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AN EVALUATION OF CLINICAL, SEROLOGIC, ANATOMOPATHOLOGIC AND IMMUNOHISTOCHEMICAL FINDINGS FOR FIFTEEN PATIENTS WITH MUCOSAL LEISHMANIASIS BEFORE AND AFTER TREATMENT

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SUMMARY

Treatment of mucosal leishmaniasis (ML) can be controlled by clinical examination and by serologic titers by the indirect immunofluorescence serologic reaction (IISR). We studied the correlation between the presence of antigen in tissue determined by immunohistochemistry, the IISR titers and the anatomopathologic findings in fifteen patients with ML before and after healing of the lesions as determined by otorhinolaryngologic evaluation, and evaluated these parameters to determine which of them could be useful during follow-up. Tissue antigens became negative in four patients (group A) after treatment, with a statistically significant reduction or negativity of IISR titers ($p<0.05$). This did not occur in patients in whom the antigen persisted after treatment (group B), suggesting that serologic follow-up should be performed together with the search for tissue antigen, a combination which, to our knowledge, has not been used in previous studies. The negativity of tissue antigens and the behavior of IIRS titers in group A patients probably indicate a lower possibility of recurrence. Upon anatomopathologic examination the inflammatory process was found to persist after treatment even in group A, suggesting that the permanence of inflammatory activity even in clinically healed lesions is possibly correlated with the presence of the antigen or of some unknown factor.

KEYWORDS: Mucocutaneous leishmaniasis; Control treatment evaluation

INTRODUCTION

New World American tegumentary leishmaniasis (ATL) is a zoonosis caused by various protozoan species of the genus *Leishmania* and is transmitted by the bite of females of different species of phlebotomine mosquitoes of the genera *Lutzomyia* and *Psychodopygus* [10]. In Brazil the disease is endemic, with approximately 30,000 cases being notified each year[7].

Three clinical forms of ATL exist in the Americas: the cutaneous form, the diffuse cutaneous form and the mucocutaneous form[5]. In the last, the mucosal lesion caused by *Leishmania (Viannia) braziliensis* can occur many years after healing of the primary cutaneous lesion. The patients are chronically infected with the parasite and may later develop mucosal leishmaniasis (ML) with subsequent deformities, including perforation of the nasal septum, different degrees of lesions on the face, nasopharynx, oropharynx, larynx and trachea. The involvement of the pharynx or larynx permits the occurrence of aspiration or airway obstruction, eventually causing death of the patient[1,3,9].

ML is usually diagnosed by clinical examination, by the Montenegro skin test, by serology using the indirect immunofluorescence method, and by anatomopathologic examination of a lesion biopsy[9]. The last test is inconclusive, except when the parasite is visualized, since a nonspecific chronic inflammatory reaction is present, accompanied or not by a granuloma, but without the presence of the agent. A confirming diagnosis can be obtained by demonstrating the leishmania antigen in tissue by immunohistochemistry[17].

The first-choice drugs for ATL treatment are pentavalent antimonials. However, when these medications are contraindicated or when treatment fails, the alternatives are pentamidine isothianate or amphotericin B[11].

Patients with ML may present recurrence months or years after treatment. Today, the parameter utilized to control the cure after healing of the mucosal lesions is mostly a clinical one, since the value of a post-treatment biopsy and of the monitoring of serologic titers has not been well established in this situation[12,13].

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The objective of the present study was to investigate the correlation between the anatomopathologic findings, the presence of the antigen in tissue (demonstrated by immunohistochemistry) and the titers of the serologic reaction in patients with ML lesions considered to have healed by clinical examination, and to identify which of these parameters could be useful for post-treatment follow-up.

MATERIALS AND METHODS

Patients. The study was conducted on nine men and six women aged on average 51 years (range: 23-76 years), seen at American tegumental leishmaniasis Outpatient Clinic of the Clinical Division of Infectious and Parasitic Diseases, University Hospital, Faculty of Medicine of the University of São Paulo. This study was originally approved by the Ethics and Research Committee of Department of Infectious and Parasitic Diseases from Faculty of Medicine of the University of São Paulo, as well by the Ethics and Research Committee of Clinical Hospital of Faculty of Medicine of the University of São Paulo. Informed consent was obtained from all participants.The method for the diagnosis of ML, patient treatment and follow-up and statistical analysis are described below.

Otorhinolaryngologic examination. The presence of ML was suspected on the basis of the presence of hyperemia, edema, granular aspect of the mucosa or septal perforation determined by rhinoscopy, direct and indirect laryngoscopy and fibroscopy when necessary.

