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THE CHANGE OF BEHAVIOUR OF TWO STRAINS OF *LEISHMANIA* AFTER CULTIVATION IN A DEFINED MEDIUM

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Attempts have been made to characterize two strains of Leishmania *that became infective to golden hamsters only after they had been maintained for several years in a chemically defined culture medium. Observations were made on the growth rates of promastigotes* in vitro, *course of infection in hamsters, morphology of amastigotes, and electrophoretic mobility patterns of eight isoenzymes. Information was obtained about the buoyant densities of n-DNA and k-DNA, and one strain was tested against monoclonal antibodies. The identity of both strains remains obscure.*

Key words: human cutaneous leishmaniasis – *in vitro* growth curves – infections in hamsters – isoenzyme patterns – n-DNA and k-DNA – monoclonal antibodies

This report deals with strains of *Leishmania* isolated in different parts of Brazil from human cases of cutaneous leishmaniasis. Both were isolated in NNN medium (Pessôa & Martins, 1982) and later adapted to LIT (Camargo, 1964). From the time the strains were isolated, attempts were made to infect laboratory animals with cultured promastigotes but with negative results. After maintenance for about five years in a chemically defined medium (Melo et al., 1985), both strains became infective to golden hamsters. Both have retained their ability to infect hamsters but remain non-infective to other experimental animals.

MATERIAL AND METHODS

Parasites – Strain BH 49 was supplied by Professor William Barbosa, Institute of Tropical Pathology, Federal University of Goiás, Goiânia, Brazil. Apart from knowing that the parasites were isolated from a human case of cutaneous leishmaniasis, no particulars of the strain were provided. The parasite arrived in Belo Horizonte in September, 1971, in NNN culture and was serially maintained in this medium and in LIT.

Strain BH 121 was isolated by one of us (P.A.M.) from a cutaneous lesion on a 54-year-old man living near the town of Engenheiro Caldas in the Rio Doce Valley of the State of Minas Gerais. After a single passage in NNN with a saline overlay, the parasites were adapted to LIT and, thereafter, maintained in both culture media.

In vitro maintenance – Screw-capped "Pyrex" test-tubes (15 x 1.5 cm) were used. Cultures were maintained in an incubator at 23 ± 0.5°C. Promastigotes grown in NNN and LIT were transferred to fresh medium at 12-day intervals. Those grown in the chemically defined medium were transferred every nine days.

Assessment of growth rates in the three culture media were made at 24-hour intervals. A Coulter Counter (Model ZB) was used.

Infectivity tests – The trials recorded herein were carried out in 1981. At that time, BH 49 had been maintained through 305 passages in NNN, 303 in LIT and 202 in the chemically defined medium (MD-29). BH 121 had been maintained through 214 passages in NNN, 212 in LIT and 201 in MD-29.

The following animals were used: one-day-old Swiss mice; five-week-old female golden hamsters; five-week-old outbred white mice; five-week-old inbred mice (C_3H, C57BL/10, BALB/c and CBA). The animals were reared in conditions that precluded exposure to the bites of phlebotomine sand flies and to contact with animals infected with *Leishmania.* Ten animals of each type were used in each trial. Test animals were inoculated with 1 x 10^7/ml of promastigotes. Flagellates harvested in the logarithmic and stationary phases of development were used in separate trials.

Baby mice were dealt with in the manner described by Adler (1963) and autopsied 24-48

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hours after inoculation. The other animals were inoculated subcutaneously at the base of the tail (mice) or in the snout (hamsters). The animals were inspected weekly. Four weeks after inoculation, and thereafter at four-week intervals, two animals of each group were killed and autopsied. Impression smears were made from lesions (when detected) or from the skin at the site of inoculation, and smears were also prepared from the liver and spleen. Smears were fixed in methyl alcohol and stained with Giemsa. They were considered negative when no amastigotes were found in 100 oil-immersion fields of each smear.

