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MICRO ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE SERODIAGNOSIS OF NEW WORLD LEISHMANIASIS*

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Abstract. Of 21 confirmed cases of New World leishmaniasis, 16 exhibited antibody to antigens of the promastigote of *Leishmania braziliensis panamensis* by the enzyme-linked immunosorbent assay (ELISA). Comparison of antibody titers obtained by ELISA with titers obtained by indirect immunofluorescence using an amastigote substrate confirmed that the sensitivities of the two techniques were within the same range ($r = 0.80$). Although sera from patients with New World leishmaniasis failed to react with antigens extracted from epimastigotes of *Trypanosoma cruzi*, sera from 39 cases of Chagas' disease were reactive with promastigotes of *L. braziliensis panamensis*. This apparent unidirectional cross-reactivity has been attributed to differences in potency of the antigenic stimulus presented in the two diseases.

Prior to 1972 and the advent of an immunofluorescent antibody assay (IFA) using the amastigote as the substrate,[1] we had no conclusive evidence that human infection with *Leishmania braziliensis* resulted in the production of humoral antibodies. The IFA procedure was reported to be specific for antibody to *L. braziliensis* and, as with other IFA techniques, it has a sensitivity of detecting antibody at the nanogram level. Nevertheless, we felt that an enzyme-linked immunosorbent assay (ELISA), such as that which has been described for visceral leishmaniasis,[2] would offer several advantages over the IFA. Specifically, the ELISA would be more economical for large-scale testing in seroepidemiologic surveys and its potential for performance under field conditions looks more promising.

MATERIALS AND METHODS

Serum collection

Sera were obtained from 21 human cases of New World leishmaniasis. Methods of diagnosis, duration of disease and extent of disease (number of lesions) are given in Table 1. An additional 30 sera of known reactivity to *L. braziliensis panamensis* by IFA were retrieved from the serum bank at the Gorgas Memorial Laboratory. Negative control sera were obtained from six United States military personnel who were recent arrivals in the Canal Zone.

Conjugate

The conjugate was the globulin fraction of a goat anti-human globulin serum labeled with alkaline phosphatase (Sigma Type VII). Methods for preparation and determination of working strength of conjugate have been described by Voller et al.[3]

Antigens

Promastigotes of *L. braziliensis panamensis*, originally isolated by Herrer et al.[4] from a two-toed sloth (*Choleopus hoffmanni*) captured in Achiote, Panama, were cultivated on slants of modified Senekjie's medium for hemoflagellates.[5] Prior to the current study, the strain had been transferred 67 times in vitro. The organisms were harvested from the slants and were washed three times in carbonate-bicarbonate buffer, pH 9.6. The final suspension was standardized to 150 μg protein/ml, disrupted by ultrasonic treatment, and stored at −4°C.

Amastigotes of *L. braziliensis panamensis* were recovered from monolayers of Vero cells which

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TABLE 1

*Clinical information for 21 human cases of New World leishmaniasis**

Patient	Age/sex	Lesions/type	Duration (mos.)	Diagnosis
EP	17 ♂	S/C	2	DE
MG	21 ♂	S/C	4	C
FD	23 ♂	S/C	?	M
DG	15 ♂	S/C	12	Neg.
RC	45 ♀	S/C	15 days	M
FM	22 ♂	S/C	12	DE, C, M
IJ	21 ♂	S/C	6	DE
IE	27 ♀	S/C	2	DE, C, M
DM	37 ♂	S/C	12	M
CM	18 ♂	S/C	2	DE, M
CC	36 ♂	S/C	7	DE
EM	32 ♂	S/C	5	DE, C, M
VV	17 ♀	M/C	18 days	DE
IH	62 ♂	M/C	4	DE, C, M
RR	21 ♂	M/C	2	DE, C
JN	53 ♀	M/C	?	M
JR	64 ♂	M/R	24	M
PA	21 ♂	M/C	1	DE
CC	54 ♂	M/C	1	DE
AD	13 ♂	M/C	3	DE
MF	12 ♂	Muco	?	M

* Abbreviations: S/C = single/cutaneous; M/C = multiple/cutaneous; M/R = multiple/recidivan; Muco = mucocutaneous; DE = direct examination of a lesion scraping; C = culture; M = Montenegro skin test.

had been infected with a human isolate (Gorgas no. L004). Methods used for cultivation and harvesting these intracellular forms were those which have been described by Walton et al.[1] The amastigotes, released from infected cells by treatment with 0.02% EDTA in veronal-buffed saline, were washed three times in carbonate-bicarbonate buffer, disrupted by ultrasonic treatment and stored at −4°C. Contamination by proteins originating from Vero cells prohibited accurate protein determinations of the amastigote preparations.

