JOURNAL OF CLINICAL MICROBIOLOGY, July 1991, p. 1429–1435
0095-1137/91/071429-07$02.00/0

Vol. 29, No. 7

Immunoblot Analysis of the Humoral Immune Response to *Leishmania donovani infantum* Polypeptides in Human Visceral Leishmaniasis

LAURENCE ROLLAND-BURGER,[1]* XAVIER ROLLAND,[1] CAROLINE W. GRIEVE,[2] AND LOÏC MONJOUR[1]

Institut National de la Santé et de la Recherche Médicale U313, Centre Hospitalo-Universitaire Pitié-Salpétrière, 91 Boulevard de l'Hopital, 75643 Paris Cédex 13,[1] and Département de Génie Biologique, Université de Technologie de Compiègne, 60200 Compiègne,[2] France

Received 28 January 1991/Accepted 24 April 1991

Using the immunoblot technique, we have compared the reactions of *Leishmania donovani infantum* polypeptides with the immunoglobulin G of human sera from patients with parasitologically proven *L. d. infantum* infection, with suspected visceral leishmaniasis, and with other leishmaniases, protozoiases, helminthiases, and fungal or bacterial diseases. A 94-kDa component reacted with all *L. d. infantum*-infected sera and with 75% of sera from patients with clinical and serological but no parasitological diagnoses. No reaction was observed with sera from patients in the other disease groups or with control sera. Studies of eight different isolates, subspecies, and species of the genus *Leishmania* demonstrated that the 94-kDa component was expressed in all strains examined.

Human visceral leishmaniasis (VL) is caused by protozoan parasites, members of the family *Trypanosomatidae*, of the *Leishmania donovani* complex, namely, *L. d. donovani* and *L. d. infantum* in the Old World and *L. d. chagasi* in the New World. The organisms are obligatory intracellular parasites which infect cells of the reticuloendothelial system. This infection is characterized clinically by fever, hepatosplenomegaly, anemia, and weight loss. A marked hypergammaglobulinemia and absence of detectable cell-mediated immunity are the principal immunological features of the disease. There are approximately 100,000 new cases each year. The disease has a high mortality rate if untreated and is often complicated by intercurrent infections. Available drugs are not always effective, side effects are not uncommon, and no vaccine exists (for a review, see reference 41).

For the diagnosis of VL, presumptive arguments are given by both clinical and epidemiological data. However, on the basis of these same criteria, other diseases have to be considered in the differential diagnosis, among them malaria, typhoid fever, tuberculosis, brucellosis, Hodgkin's disease, some leukemias, and African trypanosomiasis. Definitive diagnosis is based on the demonstration of the parasite, which may be found in aspirates of the spleen (98% positive), (sternal) bone marrow (85% positive), liver (60% positive), or lymph node (60% positive). Splenic aspiration is the most reliable method but is a high-risk procedure. Because in many cases demonstration is difficult, serologic tests have provided useful alternatives.

Antigenic similarities among various species of the genus *Leishmania* (2, 6, 8, 13, 17, 26, 28) and other species of the family *Trypanosomatidae* (1, 19) as well as phylogenetically distant organisms (9, 30, 37) have been described, and serologic cross-reactivities between VL and other diseases are well known (3, 7, 10, 14, 31). To date, several *L. donovani*-specific proteins, including species-specific (13, 17, 20, 28, 39), stage-specific (17, 28) and subspecies-specific (25) molecules, have been identified with monoclonal antibodies or polyvalent antisera. Several of these proteins elicit a strong humoral immune response during VL infection, allowing specific VL diagnosis by a competitive enzyme-linked immunosorbent assay (ELISA) (21) or a direct dot blot assay (22). Immunoblots have been useful in studies of the host serological responses during infection, and a few antigens with potential diagnostic value have been selected (11, 27, 33).

In the present study, we have examined the specificity of the human humoral immune response to *L. d. infantum* infection. For this purpose, immunoblots of different *Leishmania* isolates, subspecies, and species were analyzed with sera from patients with leishmaniases, other protozoal infections, helminthiases, and fungal or bacterial diseases. Results show that antibodies directed against a 94-kDa component are restricted to *L. d. infantum*-infected sera. The additional value of this distinct molecule in the immunodiagnosis of visceral leishmaniasis is discussed.

MATERIALS AND METHODS

Parasites and culture conditions. The following *Leishmania* strains were used in this study: *L. d. infantum* LEM497 (MCAN/GR/82/LEM497) and Gr-L4 (MHOM/GR/78/Gr-L4) (isolated from the cutaneous lesion of a dog in Zakynthos, Greece, and from the bone marrow aspirate of a patient in Salamis, Greece, respectively), *L. d. infantum* LEM75 (MHOM/FR/78/LEM 75), *L. donovani* LEM138 (MHOM/IN/DEVI), *L. tropica* LEM135 (MHOM/IQ/LV 556), *L. major* (NIH 173), *L. braziliensis guyanensis* P30 (IUMB/GF/82/CAY P30), and *L. mexicana mexicana* (LV4).

