Proteomic Approach for Characterization of Immunodominant Membrane-Associated 30- to 36-Kilodalton Fraction Antigens of *Leishmania infantum* Promastigotes, Reacting with Sera from Mediterranean Visceral Leishmaniasis Patients

Sayda Kamoun-Essghaier,1 Ikram Guizani,2 Jean Marc Strub,3 Alain Van Dorsselera,3 Kamel Mabrouk,4 Lazhar Ouelhazi,5 and Koussay Dellagi1*

*Laboratoire d’Immunologie, Vaccinologie et Génétique Moléculaire,1 and Laboratoire d’Épidémiologie et d’Ecologie Parasitaire,2 Institut Pasteur de Tunis, and Laboratoire de Biochimie Végétale et Symbiotes, Institut National de la Recherche Scientifique et Technique, Hammam-Lif,3 Tunis, Tunisia, and Laboratoire de Spectrométrie de Masse Bio-Organique (UMR 7509), Strasbourg,4 and Laboratoire de Biochimie CNRS (UMR 6560 and LIA Ingenierie Biomoléculaire), Faculté de Médecine Nord, Marseille,5 France

Received 15 January 2004/Returned for modification 4 May 2004/Accepted 6 October 2004

The aim of the present study was to identify and characterize proteins of a 30- to 36-kDa fraction of *Leishmania infantum* promastigote membranes previously shown to be an immunodominant antigen(s) in Mediterranean visceral leishmaniasis (MVL) and a consistent and reliable serological marker of this disease. By the first approach, Coomassie-stained protein bands (32- and 33-kDa fractions) that specifically reacted by immunoblotting with sera from MVL patients were excised from the gel and submitted to enzymatic digestion to generate peptides. Four peptides were sequenced, three of which were shown to be definitely associated with MVL-reactive antigens and ascribed to a mitochondrial integral ADP-ATP carrier protein from *L. major*, a putative NADH cytochrome b reductase, and a putative mitochondrial carrier protein, respectively. The second approach combined two-dimensional gel electrophoresis of membrane antigens and mass spectrometry (liquid chromatography-mass spectrometry/mass spectrometry) by using a quadrupole time-of-flight analysis. Six immunoreactive spots that resolved within a molecular mass range of 30 to 36 kDa and a pH range of 6.7 to 7.4 corresponded to four *Leishmania* products. The sequences derived from two spots were ascribed to a beta subunit-like guanine nucleotide binding protein, known as the activated protein kinase C receptor homolog antigen LACK, and to a probable member of the aldehyde reductase family. One spot was identified as a probable ubiquinol-cytochrome c reductase (EC 1.10.2.2) Rieske iron-sulfur protein precursor. The remaining three spots were identified as truncated forms of elongation factor 1α. These antigens correspond to conserved proteins ubiquitously expressed in eukaryotic cells and represent potential candidates for the design of a reliable tool for the diagnosis of this disease.

Trypanosomatid protozoans belonging to the genus *Leishmania* are obligate parasites of mammalian macrophages. The life cycle of these organisms goes through two morphologically different stages: the amastigote, which is found in the parasitophorous vacuoles of host macrophages and dendritic cells, and the promastigote, which is an extracellular flagellated form found in the gut of the sandfly vector. At least, 15 *Leishmania* species are infectious for humans and cause a wide spectrum of diseases, including cutaneous, mucocutaneous, and visceral leishmaniasis, as well as asymptomatic infections. Intermediate forms may be encountered, and the same parasite species may cause different forms of disease. Leishmaniasis are prevalent on four continents, and the World Health Organization considers leishmaniasis to be among the major infectious diseases in the world. In 1990, the World Health Organization estimated that ~350 million people were at risk of acquiring leishmaniasis and that 12 million people were infected (1). In Tunisia, as in other Mediterranean countries, several forms of leishmaniasis coexist. Among these is Mediterranean visceral leishmaniasis (MVL), which is caused by *Leishmania infantum*. MVL, also known as infantile kala azar, is a severe systemic disease which mostly affects children under the age of 5 years and which is constantly fatal if it is not rapidly diagnosed and treated. Patients with MVL usually present clinically with a combination of prolonged fever, hepatosplenomegaly, anemia, and leukopenia (5). It is characterized by high titers of both nonspecific and specific antibodies (10). Early diagnosis is of great importance for effective treatment of this potentially fatal disease. The diagnosis of MVL is based on the demonstration of amastigotes in Giemsa-stained smears of bone marrow aspirates or specimens obtained by needle puncture of the spleen (12, 44) and by growth of the parasites on Novy, McNeal, and Nicolle medium. The major drawbacks of these two classical diagnostic tests are their weak sensitivities.

