

IMMUNOLOGIC TESTS IN PATIENTS AFTER CLINICAL CURE OF VISCERAL LEISHMANIASIS

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Abstract. The results of five serologic tests (ELISA using promastigote antigen [ELISAp] and recombinant K39 [ELISArK39] and K26 [ELISArK26] antigens, indirect immunofluorescence test using promastigote antigen [IIFT], and immunochromatographic tests using the rK39 antigen [TRALd]) and of the Montenegro skin test (MST) were analyzed in 41 individuals treated for kala-azar and living in Porteirinha, Minas Gerais, Brazil. The tests were carried out 1 week to 12 years after specific treatment. All MSTs during the 8 months after treatment were negative, whereas after 1 year, 28 (84.8%) were positive. Negativity in all serologic tests was observed for 11 (26.8%) of the 41 individuals, whereas positivity in at least one test was observed for 70.3% of subjects evaluated ≥ 2 years after treatment. With respect to each exam, positivity was 38.0% for TRALd, 61.9% for ELISA rK39, 47.6% for ELISA rK26, 38.0% for ELISAp, and 40.5% for IIFT. None of the individuals presented recurrence of the disease during the 4 years of follow-up. The tests were repeated in 24 of the 41 individuals, after some time, and the results were the same in 33.3% of the cases. We conclude that serological tests for kala-azar might continue to be positive after treatment of the disease, although this does not indicate a poor prognosis or a poor therapeutic response.

INTRODUCTION

Serological tests have been widely used in the diagnosis of kala-azar when antibody titers are high and, therefore, easily detectable. However, there is no consensus regarding the behavior of different tests after specific treatment. Some investigators suggested that persistently positive tests, especially ELISA using recombinant K39 antigen, might indicate therapeutic inefficacy and a greater possibility of disease reactivation or evolution to post-kala-azar dermal leishmaniasis.^{1–6} However, other investigators have reported cases of persistently positive tests with no indication of a poor prognosis, such as the direct agglutination test, which can be positive up to 7 years after treatment, and ELISA using recombinant K39 (ELISA rK39) and immunochromatographic tests using the rK39 antigen (TRALd), which can be positive in patients 1 year after treatment.^{7–11}

The Montenegro skin test (MST) is negative in kala-azar and becomes positive after treatment.^{12,13} Some investigators believe that a positive test indicates immunity of the individual against the disease, but others have reported that, if this resistance really exists, it might not be permanent.^{14–18}

Within this context, the aim of this study was to analyze the behavior of skin and various immunologic tests used for the detection of kala-azar in individuals with clinical cure of the disease.

MATERIALS AND METHODS

Forty-one individuals with a history of human visceral leishmaniasis (VL) from the Municipality of Porteirinha, north region of the State of Minas Gerais, 640 km from Belo Horizonte, were studied. The diagnosis of kala-azar was confirmed based on the presence of the parasite in bone marrow,

and on this occasion, specific treatment was done at different localities, mainly by us at the Clemente Faria Hospital in Montes Claros.

Some time after treatment, all patients were submitted to clinical examination and serological and skin tests. After the first assessment, all these individuals were followed up clinically for an additional period of 3 years. The skin and serological tests were repeated in 24 of the 41 patients (second assessment).

In both assessments, the patients were examined and submitted for the MST and blood collection. For the evaluation of the clinical recurrence of kala-azar, we considered the classic symptoms and signs that should be confirmed to isolation or visualization of the parasite. Thinking of the possibility that kala-azar can occur in a sub-clinical form, we studied for the presence of signs and symptoms. Based on literature definitions, the patients were classified into the following categories^{19–21}:

- ≥ 3 : individuals showing three or more of the following signs or symptoms: fever, cough, adynamia, skin pallor, abdominal distension, weight loss, hepatomegaly, and/or splenomegaly;
- = 2: individuals showing two of the signs and symptoms described above;
- ≤ 1 : individuals showing only one or none of the signs or symptoms described above.

The serum samples were collected between January 1998 and May 1999 and between January and October 2001. The samples were processed in 1999 and 2001, respectively. The TRALd, ELISA rK39, and ELISA using recombinant K26 (ELISA rK26) tests were performed at the Federal University of Triângulo Mineiro (UFTM). Another aliquot preserved at -20°C at UFTM was stored on dry ice and sent to the Laboratory of Leishmaniasis and Vaccines, Institute of Biologic Sciences, Federal University of Minas Gerais (ICB/UFGM), Belo Horizonte, for ELISA and indirect immunofluorescence testing (IIFT) using promastigote antigen.

