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SEROEPIDEMIOLOGY OF CUTANEOUS LEISHMANIASIS FROM RIBEIRA DO IGUAPE VALLEY. IgM AND IgG ANTIBODIES DETECTED BY MEANS OF AN IMMUNOENZYMATIC ASSAY (ELISA)

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SUMMARY

By means of an immunoenzymatic assay (ELISA) IgM class anti-**L. braziliensis** antibodies are reported in a series of sera from clinical cases of Cutaneous Leishmaniasis and normal individuals, from Ribeira do Iguape Valley, São Paulo State, Brazil. The antibody specificity was demonstrated by absorbing the sera with heat-aggregated gamma globulin. Anti-**L. braziliensis** IgM antibodies were present in treated and untreated Cutaneous Leishmaniasis sera and in 43.21% of normal sera from the same geographical sera. IgG antibodies were present in 86.0% of clinical cases of Cutaneous Leishmaniasis.

INTRODUCTION

Among the 5 diseases studied by the UNDP/World Bank/WHO Tropical Diseases Research (TDR), Mucocutaneous Leishmaniasis serology is one of the least understood. Due to the importance of the disease in Brazil, where it occurs in low level socio-economic areas such as the one reported here, or in jungle areas where trees are felled to give rise to large-scale agriculture, it is important to establish serology patterns that will help to set up a diagnosis where clinical-epidemiological data are scanty or not available.

ELISA tests for IgG anti-**L. braziliensis** antibodies have been described by GUIMARÃES et al. [5] and in the present paper the authors describe IgM anti-**L. braziliensis** antibodies in a population from Ribeira do Iguape Valley by means of an immunoenzymatic assay (ELISA).

MATERIAL AND METHODS

Serum samples — a) Samples from Ribeira do Iguape Valley: 253 sera were tested, 172 from patients with clinical diagnosis of Cutaneous Leishmaniasis bled immediately before any attempt to treat the disease (22 of the patients were bled a second time after specific treatment), and 81 sera from the same area, from individuals who did not show a leishmanial ulcer or scar. All sera were tested by and immunoenzymatic assay (ELISA) for IgM antibodies. The 172 sera were also tested for IgG antibodies according to GUIMARÃES et al. [5].

b) Normal sera and from unrelated pathologies: One hundred and ten, 30 being from normal individuals, 20 from Paracoccidioidomycosis, 30 from Toxoplasmosis (stage II) and 30 from Malaria, were assayed for IgG antibodies.

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All sera were stored at -20°C with an equal amount of neutral glycerin (Merck) to inhibit bacterial growth and avoid protein denaturation, until tested.

Immunoenzymatic assays — The leishmania antigen was prepared as described by GUIMARÃES et al.[5] from **L. braziliensis** (strain 49) promastigotes. The parasites (a strain originally obtained from Minas Gerais State) were cultured in LIT medium (FERNANDES & CASTELLANI)[3].

Anti-IgG and anti-IgM horseradish peroxidase (Sigma Chemical Co.) conjugates were prepared as described by GUIMARÃES et al.[4].

For sensitization, for either IgM or IgG-Elisa, wells of microtiter U-shapped plastic plates (Canalco) were filled with **L. braziliensis** antigen at a protein concentration of 20 μg/ml, in carbonate-bicarbonate buffer (0.1 M, pH 9.6) and left overnight at 4°C. For use, plates were drained and washed 3 times with phosphate-buffered saline (0.01 M in phosphate, pH 7.2) containing 0.09% Tween-20. Doubling serum dilutions (starting dilution 1/20) were incubated for 1 hour at room temperature. After washing 3 times with Tween phosphate buffered saline, wells were filled with 0.2 ml of anti-IgM or anti IgG conjugate diluted in the same solution. Prior to use, the optimum dilution of either conjugate was determined by block titration in order to ensure maximum reactivity. Incubation for 1 hour at room temperature was followed by washings as described and 0.2 ml of substrate (5.2 mM, 5-aminosalicylic acid, and 1-5mM H_2O_2) were added to each well (RIUNTENBERG et al.[6]). The reaction was stopped by adding 1 M NaOH and well contents placed in a glass cuvette and read at 450 nm in a Beckman D.U. Spectrophotometer.