The *Trypanosoma cruzi* antigen was prepared from in vitro cultures of epimastigotes (Panamanian strain from patient MB, Altos del Jobo). Methods of cultivation were those which have been described by Vattuone and Yanovsky.[6]

Working strengths of antigens were determined by titrating positive and negative control sera in wells coated with serial dilutions of antigen (1–150 μg protein/ml for the promastigotes). The highest dilution of antigen which gave maximum titer with positive control serum but which failed to produce color with the undiluted negative control was chosen as the working strength.

ELISA

Wells of polystyrene microtiter plates (Linbro MVC-98) were filled with 200 μl antigen diluted in carbonate-bicarbonate buffer. After an overnight incubation at 4°C, the plates were washed three times in phosphate-buffered saline (PBS), pH 7.4, containing 0.5% Tween 20 and 0.02% sodium azide. Serial dilutions of the test sera, from 1:10 to 1:1280, were prepared in test tubes using PBS-Tween as the diluent. Two hundred microliters of each serum dilution were added to a corresponding well of the plate. After incubation at room temperature for 2 hours the plates were washed an additional three times. Two hundred microliters of conjugate, diluted in PBS-Tween, were added to each well and incubation was continued for 2 hours at room temperature. Plates were washed three more times and 200 μl p-nitrophenylphosphate in 10% diethanolamine buffer (5 mg/ml) were added. After 15 min at room temperature, the reaction was stopped by the addition of 50 μl, 3 M NaOH. Intensity of color development was rated subjectively as 0, 1+, 2+, 3+, or 4+. The highest dilution of serum exhibiting a 3+ or greater coloration was regarded as the endpoint of the titration. Titers ≥1:10 were considered as positive.

IFA

The IFA procedure, which used amastigotes as the substrate, was performed according to the methods of Walton et al.[1] Titers ≥1:8 were regarded as positive.

RESULTS

Sensitivity of the ELISA was assessed by comparing antibody titers obtained with the promastigote antigen to antibody titers of the IFA using an amastigote substrate (Table 2). If an IFA titer of ≥1:8 is considered evidence of infection with *L. braziliensis panamensis*, 10 sera were false negative by ELISA and 1 serum was false positive. Eight of these 10 false negatives had IFA titers in the lower range. Six sera from healthy U.S. military personnel were negative in both assays.

Specificity of the ELISA was evaluated by examining the reactivity of 21 sera, from confirmed cases of leishmaniasis (Table 1), against promastigote and amastigote antigens of *L. braziliensis panamensis* and an epimastigote antigen of *T.*

TABLE 2

Enzyme-linked immunosorbent assay (ELISA) titers vs. indirect fluorescent antibody (IFA) titers for antibody to Leishmania brasiliensis panamensis*

ELISA (promastigote)	IFA (amastigote)								
	<8	8	16	32	64	128	256	512	Total
<10	6†	6	2	1	0	1	0	0	16
10	0	0	0	1	1	0	0	0	2
20	0	0	1	2	2	0	0	0	5
40	1	0	0	1	2	1	0	0	5
80	0	0	0	0	0	4	1	0	5
160	0	0	0	0	0	1	1	1	3
Total	7	6	3	5	5	7	2	1	36

* Coefficient of correlation r = 0.80.
† Healthy controls.

cruzi (Table 3). Of 21 cases, 16 were positive with the promastigote antigen and only six were positive with the amastigote forms. Although the number of cases was small, a relationship between number of lesions and response was suggested. All individuals with multiple lesions, thus greater antigenic stimulus, were positive for antibody to the promastigote antigen as compared to only 58% of the cases with a single lesion. The failure to detect antibody in five cases with single lesions (EP, DG, IJ, CM, EM) could not be attributed to duration of disease. All but one of these cases (DG) were positive for organisms upon direct examination of a lesion scraping. All 21 cases were negative with the *T. cruzi* antigen.

The promastigote form of *L. braziliensis panamensis* does contain antigens which are reactive with antibodies in sera of individuals infected with *T. cruzi* (Table 4). Of 49 sera from cases of Chagas' disease, confirmed by clinical diagnosis, 39 were reactive with the leishmanial antigen.

TABLE 3

Positivity of ELISA for 21 confirmed cases of New World leishmaniasis

Diagnosis*	ELISA		
	Promastigote	Amastigote	*T. cruzi*
Cutaneous			
Multiple lesions	8/8	4/8	0/8
Single lesions	7/12	1/12	0/12
Mucocutaneous	1/1	1/1	0/1

* Diagnosis based upon direct examination, positive culture, or positive Montenegro test.

