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Comparative studies of *Leishmania* soluble antigens by crossed-immunoelectrophoresis: demonstration of specific antigens to *L. mexicana* and *L. braziliensis**

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The antigenic relationship between two species of Leishmania (L. mexicana amazonensis *and* L. braziliensis braziliensis) *was analysed using crossed-immunoelectrophoresis with an intermediate gel. Rabbit hyperimmune sera were prepared by subcutaneous inoculations of soluble antigens obtained from each species, emulsified in Freund's adjuvant. The antisera were then tested by immunodiffusion and electrophoresis; soluble antigens obtained from promastigote forms were submitted to ultrasonication and ultracentrifugation. Immunoelectrophoresis with homologous and heterologous antisera demonstrated the existence of some antigens which were species specific and some which were common to both species. The importance of these antigens is discussed.*

In the Americas, the cutaneous and mucocutaneous leishmaniases are caused by species belonging respectively to the *Leishmania mexicana* and *L. braziliensis* complexes. The cutaneous lesion is single or limited, with usually an abundance of parasites, and recovery is often spontaneous without any treatment. On the other hand, *L. braziliensis* is responsible for a disease generally referred to as American mucocutaneous leishmaniasis which is believed to be confined to the Latin American countries, e.g., in Brazil where it is called *espundia*. This disease usually produces a metastatic lesion in the mucous membrane of the nasopharyngeal cavity, with tissue destruction. In both types of leishmaniasis the organism is transmitted to man by an insect vector as the flagellated promastigote. In the vertebrate host, the parasites are restricted to the mononuclear phagocytic system as aflagellated amastigotes (*2*).

The parasites from different *Leishmania* species are almost indistinguishable morphologically, but the results of molecular and immunological analysis — such as electrophoretic patterns of isoenzymes (*9*), buoyant density of k-DNA and nuclear-DNA (*14*), serology of excreted factors (*13*), and reactions using monoclonal antibodies (*5, 11*) — have recently helped in their identification and classification. However, a general study comparing several characteristics of *Leishmania* species has not yet been carried out. For instance, it is well known that monoclonal antibodies are highly specific not only for one antigen but also for one epitope, which for certain studies is the great advantage of hybridoma technology over conventional methods using antisera. However, even the former is not the method of choice for a more general comparison between the antigenic constitution of the two parasite species because of the very high limitation in its specificity. Kohanteb et al. (*7*) compared different species of leishmanias using crossed electroimmunodiffusion and electroimmunodiffusion and found common antigens for the species studied as well as some indication of antigenic heterogeneity. However, the identification of species-specific antigens should be facilitated by preabsorption with heterologous antisera. We therefore chose the method of crossed immunoelectrophoresis with an intermediate gel employing polyclonal antibodies raised in rabbits, because it allows analysis of a high number of antigens with greater resolution than the other available immunoelectrophoretic methods. In the present study, the antigenic constitutions of strains of *L. mexicana amazonensis* and *L. braziliensis braziliensis* were compared using this technique.

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MATERIALS AND METHODS

Parasites

Two different species of tegumentary *Leishmania* were used, *L. braziliensis braziliensis* (M2903) and *L. mexicana amazonensis* (H21), which were provided by the Wellcome Parasitology Unit, Evandro Chagas Institute, Belém, PA, Brazil. The promastigotes were maintained at 26 °C by weekly passages in a biphasic medium consisting of a rabbit-blood agar base and a liquid phase of brain heart infusion (BHI-Difco).

Antigen extraction

Cells obtained from bulk growth were harvested on the seventh day, washed six times with 0.15 mol/l phosphate-buffered saline at pH 7.2, and disrupted by sonication using the Branson Sonifier Disruptor at 95 W, five times (3 minutes each). The sonicated material was ultracentrifuged at 100 000 *g* for 30 min and the supernatant was filtered through a 0.2 μm Millipore membrane. In order to minimize proteolytic degradation, all the procedures were carried out at 4 °C and the antigenic preparations were aliquoted and stored at −70 °C until use. Protein content was measured by Lowry's method (*8*) using bovine albumin as a standard.

Antisera

Antisera were obtained from 3 New Zealand rabbits after subcutaneous inoculation according to the following schedule. The first injection with the soluble antigen consisted of 1.5 mg of protein emulsified in Freund's complete adjuvant. After six weeks, the animals received the same dose of protein emulsified in Freund's incomplete adjuvant. The third dose (5 mg of protein) was given one week later, also in Freund's incomplete adjuvant. The rabbits were bled on days 5, 7 and 9 after the last injection. Antisera were tested for polyvalency and titre by immunodiffusion and electrophoresis. The sera with the highest titres from the 3 rabbits were pooled and stored at −20 °C.

