Multiple Host Defense Defects in Failure of C57BL/6 *ep/ep* (Pale Ear) Mice To Resolve Visceral *Leishmania donovani* Infection

HENRY W. MURRAY,* JUNE HARIPRASHAD, DAVID F. McDERMOTT,† AND MARK Y. STOECKLE

Department of Medicine, Cornell University Medical College, New York, New York

Received 10 July 1995/Returned for modification 2 August 1995/Accepted 18 October 1995

Euthymic C57BL/6 *ep/ep* (pale ear [PE]) mice halt the visceral replication of intracellular *Leishmania donovani* but fail to properly resolve infection. A previous study identified an isolated defect in tissue granuloma formation in these mice; CD4⁺ and CD8⁺ cell number, gamma interferon (IFN- γ) production, and macrophage antimicrobial activity in vitro were all intact. New in vivo results reported here suggest a considerably more complex immune defect, with evidence indicating (i) enhanced control over *L. donovani* after transfer of normal C57BL/6 spleen cells, (ii) a partially suppressive Th2 cell-associated response mediated by interleukin-4 (IL-4) but not reversed by CD4⁺ cell depletion, (iii) absent responses to endogenous Th1 cell lymphokines (IFN- γ and IL-2) but preserved responsiveness to endogenous tumor necrosis factor alpha, (iv) absent responses to exogenous treatment with recognized antileishmanial cytokines (IFN- γ , IL-2, IL-12, and granulocyte-macrophage colony-stimulating factor [GM-CSF]) not corrected by transfer of C57BL/6 spleen cells, and (v) a deficient response to antimony chemotherapy. Defective hepatic granuloma formation was not corrected by transfer of C57BL/6 spleen cells or by anti-IL-4 administration. While treatment with IL-2 and GM-CSF modified the tissue reaction and induced selected effector cells to encase infected tissue macrophages, no antileishmanial activity resulted. Together, these observations suggest that the failure of PE mice to resolve visceral *L. donovani* infection likely represents expression of multiple suboptimal immune responses and/or partial defects, probably involving a combination of T-cell dysfunction, a Th2 cell response, and target cell (macrophage) hyporesponsiveness.

BALB/c and C57BL/6 mice are acutely susceptible to *Leishmania donovani*, a macrophage-parasitizing intracellular protozoan which causes visceral leishmaniasis (20, 24). Despite innate susceptibility controlled by the *Lsh* gene on chromosome 1 (7), however, these euthymic mice acquire sufficient resistance to halt parasite replication 4 weeks after challenge and largely resolve visceral infection by week 8 (20, 24). This T-cell-dependent response requires both CD4⁺ and CD8⁺ cells, is expressed in the tissues by the assembly of influxing blood monocytes and T cells into granulomas at sites of parasitized macrophages, and involves multiple macrophage- and T-cell-activating endogenous cytokines (3, 14, 18, 20, 24–27). The initial acquisition of resistance to *L. donovani* requires at least four cytokines: gamma interferon (IFN- γ) and interleukin-2 (IL-2) (18, 25), Th1 cell-associated lymphokines; tumor necrosis factor alpha (TNF- α) (27); and granulocyte-macrophage colony-stimulating factor (GM-CSF) (14). The subsequent near-resolution stage of experimental visceral infection is primarily driven by endogenous TNF- α alone (27).

Although they are also euthymic, C57BL/6 *ep/ep* (pale ear [PE]) mice, a strain with a coat pigment mutation mapped to chromosome 19 (2), initially permit visceral *L. donovani* to replicate to a greater extent than in either BALB/c or C57BL/6 animals (24). In addition, despite generating an immune response sufficient to restrain visceral replication (organ parasite burdens plateau 4 weeks after challenge), PE mice nevertheless fail to resolve visceral infection at week 8 and remain persistently parasitized at high levels (24). Initial characterization of PE host defense mechanisms demonstrated that the

following were normal or intact: (i) T-cell number, percentages of CD4⁺ and CD8⁺ cells, and in vitro secretion of IFN- γ (IL-2-dependent [18]); (ii) macrophage responsiveness to IFN- γ and in vitro antimicrobial capacity; and (iii) circulating levels of antileishmanial antibody (24). In addition, although influx of CD4⁺ and CD8⁺ cells and monocytes into infected tissue was also intact (24), PE mice failed to properly assemble these cells and maintain granulomas at foci of visceral infection. This observation suggested a defect in the tissue cellular immune response and provided one explanation for the inability of these animals to successfully eradicate *L. donovani* (24). The results of our on-going analysis reported here indicate that the failure of PE mice to resolve visceral infection is probably multifactorial and considerably more complex than was originally apparent.

