Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *87* (2): 229-234, apr./jun. 1992

ANTIGENIC DIFFERENCES AMONG *LEISHMANIA AMAZONENSIS* ISOLATES AND THEIR RELATIONSHIP WITH DISTINCT CLINICAL FORMS OF THE DISEASE

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Immunoblot analysis was used to investigate antigenic differences among clinical isolates of Leishmania amazonensis *and their role in the etiology of the disease. Western blots of promastigote homogenates were analyzed with either monoclonal antibodies (MAbs) specific for the* L. mexicana *complex (M-4, M-6, M-9, and M-11) or polyclonal sera from* L. amazonensis *infected patients with the various forms of clinical disease. In the case of the MAbs, no significant variation was observed among the strains of* L. amazonensis, *isolated from cases of cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), visceral leishmaniasis (VL) or post kala-azar dermal leishmaniasis (PKDL), in either the relative mobility (Mr) or the quantitative amount (intensity) of the antigenic determinants. In the case of the sera of the infected patients, the patterns of antigenic reactivity of these strains revealed that, despite showing the presence of shared antigens, differences were observed between some of the antigenic components of the various isolates of* L. amazonensis *that were recognized by a single serum. Differences were also demonstrated between the antigenic determinants of a single isolate of* L. amazonensis *that were recognized by the different patients' sera. No apparent association was consistently found, however, between the Mr components identified in these isolates and the clinical form of the disease or the geographical area of isolation. In addition, the spectrum of antigens recognized by the sera from patients with the same clinical form were not identical; although in some instances, similar Mr antigens were shared. These results indicate that isolates of* L. amazonensis *are not antigenically identical (homogeneous) and that the immune responses (antibodies) observed among infected patients are heterogeneous.*

Key words: *Leishmania amazonensis* – Protozoa – parasitic – hemoflagellate – promastigote – immunoblot – antigenic differences

The human leishmaniases are caused by a number of different trypanosomatid protozoa in the genus *Leishmania* (Anonymous, 1990). Clinical and experimental evidence suggests that both parasite and host factors influence the course of infection. Thus, the ability of *Leishmania* to cause different clinical forms of the disease depends both on the nature of the infecting agent and on the immune response developed by the host (Mauel & Behin, 1987; Preston, 1987). This concept may explain the following facts: a) the various species of *Leishmania* that parasitize humans induce a variety of clinical manifestations; b) different leishmanial parasites may cause clinically similar disease; and c) one particular parasite species may produce distinct clinical manifestations of leihsmaniasis (for review see Grimaldi et al., 1989; Grimaldi & McMahon-Pratt, 1991). In previous study we have shown that in Bahia, Brazil *L. amazonensis* was frequently associated with human leishmaniasis and was capable of producing a wide spectrum of disease (Barral et al., 1991). We also demonstrated variation between the antigenic components of geographically distinct isolates of this species causing DCL (Leon et al., 1990). Here, we have extended these investigations

This investigation received support from PADCT/FINEP, CNPq (Brazil), and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

Received 9 September 1991.
Accepted 12 February 1992.

employing Western blot analyses, using both specific monoclonal and polyclonal antibodies, to determine the antigenic components of *L. amazonensis* isolates that were obtained from a wide range of clinical cases and to see whether any strain-related differences exist in their antigenic composition.

MATERIALS AND METHODS

Leishmania *stocks* – A total of 22 New World *Leishmania* stocks, including some of WHO reference strains, were examined in this study. Nineteen of these stocks represented isolates from patients with different clinical forms of leishmaniasis in the State of Bahia, Brazil (Table). These isolates have been typed as *L. amazonensis* by both indirect radioimmune binding assay, using specific monoclonal antibodies, and enzyme electrophoresis (Barral et al., 1991).