The informed consent was obtained from all participants of

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this study, and the project was approved in Ethical Committee of UFTM.

The program used for statistical analysis was STATISTICA 6.0. The variables were categorical, and we used the Fischer exact test, with 5% significance ($P < 0.05$).

Immunologic tests. *Montenegro skin test.* Antigen of *Leishmania (Leishmania) amazonensis* promastigotes (IFLA/BR/67/PH8), produced at the Laboratory of Leishmaniasis and Vaccines, ICB/UFGM, was used at a standard concentration of 40 µg/mL protein nitrogen diluted 1:10,000 in saline merthiolate.²² Antigen (0.1 mL) was applied intradermally to the anterior side of the left forearm. Readings were obtained 48–72 hours after injection, and the size of the papule was delimited with a ball-point pen.²³ The reactions were considered to be positive when the mean of the measurements of cross-sectional and longitudinal diameters was ≥ 5 mm.

TRAL-d (rK39). For the rapid immunochromatographic test, a kit (InBios International, Seattle, WA) consisting of paper strips coated with recombinant K39 antigen was used according to manufacturer instructions. The strips were

stored at ambient temperature (28–30°C) or refrigerated (2–8°C) and protected from humidity. The buffer solution was stored at 2–8°C.

Readings were obtained 10 minutes later according to manufacturer recommendations, and the results are reported as positive or negative.

ELISA using recombinant antigens. The assay was carried out according to the method of Badaró and others²⁴ using recombinant K39 and K26 antigens of *Leishmania (Leishmania) chagasi* produced by InBios International. The serum samples were diluted 1:50 and 1:100 and developed with peroxidase-conjugated protein A (Sigma Co., St. Louis, MO).

The cut-off was established as the mean absorbance plus 2 SD of the negative controls.

ELISA using promastigote antigen. The assay was carried out as described by Hommel and others²⁵ and Voller and others,²⁶ using as antigen *L. (L.) amazonensis* (MHOM/BR/60/BH6) promastigotes in LIT culture medium in the stationary growth phase and lysed by sonication.

Serum samples were tested at an initial dilution of 1:80.

TABLE 1
Clinical characteristics and immunological tests performed after cure in 41 subjects with human visceral leishmaniasis

No.	Age (years)	Sex	Contact with VL cases	Time after treatment	Immunological test					
					MST	TRAL-d	ELISA rK39	ELISA rK26	ELISA	IIFT
1	5	F	No	1 week	-	+	+	+	+	+
2	1	F	No	1 month	-	+	+	+	+	+
3	2	F	HVL	2 months	-	+	+	+	+	+
4	15	M	CVL	4 months	-	-	+	-	-	-
5	38	F	No	5 months	+	+	+	+	+	+
6	12	F	No	7 months	-	-	-	-	-	-
7	5	F	No	7 months	-	+	+	+	-	+
8	5	M	HVL	8 months	-	-	-	+	-	-
9	8	M	No	1 year	+	-	-	-	-	-
10	2	F	CVL	1 year	-	-	-	-	-	-
11	2	F	HVL	1 year	+	-	+	-	-	+
12	7	F	HVL	1 year	+	-	+	+	+	-
13	3	F	No	1 year	+	+	+	+	+	+
14	4	F	No	1 year	+	-	+	+	-	-
15	7	M	HVL	2 years	+	-	-	-	-	-
16	6	F	HVL	2 years	-	-	-	-	-	-
17	11	M	No	2 years	+	+	+	-	-	-
18	8	F	No	2 years	-	+	+	-	-	-
19	37	F	No	2 years	+	-	+	-	-	-
20	2	M	CVL	2 years	+	-	-	-	-	-
21	5	M	No	2 years	+	-	+	+	-	-
22	3	M	CVL	2 years	+	+	+	+	-	+
23	3	M	No	2 years	+	+	+	+	-	+
24	3	M	No	2 years	+	+	+	-	-	-
25	3	F	HVL	3 years	+	-	+	+	+	-
26	15	M	No	3 years	+	+	+	-	+	+
27	4	M	No	3 years	+	-	-	-	-	-
28	20	M	No	3 years	+	-	+	-	+	+
29	6	M	No	4 years	+	-	-	+	+	+
30	7	F	CVL	4 years	-	-	-	-	-	-
31	6	F	No	5 years	+	-	-	-	-	+
32	49	F	No	5 years	+	-	+	+	+	+
33	14	M	CVL	5 years	+	-	-	-	-	-
34	11	M	No	6 years	+	-	-	-	-	-
35	15	M	HVL	7 years	+	-	+	-	-	-
36	12	M	No	8 years	+	+	-	+	+	-
37	49	M	No	8 years	-	+	+	+	-	+
38	46	F	CVL	9 years	+	-	-	-	-	-
39	20	M	No	9 years	+	-	+	+	+	-
40	27	M	No	10 years	+	+	+	+	+	+
41	20	F	No	12 years	+	-	+	-	+	-