In all tests controls of positive, negative serum and conjugate were included. Negative serum control readings at a 1/20 dilution varied between O.D. of 0.015 to 0.000. End point titration was determined comparing individual readings to negative control readings.

Adsorption with heat-aggregated gamma-globulin — All samples that were positive in IgM-ELISA were absorbed with heat-aggregated gamma-globulin and again tested for IgM antibodies, as described above. Gamma-globulin absorption of sera was done as follows: 100 mg of a commercial preparation of human gamma-globulin (Hoechst do Brasil, Química e Farmacêutica S.A.) was dissolved in 10 ml of 0.01 M phosphate buffered saline, pH 7.2 and placed in a water-bath at 63°C for 10 min. Absorption of serum samples was done by adding 1 volume of heat-aggregated gamma-globulin to one volume of a 1/10 dilution of serum and the mixture was incubated in a water-bath at 37°C for 1 hour (CAMARGO et al.[2]).

Geometric mean titer — For the assessment of geometric mean titers, all data were transformed into $\log_{10}$ ($\times+1$) and the arithmetic average transformed into the corresponding anti-log.

RESULTS

Table I shows the sera distribution according to their reactivity in the enzymatic assays for IgG and IgM anti-**L. braziliensis** antibodies. As seen from Table I, the IgG-assay was positive in 86.0% of sera. Of these, 30.7% were IgM negative and 27.3% presented a non-specific IgM that was removed by gamma-globulin absorption of the serum. Three sera or 2.0% showed only IgM antibodies and 15 sera or 10.0% were negative for both immunoglobulins.

Among the 22 sera from the second bleeding sample, 20 sera were IgG positive and 3 sera presented IgM antibodies that were removed by gamma-globulin absorption of sera. All 22

TABLE I

IgM and IgG distribution in sera of Cutaneous leishmaniasis from Ribeira do Iguape Valley, by ELISA tests

IgM absorption with heat aggregated gamma-globulin		IgG	Number	(%)	2nd bleeding series	(%)
Before	After					
+	+	+	42	28.0	8	36.4
+	neg.	+	41	27.3	3	13.6
neg.	n.d.	+	46	30.7	9	40.9
+	+	neg.	3	2.0	2	9.1
+	neg.	neg.	3	2.0	0	0
neg.	n.d.	neg.	15	10.0	0	0
Total			150	100.0	22	100.0

Neg. = negative
+ = positive
n.d. = not done

sera were positive for at least one immunoglobulin (IgM or IgG) and 8 sera were positive for both.

IgG and IgM titer frequency distribution among the 172 sera is shown in Table II where

T A B L E II

IgG and IgM titer frequency distribution in 172 sera with clinical diagnosis of Cutaneous Leishmaniasis, by ELISA tests

Titer	IgG frequency	(%)	IgM frequency	(%)
<20	24	13.95	70	40.70
20	34	19.77	4	2.33
40	33	19.19	25	14.53
80	36	20.93	60	34.68
160	22	12.79	5	2.91
320	14	8.14	4	2.33
640	5	2.91	2	1.16
1280	1	0.58	1	0.58
>1280	3	1.74	1	0.58
Total	172	100.00	172	100.00
Geometric mean titer	145.45		74.30	

86.0% of sera had IgG titers between 1/20 and 1/160. The geometric mean titer for IgG antibodies was 145.45. For the 102 sera which were IgM positive in ELISA tests 54.60% or 94 had titers between 1/20 and 1/160. The geometric mean titer for IgM antibodies before absorption by aggregated gamma globulin was 74.30. After absorption, 15 sera showed the same titer as before, 42 differed by one serum dilution and 45 differed by two or more dilutions. The geometric mean titer after gamma globulin absorption was 45.92.

For the 81 control sera from the same geographical area, 46 were negative in the IgM-ELISA test. The remaining 35 sera had titers varying between 1/20 to 1/160. Following gamma-globulin absorption, 9 became negative and the remaining 26 had titers ranging from 1/20 to 1/80. The geometric mean titers were 7.44 before absorption and 13.29 after absorption with heat-aggregated gamma-globulin. These results are shown in Table III.

