MAJOR ARTICLE

The Predictive Validity of Naturally Acquired Delayed-Type Hypersensitivity to Leishmanin in Resistance to *Leishmania major*–Associated Cutaneous Leishmaniasis

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To accurately quantify the different outcomes of *Leishmania major* infection and to evaluate the fraction of zoonotic cutaneous leishmaniasis (ZCL) cases prevented by naturally acquired leishmanin skin test (LST) reactivity, a cohort of 470 children was followed up in 2 endemic foci, Remada and Dhiba, in southern Tunisia. During May 1997, before the ZCL emergence season, LST was performed, and results were reassessed 12 months later. Active case detection during the ZCL emergence season showed a high incidence of ZCL: 57.0% in Remada and 13.7% in Dhiba. The preventive fraction of ZCL conferred by LST reactivity increased proportionally with the reaction size before the emergence season, revealing a dose-response effect of ~70%. In addition, asymptomatic *L. major* infection appeared to be a significan form of natural immunization, particularly in the context of relatively low transmission. These finding may help in the design and evaluation of vaccines.

Leishmaniasis affects millions of people [1]. Different *Leishmania* species induce various clinical presentations ranging from asymptomatic or localized infection to disseminated visceral disease [2]. Zoonotic cutaneous leishmaniasis (ZCL) due to *L. major* infection remains an unresolved public health problem in northern Africa, the Middle East, and central Asia [3, 4]. In these regions, especially in rural areas, ZCL may cause considerable morbidity. The symptoms of the disease are rather polymorphic, ranging from benign self-limited cutaneous sores to more-protracted and extensive lesions that may cause severe disfigu ement [5]. Tools available for the control of human leishmaniasis are of poor efficacy antileishmanial drugs are toxic, and vaccines recently developed and evaluated are only marginally effective [6–9]. Field studies have revealed that asymptomatic infection also occurs in endemic areas, but the extent of this phenomenon has not been fully evaluated. People without a definit history of overt disease may show evidence of infection, as is demonstrated by a positive delayed-type hypersensitivity reaction assessed by the leishmanin skin test (LST), which is presently used to measure the prevalence of exposure to *Leishmania* species [10, 11]. Previous studies have shown that individuals with previous episodes of cutaneous leishmaniasis or asymptomatic infection have a positive LST result as well as strong immunity against the parasite and usually become resistant to a subsequent clinical infection [12, 13]. These features theoretically support the feasibility of a vaccine that could induce an immune state similar to that developed after natural infection. Recent studies, at least in the context

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of *L. major* transmission, indicate the possibility that ZCL recurs. These possible recurrences, although documented elsewhere [14, 15], were not accurately quantified particularly in the context of variable transmission pressures, which usually characterize the epidemiological profil of cutaneous leishmaniasis.

The main objectives of the present study were to accurately quantify, through a prospective survey, the different outcomes of *L. major* infection and to evaluate the preventive fraction (PF) of ZCL conferred by LST reactivity induced by natural exposure to the parasite. We demonstrate that resistance to ZCL is neither lifelong nor absolute, regardless of the intensity of the delayed-type hypersensitivity reaction to leishmanin developed by the host. The PF of ZCL increased proportionally with the size of the LST reaction before the emergence season, revealing a dose-response effect. In addition, we clearly show that asymptomatic *L. major* infection is a significan outcome in endemic areas.

SUBJECTS, MATERIALS, AND METHODS

Study Population

The study was performed within the governorate of Tataouine, in the districts of Remada (550 km south of Tunis) and Dhiba (620 km south of Tunis). Both regions are endemic for ZCL caused by *L. major* zymodeme MON25 [16, 17]. The environmental conditions in both districts were equivalent. The areas do not overlap with areas endemic for *L. tropica* infections, and no cases of visceral leishmaniasis have ever been diagnosed in the study area. Data on the incidence of ZCL have been well documented by the surveillance systems in both districts since 1991. As is shown in figu e 1, the 2 selected foci display contrasting trends in the incidence of ZCL.