Preparation of samples – Procedures for growing *Leishmania* promastigotes *in vitro* have been reported previously (Grimaldi et al., 1987). When the promastigote were in the log phase of growth in Schneiders' medium they were harvested by centrifugation (1,500 x g for 10 min, at 4°C) and washed twice in phosphate buffered saline (PBS), pH 7.3. The final pellet was resuspended in an antiproteolytic buffer containing 0.04 M NaCl, 0.01 M sodium ethylenediamine tetraacetate (EDTA), 0.001 M phenylmethylsulfonylfluoride (PMSF), 0.01 M iodoacetamide, 0.005 M 1,10-phenanthroline, and 0.01 M Tris, pH 8.0. Prior to analysis, the samples were briefly sonicated using a bath sonicator (RAI Research, Model 250 Ultrasonic cleaner) to homogenize the antigens, and then centrifuged (2,000 x g for 10 min, at 4°C). The supernatants were aliquoted and stored at –70°C until used for analysis. Protein concentration was measured by the protein assay method of Lowry et al. (1951), and all samples were resuspended to the same concentration before analysis.

Western blot analysis – The soluble antigen extracts were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 10% slab gel in non-reducing conditions, and electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell, Keene, New Hampshire, USA), according to the pro-

TABLE

Origin and identification of *Leishmania* strains used in this study

Stock code	Stock designation	Clinical form[a]	Geographic origin[b]	Species
L113	MHOM/VE/00/L54	DCL	VE, Guarico	*L. amazonensis*
L168	MHOM/BR/73/MT (D)	DCL	AM, Airão	*L. amazonensis*
L179	MHOM/BR/82/Dilma	VL	BA, E. da Cunha	*L. amazonensis*
L185	MHOM/BR/77/LTB0016	CL	BA, T. Braços	*L. amazonensis*
L322	MHOM/BR/83/JGL	DCL	MA, Cavalcante	*L. amazonensis*
L358	MHOM/BR/84/LTB662	CL	BA, C. de Pedras	*L. amazonensis*
L565	MHOM/BR/75/M4147	CL	PA, M. Dourado	*L. guyanensis*
L566	MHOM/BR/75/M2903	CL	PA, S. Carajás	*L. braziliensis*
L575	IFLA/BR/67/PH8	–	PA, Utinga	*L. amazonensis*
L579	MHOM/BR/74/PP75	VL	BA, Ituaçu	*L. chagasi*
L612	MHOM/BR/85/ASS	VL	BA, M. do Chapéu	*L. amazonensis*
L613	MHOM/BR/85/EAS	VL	BA, Ouricuri	*L. amazonensis*
L614	MHOM/BR/85/JSS	VL	BA, Jacobina	*L. amazonensis*
L615	MHOM/BR/85/MNB	CL	BA, Gandu	*L. amazonensis*
L617	MHOM/BR/85/ABC	MCL	BA, Algodão	*L. amazonensis*
L619	MHOM/BR/85/JJA	VL	BA, Jacobina	*L. amazonensis*
L624	MHOM/BR/85/LTC395	MCL	BA, Algodão	*L. amazonensis*
L626	MHOM/BR/85/JCGR	PKDL	BA, Ouricuri	*L. amazonensis*
L651	MHOM/BR/85/VS	CL	BA, Jacobina	*L. amazonensis*
L725	MHOM/BR/86/MPS	PKDL	BA, Ouricuri	*L. amazonensis*
L728	MHOM/BR/86/JGR	PKDL	BA, Ouricuri	*L. amazonensis*
L935	MHOM/BR/86/BA-106	DCL	BA, Ilhéus	*L. amazonensis*

a: CL = cutaneous leishmaniasis; VL = visceral leishmaniasis; MCL = mucutaneous leishmaniasis; PKDL = post kala-azar dermal leishmaniasis; DCL = diffuse cutaneous leishmaniasis.
b: AM = Amazonas, BA = Bahia, MA = Maranhão, and PA = Pará, Brazil; and VE = Venezuela.

