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Antibodies to Laminin in American Cutaneous Leishmaniasis

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We found that serum samples from patients with different clinical forms of American cutaneous leishmaniasis (ACL) contained immunoglobulin G and immunoglobulin M antibodies which reacted with laminin but not with various other purified connective tissue components, such as collagen types I, III, IV, and V and fibronectin. Eighty-one percent of ACL patients had high antilaminin antibody levels, with a relationship existing between ACL ulcers and antibody levels. This was not, however, the case with patients having treated and healed ACL ulcers; only 34% of these patients had elevated antilaminin antibodies. Eighty-four percent of chronic Chagas' disease patients were also found to contain antilaminin antibodies that were limited to the immunoglobulin G class, but these were not detected in patients suffering from any of 11 other infectious diseases.

It has recently been shown that humans and monkeys infected with *Trypanosoma cruzi*, the hemoprotozoan causing Chagas' disease, produce antibodies that react with extracellular structures, particularly basement membranes. The serum samples examined were found to contain high titers of antibodies to laminin but not to various other connective tissue components (15). Furthermore, enzyme-linked immunosorbent assays (ELISA) indicated that serum samples from both acute and chronic Chagas' disease patients contained antibodies to laminin and that the highest titers were present in the acute phase.

Additional experiments (15) revealed that purified antilaminin antibodies react strongly with *T. cruzi* trypomastigotes and amastigotes but show only a weak reaction with epimastigotes. It was concluded that because the trypomastigote form of *T. cruzi* encounters basement membranes during its penetration into the host, the production of a laminin-like protein or incorporation of host laminin or both may permit the parasite to interact with the host tissues (15). The second possibility has recently been supported by the demonstration that *T. cruzi* trypomastigotes emerging after intracellular replication in WOS sarcoma monolayers express a sarcoma-associated surface antigen (4).

In this report, we present results showing that serum samples from humans with different clinical forms of American cutaneous leishmaniasis (ACL) contain antibodies of various classes, which react specifically with laminin but not with other purified connective tissue components, such as collagen types I, III, IV, and V or fibronectin.

MATERIALS AND METHODS

Patients. Serum samples were examined from 125 patients, aged 3 to 66 years, with either acute or healed ACL lesions. In all cases, the evolution of the infection was at least 1 month. These subjects included 74 untreated acute ACL patients examined at random during outbreaks of the disease in Acevedo and Paez Districts of Miranda State and Torres District of Lara State, Venezuela. In addition, 29 patients from Acevedo District who had been treated with meglumine antimoniate (Glucantime; Specia Laboratories, Paris France; 50 to 60 mg/kg for 12 days, two courses) 3 months before and whose lesions were completely healed were examined. Twenty-two acute ACL patients from Acevedo and Paez Districts who had positive serological tests for Chagas' disease infection were also evaluated. Since there is no recent description of acute Chagas' disease in this area and since the serology has markedly decreased in children aged 0 to 9 years, it was inferred that a positive Chagas' disease serology meant a previous contact with ACL. In addition, some diffuse cutaneous or mucocutaneous leishmaniasis cases were studied in patients of the Instituto Nacional de Dermatología (J. Convit). Thirty healthy Venezuelan individuals and 32 patients with inflammatory diseases other than ACL (2 with histoplasmosis, 4 with lepromatous leprosy, 4 with lupus erythematosus, 2 with malaria, 5 with paracoccidioidomycosis, 2 with toxoplasmosis, 2 with sporotrichosis, 3 with chromomycosis, 2 with aspergillosis, 2 with cutaneous mycoses caused by *Trichophyton rubrum*, 2 with micetoma caused by *Nocardia brasiliensis*, and 2 with amoebiasis) from the same geographical area were also studied; all of these subjects showed a negative leishmanin reaction and were serologically negative for Chagas' disease. We also studied 37 chronic Chagas' disease patients whose clinical evaluations have previously been partially described (12) and 6 adult New York residents in good health without known parasitic diseases.