MATERIALS AND METHODS

Mice and visceral infection. C57BL/6 *ep/ep* (PE) mice, initially obtained from Jackson Laboratories (Bar Harbor, Maine), were bred under conventional conditions and used at 6 to 12 weeks of age (24). PE and normal C57BL/6 mice (Charles River Laboratories, Wilmington, Mass.) were infected via the tail vein with 10⁷ *L. donovani* amastigotes maintained in hamsters (20, 24). One day prior to challenge, some PE mice received 10⁷ nonadherent spleen cells from normal C57BL/6 animals by intravenous injection. Visceral infection was determined microscopically by using stained liver imprints, and parasite liver burdens, expressed as Leishman-Donovan units (LDU), were calculated as the number of amastigotes per 1,000 cell nuclei times liver weight (in grams) (20, 24). In mice treated with IL-2 or GM-CSF, LDU were calculated as the number of amastigotes per 500 hepatocyte nuclei times liver weight (in grams) because treatment with these cytokines increased the number of cells in the liver (14, 18). A two-tailed Student's *t* test was used to analyze differences in liver parasite burdens (14). Formalin-fixed liver sections stained with hematoxylin and eosin were used for histologic examination (24).

Anti-T-cell and anticytokine antibody treatments. With the previously described preparations, doses, and administration schedules (see table footnotes and figure legends) (11, 14, 15, 18, 25–27), mice were injected intraperitoneally (i.p.) with (i) 1 ml of hybridoma culture supernatants containing rat anti-mouse monoclonal antibody (MAb) GK1.5 (anti-CD4; ATCC TIB 207; 8 μ g of immunoglobulin G [IgG] per ml), 53.672 (anti-CD8; ATCC TIB 105; 11.6 μ g of IgG

* Corresponding author. Mailing address: Department of Medicine, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

† Present address: Tufts-New England Medical Center, Boston, Mass.

FIG. 1. (A) Course of visceral infection in C57BL/6 (●) and PE (○) mice. (B) Course of visceral infection in PE mice that had received 10^7 C57BL/6 spleen cells 1 day prior to infection (△) versus untreated PE mouse controls (○). Results are from three to six experiments and indicate means $\pm$ SEM for 9 to 21 mice at each time point. *, $P < 0.05$ versus corresponding value for control (C57BL/6 or untreated) mice.

per ml), or S4B6.1 (anti-IL-2; 12.5 μ g of IgG per ml [18, 26]); (ii) 0.2 ml of rabbit anti-mouse TNF- α antiserum (1.5×10^5 neutralizing units per ml), rabbit anti-mouse IFN- γ antiserum (10^5 neutralizing units per ml), or normal rabbit serum (14, 15, 27); or (iii) normal rat IgG (Sigma Chemical Co., St. Louis, Mo.) or rat anti-IL-4 MAb (11.B.11; generously provided by C. Reynolds, Biological Response Modifiers Program, National Cancer Institute, Frederick, Md.) (11). Fluorescence analysis of spleen cells (26) after three consecutive daily injections of anti-CD4 or anti-CD8 MAb demonstrated 90 to 93% depletion of the targeted T-cell subset.

Cytokine treatments. Treatment with the following recombinant murine cytokines was begun 2 weeks after infection (day 0) and given for 7 days: IFN- γ (2×10^7 U/mg; Amgen, Thousand Oaks, Calif.), GM-CSF (1×10^5 U/mg; Amgen), TNF- α (1.2×10^7 U/mg; Genentech, Inc., South San Francisco, Calif.), and IL-12 (7.8×10^6 U/mg; Genetics Institute, Cambridge, Mass.). Human recombinant IL-2 (7×10^6 U/mg) was also a gift from Amgen. Cytokines were administered continuously via subcutaneously implanted osmotic pumps (Alzet model 2002; Alza Corp., Palo Alto, Calif.) (13). The doses were selected on the basis of prior studies (13–15, 18, 27), and the calculated rates of release were 2×10^5 U/day for IFN- γ , 6×10^4 U/day for IL-2, 5 μ g per day for GM-CSF, 5 μ g per day for TNF- α , and 1 μ g per day for IL-12. Liver parasite burdens were determined just prior to starting treatment (day 0) and 7 days later (day 7).

Antibiotic treatment. Two weeks after infection (day 0), mice received a single i.p. injection of 100 or 500 mg of sodium stibogluconate (Pentostam; Burroughs-Wellcome, Research Triangle Park, N.C.) per kg (19) or began three every-second-day i.p. injections of 5 mg of amphotericin B (Fungizone; Bristol Myers Squibb, Princeton, N.J.) per kg (17). All mice were killed on day 7.

RT-PCR. Total RNA was prepared from liver homogenates of uninfected and infected mice (11). Cytokine mRNA expression (IFN- γ , IL-4, and IL-10) was assayed with the preparations and qualitative reverse transcription (RT)-PCR amplification procedures described in detail elsewhere (11).

IgE assay. Serum IgE was kindly assayed by enzyme-linked immunosorbent assay (ELISA) by Fred Finkelman (Uniformed Services Medical School, Bethesda, Md.) (11).