Isoenzyme studies – These were undertaken before the strains had become infective to golden hamsters. Amastigotes from hamster lesion were re-isolated in LIT and cryopreserved until enzyme examination were made. Thin-layer starch-gel electrophoresis, by methods of enzyme extraction and electrophoretic proceedures to be described in detail elsewhere (Magalhães Rocha et al., in preparation) was used to determine the mobility patterns of the following enzymes: GPI (EC 5.3.1.9); G6PH (EC 1.1.1.49); ALAT (EC 2.6.1.2); ASAT (EC 2.6.1.2); MDH (EC 1.1.1.37); ME (EC 1.1.1.40); PGM (EC 2.7.5.1); 6PGDH (EC 1.1.1.4.4).

The marker strains were: MHOM/BR/74/M2682 *(L. donovani chagasi)*; MHOM/BR/75/M2903 *(L. braziliensis braziliensis)*; MHOM/BR/70/M1176 *(L. braziliensis guyanensis)*; IFLA/BR/67/PH8 *(L. mexicana amazonensis)*; MNYC/BZ/62/M379 *(L. mexicana mexicana)*; MRHO/SU/59/P *(L. major)*. *L. donovani chagasi* was used as a marker because BH 121 was isolated in an area endemic for both visceral and cutaneous leishmaniases. An Old World stock was selected as a marker because one of us (W.M.) has isolated parasites, most probably *L. major*, from individuals returning to Brazil after being engaged in construction work in Iraq.

RESULTS

Growth rates in vitro – Fig. 1 shows rates of reproduction in three types of culture media. These results suggested that BH 121 probably belongs to the *L. mexicana* complex whereas BH 49 does not.

Infectivity tests – Promastigotes grown in NNN and LIT, and those harvested in the stationary phase in MD-29, failed to infect test animals. In animals inoculated with these materials, no macroscopic changes were detected in the skin or viscera, and no amastigotes were found in impression smears prepared from subcutaneous tissue (site of inoculation), liver or spleen.

Promastigotes of both BH 49 and BH 121 were infective to golden hamsters after being grown in MD-29 and harvested in the logarithmic phase. Lesions induced by BH 121 were first observed six weeks after inoculation; those produced by BH 49 appeared after 32 weeks. Again, the results suggested that BH 121 belongs to the *L. mexicana* complex and that BH 49 does not.

Infections in hamsters – Eight hamsters developed lesions after inoculation with BH 121, two animals having already been sacrificed before lesions were detected. In each of the animals, the lesions developed into prominent histiocytomas that later ulcerated. The lesions were rich in amastigotes which were morphologically like those that Shaw & Lainson (1976) described for the *L. mexicana* complex. Since becoming infective, BH 121 has been maintained by serial passage of amastigotes from hamster to hamster. Invariably, lesions appear soon after inoculation, large histiocytomas develop and ulceration occurs.

Lesions due to BH 49 developed in only two hamsters, but eight animals had already been sacrificed. The lesions were small and nodular, did not ulcerate, and contained small, scanty amastigotes. Similar lesions have been produced, subsequently, by serial passage of amastigotes from hamster to hamster. Most commonly, lesions contain scanty, small amastigotes but, from time to time, they are found to be rich in amastigotes of variable size.

Metastases have never been detected in hamsters infected with either strain.

The course of infection in hamsters, the form of lesion produced and the appearance of amastigotes, all support the idea that BH 121 belongs to the *L. mexicana* complex and that BH 49 does not. An unusual aspect for a member of this complex has been the consistent failure to detect metastatic lesions in hamsters infected with BH 121.

Enzyme analyses – Fig. 2 shows the electrophoretic mobility patterns of the eight isoenzymes studied. The salient features of these results are: a) The two strains differ from one another in the mobility patterns of G6PDH, ALAT, MDH and GPI; b) Both strains differ from *L. braziliensis guyanensis* in all eight enzymes; c) Both strains differ from *L. braziliensis braziliensis* in the mobility patterns of 6PGDH, PGM, ALAT, ASAT and MDH and slightly from those for ME and GPI; d) BH 49 differs from *L. mexicana mexicana* by the mobility patterns of ALAT, ASAT, G6PDH, GPI and MDH (and, possibly, by those for ME and PGM), and from *L. mexicana amazonensis* by the patterns for ALAT, PGM, G6PDH, GPI

