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Defect in the Tissue Cellular Immune Response: Experimental Visceral Leishmaniasis in Euthymic C57BL/6 *ep/ep* Mice

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In BALB/c mice, successful defense against visceral leishmaniasis is T cell dependent, expressed by tissue granuloma formation, and probably mediated by macrophages activated by cytokines, including gamma interferon (IFN-γ). C57BL/6 *ep/ep* (pale ear) mice, which reportedly exhibit impaired IFN-γ production, were challenged with *Leishmania donovani* to determine the outcome of infection in a euthymic host with an apparent defect in lymphokine secretion. In BALB/c and normal C57BL/6 mice, *L. donovani* liver burdens peaked at 2 weeks and were largely eliminated by 4 weeks. In contrast, in pale ear mice, infection progressed until after 4 weeks and persisted at high levels at 8 weeks. The failure to resolve hepatic infections was not related to deficiencies in (i) Thy-1$^+$, L3T4$^+$, or Lyt-2$^+$ T cells; (ii) IFN-γ secretion; (iii) liver tissue Ia expression; (iv) macrophage antimicrobial capacity; or (v) antileishmanial antibody production. However, despite the anticipated influx of mononuclear cells into livers, these cells were not properly focused on the parasitized Kupffer cells, the inflammatory infiltrate receded prematurely, and mature granulomas failed to develop. These results suggest that there is a cellular immune defect at the tissue level and emphasize the critical role of granuloma formation in successful resolution of systemic intracellular infections.

In prior studies in which we used a model for visceral leishmaniasis in euthymic BALB/c mice (13, 17, 22), we correlated successful acquisition of resistance to intracellular *Leishmania donovani* infection with the emergence of the following two key expressions of the cellular immune response: (i) the capacity of T cells to respond to leishmanial antigen with secretion of the activating lymphokines, interleukin-2 (IL-2), and gamma interferon (IFN-γ); and (ii) the development of mature granulomas at the tissue foci of infection. The granulomatous reaction, comprising influxing T cells and mononuclear phagocytes, encircles parasitized macrophages and contains proliferating *L. donovani* (10, 17, 22); IFN-γ appears to activate the macrophages to kill intracellular parasites (5, 11, 15, 16).

In BALB/c mice infected with *L. donovani*, the formation of leishmanicidal tissue granulomas is strictly T cell dependent (17, 22), requires both L3T4$^+$ and Lyt-2$^+$ cells (10, 21), and as judged by the effect of treatment with a neutralizing monoclonal antibody, appears to be largely under the control of IFN-γ during the initial stages of infection (21). To extend the analysis of the tissue immune response to *L. donovani*, we examined C57BL/6 *ep/ep* (pale ear) mice, a euthymic strain which reportedly displays defects in macrophage activation and control over *Listeria monocytogenes* infections (V. H. Freedman, M. Meisler, D. Lepay, W. A. Scott, and S. C. Silverstein, Fed. Proc. **43**:1800, 1984) and a defect in IFN-γ secretion (M. Kirch, V. Freedman, M. Meisler, B. Naprstek, R. Schreiber, and S. C. Silverstein, J. Leukocyte Biol. **40**:295, 1986). Although T cells from pale ear mice were capable of responding to leishmanial antigen with IFN-γ production, visceral infections did not resolve as anticipated and persisted at high levels. Our results suggest that there is a novel defect in induction of tissue granulomas in pale ear mice and emphasize the critical role of an intact cellular response at the tissue level.

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MATERIALS AND METHODS

Mice. Female and male C57BL/6 *ep/ep* (pale ear, coat pigment mutant) mice were obtained from Jackson Laboratories, Bar Harbor, Maine, bred under conventional conditions, and used for experiments when they were 6 to 8 weeks old; 20- to 30-g BALB/c, C57BL/6, and C57BL/6 *bg/bg* (beige) mice were purchased from Charles River Laboratories, Wilmington, Mass.