Cells were cultured as promastigotes at 27°C in RPMI 1640 medium (GIBCO, Paisley, United Kingdom) containing 10% heat-inactivated fetal calf serum (GIBCO), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo.), 25 mM sodium carbonate (Merck, Darmstadt, Germany), 4 mM Tricine (Merck) 100 IU of penicillin per ml, and 100 μg of streptomycin per ml (GIBCO). All strains were maintained by weekly subculture, with an initial inoculum of 2×10^5

* Corresponding author.

cells per ml, except for *L. b. guyanensis* P30 (6×10^5 cells per ml).

For the immunoblot assays, leishmanias were harvested after 7 days of in vitro growth and washed three times in cold sterile phosphate-buffered saline (PBS, pH 7.4) before cell lysis (see below).

Human sera. Sera from 15 patients with VL were obtained from the Laikon University Hospital, Athens, Greece. VL was diagnosed by the detection of Giemsa-stained amastigotes in bone marrow aspirates, by clinical evaluation (fever, splenomegaly with or without hepatomegaly), and by indirect immunofluorescence assay. *L. tropica*-infected patients (18 serum samples) were from Zakynthos, Greece. *L. b. guyanensis*-infected patients (10 serum samples) were from Institut Pasteur, Cayenne, French Guyana; eight patients had primary infections with one to seven lesions, and two had recurrences with unique lesions (patients 7 and 8). Serum samples from 12 patients infected with *L. b. braziliensis* were from Bolivia; 2 of the patients had lesions for 3 weeks (patients 3 and 8), and the others had lesions for 8 to 12 months. Sera from patients with suspected VL (24 serum samples) and sera from patients infected with other organisms (*Trypanosoma cruzi* [8 samples], *Trypanosoma gambiense* [11 samples], *Plasmodium falciparum* [16 samples], *Toxoplasma gondii* [5 samples], *Entomoeba histolytica* [3 samples], *Onchocerca volvulus* [7 samples], *Echinococcus granulosus* [9 samples], *Schistosoma mansoni* [1 sample], *Schistosoma japonicum* [1 sample], *Candida albicans* [2 samples], *Aspergillus fumigatus* [1 sample], *Cryptococcus capsulatum* [2 samples], and *Mycobacterium tuberculosis* [3 samples]) were provided by the Department of Parasitology and Tropical Medicine and the Pneumology Service of the Hospital Pitié-Salpétrière, Paris, France, and by the Laboratory of Parasitology, Hospital Henri Mondor, Créteil, France. *Paracoccidioides brasiliensis* sera (4 samples) came from the Laboratory of Mycology, Hospital Vargas, Caracas, Venezuela.

Sera from 10 healthy French blood donors without histories of leishmaniasis or trypanosomiasis infection were used as negative controls. All sera were stored at −20°C until required.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Between 1.5×10^8 and 3×10^8 promastigotes, corresponding to 1.2 mg of protein (BCA Chemical Co., Rockford, Ill.), were added to an equal volume of sample buffer consisting of 125 mM Tris HCl (pH 6.8), 6% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.05% bromophenol blue and boiled for 5 min. Samples were immediately submitted to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS, as described by Laemmli (24). Slab gels (1.5 mm thick) were used, with two lanes per comb: a 100-mm lane for the parasite sample and an 8-mm lane for mixed high- and low-molecular-mass protein standards (Pharmacia, Uppsala, Sweden).

Immunoblotting. Transfer of polypeptides from SDS-polyacrylamide gels onto nitrocellulose membranes (0.45 μm pore size; Schleicher & Schuell, Dassel, Germany) was done in a transblotting chamber (Bio-Rad, Richmond, Calif.) at 3 V/cm and 4°C for 12 h in 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol (38). After the transfer, lanes containing molecular mass standards were stained with India ink (16). For immunoblotting, nonspecific sites on 3-mm longitudinal strips were blocked with 5% skimmed milk (15) and 0.3% Tween 20 (4) in 100 mM PBS, pH 7.4, for 1 h at 22°C. After six washes of 15 min each in PBS–0.1% Tween 20, strips were incubated with serum (diluted 1:200 unless stated otherwise) for 2 h at 37°C. After the washes, immune complexes were detected either with a 1:500 dilution of horseradish peroxidase (HRP)-labeled goat anti-human immunoglobulin G (IgG), IgA, and IgM (Biosys SA, Compiègne, France), a 1:5,000 dilution of goat anti-human IgG, a 1:200 dilution of anti-human IgA, a 1:200 dilution of anti-human IgM, or a 1:200 dilution of anti-human IgE (Fc specific) (Nordic, Thilburg, The Netherlands) in washing buffer for 2 h at 22°C. To develop color, nitrocellulose strips were washed and incubated in 0.5 mg of 3,3′-diaminobenzidine per ml (Sigma) and H_2O_2 (3 μl/ml, 30 volumes) in Tris-buffered saline (10 mM Tris HCl [pH 7.4], 150 mM NaCl). The reaction was stopped with distilled water when a background color began to appear. Strips were dried and stored in the dark.