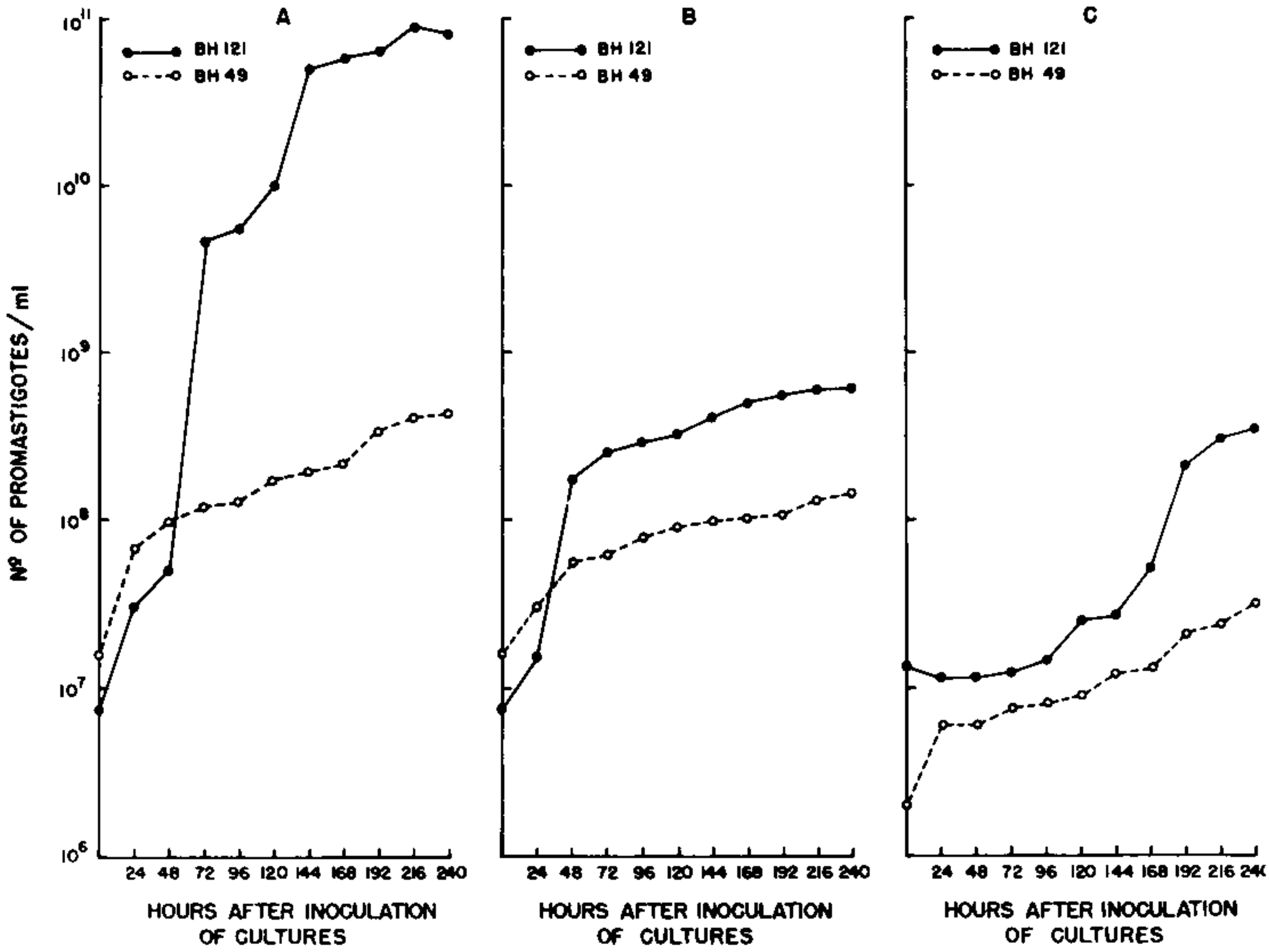

Fig. 1: Growth rates of stocks BH 49 and BH 121 (non-infective lines) in three types of cu'ture media. A-NNN; B-LIT; C-MD29.