RESULTS AND DISCUSSION

In vivo T-cell activity. The kinetic data in Fig. 1A indicate the capacity of C57BL/6 but not PE mice to promptly control and then largely resolve *L. donovani* visceral infection. Eight weeks after challenge, liver parasite burdens were 12.6-fold higher in PE mice. Since resolution of infection requires T cells (20, 26), we first tested possible T-cell dysfunction in PE mice by three approaches: (i) repopulating PE mice prior to infection with naive C57BL/6 spleen cells as a source of normal T cells, (ii) treatment with T-cell-depleting MAb to determine if visceral infection was appropriately exacerbated (26), and (iii) analyzing whether a suppressive Th2 cell-associated response had developed (1, 8, 9, 11).

As shown in Fig. 1B, transfer of C57BL/6 cells into PE mice had no effect on the course of *L. donovani* infection at week 2

TABLE 1. Effect of anti-T-cell and anticytokine antibody treatment^a

Group and treatment	Mean wk 4 parasite burden (LDU) $\pm$ SEM in liver	
	C57BL/6 mice	PE mice
A		
None	553 $\pm$ 30	1,636 $\pm$ 79
Anti-CD4	1,339 $\pm$ 82*	3,855 $\pm$ 148*
Anti-CD8	1,192 $\pm$ 71*	3,221 $\pm$ 94*
Rat IgG	471 $\pm$ 52	1,724 $\pm$ 76
B		
None	641 $\pm$ 66	1,793 $\pm$ 94
Anti-IL-2	1,407 $\pm$ 57*	2,083 $\pm$ 139
Rat IgG	798 $\pm$ 60	1,860 $\pm$ 123
Anti-IFN- γ	1,985 $\pm$ 103*	2,178 $\pm$ 126
Anti-TNF- α	2,478 $\pm$ 129*	3,650 $\pm$ 142*
Rabbit serum	972 $\pm$ 89	1,986 $\pm$ 59

^a Group A mice were treated with the indicated preparations for 3 consecutive days before infection and then twice weekly thereafter for 4 weeks. Results indicate mean $\pm$ SEM values for six mice per group from two experiments. Group B mice were treated the day before infection and then twice (anti-IL-2, rat IgG [25 μ g], and anti-IFN- γ) or thrice (anti-TNF- α and rabbit serum) weekly thereafter for 4 weeks. Results indicate mean $\pm$ SEM values for six to nine mice per group from two or three experiments. An asterisk (*) indicates that the value is significantly different ($P < 0.05$) from that for untreated mice.

or 4. Eight weeks after infection, however, there was a moderate enhancing effect, sufficient to suggest possible T-cell dysfunction. Despite this result, treatment with anti-CD4 and anti-CD8 MAb exacerbated visceral infection in PE mice and, as anticipated (26), in normal C57BL/6 mice as well (Table 1). This finding in PE mice suggested that both CD4⁺ and CD8⁺ cells were functionally active in vivo and capable of mediating positive antileishmanial effects, albeit insufficient to resolve infection. Thus, if present, a T-cell defect was likely to be either subtle or only partially expressed. Alternatively, and as considered below, the limited effect of spleen cell transfer (Fig. 1B) could also reflect macrophage unresponsiveness to T-cell-derived activating signals.

Th2 cell-associated response. CD4⁺ cell depletion exacerbated rather than enhanced control over visceral infection in PE mice (Table 1). Therefore, it seemed unlikely that the suppressive Th2 cell-associated response, well characterized in models of progressive *L. major* infection (1, 8, 9) and reversible by anti-CD4 treatment (8), was a primary factor in the failure of PE mice to control *L. donovani* infection. Nevertheless, the following results suggested that while a Th1 cell-associated response was triggered, a Th2 cell response related to IL-4 was also induced and exerted a suppressive effect. By qualitative RT-PCR assay (11), expression of IFN- γ , IL-4, and IL-10 mRNAs was induced in PE mouse liver tissue 2 weeks after infection (Fig. 2 and data not shown), and expression of each cytokine was maintained at weeks 4 and 8 (not shown). Qual-

FIG. 2. RT-PCR analysis of induction of IFN- γ mRNA expression in livers of PE and C57BL/6 (C57) mice. Glyceraldehyde-3-phosphate dehydrogenase (GAP) was used as a control for the amount of RNA in each sample. Lanes: 1, uninfected mouse; 2 and 3, two mice infected for 2 weeks; 4, PCR control (no RNA).