RESULTS

***L. d. infantum* polypeptides recognized by infection sera.** The 15 VL-infected patient sera were tested for IgM, IgG, IgA, and IgE reactivity to SDS-PAGE-separated *L. d. infantum* (LEM497) polypeptides blotted onto nitrocellulose. No

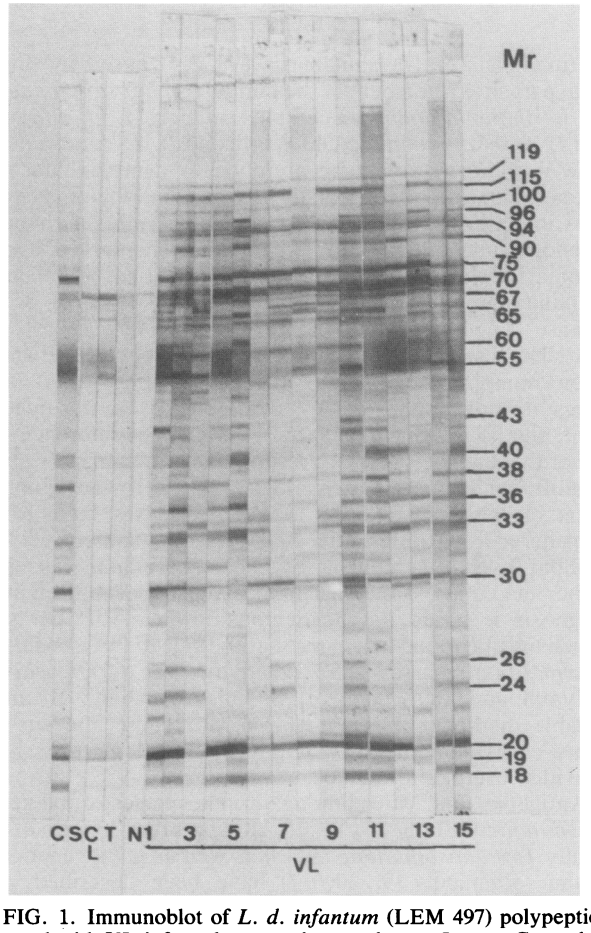

FIG. 1. Immunoblot of *L. d. infantum* (LEM 497) polypeptides reacted with VL-infected sera and control sera. Lanes: C, pool of 8 Chagas' disease sera; S, pool of 11 sleeping sickness patient sera; CL, pool of 10 *L. b. guyanensis* cutaneous leishmaniasis patient sera; T, pool of 5 toxoplasmosis patient sera; N, pool of 10 healthy negative sera; 1 through 15, individual VL Greek patient sera. The gel was 10% polyacrylamide, sera were diluted at 1:200, and HRP-labeled goat anti-human IgG was used as second antibody (1:5,000). Relative molecular masses (Mr) given in kilodaltons.