F, female; M, male; CVL, canine visceral leishmaniasis; HVL, human visceral leishmaniasis; MST, Montenegro skin test; IIFT, indirect immunofluorescence test.

Based on these tests, the cut-off for anti-*Leishmania* IgG reactivity was established using 40 sera obtained from truly negative individuals or controls negative for American tegumentary leishmaniasis and stored at the Laboratory of Leishmaniasis and Vaccines. The cut-off was established based on the mean absorbance of these sera plus 2 SD.

Indirect immunofluorescence test. The assay was performed as described by Camargo *L. (L.) amazonensis* (MHOM/BR/60/BH6); promastigotes in LIT culture medium in the exponential growth phase were used as antigen.²⁷ Fluorescein isothiocyanate-labeled human IgG anti-globulin obtained from rabbit immune serum was used as conjugate (Biomanguinhos, Rio de Janeiro, Brazil).

All samples showing positivity at a dilution $\geq 1:40$ were considered to be reactive.

RESULTS

General features. This series included 21 male and 20 female subjects. At the time of first assessment, 12 patients were 0–4 years old, 12 were 5–9 years old, 8 were 10–19 years old, and 9 were > 19 years. The time between treatment and the first assessment was up to 1 year in 14 patients, 2 years in 10 patients, 3 years in 4 patients, and ranged from 4 to 12 years in 13 patients. Of the 41 subjects, 26 (63.4%) had no history of domiciliary contact with human or canine VL, 7 (17.1%) had contact with canine VL, and 8 (19.5%) had contact with human VL (Table 1). With respect to symptoms, 34 (82.9%) subjects showed only one or none, 5 (12.2%) showed two, and only 2 (4.9%) showed three or more. These two patients with three symptoms were examined until 6 months after the treatment and had regression of symptoms.

TABLE 2

Immunological tests in second assessment in 24 subjects with human visceral leishmaniasis

No.	Time after first exam (years)	Immunological test					
		MST	TRAL-d	ELISA rK39	ELISA rK26	ELISA	IIFT
31	1	+	-	+	+	-	-
11	1	+	-	+	-	-	+
13	1	+	+	+	+	+	+
14	1	+	-	+	-	-	+
25	1	+	-	-	-	+	+
30	1	+	-	-	-	-	-
41	1	+	+	+	-	+	+
39	1	+	-	+	+	+	+
2	2	+	-	-	-	+	+
12	2	+	-	+	+	-	+
10	2	-	-	-	+	-	-
34	2	+	-	-	-	-	-
6	3	+	-	-	-	-	-
29	3	-	-	-	-	+	+
27	3	+	-	-	-	-	+
20	3	+	-	+	+	-	-
8	4	+	-	-	+	+	+
9	4	+	-	-	+	-	-
15	4	+	-	-	-	-	-
16	4	+	-	-	-	-	-
38	4	+	-	-	-	-	-
18	4	+	-	+	-	-	+
19	4	+	-	+	+	+	+
36	4	+	+	+	+	+	+

F, female; M, male; CVL, canine visceral leishmaniasis; HVL, human visceral leishmaniasis; MST, Montenegro skin test; IIFT, indirect immunofluorescence test.

In the second assessment, 23 (95.8%) of the 24 examined patients had no symptoms. The seven patients that had two or more symptoms in the first assessment reported that these symptoms disappeared without treatment. In addition, none of the subjects showed clinical recurrence of kala-azar or lesions compatible with the diagnosis of post-kala-azar dermal leishmaniasis during follow-up. We concluded that these symptoms had no relation with kala-azar diagnosis. Therefore, we did not look for *Leishmania* in anyone after treatment, considering it does not justify the invasive procedure.