Several serological tests are of diagnostic value. Among these are the indirect immunofluorescence assay (4), the direct agglutination test (19), enzyme-linked immunosorbent assays (ELISAs) with whole parasites or crude antigens (3, 13, 23), or immunoblot analysis (13, 22, 54, 61). These methods proved to
be more sensitive than the existing invasive techniques for the diagnosis of MVL. One drawback of serological assays with whole parasites is the existence of cross-reactivity with other pathogens, including Trypanosoma cruzi, mycobacteria, malaria parasites, or amoebae, which are coendemic with Leishmania in many parts of the world (7, 51). The performance of serodiagnostic assays could be improved by using purified or recombinant leishmanial antigens, such as gp63 (40, 41, 56), Hsp70 (30, 48), p94 (53), gp70 and p72 (24), p32 (61), rK39 (2, 11), r gene B protein (rGBP) (15, 31), H2A and H2B (31, 57, 58, 59), rLACK (31), and the promastigote surface antigen 2 (rPSA-2) (18, 31, 37), or synthetic peptides (14, 48) and antigens from promastigote-conditioned media (33).

Previous work in our laboratory has characterized a 32-kDa fraction (P32) of L. infantum promastigote membranes which consistently reacts on Western blots with sera from MVL patients but not with sera from patients with zoonotic cutaneous leishmaniasis (ZCL) (61). Interestingly, the P32 antigen(s) did not react with sera from patients with other infectious diseases, such as toxoplasmosis, chancroidosis, and tuberculosis. When the P32 band was electroeluted and used in an ELISA, the assay had good performance in terms of specificity and sensitivity (94% each) and showed some cross-reactivity only with sera from patients with Chagas’ disease. Only 1.4% false-positive results were observed when P32 was used, whereas 19 and 7.3% false-positive results were observed when crude membrane and soluble antigens were used, respectively. Moreover, the antibody response to the P32 antigen appeared to be specific with samples from patients with overt disease compared to the specificity of the response with samples from asymptomatic subjects.

These results stressed the usefulness of this antigenic fraction for the diagnosis of visceral leishmaniasis in the Mediterranean region and Asia, where trypanosomiases are absent, and prompted us to characterize the polypeptides composing the P32 fraction using biochemical and biophysical approaches.

MATERIALS AND METHODS

Sera. Nine serum samples from MVL patients that strongly reacted with P32 were pooled in equal ratios (by volume) and are designated the MVL serum pool, which was used in this study as the positive test serum sample. Ten serum samples from ZCL patients unreactive with P32 were also selected and were pooled for use as negative serum controls (ZCL serum pool).

Parasites. The antigens used in the study were prepared from an isolate (MHOM/TN87/KA412; Zymodeme MON-1) from a Tunisian patient with MVL. Promastigotes were grown at 26°C in RPMI 1640 medium (Sigma, St. Louis, Mo.). The lysate was maintained on ice for 20 min. The cell suspension was further disrupted with a Dounce homogenizer (60 strokes of pestle A; Kontes, Vineland, N.J.) and four cycles of sonification (Vibra Cell sonicator; Sonics & Materials Inc., Danbury, Conn.) of 10 s each. A first centrifugation was performed at 1,500 × g for 10 min at 4°C to remove unbroken parasites and nuclei. The supernatant (S1) containing the protein extracts was kept in ice. The pellet (P1) was resuspended in 2 ml of LBI and was then subjected to a second set of separations and centrifugation at 1,500 × g. The final pellet was discarded, and the supernatants (S1, etc.) were pooled (total antigens) and centrifuged at 10,000 × g for 30 min at 4°C to generate a supernatant (soluble antigens) and a pellet fraction (MBAs). The P1 supernatant, which contained the crude MBAs, was resuspended in 5 ml of Tris-buffered saline (10 mM Tris-HCl [pH 7.4], 150 mM NaCl) supplemented with protease inhibitors, as described above. Aliquots (0.5 mg/ml) of the crude MBA preparation were conserved at −80°C until use.