Visceral infection and hepatic tissue analysis. To establish visceral infections, mice were infected intravenously with 10^7 *L. donovani* amastigotes (1 Sudan strain) obtained from infected hamster spleens (17). Liver parasite burdens, expressed as Leishman-Donovan units (LDU), were determined microscopically by using Giemsa-stained tissue imprints (17). Histologic responses to infection were evaluated by using Formalin-fixed liver sections stained with hematoxylin and eosin (17, 22). Using an ×63 objective, we first determined the number of discrete, amastigote-containing foci of infection present in 50 consecutive microscopic fields selected at random from each of two tissue sections of each liver (17, 22). The cellular reaction (granuloma formation) at each focus of infection was examined and then scored as indicated below (see Table 4, footnote *c*) by using a previously described grading system (17, 22). Frozen sections of paraformaldehyde–lysine–peroxidate-fixed livers were also stained by a monoclonal antibody-immunoperoxidase technique (10) for expression of the following surface markers (rat anti-mouse monoclonal antibody [specificity]): F4/80 (Kupffer cells, macrophages), M1/70 (C3bi receptor on monocytes, macrophages), B21.2 (I-A^{b,d} class II determinants), GK 1.5 (L3T4$^+$ cells), and 53-6.72 (Lyt-2$^+$ cells) (10).

T-cell enumeration. As previously described (22), spleen cells were incubated with rat monoclonal antibody and labeled with fluorescein isothiocyanate-labeled mouse anti-rat immunoglobulin G. The percentages of cells which expressed surface Thy-1, L3T4, and Lyt-2 were measured by using a model Epic 752 System apparatus (Coulter Electronics, Hialeah, Fla.) (22).

Assay for IFN-γ. A standard cytopathic effect inhibition bioassay, in which L929 cells and vesicular stomatitis virus were used (17), was employed to measure the IFN-γ activity of supernatants from duplicate cultures of spleen cells (5×10^6 cells per ml) stimulated for 48 h with optimal concentrations of either concanavalin A (5 μg/ml) or soluble *L. donovani* antigen (100 μg/ml) (17). The IFN-γ activity detected in the bioassay was comparable to the activity measured in an enzyme-linked immunosorbent assay in which an anti-murine IFN-γ monoclonal antibody was used (20).

Measurement of antileishmanial antibody. Soluble *L. donovani* antigen, prepared from promastigote sonic extracts (1), was used in an enzyme-linked immunosorbent assay to detect antileishmanial antibody in the serum. A titer of ≥1:640 was considered a positive test result (1).

Macrophage activities. To assess intracellular antimicrobial activity, cover slips containing resident peritoneal macrophages were cultivated overnight in medium alone or in medium containing 100 U of recombinant murine IFN-γ (Genentech, Inc., South San Francisco, Calif.) (16) per ml and then challenged with the intracellular test organism *Toxoplasma gondii* (14, 16). Antimicrobial effects were determined for duplicate cover slips by microscopic enumeration of the number of *T. gondii* per 100 macrophages immediately after a 30-min challenge (time zero) and 18 h later. The fold increase in replication was calculated by dividing the number of *T. gondii* per 100 cells present at 18 h by the number present at time zero (14). We attempted to use *L. donovani* as the in vitro test pathogen; however, during the course of these experiments intracellular amastigotes did not consistently survive well within unstimulated peritoneal macrophages from either uninfected or infected BALB/c or pale ear mice. However, the same macrophages from the same mice readily supported *T. gondii* replication (see Table 3). Although this in vitro result with *L. donovani* was unexpected (13, 16), the same amastigotes nevertheless produced the anticipated levels of visceral infection in both BALB/c (12, 13, 17, 21, 22) and C57BL/6 mice (17), caused persistent infections in pale ear mice, and induced fatal infections in hamsters.

Macrophage respiratory burst activity was examined on triplicate cover slips by determining the qualitative reduction of Nitro Blue Tetrazolium 1 h after either ingestion of opsonized zymosan particles or triggering with 100 ng of phorbol myristate acetate per ml (12).