cedure described by Towbin et al. (1979). The nitrocellulose strips were then incubated overnight at 4°C with either 1/4 dilution of cell culture supernatants containing the MAbs or anti-*L. amazonensis* polyclonal antibodies. The MAbs used (M4, IX-1F9-D8; M6, LXVII-4C7-B8; M9, XLV-2B5-H7; and M11, XLV-1D11-E11) have been characterized elsewere (McMahon-Pratt et al., 1985; Grimaldi et al., 1987) and are specific for members of the *L. mexicana* complex. The anti-*L. amazonensis* immune sera were obtained from patients infected with this parasite, but with different clinical forms of leishmaniasis (Table). Following incubation with the primary antibody, the strips were washed three times in wash buffer (PBS containing 0.05% Tween-20; Fisher Scientific), and incubated with either rabbit anti-mouse or anti-human immunoglobulin G conjugated to peroxidase (Sigma), for 1 h. After rinsing with wash buffer, the strips were incubated for 15 min in a saturated solution of 3,3'-diaminobenzidine in the Tris buffer, pH 7.4, containing 0.01% H_2O_2 (Graham & Karnovsky, 1966).

RESULTS

Identification of L. amazonensis *antigens by MAbs* – The *L. mexicana* group-specific antigenic determinants were recognized by MAbs in all *L. amazonensis* strains analyzed (Figs 1, 2). Both pools of MAbs (M9 and M11; M4 and M6) recognized multiple components in all *L. amazonensis* strains analyzed, but there was no binding of these *L. mexicana* complex-specific MAbs with *L. guyanensis, L. braziliensis,* or *L. chagasi.* As seen from Figs 1 and 2, these MAbs could detect no significant variation among the strains of *L. amazonensis* isolated from cases of CL, MCL, DCL, VL, or PKDL, in either the relative mobility (Mr) or the quantitative amount (intensity) of the antigenic components.

Characterization of L. amazonensis *antigens by immune sera* – Figs 3 and 4 show that the Western blots prepared from the *L. amazonensis* strains, reacted with polyclonal sera obtained from *L. amazonensis* infected patients. Although the analysis indicated extensive cross-reactivity, differences were also observed between the antigenic components of the various isolates of *L. amazonensis* that were recognized by a single serum, and between the antigenic fractions of a single isolate of *L. amazonensis* that were recognized by the different patients' sera. Inspection of these blots revealed no correlation, however, between the antigenic components present in the various strains of *L. amazonensis* and the clinical form of the disease or the geographical area of isolation. Although certain Mr components present in individual strains were recognized by sera from all patients grouped by clinical presentation, the spectrum of antigens recognized by these groups of sera were not identical.