FIG. 3. Effect of anti-IL-4 MAb treatment on visceral infection in PE mice. Starting 1 day before infection and once weekly thereafter, mice received no treatment (○) or were injected with 1 mg of anti-IL-4 (●) or rat IgG (△). Results are means $\pm$ SEM for nine mice per group at week 4 (three experiments) and six to seven mice per group at week 8 (two experiments). *, $P < 0.05$ versus untreated controls.

itative PCR results for IFN- γ , IL-4, and IL-10 mRNA expression were similar in infected C57BL/6 mice (Fig. 2 and not shown). However, PE but not C57BL/6 mice showed a clear increase in serum levels of IgE (used as a marker for the presence of IL-4 [11]), and only PE mice responded to treatment with anti-IL-4 MAb. In two experiments, serum IgE levels (mean $\pm$ standard error of the mean [SEM]) for uninfected and 4- and 8-week-infected PE mice were 0.12 ± 0.04 , 4.3 ± 0.9 , and 6.0 ± 0.7 μ g/ml, respectively (four to nine mice per time point); corresponding values in C57BL/6 mice were 0.05 ± 0.02 , 0.91 ± 0.3 , and 1.2 ± 0.4 μ g/ml (four to six mice per time point). The results induced by once-weekly treatment with anti-IL-4 MAb are shown in Fig. 3. In anti-IL-4-treated PE mice, liver parasite burdens at week 4 (934 ± 41 LDU) were 50% lower than in rat IgG-treated mice ($1,860 \pm 102$). This effect was more fully developed after continued MAb treatment and led to partial resolution of visceral infection at week 8. At this time point, serum IgE was reduced to 2.6 ± 0.8 μ g/ml in treated mice versus 6.0 ± 0.7 μ g/ml in PE controls (four to six mice per group). Anti-IL-4 administration had no effect at week 4 or 8 in C57BL/6 controls (two experiments; data not shown), consistent with prior results in self-curing BALB/c mice [11].

We concluded from these three sets of experiments that while both CD4⁺ and CD8⁺ cells were active in vivo in PE mice (Table 1), a suppressive Th2 cell-associated response involving IL-4 (Fig. 3) likely contributed to both the higher parasite burden at week 4 and the failure to resolve visceral infection at week 8. The immunopathogenic role, participating cells, and potency of this Th2 cell response, however, appeared to differ from that induced by *L. major* in BALB/c mice (8, 9). In the *L. major* model, infection is unrestrained and ultimately fatal, serum IgE levels are appreciably higher, and abolishing the Th2 response by CD4⁺ cell depletion or neutralizing endogenous IL-4 results in cure of infection (8, 9, 23). Instead, our observations in PE mice are more akin to those reported for C57BL/10 mice infected with *L. amazonensis* (1). In that model, the Th2 cell-associated response which involves IL-4 also appears to exert an incomplete suppressive effect on host defense (1). Thus, our results support the notion that the triggering of a Th2 cell response may not necessarily induce unrelenting *Leishmania* infection (1, 11, 12).

Activity of and response to endogenous antileishmanial cytokines. In previous studies, treatment with anticytokine antibodies was used to identify IFN- γ , IL-2, and TNF- α as endogenous participants in the initial acquisition of resistance to *L. donovani* in BALB/c mice (18, 25, 27). These studies also indicated that the subsequent resolution of visceral infection was primarily dependent upon TNF- α alone (18, 25, 27). One of the effects of an established Th2 cell response is suppression of production of or inhibition of target cell responsiveness to activating cytokines, including IFN- γ , IL-2, and TNF- α (6, 10). Therefore, groups of PE mice were next injected before and after *L. donovani* challenge with anti-IFN- γ , anti-IL-2, or anti-TNF- α to determine if, as judged by exacerbation of visceral infection, these three selected pro-host defense cytokines were active in vivo. While each of these cytokine-depleting preparations promoted visceral infection in C57BL/6 controls (Table 1), neither anti-IFN- γ nor anti-IL-2 induced an effect in PE mice. These data suggested either that these two endogenous Th1 cell cytokines were not properly secreted in PE mice or that, if produced, the effector cells for these cytokines (for example, the Kupffer cell or influxing blood monocyte for IFN- γ [3]) were unresponsive. Since, however, (i) spleen cells from infected PE mice respond normally to *L. donovani* antigen in vitro with IFN- γ secretion (24), which is IL-2-dependent (18), (ii) tissue Ia expression (largely IFN- γ dependent [25]) is intact in infected PE mouse liver (24), and (iii) IFN- γ mRNA is induced (Fig. 2), a defect in endogenous IFN- γ (and IL-2) production seemed unlikely.

While target cell (macrophage) unresponsiveness to endogenous activating cytokines (e.g., IFN- γ) appeared possible, we excluded a generalized defect because injections of anti-TNF- α clearly exacerbated visceral infection at week 4 (Table 1). These latter results provided evidence that at least one recognized antileishmanial cytokine, involved in macrophage activation and leishmanicidal activity (27), was indeed produced in vivo and that PE mouse target cells could be partially activated. In one experiment carried out to week 8, mean liver parasite burdens in PE mice treated three times a week with anti-TNF- α ($7,119 \pm 294$ LDU) were >4-fold higher than in rabbit serum-treated controls ($1,512 \pm 126$ LDU) (three to four mice per group). Despite responsiveness to endogenous TNF- α (which, in self-curing BALB/c mice, appears to be sufficient to mediate resolution of visceral infection [27]), PE mice nonetheless remained persistently parasitized. Thus, factors in addition to endogenous TNF- α may be required for the conversion of *L. donovani* infection to a chronic, low-level state by week 8.