RESULTS

Course of in vivo infection. As Fig. 1 shows, *L. donovani* replicated freely within the liver macrophages of BALB/c and pale ear mice during the first 2 weeks after infection. Thereafter, however, BALB/c mice acquired sufficient resistance to inhibit visceral replication and reduce their liver parasite burdens. In contrast, the liver burdens in pale ear mice continued to increase during the first 4 weeks and had not declined appreciably at 8 weeks. In subsequent experiments, we compared the course of visceral infection in pale ear mice with that in the parent C57BL/6 (black) strain. While pale ear mice again failed to appropriately control *L. donovani* infection (Fig. 2), C57BL/6 mice readily developed resistance after 2 weeks in a fashion similar to resistance development in BALB/c mice. In a single experiment performed in parallel with the experiments whose results are shown in Fig. 2, mice belonging to another homozygous coat pigment mutant strain, natural killer cell-deficient C57BL/6 *bg/bg* (beige) mice, were also challenged with *L. donovani*. The liver parasite burdens in three beige mice at both 2 and 4 weeks after infection (338 ± 57 and 16 ± 3 LDU, respectively) were comparable to those in C57BL/6 mice. These results agree with previous observations (8, 17), indicating that beige mice control *L. donovani* in the liver after 2 or 3 weeks of infection.

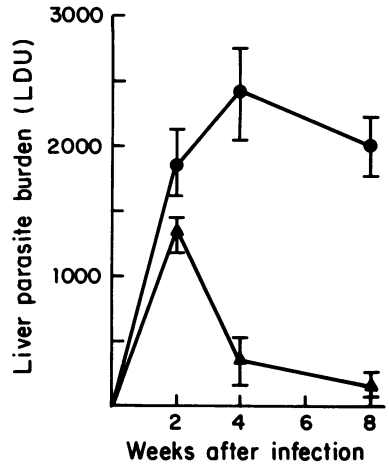

FIG. 1. Course of *L. donovani* infection in BALB/c (▲) and pale ear (●) mice. The results shown (mean ± standard error of the mean for three mice) are from one experiment and are representative of the results of the four experiments which were performed.

T-cell populations and function. Since successful resistance to *L. donovani* and resolution of visceral infection are

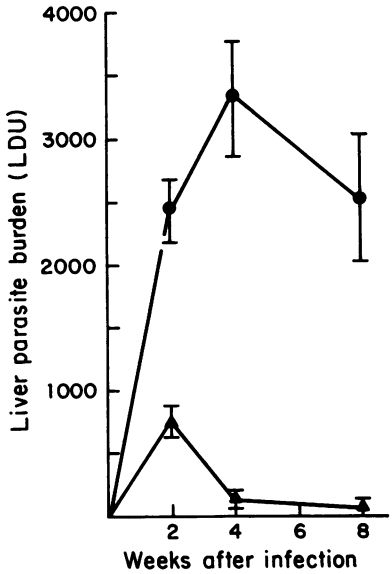

FIG. 2. *L. donovani* infection in normal C57BL/6 (▲) and pale-ear (●) mice. The results shown (mean ± standard error of the mean for three mice) are from one experiment and are representative of the results of the three experiments which were performed.

TABLE 1. Immunofluorescent quantification of spleen T cells[a]

Mice	% of spleen cells that were:		
	Thy-1$^+$	L3T4$^+$	Lyt-2$^+$
BALB/c	27 ± 1	27 ± 1	7.5 ± 2
Pale ear	26 ± 1	21 ± 2	8.2 ± 3

[a] Measurements were made 4 weeks after *L. donovani* infection. The values are means ± standard errors of the means for a total of four mice from two experiments.

