LOSS OF LEISHMANIN SKIN TEST ANTIGEN SENSITIVITY AND POTENCY IN A LONGITUDINAL STUDY OF VISCERAL LEISHMANIASIS IN BANGLADESH

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Abstract. Annual leishmanin skin test (LST) surveys were conducted in a visceral leishmaniasis-endemic Bangladeshi community from 2002 through 2004, using Leishmania infantum antigen from the same manufacturer and batch. In 2002, 530 (35%) of 1,532 had positive LST results; the prevalence increased with increasing age. The LST result was positive in 24 (51%) of 47, 18 (72%) of 25, and 11 (85%) of 13 kala-azar patients treated in the previous 1–11, 12–23, and 24–35 months. A positive LST result in 2002 was associated with protection against subsequent kala-azar (P < 0.0001). In 2003–2004, decreased antigen sensitivity was observed. Among 686 participants, 34% were LST-positive in 2002, 29% in 2003, and 19% in 2004. Of 63 cured kala-azar patients, 70% were positive in 2002, 53% in 2003, and only 30% in 2004. Among 171 participants tested with both antigens, L. infantum study antigen sensitivity was 70% compared with L. amazonensis antigen. Our data underscore the need for better production, standardization, and documentation of sensitivity, potency, and stability of leishmanin antigens.

INTRODUCTION

The leishmanin skin test (LST) is widely used as an epidemiologic tool to characterize populations in leishmaniasis-endemic areas.1,2,3 The LST was introduced in 1926 by Montenegro as a diagnostic tool for cutaneous and mucocutaneous leishmaniasis,4 and was first used in visceral leishmaniasis (VL)–endemic areas by Manson-Bahr and others in Kenya.5 A positive LST result indicates exposure to Leishmania and is generally thought to reflect a durable cell-mediated immune response; positive responses have been documented to last up to 20 years after exposure to the parasite.6 The LST result is usually negative in active kala-azar (symptomatic VL) patients, and becomes positive several months or longer after resolution of symptoms in a proportion of patients.7,8 In the absence of a history of symptomatic leishmaniasis, a positive LST result is presumed to indicate prior asymptomatic infection and species-specific protection from leishmanial disease in the future.9,10 Thus, in the setting of a survey, the proportion LST positive is believed to reflect the cumulative leishmanial exposure experienced by the community, while those who remain LST negative indicate the susceptible segment of the population. We report here the results of LST surveys conducted annually for three consecutive years in a VL-endemic community in rural Bangladesh.

METHODS

The study site was a village in Fulbaria subdistrict, Mymensingh District, with a high incidence of kala-azar reported in government surveillance data during the two years prior to initiation of the study. The epidemiologic methods are described in detail elsewhere.11 Briefly, we conducted three annual house-to-house surveys in January–April of 2002, 2003, and 2004 that included ascertainment of past and current kala-azar cases and application of the LST among all consenting participants ≥ 3 years of age. The protocol was reviewed and approved by the ICDDR,B Research and Ethical Review Committees and the Institutional Review Board of the Centers for Disease Control and Prevention. Written informed consent was obtained from all adult participants and the parent or guardian of all participating children. Assent was also obtained from children ≥ 7 years of age.

We defined a past case of kala-azar as an illness with ≥ 2 weeks of fever, plus at least one of the following: weight loss, abdominal fullness, and/or skin darkening, with clinical improvement after anti-leishmanial treatment. We defined a current case of kala-azar as illness meeting the above definition, plus 1) physical examination consistent with kala-azar (splenomegaly and/or hepatomegaly, with or without measured fever, evidence of weight loss, skin darkening, and/or jaundice), and 2) positive rK39 enzyme-linked immunosorbent assay result and/or rK39 dipstick test result (Inbios International, Seattle, WA), assays for IgG antibodies to Leishmania that have high sensitivity and good specificity for active kala-azar in South Asia.12,13

The skin test antigen used in the 2002–2004 surveys was provided by the Istituto Superiore di Sanità (Rome, Italy). The antigen was prepared from recently transformed Leishmania infantum promastigotes (World Health Organization reference strain MHOM/TN/80/IPT1) grown in Schneider’s medium at 22.5°C and harvested at the log phase of growth (4–5 days).7 The parasites were washed four times in pyrogen-free saline and resuspended in saline plus 0.5% distilled phenol (v/v) to obtain a final concentration of 5 × 10⁶ organisms/mL. Antigen preparation followed the safety procedures prescribed by the World Health Organization and the Italian Official Pharmacopoeia, and the antigen was provided as a ready-to-use liquid suspension.7 All L. infantum antigen was from the same batch, originally prepared in 1999, but provided to us each year from Rome. The antigen was stored under refrigeration and kept on ice packs during fieldwork. In April 2004, we also tested a subset of participants simultaneously with a second skin test antigen prepared from heat-killed L. amazonensis promastigotes (IFLA/BR/67/PH8) suspended in saline with 0.4% phenol.12 This antigen is available commercially from BioManguinhos (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil). The subset of participants to be tested with two antigens were chosen from those not yet

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tested in January–March 2004 who had a known positive LST result in either 2002 or 2003. All such participants were identified and the tests applied as long as the supply of both antigens lasted.

