T-Cell Responses to Immunodominant LACK Antigen Do Not Play a Critical Role in Determining Susceptibility of BALB/c Mice to *Leishmania mexicana*

FABIOLA AGUILAR TORRENTERA,[1,2]† NICOLAS GLAICHENHAUS,[3]
JON D. LAMAN,[4] AND YVES CARLIER[1]*

*Laboratoire de Parasitologie, Université Libre de Bruxelles (ULB), Brussels, Belgium[1]; Institut de Pharmacologie
Moléculaire et Cellulaire, CNRS, Valbonne, France[3]; Department of Immunology, Erasmus University
Rotterdam (EUR), Rotterdam, The Netherlands[4]; and Departamento de Inmunologia, Escuela
Nacional de Ciencias Biologicas, Instituto Politecnico Nacional, Mexico City, Mexico[2]*

Although BALB/c mice develop lesions when infected with *Leishmania mexicana*, the mechanisms which are responsible for susceptibility to this parasite have not been elucidated. In contrast, susceptibility of BALB/c mice to *Leishmania major* has been shown to depend on the early production of interleukin-4 (IL-4) by T cells which react to the parasitic LACK antigen. Here, we demonstrate that the lesions induced by *L. mexicana* are delayed compared to those induced by *L. major* but rapidly develop at later time points. Interestingly, while LACK-tolerant BALB/c-derived IE-LACK transgenic mice were resistant to *L. major*, they were susceptible to *L. mexicana* and developed lesions similar to those observed in wild-type BALB/c mice. The latter result was observed despite the fact that (i) LACK was expressed by *L. mexicana*, (ii) splenocytes from BALB/c mice were able to stimulate LACK-specific T-cell hybridoma cells when incubated with live *L. mexicana* promastigotes, and (iii) LACK-specific T cells contributed to IL-4 production in *L. mexicana*-infected BALB/c mice. Thus, in contrast to what was observed for *L. major*-infected mice, LACK-specific T cells do not play a critical role in determining susceptibility to *L. mexicana*. Although BALB/c mice are susceptible to both *L. major* and *L. mexicana*, the mechanisms which are responsible for susceptibility to these parasites are likely to be different.

Mice have been widely used as an animal model to study human leishmaniasis. In many cases, the outcome of the disease is different depending on the strains which are infected. In the case of mice infected with *Leishmania major*, it has been shown that CBA, C3H, C57BL/6, and B10.D2 mice develop cutaneous lesions that spontaneously heal, while BALB/c, SWR/J, DBA/2, and A/Jax mice develop nonhealing cutaneous lesions which are susceptible to dissemination (4, 20). Several studies have shown that the early production of interleukin-4 (IL-4), which is induced in the draining lymph nodes (LNs) of BALB/c mice 16 h after infection with *L. major*, is one of the critical events leading to the development of a counterprotective antiparasite Th2 response (5, 10, 12–14). IL-4 is produced by CD4⁺ T cells which express a highly restricted Vβ4 Vα8 receptor repertoire (11). These cells recognize a single parasite determinant derived from the antigen LACK (leishmania receptor for activated C kinase, for its homology with the mammalian receptors for activated C kinase [RACK]), a protein which is conserved among different *Leishmania* spp. and which accounts for 0.03% of the proteins expressed by *Leishmania* promastigotes or amastigotes (14, 17, 18). The critical role of the LACK-specific T cells in determining susceptibility to *L. major* is further demonstrated by the observation that BALB/c mice which have been made tolerant to LACK are resistant to infection (9, 11).

Although studies have shown that IL-4 is critical for susceptibility to *Leishmania mexicana* (23–25), it is not known whether LACK-specific T cells play a critical role in the animal model for this infection. To address this issue, we have compared the courses of *L. mexicana* and *L. major* infections in wild-type (WT) BALB/c mice and in BALB/c-derived IE-LACK transgenic mice which are tolerant to LACK as the result of its constitutive expression in the thymus. In contrast to what was observed for *L. major*-infected mice, we found that LACK-specific T cells do not seem to play a critical role in determining the susceptibility of BALB/c mice to *L. mexicana* infection.