T cell dependent (17, 22) and require the presence of both L3T4$^+$ and Lyt-2$^+$ cells (22), the failure of pale ear mice to clear infections suggested the possibility that there is a quantitative or functional T-cell defect which might include impaired IFN-γ production (Kirch et al., J. Leukocyte Biol. 40:295, 1986). In uninfected BALB/c and pale ear mice, however, the mean numbers of total cells per spleen were similar (7.9×10^7 and 8.5×10^7 cells, respectively; $n = 3$), as were the percentages of Thy-1$^+$ cells (40 and 38%, respectively; $n = 3$). As Table 1 shows, the percentages of Thy-1$^+$, L3T4$^+$, and Lyt-2$^+$ cells in the spleens of infected mice were also comparable. In addition, at both 4 and 8 weeks after infection, spleen cells from pale ear mice readily responded to a mitogen and a specific antigen with the secretion of IFN-γ (Table 2). The capacity to generate IFN-γ appeared to exclude the possibility of a generalized T-cell defect since spleen cell production of *L. donovani* antigen-induced IFN-γ in this model is T cell dependent (e.g., absent in athymic mice [17]), involves both L3T4$^+$ and Lyt-2$^+$ cells (22), and requires IL-2 (Squires and Murray, Clin. Res. 37:441, 1989).

B-cell function. Although the humoral response to infection is not thought to play a significant role in host defense against visceral leishmaniasis (18), we also examined B-cell function in pale ear mice by measuring serum levels of antileishmanial antibody. At 4 weeks after infection, serum antibody was not detected in BALB/c, C57BL/6, or pale ear mice. By 8 weeks after infection, antibody titers were ≥1:640 in all mice tested, and in two experiments the mean titers were similar for the three strains (BALB/c mice, 2,432 ± 687; C57BL/6 mice, 3,904 ± 1,589; pale ear mice, 3,931 ± 1,145 [means ± standard errors of the means of reciprocals of titers; five to seven mice per group]).

Macrophage function. To explore the possible presence of an intrinsic macrophage defect or a defect in achieving the activated microbicidal phenotype, we next examined peritoneal macrophages from infected pale ear mice. As Table 3 shows, pale ear mouse macrophages responded to soluble (phorbol myristate acetate) and ingestible (zymosan) stimuli with respiratory burst activity (Nitro Blue Tetrazolium reduction [12]) and once stimulated with IFN-γ, readily acquired the capacity to kill or inhibit the intracellular protozoan *T. gondii*. The mechanisms utilized by the IFN-γ-activated macrophages to kill or inhibit *T. gondii* and *L. donovani* are similar (11).

TABLE 2. In vitro spleen cell production of IFN-γ[a]

Weeks after infection	IFN-γ production (U/ml)			
	Pale ear mice		BALB/c mice	
	ConA stimulated	Antigen stimulated	ConA stimulated	Antigen stimulated
4	160 ± 68	144 ± 79	160 ± 68	34 ± 21
8	122 ± 34	96 ± 44	224 ± 127	96 ± 27

[a] Spleen cells from infected mice were stimulated for 48 h with either concanavalin A (ConA) or *L. donovani* antigen. The values are from three experiments at each time point and are the means ± standard errors of the means of the reciprocals of the IFN-γ titers. In a single experiment, concanavalin A- and leishmanial antigen-stimulated spleen cells from C57BL/6 mice that were infected for 8 weeks produced 128 U of IFN-γ per ml in response to either stimulus.

TABLE 3. Macrophage oxidative and antimicrobial activities[a]

Mice	Nitro Blue Tetrazolium reduction assay (% of cells positive)		Anti-*T. gondii* activity assay (fold increase in *T. gondii* per 100 cells)	
	Phorbol myristate acetate stimulus	Zymosan stimulus	Control	IFN-γ stimulated
BALB/c	81 ± 3	85 ± 4	6.8 ± 0.2	0.9 ± 0.1
Pale ear	90 ± 2	82 ± 3	5.6 ± 1.1	0.7 ± 0.1

[a] Peritoneal macrophages from mice that were infected for 4 weeks were cultivated overnight prior to assay in medium alone (for the Nitro Blue Tetrazolium assay) or in medium alone or medium containing 100 U of IFN-γ per ml (for the *T. gondii* infection assay). The values are means ± standard errors of the means from three experiments. An increase of more than onefold indicated that there was parasite killing.