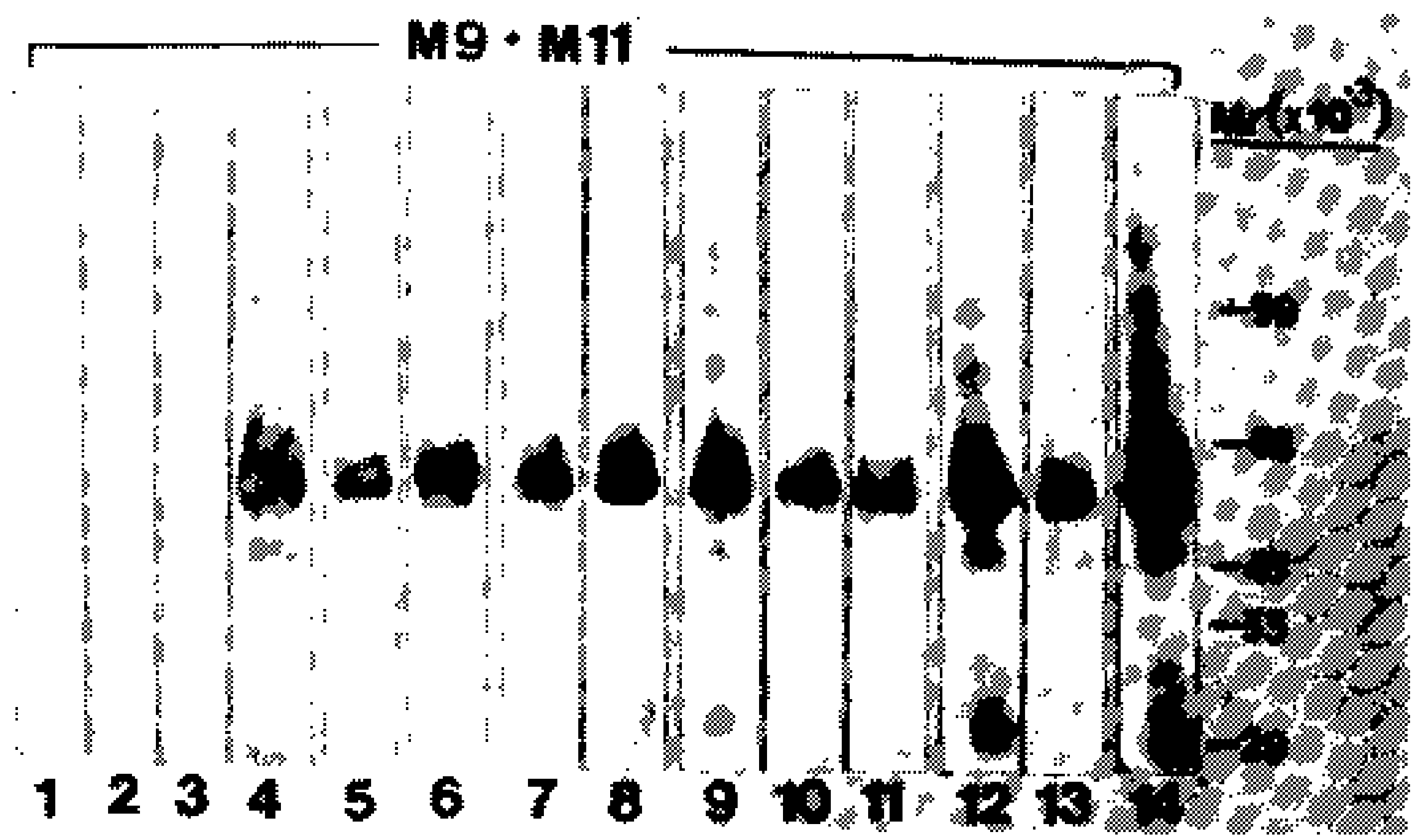

Fig. 1: Western blot analyses of promastigote homogenates of *Leishmania amazonensis* strains (sse Table for information about their origin), using the specific monoclonal antibodies M9 and M11. The following strains were used as antigens: track 1: L565; track 2: L579; track 3: L566; track 4: L575; track 5: L651; track 6: L185; track 7: L624; track 8: L617; track 9: L626; track 10: L725; track 11: L728; track 12: L179: track 13: L614; and track 14: L935. The strains L565 (*L. guyanensis*), L579 (*L. chagasi*) and L566 (*L. braziliensis*) were utilized as negative controls.