Children in primary school ($n = 470$) participated in the study, and informed consent was obtained from their parents. A total of 274 children were from Remada (mean ± SD age, 9.674 ± 2.016 years [range, 6.0–14.7 years]; male:female ratio, 0.99), and 196 were from Dhiba (mean ± SD age, 9.624 ± 2.307 years [range, 6.2–14.6 years]; male:female ratio, 1.15). This study was approved by the ethical committee at the Pasteur Institute of Tunis.

Clinical Management and Diagnosis of ZCL

Study design. At enrollment, during April 1997, a physical examination and a detailed skin examination were performed on each participant. The history of ZCL was assessed on the basis of the presence of typical scar(s), and then the LST was administered. Active case detection of ZCL was achieved between October 1997 and March 1998 (during the ZCL emergence season in Tunisia) by visiting the study population at their schools 4 times during this period. The diagnosis of ZCL was established by the same dermatologist on the basis of clinical and epidemiological criteria [18]; the patient was then referred to the local health care center for case management, in accordance with the present instructions of the Ministry of Health. In cases of nontypical lesions, a parasitological confi mation of the disease, based on positive direct smear and/or culture, was required. A second LST was administered to all participants during April 1998, just before the next emergence season.

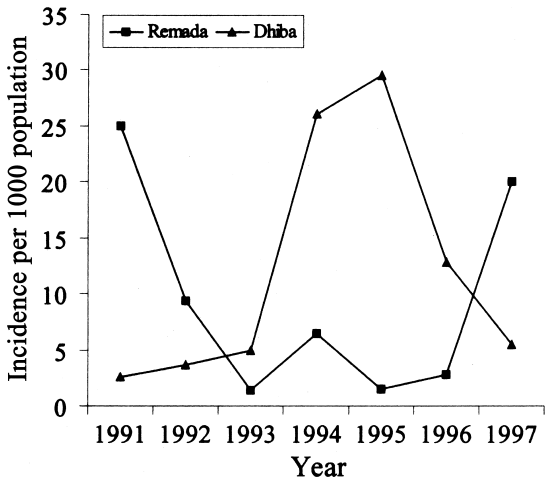

Figure 1. Temporal evolution of the incidence of zoonotic cutaneous leishmaniasis (ZCL) in Remada and Dhiba, 1991–1997. The incidence was assessed using the total no. of ZCL cases in the district as the numerator and the total population of the district as the denominator. Data were supplied by the Unit of Epidemiology, Regional Directorate of Health of Tataouine, Ministry of Health, Tataouine, Tunisia.

LST. The LST was performed by intradermal injection of 100 μL of leishmanin (gift from M. Gramiccia, Instituto Superiore di Sanita, Rome, Italy) suspension containing 5×10^6 *L. major* promastigotes/mL in 0.5% phenol saline. The induration was measured along 2 diameters by use of the ballpoint technique 72 h after testing [19].

Parasitological evaluation. To confi m the diagnosis of ZCL for a fraction of patients and to type the parasites, serous dermal flui was collected from the border of the lesions and was used to prepare May-Grünwald-Giemsa–stained smears and to inoculate Novy, Neal, and Nicolle medium. Parasite stock was then expanded in RPMI 1640 medium containing 15% heat-inactivated calf serum and was typed by isoenzymatic analysis (performed by J.-P. Dedet and F. Pratlong, Centre National de Référence sur les Leishmanioses, Service d'Identificatio Enzymatique des *Leishmania,* Montpellier, France).