Montenegro test. Antigenic material consisting of promastigote forms of *Leishmania major* was applied intradermally on the anterior surface of the forearm in the amount of 0.1 ml and a reading was taken 48 hours later; nodules with an induration exceeding 5mm were considered to be positive.

Anatomopathologic examination of material obtained from a lesion biopsy. Biopsy material was stained with hematoxylin-eosin and the following epithelial aspects were examined: presence or absence of hyperplasia, acanthosis, ulceration and necrotic plug. The following stromal aspects were examined: acute inflammatory process, nonspecific inflammatory process without evident plasmacytosis, nonspecific inflammatory process with increased plasmacytosis, chronic granulomatous process, necrosis, and presence of amastigote forms.

Immunohistochemical reaction (IHR) [17]. The biopsy material was cut into 4 μm sections, cleared with xylene and dehydrated in a decreasing alcohol series. The slides were incubated with a 3% hydrogen peroxide solution to block endogenous peroxidase and then incubated with polyclonal anti-leishmania antibody, obtained by inoculation of *Leishmania (Leishmania) amazonenses* in hamsters, at 1:1000 dilution in bovine serum albumin. The material was washed in 0.01 M phosphate buffer solution (PBS), pH 7.2, and incubated with the secondary anti-mouse immunoglobulin antibody at 1:1000 dilution. After washing in PBS, the sections were incubated with the streptavidine-biotin complex at 1:1000 dilution. The reaction was developed with diaminobenzidine dissolved in PBS, with H_2O_2 added. The sections were then counterstained with hematoxylin, washed, dehydrated in a growing alcohol series and xylene and mounted on resin.

Indirect immunofluorescence serologic reaction (IISR). The antigen was prepared from promastigote forms of *Leishmania (Leishmania) amazonensis* obtained in NNN medium (solid phase) complemented with LIT (liquid phase). Starting on the sixth day of culture, these forms were washed in PBS and left in 2% formalin in PBS. On the following day, the forms were again washed three more times in PBS and placed on appropriate slides for IISR containing 60 forms per microscopic field (400X magnification). The slides were incubated with sera serially diluted from 1:2 to 1:1024 in a moist chamber and washed three times with PBS. Fluorescent human anti-IgG conjugate (BIOLAB S.A., Brazil) was then added and the slides were mounted with buffered glycerin and coverslipped. Readings were taken with an epiillumination fluorescence microscope (Olympus, Japan) equipped with a halogen lamp and a 40X objective. All slides were processed with a positive and a negative control serum. Sera that produced fluorescence at $\geq$32 titer both on the membrane and on the flagellum of the parasite were considered to be positive and those that did not produce any fluorescence were considered to be negative.

Patients treatment. Seven patients were treated with N-methyl glucamine antimoniate at the intravenous dose of 20 mg/kg pure antimonium daily for 30 days. The remaining patients received intravenous pentamidine isothionate at the dose of 4 mg/kg on alternate days until the lesions healed.

Patients follow-up. After the end of treatment and when the lesions had healed, the patients were followed up always by the same otorhinolaryngologist. After a varying period of follow-up, a biopsy was taken from the same region as the biopsy taken for diagnosis, for anatomopathological examination and repetition of the immunohistochemical reaction (IHR). During the follow-up of this paper, the IISR was being taken for almost each three months, after the end of the treatment. The last was taken in the same date in which the control was done.

Statistical analysis. Data were analyzed statistically by the Student t-test, the Fisher exact test and the Pearson chi-square test.

RESULTS

Thirteen of the fifteen patients had lesions only in the nasal septum, one in the nasal septum and oropharynx, and one only in the oropharynx. Before treatment patients presented IHR positivity in the lesion biopsy (Fig.1). After treatment, negativity occurred in four patients. At this time, the patients were divided into two groups for statistical comparison between the anatomopathologic changes and the evolution of serologic titers: group A (antigen negativity after treatment) and group B