Tissue reaction. Unable to identify an apparent T-cell, B-cell, or macrophage defect to explain the failure of pale ear mice to resolve visceral infection, we next examined the cellular immune response at the tissue level by using a method previously used for BALB/c mice (10, 17, 22).

During the first 2 weeks after *L. donovani* infection, livers of BALB/c mice contained single or fused parasitized Kupffer cells and showed a modest influx of mononuclear cells. By 2 to 4 weeks after infection, most infected foci exhibited early granuloma formation with well-localized cellular accumulations (Fig. 3A). After 4 weeks, fully developed mature granulomas were abundant (Table 4 and Fig. 3B). As shown in a previous study (10), mature granulomas consisted of a core of fused, parasitized Kupffer cells (Ia$^+$, F4/80$^+$) surrounded by an organized lymphocyte mantle comprising M1/70$^+$ cells and equal numbers of both L3T4$^+$ and Lyt-2$^+$ cells. At 8 weeks after challenge, most granulomas in BALB/c mice were devoid of amastigotes, which correlated with the >80% decline in liver parasite burdens. Similar events occurred in the hepatic tissue of normal C57BL/6 mice (Table 4).

In pale ear mice, the expression of Ia and F4/80 in infected foci and uninvolved liver parenchyma was indistinguishable from the expression in BALB/c mice 4 weeks after infection (data not shown). However, the orderly progression of the tissue immune response and the formation of granulomas failed to develop in pale ear mice. Although influxing mononuclear cells were plentiful at 4 weeks and included M1/70$^+$ cells and comparable numbers of L3T4$^+$ and Lyt-2$^+$ cells (data not shown), the cellular infiltrate was diffusely distributed (Fig. 3C) and not focused on the appropriate target, the parasitized Kupffer cells (Fig. 3D). In particular, the organized lymphocyte mantle, which defines a mature granuloma in this model (17, 22), essentially failed to evolve, and overall, only 8% of infected foci in pale ear mice were sufficiently well developed to be scored as mature granulomas 8 weeks after infection (Table 4).

DISCUSSION

Challenging pale ear mice with *L. donovani* resulted in visceral parasite burdens that persisted at high levels during a period in which other strains of innately susceptible mice