Statistical Methods

Statistical associations of categorical variables were based on χ^2 tests. Means of continuous variables were compared using pooled *t* tests. The PF of ZCL attributed to LST-positive reactions was calculated using the following formula: $PF = I_0 - I_1/I_0 = 1 - RR$ [20], where I_0 is the incidence of ZCL in LST-negative participants, I_1 is the incidence of ZCL in LST-positive participants,

and RR is the relative risk of developing ZCL. The force of infection (FOI) was defined as the instantaneous per capita rate of conversion from a negative to a positive LST result and was calculated using the following formula:

$$\lambda = -\frac{\ln\left(\frac{S_2}{S_1}\right)}{t_2 - t_1},$$

where λ is the per capita rate of infection, S_1 is the size of the LST-negative population at time t_1 (1997), and S_2 is the size of the LST-negative population at time t_2 (1998). The rate of reversion was defined as the instantaneous per capita rate of reversion from a positive to a negative LST result and was calculated using the following formula:

$$\rho = -\frac{\ln\left(\frac{Y_2}{Y_1}\right)}{t_2 - t_1},$$

where ρ is the per capita rate of reversion, Y_1 is the size of the LST-positive population at time t_1, and Y_2 is the size of the LST-positive population at time t_2.

RESULTS

Prevalence of LST-positive reactions and typical ZCL scars at baseline. In April 1997, at the first study visit, the overall prevalence of LST-positive reactions (≥5 mm) was 41.4%. It was significantly higher in Dhiba than in Remada: 48.5% (95/196) versus 37.2% (102/274) ($\chi^2 = 5.933$; $P = .015$). However, a history of ZCL, assessed on the basis of the presence of typical scar(s), showed no significant difference in prevalence between the 2 areas: 37.4% (73/195) in Dhiba and 38.5% (105/273) in Remada. Unsurprisingly, in both areas, a strong association was found between the presence of typical ZCL scars and a positive LST result, with an observed agreement of 72.3% in Dhiba ($\chi^2 = 40.84$; $P < .0001$) and 69.0% in Remada ($\chi^2 = 15.77$; $P < .0001$).

Effect of a transmission cycle on the emergence of ZCL. During the ZCL emergence season, the study cohort was visited 4 times and was thoroughly examined by the same dermatologist for the presence of active cutaneous sores. Effective follow-up with measurement of all variables was possible for 454 participants (96.6%). The diagnosis of ZCL was based on clinical evaluation of the skin lesions. Detection of parasites was undertaken for only 11 participants, to confirm the diagnosis of ZCL, and culture and typing were also performed. For 8 participants, we were able to detect parasites (by use of direct smears and/or culture). Despite the failure to do so for the 3 remaining participants, they were considered to have ZCL on the basis of the clinical evaluation.

The incidence of ZCL was very high, with 180 new cases (in 39.6% of participants). The incidence was significantly higher in Remada than in Dhiba: 57.0% (155/272) versus 13.7% (25/182) ($\chi^2 = 85.24$, 1 *df*; $P < .001$). Similarly, the proportion of participants with multiple lesions was higher in Remada (88/155 [56.8%]) than in Dhiba (9/25 [36.0%]) ($\chi^2 = 3.74$; $P = .053$).