Leishmanin tests. Leishmanin was produced by autoclaving *Leishmania braziliensis* subsp. *guyanensis* BG (3), cultured in minimal essential medium supplemented with 2.5% fetal calf serum, as previously described (2). This preparation was more potent as an antigen than the same promastigotes grown in biphasic media (unpublished observations), and after cutaneous tests in several human volunteers, the final parasite concentration was adjusted to 6.5×10^6 organisms per ml. The diameter of the induration resulting from the intracutaneous injection of 0.1 ml was measured after 24 and 48 h, ≥10 mm usually being considered as a positive reaction. This leishmanin caused no appreciable reactions when tested in the skin of 142 healthy individuals who lived in a nonendemic area and who had no prior history of serious protozoan or bacterial infection.

Chagas' disease serology. Quantitative complement fixation and indirect hemagglutination tests were performed as previously described (11). Titers higher than 1:16 and 1:256 were considered as positive for the complement fixation and indirect hemagglutination tests, respectively. It was verified that all the Chagas' disease patients studied were positive by both serological tests.

Antigens. Laminin was extracted from the Engelbreth-

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Holm-Swarm (EHS) tumor grown in C57BL mice and was isolated as previously described (17).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the laminin preparation revealed the presence of two chains with molecular weights of 400,000 and 200,000. In addition, antibodies raised in various rabbits and subsequently purified were found to precipitate the laminin in a single line of identity. Fibronectin was obtained from Collaborative Research, Inc., Waltham, Mass.

ELISA. The ELISA performed here employed peroxidase-labeled antiglobulin (conjugate) for the indirect detection of antibody. Laminin was suspended in 0.01 M carbonate buffer, pH 9.6, at a concentration of 1 μg/ml. A sample of this solution (200 μl) was placed in each well of a 96-well flat-bottom polystyrene microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated at 4°C for 48 h. The excess antigen was then removed by aspiration, and the wells were filled with phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20. This process was repeated three times. Study serum samples were then added to the wells. Each serum sample was diluted in a buffer consisting of PBS containing 0.05% Tween 20 and 0.02% NaN_3, 200 μl being added to triplicate wells of antigen. Each plate also had internal controls consisting of (i) serial dilutions of pooled human Chagas' disease serum samples, (ii) fixed dilutions of pooled human leishmaniasic serum samples, and (iii) fixed dilutions of normal human serum samples. Considering the results of titrations performed in preliminary studies, a 1:1,600 dilution of the test serum samples was chosen for the analysis presented in this study.

The plates were then incubated for 4 h at room temperature in a humid box. After further washing, except when indicated, 200 μl of freshly diluted (1:2,000) peroxidase-labeled polyvalent goat anti-human immunoglobulin conjugate was added in PBS-Tween. The plates were incubated for 16 h at 4°C, and after three washes with PBS-Tween, 200 μl of an 0.003% H_2O_2–0.001% *o*-dianisidine solution in PBS (pH 6.0) was added as a substrate-chromogen. The plates were next incubated in the dark at 25°C for 60 min. The reaction was stopped by adding 10 μl of 5 N HCl, and the optical density at 405 nm (OD_{405}) was read with a MicroELISA reader (Dynatech). Under previous experimental conditions, enzyme activity was proportional to the incubation time up to an OD of about 2,800.

Absorption procedures. Some of the antilaminin antibody-positive serum samples were retested by ELISA after being absorbed with either blood group O or blood group A human erythrocytes (RBC) (2 volumes of packed cells per volume of serum diluted 1:2 in PBS), guinea pig RBC (same ratio as with human RBC), acetone powder of human liver, heart, and skeletal muscle (100 mg/ml of serum diluted 1:4 in PBS), or *T. cruzi* epimastigotes or *Leishmania mexicana* or *L. braziliensis* promastigotes (grown as previously described [2], washed twice in PBS, and mixed at 10^8 parasites per ml of serum diluted 1:4 in PBS).