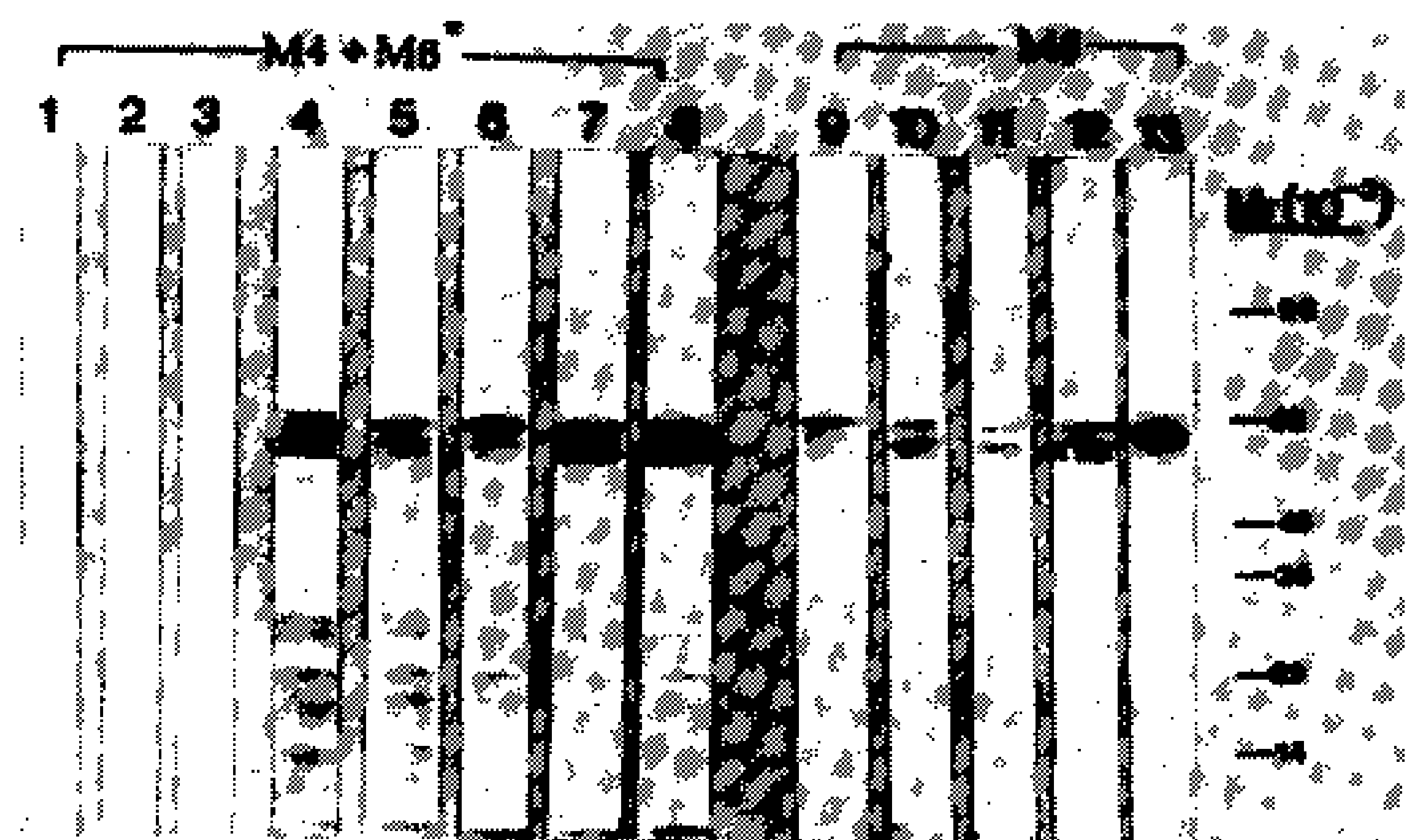

Fig. 2: Western blot analyses of promastigote homogenates of *Leishmania amazonensis* strains (see Table for information about their origin), using the specific monoclonal antibodies M4 and M6. The following strains were used as antigens: track 1: L565; track 2: L579; track 3: L566; track 4: L575; track 5: L612; track 6: L613; track 7: L614; track 8: L725; track 9: L179; track 10: L619; track 11: L626; track 12: L651; and track 13: L185. The strains L565 (*L. guyanensis*), L579 (*L. chagasi*) and L566 (*L. braziliensis*) were utilized as negative controls.