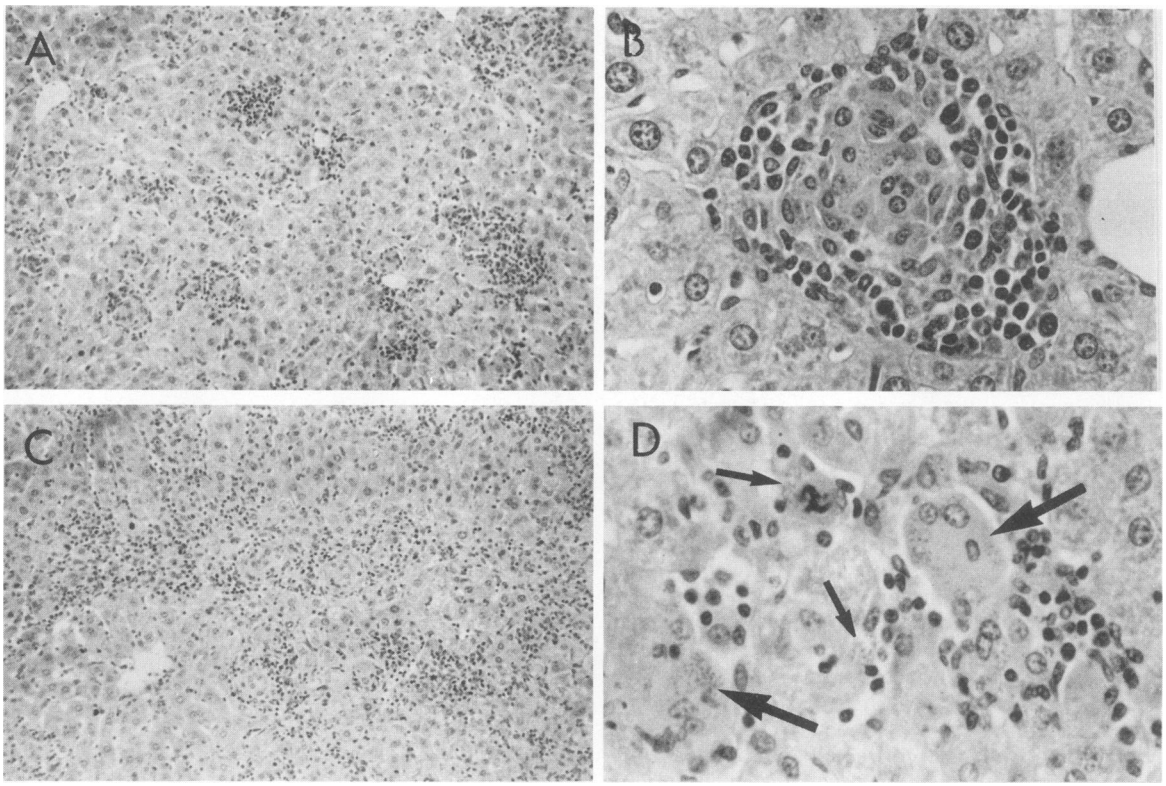

FIG. 3. Representative histologic sections of livers from BALB/c mice (A and B) and pale ear mice (C and D) that were infected for 4 weeks. (A) Well-localized cellular accumulations at sites of infection in BALB/c mice. Magnification, ×50. (B) Fully developed granuloma in BALB/c mice with a central core of fused, parasitized Kupffer cells surrounded by an organized mononuclear cell mantle. Magnification, ×500. (C) Mononuclear cells were abundant in pale ear mice but diffusely distributed. Magnification, ×50. (D) Four infected foci in pale ear mice comprising both fused (large arrows) and single (small arrows) parasitized Kupffer cells. Infiltrating mononuclear cells were not appropriately focused on the infected cells. Magnification, ×500.

(BALB/c, C57BL/6) resolved their infections. The failure to reduce liver burdens occurred despite (i) a full numerical complement of T cells, (ii) an intact T-cell capacity to respond to *L. donovani* antigen with the secretion of the macrophage-activating lymphokine IFN-γ (11), (iii) apparently normal macrophage antimicrobial mechanisms, and (iv) the production of specific antileishmanial antibody. However, pale ear mice were capable of successfully preventing the unchecked visceral replication of *L. donovani* that occurs in T-cell-deficient nude mice (17, 22). Since acquired resistance to *L. donovani* is T cell dependent (17, 22), the presence of functional (albeit seemingly misdirected) T cells may have permitted pale ear mice to exert an incomplete (leishmanistic) host defense response.

TABLE 4. Liver histologic responses to *L. donovani* infection[a]

Mice	No. of weeks after infection	Liver burden (LDU)	No. of infected foci[b]	% of infected foci with[c]:			No. of uninfected granulomas[b]
				No tissue reaction	Early granulomas	Mature granulomas	
BALB/c	4	737	68	8	60	32	11
	8	147	25	2	49	49	20
C57BL/6	4	150	74	6	70	24	9
	8	64	30	2	52	46	22
Pale ear	4	2,419	121	22	75	3	0
	8	2,017	94	15	77	8	3

[a] Liver imprints and histologic sections were examined after 4 and 8 weeks for amastigote liver burdens and tissue reactions. The values are from two experiments, and for each time point the value is the mean derived from an examination of two random tissue sections from each of the livers of a total of four mice. The standard error of the mean was <10% for each value.

[b] Per 50 ×63 microscopic fields.

[c] After we determined the number of discrete infected foci per 50 ×63 microscopic fields, the tissue reaction at each focus was scored as previously reported (2), as follows: no tissue reaction (single infected Kupffer cell with no mononuclear cell infiltrate), early granuloma (fused infected Kupffer cells with some mononuclear cell infiltrate), mature granuloma (core of fused infected Kupffer cells surrounded by an organized mononuclear cell infiltrate), or uninfected granuloma (resolving, devoid of amastigotes).