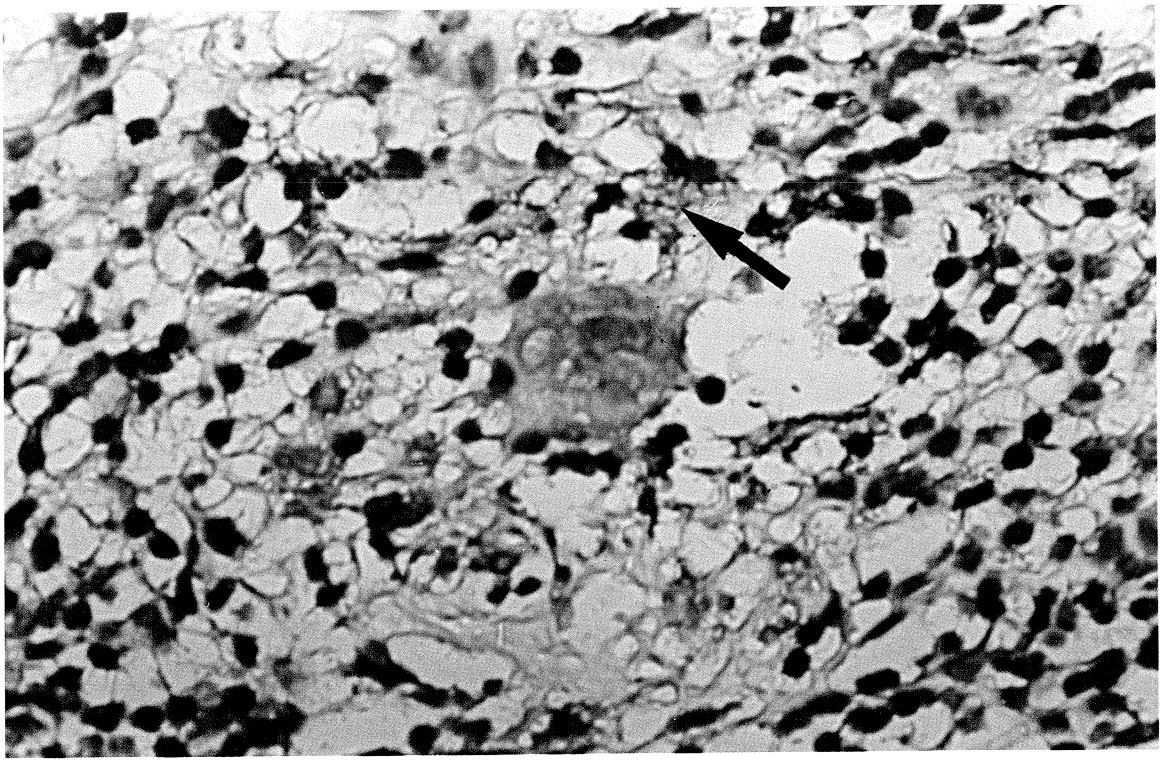

Fig. 1 – The biopsy of nasal septum that was carried out, before treatment: positive IHR for leishmania antigens and stromal granulomatous process.

(persistence of leishmania antigen positivity after treatment). The figures of the patients from groups A and B with regard to age, initial or relapsing clinical manifestations, drug used to treatment, duration of treatment, date of second biopsy and intervals between first and second biopsy are shown at table 1. The range of the time between the end of treatment, with clinical evidence of healing lesions, and the biopsy of these ones for anatomopathologic examination, associated with IHR of group A was 22±0.8 (range:21-23 months) and of group B was 18.4±13.7 (range 4-42 months), this difference was not statistically significant (Table 2).

As to the anatomopathologic findings, the only significant difference between group A and group B was the presence of a necrotic plug before treatment in group B. There were significant differences in hyperplasia, acanthosis, ulceration, and acute inflammatory processes ($p<0.05$) between the pre-treatment and post-treatment evaluation, but only within and not between groups (Table 3). No significant differences were observed in the nonspecific inflammatory process without plasmacytosis, in the nonspecific process with increased plasmacytosis, or in the chronic granulomatous inflammatory process (Table 4). The evaluation of pre-treatment and post-treatment serologic titer and IHR from groups A and B are shown at table 5. Amastigote forms were observed in one patient from group B before treatment and in two patients from the same group after treatment.

All patients presented a positive IISR before treatment. After treatment there was a decrease or negativity in all 4 patients who presented a negative immunohistochemical reaction (group A), a result that was statistically significant (Table 6).

DISCUSSION

The IHR has been important the most to confirm the diagnosis of LM, since the histopathologics findings have usually shown a non-specific chronic inflammation and/or granulomatous reaction with few parasites and difficult to find in LM. In the sample of patients studied, the positivity of IHR was 100% before treatment. Nonetheless the IHR positivity has ranged from 62% to 75% in some studies published[4].

