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ANTIGENIC STANDARDIZATION FOR MUCOCUTANEOUS LEISHMANIASIS IMMUNOFLUORESCENCE TEST

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SUMMARY

Promastigotes of *L. braziliensis* (strain 6, Belo Horizonte) were grown in semi-solid NN medium for 4, 8, 9, 10, 11 and 13 days. Positive IF was seen with sera from mucocutaneous leishmaniasis patients with parasites grown for 8, 9 and 10 days although only for 8 days' old parasites, uniformity of staining was observed. The same uniform staining was seen when 8 days' old parasites were submitted to 2% (v/v) formalin treatment. Slides were kept at −20°C for 3 years without loss in antigenicity. Specificity was assayed with sera from several pathologic conditions. Cross-reactions occurred with sera from American Trypanosomiasis and Kala-Azar.

INTRODUCTION

Fluorescent tests to detect levels of humoral antibodies in muco-cutaneous leishmaniasis have been reported in the literature (BRAY & LAINSON [1]; CAMARGO & REBONATO [2]). So far there has been no attempt recorded in the literature, to standardize the preparation of the antigen in order to obtain both sensitive and specific results.

Culturing of parasites, if mentioned, has been of 7 days (CAMARGO & REBONATO [2]; BRAY & LAINSON [1]) 6 days (NERY-GUIMARÃES et al. [5] and between 6 and 9 days (CHIARI [3]). Usually, the antigen has been washed in 0.15 M NaCl and applied to slides without previous fixation, but CAMARGO & REBONATO [2], have used 1% formalin to preserve the antigen. WALTON et al. [8] were unable to use the IFA test with leptomonads from *in vitro* cultures of *L. braziliensis* as antigen, due to lack of specificity and sensitivity of the test. The Authors proposed that leishmanial forms grown instead in cell monolayers, would provide a more suitable antigen for this purpose.

Since we have observed variations in the antigenicity of culture forms of *L. braziliensis* in fluorescence tests, we have attempted to establish in the present study the necessary parameters in order to obtain an antigen which would ensure sensitive and specific reactions against antibodies present in sera of muco-cutaneous leishmaniasis patients.

We have therefore studied the optimal period of *in vitro* growth of the parasite, the time and concentration of added formalin, the length of time the slides could be stored without loss of antigenicity and the occurrence of cross-reactivity with sera from several pathological conditions.

MATERIAL AND METHODS

Culture and preparation of antigens: Promastigote forms of *L. braziliensis* (strain 6) obtained from the laboratory of Prof. Marcelo Vasconcelos Correa, Belo Horizonte, Brasil, were cultivated in semi-solid NN medium (PACKCHANIAN & SWEETS Jr. [6]).

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The parasites were cultivated in stoppered glass tubes at room temperature (about 22°C). The supernatants were removed to new glass tubes, centrifuged at 3,000 g/10 minutes. Supernatant was discarded and the pellet washed twice with 0.15 NaCl, resuspended in saline-diluted formalin and left for different periods of time at room temperature. The parasites were centrifuged at 3,000 g/10 minutes and washed twice in 0.15M NaCl and the supernatants discarded. The pellet resulting from the final centrifugation was resuspended in 0.15M NaCl, the volume of this solution being adjusted so as to yield 20-25 parasites per microscope field (500 ×).

Preparations of slides — one drop of the solution was placed on microscope slides, excess liquid sucked off and the slide left to dry at room temperature for one hour. The dried slides were kept at −20°C until use.

Fluorescent test — serum samples from patients with muco-cutaneous leishmaniasis were distributed in small aliquots and kept at −20°C. Throughout the study, samples from the same patients were used and for each experiment, an aliquot of those sera was thawed and diluted two-fold from an initial dilution of 1/20. Slides were allowed to reach room temperature for 5 minutes. One drop of each dilution was placed over the antigen and incubated in a moist chamber at 37°C for 30 minutes. The slides were washed twice in PBS (0.01M phosphate buffered 0.15M NaCl, pH 7.2) for 5 minutes each time. The slides were covered with FITC — antihuman gammaglobulin (weight-ratio F : P = 9.6) diluted with PBS containing 0.2% Evans Blue and incubated in a moist chamber at 37°C for 30 minutes. They were washed twice in PBS for 5 minutes each time and covered with buffered glycerin pH 8.0 and coverslip and examined the same day on a Zeiss Fluorescent microscope provided with dark field condenser, HBO-200 as the light source, KP-500 as exciter filter and 50 (Zeiss) as barrier filter. In all experiments 1/20 normal serum was included as negative control.

For reactive serum samples, parasites displayed a bright-green peripheral stain with a dull fluorescence of the cytoplasm. For non-reactive sera, parasites remained as dim red spots. Titers of serum samples were given as the highest dilutions still producing any discernible fluorescent rim around the parasites.

