 antibody treatment of infected mice which had already developed an ineffective Th2 response would be more effective if the antigen load responsible for continual stimulation of the Th2 type population was decreased. We demonstrated that a dose of anti-IL-4 antibody (2.5 mg per injection) which was ineffective in altering the outcome of disease when administered during the 3rd and 4th weeks postinfection can arrest lesion development, and in some cases promote healing, in *L. major*-infected BALB/c mice when administered with an antileishmanial drug, Pentostam. Importantly, the dose of Pentostam used in these studies did not promote cure, although it did temporarily suppress lesion development. Even doubling the dose and duration of Pentostam therapy did not affect the ultimate outcome of the infection (data not shown). Thus, the reduction in parasite burden that resulted from treatment with this dose of Pentostam was not sufficient to permanently affect the expression of the antileishmanial response in a favorable manner, whereas reduction of the parasite burden in treated mice in which the function of IL-4 was inhibited resulted in a dramatically increased protective response. The elevated levels of IFN-γ, a cytokine produced during a Th1 response, and decreased levels of IgE in serum, an indicator of a Th2 response, noted in mice after treatment with Pentostam and two 2.5-mg injections of anti-IL-4 antibody demonstrated that the antiparasite immune response was modified in these mice.

Not all mice treated with Pentostam and the low dose of

anti-IL-4 antibody responded equally well to treatment, and for this reason, we examined the nature of the immunological responses of individual mice. In general, we observed a very strong correlation between healing (small lesion size), elevated production of IFN-γ, and low levels of IgE in serum. In a previous study in which cytokine production by multiple strains of mice with various degrees of susceptibility to *L. major* infection was examined, the amount of IL-4, but not IFN-γ, produced in chronically infected mice correlated with the severity of disease (12). Although we also noted a positive correlation between levels of IL-4 produced and lesion size, in this study a closer correlation existed between lesion size, IgE levels in serum, and the amount of IFN-γ produced by spleen cells.

In a second series of experiments, mice were again treated with Pentostam, with and without the high dose of anti-IL-4 antibody or control antibody (5 mg per injection). In contrast to results obtained for a previously published report (4), mice treated with this dose of anti-IL-4 antibody alone resolved their infections. Our result was unexpected, given the fact that a 2.5-mg dose of 11B11 failed to significantly alter the course of disease in mice when given at 2 and 3 weeks postinfection. Additionally, in a previous study, a single 10-mg injection, or weekly 10-mg injections of 11B11 beginning at 2 weeks postinfection, failed to promote cure, although further lesion development was halted by either of these treatments (4). These later results suggest that depletion of IL-4 can affect the induction of a Th2 response but cannot fully reverse established Th2 responses. In our study, the healing rather than control of lesions as previously noted (4) of 2-week-infected, anti-IL-4-treated mice may be due to the use of a different strain of *L. major* or different parasite inocula, although the actual infective doses of organisms are difficult to compare, since we used purified metacyclic, as opposed to stationary-phase, promastigotes. An increased dose of 11B11 might have led to a cure in the study mentioned above (4), or alternatively, the Th2 response of the mice infected for 2 weeks in our experiments may have not been completely developed, although published studies have shown that IL-4 production does not increase significantly after 2 weeks of infection (20). Nevertheless, in our experiments, mice receiving both anti-IL-4 antibody and Pentostam exhibited still greater resistance than mice treated with antibody or Pentostam alone, as evidenced by threefold-smaller lesions at 6 weeks postinfection.

Since all mice treated with 5-mg injections of anti-IL-4 antibody healed, we did not observe a more intense Th1 response for the group of animals treated with anti-IL-4 plus Pentostam than for the group treated with antibody alone, as was noted in the first series of experiments. However, it is probable that the combined treatment, which promoted more rapid healing, similarly potentiated the Th1 response during the active infection, since such an effect was observed in the first series of experiments in which the low dose of anti-IL-4 antibody was used.

Although it is clear from the present study that anti-IL-4 antibody and Pentostam can synergize to inhibit the progression of established cutaneous leishmaniasis and promote Th1-like responses, the mechanism underlying this effect remains undefined. Clearly, inhibition of IL-4 function during infection would be beneficial to the host, since IL-4 drives Th2 differentiation (4, 9, 18, 19) and is known to inhibit IFN-γ-mediated killing of *Leishmania* spp. in macrophages, an activity linked to the suppression of NO synthesis by the infected host cell (10). However, a synergistic and not an additive effect was observed when mice receiving anti-IL-4 antibody were also treated with Pentostam.

Anti-IL-4 antibody and Pentostam may act to modify the antileishmanial response at the level of the macrophage in a number of ways. Pentostam treatment, via its capacity to destroy intracellular parasites, may affect the production of a number of macrophage factors which could influence the development of Th1 responses such as IL-10, IL-12, and transforming growth factor β. In addition, new parasite antigens that had previously been unavailable for processing may be presented by macrophages following treatment with Pentostam, and the resulting death of the amastigotes within infected cells might in turn result in the expansion of a new population of *Leishmania*-specific T cells.

It is possible that the reduction in the parasite burden that occurs as a result of the effects of Pentostam decreases the intensity of the Th2 response by effectively limiting the amount of antigen available to drive the response. In the absence of IL-4, and in an environment where the parasite population is decreased, the low levels of IFN-γ produced at this time might increase effective activation of macrophages to kill amastigotes and preferentially stimulate Th1 development. The role of IFN-γ in the effectiveness of this treatment may be particularly important, considering the fact that IFN-γ, which inhibits the proliferation of Th2 cells (7), is also known to synergize with Pentostam to kill *Leishmania* spp. in infected host cells (13). The production of IFN-γ is slightly increased in *L. major*-infected mice treated with Pentostam alone shortly after the termination of treatment, and therefore, this increase may further contribute to the development of a protective immune response following therapy with Pentostam and anti-IL-4 antibody.

Although the mechanism responsible for the effectiveness of Pentostam and anti-IL-4 treatment remains undetermined, alterations created by concurrently inhibiting IL-4 and decreasing parasite numbers appear to create an environment in which the balance of helper T-cell responses, heavily biased toward a Th2 profile prior to treatment, can be regulated toward the establishment of a protective Th1-like response. Whether or not Pentostam influences the antileishmanial response by destroying intracellular parasites, and thus reducing the antigen load, or by altering the physiology of infected macrophages in terms of their ability to produce regulatory cytokines, it is clear that reduction of the parasite burden concurrent with inhibition of the effects of IL-4 in an active infection has a marked effect on the potentiation of the Th1 type response and the development of resistance to *L. major* in this model.

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