and MDH; e) BH 121 differs from *L. mexicana mexicana* in the patterns of ALAT, ASAT, GPI and MDH, and from *L. mexicana amazonensis* by ALAT, PGM, G6PDH, GPI and MDH; f) Both strains are readily distinguished from *L. donovani chagasi* and *L. major.*

Additional data – Studies undertaken at the Liverpool School of Tropical Medicine have shown that BH 49 has a buoyant density of 1.718 g/ml for n-DNA and 1-704 g/ml for k-DNA; its MDH mobility pattern conforms with Type III of Gardener et al. (1974). BH 121 has a buoyant density of 1.718 g/ml for n-DNA and 1.704 g/ml for k-DNA and, according to Brazil (1976) conforms to MDH Type XII.

Investigations at the Wellcome Parasitology Unit, Belém, Pará State, using eleven *Leishmania* species and subespecies specific monoclonals, have shown that BH 49 gives a positive reaction to a monoclonal antibody that is group specific for *L. mexicana* but is also positive for *L. major* and another that is an *L. major* specific monoclonal (Dr. J. J. Shaw, in litt.).

DISCUSSION

Strains BH 49 and BH 121 have but few features in common: from the time of isolation, both failed to infect laboratory animals; promastigotes cultured in NNN and LIT remain non-infectious; and, after maintenance for five years in the chemically defined medium MD-29, promastigotes harvested in the logarithmic phase (but not those in the stationary phase) became infective to golden hamsters (but not to other test animals). Apart from these similarities, the two strains are so different from one another that it is most convenient to discuss them separately.

Monoclonal antibody studies on BH 49 gave positive results for both *L. mexicana* and *L. major* but the parasites cannot be placed in either of these species when the other evidence is taken into account. Growth rates in culture media, the behaviour of the organisms in hamsters (slowly developing, nodular, non-ulcerating lesions containing scanty, small amastigotes), and a k-DNA buoyant density of 1.704 g/ml are inconsistent for a member of the *L.*