The LST was applied and read following standard methods: 0.1 mL of antigen was injected intradermally on the volar surface of the forearm; 48–72 hours later, induration was measured in two perpendicular directions using the ball-point pen method. Following the international consensus definition, the LST result was considered positive if the mean of the two measurements was ≥ 5 mm. We considered a study subject to have lost LST reactivity if the LST result changed from positive one year to negative in a subsequent year, and there was at least a 5-mm decrease in the mean measurement. For example, if a person had 7 mm of induration in 2002, he would have to have had a reading of ≤ 2 mm in 2003 to be considered as having LST loss. We defined sensitivity as the proportion of persons with known cured *Leishmania* infection with a positive LST reaction. We assessed potency by comparing the induration size resulting from testing with *L. infantum* and *L. amazonensis* antigens among the subset of participants described above. Data were analyzed using SAS version 9.1 (SAS Institute, Cary, NC). We compared the frequency of positive LST results for different survey years and antigens using the McNemar test. We compared the size of the response for the *L. infantum* and *L. amazonensis* antigens using the Wilcoxon signed rank test for the mean difference between the two tests for each individual.

**RESULTS**

At the time of the 2002 survey, there were 1,763 people ≥ 3 years of age eligible for the LST survey: 1,659 with no history of kala-azar, 89 individuals who had been treated for kala-azar in the previous 3 years, and 15 currently ill, untreated kala-azar patients. Leishmanin skin tests were placed for 1,620 (92%) of these people and read 48–72 hours later in 1,532 (94% of those placed). Thirty-seven people (2%) declined participation; 106 others (6%) were not available in the village at the time of the survey. Of the 1,532 people with LST readings in 2002, 530 (35%) had a positive response to intradermal testing with the *L. infantum* antigen. The percentage of participants with a positive LST response increased steadily with age, from 17% of children < 10 years of age to 59% of participants ≥ 60 years of age (Figure 1). Treated kala-azar patients were more likely to have a positive LST result than those with no history of kala-azar (53 of 85, 62%) versus 476 of 1,432, 33%; *P < 0.0001*). One of 15 active kala-azar patients had a positive LST reading (6.5 mm). The proportion of treated kala-azar patients with positive LST reactions increased with increasing time since treatment: the LST result was positive in 24 (51%) of 47, 18 (72%) of 25, and 11 (85%) of 13 patients treated in the previous 1–11, 12–23, and 24–35 months, respectively (χ² for linear trend = 6.1, *P < 0.05*). A positive LST result in 2002 was associated with a strong protective effect against subsequent development of kala-azar during the follow-up period (1 of 476 LST-positive versus 43 of 956 LST-negative participants; relative risk = 0.05, 95% confidence limits = 0.01, 0.35, *P < 0.0001*).

In the 2003 survey, we observed the unexpected loss of LST reactivity in 150 (30%) of 499 people who had positive responses in 2002. We instituted especially stringent quality control of the LST field methods, including review of application and reading techniques, and duplicate readings of a subset of tests by independent observers. Reading reproducibility was good and no systematic errors were identified. In the first month of the 2004 survey, the rate of LST reactivity loss was 48% (119 of 246) compared with responses in 2002, leading us to suspect substantial loss of antigen quality. Among the 686 participants tested during all 3 years, the proportion with positive LST results decreased from 34% in 2002 to 29% in 2003 and 19% in 2004. Of 63 clinically cured kala-azar patients tested in all three years, 70% were positive in 2002, 53% in 2003, and only 30% in 2004. Only one subject with loss of LST reactivity was subsequently diagnosed with kala-azar; this young man had an LST reading of 6 mm in 2002, a reading of 0 mm in March 2003, and a diagnosis of kala-azar a few weeks later.

In April 2004, we chose 171 study subjects who were known to have had a previous positive LST result in 2002 or 2003, and had not yet been tested in January–March 2004. Of the 171, 147 had a positive LST result in 2002 but a negative LST result in 2003, while the other 24 had a negative or missing LST result in 2002 and a positive LST result in 2003. The mean induration of the previous positive LST was 8.1 mm. We tested these participants simultaneously with the *L. infantum* antigen on the right arm and the *L. amazonensis* antigen on the left arm. Of the 171 participants, 22 were skin test negative by both antigens, 97 positive by both, 45 positive by *L. amazonensis* antigen but negative by *L. infantum* antigen, and 7 positive by *L. infantum* antigen but negative by *L. amazonensis* antigen (*P < 0.001* by McNemar test). The sensitivity of the *L. infantum* antigen in 2004 was 70% compared with *L. amazonensis* antigen. The mean induration size was 5.9 mm by *L. infantum* antigen and 9.6 mm by *L. amazonensis* antigen (Figure 2; *P < 0.001*), demonstrating that the *L. infantum* antigen in 2004 was approximately 61% as potent as the *L. amazonensis* antigen for this study population.