Taken together, our observations suggest that a defect in the cellular immune response at the tissue level may be responsible for the inability of pale ear mice to resolve visceral infections. This proposed defect appears to be subtle, however, since the components thought to be involved in effective tissue granuloma formation (2, 10, 17, 22) were all attracted to and/or well-represented in the livers of infected pale ear mice ($L3T4^+$ and $Lyt\text{-}2^+$ cells, $M1/70^+$ cells, class II MHC products [Ia], and parasitized macrophages) (10, 17, 22). Nevertheless, the orderly assembly of the cellular components around the critical targets (the parasitized Kupffer cells [10, 17, 22]) and the maintenance of the components at infected foci both appeared to be impaired. Therefore, our findings raise the possibility that there is production of an inhibitor or, alternatively, a defect in the generation of or responsiveness to local cellular or soluble stimuli responsible for attracting inflammatory cells to the specific site of infection. Such signals are presumably in-

volved in both initiating tissue granulomas and sustaining their cellular composition and configuration. Indeed, at a number of infected foci in pale ear mice that were infected for 8 weeks, the cellular infiltrate was actually diminished compared with cellular infiltrate in mice that were infected for 4 weeks, suggesting that the granulomatous process was prematurely and inappropriately receding. Under these conditions, critical cell-cell and cytokine-cell interactions may not have had the opportunity to develop.

Additional experiments are now under way in which pale ear mice are being used to analyze the tissue immune response by examining (i) possible inhibitors of chemotaxis; (ii) the potential role of suppressor cells (12) or suppressive cytokines, such as IL-3 (4) and IL-4 (9, 19); (iii) deficient secretion of other cytokines that are possibly involved in granuloma formation and/or antileishmanial activity (tumor necrosis factor [7, 23], granulocyte-macrophage colony-stimulating factor [24; J. Cervia, H. Rosen, M. Narachi, and H. Murray, Clin. Res. **38**:236A, 1990]); and (iv) granuloma induction in response to stimuli unrelated to *L. donovani*.

While we await the results of these experiments, our current findings suggest the following conclusions. First, although IFN-γ appears to play an important role in inducing macrophages to kill *L. donovani* (5, 11, 15, 16) and in the formation of *L. donovani*-induced granulomas (21), there may also be T-cell-dependent but IFN-γ-independent mechanisms that contribute to both of these activities (21). These latter mechanisms may be defective in pale ear mice. Second, given the intact production of IFN-γ by pale ear mouse spleen cells and the presence of Ia expression in the livers of infected pale ear mice, a primary defect in accessory cell function seems unlikely (6, 11). Similarly, since leishmanial antigen-induced IFN-γ secretion is also dependent upon the presence of IL-2 (Squires and Murray, Clin. Res. **37**:441, 1989), our data do not appear to implicate deficient IL-2 production in pale ear mice. Third, the possibility of a defect in local generation of IFN-γ also seems remote because tissue Ia expression is largely (but not exclusively) IFN-γ dependent (3, 11, 21), and it seems improbable that influxing peripheral blood-derived but not splenic T cells are defective in lymphokine secretion. Finally, a primary defect in the responsiveness of pale ear Kupffer cells to cytokines (or other mechanisms which may induce macrophage antileishmanial effects [25]) also appears unlikely since intracellular hepatic infection does not proceed in an uninhibited fashion, as it does for example, in nude mice (17, 22).

Together, these observations extend the spectrum of immunologic defects in visceral leishmaniasis to the tissue level and emphasize the importance of the orderly assembly of T cells and macrophages into granulomas in the control of systemic intracellular infections. Of additional importance is our finding that the pale ear mutation may provide a model that is useful for identifying the mechanisms that govern the tissue arrangement of T cells and macrophages and for characterizing the roles of these cells in inducing and maintaining antimicrobial activity within granulomas.

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