Progression of Visceral Leishmaniasis Due to *Leishmania infantum* in BALB/c Mice Is Markedly Slowed by Prior Infection with *Trichinella spiralis*

Déborah Rousseau,1 Yves Le Fichoux,1 Xavier Stien,2 Isabelle Suffia,1 Bernard Ferrua,1 and Joanna Kubar1

1 Groupe de Recherche en Immunopathologie de la Leishmaniose, Laboratoire de Parasitologie, Faculté de Médecine, 06107 Nice Cedex 2, France
2 Laboratoire de Toxicologie, 06107 Nice Cedex 2, France

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We investigated in BALB/c mice the influence of the immunological environment created by the nematode *Trichinella spiralis* on the course of visceral leishmaniasis due to *Leishmania infantum*. On the day of Leishmania inoculation (day 0), mice, *T. spiralis* infected 7 days earlier, presented increased gamma interferon (IFN-γ), interleukin-4 (IL-4), and IL-5 mRNA levels locally and systemically and increased the potential of spleen cells to synthesize IFN-γ and IL-4 after activation in vitro. Eighteen days after *Leishmania* inoculation (day 18), corresponding to the acute phase of leishmaniasis, the hepatic amastigote burden in mice coinfected with *L. infantum* and *T. spiralis* (LT mice) was significantly lower (*P < 0.001*) than that in mice infected with *L. infantum* only (L mice). IFN-γ and IL-4 mRNAs were overexpressed in livers of LT and L mice. On day 70, corresponding to the chronic phase, the spleen amastigote load was significantly lower (*P < 0.004*) in LT mice than it was in L mice. Splenic IFN-γ transcripts were overexpressed in both L and LT mice. After Leishmania-specific in vitro stimulation, cytokine production was enhanced in both groups, but spleen cells from LT mice produced significantly more IFN-γ than did spleen cells from L mice. Our data (i) generalize previous results indicating the lack of a clear-cut correlation between the outcome of murine visceral leishmaniasis and the type of cytokine pattern and (ii) demonstrate that in LT mice, leishmaniasis takes a markedly milder course than it does in L mice, providing information on the potential consequences of coinfection in a mammalian host.

MATERIALS AND METHODS

Mice and experimental protocol. Five-week-old female BALB/c mice (Iffa Credo, L’Arbresle, France), maintained in a positive pressure chamber (Prunelle, France), were used for experimentation at 7 to 8 weeks of age. Mice were split into following groups of 10 animals: (i) mice infected with *L. infantum* only (T mice), (ii) mice coinfected with *T. spiralis* only (T mice), (iii) mice coinfected with *L. infantum* and *T. spiralis* (LT mice), and (iv) noninfected littermates (used as age-matched controls). First, two groups of mice (T and LT mice) were infected with *T. spiralis* larvae; next, 7 days later, two groups of mice (L and LT mice) were infected with *L. infantum*. Hereafter, the day of infection with *L. infantum* is termed day 0. Eighteen and seventy days after *L. infantum* inoculation, five mice from each group were anesthetized (sodium thiopental [50 μg/g of body weight]) and sacrificed. Livers, spleens, and diaphragms were taken under sterile conditions and weighed. Blood samples for serum separation were obtained from the orbital chamber and pooled within each group. Blood samples for the preparation of peripheral blood mononuclear cells (PBMC) were drawn from the vena cava and also pooled.

Parasites and infection. *L. infantum* MON1 (MHOM/FR/94/LPN101) was maintained by serial passages in Syrian hamsters. The promastigote form was cultured under standard conditions (39) and used for infection after three in vitro passages and 2 weeks of growth.