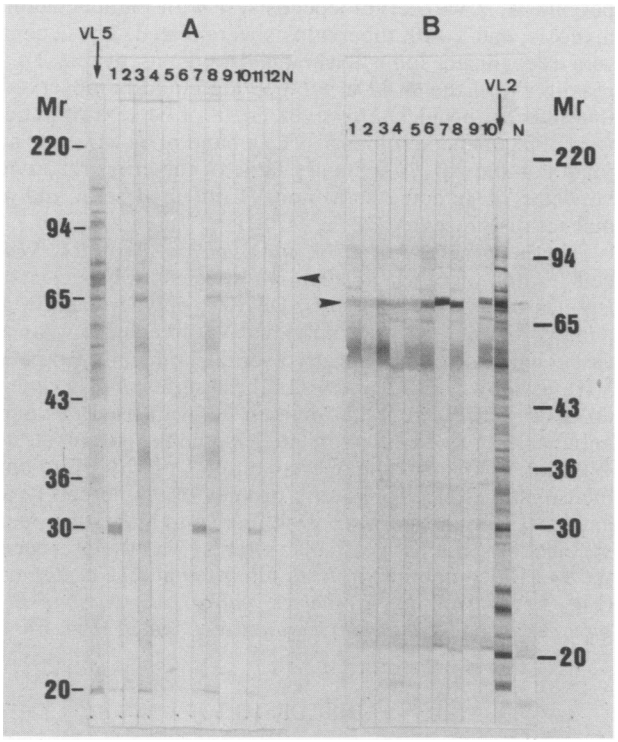

FIG. 2. Immunoblot of *L. d. infantum* (LEM 497) polypeptides reacted with leishmaniasis-infected (but not VL-infected) sera. Lanes: A1 through A12, individual *L. b. braziliensis* patient sera; B1 through B10, individual *L. b. guyanensis* patient sera; VL5 and VL2 are as shown in Fig. 1; N, pooled negative sera. Gel was 10% polyacrylamide, sera were diluted at 1:200, and HRP-labeled goat anti-human IgG was used as second antibody (1:5,000).

IgA or IgE was detected, and only one patient (VL5) had some IgM (not shown).

The response of IgG, the predominant Ig class in VL-infected patient sera, to *L. d. infantum* polypeptides revealed variability of the antigen-binding patterns of the different sera (Fig. 1). Ten bands were common to all VL-infected sera, corresponding to relative molecular masses of 119, 94, 75, 70, 67, 60, 38, 30, 20, and 18 kDa; 13 major polypeptides of 115, 100, 96, 65, 90, 55, 43, 40, 36, 33, 26, 24, and 19 kDa were occasionally identified (lanes 1 through 15). Pooled negative sera could recognize some leishmanial antigens of 70 and 55 kDa (lane N); the same pattern was observed with a pool of 10 healthy Greek sera (not shown). These antigens might be cross-reacting with other infective agents, such as mycobacteria.

Sera from patients with Chagas' disease recognized about 30 antigens with molecular masses ranging between 18 and 119 kDa (lane C). Sera from patients with sleeping sickness (lane S), cutaneous leishmaniasis from *L. b. guyanensis* (lane CL), and toxoplasmosis (lane T) identified a smaller number of components of 55 to 96 kDa.

Selection of antibody for identification of VL-infected sera. For a more complete analysis of cross-reactive antibodies present in control sera, immunoblotting with individual samples was performed.

Sera from 18 *L. tropica*-infected Greek patients did not recognize any *L. d. infantum* (LEM497) antigens, even at a 1:50 dilution (not shown). *L. b. braziliensis*-infected sera primarily identified antigens of 70, 65, 55, 40, 38, 30, and 20 kDa (Fig. 2A, lanes 1 through 12); *L. b. guyanensis*-infected sera reacted with the 70- and 65-kDa antigens and the antigens of the highly dispersed 60- to 55-kDa region (Fig. 2B, lanes 1 through 10). Thus, the 70-kDa (Fig. 2, arrows) and 65-kDa antigens were common major antigens identified by patient sera infected with these two *L. braziliensis* subspecies. The seroreactivity patterns of *L. b. braziliensis* were distinguishable from those of *L. d. infantum* by the absence of immunostaining of the 119-, 115-, 94-, and 36-kDa bands; *L. b. guyanensis*-infected sera differed from *L. d. infantum*-infected sera in the high (above 100-kDa)- and low (below 55-kDa)-molecular-mass regions.

Sera from patients with Chagas' disease strongly reacted with most of the *L. d. infantum* polypeptides recognized during *L. d. infantum* infection, but not with the 94- and 43-kDa polypeptides (Fig. 3A, lanes 1 through 8); sera from African trypanosomiasis patients did not react with the 94-kDa polypeptide (Fig. 3B, lanes 1 through 11), whereas the 43-kDa polypeptide was stained (lanes 2 through 6). Thus, the 94-kDa polypeptide was not identified by sera infected with Chagas' disease or African trypanosomiases. Moreover, neither *L. b. braziliensis*-infected nor *L. b. guyanensis*-infected sera could recognize this antigen, as can be seen in Fig. 2.

