The relationship between severity of ulcerated lesions and immune responses in the early stage of cutaneous leishmaniasis in Ecuador

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The relationship was examined between the severity of ulcerated lesions and immune responses in 19 Ecuadorian patients in the early stages of New World cutaneous leishmaniasis. As an immunological assay, the humoral immune response was assessed by enzyme-linked immunosorbent assay (ELISA) and the cell-mediated response by delayed type skin test for leishmanial antigen (leishmanin test). There was a statistically significant correlation ($r=0.61$, $P<0.01$) between the total area of ulcerated lesions and the reciprocal titre of ELISA in identical subjects. However, no significant difference was observed in the ELISA titre between patients with a single lesion and those with multiple lesions ($\chi^2=7.06$, $df=5$, $P>0.2$). A correlation ($r=0.48$, $P<0.05$) was observed between the area of lesions and the intensity of leishmanin test; further, the ELISA titre also correlated to the intensity of leishmanin test in the identical subjects ($r=0.71$, $P<0.01$). These results suggest that the severity of ulcerated lesions relates to the activation of both the humoral and cell-mediated immune systems in the early stage of New World cutaneous leishmaniasis.

New World cutaneous leishmaniasis is widely distributed in Central and South America, where it is a considerable health hazard (Molyneux and Ashford, 1983). Serological examinations and delayed type skin tests have been performed recently, for the diagnosis of the cutaneous leishmaniasis in addition to parasitological examination by skin biopsy (Bray, 1980). However, little is known about the correlation between the severity of ulcerated leishmanial lesions and immune responses in natural infections with New World leishmaniasis. We therefore looked for a possible correlation between the area of ulcerated lesions and the humoral immune response by ELISA and the cell-mediated one by skin test, employing parasitologically confirmed leishmaniasis patients in Ecuador.

MATERIALS AND METHODS

Patients

The parasitological examination was carried out on subjects with ulcerated dermal lesions who visited the Instituto Nacional de Higiene y Medicina Tropical, Guayaquil, Ecuador. Biopsies were taken from the indurated edges of ulcerated lesions. Impression smears were dried, and then stained with Wright's solution. Microscopical examination of these specimens revealed amastigotes of *Leishmania braziliensis s.l.* from the lesions of 19 subjects, 18 males and one female, ranging from 17 to 38 years of age. All subjects examined were infected during their short visit to the endemic area of leishmaniasis, and the duration of ulceration ranged from one to two months from the onset.

Antigen

Promastigotes of *Leishmania* sp., isolated by Hashiguchi *et al.* (1985) from *Bradypus variegatus ephippiger* captured in Naranjal, Ecuador, were cultured on slides of modified Senekjic's medium (Herrer *et al.*, 1966). The promastigotes were washed three times with physiological saline and were ruptured by ultrasonic treatment in 0.05 M carbonate bicarbonate buffer (CBB), pH 9.6, containing 0.02% sodium azide for the enzyme-linked immunosorbent assay (ELISA), and homogenized in physiological saline for the skin test. These homogenates were centrifuged at 10 000 *g* for 30 minutes at 4°C. Supernatants were used as a crude antigen. The concentration of protein was determined by the method of Lowry *et al.* (1951).

Elisa

The ELISA procedure was slightly modified from that described by Korenaga *et al.* (1983). EIA microtitration plates (Flow Laboratories Inc., U.S.A.) were coated overnight at 4°C with 100 µl of the antigen solution diluted in CBB. The plates were washed three times with 0.05% Tween 20 in saline: 100 µl of test sera, serially diluted from 1:10 to 1:320 in 0.05% Tween 20 in 0.02 M phosphate buffered saline, were added to each well and the plates were incubated at 37°C for 30 minutes.

After washing, 100 µl of conjugate diluted in 0.05 M PBS-T were added. The conjugate was horseradish peroxidase-goat anti-human heavy and light chain IgG (Miles Laboratories Inc., U.S.A.). The substrate was 0.1 mg of orthophenyldiamine (OPD) per ml and 0.03% of H₂O₂ in 0.05 M acetate buffer, pH 4.5: 150 µl were added to each well and the plates were incubated at 25°C in the dark. The enzyme reaction was stopped by the addition of 50 µl of 4 N H₂SO₄. The absorbance was read by a spectrophotometer (Corona MTP-12, Nissey Sangyo, Japan) at 500 nm. For the determination of antigen and conjugate concentrations, serial dilutions of antigen from 60 µg ml⁻¹ to 1 µg ml⁻¹, and of conjugate from 1:50 to 1:2000, were reacted with several dilutions of the standard positive and negative sera. From these pre-experiments it was shown that the best concentration of antigen was 20 µg ml⁻¹ protein, and that of conjugate was 1:500. The diluted serum sample thus tested was determined as its reciprocal titre when the ELISA value was > 0.36.

Delayed Type Skin Test

The concentration of antigen was adjusted to 40 µg ml⁻¹ of saline containing 0.005% Merthionate. Both the antigen and control solutions were passed through the microporous membrane filter (0.45 µm) for sterilization. A skin test (leishmanin test) was performed by inoculation of 0.1 ml of solution into the inner surface of the forearm of patients and control volunteers. After 48 hours, the size of induration was measured.

To examine non-specific cell-mediated immune response, intradermal injection of 0.1 ml of purified protein derivatives (PPD) from *Mycobacterium tuberculosis* PT 23 (equivalent to

0.005 µg of PPD-s) was performed. After 48 hours, an induration of 5 mm or more in diameter was considered as a positive reaction to the test.