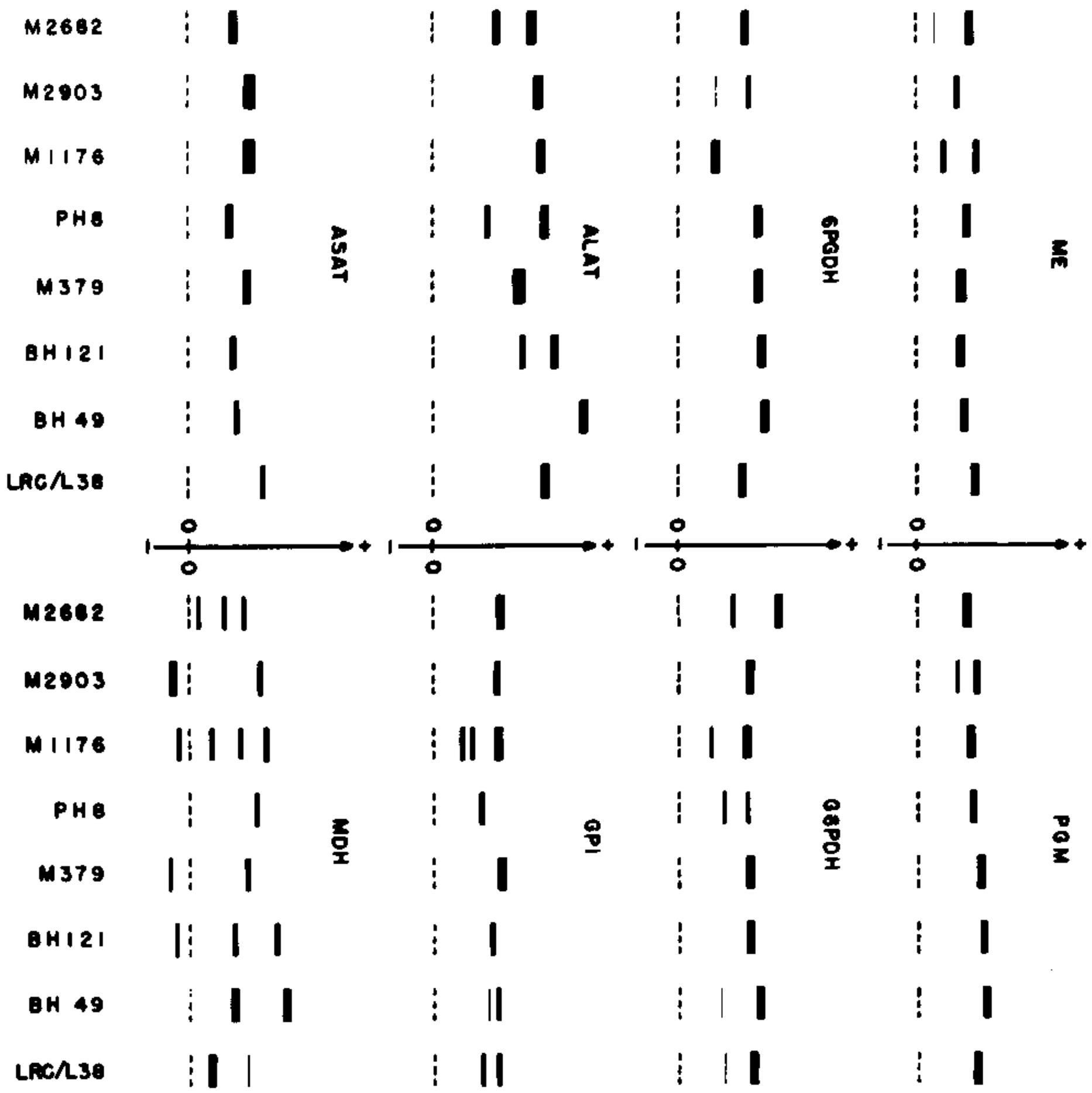

Fig. 2: Diagrams summarizing the electrophoretic patterns of eight enzymes. Reading from left to right: *L. donovani chagasi; L. braziliensis braziliensis; L. braziliensis guyanensis; L. mexicana amazonensis; L. mexicana mexicana;* BH 121; BH 49; *L. major.*

mexicana complex. Multiplication rates in cultures, the appearance of lesions on hamsters, morphology of amastigotes, and distinction from the WHO recommended marker stock for *L. major* by the mobility patterns of seven enzymes, all separate BH 49 from *L. major.* The behaviour of this strain in culture and hamsters, together with the morphology of amastigotes, suggests that BH 49 is more akin to the *L. braziliensis* complex. However, it differs considerably from the marker stocks for *L. braziliensis braziliensis* and *L. braziliensis guyanensis.* With a k-DNA buoyant density of 1.704 g/ml, it is well outside the normal range (1.688 - 1.694 g/ml) for members of the *L. braziliensis* complex (Chance, 1979). The behaviour of the parasites in hamsters, together with the results of enzyme studies, excludes BH 49 from the *L. donovani* complex.

Some characteristics of strain BH 121 (growth in cultures, lesions on hamsters, morphology of amastigotes) suggest that it could be a member of the *L. mexicana* complex. However, its enzyme characteristics are quite different from those of *L. mexicana mexicana* and *L. mexicana amazonensis.* BH 121 also differs from an isolate from Três Braços, State of Bahia (Cuba et al., 1984) that differs from *L. mexicana amazonensis* only in the mobility pattern of G6PD, from an isolate obtained from a rodent captured near São João de Jacutinga, Caratinga, State of Minas Gerais, which was identified by Lopes et al. (1984) as *L. mexicana mexicana,* and from four other rodent isolates from São João de Jacutinga that differ from *L. mexicana amazonensis* only in the pattern for ALAT and MDH (Magalhães Rocha et al., in preparation). Doubts about placing BH 121 in the *L. mexica-*

na complex arise because the buoyant density for k-DNA is somewhat higher than the normal range for members of this species (Chance, 1979) and because of the consistent absence of metastases in infected hamsters.