BALB/c male mice (8 to 12 weeks old) were purchased from Banting & Kingman Universal Limited (Hull, United Kingdom). IE-LACK transgenic mice have been previously described (9). *L. mexicana* (strain MHOM/BZ/82/BEL21) and *L. major* (strain WHOM/IR/−173) promastigotes were grown in RPMI medium (Life Technologies, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Life Technologies), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Promastigote lysates were prepared by 10 cycles of freezing (in liquid nitrogen) and thawing (in a water bath at 37°C), and their endotoxin contents were lower than 0.20 pg/10⁶ promastigotes as determined by the *Limulus* amebocyte lysate-Coatest endotoxin assay (Chromogenix, Mölndal, Sweden). The LACK-derived peptide, previously identified as an immunodominant epitope in BALB/c mice (amino acids 161 to 172, of sequence SLEHPIVVSGSW) (17), was obtained from Chiron Mimotopes (Paris, France) and from P. Gourlet (Laboratory of Bio-

* Corresponding author. Mailing address: Laboratoire de Parasitologie, Faculté de Médecine, ULB, CP 616, Route de Lennik 808, B-1070 Brussels, Belgium. Phone: 32 2 555 62 55. Fax: 32 2 555 61 28. E-mail: ycarlier@ulb.ac.be.
† Present address: Escuela Nacional de Ciencias Biologicas, Instituto Politecnico Nacional, Mexico City, Mexico.
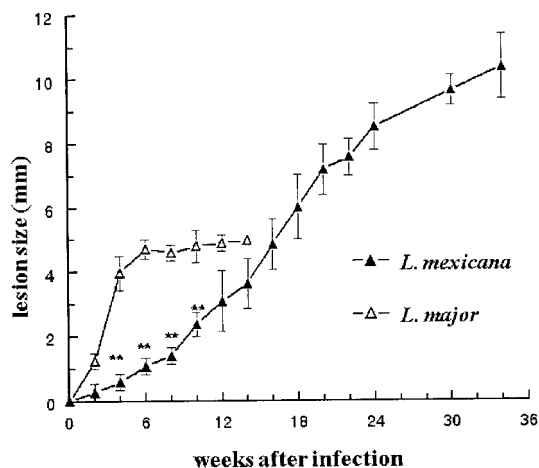
FIG. 1. Sizes of primary footpad lesions of BALB/c mice infected with *L. mexicana* or *L. major*. Results are from one representative experiment performed with 28 (*L. mexicana*) and 15 (*L. major*) animals and are expressed as means ± SEM. **, $P < 0.001$ (Student's *t* test, comparing *L. mexicana* and *L. major* infections). Three other experiments using 15, 16, and 12 animals infected with *L. mexicana* displayed similar results. This was also the case for another experiment including 10 mice infected with *L. major*.

chemistry, Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium). The LACK-specific T-cell hybridoma LMR16.2, producing IL-2 upon recognition of the immunodominant *L. major* LACK epitope in an I-A^d major histocompatibility complex class II-restricted fashion, was used as previously described (17). IL-2 and IL-4 levels were determined by enzyme-linked immunosorbent assays using commercially available kits (duoset from Genzyme Diagnostics, Cambridge, Mass.). The limit of detection was 5 pg/ml for both cytokines.

Diseases induced by *L. mexicana* and *L. major* in BALB/c mice. Although BALB/c mice are known to be susceptible to both *L. major* and *L. mexicana* (1), the diseases induced by these two species have not been compared under the same experimental conditions. In our study, *L. mexicana* or *L. major* promastigotes were injected into the left footpad of BALB/c mice. Each mouse received 10^7 stationary-phase promastigotes in a final volume of 25 μl of RPMI medium. The contralateral right footpad received an identical volume of RPMI medium, without parasites, as an internal control. Footpad thicknesses were measured with a metric caliper, and the difference between both measurements corresponded to lesion size. As shown in Fig. 1, while *L. mexicana* induced slowly progressive primary lesions which reached 10 to 11 mm after 34 weeks, *L. major* induced the rapid development of lesions which were 4 to 5 mm thick at 4 weeks postinfection (wpi). The cutaneous lesions induced by *L. major* became ulcerated more frequently and earlier than those induced by *L. mexicana*. Likewise, *L. major*, but not *L. mexicana*, induced spontaneous losses (mutilations) of necrotic footpad lesions which could not be measured after 14 weeks (Fig. 2A). While both *L. mexicana* and *L. major* induced secondary lesions which appeared in the contralateral posterior or anterior footpads, the eyelids, the nostrils, the ears, and the base of the tail, these lesions developed later in mice infected with *L. mexicana* than in those infected with *L. major* (Fig. 2B). Thus, while BALB/c mice