To obtain integral MBAs, one aliquot (500 μg) of crude MBAs was centrifuged at 2,000 × g for 10 min at 4°C. The pellet was resuspended in 1 ml of 10 mM Tris-HCl (pH 11)+2 mM EDTA–30% sucrose and was then incubated at 4°C under agitation for 1 h. After one freeze-thaw cycle, the L. infantum integral membranes were purified by ultracentrifugation at 90,000 × g for 4 h at 4°C in a Beckman L7 ultracentrifuge with an SW28.1 rotor. The peripheral MBAs were collected in the supernatant (S0.0001), and the integral MBAs (IMAs) were sedimented in the pellet (P0.0001). Protein yields were estimated by the Bradford assay (9), and the fractions obtained at two different centrifugation steps (at 50,000 and 90,000 × g) were analyzed by immunoblotting of one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gels with the MVL serum pool.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed under denaturing and reducing conditions with a 15% acrylamide–3% bisacrylamide gel, as described by Laemmli (28). Two vertical electrophoresis systems were used: (i) a mini-system (8 by 6.5 by 1 mm thick; Mini Protein II; Bio-Rad Laboratories, Richmond, Calif.) for analysis purposes and (ii) a standard-size electrophoresis apparatus (14 by 16 cm by 1.5 mm thick; LKB Instruments) for preparative purposes. The gels were stained with Coomassie blue Gold (0.25% [wt/vol]; Fluka, Lyon, France) or electroblotted.

Molecular mass markers. The molecular mass standards (Bio-Rad Laboratories) used were phosphorylase b (97,400 kDa), bovine serum albumin (66,200 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21,500 kDa), and lycosyme (14,400 kDa).

Immunoblotting. Once the molecular mass–dimensional gel electrophoresis (2DE) was finalized, the polyacrylamide gels were electrodified onto 0.45-μm pore-size polyvinylidene difluoride membranes (Amersham, Les Ulis, France) for 2 h and 30 min at 50 V and 4°C by using 10 mM 3-(cyclohexylaminol)-1-propanesulfonic acid (pH 11) containing 10% methanol as the transfer buffer, as described by Matsudaira (34), without any membrane staining. The membranes were then incubated for 2 h at room temperature under agitation in 1× phosphate-buffered saline (PBS)–1% Tween 20–5% milk for 1 h. The membranes were then washed in PBS-Tween 0.01% Tween 20 (wt/vol), respectively. The enzyme/protein ratios were those recommended by the supplier. The two mixtures were cut apart from six preparative SDS-polyacrylamide gels and were then incubated for 1 h at room temperature under agitation in 1× phosphate-buffered saline (PBS)–1% Tween 20–5% milk for 1 h. The membranes were then relaxed in 1× phosphate-buffered saline (PBS)–1% Tween 20 for 1 h at room temperature. After three washes in PBS-Tween, the different antigen–antibody complexes were detected after incubation with peroxidase-conjugated anti-human sheep or anti-rabbit donkey immunoglobulin (Amersham Life Science International plc, Little Chalfont, United Kingdom) diluted 1/1,000 or 1/2,000 in PBS-Tween, respectively. The peroxidase enzymatic activity was revealed by incubation in 0.05% 3,3′-diaminobenzidine (Sigma)-0.03% H2O2 in 50 mM Tris-HCl (pH 7.6). In the case of 2DE, the proteins were detected by an enhanced chemiluminescence assay, according to the instructions of the supplier (Amersham, Les Ulis, France), at different exposure times.

Detergent solubilization of the antigenic P32 fraction. To further determine the nature of the association between antigenic P32 fraction with the membranes and their association after treatment at pH 11, equal aliquots of the P0.0001 pellet (100 μg) were resuspended in 1 ml of PBS (pH 7.2) containing one of the following detergents: 1% Triton X-100 (TX-100), 1% Tween 20–5% milk, 1% digitonin (Dig), 1% 3-cholamidopropyl(dimethylamino)·1-propanesulfonate (CHAPS), 1% sodium cholate, 1% octyl-β-D-glucopyranoside (OG), or 0.5% lauryldimethylamine oxide (LDAO). All samples were incubated for 1 h at 4°C under vigorous agitation and were centrifuged at 90,000 × g in a Beckman L7 ultracentrifuge with an SW41.1 rotor for 1 h at 4°C. The supernatants were submitted to electrophoresis and were subsequently immunoblotted.