Data analyses. Each serum was assayed in triplicate, and the arithmetic mean of the OD_{405} readings was used in all analyses. The mean and standard deviation OD readings at serum dilutions of 1:1,600 were calculated for each of the eight study populations. Each of the six groups of patients was compared with the Venezuelan control group by calculation of the standard error of the difference between the means.

Reagents. Minimal essential medium and fetal calf serum were purchased from GIBCO Laboratories (Grand Island, N.Y.). Peroxidase-labeled polyvalent goat anti-human immunoglobulins and specific anti-human immunoglobulin G (IgG), IgA, and IgM were from the Institut Pasteur, Paris, France. All other chemicals were of reagent grade.

RESULTS

General characteristics of the study populations. Except for the group with treated and healed ACL ulcers, there were close similarities in the mean ages and percentages of male subjects among the other groups, namely the Venezuelan and U.S. control groups and the groups with ACL ulcers, recently closed ACL ulcers, ACL ulcers plus Chagas' disease infection, chronic Chagas' disease, and other inflammatory disease patients. The group with treated and healed ACL ulcers had a different age and sex profile because it was composed mostly of individuals who did not attend the rural medical center during the sampling of the general population.

The clinical evolution times of ACL ulcers (that is, the time lapses between the first clinical manifestations of the ulcers and the time that patients were studied) were 3.8 ± 1.9, 4.4 ± 1.7, 6.4 ± 7.7, and 7.9 ± 8.6 months for the groups with recently closed ACL ulcers, ACL ulcers plus Chagas' disease infections, ACL ulcers, and treated and healed ACL ulcers, respectively.

Significant differences in leishmanin reaction diameters were found among ACL patients, Venezuelan controls, and U.S. controls; 8% of the control Venezuelan population from the endemic area had leishmanin reaction diameters greater than 10 mm, compared with none in U.S. controls. Furthermore, leishmanin reaction diameters were significantly ($P < 0.05$) greater in patients with ulcers or recently closed ACL ulcers than in those with treated and healed ACL lesions. It was found that 91% of the patients with ulcers or recently closed ACL ulcers had leishmanin reaction diameters greater than 15 mm, compared with only 49% of the patients with treated and healed ACL ulcers.

With height and weight used as clinical criteria, no evidence of malnutrition was found in the Venezuelan populations studied.

ELISA analysis of Venezuelan and U.S. controls, patients with different clinical forms of ACL, and Chagas' disease patients. The results of the ELISA for antilaminin antibody are shown in Fig. 1 and Table 1. It can be seen by comparing the two control groups that Venezuelan subjects had higher mean antibody levels than the U.S. controls. The spread seen in Fig. 1 and the large standard deviations noted indicate the considerable variation in the antilaminin antibody responses of different patients with ACL or Chagas' disease. The OD_{405} of 0.534 represents the upper limit of normality, defined as the mean plus two standard deviations, of the Venezuelan control group.

ACL. With an OD_{405} of 0.534 as noted above, 81% of the active ACL patients (that is, those having ulcers or recently closed ulcers) and 34% of the patients with treated and healed ACL ulcers had higher antilaminin levels than normal. The ACL ulcer group contained individuals with extremely elevated values ($OD_{405} > 1,600$), which represented 23% of the total ACL patient groups. Such values were not found in the Venezuelan or U.S. controls or in the inflammatory disease patients. No correlation was seen between the time course of the disease and the antilaminin antibody titers (data not shown).