RESULTS

A total of 36 ulcerated lesions observed in 19 patients were all of the cutaneous type. The areas of the lesions ranged from 0.2 to 7.1 cm². The frequency and location of lesions on the body was as follows: 27 lesions on arms, four on legs, two on ears, two on the neck and one on the abdomen. Eleven patients harboured a single lesion, and the remainder harboured multiple lesions, the numbers of which ranged from two to six.

TABLE 1
The reciprocal titre of sera determined by the enzyme-linked immunosorbent assay (ELISA) using Leishmania sp. promastigote antigen

Groups examined	Reciprocal titre by ELISA						
	<10	10	20	40	80	160	320
Leishmaniasis*		3	5	7	1	2	1
Ecuadorian healthy control	14	5					
Japanese healthy control	10						

*Diagnosed as cutaneous leishmaniasis by the biopsy of the ulcerated lesions.

The reciprocal ELISA titres of sera from 19 leishmanial patients confirmed parasitologically, and from 19 healthy Ecuadorians and ten healthy Japanese as controls, are shown in Table 1. ELISA titres of all the patients were 10 or more. On the other hand, the control titres were less than 10 in 14 of the Ecuadorians and all ten Japanese, and 10 in five of the Ecuadorians.

In the leishmanin test, an induration of 5 mm or more in diameter was observed in 11 (57.9%) of 19 leishmanial patients as compared to seven (17.5%) of 40 Ecuadorian controls. There was no significant difference in the positive rates of the skin test by PPD between leishmanial patients with a leishmanin test of less than 5 mm (six positives out of eight by PPD), patients with a leishmanin test of 5 mm or more (ten out of 11), and Ecuadorian controls (30 out of 40).

The correlation between the total area of ulcerated lesions per person from 19 leishmanial patients and their ELISA titres is shown in the Figure. There was a significant correlation between the two parameters ($r=0.61$, $P<0.01$). However, no significant difference was observed in the ELISA titres between subjects with a single lesion and those with multiple lesions ($\chi^2=7.06$, $df=5$, $P>0.2$).

There was a correlation ($r=0.48$, $P<0.05$) between the area of lesions and the size of the induration of the leishmanin test in individuals. No significant difference was observed in the leishmanin test between subjects with a single lesion and those with multiple lesions ($\chi^2=4.48$, $df=3$, $P>0.2$).

As shown in Table 2, the ELISA titre also correlated significantly with the size of the induration of the leishmanin test in the same subject ($r=0.71$, $P<0.01$).

Fig. Correlation between the total area of ulcerated lesions and the reciprocal titre of the sera determined by ELISA in subjects with cutaneous leishmaniasis. ●, Single lesion; ○, multiple lesions. Correlation coefficient $r=0.61$, $P<0.01$.

TABLE 2

*Correlation between the reciprocal titre of sera determined by the enzyme-linked immunosorbent assay (ELISA) and the induration size following the leishmanin test in subjects with cutaneous leishmaniasis**

Diameter (mm) of induration by leishmanin test†	Reciprocal titre by ELISA					
	10	20	40	80	160	320
<5	3	4	1			
5-7.5		1	1		1	
7.5-10			3	1		
10 ≤			2		1	1

*Correlation coefficient $r=0.71$, $P<0.01$.

†The induration was measured at 48 hours post-injection.

DISCUSSION

Our study indicates that there are correlations between the severity of ulcerated lesions, the reciprocal titre of ELISA and the intensity of the leishmanin test in early-stage patients with New World cutaneous leishmaniasis in Ecuador.

It has been reported that antibodies of patients infected with New World leishmaniasis were not developed to the same degree as in those with visceral leishmaniasis (Mauel and Behin, 1982). The present procedure, which detects specific IgG antibody by ELISA, can

distinguish leishmaniasis patients from healthy controls, but the titres of patients were not so high as reported previously by Anthony *et al.* (1980). IgG antibody assessed by ELISA was correlated to the total area of ulcerated lesions per person. Anthony *et al.* (1980) suggested that there was a relationship between the number of lesions per person and the ELISA titre of New World leishmaniasis, using promastigote antigen. In their study, the ELISA positive rate of subjects with multiple lesions was higher than that of those with a single lesion. However, no such relationship was observed in the current examination.

It is well known that the leishmanin test is useful for the diagnosis of New World cutaneous leishmaniasis (Bray, 1980). In our leishmanin test the induration of eight cases out of 19 was less than 5 mm, though the same test revealed strong reactivity in four subjects with larger lesions in whom the history of leishmaniasis was more than ten months (data not shown). On the other hand, a non-specific depression of cell-mediated immune response, such as found in Kala-azar (Rees *et al.*, 1981), was not shown by the skin test with PPD in our patients. From the results obtained, it is suggested that subjects with low reactivity to the leishmanin test, and whose total areas of ulcerated lesions are small, are in the early stages of *Leishmania* infection and have not yet strongly activated the cell-mediated immune system, nor the humoral immune system, as revealed by their low ELISA titre (Table 2). It is interesting that the ELISA titre correlated with the intensity of the leishmanin reaction in the same patient. In Kala-azar, high levels of specific antibodies are produced, while cell-mediated immunity is not specially detectable (Mauel and Behin, 1982). In the early stages of infection, New World cutaneous leishmaniasis might activate both the humoral and cell-mediated systems at the same time.

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