were susceptible to both *L. major* and *L. mexicana* infections, the courses of disease induced by these two parasites were markedly different.

Stimulation of a LACK-specific T-cell hybridoma by BALB/c splenocytes incubated with *L. mexicana* live promastigotes. To determine if antigen-presenting cells could process the *L. mexicana* LACK antigen and present it to T cells, splenocytes from BALB/c mice were incubated in 96-well plates (Nunc, Roskilde, Denmark) with the LACK-specific T-cell hybridoma LMR16.2 and *L. mexicana* or *L. major* live or lysed promastigotes. In some wells, splenocytes (5×10^5/well) were incubated with either live *L. mexicana* or *L. major* promastigotes for 6 h at 37°C. Other splenocyte-containing wells received *L. mexicana* or *L. major* lysate (at the corresponding parasite/splenocyte ratios used for live parasites) or the LACK-derived peptide (at concentrations ranging from 0.2 to 25.6 μM). The LMR16.2 hybridoma cells (10^5/well) were added to each well. Twenty hours later, cellular supernatants were harvested for subsequent measurement of IL-2 levels.

LACK peptide (data not shown) and both *L. major* and *L. mexicana* promastigotes (Fig. 3) induced the LMR16.2 hybridoma cells to secrete IL-2 in a dose-dependent manner. The fact that splenocytes incubated with *L. mexicana* promastigotes could stimulate the LMR16.2 T-cell hybridoma recognizing specifically the *L. major* LACK antigen suggested that the
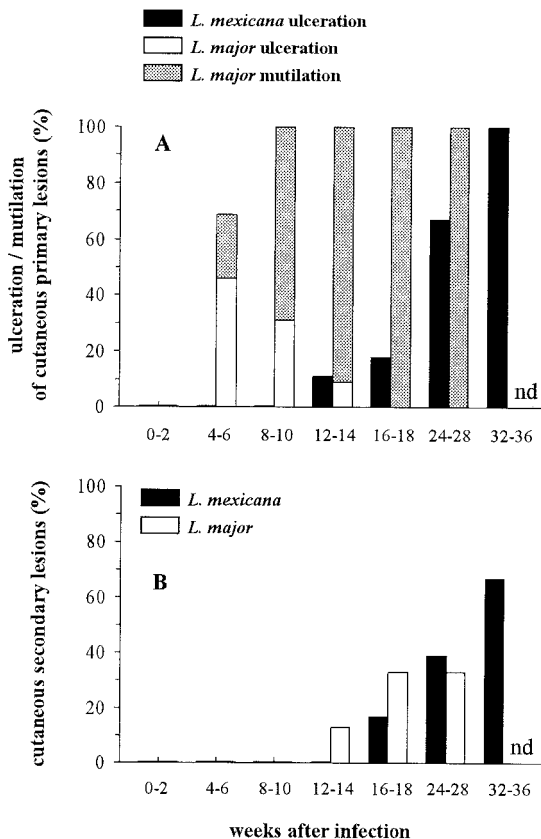