From studying the various clinical forms of ACL (Table 1), it was found that mucocutaneous leishmaniasis patients had the highest antilaminin antibody levels ($P < 0.005$) among the ACL groups, whereas patients with diffuse and

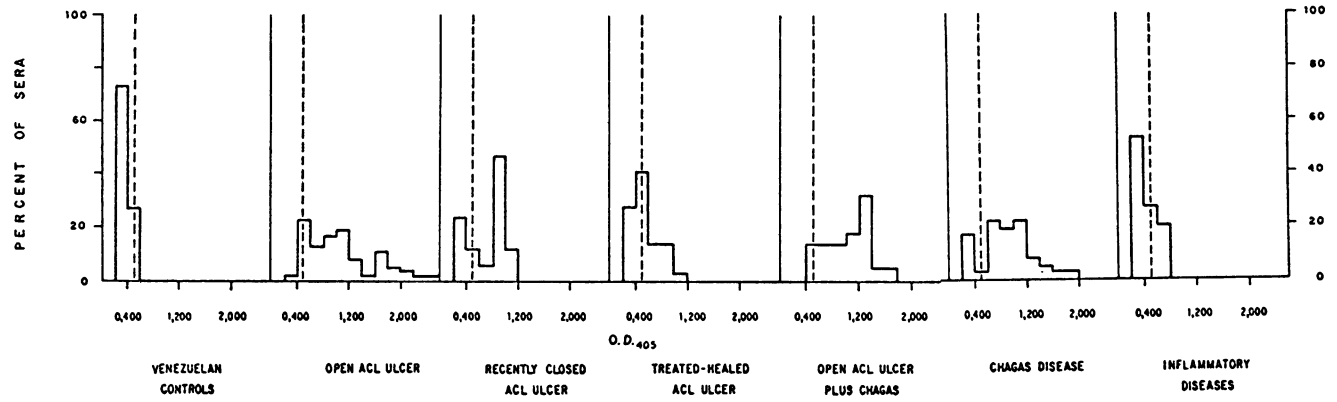

FIG. 1. ELISA results on serum samples from 194 patients with ACL, Chagas' disease, and inflammatory diseases, and from 30 Venezuelan controls. Each serum specimen is from a different individual. The dotted line represents the upper limit of normal as defined in the text.

localized cutaneous leishmaniasis had comparably elevated levels when compared with control patients ($P < 0.005$).

Chagas' disease patients. Eighty-four percent of chronic Chagas' disease patients had antilaminin antibody levels higher than normal, with a significantly higher mean level compared with Venezuelan controls. These levels were, however, similar to those found in patients with active ACL. This occurred despite the acute state of ACL (Table 1). Only 5.4% of Chagas' disease patients had OD_{405} values higher than 1,600.

Chagas' disease infection plus ACL. Since it is possible that the ACL patients also have Chagas' disease, we evaluated beforehand by serological methods the presence of anti-*T. cruzi* antibodies. ACL patients having these antibodies were excluded from the three ACL patient groups (Table 1) and formed a mixed group (ACL plus Chagas' disease infection). We found that 22 of the 125 ACL patients (17.6%) were also serologically positive for Chagas' disease, 86% of these patients having an OD_{405} greater than the upper limit of normality.

Inflammatory diseases. An interesting finding was that 32 patients having acute or chronic inflammatory diseases had a mean OD_{405} that was not significantly different from that of control patients ($P > 0.10$), 73% having OD_{405} values below 0.534. This contrasts with the findings for acute ACL and chronic Chagas' disease.

Immunoglobulin class distribution of antilaminin antibody. Table 2 shows that the antilaminin antibodies were mainly of IgG class in chronic Chagas' disease patients but were IgG and IgM in acute ACL patients, with lower reactivity being detected in IgA.

Specificity of antilaminin response. Table 3 shows that serum samples from patients with ACL or chronic Chagas' disease did not react significantly with collagen types I, III, IV, and V or with fibronectin.

Absorption of antilaminin antibody-positive serum samples. Attempts to absorb antilaminin antibody-positive serum samples by incubation with *T. cruzi* epimastigotes, *L. mexicana* AZV or *L. braziliensis* promastigotes, human organ acetone powder, human blood group O and A, and guinea pig RBC failed to decrease OD_{405} values (data not shown).