Peptide synthesis. The two prominent bands (P32 and P33) that were revealed by Coomassie blue staining and that were reactive with the MVL serum pool but not the ZCL serum pool were cut apart from six preparative SDS-polyacrylamide gels and digested with sequencing-grade lysine C protease (Boehringer Mannheim) and trypsin (Sigma) in 100 mM Tris-HCl (pH 8.8) with 0.003% SDS or 0.01% Tween 20 (wt/vol), respectively. The enzyme/protein ratios were those recommended by the supplier. The two mixtures were incubated for 18 h at 30°C. The peptides generated from the P32 and P33 bands were purified on a C18-DEAE high-pressure liquid chromatography (HPLC) column by using linear gradients of 2 to 35% and 2 to 45% acetonitrile in 0.1% trifluoroacetic acid over 40 min, respectively. Four different peptides (peptides P1, P2, P3, and P4), which contained the sequences 12, 15, 12, and 9 residues derived from the enzymatic digestion of the P32 and P33 bands, respectively, were selected for amino acid sequencing on the basis of their peak homogeneities and prominence. The peptides were sequenced with an ABI 473 apparatus (Applied Biosystems, Foster City, Calif.). The four peptides sequenced were then chemically assembled by the solid-phase method.
The gel digestion procedure was carried out as described by Rabilloud et al. (49). Digestion and analysis by liquid chromatography (LC)-mass spectrometry (MS). From the gels and stored in 100 μl of HPLC-grade water at 4°C until subsequent acidolysis, and (iii) mass determination by matrix-assisted laser desorption ionization–time of flight mass spectrometry.

Peptide-KLH conjugation and rabbit immunization. The purified peptides were conjugated to keyhole limpet hemocyanin (KLH; Sigma) through an N-terminal α-amino group by using a homobifunctional reagent, glutaraldehyde (Sigma), by the procedure described by Pfaff et al. (45). To conjugate peptides (250 μg/0.5 ml) were mixed with complete Freund’s adjuvant at a 1:1 ratio and were inoculated intradermally into rabbits. Three booster doses were administered at days 30, 60, and 90 by subcutaneous injection of the antigen admixed with incomplete Freund’s adjuvant (by volume). The rabbits were bled before immunization and 10 days after each boost. Antibody titers were determined by ELISA.

ELISA. Rabbit sera with antibodies to the P32- or P33-derived synthetic peptides and the MVL serum pool were checked for their ability to react with the immunizing peptides by enzyme-linked immunosorbent assay (ELISA) as described by Voller et al. (62) and Pfaff et al. (45), with some modifications. Polyacrylamide gel plates (Nunc) were coated overnight at 4°C for 2 or 2 h at 37°C with 50 μl of synthetic peptides (10 μg/ml) diluted in PBS (10 mM; pH 7.4). Excess coating buffer was flicked off, and nonspecific binding sites were blocked with 5% skim milk in PBS containing 0.1% Tween 20 (PBS-T-M) for 1 h at 37°C. After three washes with 0.1% Tween 20 in PBS (PBS-T), the plates were incubated for 2 h at 37°C in the presence of 50 μl of antibody (antiserum taken from the different rabbit sera (dilutions from 1/100 to 1/100)) or the MVL serum pool (diluted 1/100 in PBS-T-M). Unbound antibodies were washed off five times, as described above, and peroxidase-conjugated donkey anti-rabbit or sheep anti-human immunoglobulin (Amersham Life Science International plc, Little Chalfont, United Kingdom) diluted 1/2,000 and 1/1,000 in PBS-T, respectively, were added, followed by incubation for 1 h at 37°C. Unbound conjugate was washed off six times, and then 100 μl of orthophenylenediamine (1 mg/ml [w/v]; Sigma) dissolved in citrate buffer (100 mM; pH 5.0) containing 0.03% (v/v) hydrogen peroxide was added. The plates were incubated for 20 min at room temperature in the dark, and the reactions were stopped by the addition of 50 μl of a 4 N sulfuric acid solution to each well. The optical density at 492 nm was measured in an ELISA reader (Multiskan; Tintek, Helsinki, Finland), and the titers were determined when necessary.

2DE. Nonequilibrium pH gradient electrophoresis (NEPHGE)—SDS-PAGE (2DE) was performed as described by O’Farrell et al. (39), with minor modifications (43). Briefly, the proteins were separated in the first dimension by using ampholytes at two ratios, 1:1.2 and 1:0.3. The run was carried out in the opposite direction compared to that used for typical isoelectric focusing. Phosphoric acid (0.011 M) was placed in the upper chamber, and NaOH (0.1 M) solution was placed in the lower one. The connections to the power supply were also reversed. The optimized migration conditions corresponded to 200 V for 30 min and then 300 V for 30 min and 400 V for 6 h at 20°C. Separation in the second dimension (SDS-PAGE) was performed as described above.