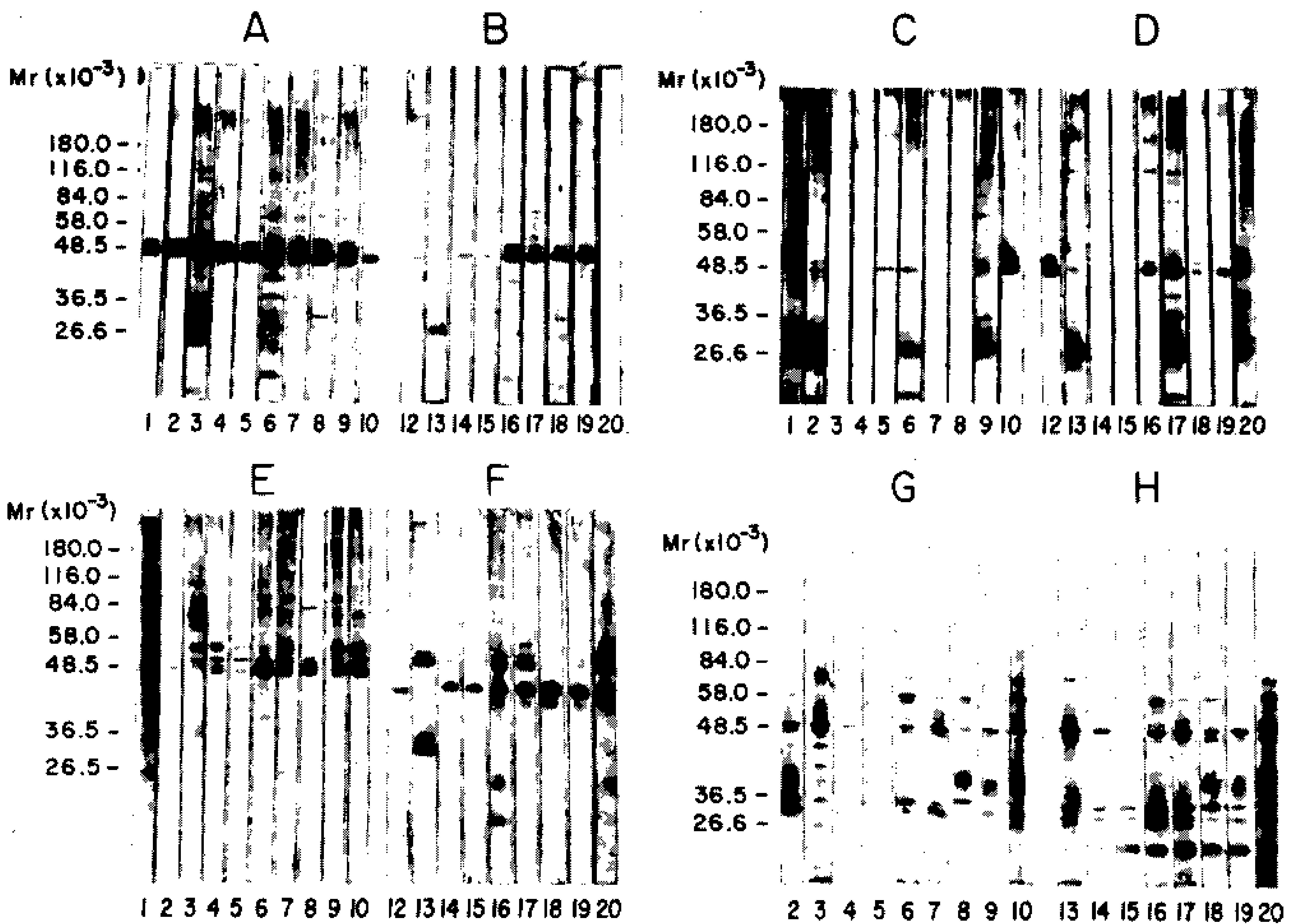

Fig. 3: Western blot analyses of promastigote homogenates of Leishmania amazonensis strains (see Table for information about their origin), using *L. amazonensis* specific polyclonal antibodies [immune sera obtained from patients with: tracks 2 and 12, VL (case # 1); 3 and 13, VL (case # 2); 4 and 14, PKDL (case # 3); 5 and 15, PKDL (case # 4); 6 and 16, DCL (case # 5); 7 and 17, MCL (case # 6); 8 and 18, MCL (case # 7); 9 and 19, CL (case # 8)], and anti-*L. chagasi* (track 1) and anti-*L. braziliensis* (tracks 10 and 20) immune sera. The following strains were used as antigens: A: L561; B: L358; C: L728; D: L725; E: L624; F: L617; G: L179; and H: L935.