DISCUSSION

One of the primary goals of this study was to determine whether ACL patients had antilaminin antibodies. Our results indicate that in fact humans infected with either of two different members of the family *Trypanosomatidae* have high levels of such antibodies. In our study, the presence of

TABLE 1. Antilaminin antibody levels and leishmanin reactivity in healthy controls and in study groups of patients

Study group[a]	Leishmanin reaction (maximum diam, mm)	OD_{405}[b]	P[c]
Venezuelan controls (30)	1.90 ± 0.26	0.360 ± 0.087	
U.S. controls (6)	1.00 ± 0.09	0.253 ± 0.031	>0.05
Cutaneous ACL (74)	18.51 ± 2.37[d]	1.003 ± 0.410	<0.005
Mucocutaneous ACL (5)	26.67 ± 2.74[d]	1.429 ± 0.205	<0.005
Diffuse cutaneous ACL (5)	1.12 ± 0.08	1.074 ± 0.405	<0.005
Recently closed ACL ulcer (17)	17.68 ± 1.72[d]	0.923 ± 0.291	<0.005
Treated and healed ACL ulcer (29)	12.23 ± 2.07[d]	0.536 ± 0.232[e]	>0.05
ACL ulcer plus Chagas' disease infection (22)	18.29 ± 1.84[d]	1.047 ± 0.364	<0.005
Chronic Chagas' disease (37)	1.30 ± 0.24	0.915 ± 0.390	<0.005
Inflammatory diseases (32)	1.97 ± 0.16	0.414 ± 0.181	>0.05

[a] Number of patients is shown in parentheses. All patients produced negative serological tests for Chagas' disease except those in the groups with ACL ulcer plus Chagas' disease and chronic Chagas' disease.
[b] Mean ± standard deviation at a serum dilution of 1:1,600.
[c] Determined in comparison with the Venezuelan control group.
[d] $P < 0.001$ compared with controls.
[e] $P < 0.05$ compared with other ACL groups.

TABLE 2. Distribution of antilaminin antibodies among serum immunoglobulin classes[a]

Study group (no. of patients)	OD_{405}			
	Total immunoglobulin	IgA	IgG	IgM
Controls (6)	0.423 ± 0.092	0.240 ± 0.031	0.310 ± 0.053	0.416 ± 0.048
ACL ulcer (6)	1.476 ± 0.432	0.595 ± 0.156	1.665 ± 0.287	0.958 ± 0.257
Chagas' disease (6)	0.852 ± 0.204	0.341 ± 0.085	1.018 ± 0.301	0.537 ± 0.144

[a] Peroxidase-conjugated goat anti-human immunoglobulin sera were used at the following concentrations: total immunoglobulin, 1:2,000; IgA, 1:500; IgG, 1:1,500; IgM, 1:2,000.

such antibodies was demonstrated by ELISA. The laminin used in the test was purified from EHS tumors of C57BL mice, but as shown by Szarfman et al. (15), human amnion laminin can block the reactivity to mouse laminin, suggesting their antigenic identity.

We found that 81% of active ACL patients and 84% of chronic Chagas' disease patients had high antibody levels compared with the upper limit of normality for healthy Venezuelan subjects. Furthermore, in ACL there was a relationship between patients with ulcers and very high antibody levels. This was not, however, the case for patients with treated and healed ACL ulcers, only 34% of whom had elevated antilaminin levels. In addition, in chronic Chagas' disease patients, the levels were similar to those found in acute ACL patients.

Interestingly, mucocutaneous leishmaniasis patients had higher antilaminin antibody levels than patients with localized cutaneous lesions, as has also been described for total immunoglobulin and antiparasite antibody levels (5, 6, 8, 18).