Assuming that LST reactivity and the presence of typical ZCL scars confer some resistance to reinfection, we refine these estimates by evaluating the incidence of ZCL and considering the immune status of the study population before the ZCL emergence season. The RR of developing ZCL was also derived for these categories for each district. In LST-negative participants (reaction size, <5 mm), the incidence was 71.5% (123/172) and 20.9% (19/91) in Remada and Dhiba, respectively ($\chi^2 = 61.42$, 1 *df*; $P < .001$). In LST-positive participants, who were assumed to be resistant, we found a high incidence of 32.0% (32/100) in Remada and 6.6% (6/91) in Dhiba ($\chi^2 = 19.3$, 1 *df*; $P < .001$), indicating that LST positivity is not fully protective against reinfection, especially in Remada. Similarly, in participants without typical ZCL scars, the incidence was 69.0% (116/168) in Remada and 19.1% (21/110) in Dhiba ($\chi^2 = 66.37$, 1 *df*; $P < .0001$). In participants with typical ZCL scars, the incidence was 37.9% (39/103) in Remada and 5.6% (4/72) in Dhiba ($\chi^2 = 23.87$, 1 *df*; $P < .001$), indicating a high proportion of recurrence. However, these parameters showed evidence of relative protection against developing ZCL. Indeed, the RRs of developing ZCL in LST-positive participants compared with LST-negative participants (RR, 0.447 in Remada [$P < .001$] vs. 0.316 in Dhiba [$P = .005$]) and in participants with typical ZCL scars compared with participants without scars (RR, 0.548 in Remada [$P < .001$] vs. 0.291 in Dhiba [$P = .009$]) were significantly <1. Table 1 details the incidence of ZCL according to the baseline status of LST reaction and the presence of typical ZCL scars. The data illustrate a decreasing trend in the incidence, with the most susceptible being LST-negative participants without typical ZCL scars (the reference group) and the most resistant being LST-positive participants with typical ZCL scars.

LST reactivity and the PF of ZCL after 1 emergence season. We then treated LST reactivity as a continuous variable, to calculate its capacity to predict resistance to ZCL. LST-negative participants were treated as the reference group against which the RR of developing ZCL was estimated according to the increasing size of the LST reaction. As is shown in table 2, protection against developing ZCL increased proportionally with the size of the LST reaction before the emergence season. In addition, the intensity of the LST reaction confirmed a dose-response effect of protection in Remada and Dhiba, and no full protection was reached in participants with the largest LST reactions (>7 mm). It is important to point out that the PFs of ZCL attributed to LST reactivity were similar in Remada and Dhiba and was ~70% for participants with the largest LST reactions.

Table 1. Incidence of zoonotic cutaneous leishmaniasis (ZCL) according to baseline immune status.

Baseline immune status	Remada			Dhiba		
	Total participants, no.	Participants with ZCL, no.	Incidence of ZCL, %	Total participants, no.	Participants with ZCL, no.	Incidence of ZCL, %
LST negative/scar negative	128	101	78.9	75	17	22.7
LST negative/scar positive	43	22	51.2	16	2	12.5
LST positive/scar negative	40	15	37.5	35	4	11.4
LST positive/scar positive	61	17	27.9	56	2	3.6
Total	272	155	57.0	182	25	13.7

NOTE. In Remada, $\chi^2 = 52.98$, 3 *df; P* < .001. In Dhiba, $\chi^2 = 10.11$, 3 *df; P* = .017. LST, leishmanin skin test; scar negative, absence of typical ZCL scar; scar positive, presence of typical ZCL scar.

Changes in LST reactivity after 1 ZCL emergence season. To evaluate the temporal changes of transmission parameters after 1 ZCL emergence season, and under the assumption that LST conversion is an indirect indicator of *L. major* infection, a second LST was administered in April 1998, 1 year after enrollment. Regardless of the initial LST reactivity, the size of the LST reaction increased overall in participants in both areas, likely reflectin the boosting effect of infecting sand fl bites. However, the increase was significantl higher in participants in Remada (mean reaction size, 9.10 mm in 1998 vs. 3.08 mm in 1997; matched *t* test, 25.49, 269 *df; P* < .0001) than in Dhiba (mean reaction size, 5.91 mm in 1998 vs. 4.43 mm in 1997; matched *t* test, 5.50, 176 *df; P* < .001). A scatterplot of the mean size of the LST reactions in 1997 and 1998 in each area, which took into consideration the development of lesions in the meanwhile, was produced (figu e 2*A* and 2*B*). This showed different patterns in the 2 areas. In Dhiba, the population segregated into 2 groups: 1 composed of naive individuals with no LST reactivity in 1997, in whom most of the lesions developed, and 1 composed of immune individuals whose LST reaction increased significantl in size, most of whom did not develop lesions. In Remada, a significan proportion of the lesions emerged in LST-positive participants.