Response to exogenous antileishmanial cytokines. To determine if the failure of PE mice to respond to IFN- γ and IL-2 in exogenous form extended to exogenous IFN- γ and IL-2, 2-week-infected animals were treated with cytokine preparations which in parallel studies induced clear killing of visceral *L. donovani* in BALB/c mice (13, 18). Despite activity in C57BL/6 controls, neither IFN- γ nor IL-2 treatment induced an antileishmanial effect in PE mice (Table 2). Since responses to IFN- γ and IL-2 require host T cells (16, 18), these experiments were repeated in PE mice which had received C57BL/6 spleen cells. Cell transfer, however, did not restore responses to either cytokine or to the other cytokines also tested (Table 2). While treatment with IL-12, a recognized IFN- γ inducer (15), resulted in activity in C57BL/6 controls, it also had no effect in PE mice. Exogenous TNF- α did not induce antileishmanial activity in either C57BL/6 or PE mice, an observation already made for BALB/c mice and attributed to previously sufficient levels of endogenous TNF- α (27). Finally, we treated PE mice with GM-CSF, which induces three linked T-cell-

TABLE 2. Effect of cytokine treatment^a

Group and treatment	Day	Mean parasite burden (LDU) $\pm$ SEM in liver ^b		
		C57BL/6 mice	PE mice	Repopulated PE mice
A				
None	0	823 $\pm$ 50	1,040 $\pm$ 71	967 $\pm$ 105
	7	1,125 $\pm$ 93	1,905 $\pm$ 150	2,036 $\pm$ 187
IFN- γ	7	426 $\pm$ 86*	2,379 $\pm$ 132	2,511 $\pm$ 142
TNF- α	7	1,254 $\pm$ 79	1,617 $\pm$ 69	2,290 $\pm$ 172
IL-12	7	656 $\pm$ 82**	1,835 $\pm$ 119	NT
B				
None	0	1,601 $\pm$ 56	2,295 $\pm$ 133	1,731 $\pm$ 57
	7	2,068 $\pm$ 102	4,240 $\pm$ 219	3,905 $\pm$ 269
IL-2	7	891 $\pm$ 99*	4,023 $\pm$ 243	3,215 $\pm$ 296
GM-CSF	7	1,280 $\pm$ 71**	5,057 $\pm$ 310	3,790 $\pm$ 150

^a Two weeks after infection (day 0), osmotic pumps were inserted, and cytokine treatment was given continuously for 7 days with IFN- γ (2×10^5 U/day), TNF- α (5 μ g/day), IL-12 (1 μ g/day), IL-2 (6×10^4 U/day), or GM-CSF (5 μ g/day). Repopulated PE mice received 10^7 C57BL/6 spleen cells 1 day prior to infection. Values are means $\pm$ SEM for 6 to 12 mice per group from two to three experiments. LDU are expressed per nucleated cell for group A and per hepatocyte for group B (see Methods).

^b *, significantly different ($P < 0.05$) from both day 0 and day 7 results in untreated controls; **, significantly different ($P < 0.05$) only from day 7 value in untreated controls. NT, not tested.

dependent effects in this model: striking increases in the peripheral leukocyte count, markedly enhanced influx of monocytes and neutrophils into infected liver, and moderate antileishmanial activity (14). In both C57BL/6 and PE mice, 7 days of GM-CSF treatment comparably increased the peripheral leukocyte count by eight- to nine-fold, to $92.1 \times 10^3 \pm 10.3 \times 10^3$ and $124.0 \times 10^3 \pm 13.9 \times 10^3$ per mm³, respectively (six mice per group). In parallel, while GM-CSF treatment also resulted in clear accumulation of myelomonocytic cells at foci of *L. donovani* infection in the livers of both PE and C57BL/6 animals (see below), antileishmanial effects were induced only in C57BL/6 mice (Table 2).

Response to antileishmanial chemotherapy. Although both antimony and amphotericin B exert leishmanicidal activity in vivo (17, 19), responsiveness to antimony requires the presence of host T cells (either CD4⁺ or CD8⁺ cells [19]), whereas amphotericin B is fully active in T-cell-deficient nude mice (17). As shown in Table 3, PE and C57BL/6 mice responded comparably to amphotericin B. In contrast, while antimony at both 100 and 500 mg/kg readily induced leishmanicidal effects

in C57BL/6 mice, PE animals showed no response to 100 mg/kg, and treatment with 500 mg/kg induced only leishmanicidal activity. Repopulating PE mice with C57BL/6 spleen cells 1 day before infection did not correct the defective response to antimony.