RESULTS

Period of culture growth — The titer of the standard sera varied according to the age of culture of the parasites, as shown in Table I.

In days 4, 9, 10 and 13, an irregular staining of the parasites was seen in all dilutions, with brightly stained ones along with non-stained parasites. In days' 8 there was uniformity of staining in all dilutions.

Titers of sera of muco-cutaneous leishmaniasis did not differ significantly for 8 days old *L. braziliensis* treated with formalin for periods of 30 minutes, 60 minutes, 4 hours, 7 hours, 24 hours and with formalin concentrations of 1%, 2% or 3%. However, a more uniform staining of parasites resulted when these were treated with 2% formalin for 24 hours.

Slides could be kept at −20°C for long periods of time (Table II).

Specificity of the IF tests — The *L. braziliensis* antigen was tested against sera from 5 chronic Hepatitis, 1 Systemic Lupus Erythematosus, 1 Hashimoto's Thyroiditis, 9 acute Toxoplasmosis, 10 Schistosomiasis, 7 Malaria, 11 American Trypanosomiasis and 9 Kala-Azar patients.

Except for sera from American Trypanosomiasis and Kala-Azar patients all other reactions were negative at a 1/20 dilution. In 11 cases of Chagas' disease with positive immunofluorescence tests with *T. cruzi* antigen, negative results were seen with *L. braziliensis* antigen in 2, and positive in 9 but titers were always lower than with the homologous antigen, by one or more frequently by two dilutions.

For Kala-Azar sera the titer was the same in 5 out of 9 cases for both homologous

TABLE I

Maximum titer of sera according to age (in days) of *L. braziliensis* culture

Serum samples	Days of culture					
	4 d.	8 d.	9 d.	10 d.	11 d.	13 d.
132	1/40	1/640	1/1280	1/2560	1/2560	1/80
127	1/40	1/160	1/160	1/80	1/80	1/20
1	1/40	1/320	1/160	1/80	1/80	neg.
137	1/80	1/160	n.d.	1/160	1/160	1/40
155	1/160	1/320	1/160	1/160	n.d.	1/160
161	1/320	1/1280	1/2560	n.d.	1/640	1/160

neg. = negative

n. d. = not done

TABLE II

Titers of positive standard sera for 8 days old *L. braziliensis* after 2% formalin treatment kept at −20°C for varying periods of time

Sera	Unfrozen	Freezing time						
		3 hs	14 hs	24 hs	5 d.	10 d.	45 d.	3 years
P_1	1/160	1/160	1/80	1/80	1/160	1/320	1/320	1/320
P_2	1/80	1/80	1/160	1/160	1/160	1/160	1/160	1/160

(*L. donovani*) and heterologous antigens (*L. braziliensis*). In 2, the titer with the heterologous antigen was higher by at least 2 dilutions, and in 2 it was lower.

DISCUSSION

Table I shows endpoint titrations of standard sera when tested against *in vitro* grown *L. braziliensis*. Uniformity of staining was obtained only when 8 days' old parasites were used. The same did not occur with 9, 10 and 11 days' old *L. braziliensis*. Due to this, day 8 was chosen as optimum for preparation of the antigen. For the same reason a 2% formalin concentration was selected to preserve the parasites.

Cross-reactions were seen only with American trypanosomiasis and Kala-Azar sera, as observed before in the literature (BRAY & LAINSON [1]; NERY-GUIMARÃES et al. [5]; CHIARI [3]).

As seen from our results, both sensitive and specific immunofluorescence tests were obtained from *in vitro* grown *L. braziliensis* strain 6, provided that the parasites are cultured for an optimal period of time and adequately preserved by means of formalin-treatment.

The fact that the antigen was stable for at least 3 years makes it very usefull for serologic studies besides the fact that the *in vitro* culturing of parasites is a more economical and less time-consuming procedure compared to the one proposed by WALTON et al. [8].

The need remains to extend these findings to other strains of *L. braziliensis*.

RESUMO

Padronização antigênica para a leishmaniose cutâneo-mucosa na reação de imunofluorescência

Formas promastigotas de *L. braziliensis* (cepa 6, Belo Horizonte) foram cultivadas em meio semi-sólido NN por 4, 8, 9, 10, 11 e 13 dias. Obtiveram-se reações de imunofluorescência positivas com parasitas cultivados por 8, 9 e 10 dias usando-se soro de pacientes com leishmaniose cutâneo-mucosa, embora houvesse uniformidade de coloração somente com parasitas cultivados por 8 dias.

A mesma uniformidade de coloração foi obtida quando o antígeno foi preservado por tratamento com formalina a 2% (v/v).

O antígeno colocado em lâmina de microscópio foi mantido a −20°C por 3 anos sem perda de antigenicidade.

A especificidade da reação foi verificada com soro de diferentes moléstias. Reações cruzadas foram observadas com soro de pacientes com moléstia de Chagas e Calazar.

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