It is noteworthy that 19% of active ACL patients had normal antilaminin antibody levels. These results fit perfectly well with those of Anthony et al. (1), who also found by ELISA that 24% of clinically confirmed ACL patients had no *L. braziliensis* promastigote antibodies. The reasons for this minimal or absent humoral response in some acute ACL patients remains to be established, but it must be stressed that these patients were all leishmanin positive and had no clinical evidence of malnutrition.

Antilaminin antibodies were mainly of the IgG class in chronic Chagas' disease patients but were IgG and IgM in acute ACL patients. Only low reactivity was detected for IgA in both clinical conditions. This was not unexpected, given the different conditions, acute and chronic, of the ACL and Chagas' disease patients, respectively. Interestingly, no antibodies were found in these patients against collagen types I, III, IV, and V or fibronectin, suggesting that among the different components of basement membranes, these antibodies only reacted with laminin. Our results are, therefore, similar to those recently reported for humans and monkeys infected with *T. cruzi* or *Trypanosoma rhodesiense* (15).

The presence of these antibodies is apparently due to the *Trypanosomatidae* infection specifically since these antibodies were not detected in 11 different infectious diseases (histoplasmosis, lepromatous leprosy, sporotrichosis, aspergillosis, cutaneous mycoses, micetoma, paracoccidioidomicosis, malaria, chromomycosis, toxoplasmosis, and amoebiasis) or in lupus erythematosus. These results suggest that not all inflammatory diseases increase antilaminin antibody levels and also that discrete foci of tissular destruction are not responsible for the increase in antilaminin antibody found in these two parasitic infections.

It is noteworthy that chronic Chagas' disease patients also suffering acute ACL were found, a situation already reported in the literature (13). The interesting feature of these clinical cases is that although chronic Chagas' disease patients have high levels of antilaminin antibodies, these immunoglobulins do not appear to be related to immune protection since these patients also developed acute ACL. It should be noted here that even antiparasite antibodies appear to have little protective activity against these diseases (18).

The presence of high levels of antilaminin antibodies in ACL infection also makes it improbable that these antibodies have a role in the production of the endocardial fibrosis found in Chagas' disease (10) and in African trypanosomiasis (14) since no such cardiac damage has been reported in American leishmaniasis.

Attempts to absorb antilaminin antibodies with acetone powder of human organ material (heart, skeletal muscle, and liver), *T. cruzi* epimastigotes, *L. mexicana* and *L. braziliensis* promastigotes, and human and guinea pig RBC were unsuccessful, suggesting that antilaminin antibodies are different from EVI (endocardium, blood vessels, and interstitium) antibodies, which have been implicated in the pathogenesis of cardiac, skeletal muscle, and neural lesions in Chagas' disease (7, 9).

With regard to the mechanism of production of these antilaminin antibodies, at least two hypothesis can be formulated: (i) members of the *Trypanosomatidae* family possess a particular protein that cross-reacts with host laminin, and (ii) *T. cruzi* trypomastigotes may incorporate blood vessel laminin into their surfaces by a mechanism similar to that recently reported for other cell host proteins (4). This may result in a modification of the structure of the laminin, thus rendering it antigenic.

Szarfman et al. (15) have, in fact, found that trypomastigote and amastigote forms of *T. cruzi* have a higher reactivity with purified antilaminin antibodies from infected monkeys

TABLE 3. ELISA levels for antibodies to different connective tissue components

Study group (no. of patients)	OD_{405}					
	Collagen type:				Laminin	Fibronectin
	I	III	IV	V		
Controls (4)	0.210	0.250	0.280	0.190	0.242	0.241
ACL (4)	0.398	0.416	0.408	0.174	0.900	0.267
Chronic Chagas' disease (4)	0.449	0.400	0.409	0.128	1.064	0.259

than do the epimastigote forms that do not have contact with mammalian host cells.

In conclusion, the possibility of antilaminin antibodies participating in autoimmune reactions is interesting, but such responses have not been demonstrated in human ACL, although they do seem to exist in Chagas' disease (16).

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