- Corresponding author. Mailing address: Groupe de Recherche en Immunopathologie de la Leishmaniose, Laboratoire de Parasitologie, Faculté de Médecine, Ave. de Valombrose, 06107 Nice Cedex 02, France. Phone: 33 4 93 37 76 84. Fax: 33 4 93 37 76 84. E-mail: kubar@unice.fr.
passages as stationary-phase cells from 7-day-old cultures (2.5 × 10^6 promastigotes/mL). After two washings (15-min sedimentation at 2,500 g × 4°C), promastigotes were resuspended in 0.9% NaCl at 2 × 10^6 cells/mL and injected into the caudal vein at 10^5 promastigotes/mouse. The evaluation of Leishmania infections induced by blinded mice enumeration (liquid nitrogen)-treated and finally by eight sonication (1 min) on ice. The suspensions were centrifuged (15,000 × g × 4°C for 15 min), and the supernatants were stored at −80°C. The protein concentrations were determined with a MicroBCA protein assay reagent kit (Pierce).

Detection of anti-T. spiralis IgE. Specific IgE in the sera of infected and control mice was detected by a classic ELISA procedure. Briefly, wells of high-binding-capacity microtiter plates (Greiner) were coated overnight with 1 μg of T. spiralis soluble exoesterase buffer (liquid nitrogen)-treated L. infantum and finally by eight sonication (1 min) on ice. The suspensions were centrifuged (15,000 × g × 4°C for 15 min), and the supernatants were stored at −80°C. The protein concentrations were determined with a MicroBCA protein assay reagent kit (Pierce).

RESULTS

Clinical evaluation of infected and coinfected mice. Wasting, hepatomegaly, and splenomegaly are known aspects of human visceral leishmaniasis. The weights of L. infantum-infected BALB/c mice (L. mice) followed the standard weight curve. No hepatomegaly or splenomegaly was observed in either group, although liver weight to standard deviation [SD] was 1.3 ± 0.2 g). In contrast, splenic weights increased from 140 ± 32 mg on day 0 to 198 ± 37 and 148 ± 34 mg on day 18 and to 442 ± 63 and 249 ± 131 mg on day 70 in L mice (6.6 ± 0.1 LDU [range, 0.2 to 0.3 LDU]; LT mice, 0.5 ± 0.1 LDU [range, 0.2 to 0.3 LDU]; LT mice, 0.5 ± 0.1 LDU [range, 0.2 to 0.3 LDU]; LT mice, 0.5 ± 0.1 LDU [range, 0.2 to 0.3 LDU]; LT mice, 0.5 ± 0.1 LDU [range, 0.2 to 0.3 LDU]).

Parasite burdens in infected and coinfected mice. The L. infantum loads in the livers and spleens of L and LT mice are shown in Fig. 1. Eighteen days after L. infantum inoculation (day 18), the hepatic amastigote load (Fig. 1A) of LT mice (mean ± SD, 33.4 ± 10.0 LDU; range, 23 to 44 LDU) was significantly lower (P < 0.001) than that of L mice (116.9 ± 34.0 LDU; range, 73 to 150 LDU). On day 70, the hepatic load of L mice decreased as expected (references 23 and 45 and our unpublished observations). In L mice, it was 16.1 ± 7.9 LDU (range, 9 to 24 LDU), roughly the same as that in LT mice (26.8 ± 19.2 LDU; range, 15 to 49 LDU). Unlike in the liver, in the spleen L. infantum multiplication continued between days 18 and 70 (references 36 and 45 and our unpublished observations). On day 18, the splenic parasite burden was very low and statistically not different between these two groups (L mice, 0.2 ± 0.1 LDU [range, 0.2 to 0.3 LDU]; LT mice, 0.5 ± 0.5 LDU [range, 0.1 to 1.5 LDU]). It increased notably on day 70 in the spleens of L mice (6.6 ± 2.4 LDU; range, 5 to 9 LDU), and at that time it was significantly higher (P = 0.004) than the leishmanial burden in LT mice (0.8 ± 0.9 LDU; range, 0 to 2 LDU).