The second LST also permitted us to estimate prospectively the mean FOI after a ZCL emergence season in participants who initially had negative LST results, the rate of LST reversion, and the proportion of participants whose LST result converted and did not have lesions (asymptomatic infection). In agreement with the incidence of ZCL, the FOI was higher in Remada ($\lambda = 1.77$/year) than in Dhiba ($\lambda = 0.20$/year). Conversely, the rate of reversion in LST-positive participants who had negative LST results 1 year later was significantl higher in Dhiba ($\rho = 0.10$/year) than in Remada ($\rho = 0.01$/year).

Asymptomatic infection was evaluated in LST-negative participants without typical ZCL scars who converted their LST reactivity and did not have lesions during the follow-up period. This proportion was 8.0% (6/75) in Dhiba and 11.7% (15/128) in Remada (not significant) Interestingly, the ratio of asymptomatic infection to ZCL was higher in Dhiba (6/17 [35.2%]), where the FOI is low, than in Remada (15/101 [14.9%]), where the FOI is high, although the difference between the 2 areas did not reach statistical significance

DISCUSSION

During this era of the development and evaluation of anti-*Leishmania* vaccines, important parameters related to natural infection with *Leishmania* parasites, particularly in the context of *L. major*–associated ZCL, are still ill defined Individuals with previous cutaneous leishmaniasis or asymptomatic infection have been thought to be resistant to reinfection. To provide a thorough description of the natural history of *L. major* infection, we conducted a prospective study with tight follow-up. We took advantage of 2 foci with contrasting endemicity to measure the

Table 2. Relative risk (RR) and preventive fraction (PF) of zoonotic cutaneous leishmaniasis (ZCL) lesions according to intensity of leishmanin skin test (LST) reaction.

LST reaction size, mm	Remada				Dhiba			
	Participants with ZCL (*n* = 155)	Participants without ZCL (*n* = 117)	RR (95% CI)	PF, %	Participants with ZCL (*n* = 25)	Participants without ZCL (*n* = 157)	RR (95% CI)	PF, %
0	102	31	Reference	…	18	70	Reference	…
>0 to ≤5	26	27	0.64 (0.48–0.85)	36	1	3	0.82 (0.14–4.69)	18
>5 to ≤7	15	25	0.49 (0.32–0.74)	51	2	19	0.47 (0.12–1.85)	53
>7	12	34	0.34 (0.21–0.56)	66	4	65	0.28 (0.10–0.80)	72

NOTE. CI, confidence interval; PF = 1 − RR.

