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Identification of a major 72 kilodalton surface antigen in twelve isolates of *Leishmania braziliensis braziliensis*

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The study of the surface antigens of *Leishmania braziliensis braziliensis* revealed a great homogeneity among ten strains isolated from Bolivia and two reference strains from Brazil and Belize. A 72 kDa major protein, present in all *L. b. braziliensis* strains, was recognized by both cutaneous and mucocutaneous human sera, but was not recognized by Kala-azar and chagasic sera. No cross-reactive antigens were found among strains of *Leishmania braziliensis guyanensis, Leishmania braziliensis panamensis, Leishmania mexicana amazonensis* and *Leishmania donovani chagasi* testing these strains with hamster and human anti-*L. b. braziliensis* sera. Moreover, these strains possessed major antigens with molecular weights different from those of *L. b. braziliensis* strains. A microheterogeneity of *L. b. braziliensis* surface antigens was detected for the high molecular weight antigens and seemed to be related to the isoenzymic microheterogeneity.

Key words: *Leishmania braziliensis braziliensis*; Cell surface iodination; Immunoprecipitation; Surface antigen

Introduction

Leishmania are protozoan parasites belonging to the family Trypanosomatidae. They are responsible for a wide variety of diseases which affect man and other mammals in different parts of the world. In the New World, *Leishmania chagasi* and *Leishmania mexicana* species are associated with human visceral and human cutaneous leishmaniasis, respectively. *Leishmania braziliensis* species constitutes the main etiologic agent of cutaneous and mucocutaneous leishmaniasis [1].

Biochemical technologies for the analysis of isoenzyme patterns [2–5], buoyant density of nuclear and kinetoplast DNA [6], as well as immunological methods using monoclonal antibodies [7–10] have been used to classify *Leishmania* species. However, the use of these methods has not resulted in a definitive classification of New World *Leishmania* species and subspecies. As a new tool, the study of surface antigens of *Leishmania* promastigotes may contribute taxonomic data and enhance our understanding of pathogenicity of certain *Leishmania* subspecies.

Many surface antigen studies of Old World *Leishmania* species including *L. tropica* [11–13], *L. major* [14], *L. donovani* [15,16] and New World *Leishmania* species such as *L. donovani chagasi* [16], *L. mexicana* [17], and *L. braziliensis* [18,19] have been reported. The existence of a cross-reacting antigen of about 65 kDa has been reported in several Old and New World *Leishmania* species [15,19–22].

Few reports [20,22] include precise data on the

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Abbreviations: Iodo-Gen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; EDTA, ethylenediaminetetraacetate; PBS, phosphate-buffered saline; MDH, malate dehydrogenase; ICD, isocitrate dehydrogenase; ME, malic enzyme; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HMW, high molecular weight.

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surface antigenicity of the subspecies *L. braziliensis guyanensis*, *L. braziliensis panamensis* and, more importantly, *L. braziliensis braziliensis* which produces the most serious leishmaniasis in the New World countries, including Bolivia [23].

In order to compare the surface antigens of *L. b. braziliensis* with those of other New World leishmanias, we have performed a study of twelve *L. b. braziliensis* strains (ten from Bolivia, one from Brazil and one from Belize), and of *L. b. panamensis*, *L. b. guyanensis*, *L. mexicana amazonensis* and *L. donovani chagasi* strains.

Material and Methods

Parasites. Promastigotes of ten *L. b. braziliensis* strains were isolated from various regions of Bolivia, as shown in Table I. Two strains (LPZ-595 and LPZ-704) were isolated from sandflies (*Psychodopygus llanosmartinsi* and *Psychodopygus yucumensis*), while eight others were isolated from primary cutaneous lesions of Bolivian patients. All strains were characterized by isoenzyme analysis [23].

Seven reference strains were used: a *L. b. braziliensis* strain from Brazil (MHOM/BR/75/M-2904) [24], a *L. b. braziliensis* strain from Belize (MHOM/BZ/83/BEL-53) [24], a *L. b. guyanensis* strain (CHO/PA/76/M-5378) [25], a *L. b. panamensis* strain (MHOM/PA/75/M-4039) [25], a *L. m. amazonensis* strain (IFLA/BR/67/PH-8) [24], a *L. d. chagasi* strain (MHOM/BR/00/M-2682) [24] and a *Trypanosoma cruzi* strain (Tehuentepec).