On day 18 (25 days after T. spiralis ingestion), the ML burden in the diaphragms of LT mice was notably lower than that of T mice (4.7 ± 1.1 and 249 ± 3.7 ML/mg of diaphragm, respectively) and still significantly lower by day 70 (18.1 ± 2.7 and 28.6 ± 4.3 ML/mg, respectively).

Immunological status of mice at the time of infection with L. infantum. The immunological statuses of T mice and uninfected controls were determined on the day of L. infantum inoculation. The mRNA expression levels of type 1 and type 2

Antigen preparation. Leishmanial antigen preparation for functional studies was carried out as described previously (39). T. spiralis ML were suspended at 5,000 ML/mL in water containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1,000 μg of aprotinin per ml) and disrupted first in a Dounce homogenizer, then by five cycles of freeze (liquid nitrogen)-thaw (37°C) and finally by eight sonication (1 min) on ice. The suspensions were centrifuged (15,000 × g × 4°C for 15 min), and the supernatants were stored at −80°C. The protein concentrations were determined with a MicroBCA protein assay reagent kit (Pierce).
cytokines were assessed in blood, liver, and spleen samples by RT-PCR, and in the vitro capacity of spleen cells to produce cytokines was measured by ELISAs. Figure 2 shows that IFN-γ mRNAs were overexpressed in blood and liver samples and that IL-4 transcripts were overexpressed in blood, liver, and spleen samples of T mice compared to the levels in controls; IL-5 mRNAs were detected only in infected animals (the SI is indicated in arbitrary units). Figure 3A and C show that under polyclonal in vitro stimulation to gauge the potential of preinfected cells for development into either Th1 or Th2 cells, the production levels of both IFN-γ and IL-4 in T mice were much higher than those in control mice.

Evolution of cytokine mRNA expression in livers and spleens of infected and coinfecte mice. The in situ cellular responses to disease in the livers and spleens of L and LT mice were assessed in terms of IFN-γ, IL-4, and IL-5 mRNA expression by RT-PCR. Note that no direct comparison between cytokine mRNA levels in the liver and spleen should be made here; in these series of experiments, the RT-PCR analyses of RNAs from these two organs were performed independently. Figure 2 shows that on days 18 and 70 in the liver, IFN-γ mRNAs were overexpressed for L and LT mice, whereas IL-4 mRNAs were more greatly increased for LT mice. In the spleen, the most striking feature was overexpression of IFN-γ mRNAs in L and LT mice on day 70, compared with moderate increases in IFN-γ mRNAs on day 18 and in type 2 (IL-4 and IL-5) transcripts.

Capacity of in vitro-activated spleen cells to produce type 1 and type 2 cytokines. The capacity of spleen cells to respond to Leishmania-specific in vitro stimulation was analyzed by measuring IFN-γ and IL-4 production. Spleen cells were activated by 48-h incubation with live promastigotes (at 1 × 10^6, 2 × 10^6, and 4 × 10^6 parasites/ml) or total leishmanial antigens (at 10, 20, and 80 μg/ml), and the cytokine levels were measured in cell lysates. On day 18, there was no detectable cytokine production induced by cell stimulation in either group, whereas on day 70, spleen cells did respond to specific activation. Figure 3B and D show that on day 70, after stimulation with live parasites cells from L mice produced markedly more IFN-γ than did cells from LT mice. Promastigote-induced IL-4 secretion was slightly higher for L mice than it was for LT mice, and both were higher than that for controls. The same profiles and orders of magnitude for cytokine production were induced by total leishmanial antigens (not shown). Cells from all groups of infected mice showed basal cytokine levels that were greater than those of controls, probably due to the presence of parasites in spleen cell preparations (estimated to be 0.5 × 10^6 to 1 × 10^6/well for L mice and 1 × 10^6 to 1.5 × 10^6/well for LT mice [corresponding to 0.2 and 0.03 μg of leishmanial proteins, respectively]). We found by cytofluorimetry that T lymphocytes, B lymphocytes, and macrophages represented 55 to 65, 30 to 40, and 10 to 18% of spleen cells, respectively, and there was no major differences between the results for various experimental groups of mice.