FIG. 2. Frequencies of ulcerated cutaneous primary lesions and mutilations of footpads (A) and cutaneous secondary lesions (B) during the course of *L. mexicana* and *L. major* infections in BALB/c mice. Results are from the same experiment described for Fig. 1. nd, not determined.
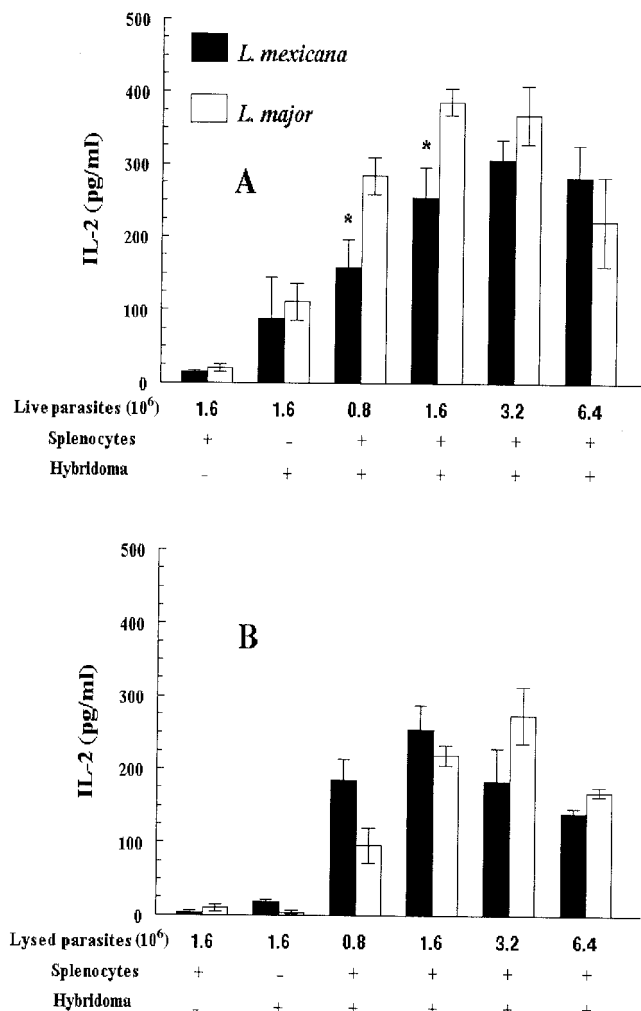
FIG. 3. IL-2 secretion by LMR16.2 hybridoma cells in response to *L. major* and *L. mexicana* promastigotes. Splenocytes from BALB/c mice were incubated with LMR16.2 hybridoma cells and with live (A) or lysed (B) *L. mexicana* or *L. major* promastigotes. Results are expressed as means ± SEM of IL-2 levels released in culture supernatants for three experiments. *, $P < 0.05$ (Student's t test, comparing *L. mexicana* and *L. major*).

antigenic determinant which was recognized by these LACK-specific cells was conserved between the *L. mexicana* and *L. major* LACK proteins.

Interestingly, while the *L. mexicana* and *L. major* lysed promastigotes did not differ in the ability to stimulate LMR16.2 hybridoma cells (Fig. 3B), *L. mexicana* live promastigotes induced less IL-2 than *L. major* live promastigotes (Fig. 3A). Though slight, such differences were significant and might be related to differences in the proportion of metacyclic-like promastigotes (the parasitic form infecting cells) within the *L. major* and *L. mexicana* stationary-phase populations (22) or to differences in the intracellular development of both parasites, interfering with antigen presentation (19). Indeed, the tight binding of *L. mexicana* amastigotes to the membrane vacuole of infected macrophages, which is not seen with *L. major* amastigotes (2), and the sequestration of major histocompatibility complex class II molecules at this point of the membrane (rendering difficult their egress from the parasitophorous vac-

uole) (21), as well as the production of large amounts of cysteine proteases by *L. mexicana* (8, 16) but not *L. major*, are likely to reduce antigen processing and presentation of the *L. mexicana* LACK antigen.

Molecular identification of the LACK epitope in *L. mexicana*. In order to confirm that the antigenic epitope recognized by the LACK-specific cells was conserved between the *L. mexicana* and *L. major* LACK proteins, cDNA was prepared from RNA of *L. mexicana* promastigotes, and the sequence corresponding to the immunodominant LACK epitope was amplified by PCR using the following primers: LM12 (TCCA TCCGCATGTGGGACCTG) and LM13 (CCCGTTCACG TTCCATACTTTG) (17). The nucleotide sequence of the *L. mexicana* PCR products, determined using standard procedures, was identical to that of the corresponding *L. major* region (data not shown). This confirms that the parasite LACK antigen is well conserved among the different studied *Leishmania* species, including *L. major*, *Leishmania amazonensis*, *Leishmania donovani*, *Leishmania chagasi* (17), and *L. mexicana*.