The promastigotes were adapted to and maintained in LIT with 15% (v/v) of heat inactivated fetal bovine serum or modified N.N.N. medium [26], both with gentamycin 0.1 mg ml^{-1}.

Human and hamster sera. Sera of five Bolivian patients with cutaneous leishmaniasis and five patients with mucocutaneous leishmaniasis were collected in our laboratory. All the patients were clinically diagnosed and the sera exhibited positive serology for *L. b. braziliensis*. In addition, sera of four African patients with visceral leishmaniasis (Pasteur Institute of Dakar) and two Bolivian patients with Chagas' disease were used. Diagnoses of these six patients were serologically and parasitologically confirmed. Finally, two sera of healthy human subjects were also used.

Hamster antiserum to *L. b. braziliensis* was collected one year after foot pad inoculation [27] of viable promastigotes of the strain MHOM/BO/84/LPZ-688. Normal hamster serum was used as control.

Isoenzymic electrophoresis. Samples of *L. b. braziliensis* promastigotes were prepared as previously described [23]. Electrophoresis was carried out on cellulose acetate plates (Helena Laboratories). The 13 enzyme systems assayed were: malate dehydrogenase (EC 1.1.1.37; MDH), malic enzyme (EC 1.1.1.40; ME), isocitrate dehydrogenase (EC 1.1.1.42; ICD), 6 phosphogluconate dehydrogenase (EC 1.1.1.44; 6PGDH), glucose 6 phosphate dehydrogenase (EC 1.1.1.49; G6PDH), glutamate dehydrogenase (NAD^+) (EC 1.2.1.2; GDH NAD^+), glutamate dehydrogenase ($NADP^+$) (EC 1.4.1.2; GDH $NADP^+$), glutamate oxaloacetate transaminase (EC 2.6.1.1; GOT), phosphoglucomutase (EC 2.7.5.1; PGM), peptidase (L-leucyl-leucyl-leucine as substrate) (EC 3.4.1.1; PEP), aconitate hydrolase (EC 4.2.1.3; ACON), mannose phosphate isomerase (EC 5.3.1.8; MPI) and phosphoglucose isomerase (EC 5.3.1.9; PGI). The assays were performed according to the method of Lanham et al. [28] as modified by Tibayrenc and Le Ray [29].

TABLE I

Sources among Bolivian *L.b.braziliensis* strains

Strains	Source of isolate	Number of cutaneous lesions	Mucous involvement	Geographical origin
MHOM/BO/82/LPZ-13	Human	10	Yes	North-Yungas
MHOM/BO/82/LPZ-17	Human	1	No	Alto-Beni
MHOM/BO/83/LPZ-155	Human	1	No	Beni
MHOM/BO/83/LPZ-355	Human	1	No	Alto Beni
MHOM/BO/84/LPZ-440	Human	1	No	Alto Beni
ILMA/BO/84/LPZ-595	Sandfly	–	–	Alto Beni
MHOM/BO/84/LPZ-662	Human	3	No	Alto Beni
MHOM/BO/84/LPZ-688	Human	50	Yes	North Yungas
IYUC/BO/84/LPZ-704	Sandfly	–	–	Alto Beni
MHOM/BO/84/LPZ-714	Human	1	No	Beni

Surface iodination. Promastigotes were collected in their stationary growth phase at a density of approximately 2×10^7 cells ml^{-1} and were harvested by centrifugation at $400 \times g$ for 15 min. The cell pellets were resuspended and washed twice in Hanks–Wallace balanced salt solution and once in phosphate buffer pH 7.2 (PBS), 0.15 M NaCl, both containing 100 U of aprotinin (Sigma Chemical Co., St Louis, MO, U.S.A.). The final washed pellets were adjusted to 5×10^8 cells ml^{-1} in PBS pH 7.2.

Surface iodination by Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenyl-glycoluril; Pierce Chemical Co., Rockford, IL, U.S.A.) was carried out according to Lemesre et al. [16].