IgE response. Anti-T. spiralis IgE and anti-L. infantum IgE in pooled serum of five mice from each experimental group were measured by ELISA. Specific anti-T. spiralis larva IgE levels, expressed as optical density readings, were as follows (mean ± SD): 0.10 ± 0.01 on day 0 for T mice; 0.19 ± 0.01 and 0.14 ± 0.001 on day 18 for T and LT mice, respectively; 0.28 ± 0.003 and 0.40 ± 0.004 on day 70 for T and LT mice, respectively; 0.070 ± 0.01 for L mice and controls. Specific anti-L. infantum IgE was not detectable in any group of mice.

DISCUSSION

We studied the influence of the immunological environment created by preinfection with the nematode T. spiralis on the course of murine visceral leishmaniasis. In general, intracellular parasitism is associated mainly with type 1 cytokine responses, whereas extracellular helminths induce predominantly type 2 responses (12, 19, 35, 42). Our working assumption was that the course of leishmaniasis in mice infected first with T. spiralis and then 1 week later with L. infantum (LT mice) would be more severe than that in mice infected with Leishmania only (L mice). Unexpectedly, in LT mice, both pathologies, leishmaniasis and trichinellosis, appeared to take a markedly milder progression than in simply infected L or T mice.

On the day of Leishmania inoculation, mice infected with T. spiralis 7 days earlier presented a hyperstimulated immunological state, compared to that of controls. It involved both type 1 (IFN-γ) and type 2 (IL-4 and IL-5) cytokines. It was manifest both locally (in the liver and spleen) and systemically (in the blood). It was characterized at the transcriptional level by overexpression of IFN-γ, IL-4, and IL-5 mRNAs and at the protein level by the potential of spleen cells to synthesize IFN-γ and IL-4 after in vitro polyclonal stimulation. This apparent lack of cross-regulation between type 1 and type 2 activities in early infection with T. spiralis (8, 14, 28) suggests...
that cells other than those which belong to Th1 and Th2 subpopulations contribute to cytokine production (12, 19).

The next assessment was carried out 18 days after Leishmania inoculation. Day 18 of leishmaniasis in BALB/c mice (Lsh H-2d) infected with viscerotropic Leishmania species corresponds to the acute phase of disease, as defined by hepatic parasite proliferation (4). We found that although on day 18 the amastigote burden in the livers of L mice was indeed high, in the livers of LT mice it was significantly lower (P < 0.001). Our analysis of the immune state in the liver at the same stage of the disease (day 18) showed that both IFN-γ and IL-4 mRNAs were overexpressed by L and LT mice.

We then showed that preinfection of mice with T. spiralis continued to exert its protective effect on the course of leishmaniasis beyond the acute phase. At the end of the eighth or ninth week after infection, the hepatic parasite counts in BALB/c mice drop to very low levels (23, 45). This stage of disease is referred to as the chronic or recovery phase (4). During this phase, however, Leishmania organisms do proliferate in the spleen and the splenic burden steadily increases (references 36 and 45 and our unpublished observations). We showed that in contrast to L mice, which allowed notable parasite multiplication between days 18 and 70, LT mice delayed, in fact almost prevented, amastigote proliferation; the splenic load of LT mice, as measured 10 weeks after Leishmania infection, was significantly lower (P = 0.004) than that of L mice. A concurrent analysis of the cellular immune response in the spleen on day 70 showed that IFN-γ mRNA was overexpressed in both L and LT mice. In vitro cytokine production after Leishmania-specific stimulation was also enhanced in both groups, but spleen cells from L mice showed a much higher capacity to produce IFN-γ than did cells from LT mice.