The protein concentration was evaluated by the Bradford protein assay (9), as modified by Ramagli and Rodríguez (50).

Analysis of 2DE polypeptide patterns. For the sake of reproducibility between successive migrations, equivalent amounts of the L. infantum promastigote MVBs were run under identical conditions. The immunoblots revealed by the MVL serum pool were compared to the patterns on Coomassie blue-stained gels by visual comparison of the relative intensities and positions of the spots corresponing to each polypeptide. The immunodominant antigens corresponding to abundant spots were identified on the gels and were considered for further analysis. Similarly, immunoblots of MBAs separated in a narrow pH range (at pH 3 to 10, at pH 5 to 7, and at pH 6 to 8) by NEPHGE–SDS-PAGE, which revealed polyclonal rabbit anti-P1 peptide, anti-P2 peptide, or anti-P3 peptide serum, were compared to the immunoblots revealed by the MVL serum pool.

Protein identification by LC-MS/MS. MBAs were separated by NEPHGE–SDS-PAGE as described above. Six preparative gels were stained with Coomasie blue. Visible polypeptides of interest in the 30- to 36-kDa region were excised from the gels and stored in 100 μl of HPLC-grade water at 4°C until subsequent digestion and analysis by liquid chromatography (LC)-mass spectrometry (MS). The gel digestion procedure was carried out as described by Rabilloud et al. (49), MS. The MS and MS/MS mass measurements were performed with a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF 2; Micromass Ltd., Manchester, United Kingdom) equipped with a z-spray ion source and the liquid junction. The instrument consists of an electrospray ionization source, a quadrupole mass filter operating as a variable band-pass device, a hexapole collision cell, and an orthogonal acceleration time-of-flight mass analyzer. The time-of-flight mass analyzer is used to acquire data in both the MS and the MS/MS modes.

Data processing and data analysis. Processing of the LC-MS/MS data was done automatically with the ProteinLynx process module (Micromass Ltd.). Data analysis was done with Global Server (Micromass, Ltd.) and Mascot (Matrix Science Ltd., London, United Kingdom) software by comparison with the data in the National Center for Biotechnology Information (NCBI) nonredundant database.

Nano-LC-MS/MS. A CapLC system (Micromass Ltd.) was used for sample injection and preconcentration. Sample preconcentration and desalting were done on a precolumn cartridge packed with a 5-μm 100-Å C18 PepMap stationary phase (LC-Packings); the cartridge had a length of 1 mm and an inner diameter of 300 μm, and the flow rate was 30 μl/min for 3 min. The loading solvent for sample preconcentration and cleanup consisted of 0.1% formic acid in water. After cleanup, the preconcentration system was switched (Stream Select) and the column was placed in-line with the analytical column. The bound peptides were backflushed for elution from the precolumn onto the analytical column. The peptides were separated on a column (15 cm by 75 μm [inner diameter]) packed with 3-μm 100-Å C18 PepMap (LC-Packings) stationary phase. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Elution was performed at a flow rate of 200 nl/min with a 5 to 45% gradient of solvent B over 35 min, followed by elution with 95% solvent B for 5 min, and the column was reequilibrated for 20 min with 100% solvent A.

Bioinformatics analyses. The sequences generated in this study were submitted to searches for homologies with other sequences by using the BLAST servers of NCBI and the Leishmania Friedlin Genome Project (www.sanger.ac.uk/projects/L_major). Alignments were generated with the Clustal W program (www.ebi.ac.uk/clustalw). Data relating to Leishmania genes or products were extracted from the L. major Gene Database (www.genedb.org/geneb/Leish/index.jsp) or from the NCBI and Swiss-Prot TrEMBL databases (www.ncbi.nlm.nih.gov and www.uniprot.org).