Histologic responses in PE mice. In each of the preceding experiments, PE mouse liver sections were examined to determine what, if any, effects the various treatments employed induced in infected tissues. Transfer of 10^7 C57BL/6 spleen cells had no effect on deficient PE mouse hepatic granuloma formation (24) (not shown). Similarly, despite enhancing control over *L. donovani*, anti-IL-4 treatment also failed to enable PE mice to assemble organized, mature granulomas. However, PE mice readily responded to treatment with IL-2 and GM-CSF, with obvious modification of the tissue reaction. This response was expressed by enhanced influx into the liver of mononuclear cells (IL-2) (18) and myelomonocytic cells (GM-CSF) (14), with marked cellular accumulation at sites of *L. donovani* infection (Fig. 4). However, despite the induction of these effector cells (3, 14, 18) to encase parasitized Kupffer cells, these effects directed by IL-2 and GM-CSF did not result in antileishmanial activity (Table 2). Since both IFN- γ -secreting, IL-2-stimulated mononuclear cells and influxing blood monocytes are involved in defense against *L. donovani* (3, 18), the ability of IL-2 and GM-CSF to deliver these cells but not to induce antileishmanial activity also suggested the possibility of an additional defect in PE mice: target cell (Kupffer cell) unresponsiveness or deactivation within the infected tissue focus.

As judged from the results reported here, the previously identified lesion in granuloma assembly in PE mice (24) probably represents just one expression of what appears to be a complex, multifactorial immune defect in this host's antileishmanial defense mechanisms. PE mice have also been reported to display impaired in vivo control over *Listeria monocytogenes* (7a) and to show reduced natural killer (NK) cell activity and macrophage lysosomal enzyme secretion in vitro (2, 21). Neither of these two defects seems likely to account for impaired antileishmanial activity, however, since (i) NK cell-deficient C57BL/6 beige mice control infection by week 4 (20, 24) and (ii) intracellular *L. donovani* organisms readily survive and replicate within fused phagolysosomes (4).

Since PE mice are able to prevent the unrestrained replication of *L. donovani* seen in T-cell-deficient nude mice (20), their immune defect(s) at the level of the T-cell or macrophage seems to be a partial one, expressed primarily by the failure to resolve visceral infection. While this on-going analysis of PE

TABLE 3. Response to antileishmanial chemotherapy^a

Treatment	Day	Mean parasite burden (LDU) $\pm$ SEM in liver ^b		
		C57BL/6 mice	PE mice	Repopulated PE mice
None	0	852 $\pm$ 60	1,038 $\pm$ 54	811 $\pm$ 26
	7	1,094 $\pm$ 93 (0)	2,088 $\pm$ 126 (0)	1,786 $\pm$ 130 (0)
Antimony	7	522 $\pm$ 70** (39)	2,294 $\pm$ 133 (0)	1,679 $\pm$ 103 (0)
	7	178 $\pm$ 41* (79)	971 $\pm$ 151** (7)	999 $\pm$ 95** (0)
Amphotericin B	7	195 $\pm$ 27* (77)	394 $\pm$ 64* (62)	NT

^a Two weeks after infection (day 0), mice received a single injection of antimony (100 or 500 mg/kg) or three every-second-day injections of amphotericin B (5 mg/kg), and LDU were determined after 7 days (day 7). Data are from two to four experiments, and results indicate mean $\pm$ SEM values for groups of 9 to 12 C57BL/6 mice, 6 to 15 PE mice, and 6 repopulated PE mice (as in Table 2).

^b Values in parentheses represent percent killing, calculated as (day 0 LDU - day 7 LDU)/(day 0 LDU) $\times$ 100. *, Significantly different ($P < 0.05$) from day 0 and day 7 values in untreated controls; **, significantly different ($P < 0.05$) only from day 7 value in untreated controls. NT, not tested.

FIG. 4. Histologic responses in livers of PE mice 3 weeks after *L. donovani* infection. (A) Untreated PE controls show ill-focused mononuclear cell infiltrate with no organized granuloma formation surrounding heavily parasitized Kupffer cells (arrows). (B and C) PE mice treated with IL-2 (B) or GM-CSF (C) during the preceding 7 days demonstrated markedly enhanced influx of mononuclear cells (B) and myelomonocyte cells (C), respectively, but encased Kupffer cells remain heavily infected. Magnification, $\times 200$.

mice has not pinpointed one specific underlying mechanism to account for each of the defects documented here, a partially suppressive, deactivating Th2 cell-associated response, involving IL-4 and perhaps other cytokines such as IL-10 (6, 10, 22), seems likely to be one such factor. Indeed, in additional experiments in which exogenous IFN- γ had no effect in PE mice (Table 4), combination treatment with anti-IL-4 plus IFN- γ induced leishmanistatic activity. Since production of IFN- γ by PE mouse spleen cells is intact in vitro (24), the results in Table 4 suggest that in this model, IL-4 may preferentially act to inhibit responsiveness to rather than secretion of IFN- γ . The failure to respond properly to antimony chemotherapy may also reflect separate deactivating effects of IL-4, since a T-cell-dependent mechanism is required for in vivo responsiveness to antimony (19).