Of 38 (92.7%) patients with treatment information, 21 (55.3%) used meglumine antimonate (Glucantime, Rhône-Poulenc Rorer, Mexico), 15 (39.5%) used amphotericin B, and 2 (5.2%) used a combination of meglumine antimonate and amphotericin B. It was not possible for us to access the registers of three patients, because they had been assisted in other health units. The period of treatment ranged from 20 to 30 days (median: 25.3 days) with meglumine antimonate, from 10 to 21 days (median: 14.2 days) with amphotericin B, and 7 days in the two patients treated with the both drugs. We did not observe any relation between the drug used, the period of treatment, and the results of the immunologic tests.

Montenegro skin test. Analysis of the MST in the first assessment showed that 7 of the 8 tests were negative in the first year after treatment, whereas between 1 and 2 years, 3 tests were negative and 13 were positive. Three or more years after treatment, 15 subjects showed positive tests, and only two patients tested negative.

Conversions in the MST were observed. Four patients that were negative in the first assessment converted to positive in the second about 2 years or more after the treatment. From 23 tests done in the second assessment, only 1 was negative. This patient was positive in the first exam and converted to negative.

After the first year after treatment, 84.8% of the skin tests in the first assessment and 95.4% in the second were positive, but this difference didn't have statistical significance.

Serologic tests. Regarding the serologic tests for kala-azar in the first assessment, negativity in all tests was observed for 11 subjects. One of them had < 1 year since treatment, five had between 1 and 2 years, and five had been treated 3 years or more ago. From the 41 studied, 5 individuals showed a positive reaction in one test, 7 in two, 6 in three, 6 in four, and 6 subjects tested positive in all tests. After 1 or more years after treatment, 69.7% of the subjects evaluated showed a positive reaction in at least one test. For each test, positivity was 36.6% for TRALd, 63.4% for ELISA rK39, 46.3% for ELISA rK26, 36.6% for ELISAp, and 39.0% for IIFT. Only

TABLE 3

Comparison of the positives percentage of the serological tests between the first and second assessment

Serological test	Number (%) of positives		Statistical analysis
	First assessment [N = 41 (100)]	Second assessment [N = 24 (100)]	
TRALd	15 (36.6)	3 (12.5)	<i>P</i> < 0.05
ELISA rK39	26 (63.4)	11 (45.8)	NS
ELISA rK26	19 (46.3)	10 (41.6)	NS
ELISAp	15 (36.6)	9 (37.5)	NS
IIFT	16 (39.0)	14 (58.3)	NS

NS, no significance.

one of the seven individuals with a positive TRALd test did not test positive by ELISA rK39. The ELISA rK39 was positive in 11 individuals that were found TRALd negative. No correlation was observed between the presence or absence of symptoms and the results of the immunologic tests.

Repeating the tests, we observed that eight (33.3%) had the same results: six of them were negative for all the tests (Table 2). Similarly, serologic results of the second assessment tended to be the same as the first assessment, except in five patients where there was an increase in positive tests. It did not have significant statistical difference between the proportion of positives in each test when we compared the first and the second assessment, except to the TRALd (Table 3).

DISCUSSION

A high conversion rate from a negative to positive MST was shown 1 year after specific treatment of human VL, in agreement with the literature.^{12,13,28} All serological tests were less frequently positive than before treatment (unpublished data). In contrast to the findings of some investigators, from a clinical point of view, the lack of negativity did not indicate a poor therapeutic response or poor prognosis because none of the patients presented recurrence during follow-up.¹⁻⁶ The signs or symptoms that led to the diagnosis of symptomatic or oligosymptomatic kala-azar in five patients showed no correlation with the results of the serological exams and did not seem to be related to kala-azar.

The meaning of positive serologic tests after treatment of kala-azar in clinically cured patients remains unknown. One explanation might be the fact that individuals with VL do not show complete elimination of the parasite, as shown for 11 (85%) individuals evaluated by a liver biopsy 1-11 months after treatment.²⁹ This agrees with the opportunistic occurrence of the disease as observed in patients with AIDS.³⁰ On the other hand, because these individuals continued to live in a kala-azar endemic area, we can not determine or dismiss the influence of re-infection in the serological results. Finally, we think that the positive serologic reactions (including with recombinant antigens) in patients treated for kala-azar frequently did not indicate poor therapeutic response or prognosis.

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