RESULTS

Characterization of serum pool used in our study. In previous studies we have shown, using Western blots of L. infantum parasite MBAs, that a P32 antigen(s) is recognized by 95% of serum samples from patients with MVL but not by sera from patients with ZCL. In order to characterize this antigenic fraction further, we selected 9 serum samples from MVL patients and 10 serum samples from ZCL patients. They were characterized individually for their reactivities to the Leishmania MBAs and were then pooled and tested. All sera from patients with MVL reacted with bands at 32 and 33 kDa and to a lesser extent with bands at 30 kDa (seven of nine samples) and 36 kDa (four of nine samples). The reactivity of the MVL serum pool was representative of those of the individual serum samples (Fig. 1, lane a). As expected, the 10 serum samples from patients with ZCL and the ZCL serum pool did not react with bands in the 30- to 36-kDa region; this pool of sera was considered the negative control in this study (Fig. 1, lane b). Given the hypothesis that the antigenic fraction of interest is constituted by different proteins, at this step the MVL serum pool was considered a good tool for analysis of the reactivities of sera from different patients by using the laborious techniques of this study.
Enrichment and solubilization of membrane-associated 30- to 36-kDa *L. infantum* antigens. The first step aimed at determining the association of the antigens to cell membranes. In order to enrich the crude membrane preparations for the 30- to 36-kDa antigens recognized by the MVL serum pool and to solubilize them, MBA extracts were submitted to different treatments and were analyzed by one-dimensional SDS-PAGE and immunoblotting. In order to remove peripheral proteins, the MBAs were first treated at pH 11. As shown in Fig. 2, alkaline treatment did not extract the bulk of reactive antigens but did partially solubilize P36, suggesting that most reactive components could be considered integral membrane proteins (Fig. 2A). The insoluble fraction obtained after incubation at pH 11 was further treated with one of the following detergents: LDAO (0.5%), sodium cholate (1%), OG (1%), Dig (1%), TX-100 (1%), or CHAPS (1%). As illustrated in Fig. 2B, only LDAO partially solubilized the P32 antigen(s).

These results indicate that the P32 band has a heterogeneous composition and that some of the antigens in the area of 30 to 36 kDa either are integral proteins or are strongly complexed to the membranes. Furthermore, one could not exclude the possibility that isoforms within each individual band migrated at a given electrophoretic position. The difficulties in enriching the samples for these antigens or solubilizing them...
led us to use alternative approaches to characterize the 30- to 36-kDa antigens.

Microsequencing analysis of the 32- and 33-kDa bands. The 32- and 33-kDa protein bands resolved on SDS-polyacrylamide gels were stained with Coomassie blue and were then cut out from the gel and digested with lysine C protease and trypsin, respectively. Four peptides, peptides P1 and P2 from the 32-kDa band and peptides P3 and P4 from the 33-kDa band, were selected for sequencing. The peptide sequences were as follows: P1, KLLVQNQGEMIK; P2, KAPSEWWMGGVM/GFVNK; P3, KLGQGISLIMIK; and P4, KDLVPLWGR. On the basis of these results, the four peptides were chemically synthesized, coupled to KLH as a carrier, and then inoculated into rabbits to produce polyclonal antisera. The resulting antisera were tested by immunoblotting with MBAs (Fig. 3). The anti-P1 and anti-P2 sera strongly reacted with a major band at 33 kDa and to a lesser extent with an additional band at 30 kDa. The anti-P3 sera specifically reacted with a 35-kDa band. The anti-P4 sera did not react with any membrane protein in the range of 30 to 36 kDa and was not further analyzed. These results were confirmed by immunoblots of two-dimensional gels (Fig. 4). As shown in Fig. 4, each of the polyclonal anti-P1, anti-P2, and anti-P3 sera reacted very specifically with proteins migrating within the pH range of 7.2 to 8.10 at the sizes observed on one-dimensional gels: 33, 33, and 35 kDa, respectively. It seems clear that each of the patterns observed contributes to the reactivity of the MVL serum pool in the 30- to 36-kDa region. However, the reactivities of the anti-P1 and anti-P2 sera appeared to be stronger than the reactivity of the anti-P3 sera.

Furthermore, each of the P1, P2, and P3 peptides tested by ELISA strongly and specifically reacted with sera from patients with MVL, whereas they did not react with sera from patients with ZCL or normal human sera (data not shown).

Finally, the sequences of the P1, P2, and P3 peptides were analyzed by using the regular BLAST option and the search for short nearly exact matches BLAST option in the NCBI nr database. Significant matches with known products were observed with P1 and P3 when the second option was used. P1 presented a 100% identity with an NH₂-terminal sequence (residues 48 to 59 [KLLVQNQGEMIK]) of L. major mitochondrial ADP-ATP carrier proteins (Swiss-Prot database accession no. Q9N647), which have a predicted molecular mass of 35 kDa and an isoelectric point of 10.1.