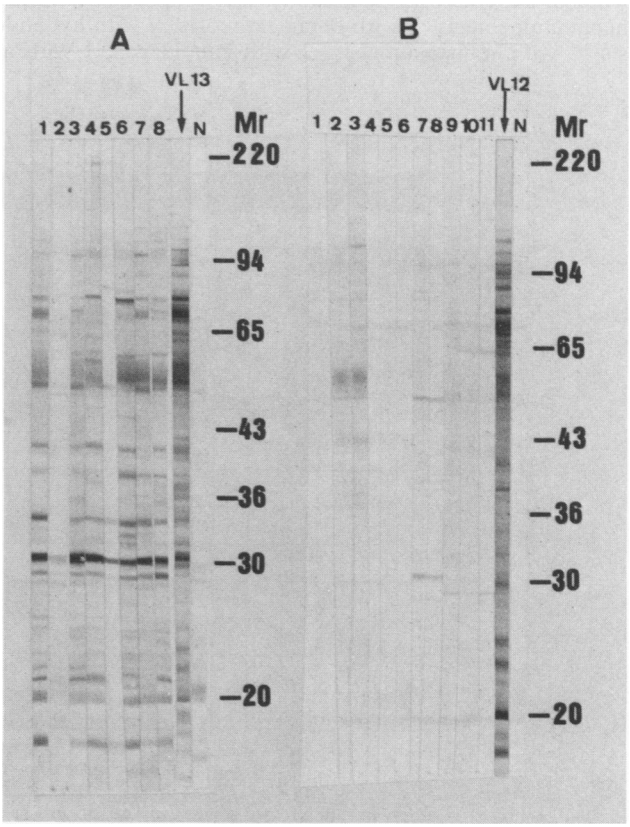

FIG. 3. Immunoblot of *L. d. infantum* (LEM497) polypeptides reacted with sera from patients with protozoal infections other than leishmaniases. Lanes: A1 to A8, individual *T. cruzi* patient sera; B1 to B11, individual *T. gambiense* patient sera; VL13 and VL12 are as shown in Fig. 1; N, pooled negative sera. Gel was 10% polyacrylamide, sera were diluted at 1:200, and HRP-labeled goat anti-human IgG was used as second antibody (1:5,000).

TABLE 1. IgG reactivity of some non-VL-infected sera to *L. d. infantum* (LEM497) polypeptides

Infection	Patient no.[a]	No. of patient sera reacting with following *L. d. infantum* polypeptides[b] (kDa)											
		115	100	96	90	75	70	60	55	40	38	33	30	20
Malaria	16		2	8	1	3	1		3				1	1
Toxoplasmosis	5			1				1					1	
Hepatic amebiosis	3													
Onchocercosis	7								1				1	
Hydatidosis	9			1		1				1				5
Schistosomiasis	2		2						1					1
Candidiasis	2	1	1										2	
Aspergillosis	1													
Cryptococcosis	2								1					
Paracoccidioidomycosis	4		1	2		1	1		1		2	3		3
Tuberculosis	3			1	1		2		2	1			1	

[a] All sera were tested at 1:200 dilution except for tuberculosis sera (1:100). HRP-labeled goat anti-human IgG was used as second antibody (1:5,000).

[b] *L. d. infantum* polypeptides separated by 10% polyacrylamide gels and blotted onto nitrocellulose membrane as antigens.

In order to determine whether anti-94-kDa-polypeptide antibodies were restricted to *L. d. infantum*-infected patient sera, LEM497 blots were tested with more individual control sera and sera from patients suspected of having VL. Sera from 16 patients with malaria, 5 with toxoplasmosis, 3 with hepatic amebiasis, 7 with onchocerocosis, 9 with hydatidosis, 2 with schistosomiasis, 2 with candidiasis, 1 with aspergillosis, 2 with cryptococcosis, 4 with paracoccidioidomycosis, and 3 with tuberculosis were tested. These control sera recognized some leishmanial antigens (Table 1). No reaction with the 94-kDa polypeptide could be observed in the different profiles. However, in 18 of 24 sera of patients with clinical and serological VL, a band of 94 kDa was seen (data not shown). A summary table of the cross-reactivities between VL-infected sera, non-VL-infected sera, and normal sera is presented in Fig. 4.

Species specificity of *L. d. infantum* polypeptides. Whole cell lysates of other *Leishmania* species, subspecies, and isolates were separated by SDS-10% polyacrylamide gels and immunoblotted with individual VL-infected sera as well as pooled control and negative sera. The results for patient VL15 is shown (Fig. 5 and 6). In these series of experiments, an HRP-labeled goat anti-human Ig was used. Common antigens were identified in all leishmanial strains tested. With immunoblot technique conditions and by using unidimensional gel electrophoretic profiles of whole cell lysates and total immune sera, we were unable to find (sub)species-specific antigens. Of particular interest was the presence of the 94-kDa component in *L. d. infantum* and *L. d. donovani* (Fig. 5) as well as in the *L. major*, *L. tropica*, *L. b. guyanensis* and *L. m. mexicana* strains tested (Fig. 6).