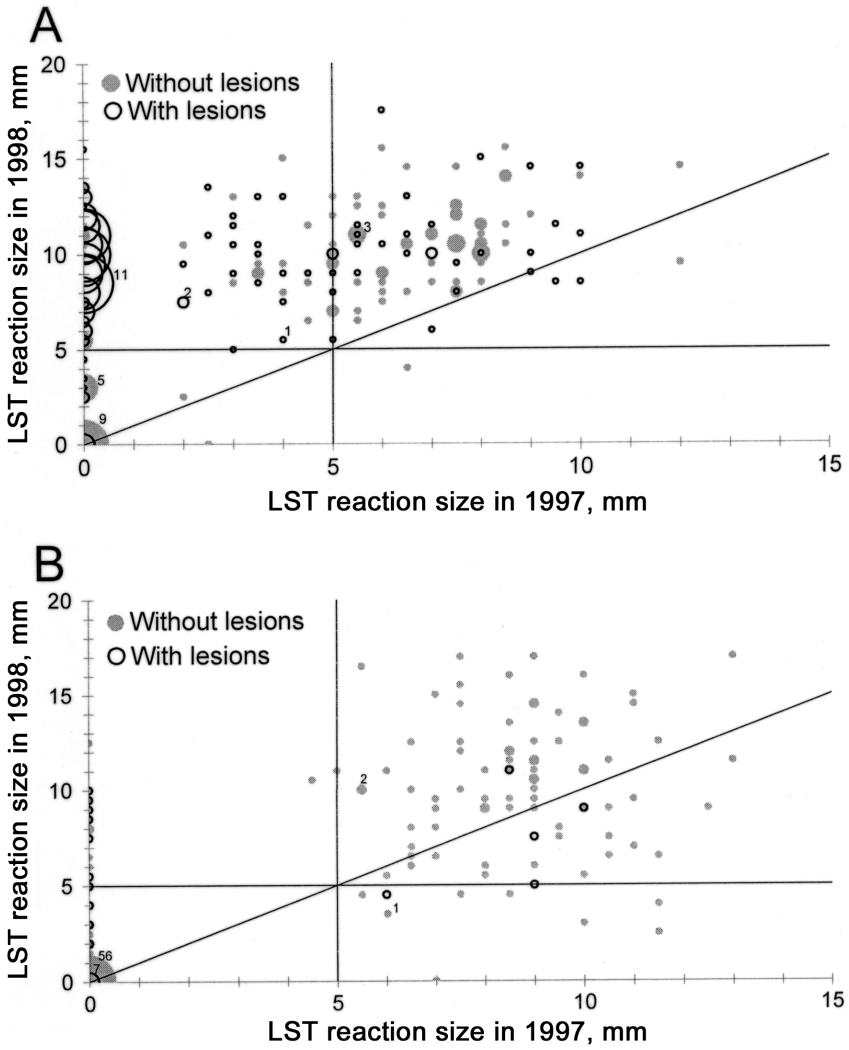

Figure 2. Scatterplot showing the sizes of leishmanin skin test (LST) reactions by year. *A,* Data collected in Dhiba. *B,* Data collected in Remada. White circles indicate participants with zoonotic cutaneous leishmaniasis (ZCL) diagnosed during follow-up, and shaded circles indicate participants without ZCL diagnosed during follow-up.

incidence of ZCL, the rates of asymptomatic infection and recurrence, and the efficac of LST reactivity in protection against developing ZCL.

We demonstrated that resistance to ZCL is neither lifelong nor absolute, regardless of the intensity of the immune response against the parasite (size of the LST reaction) or the history of symptomatic disease. However, individuals with a history of ZCL or asymptomatic infection exhibit resistance to reinfection that increases in proportion to the size of the LST reaction. Interestingly, the PF of ZCL conferred by naturally acquired infection seems to be independent of transmission pressures and was ~70%. Furthermore, we have clearly shown that asymptomatic *L. major* infection constitutes a relatively frequent mode of natural immunization and that the ratio of asymptomatic infection to patent ZCL may reach approximately one-third, especially in the context of low transmission rates.

The differences in LST prevalence between the 2 foci at baseline are most likely explained by the epidemic peak of ZCL that took place during previous epidemiological years in Dhiba, as is supported by data from the surveillance system. Fluctuations in the incidence of ZCL in Remada and Dhiba reveal the true cyclic evolution of the disease, which depends on the joint effect of the intensity of transmission, as determined by the vector and rodent densities, and the pool of susceptible humans, as determined by birth and immigration patterns.

The LST is an indicator of the delayed-type hypersensitivity reaction to the parasite and reflect a type 1 $CD4^+$ cell–specifi immune response [21, 22]. This adaptive immune response develops during acute or healing localized cutaneous leishmaniasis and is thought to constitute the basis of resistance to reinfection. Therefore, this indicator is usually used as an immune correlate for protection in the context of evaluations of anti-