Studies have begun to determine the growth patterns of the two strains in *Lutzomyia longipalpis* ("one spot" strains) but the preliminary results suggest that this is an unproductive line of inquiry. A total of 213 female flies have fed directly on lesions of hamsters infected with BH 49. Mortality was heavy in the period immediately following blood feeding. Only 79 flies survived long enough to be dissected 3-9 days after exposure to infection and none contained promastigotes or paramastigotes. In experiments with BH 121, female sand flies fed directly on large hamster lesions rich in amastigotes but only a small proportion developed infections. All infected flies contained but a few promastigotes, which were confined to the midgut.

From the available evidence, it is reasonable to suspect that BH 49 and BH 121 represent new taxa but it would be premature to define them as such before comparing them with a wider range of recommended marker stocks. For the present, it can only be recorded that they are, indeed, unusual strains of *Leishmania*.

RESUMO

Mudanças no comportamento de duas cepas de *Leishmania* após cultivo em meio definido – Duas cepas de *Leishmania* originalmente isoladas *in vitro* de casos humanos de leishmaniose cutânea e que *ab initio* não infectaram animais de laboratório, tornaram-se infectantes para hamsters após serem mantidos por vários anos em meio de cultura quimicamente definido.

Foram realizadas observações sobre o crescimento de promastigotas *in vitro*, curso da infecção em hamsters, morfologia das amastigotas, mobilidade eletroforética de oito enzimas solúveis. Foram obtidas informações sobre a densidade de flutuação do n-DNA e do k-DNA e uma das cepas foi testada contra anticorpos monoclonais. Ambas as cepas permanecem sem identificação precisa.

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REFERENCES

ADLER, S., 1963. The behaviour of a strain of *Leishmania* (originating from the sandfly *Phlebotomus martini*) in mice inoculated during suckling. *Israel J. Exp. Med., 11* :35-36.

BRAZIL, R.P., 1976. *Comparative studies on the culture forms of two isolates of* Leishmania *from Brazil.* Thesis. University of Liverpool, 49 p.

CAMARGO, E.P., 1964. Growth and differentiation in *Trypanosoma cruzi*. Origin of metacyclic trypanosomes in liquid media, *Res. Inst. Med. Trop. S. Paulo, 6* :93-100.

CHANCE, M.L., 1979. The identification of *Leishmania,* p. 55-74. In A.E.R. Taylor & R. Muller. *Problems in the identification of Parasites and their vectors.* Oxford, Blackwell Scientific Publication.

CUBA, C.A.C.; BARRETTO, A.C. & MARSDEN, P.D., 1984. *Leishmania mexicana:* aspectos taxonómicos y rareza de la infección humana em Três Braços, Bahia, Brasil. *Rev. Soc. Bras. Med. Trop., 17* :115-122.

GARDENER, P.J.; CHANCE, M.L. & PETERS, W., 1974. Biochemical taxonomy of *Leishmania.* II. Electrophoretic variation of malate dehydrogenase, *Ann, Trop. Med. Parasit., 68* :317-325.

LOPES, U.G.; MOMEN, H.; GRIMALDI, G. Jr.; MARZOCHI, M.C.A.; PACHECO, R.S. & MOREL, C.M., 1984. Schizodeme and zymodeme characterization of *Leishmania* in the investigation of foci of visceral and cutaneous leishmaniasis. *J. Parasit., 70* :89-98.

MAGALHÃES-ROCHA, N.M.; MELO, M.N.; BABÁ, E.H.; WILLIAMS, P.; DIAS, M.; COSTA, C.A. da; MICHALICK, M.S.M. & MAYRINK, W. *(in preparation).* Characteristics of *Leishmania* isolated from wild rodents captured in the Rio Doce Valley, State of Minas Gerais, Brazil.

MELO, M.N.; AZEVEDO, H.P.; ROITMAN, I. & MAYRINK, W., 1985. A new defined medium for cultivating *Leishmania* promastigotes. *Acta Tropica, 42* :137-141.

PESSÔA, S.B. & MARTINS, A.V. 1982. *Parasitologia Médica,* 11a. Edição. Rio de Janeiro. Guanabara Koogan S.A.

SHAW, J.J. & LAINSON, R., 1976. Leishmaniasis in Brazil. XI. Observations on the morphology of the *braziliensis* and *mexicana* complex. *J. Trop. Hyg., 79* :9-13