TABLE 4

ELISA titers for antibody to Leishmania braziliensis panamensis *in sera of villagers infected with* Trypanosoma cruzi*

ELISA—*T. cruzi*†	ELISA—*L. braziliensis* (promastigote)						
	<10	10	20	40	80	160	Total
10	1	0	0	0	0	0	1
20	2	2	1	2	1	1	9
40	3	0	1	0	0	1	5
80	1	1	0	1	1	1	5
160	1	1	4	2	1	1	10
320	0	0	0	3	4	3	10
640	2	0	0	0	1	1	4
1,280	0	0	0	1	2	2	5
Total	10	4	6	9	10	10	49

* Correlation coefficient r = 0.47.
† Confirmed cases of Chagas' disease by clinical diagnoses.

The correlation coefficient (r = 0.47) is significant at $P = 0.001$. Of the nine cases in which the heterologous titer equaled or was greater than the homologous titer, all were also positive with the amastigote of *L. braziliensis panamensis* in the ELISA and five were positive in the amastigote IFA.

DISCUSSION

The specificity of the IFA procedure for detection of an antibody response following infection with *L. braziliensis* is dependent upon the source of antigen. When promastigotes are used as the antigen, serologic cross-reactivity between related and unrelated species occurs.[7-8] This cross-reactivity can be eliminated, however, by using intracellular amastigotes as the antigenic substrate.[1, 9, 10] Nevertheless, as has been pointed out by others,[9] cultivation of adequate quantities of leishmanial amastigotes requires much more expertise in the mechanisms of tissue culture than does the simple procedure of cultivating promastigotes on slants of Senekjie's medium. These authors reported on an alternative procedure whereby amastigotes were recovered from cutaneous lesions of laboratory infected hamsters. Although this method was reported to be "simple" in comparison to tissue culture, it is still far more complex than any of the conventional techniques for recovery of promastigotes.[5, 11] Furthermore, even if adequate quantities of the amastigote substrate could be made available to rural health centers of Central and South America, the requirement of a fluores-

cent microscope precludes the use of IFA as a field assay. Development of an ELISA with a specificity and sensitivity comparable to that of IFA would provide us with a simple and economical assay which, although subjective in interpretation, could be adapted for large-scale seroepidemiologic surveys in the field.

The sensitivity of the ELISA, as described here, approaches that of the IFA. The coefficient of correlation between the two sets of quantitative antibody titers was 0.80. The minor discrepancies in titers may be associated with the source of antigen, since for the IFA an amastigote substrate from a human isolate was used while a promastigote antigen from a strain isolated from a two-toed sloth was used for the ELISA.

Our failure to develop a sensitive ELISA by means of an amastigote antigen was probably a consequence of our inability to eliminate contaminating cell culture proteins from our antigen preparation. These proteins compete for available sites on the polystyrene surface, thus prohibiting adequate coating with leishmanial antigens and resulting in false negative assays. This problem will be alleviated following development of more efficient means for recovering amastigotes from cell cultures and/or skin lesions. In future studies consideration should be given to using amastigotes from Vero cell cultures infected with sloth isolates, and possibly promastigote cultures of the human strain.

Sera from 21 cases of confirmed New World leishmaniasis were negative for antibody in the ELISA developed with a saline-soluble extract of *T. cruzi* epimastigotes as the antigen. Since 16 of these sera were reactive with the promastigotes of *L. braziliensis panamensis* it was thought that the requirement for specificity had been satisfied. However, when sera from 49 confirmed cases of Chagas' disease were examined for antibodies reactive with promastigotes of *L. braziliensis panamensis*, 39 were positive. The apparent unidirectional cross-reactivity is probably a consequence of differences in potency of the antigenic stimulus. Although the two species share common antigens *T. cruzi* is the more potent immunogen, eliciting a profound antibody response to both major and minor antigenic determinants. In contrast, the antibody response to *L. braziliensis panamensis* is weak. If the shared antigens represent minor determinant groups, the ensuing response may be below the threshold of the sensitivity of our assay.

In summary, the need for a good field assay for measuring the antibody response to *L. braziliensis* is obvious. The IFA technique satisfies the requirements of specificity and sensitivity, but it is not readily adaptable to large-scale seroepidemiologic studies. The ELISA procedure fulfills the requirement of sensitivity, but fails with respect to specificity. Future efforts should be directed toward development of improved assays using specific antigens recovered from the heterologous mixture which is now an integral part of our assay system. Development of an assay designed to detect antibody to the specific antigen which elicits a protective immune response could have far-reaching implications in our efforts to control the disease and should be our primary goal.

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