The present study permitted us to observe originally that Leishmanian antigen may persist in tissue even after ML lesions have healed, as detected by IHR. The presence of amastigote forms or of the leishmania antigen after treatment may reflect reinfection or persistence of the agent even after the lesion heals. The phenomenon of parasite persistence is a proven fact in visceral leishmaniasis and in the cutaneous form of ATL[2,16]. In the cutaneous form the immune response is mainly characterized by a Th1 type response, with the predominance of gamma-interferon and with low levels of interleukins 4, 5 and 10. The skin lesion heals with or without treatment within a few months and cure usually leads to permanent protection. In contrast to the cutaneous form, ML is characterized by exacerbation of the immune response, causing a destructive tissue lesion directed at the parasite or some of its antigens[18]. In the present study we observed the permanence of the parasite after treatment in 2 patients (13%) and of its antigen in 11 (73%), even though the mucosal lesions had completely healed.

TABLE 1
Characterization of the patients from groups A and B

Group	Pat. No.	Initials	Age (years)	Manifestation*	Treatment	Duration of treatment (days)	Date of 2nd biopsy	Intervals 1st and 2nd biopsy (months)
	1	JFS	68	initial	pentamidine	22	05/12/97	22
A	2	FSS	42	initial	antimoniate	30	06/27/97	23
	3	OP	56	initial	pentamidine	14	07/15/97	21
	4	MBS	47	initial	antimoniate	30	07/28/97	22
	5	OR	76	initial	pentamidine	28	05/21/97	22
	6	DSS	71	initial	pentamidine	35	05/12/97	23
	7	MLF	38	initial	pentamidine	30	08/19/97	11
	8	EPS	58	relapsing	pentamidine	36	08/05/97	4
B	9	GFV	61	initial	antimoniate	30	04/07/97	42
	10	DMG	23	initial	pentamidine	36	04/23/97	39
	11	VM	51	initial	antimoniate	30	05/12/97	9
	12	JFM	40	initial	antimoniate	30	08/01/97	31
	13	TC	63	initial	pentamidine	23	08/18/97	12
	14	AJR	34	relapsing	antimoniate	30	04/23/97	8
	15	ISS	39	initial	pentamidine	39	03/11/97	4

* ML as the first episode or relapse

TABLE 2
Comparison between groups A and B

	Groups:	A (n=4)	B (n=11)	Comparison between groups*
Age	mean ± sd	53.2 ± 11.4	50.4 ± 16.8	n.s.
(years)	range	42 – 68	23 – 76	
Duration of treatment	mean ± sd	24.0 ± 7.7	31.5 ± 4.5	n.s.
(days)	range	14 – 30	23 – 39	
Intervals 1st and 2nd biopsy	mean ± sd	22.0 ± 0.8	18.6 ± 13.7	n.s.
(months)	range	21 – 23	4 – 42	

* Student t test (independent samples)

Our patients were from an endemic area which they had left before the appearance of the mucosal lesion and were living in the urban region of the city of São Paulo, a fact that makes the hypothesis of reinfection unlikely. Another aspect to be emphasized, in order to explain the persistence of antigen in group B, is that the dead parasites had probably not degenerated yet or the tissue fixed-antigens still had persisted after treatment. Although, this seems unlikely since the biopsy taken after treatment was obtained on average after 18.6±13.7 months (range:4 to 42 months), a time probably sufficient for the disappearance of dead amastigotes or of their antigens. Recurrence after lesion healing has been reported after treatment with N-methyl glucamine antimoniate, pentamidine isothionate and amphotericin B[4,9,14]. The recurrence of ML months or years after treatment is probably due to the inability of the medications and of the immune system to fully eliminate the parasite or its antigens. Perhaps the presence of amastigote forms or of the antigen in some of our patients after treatment (group B) may favor future recurrence, and therefore we believe that these patients should followed up for as long as possible so that treatment may be instituted at the first sign of recurrence of the infection. In cases in which negativity of the antigen and of the amastigote forms occurred (group A), the possibility of parasite activation may possibly be lower.