The IgG-ELISA geometric mean titers of the 30 normal sera and 80 unrelated pathology sera were 15.05 and 14.05, respectively.

T A B L E III

IgM anti-**L. braziliensis** titer frequency distribution in 81 control sera, by ELISA tests

Titer reciprocal	Heat aggregated gamma-globulin Before absorption frequency	(%)	After absorption frequency	(%)
<20	46	56.79	9	25.71
20	24	29.63	11	31.43
40	6	7.41	13	37.14
80	3	3.70	2	5.72
160	2	2.47	0	0
Total	81	100.00	35	100.00
Geometric mean titer	7.44		13.29	

Table IV shows IgM and IgG anti-**L.braziliensis** titers in second bleedings from 22 patients from Ribeira do Iguape Valley series.

T A B L E IV

IgM and IgG anti-**L. braziliensis** titers in second bleedings from 22 patients, by ELISA test

Initials	IgM titer before and after gammaglobulin absorption				IgG titer	
	1st sample		2nd sample		1st sample	2nd sample
B.P.S.	80	40	40	40	320	20
M.E.J.	40	40	80	40	80	40
M.J.M.	320	160	160	80	320	160
J.P.F.	320	160	160	80	>1280	160
V.A.M.	160	80	80	neg.	320	40
M.D.M.R.	neg.	neg.	neg.	neg.	40	20
J.P.B.	neg.	neg.	neg.	neg.	40	80
Le.C.P.	neg.	neg.	neg.	neg.	80	40
Li.C.P.	neg.	neg.	neg.	neg.	80	20
H.C.M.	neg.	neg.	neg.	neg.	160	160
O.G.D.	neg.	neg.	neg.	neg.	40	20
M.A.R.	neg.	neg.	40	40	320	neg.
J.C.L.	80	20	neg.	neg.	40	20
M.B.M.	80	40	neg.	neg.	320	80
N.R.P.	80	neg.	80	40	80	neg.
J.A.L.	40	neg.	80	20	320	20
A.L.	neg.	neg.	80	40	160	40
M.R.P.B.	40	40	neg.	neg.	40	20
N.C.M.	neg.	neg.	80	40	80	20
A.F.R.O.	neg.	neg.	80	80	160	80
M.T.S.	neg.	neg.	neg.	neg.	640	160
O.F.	neg.	neg.	40	20	320	40

Neg. = negative

DISCUSSION

Cutaneous Leishmaniasis constitutes a public health problem in Ribeira do Iguape Valley, São Paulo State, Brazil. In 1979, of 213 cases that occurred in the State, 193 or 95.42% were found in that region, of which, the majo-

rity was represented by the cutaneous form of the disease (183 out of 193). The disease occurs in children from 6 to 14 years of age as well as in adults and is equally distributed between males and females and between rural workers and housewives, suggesting that the sand-fly habitat is near the houses[7]. Also, this is a region where the forest was felled to allow for the development of cultures such as bananas in the low lands and tea on the high ones.

Cutaneous Leishmaniasis in Ribeira do Iguape Valley presents IgM and IgG in varying proportions. IgM was present in 59.30% of 172 clinical cases of Cutaneous Leishmaniasis of which 31.98% were represented by specific anti-**L. braziliensis** IgM, as shown by results found after absorption with heat-aggregated gamma-globulin.

Non-specific IgM was present in 27.33% of the clinical cases and in 43.20% of the control sera before absorption with heat aggregated gamma-globulin. Of these, 25.71% (9 sera) showed titers lower than 1/20 following gamma-globulin absorption, as seen in Table III. As shown by CAMARGO et al.[1] this is a common finding in populations submitted to multiple infections, parasitic and otherwise, due to their low socio-economic level as it is the case in Ribeira do Iguape Valley.