Peptide P3 presented an 83% identity with a Trypanosoma brucei brucei protein (residues 274 to 285 [KLGQGISLIMIK]) of 37 kDa that corresponded to the glycosomal glycerol-3-phosphate dehydrogenase (Swiss-Prot database accession no. P90593). However, the alignment of the last peptide to the glycerol-3-phosphate dehydrogenase proteins described for T. brucei brucei (Swiss-Prot database accession no. P90593), L. major (Swiss-Prot database accession no. LmjF10.0510), L. mexicana (Swiss-Prot database accession no. P90551), and T. brucei rhodesiense (Swiss-Prot database accession no. Q26756) did not confirm that P3 corresponds to a peptide derived from the glycerol-3-phosphate dehydrogenase protein from L. infantum. Submission of the sequences of the three peptides to the Omiblast server of the L. major Genome Project confirmed that P1 is actually part of the ADP-ATP carrier protein (100% identity; E = 2e−005). Peptide P2 showed a 93% identity (E = 2e−006) with residues 152 to 166 of a putative NADH-cytochrome b₅ reductase protein, which has an expected molecular mass of 32.290 kDa and a pI of 8.55. Residues 2 to 10 of P3 presented 100% identity (E = 0.0015) with residues 62 to 70 of a predicted putative mitochondrial carrier protein, which has an expected molecular mass of 34.661 and a pI of 9.5. These three proteins are most likely localized in the inner mitochondrial membrane and would correspond to integral MBAs.

The significant E values observed for these hits, together with the percent identities, the observed concordance of the protein sizes with the predicted ones, and the comparable reactivities of the antipeptides to the MVL sera, led us to conclude that mitochondrial carrier proteins and cytochrome b₅ reductase proteins are the targets of the humoral immune response during MVL. Furthermore, these results confirm our initial hypothesis about the heterogeneous content of the 30- to 36-kDa antigenic fraction.

NEPHGE–SDS-PAGE analysis of the 30- to 36-kDa antigens reacting with the MVL serum pool. In order to further resolve the components of membrane-associated antigens migrating by one-dimensional electrophoresis in the 30- to 36-kDa molecular mass range, MBA preparations were submitted to NEPHGE over a pH range of 5.85 to 8.10, followed by SDS-PAGE analysis. Several gels were run in parallel and were either stained with Coomassie blue or transferred onto polyvinylidene difluoride membranes for incubation with the MVL.
serum pool. At least 14 polypeptides in the size range of interest were resolved within a pH range of 5.85 to 7.6 and reacted with the MVL serum pool but not with the ZCL serum pool. Among these 14 polypeptides, spots for 6 of them were also detectable on Coomassie blue-stained gels and were considered to be present in amounts suitable for further analysis by LC-MS/MS (Fig. 5).

Molecular characterization of the 30- to 36-kDa antigens reactive with the MVL serum pool by LC-MS/MS analysis. The six immunodominant and abundant spots revealed by NEPHGE–SDS-PAGE analysis were excised from the gels and submitted to LC-MS/MS analysis. The mass spectra obtained from each spot provided signals sufficient to search databases by use of the ProteinLynx process. Significant hits were observed for all spots, providing rates of sequence coverage ranging from 8 to 23% (Table 1). The results were further confirmed by sequence analysis of a selection of the matching peptides.