An attempt at establishing a direct correlation between parasitological and immunological states at a given time would probably be unreliable. For instance, on day 70 the splenic amastigote load of L mice was high in spite of high IFN-γ levels and that of LT mice was low in spite of high IL-4 levels. However, the parasite burden, as determined at a given time, is more the result of all previous immune responses rather than a direct reflection of the concurrent immunological environment. The protection from L. infantum manifested by LT mice on days 18 and 70 may have been due partly to high levels of IFN-γ present locally and systemically on day 0, but it has previously been shown that in murine visceral leishmaniasis IFN-γ is not a unique protective cytokine (41). A recent study (45) of cytokine responses at 6 weeks after L. infantum infection of BALB/c mice showed that in vitro-stimulated spleen cells produced IFN-γ, whereas T cells isolated from liver gran-
ulomas did not. The authors concluded that the absence of IFN-\(\gamma\) in the liver correlated with high amastigote burden and that its presence in the spleen correlated with increased parasite killing. However, at the time of those analyses, although the hepatic load was higher than that in the spleen, it was in its decreasing phase, whereas the contrary was true for the splenic load (low but increasing). An interesting model (22), in which BALB/c mice were presensitized with injections of heat-killed \(L. major\) promastigotes, was recently reported. That manipulation induced a cross-reactive Th2 response which exacerbated visceral leishmaniasis (22), but our model of coinfection with two live unrelated pathogens enhanced both the Th1 and Th2 early environments and slowed the progression of the disease. The data reported here are relative to a particular protocol in which \(T. spiralis\) inoculation was carried out at the end of the intestinal phase and the beginning of the larva migration phase of trichinellosis (6). Whether the chronic phase of trichinellosis has an effect on the course of leishmaniasis is unknown. In yet another model of coinfection (1), \(L. tropica\)-infected BALB/c mice reportedly had the ability to partially inhibit \(T. gondii\) replication in vivo.

One of our present hypotheses on mechanism(s) of \(T. spiralis\)-induced modulation of the course of visceral leishmaniasis is based on a recent work by Vouldoukis et al. (44). They showed that ligation of the low-affinity receptor for IgE (FcεRII/CD23) induced the killing of \(L. major\) amastigotes due to the generation of nitric oxide in human macrophages in vitro. Moreover, induction of receptor CD23 by IL-4 on human monocytes has been previously shown (43). Whether these mechanisms hold in vivo and in other animal models and whether the ligation of CD23 induces the killing of \(Leishmania\) species other than \(L. major\) remain to be established. In our model, \(T. spiralis\)-specific IgE was produced quite early in infection. The concomitant presence in LT mice of specific IgE and the corresponding \(T. spiralis\) antigens could induce the formation of immune complexes, CD23 ligation, and subsequent amastigote killing. If this mode of action proves to be real and can be generalized, then coinfection with other helminths may also bring about modulation of the course of leishmaniasis.

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![FIG. 3. Capacity of spleen cells to synthesize IFN-\(\gamma\) (A and B) and IL-4 (C and D) after polyclonal (A and C) or \(Leishmania\)-specific (B and D) in vitro stimulation. (A and C) Spleen cells isolated on day 0 from five T mice and five age-matched naive controls were activated by incubation for 48 h with the indicated concentrations of concanavalin A (Con A) and phorbol 12-myristate 13-acetate (PMA) (2.5 \(\times\) 10\(^6\) cells/ml), and cytokine synthesis was measured in duplicate in cell lysates by sandwich ELISAs. The results shown are from one representative experiment of the two performed and are means \(\pm\) SDs. (B and D) Spleen cells were isolated from L, LT, and age-matched naive mice 70 days after \(L. infantum\) inoculation and activated by increasing concentrations of promastigotes (proM), as indicated. Sandwich ELISAs were carried out after 48 h of incubation; the results shown are from one representative experiment of the two performed and are means \(\pm\) SDs of duplicate determinations.

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