DISCUSSION

In this study, we have examined in immunoblots the specificity of the serum antibodies during *L. d. infantum*

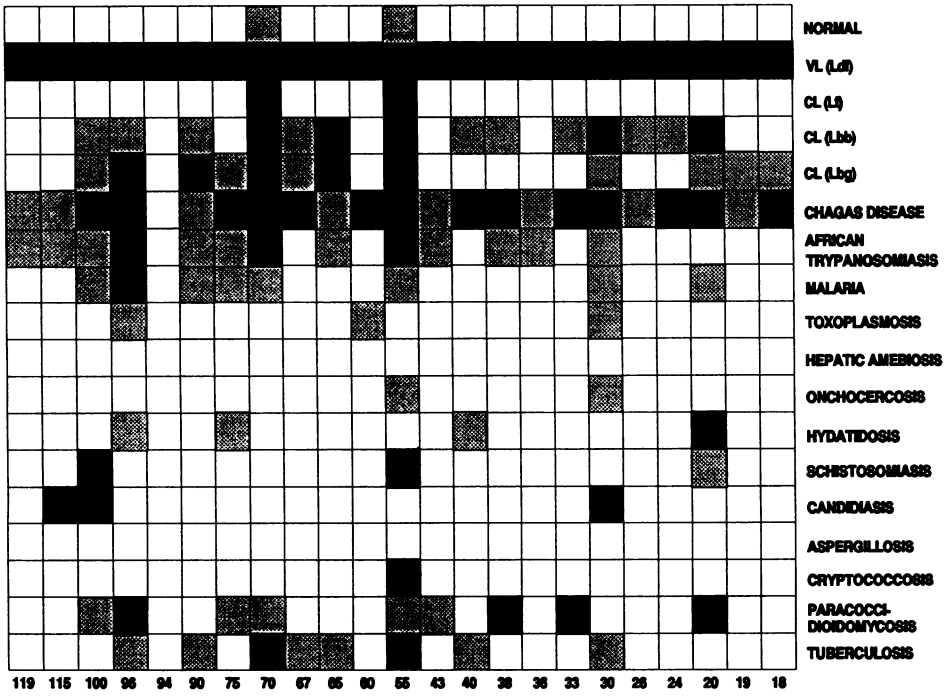

FIG. 4. Representation of the cross-reactivities between VL-infected (15 sera), non-VL-infected (113 sera), and normal (pool of 10 healthy individuals) sera for 23 *L. d. infantum* (LEM497) polypeptides. The frequency of recognition by a kind of sera for a particular antigen is ≥50% (■), <50% (▨), or 0% (□). CL, cutaneous leishmaniasis; Lbb, *L. b. braziliensis*; Lbg, *L. b. guyanensis*. All sera tested at 1:200 dilution, except *M. tuberculosis* sera at 1:100 and *L. tropica* sera at 1:50.

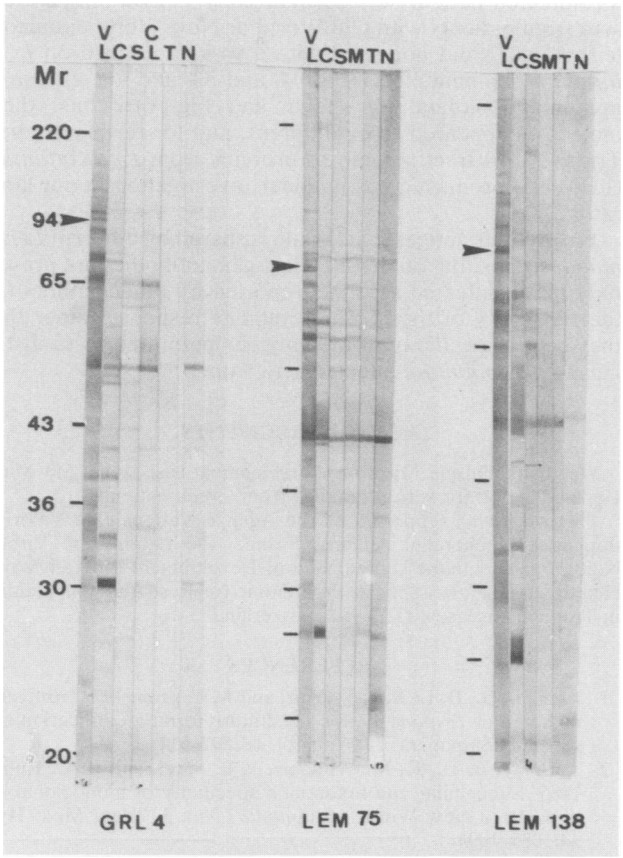

FIG. 5. Immunoblot of polypeptides from different L. donovani subspecies reacted with VL-infected and control sera. Lanes: VL, individual VL15 patient sera, as in Fig. 1; C, pool of 8 Chagas' disease patient sera; S, pool of 11 sleeping sickness patient sera; CL, pool of 10 *L. b. guyanensis* cutaneous leishmaniasis patient sera; M, pool of 12 malaria patient sera; T, pool of 5 toxoplasmosis patient sera. Gel was 10% polyacrylamide, sera were diluted at 1:200, and HRP-labeled goat anti-human Ig(G,A,M) was used as second antibody (1:5,000).

infection by using as antigens promastigote polypeptides separated electrophoretically by SDS-PAGE.