Immunoprecipitation. Immunoprecipitation was carried out by the method of Kessler [30]. 10 µl of a detergent extract of labeled promastigotes was diluted in 700 µl of 10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.5% (v/v) Nonidet-P40, 100 U of aprotinin ml^{-1}, and incubated with 200 µl of immune or normal serum for 1 h at 4°C with constant agitation. Immune complexes were absorbed for 3 h at 4°C with 10 mg of protein-A Sepharose 4B-CL (Pharmacia, Uppsala, Sweden), suspended in the buffer mentioned above. Absorbed antigens were eluted by suspending washed Sepharose pellets in 50 µl sodium dodecyl sulfate (SDS) containing slab gel buffer [22] with 10 mM dithiothreitol and boiled for 15 min. After centrifugation, the supernatants were stored at −20°C for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Gel electrophoresis and autoradiography. Samples were separated by 7% SDS-PAGE on slab gels as previously described [31]. Coomassie Blue-stained gels were dried under vacuum and autoradiographed using X-Omat AR Film (Eastman-Kodak, Rochester, NY, U.S.A.) in conjunction with Cronex intensifying screens (Dupont de Nemours, Wilmington, U.S.A.), for exposures of 1–7 days at −70°C.

Results

Surface iodination. Quite similar autoradiographic patterns were obtained after growing parasites in the two different culture media (LIT and modified N.N.N. medium). Since the modified N.N.N. medium provided optimal culture conditions for the *L. b. braziliensis* promastigotes, this medium was preferentially used.

The autoradiographic patterns resulting from the electrophoresis of surface-labeled promastigotes of *Leishmania* are shown in Fig. 1. All the *L. b. braziliensis* strains (ten Bolivian strains and two strains from Brazil and Belize) revealed nearly identical patterns with ten to thirteen protein bands with molecular weights ranging from 25 000 to 200 000. At least eight proteins with similar molecular weights lower than 150 000 appeared in each *L. b. braziliensis* strain. A 72 kDa protein band was major in all the strains, and a 55, 58 kDa protein doublet was pronounced in strains LPZ-17, -155, -662, -704 and -714. The lowest molecular weight bands (less than 30 kDa) routinely lacked distinct resolution which may reflect groups of comigrating molecules.

Differences between *L. b. braziliensis* surface proteins were detected for high molecular weight proteins (HMW proteins) ranging from 150 to 200 kDa (Fig. 1).

With respect to surface proteins of the other New World leishmanias, we observed different patterns of major surface components. Indeed, *L. m. amazonensis* strain exhibited a profile with one very major band of 63 kDa, while *L. b. guyanensis*, *L. b. panamensis* and *L. d. chagasi* strains revealed major protein bands of 150, 130, 74 and 67 kDa; 60, 61, 58, 55 and 45 kDa; 65, 60, 40 and 38 kDa, respectively (Fig. 1).

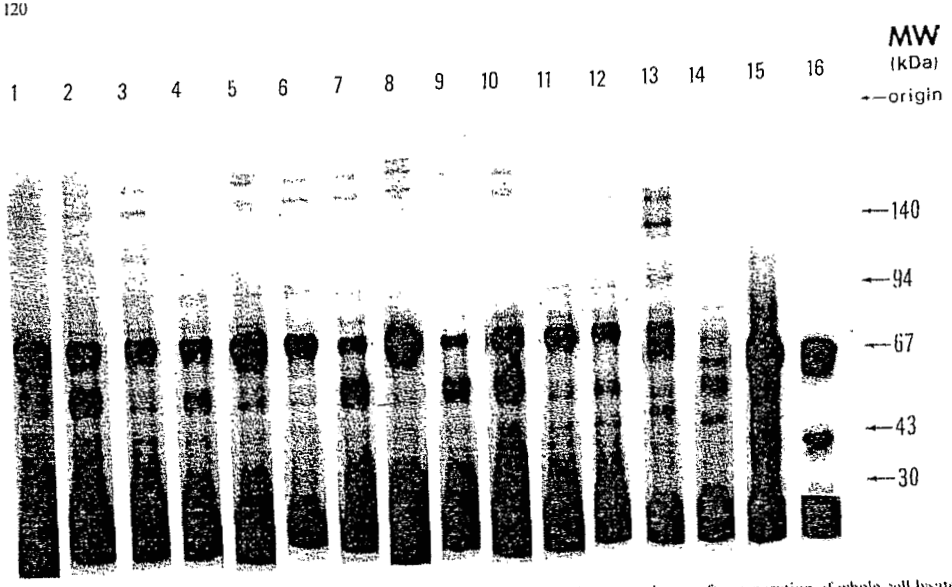

Fig. 1. Autoradiographic patterns of iodinated surface proteins of *Leishmania* promastigotes after separation of whole cell lysates by SDS-PAGE. The *Leishmania* strains were: lanes 1–10, Bolivian *L. b. braziliensis* LPZ-13, -17, -155, -355, -440, -595, -662, -688, -704, -714; lanes 11 and 12, WHO reference *L. b. braziliensis* M-2904 and BEL-53; lane 13, *L. b. guyanensis* M-5378; lane 14, *L. b. panamensis* M-4039; lane 15, *L. m. amazonensis* PH-8 and lane 16, *L. d. chagasi* M-2682. The arrows indicate the migration of protein standards.