Crossed-immunoelectrophoresis

For crossed-immunoelectrophoresis (*3*), glass plates (9 × 6 cm) were covered with a slab (6 × 1.5 cm) of agarose 1.2% in Tris barbital buffer at pH 8.6 (ionic strength 0.02) and the soluble antigen (0.16 mg of protein) was applied to the first dimension gel and submitted to electrophoresis (8 V/cm for 50 min at 4 °C) so that the antigens migrated towards the anode. Second dimension electrophoresis was carried out in a 1.2% agarose gel in the same buffer, in which the antiserum had been diluted (2 V/cm for 18 h at 4 °C). The slab containing the antigens separated in the first dimension was located on the glass plate in a way that permitted the antigens to migrate both towards the anode (upper side of the plate) and the cathode (lower side of the plate) (Fig. 1). The homologous and the heterologous antisera were compared by crossed-immunoelectrophoresis with an intermediate gel (*1*). In this case, a 1-cm slab containing no antiserum was introduced between the 3-cm slab of intermediate (heterologous antiserum) and the 3.5-cm slab of the upper (homologous antiserum) gels, in order to improve the resolution. The slab containing the antigens separated in the first dimension was located at the cathodic pole to permit migration towards the anode.

After electrophoresis the plates were washed, dried, and stained with Coomassie brilliant blue R-250.

RESULTS

The soluble extracts of the two species of *Leishmania*, belonging to different complexes, were submitted to crossed-immunoelectrophoresis in order to determine their antigenic profiles. The immunoelectrophoresis performed with the homologous and the heterologous antisera demonstrated the existence of both similarities and differences between them.

In the homologous system, we found 28 and 27 precipitin arcs for *L. braziliensis braziliensis* and *L. mexicana amazonensis*, respectively, all of them of anodic migration. In addition to the anodic arcs, *L. braziliensis braziliensis* presented another arc, with migration to the cathodic pole (Fig. 1).

A marked antigenic similarity between the species was revealed when their soluble extracts were reacted with the heterologous antisera. In this system, an intermediate gel containing the heterologous antiserum was used between the upper gel mixed with the homologous antiserum and the gel with the electrophoretically separated antigen. In the intermediate gel 15 and 13 common antigens were detected when the soluble antigens of *L. braziliensis braziliensis* and *L. mexicana amazonensis*, respectively, were subjected to electrophoresis (Fig. 2 and Table 1). The antigens, which were detected exclusively in the upper gel, were considered to be specific to each species. We observed 7 of them for the *L. braziliensis braziliensis* soluble antigens; 5 of these particular antigens (No. 3 to 7, Fig. 2 top) had the highest electrophoretic mobilities while 2 (No. 1 and 2, Fig 2 top) had intermediate mobility. When the soluble antigens of *L. mexicana amazonensis* were analysed by crossed-

Table 1. Antigens in extracts of *Leishmania braziliensis braziliensis* (*L.b.b.*) and *Leishmania mexicana amazonensis* (*L.m.a.*) as detected by crossed-immunoelectrophoresis and by crossed-immunoelectrophoresis with intermediate gel

Antiserum in the upper gel	Number of precipitin lines observed in the upper gel, with the following antigens:			
	L.b.b	*L.b.b* pre-absorbed with anti-*L.m.a* [a]	*L.m.a*	*L.m.a.* pre-absorbed with anti-*L.b.b* [a]
Anti-*L.b.b.*	28	7 [d]	13 [b]	ND [c]
Anti-*L.m.a.*	15 [b]	ND [c]	27	2 [d]

[a] Heterologous antiserum dissolved in the intermediate gel.

[b] Lines corresponding to common antigens.

[c] Not done.

[d] Lines corresponding to specific antigens

immunoelectrophoresis using the anti-*L. braziliensis braziliensis*-containing intermediate gel, 2 particular antigens were found (Table 1); these antigens (No. 1 and 2, Fig. 2 bottom) were among those with the highest electrophoretic mobility.

DISCUSSION

The taxonomic separation of *Leishmania* into well defined species and the characterization of the relevant antigens leaves much to be desired. The complexity of the situation led various investigators to search for simple ways of classifying these parasites on an immunological basis. Antigenic differences among a number of *Leishmania* species have been detected by a variety of methods, but the nature and number of such species-specific antigens are still unknown.