Blot analyses showed that IgG from negative pooled sera recognized the 70- and 55-kDa antigens, and the same pattern was obtained with IgM (not shown); this recognition may be related to the antibody-dependent lethal effect of normal human sera on *L. d. chagasi* promastigotes, observed in vitro (32). IgG of VL-infected sera reacted variably with the majority of *L. d. infantum* polypeptides, probably reflecting differences in genetic background (in particular human leukocyte antigen phenotypes) and/or disease stages of the patients. This antibody heterogeneity could explain the presence of "nonspecific" arches obtained in the counterimmunoelectrophoresis diagnostic test. Immunoblotting seems more sensitive than indirect immunofluorescence assay, since bands can be visualized with sera diluted 1:400 and sometimes even >1:25,600 (depending of the bands) (data not shown), demonstrating high levels of *Leishmania*-specific IgG antibodies in the infection.

Cross-reactivities between leishmaniases and trypanosomiases have been shown by immunoblotting (11, 33). The present study confirms and extends these observations. Several antigens have been described which discriminate between the humoral immune response in *T. cruzi* and *Leishmania* infections. Dos Santos et al. (11) identified two *L. d. chagasi* polypeptides of 123 and 119 kDa, expressed in different strains of *Leishmania* spp., reacting with all VL-infected but not *T. cruzi*-infected patient sera. Reed et al. (33) reported that *L. d. chagasi* antigens in the 62- to 66-kDa region were recognized by sera of all individuals with *L. d. chagasi* or *L. m. amazonensis* infections but not by sera of individuals in the other disease groups or by control sera. In our study with *L. d. infantum* polypeptides, three components of 119, 115, and 65 kDa could discriminate between leishmaniasis- and trypanosomiasis-infected sera; moreover, immunoblots could distinguish among sera infected with *L. d. infantum*, *L. b. braziliensis*, and *L. b. guyanensis*, since antigens of 119, 115, 94, 60, 43, and 36 kDa were not recognized by sera infected with *L. b. braziliensis* or *L. b. guyanensis* (Fig. 2 and 4). Similarly, low-molecular-mass *L. d. chagasi* antigens were not immunostained by the New World cutaneous leishmaniasis-infected sera studied by Dos Santos et al. (11), and the 32- to 35-kDa region, which corresponds to the 36-kDa component described here, has been shown by Reed et al. to distinguish between *L. m. amazonensis* and *L. d. chagasi* infections (33).

In previous studies, a major surface glycoprotein, termed gp63, was identified on several *Leishmania* species (6, 8, 12, 13, 26). gp63 has been described as being important in cell-cell (host-parasite) interaction (35), cell infectivity (23, 40), specific diagnosis (18), and immunoprotection (34, 42). Sera infected with cutaneous, mucocutaneous, or visceral leishmaniasis also identified this molecule (8, 11, 18, 25, 26, 33). In our study, the *L. d. infantum* 67- to 60-kDa region included five to six distinct bands, which were recognized by

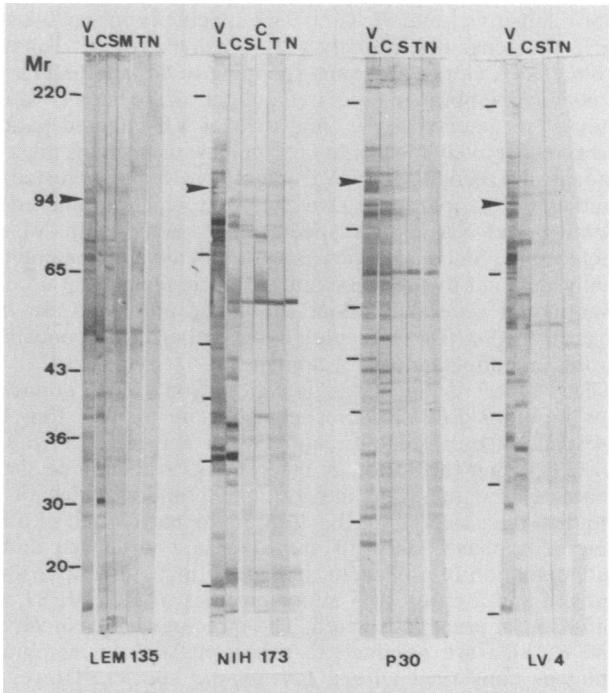

FIG. 6. Immunoblot of polypeptides from different *Leishmania* species (other than *L. donovani*) reacted with VL-infected and control sera. Conditions are the same as described in the legend to Fig. 5.