Immunoprecipitation of surface iodinated promastigotes. Immunoprecipitation of *L. b. braziliensis* detergent extracts by the human cutaneous and mucocutaneous sera and by the hamster antiserum revealed similar SDS-PAGE patterns (Fig. 2). Few antigens were recognized by the sera, among which were the 72 kDa protein and all HMW proteins (Fig. 2). For the 55, 58 kDa protein doublet and the low molecular weight proteins (less than 30 kDa), no recognition was noted. A 40 kDa curved band was encountered in all immunoprecipitation profiles. This band may correspond to immunoglobulin heavy chains, slightly iodinated during immunoprecipitation.

L. b. guyanensis, *L. b. panamensis*, *L. m. amazonensis* and *L. d. chagasi* detergent extracts were tested with the anti-*L. b. braziliensis* hamster sera, and appeared to react very weakly compared with *L. b. braziliensis* (Fig. 3a). Same results were obtained with all the cutaneous and mucocutaneous human sera.

Although the chagasic sera strongly precipitated *T. cruzi* antigens, a weak recognition of the 72 kDa and HMW antigens of *L. b. braziliensis* was observed (Fig. 3b). Likewise, while the sera of Kala-azar patients strongly precipitated the 65, 40 and 38 kDa major proteins of *L. d. chagasi*, these sera, as well as normal human sera, precipitated slightly the 72 kDa protein of *L. b. braziliensis*.

Isoenzymic and antigenic heterogeneity of L. b. braziliensis. Isoenzymic studies have been performed on *L. b. braziliensis* by using thirteen enzyme systems. Ten showed similar patterns for all the strains (not shown), and only MDH, ICD and ME enzymes showed variations between strains (Fig. 4a). MDH, ICD and ME enzyme systems define five different patterns (I–V). Pattern I is common to the two WHO reference strains (M-2904 and BEL-53) and to strains LPZ-13, -17, -155, -355. Pattern II is common to strains LPZ-595, -662, -714, and patterns II, IV and V correspond to strains LPZ-440, -688 and -704, respectively.

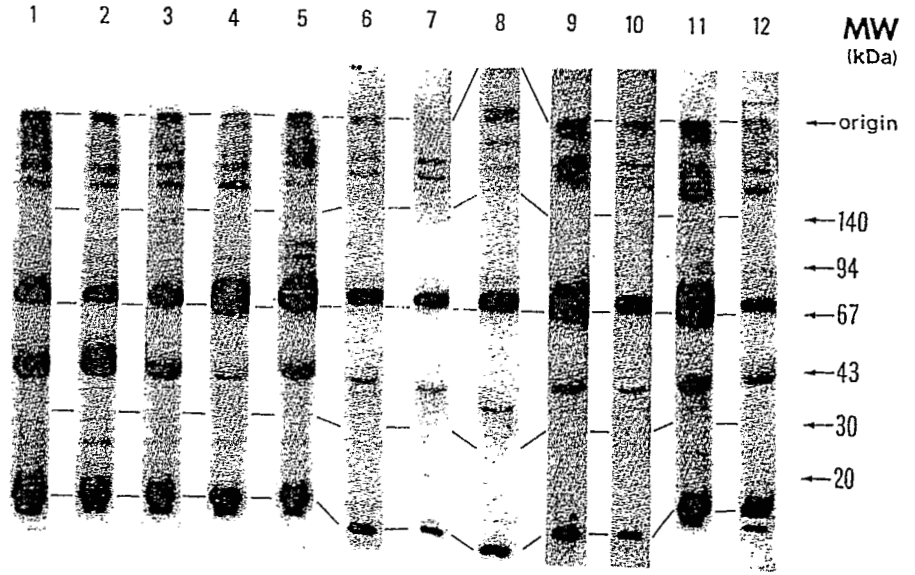

Fig. 2. Autoradiographic patterns of iodinated surface proteins precipitated from detergent extracts of *L. b. braziliensis* strains with sera of patient with mucocutaneous-leishmaniasis after separation by SDS-PAGE. Lanes 1–12, *L. b. braziliensis* strains LPZ-13, -17, -155, -355, -440, -595, -662, -688, -704, -714, M-2904 and BEL-53. The arrows indicate the migration of protein standards.