The results of our experiments using crossed-immunoelectrophoresis with an intermediate gel to analyse the etiological agents of cutaneous and mucocutaneous leishmaniasis (*L. mexicana amazonensis* and *L. braziliensis braziliensis*) showed that several antigens are shared by both species. The presence of these common antigens does not necessarily imply that cross-protection can be readily achieved. In fact, as demonstrated before (*10*), human infections by *L. mexicana* do not protect against *L. braziliensis* although the latter induces protection against the former. We also detected the presence of antigens particular to each species being studied; some of these antigens seem to be quantitatively important, such as *L. braziliensis braziliensis* No. 3 and 5 (Fig. 2 top). However, since the crossed-immunoelectrophoresis technique is not quantitative, no definite conclusion can be drawn on their relative amounts.

As there is evidence for a certain degree of cross-reaction between *L. donovani* and *Mycobacterium tuberculosis* (*15*), which is a component of the adjuvant employed in this study, it is possible that some of the common antigens reported here reflect in fact mycobacterial cross-reactivity. However, this would not cause the antigens to be wrongly identified as particular antigens, and there can be no doubts about this.

On the other hand, although crossed-immunoelectrophoresis with intermediate gel is a highly sensitive method for the analysis of complex mixtures, this technique reflects only the potency and multispecificity of the antisera used. In the present study, this limitation was somehow minimized by using pooled antisera from three rabbits which produced the highest antibody titres and the most heterogeneous antibody responses, in terms of leading to maximum numbers of lines in double immunodiffusion. Moreover, in our work comparing promastigote forms of the two species, we found for *L. mexicana* a similar number of precipitin arcs as described by Hunter & Cook. in an homologous system (*6*). Thus, we have an indication that any variation due to the immune response of the immunized rabbits was negligible. As we used only one stock of each parasite, we cannot exclude antigenic variation due to heterogeneity of the parasite within a taxon in a given geographic area. However, previous reports have indicated stability of antigenic patterns for *Leishmania* (*4*).

Studies analysing the antigenic make-up of *Leishmania* species are generally carried out using the promastigote form, because the amastigote form is difficult to be obtained pure in the quantities needed for analytical studies. Hunter & Cook. (*6*) compared the promastigote and amastigote forms of *L. mexicana* and detected considerable cross-reactivity; these findings suggest that a large number of antigens are shared by the infective promastigote form and the amastigote form, which is present in the vertebrate

host during the development of the disease. Therefore, the study of antigens extracted from the infective promastigote form should be of great value for immunoprophylaxis and immunopathology.

Furthermore, immunological studies of the cross-reaction between *Leishmania* spp. and *Trypanosoma cruzi*, which are co-endemic in some areas of Brazil, have been carried out. Based on our results and those of Morgado et al. (*12*) with *T. cruzi*, we tried to compare these parasites, again using crossed-immunoelectrophoresis with an intermediate gel; in preliminary results, we found that approximately 20% of the soluble antigens of *T. cruzi* were shared by the parasites of the genus *Leishmania*.

Many problems still remain to be solved in leishmaniasis research. We believe the isolation and characterization of the specific antigens described here might be useful for taxonomical, clinical and immunological investigations.

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RÉSUMÉ

ÉTUDES COMPARATIVES PAR IMMUNOÉLECTROPHORÈSE CROISÉE D'ANTIGÈNES SOLUBLES DE *LEISHMANIA*: MISE EN ÉVIDENCE DES ANTIGÈNES SPÉCIFIQUES DE *L. MEXICANA* ET DE *L. BRASILIENSIS*

La relation antigénique entre deux espèces de *Leishmania* (*L. mexicana amazonensis* et *L. brasiliensis brasiliensis*) a été analysée par immunoélectrophorèse croisée avec un milieu gélifié intermédiaire. Des sérums hyperimmuns de lapin ont été préparés par inoculation sous-cutanée d'antigènes solubles obtenus à partir de chaque espèce et émulsionnés dans l'adjuvant de Freund. Puis les immunsérums ont été éprouvés par immunodiffusion et électrophorèse; les antigènes solubles obtenus à partir de formes promastigotes ont été soumis aux ultrasons et à l'ultracentrifugation. L'immunoélectrophorèse avec des immunsérums homologues et hétérologues a révélé l'existence de certains antigènes spécifiques d'espèce et d'autres communs aux deux espèces.