L. d. infantum-infected patient sera; sera infected with *L. b. guyanensis* or *L. b. braziliensis* reacted with several bands which have the same electrophoretic mobilities as those recognized by VL-infected sera. However, some of these antigens were identified by patient sera infected with *T. cruzi*, *T. gambiense*, and *M. tuberculosis* (Fig. 3 and 4). Slight reactivity in the 65- to 68-kDa region of *L. d. chagasi* lysates have been seen with sera infected with Chagas' disease and tuberculosis (33). This region is heterogeneous; indeed, technical approaches, including cell-surface radiolabeling, detergent extraction, and immunoprecipitation with immune sera or monoclonal antibodies, demonstrated that the surface protein of *L. d. infantum* promastigotes are different from those of the two other subspecies of the *L. donovani* complex, *L. d. donovani* and *L. d. chagasi*, which are very similar. *L. d. infantum* had a 62-kDa membrane component recognized neither by *L. d. infantum*-infected human sera nor by murine antibodies against the surface of *L. d. donovani* or *L. d. chagasi* (25).

In this study, a 94-kDa antigenic component was recognized by 100% of sera from clinically and parasitologically confirmed VL patients and by 75% of sera from suspected VL cases. It was never identified by 113 control sera tested (100% specificity), corresponding to 14 different non-VL infections, and no false positives were observed. Recently, a number of studies have been carried out to select *L. donovani* antigens and develop assays for VL serodiagnosis. A competitive-inhibition ELISA has been described, employing VL-infected human sera and *L. donovani*-specific monoclonal antibodies directed against two membrane antigens termed gp70-2 and dp72 (20). Studies using crude parasite antigens demonstrated that >90% of VL patients produce antibodies to these proteins, and no false positives were observed; by using purified antigens, >93% were correctly diagnosed as VL, with less than 4% false positive (21). A direct dot blot assay has been developed that has 90 and 100% sensitivity and 98.8 and 93% specificity for gp70-2 and dp72 antigens, respectively (22). Fusion proteins isolated from cDNA clones and corresponding to 60- and 63-kDa *L. donovani* membrane-associated antigens have been isolated; they were detected by 95 and 94% of VL-infected patient sera, respectively (5, 18). In fact, numerous proteins must be specifically recognized by VL-infected sera, as suggested by studies of Sheppard and Dwyer (36) in which a hundred *L. donovani* cDNA clones specifically reacted with VL-infected sera. Since not every patient will always immunologically respond to the same antigen(s), it seems that a combination of several of them should be employed. In this regard, the 94-kDa molecule described here may contribute to the immunodiagnosis of human VL.

This 94-kDa antigen was present not only in *L. donovani* but in other *Leishmania* species lysates, suggesting the presence of cross-reactive antigens or epitopes. Murray et al. (29), using the Triton X-114 separation technique, have characterized the total integral membrane proteins of *L. major* promastigotes; of the 40 proteins partitioned into the detergent phase, only 10 are externally oriented, and a subpopulation of 6 of them, including the abundant gp58-63 antigen and a group of 5 minor proteins of 105, 94, 89, 81, and 42 kDa, are glycosylated. The three molecules of 94, 89, and 81 kDa are assumed to represent surface membrane antigens conserved among *Leishmania* species. However, the 94-kDa antigen was not recognized by a pool of sera from patients with VL caused by *L. d. chagasi*, but it was identified by sera from patients with cutaneous leishmaniasis. These results are different from those described here; VL-infected patient sera recognized a 94-kDa antigen, but sera from patients with Old World or New World cutaneous leishmaniasis did not react with a 94-kDa antigen on *L. d. infantum* immunoblots (Fig. 4 and 6) and on *L. major* immunoblots (data not shown and Fig. 6). Thus, these molecules appeared to be different, and for further studies, Triton X-114 fractionation of proteins from *L. d. infantum* (LEM497) promastigotes is under investigation in our laboratory.

Finally, this antigenic molecule is shared by different *Leishmania* strains, but anti-94-kDa-antigen antibodies are present only in VL-infected sera. As reported by other authors (5), the specificity of the humoral immune response rather than the species specificity of the antigens permitted us to distinguish *L. d. infantum*-infected sera from other sera.

ACKNOWLEDGMENTS

We thank Fatima Mansrour for technical assistance and Maria Hadziandoniou for serum samples from Greek patients.

This work was supported by the Agence Nationale de Valorisation de la Recherche, Amiens, France, the Laboratoire Virbac, Nice, France, and the United Nations Development Program/World Health Organization/Special Programme for Research and Training in Tropical Diseases, Geneva, Switzerland.

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