In the 22 sera that represented second bleedings from clinically positive cases as shown in Table I, the presence of both immunoglobulins increased to 36.4% and the presence of non-specific IgM decreased to 13.6%. The presence of one immunoglobulin in the absence of the other increased in this series. IgG without IgM was 40.9% (30.7% in the first bleeding series). The presence of both immunoglobulins increased in the second bleeding series from 28.0 to 36.4%.

As seen from Table IV, IgM distribution upon a second bleeding did not follow a discernible pattern. Seven cases out of 22 never showed any IgM. In 4, titers were the same in the first and second bleedings, in 4, titers fell to negative and in 7, titers increased from negative to some positivity. IgG was present in 90.0% of sera. IgG distribution was such that 10 sera showed the same titer as in the first bleeding or differed by one 2-fold dilution and 12 showed titers reduced by 2 or more 2-fold dilutions. The data reported here corroborate the findings by WALTON[8], that the antibody response in Cutaneous Leishmaniasis is short-lived, due to either healing of ulcers or treatment.

As seen from Fig. 1, in the diseased population and in the group comprised of 57 indi-

IgM TITER DISTRIBUTION IN CUTANEOUS LEISHMANIASIS SERA FROM RIBEIRA DO IGUAPE VALLEY, BY ELISA TESTS

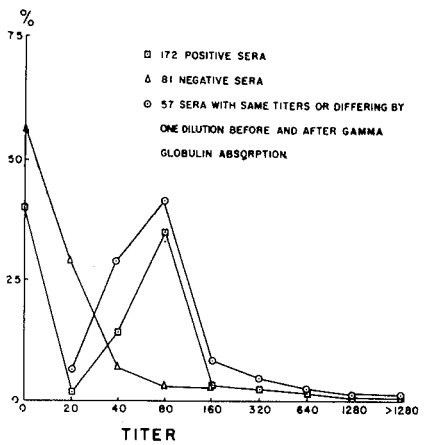

Fig. 1 — IgM antibodies titer distribution in Cutaneous Leishmaniasis

viduals, who, following absorption with heat-aggregated gamma globulin showed titers equal or differing by one dilution from the previous one, IgM antibodies show a Gaussian distribution whereas the 81 normal sera distribution assumed an assymptotic curve to the x and y axes, compatible with naturally occurring antibodies in a population. Also the IgM geometric mean titer was 74.31 for the clinically positive cases compared to 7.44 for normal sera before absorption with heat-aggregated gamma-globulin or to 13.29 after absorption. In the Ribeira do Iguape serum series IgM-ELISA (before adsorption) had a sensitivity of 0.5930, a specificity of 0.4321 and a predictive value of 0.6892. After absorption and accepting the cut-off titer of 1:40 (as seen from Figure 1) these values were 0.5697, 0.8148 and 0.8672, respectively. IgG-ELISA had a sensitivity of 0.8663, a specificity of 0.7545 and a predictive value of 0.8466.

In face of the high specificity shown by IgM-ELISA after absorption and the high sensitivity of IgG-ELISA it is recommended in order to reach a surer serological diagnosis of Cutaneous Leishmaniasis to perform both tests. An IgM-ELISA test with titers below 1:40 would quite certainly rule out Cutaneous Leishmaniasis and an IgG-ELISA with titers above 1:20 would be highly indicative of Cutaneous Leishmaniasis.

For IgG-ELISA the cut-off titer should be a 1/20 dilution in view of the findings for 30 normal sera and 80 sera from unrelated pathologies.

RESUMO

Soroepidemiologia de Leishmaniose cutânea no Vale do Ribeira. Anticorpo IgM e IgG detectados por enzima imunoensaio (ELISA)

A presença de anticorpos de classe IgM é descrita numa série de soros de leishmaniose cutânea e de indivíduos normais do Vale do Ribeira, Estado de São Paulo, Brasil. A especificidade do anticorpo foi demonstrada pela absorção de soros IgM-positivos com gamaglobulina agregada pelo calor. Os anticorpos IgM anti-**L. braziliensis** estavam presentes em casos não tratados e tratados de leishmaniose cutânea e em 43,21% dos soros de indivíduos normais da mesma área. Anticorpos de classe IgG estavam presentes em 86,0% dos casos clínicos de leishmaniose cutânea.

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