The use of IISR for post-treatment follow-up in ATL has been the subject of several studies, especially on the cutaneous

TABLE 3
Pre-treatment and post-treatment statistical significant anatomopathological findings

Parameters	Groups		Pre-treatm. No. (%)	Post-treatm. No. (%)	Comparison pre x post *
Acute inflammatory process	A	Yes	4 (100%)	3 (75%)	n.s.
		No	-	1 (25%)	
	B	Yes	11 (100%)	7 (63.6%)	p<0.05
		No	-	4 (36.4%)	
Hyperplasia	A	Yes	3 (100%)	-	n.s.
		No	-	2 (100%)	
		N.D.	1	2	
	B	Yes	8 (88.9%)	3 (30%)	p<0.05
		No	1 (11.1%)	7 (70%)	
		N.D.	2	1	
Acanthosis	A	Yes	-	4 (100%)	p<0.05
		No	3 (100%)	-	
		N.D.	1	-	
	B	Yes	2 (22.2%)	7 (70%)	n.s.
		No	7 (77.8%)	3 (30%)	
		N.D.	2	1	
Ulceration	A	Yes	3 (100%)	1 (25%)	n.s.
		No	-	3 (75%)	
		N.D.	1	-	
	B	Yes	11 (100%)	5 (45.5%)	p<0.05
		No	-	6 (54.5%)	
Necrotic plug ***	A	Yes	2 (50%)	-	n.s.
		No	2 (50%)	4 (100%)	
	B	Yes	11 (100%)	5 (45.5%)	p<0.05
		No	-	6 (54.5%)	
Comparison between groups**:			n.s.	n.s.	

* Fisher exact test

** Mann-Whitney test

*** Comparison between groups significant at pre-treatment for **Necrotic Plug** ($p < 0.05$)

ND= not determined

form of the disease and these studies have demonstrated a fall in antibody titers or even negativity of the reaction after treatment, although methodologic differences especially with respect to the antigen used and the lack of serial follow-up impair a comparison[6,19]. Theoretically, eradication of the parasite after successful treatment can eliminate the antigenic stimulus for the production of antibodies and consequently lead to a decline in the reaction titers. The number of studies on ML is much smaller but OLIVEIRA et al. observed a lack of correlation between the clinical situation of the patients and IISR titers[13]. To our knowledge, there are no studies on a possible correlation between the IHR and IISR titers. In the present study, the patients whose results for the antigen in tissue were negative by IHR presented a decrease or negativity of antibody titers after treatment, a phenomenon that was statistically significant compared to the patients who continued to be antigen-positive after treatment. In conclusion, there was an association between the disappearance of tissue antigen and the fall or negativity of serologic titers, a fact that did not occur in patients who continued to show antigen positivity, suggesting that follow-up by IIRS should be performed together with the search for tissue antigen, and also indicating the possibility of recurrence is probably lower in these patients.

Previous studies on the histologic features of cutaneous and mucosal leishmaniasis have attempted to establish a histologic

TABLE 4
Pre-treatment and post-treatment anatomopathological findings without statistical significance

Parameters	Groups		Pre-treatm. No. (%)	Post-treatm. No. (%)	comparison pre x post *
Nonspecific inflammatory process without evident plasmacytosis	A	Yes	-	-	-
		No	4 (100%)	4 (100%)	
	B	Yes	1 (9.1%)	-	n.s.
		No	10 (90.9%)	10 (100%)	
		N.D.	-	1	
Nonspecific inflammatory process with evident increased plasmacytosis	A	Yes	4 (100%)	3 (75%)	n.s.
		No	-	1 (25%)	
	B	Yes	10 (90.9%)	11 (100%)	n.s.
		No	1 (9.1%)	-	
Chronic granulomatous inflammatory process	A	Yes	2 (50%)	-	n.s.
		No	2 (50%)	4 (100%)	
	B	Yes	4 (36.4%)	2 (18.2%)	n.s.
		No	7 (64.6%)	9 (81.8%)	
Necrosis	A	Yes	-	-	-
		No	4 (100%)	4 (100%)	
	B	Yes	4 (36.4%)	2 (18.2%)	n.s.
		No	7 (64.6%)	9 (81.8%)	
Amastigote forms	A	Yes	-	-	-
		No	4 (100%)	4 (100%)	
	B	Yes	1 (9.1%)	2 (18.2%)	n.s.
		No	10 (90.1%)	9 (81.8%)	
Comparison between groups**:			n.s.	n.s.	