Leishmania vaccines [7–9, 23, 24]. The firs LST, administered before the ZCL emergence season in 1997, revealed a high proportion of LST-positive participants (48.5% in Remada and 37.2% in Dhiba) in both areas and a good concordance with the history of ZCL (presence of typical scars). After 1 emergence season, the number of new ZCL cases and the changes in LST positivity demonstrated the contrasting endemicity of disease in the 2 foci: the endemicity was higher in Remada than in Dhiba. Differences included the incidence of symptomatic cases (57.0% vs. 13.7%), the proportion of participants with multiple lesions (56.8% vs. 36.0%), and the proportion of disease recurrence (37.9% vs. 5.6%). These results were in agreement with the higher FOI in Remada. In addition to its protective role against ZCL, LST reactivity might interfere with disease severity, including LST reaction size and healing duration. These parameters should be examined in future studies. The present study offered the opportunity to quantify the efficac of naturally acquired LST reactivity in conferring resistance to ZCL and to evaluate the proportion of asymptomatic infection in contrasting foci of *L. major* endemicity.

Two primary finding emerged from the present study. First, despite significan differences in the incidence of ZCL and in the FOI between the 2 areas, the efficac of LST reactivity in conferring protection against ZCL was similar and was independent of the intensity of LST reactivity before the ZCL emergence season. Importantly, protection was conferred even in participants with low reactivity (reaction size, 2 mm); it increased in proportion to the size of the LST reaction. This findin is very important to consider for the threshold of LST positivity (typically 5 mm). Second, we demonstrated that asymptomatic infection by *L. major* is a significan event in endemic areas, with a ratio of asymptomatic infection to ZCL that seems to be higher where the endemicity is lower (35.2% in Dhiba vs. 14.9% in Remada). The latter result suggests that the number of infecting bites is a key determinant in the outcome of the infection. In the context of a high FOI, contact with infected sand flie favors development of the full-blown disease with multiple lesions at the individual level and a high incidence of overt disease at the population level. In contrast, when infecting bites are few, the infection is more typically asymptomatic, and a higher proportion of immune individuals revert their LST reactivity, which leads to a low incidence of ZCL. This findin supports the relevance of control measures that reduce the number of infecting bites, such as the use of bed nets, to favor the development of the scenario leading to asymptomatic infection and natural immunization. Further research is required to quantify these threshold values in the context of *L. major* transmission. The increase in LST reactivity may be due, at least in part, to the sensitization conferred by the initial LST reaction. However, in the present study, the results of the LST did not have a significan effect on the size of the reaction. Indeed, a significan proportion of participants in the present study who initially had LST-negative results had negative results after a second LST.

In the present study, the predictive validity of LST reactivity in resistance to ZCL was assessed in the context of natural infection. It is not guaranteed that the same results will be reproduced in the context of artificia acquisition of LST reactivity after vaccination. Recent trials evaluating heat-killed anti-*Leishmania* vaccines revealed no vaccine-induced protection, despite a significan proportion of LST conversions in vaccinated individuals. Obviously, the specifi delayed-type hypersensitivity reaction induced by killed vaccine is generally not predictive of protection against ZCL, despite a lower incidence of disease in individuals with positive LST results [7, 8]. However, in a study by Armijos et al. [9], LST conversion was not associated with protection. The apparent contrast between the efficac of naturally acquired LST reactivity and that conferred by killed vaccines in protection against ZCL stresses the urgent need for definin the immunological basis of resistance to infection with *Leishmania* parasites. With regard to the T cell subsets involved, the important role of $CD4^+$ Th1 cells, which constitute the basis of LST reactivity, has been a consistent findin in the experimental mouse model [25]. However, $CD8^+$ T cells are also required for immunity [26, 27]. These cells may contribute to resistance to *Leishmania* infection by interferon-γ release and cell-mediated killing of parasite-infected macrophages. Recent evidence obtained by our group [28] and others [29, 30] suggests that human $CD8^+$ T cells may also play a role in acquired immunity against *Leishmania* parasites. This effector mechanism, involving $CD8^+$ cytotoxic T lymphocytes, is possibly not induced by killed *Leishmania* parasites.

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