Etant donné que des observations témoignent d'un certain degré de réactivité croisée entre *L. donovani* et *Mycobacterium tuberculosis* qui est un composant de l'adjuvant employé dans cette étude, il est possible que quelques-uns des antigènes communs signalés ici traduisent en fait une réactivité croisée mycobactérienne. Toutefois, cela ne devrait pas conduire à une identification erronée des antigènes spécifiques, aucun doute n'étant possible à ce sujet.

La recherche sur la leishmaniose est confrontée à de nombreux problèmes non encore résolus. Nous pensons que l'isolement et la caractérisation des antigènes spécifiques décrits ici pourraient être utiles aux recherches taxonomiques, cliniques et immunologiques.

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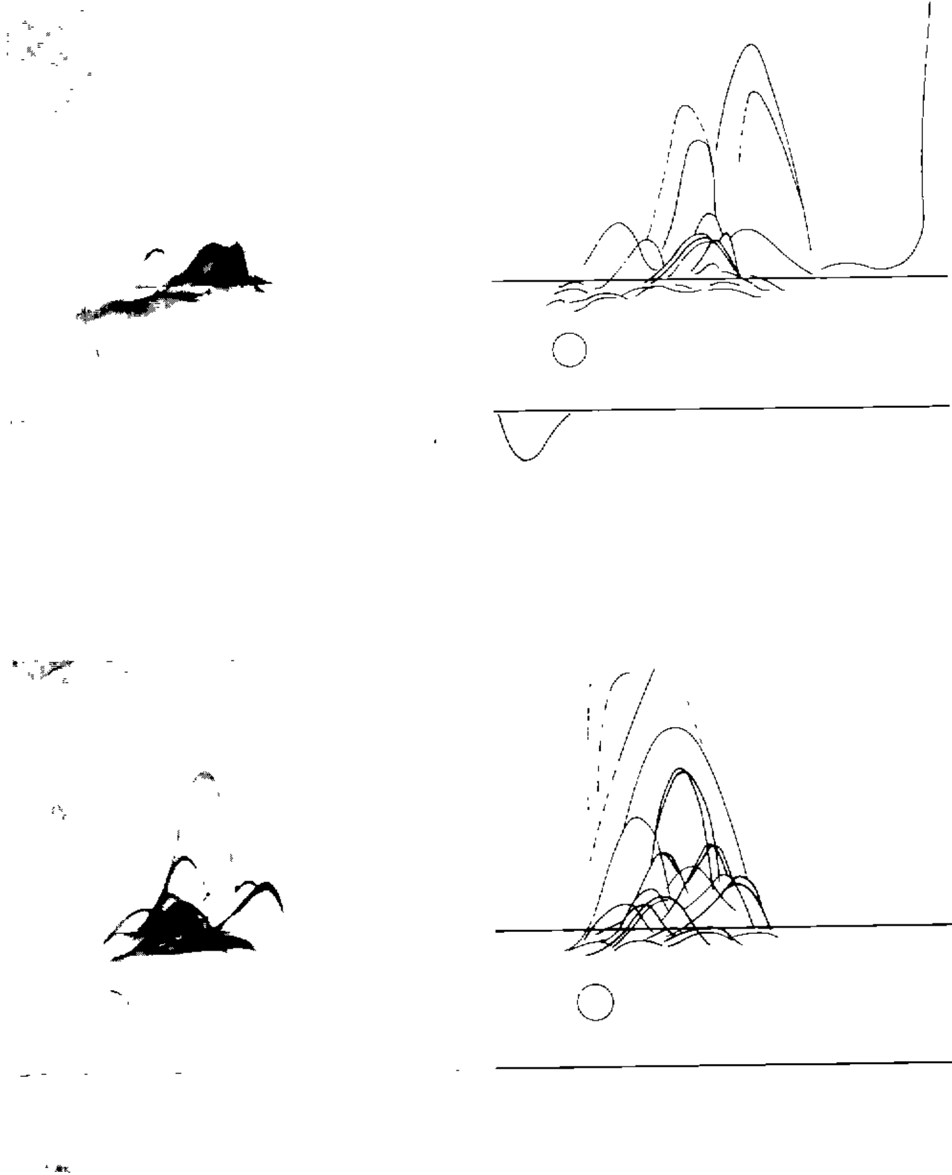

Fig. 1. Crossed-immunoelectrophoresis showing the differences in the patterns of precipitin arcs between soluble extracts of *Leishmania braziliensis braziliensis* (top figures) and *L. mexicana amazonensis* (bottom figures) and their respective homologous antisera.