As previously noted, *L. b. braziliensis* strains exhibit a HMW antigen variability. Eleven antigens, classified from a to k, define four different patterns among *L. b. braziliensis* antigenic profiles (Fig. 4b). The protein doublet f-j was found in strains M-2904, BEL-53, LPZ-13, -17, -155 and -355 (isoenzyme pattern I), and the doublet d-h in strains LPZ-595, -662, -704 and -714 (isoenzyme patterns II and V). Strains LPZ-440 and LPZ-688 possessed the proteins b-e-i and a-c-g-k, respectively (isoenzyme patterns III and IV).

Discussion

Our results show unambiguously a large antigenic homogeneity among the twelve *L. b. braziliensis* strains from Bolivia, Brazil and Belize. All these strains have in common about 90% of their surface antigens including a 72 kDa major component. Moreover, *L. b. braziliensis* strains present a great isoenzymic homogeneity as previously described by Desjeux et al. [23] and reported above.

Nevertheless, an isoenzymic and antigenic microheterogeneity is observed in *L. b. braziliensis* subspecies. The isoenzyme systems MDH, ICD and ME allow differentiation of two main groups of strains which also exhibit different HMW patterns. A relationship seems to exist between isoenzymic and antigenic expressions. There seems to be no relation between the geographical origin of the *L. b. braziliensis* strains and these microheterogeneities, since the strains from Brazil and Belize are identical to several strains from Bolivia. In addition, no relation exists between antigenicity and pathologic manifestations, since strains isolated from patients with the same type and number of lesions belong to either of the above-mentioned groups of *L. b. braziliensis*

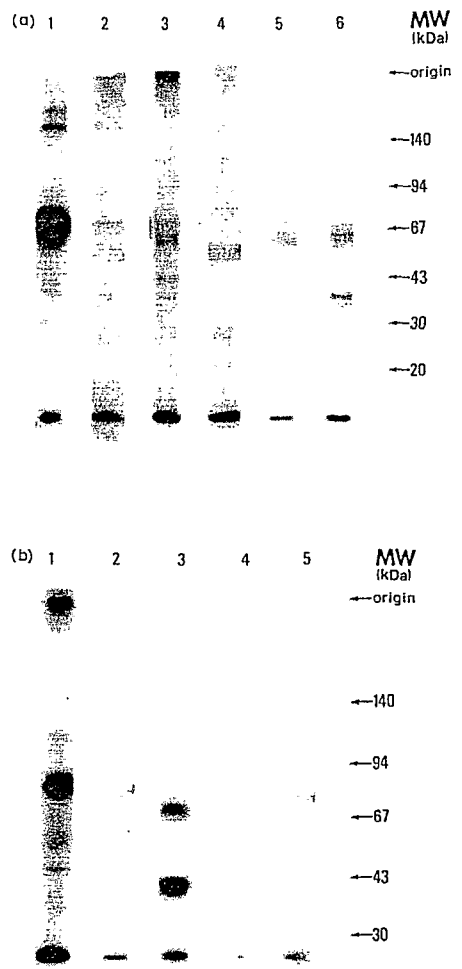

Fig. 3. (a) Autoradiographic patterns of surface proteins of different New World leishmanias precipitated by the hamster sera after separation by SDS-PAGE: lanes 1 and 2, *L. b. braziliensis* M-2904 precipitated by the anti-*L. b. braziliensis* and normal hamster sera; lanes 3–6, *L. b. guyanensis* M-5378, *L. b. panamensis* M-4039, *L. m. amazonensis* PH-8 and *L. d. chagasi* M-2682 precipitated by the anti-*L. b. braziliensis* hamster serum. (b) Autoradiographic patterns of surface proteins of: lanes 1 and 2, *T. cruzi* Tehuentepec, *L. b. braziliensis* M-2904 precipitated by a human chagasic serum; lanes 3 and 4, *L. d. chagasi* M-2682, *L. b. braziliensis* M-2904 precipitated by a human Kala-azar serum, and lane 5, *L. b. braziliensis* M-2904 precipitated by a normal human serum. The arrows indicate the migration of protein standards.

strains, and similar antigenic recognition was observed with cutaneous and mucocutaneous human sera. However, it should be mentioned that strain LPZ-688 which possesses specific HMW antigens, was collected from a Bolivian patient with more than 50 cutaneous lesions.