* Fisher exact test

** Mann-Whitney test

ND= not determined

and evolutionary classification of the disease[5,8]. With respect to its usefulness as a parameter for post-treatment evaluation, the absence of a cell infiltrate in the lesion could represent a criterion of cure, although today it seems that a biopsy of a healed lesion may present an infiltrate months or even years after treatment[11,15]. In the present study we evaluated separately the changes that occurred in the epithelium and in the stroma in an attempt to characterize a pattern that could be used as a criterion for post-treatment evaluation. We observed changes such as the disappearance of the necrotic plug in the individuals who were negative to the IHR (group A) compared to the group that continued to be positive to IHR after treatment (group B), and this was the only parameter showing a significant difference ($p<0.05$) between groups. The other alterations observed after treatment for each group were the absence of acanthosis in group A and the absence of ulceration, of a necrotic plug and of an acute inflammatory process in group B. On the basis of these findings, we conclude that it was not possible to characterize a unique histologic picture after treatment since other histologic features such as a nonspecific inflammatory process without evident plasmacytosis, a nonspecific inflammatory process with increased plasmacytosis, a chronic granulomatous inflammatory process and necrosis did not show significant differences, indicating that the inflammatory process can continue to be active even after lesions have healed, as detected by clinical examination. There was no correlation between healing of the lesions, as determined by otorhinolaryngologic evaluation, and the anatomopathologic findings. Thus, we believe that the clinical criterion is limited in terms of the determination of ML activity since the inflammatory process may persist in apparently healed lesions, probably due to antigen persistence or to some unknown factor since, as observed in group A, the inflammatory infiltrate can persist in the absence of antigen and amastigote forms.

TABLE 5
Pre and post-treatment comparison as to the serologic titers and IHR results

Group	Pat. No.	Initials	Serologic Titer Pre-treatm.	Serologic Titer Post-treatm.	IHR Pre-treatm.	IHR Post-treatm.
	1	JFS	1/256	1/64	positive	negative
A	2	FSS	1/256	1/32	positive	negative
	3	OP	1≥1024	negative	positive	negative
	4	MBS	1/64	negative	positive	negative
	5	OR	1/512	1/512	positive	positive
	6	DSS	1≥1024	1≥1024	positive	positive
	7	MLF	1/32	1/32	positive	positive
	8	EPS	1/64	1/64	positive	positive
B	9	GFV	1/256	1≥1024	positive	positive
	10	DMG	1/32	negative	positive	positive
	11	VM	1/64	1/64	positive	positive
	12	JFM	1/128	negative	positive	positive
	13	TC	1/64	1/64	positive	positive
	14	AJR	1/512	1/512	positive	positive
	15	ISS	1/64	1/64	positive	positive

TABLE 6
Changings of serologic results from groups A and B

Groups	A No. (%)	B No. (%)	Comparison between groups*
negative/decrease	4 (100%)	2 (18.2%)	
no change	-	8 (72.7%)	p<0.05
increased	-	1 (9.1%)	
total	4 (100%)	11 (100%)	

* Pearson Chi-square test

The fifteen patients continue to be under follow-up. Serologic titers did not change appreciably compared to the data reported previously: with respect to clinical evolution, reactivation of the lesion occurred in a patient belonging to group B, patient number fourteen (Table 1), who is currently treated with N-methyl glucamine antimonial. The others fourteen patients have been in good condition of health, and all of them have kept on their ML lesions clinically healed.

RESUMO

Uma avaliação entre os achados clínicos, sorológicos, anatomopatológicos e imuno-histoquímicos de quinze pacientes com leishmaniose mucosa antes e após o tratamento

O controle de tratamento da leishmaniose mucosa (LM) pode ser realizado pelo exame clínico e o acompanhamento dos títulos sorológicos da reação de imunofluorescência indireta (RIFI). Estudamos a correlação entre a presença de antígeno no tecido através da reação de imuno-histoquímica, os títulos da reação de imunofluorescência indireta e os achados anatomopatológicos, em quinze pacientes com LM, antes e após as lesões estarem cicatrizadas pela avaliação otorrino-laringológica, e avaliamos qual destes parâmetros pode ter utilidade no seguimento. Após a terapêutica houve negativação do antígeno tecidual em quatro doentes (grupo A), sendo a redução ou negativação dos títulos da RIFI estatisticamente significante (p<0.05), o que não ocorreu nos doentes, em que houve permanência do antígeno posteriormente ao tratamento (grupo B), sugerindo que o acompanhamento sorológico deva ser realizado conjuntamente com a pesquisa do antígeno tecidual, associação que não conhecemos ter sido utilizada anteriormente em outro estudo. A negativação do antígeno tecidual e o comportamento dos títulos da RIFI nos doentes do grupo A, provavelmente indicam menor possibilidade de recidiva. Ao

exame anatomo-patológico, o processo inflamatório persistiu após a terapêutica, mesmo no grupo A, sugerindo que a permanência da atividade inflamatória em lesões clinicamente cicatrizadas, possivelmente correlaciona-se com a presença do antígeno ou a algum fator desconhecido.

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