TABLE 4. Response to exogenous IFN- γ in anti-IL-4-treated PE mice^a

Treatment	Mean parasite burden (LDU) $\pm$ SEM in liver ^b	
	Day 0	Day 7
None	1,172 $\pm$ 69	2,133 $\pm$ 102
IFN- γ		2,368 $\pm$ 114
Anti-IL-4		1,906 $\pm$ 86
Anti-IL-4 + IFN- γ		992 $\pm$ 88*
Rat IgG + IFN- γ		1,999 $\pm$ 110

^a Two weeks after infection (day 0), PE mice were treated as indicated with IFN- γ alone (2×10^5 U/day for 7 days), anti-IL-4 alone (single injection of 1 mg on day 0), or single injections of anti-IL-4 or rat IgG (1 mg) on day 0 plus IFN- γ for 7 days. Results indicate means $\pm$ SEM for six mice per group from two experiments.

^b *, $P < 0.05$ versus day 7 LDU value for untreated control.

The inability of transferred C57BL/6 spleen cells to reconstitute responses to antimony or exogenous cytokines or granuloma formation in PE mice supports the idea of active suppression and/or hyporesponsiveness in the tissues rather than overall, intrinsic T-cell dysfunction. In addition, T-cell depletion exacerbated visceral infection, and PE mice showed intact histologic responses (cell influx and accumulation) to IL-2 and GM-CSF and pronounced peripheral leukocytosis (GM-CSF), responses which require functional T cells (14, 18). While Kupffer cells appeared to be responsive to at least one endogenous antileishmanial cytokine, TNF- α , this effect was selective (e.g., no response was induced by anti-IFN- γ or anti-IL-2), and TNF- α by itself was not sufficient to lead to resolution of infection. The possibility of partial macrophage deactivation or a partial defect in target cell (Kupffer cell) responsiveness also seems quite pertinent in PE mice in view of the failure to respond to either exogenous or endogenous macrophage-activating cytokines (IFN- γ [13, 25] and GM-CSF [14]), inducers of endogenous IFN- γ (IL-2 and IL-12 [15, 18]), or the cytokine-mediated delivery of mononuclear and myelomonocytic effector cells directly to the infected tissue focus.

ACKNOWLEDGMENTS

We are grateful to Fred Finkelman for performing the IgE assays; to Amgen (Bibiana Aguero and Homa Yeganegi) for providing IFN- γ , GM-CSF, and IL-2; to the Genetics Institute (Joseph Sypek) for IL-12; and to Genentech, Inc., for providing TNF- α .

This work was supported by NIH research grants AI16963 and GM46890, a New York Heart grant-in-aid, Council for Tobacco Research grant 3260R1, and American Cancer Society grant JFRA-386.

REFERENCES

1. Afonso, L., and P. Scott. 1993. Immune response associated with susceptibility of C57BL/10 mice to *Leishmania amazonensis*. *Infect. Immun.* **61**: 2952–2959.
2. Brown, J. A., E. K. Novak, and R. T. Swank. 1985. Effects of ammonia on processing and secretion of precursor and mature lysosomal enzyme from macrophages of normal and pale ear mice: evidence for two distinct pathways. *J. Cell Biol.* **100**:1894–1904.
3. Cervia, J., H. Rosen, and H. W. Murray. 1993. Effector role of blood monocytes in experimental visceral leishmaniasis. *Infect. Immun.* **61**:1330–1333.
4. Change, K.-P., and D. M. Dwyer. 1978. *Leishmania donovani*: hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes. *J. Exp. Med.* **147**:515–528.
5. Chatelain, R., K. Varkila, and R. L. Coffman. 1992. IL-4 induces a Th2 response in *Leishmania major*-infected mice. *J. Immunol.* **148**:1182–1187.
6. Coffman, R. L., K. Varkila, P. Scott, and R. Chatelain. 1991. Role of cytokines in the differentiation of CD4⁺ T cell subsets in vivo. *Immunol. Rev.* **123**:190–207.
7. Crocker, P. R., J. M. Blackwell, and D. J. Bradley. 1984. Expression of the natural resistance gene *Lsh* in resident liver macrophages. *Infect. Immun.* **43**: 1033–1040.