**DISCUSSION**

The unanticipated loss of leishmanin antigen sensitivity and potency over the three years of study diminished our ability to
complete one of our study objectives, which was to measure the incidence of subclinical leishmanial infection. The sensitivity of the antigen in 2002 appears to have been acceptable; the rates of LST reactivity in 2002 among our cured kala-azar patients (62% overall and 85% for those treated more than two years earlier) were well within the range reported in the literature. Indeed, previous studies report prevalences of positive LST results among cured kala-azar patients ranging from 30% to 80%.

Our results suggest that the full expression of the delayed hypersensitivity response after treatment of kala-azar may take several years. The delay in conversion to a positive skin test result in our data was somewhat longer than reported in an earlier study from India, in which 87% of kala-azar patients were LST positive eight months after treatment. However, in the Indian study, patients were tested at monthly intervals, raising the possibility of boosting by the leishmanin antigen itself. Other studies that tested kala-azar patients shortly after clinical resolution of symptoms indicate that only 20% were LST positive at 2 months and 36% at 3 months, figures not dissimilar to the rate in our data of 51% positive within 11 months. Nevertheless, the L. infantum antigen used in our surveys may have already had suboptimal sensitivity in 2002. Our data also support the contention that a positive LST result reflects an effective cell-mediated immune response: we found that LST reactivity in 2002 was strongly protective against kala-azar during the subsequent two years. In 2003, when we first noticed the loss of reactivity in formerly LST-positive subjects, we examined several hypotheses, including differences in LST application and reading technique, and the possibility that the level of immune responsiveness had decreased in the study population due to increased rates of malnutrition or intercurrent infection. However, our field evaluation revealed reliable LST technique and good reading reproducibility, community informants reported no epidemics and slightly better rice harvest in 2003–2004 than 2002, and in our epidemiologic data, the reported frequency of meat, fish, and dairy food intake showed no changes over time (Bern C and others, unpublished data).

Thus, the explanation that best fits the data is that the sensitivity and potency of the antigen decreased from 2002 to 2004. Debates over choice of LST antigen have generally revolved around the question of homologous versus heterologous antigen, with most investigators concluding that homologous antigens tend to be more sensitive. However, there is extensive cross-reactivity of patient responses to heterologous Leishmania species, and the method of antigen preparation also seems to be important. For example, a study in Brazil demonstrated that soluble L. chagasi antigen was significantly more sensitive than suspended whole promastigotes of the same species for skin testing of cured kala-azar patients (97% versus 45%). Indeed, the L. chagasi whole promastigote preparation appeared to be less sensitive than a soluble preparation of L. amazonensis (82% sensitivity in a separate analysis in the same report). Because we lacked the resources to develop our own leishmanin and there is no readily available source of South Asian L. donovani skin test antigen, we chose to use the only available preparation from the L. donovani species complex, an L. infantum whole promastigote antigen suspension available through the World Health Organization Collaborating Center in Rome. This antigen is one of the most widely used leishmanin preparations in the world, with results reported from studies comprising more than 14,000 subjects over the past 15 years. Nevertheless, in our 2004 survey, the L. infantum antigen was significantly less sensitive and less potent than the heterologous L. amazonensis antigen, highlighting the dilemma of how to maintain and document comparable leishmanin performance over time and from batch to batch. Although we maintained the liquid antigen at refrigerator temperature while it was in our hands, we do not have information on its temperature during shipment from Italy to the United States, nor whether higher temperatures might have had an effect on the antigen characteristics. However, one study suggested that there was no loss of leishmanin antigen sensitivity after autoclaving at 120°C for 20 minutes.

Because most leishmanin skin testing is performed for epidemiologic and public health purposes in relatively poor, disease-endemic countries, the commercial market is limited and few resources are available to those who manufacture leishmanin antigen. Methods of standardized production and antigen testing have proved elusive. Indeed, in the 1990s, the U.S. military planned to develop standardized leishmanin skin test antigens eligible for Food and Drug Administration approval, but this program did not yield any products, and efforts have now shifted to the adaptation for leishmaniasis of commercially available in vitro tests of cell-mediated immunity already in use for other diseases (Magill A, unpublished data). However, for VL control programs in VL-endemic countries, commercial in vitro tests are unlikely to be affordable. As the countries of South Asia initiate ambitious programs to eliminate kala-azar, leishmanin skin testing will take on increased importance as one of the few practical methods to quickly characterize leishmanial exposure and infection rates in communities. Our data underscore the need for better methods of production, standardization, and documentation of sensitivity, potency and stability of leishmanin antigens.

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