Susceptibility to *L. mexicana* of BALB/c mice made tolerant to LACK protein. Since LACK-specific T cells play a critical role in determining susceptibility to *L. major*, we sought to determine if this was also the case for *L. mexicana* infection. To this end, IE-LACK transgenic and WT BALB/c mice were injected with 10^7 promastigotes. In agreement with previous results (9), the lesions induced by *L. major* were much smaller in IE-LACK transgenic mice than in WT BALB/c mice (Fig. 4B). In contrast, the lesions induced by *L. mexicana* were similar in transgenic and nontransgenic animals (Fig. 4A). Likewise, parasite loads in the footpads and in the draining popliteal LNs (measured after tissue homogenization by staining released amastigotes with acridine orange [Sigma, Bornem, Belgium], as previously reported for *Plasmodium* and *Leishmania* promastigotes [3, 15]), as well as the frequencies of both necrotic and secondary cutaneous lesions, were similar for IE-LACK transgenic and WT BALB/c mice (Table 1). Thus, despite the fact that BALB/c splenocytes were able to stimulate LACK-specific T cells when incubated with *L. mexicana* promastigotes, IE-LACK transgenic mice tolerant to the LACK antigen were as susceptible as WT BALB/c mice to *L. mexicana* infection. This might be related to the reduced ability of *L. mexicana* live promastigotes to stimulate LACK-specific T cells, as shown in our experiment with LMR16.2 hybridoma cells. Moreover, since the processing of LACK by infected macrophages requires the destruction of the internalized parasites (6, 19), differences in the innate immune responses to *L. mexicana* and *L. major* and in the subsequent early production of cytokines activating the parasiticidal function of macrophages may also account for this result.

IL-4 secretion by splenocytes of IE-LACK transgenic and WT BALB/c mice infected with *L. mexicana*. Although IE-LACK transgenic mice were as susceptible as WT BALB/c mice to *L. mexicana*, we sought to determine if LACK-specific T cells contributed to IL-4 secretion in *L. mexicana*-infected mice. To this end, splenocytes of IE-LACK transgenic and WT BALB/c mice infected with 10^7 *L. mexicana* promastigotes were harvested at 0, 2, 6, and 14 wpi and incubated in 24-well plates (Nunc) (3×10^6 cells/800 µl/well) at 37°C for 48 h in a 5% CO_2 humidified atmosphere, with or without concanavalin
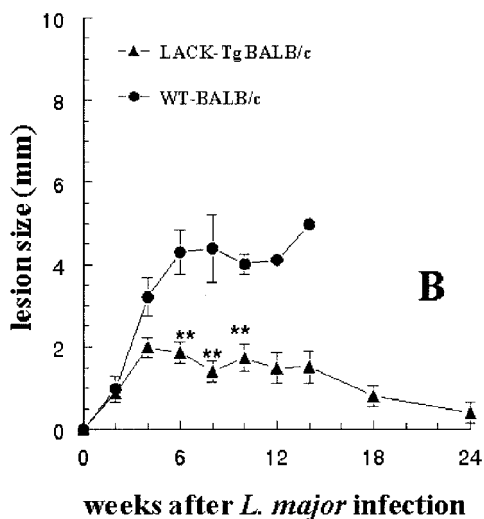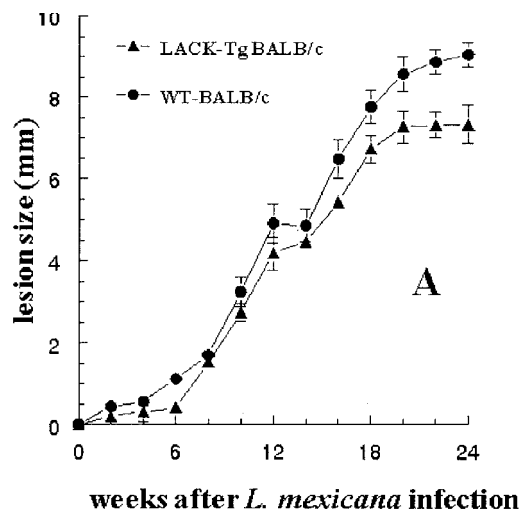
TABLE 1. Parasitic loads and frequencies of lesions in mice infected with *L. mexicana*