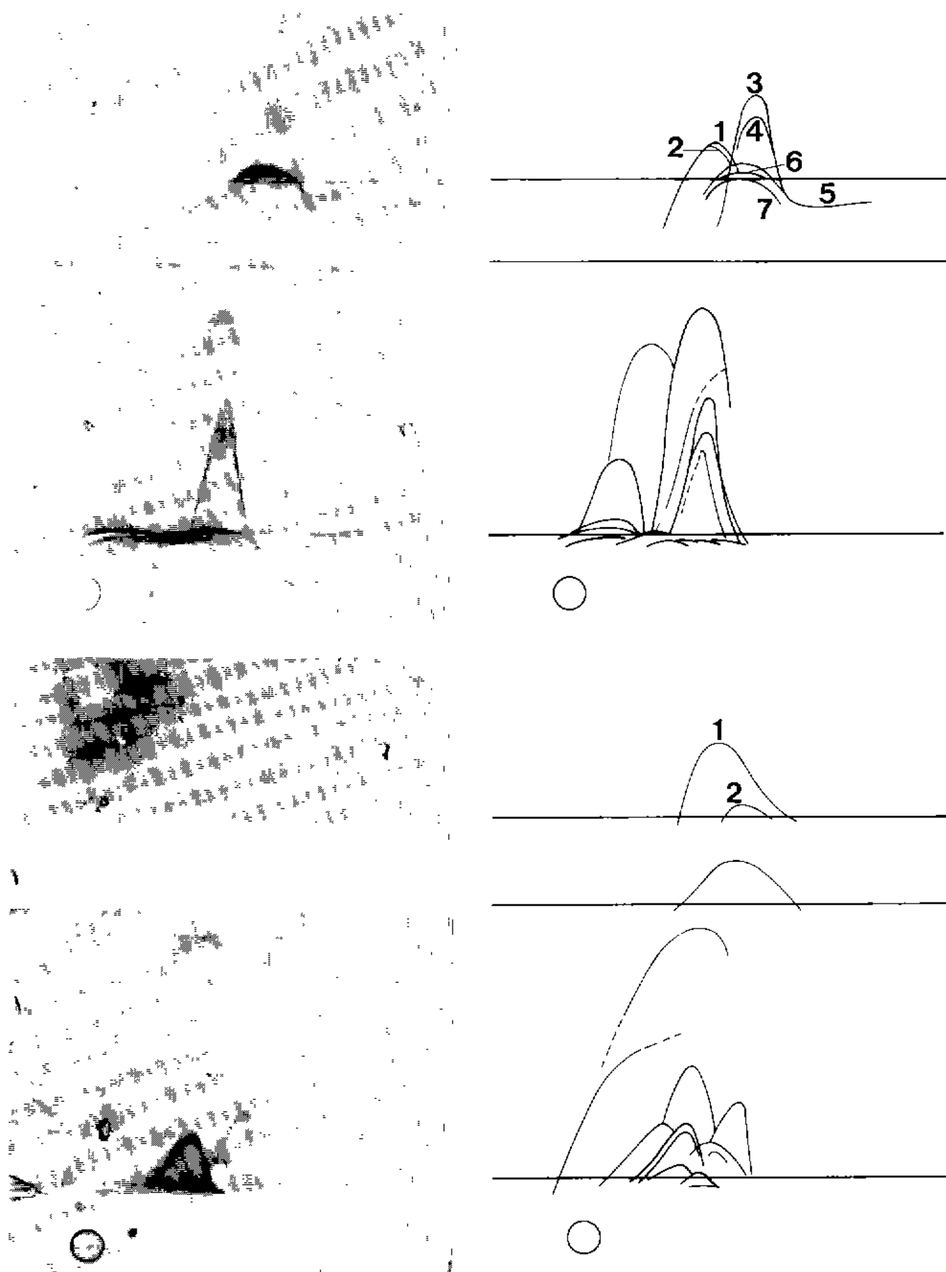

Fig. 2. Analysis of the reaction between *Leishmania braziliensis braziliensis* soluble extract with its homologous antiserum (top figures) after absorption with the anti-*L. mexicana amazonensis* serum in the intermediate gel (arcs numbered 1 to 7 correspond to particular antigens) and *L. mexicana amazonensis* soluble extract with its homologous antiserum (bottom figures) after absorption with the anti-*L. braziliensis braziliensis* serum in the intermediate gel (arcs numbered 1 to 2 correspond to particular antigens).

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