DISCUSSION

Recent taxonomic studies of *Leishmania* isolates from a variety of clinical conditions and geographical regions indicate that some of them are capable of producing a broader spectrum of illness than previously realized (for review see Grimaldi et al., 1989; Grimaldi & McMahon-Pratt, 1991). The fact that a particular *Leishmania* species (Barral et al., 1991) or strain (Convit et al., 1972) can cause different clinicopathological manifestations, implies that host factors appears to be important in determining the outcome of infection. Immunogenetic factors expressed in some races or individuals, like those defined in the murine model system (Howard, 1985; Blackwell & Alexander, 1986), are likely to play a role in determining some of these variations in the clinical manifestation of the disease seen in human leishmaniasis. There is evidence that the particular infecting species or clone of *Leishmania* also appears to influence the course of infection (Handman et al., 1983, 1987).

We have previously shown that *L. amazonensis* can be frequently associated with human leishmaniasis and that this species is able to cause distinct clinical presentations of the disease (Barral et al., 1991). Here we expanded these investigations in order to characterize and better define the contribution of parasite versus host to the range of disease/tropism associated with *L. amazonensis* infection in humans. Antigenic analysis of *L. amazonensis* isolates, using Western blots and immune sera, revealed many antigenic components in these parasites. We were also able to demonstrated that the *L. amazonensis* isolates associated with distinct clinical forms were not antigenically identical (homogenous). In addition, results obtained in a parallel study showed that serum from a single individual infected with *L. amazonensis* could recognize many