Several authors report major surface antigens of about 65 kDa, common to several Old World and New World leishmanias including *L. b. braziliensis*, that crossreact with Kala-azar and cutaneous sera [15,19–22]. In particular, Lepay et al. [15] report that a 65 kDa major antigen of *L. donovani* was immunoprecipitated by both New World Kala-azar and cutaneous sera. However, no precise data are provided about cutaneous sera, except that they came from *L. braziliensis*-infected patients. The infection of patients by *L. b. guyanensis* or *L. b. panamensis* strains cannot be excluded and could explain the cross-reactivity. More recently, Colomer-Gould et al. [22] describe a 65 kDa surface antigen which is recognized by both Kala-azar and cutaneous sera, and is present in several New World and Old World leishmanias including *L. b. braziliensis*.

Nevertheless, the present study reports the existence of a 72 kDa major surface antigen in twelve different isolates of *L. b. braziliensis*, which is not recognized by Kala-azar and chagasic sera and not found in other New World leishmanias. Some recent data could explain, in part, these discrepancies. In a preliminary report, we have shown that using high-reticulated SDS-PAGE gels (10–12%), the 55, 58 kDa protein doublet is not well separated from the major *L. b. braziliensis* surface antigen [32]. This can induce mistakes in the molecular weight determination. Moreover, Etges et al. [33] have demonstrated that the major radioiodinated surface protein (62–65 kDa) of promastigotes of different *Leishmania* species has a protease activity. Surprisingly, the same authors have observed that the protease activity found in a protein of 63 kDa of

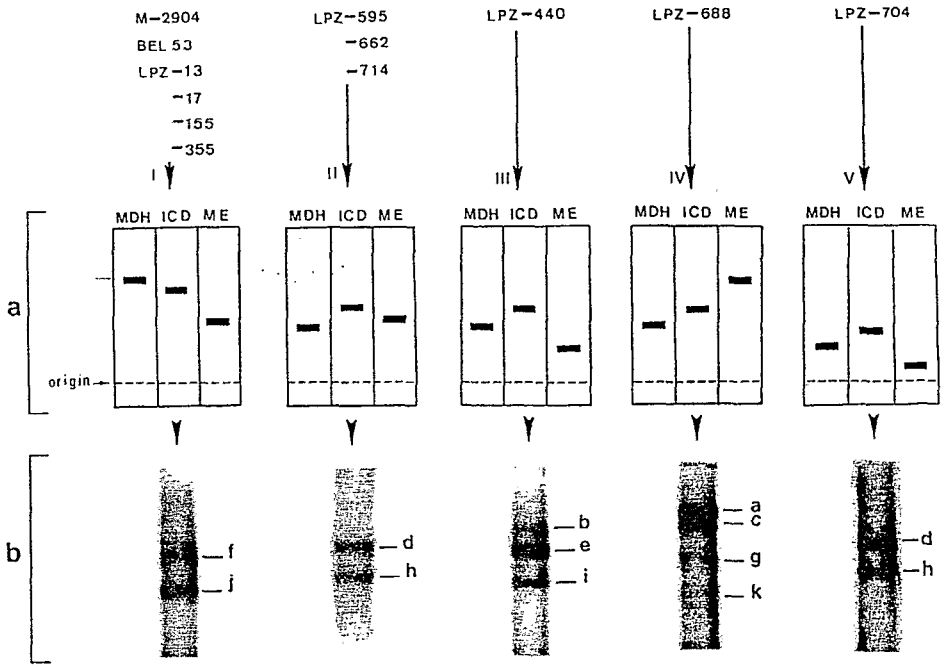

Fig. 4. Isoenzymic and antigenic variability among *L. b. braziliensis* strains: (a) 5 isoenzymic patterns of *L. b. braziliensis* strains (I–V) defined by MDH, ICD and ME isoenzyme systems; (b) 4 HMW antigen patterns defined by eleven antigen bands (a–k).

L. b. braziliensis did not correspond to its major radioiodinated surface protein which possesses a higher molecular weight [34].

A study using more strains of different regions of the New World seems to be essential to assess for the universal presence of the 72 kDa antigen among *L. b. braziliensis* subspecies. This component could be of particular biological and medical importance since its partial purification could facilitate a specific diagnosis of *L. b. braziliensis* leishmaniasis.

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