- 7a. **Freedman, V. H., M. Meisler, D. Lepay, W. A. Scott, and S. C. Silverstein.** 1984. Failure to elicit activated macrophages in light and pale ear mutant mice after treatment with BCG. *Fed. Proc.* **43**:1800. (Abstract.)
8. **Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman, and R. M. Locksley.** 1989. Reciprocal expression of interferon- γ or interleukin-4 during the resolution or progression of murine leishmaniasis: evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* **169**:59–72.
9. **Heinzel, F. P., M. D. Sadick, S. S. Mutha, and R. M. Locksley.** 1991. Production of interferon- γ , interleukin-2, interleukin-4, and interleukin-10 by CD4⁺ lymphocytes in vivo during healing and progressive murine leishmaniasis. *Proc. Natl. Acad. Sci. USA* **88**:7011–7015.
10. **Howard, M., and A. O'Garra.** 1992. Biological properties of interleukin-10. *Immunol. Today* **13**:198–200.
11. **Miralles, G. D., M. Y. Stoeckle, D. F. McDermott, F. D. Finkelman, and H. W. Murray.** 1994. Induction of Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis. *Infect. Immun.* **62**:1058–1063.
12. **Morris, L., A. Troutt, A. Kelson, E. Handman, and T. Aebischer.** 1993. Interleukin-4 but not gamma interferon production correlates with severity of murine cutaneous leishmaniasis. *Infect. Immun.* **61**:3459–3465.
13. **Murray, H. W.** 1990. Effect of continuous administration of interferon- γ in experimental visceral leishmaniasis. *J. Infect. Dis.* **161**:992–994.
14. **Murray, H. W., J. S. Cervia, J. Hariprasad, A. P. Taylor, M. Y. Stoeckle, and H. Hochman.** 1995. Effect of granulocyte-macrophage colony-stimulating factor in experimental visceral leishmaniasis. *J. Clin. Invest.* **95**:1183–1189.
15. **Murray, H. W., and J. Hariprasad.** 1995. Interleukin-12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. *J. Exp. Med.* **181**:387–391.
16. **Murray, H. W., J. Hariprasad, B. Aguero, T. Arakawa, and H. Yeganegi.** 1995. Intracellular antimicrobial response of the T cell-deficient host to cytokine therapy: effect of interferon- γ in experimental visceral leishmaniasis in nude mice. *J. Infect. Dis.* **171**:1309–1313.
17. **Murray, H. W., J. Hariprasad, and R. Fictl.** 1993. Treatment of experimental visceral leishmaniasis in a T-cell-deficient host: response to amphotericin B and pentamidine. *Antimicrob. Agents Chemother.* **37**:1504–1505.
18. **Murray, H. W., G. D. Miralles, M. Y. Stoeckle, and D. F. McDermott.** 1993. Role and effect of interleukin-2 in experimental visceral leishmaniasis. *J. Immunol.* **151**:929–934.
19. **Murray, H. W., L. Oca, A. M. Granger, and R. D. Schreiber.** 1989. Successful response to chemotherapy in experimental visceral leishmaniasis: requirement for T cells and effect of lymphokines. *J. Clin. Invest.* **83**:1253–1259.
20. **Murray, H. W., J. J. Stern, K. Welte, B. Y. Rubin, S. M. Carriero, and C. F. Nathan.** 1987. Experimental visceral leishmaniasis: production of interleukin-2 and interferon- γ , tissue immune reactions, and response to treatment with interleukin-2 and interferon- γ . *J. Immunol.* **138**:2290–2296.
21. **Orn, A., E. M. Hakansson, M. Gidlund, U. Ramstedt, I. Axberg, and L. G. Lundin.** 1982. Pigment mutations in the mouse which also affect lysosomal functions lead to suppressed natural killer cell activity. *Scand. J. Immunol.* **15**:305–310.
22. **Reiner, S. L., S. Zheng, Z. Wang, L. Stowring, and R. M. Locksley.** 1994. *Leishmania* promastigotes evade triggering interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4⁺ cells during initiation of infection. *J. Exp. Med.* **179**:447–456.
23. **Sadick, M. D., F. P. Heinzel, B. J. Holaday, R. T. Pu, R. S. Dawkins, and R. M. Locksley.** 1990. Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody: evidence for a T cell-dependent, interferon- γ -independent mechanism. *J. Exp. Med.* **171**:115–127.
24. **Squires, K. E., M. Kirsch, S. C. Silverstein, A. Acosta, J. J. McElrath, and H. W. Murray.** 1990. Defect in the tissue immune response: experimental visceral leishmaniasis in euthymic C57BL/6 *ep/ep* mice. *Infect. Immun.* **58**:3893–3898.
25. **Squires, K. E., R. D. Schreiber, M. J. McElrath, B. Y. Rubin, S. L. Anderson, and H. W. Murray.** 1989. Experimental visceral leishmaniasis: role of endogenous IFN- γ in host defense and tissue granulomatous response. *J. Immunol.* **143**:4244–4249.
26. **Stern, J. J., M. J. Oca, B. Y. Rubin, S. L. Anderson, and H. W. Murray.** 1988. Role of L3T4⁺ and Lyt-2⁺ cells in experimental visceral leishmaniasis. *J. Immunol.* **140**:3971–3975.
27. **Tumang, M., C. Keogh, L. Moldawer, D. Helfgott, J. Hariprasad, and H. W. Murray.** 1994. Role and effect of TNF- α in experimental visceral leishmaniasis. *J. Immunol.* **153**:768–772.

Editor: J. M. Mansfield