wpi	Mouse group[c]	Parasitic load[a] (10^3/mg [mean ± SEM]) in:		Ulcerations[b] (no. present/ total) (%)	Secondary lesions[b] (no. present/ total) (%)
		Foot pad	LN		
2	IE-LACK	200 ± 19	18 ± 1	0/12 (0)	0/12 (0)
	WT	125 ± 63	15 ± 4	0/12 (0)	0/12 (0)
6	IE-LACK	185 ± 27	48 ± 4	0/10 (0)	0/10 (0)
	WT	97 ± 19	20 ± 3	0/10 (0)	0/10 (0)
14	IE-LACK	835 ± 91	152 ± 7	2/7 (29)	0/7 (0)
	WT	685 ± 108	148 ± 19	2/6 (33)	0/6 (0)
24	IE-LACK	3,456 ± 132	1,456 ± 196	4/4 (100)	2/4 (50)
	WT	2,169 ± 303	1,483 ± 74	3/3 (100)	2/3 (67)

[a] Determinations of parasitic loads at each time point were performed for two or three animals infected at the same time as those from the experiment described for Fig. 4.

[b] Data on ulcerations and secondary lesions were derived from the mice from the experiment described for Fig. 4.

[c] No statistical differences were seen between IE-LACK transgenic and WT BALB/c mice.

FIG. 4. Sizes of primary footpad lesions during the course of *L. mexicana* (A) or *L. major* (B) infection in IE-LACK (LACK-Tg-BALB/c) and WT BALB/c mice. Results are expressed as means ± SEM. Twelve male mice per group were inoculated. **, $P < 0.001$ (Student's *t* test, comparing IE-LACK and WT animals).

A (8 μg/well; Sigma) or *L. mexicana* extracts (10^6 lysed parasites/well). Supernatants of unstimulated or stimulated cells were collected for subsequent determination of IL-4 levels. Concanavalin A stimulated splenocytes to produce IL-4 (100 to 390 pg/ml) at each time point for both mouse groups. Neither splenocytes from naive animals nor those from mice which had been infected 2 or 6 weeks earlier produced IL-4 in response to *L. mexicana* lysates. This agrees with previous studies which have shown that IL-4 transcripts are undetectable in the LNs and the spleens of *L. mexicana*-infected mice 1 and 7 days after infection (7). In contrast, when mice were analyzed 14 weeks after infection, splenocytes from both IE-LACK transgenic and WT BALB/c mice secreted IL-4 in response to par-

asite extracts. However, splenocytes from IE-LACK transgenic mice secreted significantly less IL-4 than those from WT BALB/c mice. (Means ± standard errors of the means [SEM] for stimulated cells were as follows: IE-LACK mice, 56 ± 9 pg/ml; WT mice, 179 ± 65 pg/ml [$P < 0.05$ by Student's *t* test, $n = 3$]. The results for unstimulated cells were as follows: IE-LACK mice, 11 ± 8 pg/ml; WT mice, 29 ± 15 pg/ml.) Such a difference suggests that LACK-specific T cells contributed to the production of IL-4 in *L. mexicana*-infected WT mice.

In conclusion, despite BALB/c splenocytes being able to stimulate LACK-specific T cells, which contribute to the production of IL-4 in *L. mexicana*-infected mice, and despite the fact that IL-4 was shown to be required for susceptibility to *L. mexicana* (23), IE-LACK transgenic mice tolerant of LACK antigen were as susceptible as WT BALB/c mice to *L. mexicana* infection. This indicates that LACK-specific T cells do not play a critical role in determining susceptibility to *L. mexicana*. This result is in striking contrast to those obtained in the case of BALB/c mice infected with *L. major*. It is tempting to hypothesize that such a difference preventing the development of an early counterprotective antiparasite Th2 response contributes to the slower development of lesions in *L. mexicana*-infected animals. It also suggests that the mechanisms which determine susceptibility to these two species are markedly different.

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