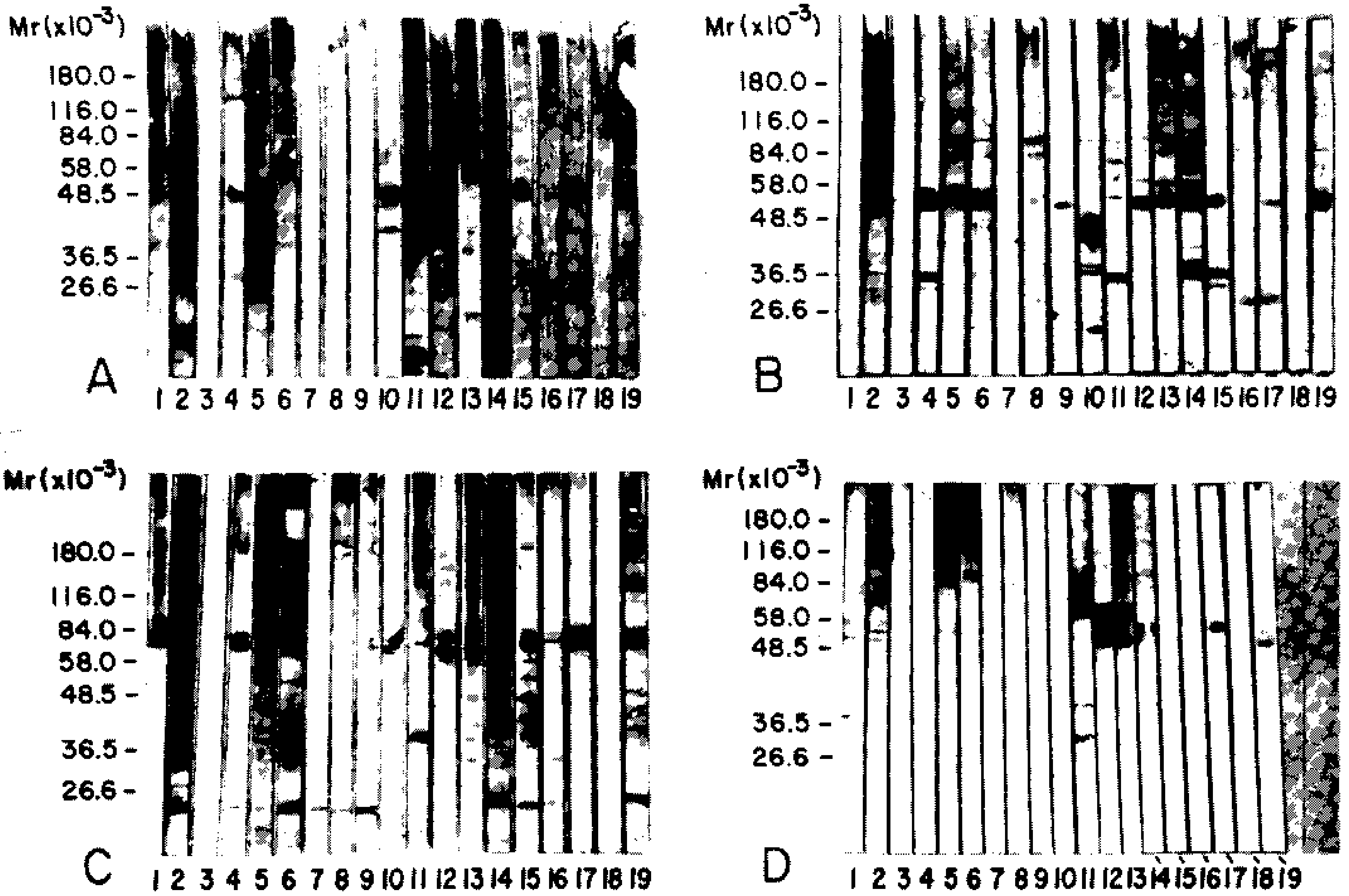

Fig. 4: Western blot analyses of promastigote homogenates of *Leishmania amazonensis* strains (see Table for information about their origin), using *L. amazonensis* specific polyclonal antibodies [immune sera obtained from patients with: A: CL (case # 8); B: MCL (case # 7); C: DCL (case # 5); and D: PKDL (case # 3)]. The following strains were used as antigens: track 1: L179; track 2: L613; track 3: L614; track 4: L619; track 5: L575; track 6: L612; track 7: L626; track 8: L725; track 9: L728; track 10: L935; track 11: L322; track 12: L168; track 13: L113; track 14: L617; track 15: L624; track 16: L651; track 17: L185; track 18: L358; and track 19: L615.

different epitopes from geographically distinct isolates of this parasite causing DCL in humans (Leon et al., 1990). It is possible that some of these molecules within the complex may represent proteolytic breakdown products (spurious bands) of larger precursor molecules by residual activity of proteases commonly found in *Leishmania* such as gp63, a metalloproteinase (Chaudhuri et al., 1989; Chang et al., 1990). However, since the results were relatively reproducible in different experiments, we assumed that these proteolytic enzymes were substantially inactivated by the protease inhibitors used for sample preparation. Differential composition/expression of immunogenic molecules, particularly of surface lipophosphoglycan and other related surface inositol glycolipids may occur in heterogenous *Leishmania* populations (for review see Chan et al., 1990). Since the heterogeneity of these *L. amazonensis* strains was also confirmed in a parallel study by their distinct kinetoplast deoxyribonucleic acid restriction enzyme profiles (Grimaldi, unpublished observations) this may explain the variation observed between the antigenic components of some isolates of *L. amazonensis* that were recognized by a single serum. Although these antigenic differences did not show a distinct association with the clinical forms of the disease, they are likely to play a role in determining some of the variations in the course of infection found in those patients.

The immune sera used were obtained from patients infected with *L. amazonensis,* but with distinct clinical forms of the disease. Sera from different individuals recognize different antigens from the same isolate, showing an heterogenous antibody response observed among infected patients with *L. amazonensis.* The variation observed in both the humoral response and clinical manifestations may also in part be explained by the various genetic factors expressed in different individuals, as discussed above.

ACKNOWLEDGEMENTS

To Dr Diane McMahon-Pratt (Yale University School of Medicine, New Haven, CT, U.S.A.) for providing the monoclonal antibodies.

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