Four *Leishmania* proteins were unambiguously identified for the six spots selected, and these corresponded to products functionally associated with parasite cell membranes, some of which belong to the glycosomal or the mitochondrial compartment. For spots d and f, with molecular masses of 34 and 31 kDa, respectively, two proteins were concomitantly identified within each spot as guanine nucleotide binding protein beta subunit-like protein, known as the activated protein kinase C receptor homolog LACK antigen (17, 36), and a probable aldehyde reductase (55). Spot e, which had a Mowse score of 155 and a sequence coverage of 17%, corresponded to a putative *T. brucei* Reiske iron-sulfur protein precursor (EC 1.10.2.2.), which is imported to the mitochondrion to be part of the ubiquinol-cytochrome c reductase complex (46). The search for a homolog in the *Leishmania* gene database allowed the identification of a product encoded by chromosome 35. This protein bears 297 residues, has 84.5% identity with the *T. brucei* product, and has an expected molecular mass and a pI of 33.634 kDa and 6.03, respectively. The matching peptides of spot e mapped to identical parts of the proteins from both organisms, confirming the identity of this spot. In the case of the polypeptides a, b, and c, which had apparent molecular masses of 32, 32, and 31.5 kDa, respectively, LC-MS/MS analysis provided a significant hit for elongation factor 1/H9251 from *L. donovani* promastigotes. This protein is actually described as being a highly abundant protein in eukaryotic cells and has a molecular mass of 49 to 50 kDa (21, 38), significantly larger than the 32 kDa characterized in our study. This prompted us to check that the elongation factor in these preparations is also identified at its expected size (49 to 50 kDa) and that it is reactive with the MVL serum pool. Four spots (spots g, h1, h2, and h3) that strongly reacted with the MVL serum pool within the 49- to 50-kDa range and with pHs at the end of migration of 6.9, 7.45, 7.5, and 7.6, respectively, which are closer to the expected pI of 9.03 than the other spots at this size, were selected for LC-MS/MS analysis (Fig. 5). As expected, these spots were identified as elongation factor 1a. The matching peptides identified by LC-MS/MS were aligned to their corre-
sponding Leishmania counterparts. Interestingly, peptides of spots d, e, f, g, h₁, h₂, and h₃ matched sequences which were located at the NH₂-terminal, central, or COOH-terminal parts of the corresponding Leishmania proteins (data not shown). However, spots a, b, and c provided peptides which matched only the N-terminal part of elongation factor 1/H9251 (Fig. 6). The fact that the latter polypeptides had molecular masses consistently smaller than expected suggested that spots a, b, and c corresponded to truncated elongation factors. Consultation of the gene database specific for L. major confirmed this hypothesis. Indeed, besides the gene coding for the 49- to 50-kDa elongation factor 1α (Fig. 6), the products of which have expected sizes at 36.4, 34.5, and 30.6 kDa; these correspond to various lengths of the N-terminal part of the protein.

In conclusion, we have identified in the present study seven immunodominant Leishmania products that are involved in the humoral immune response of MVL patients and that are non-reactive with sera from patients with ZCL. These seven proteins are part of the 30- to 36-kDa complex that strongly reacts in Western blots with sera from patient with MVL.

**DISCUSSION**

Methods for the detection of antileishmanial antibodies in sera are generally considered to provide much better sensitivities (80% or greater) than the two methods considered the “gold standards” for the diagnosis of visceral leishmaniasis, namely, microscopy and culture. However, serological assays based on the use of the whole organism or crude leishmanial or semipurified leishmanial antigens are associated with false-positive results due to cross-reactivity with other microorganisms (26). Furthermore, these antigens are not well defined; thus, variations in purification conditions might qualitatively influence the performance of the test, particularly parameters like stability, sensitivity, and reproducibility. Therefore, there is a need to use well-defined and reliable diagnostic tests based on Leishmania-specific peptides or antigens (2, 11, 14, 48, 56). The new generation of diagnostic tests should also be affordable and easy to implement in remote, poor, and less developed rural areas where leishmaniasis is endemic.

The use of patient serum with screening systems like Western blot assays or expression libraries has allowed the selection of semipurified or recombinant antigens (13, 16, 22, 54, 56, 61). In this study, we targeted semipurified P32, which is a fraction known to have good statistical performance in ELISAs (61). Considering that this fraction may actually contain several antigens, our aim here was to further characterize its components using two proteomic approaches. To our knowledge, this is the only study that has used such approaches for the identification of Leishmania immunodominant antigens, which opens the way to the identification of novel potential serodiagnostic targets.

Each of the two approaches developed presented advantages and drawbacks. The microsequencing of peptides derived from the digestion of the immunodominant bands purified by one-dimensional electrophoresis was a lengthy procedure. It allowed indirect identification of the antigens on the basis of a
search for the identities of a short peptide sequence by the use of protein sequences in databases; the significance of the hits observed was defined by the relative score and E value. A priori, the relationship between the different peptides sequenced for a given band was difficult to establish, as the bands had heterogeneous polypeptide contents. For the same reason, it was also difficult to ascertain whether the peptides identified were immunogenic and contributed to elicitation of the polyclonal humoral immune response during the course of the disease. These properties were assessed upon the chemical synthesis of the peptides, their coupling, the generation of rabbit polyclonal sera, and comparison of the antipeptide and MVL serum reactivities on immunoblots of MBAs separated by one-dimensional or two-dimensional electrophoresis. Only three of the four peptides sequenced were shown to bear antigenic properties and to correspond to targets of the humoral immune response in MVL patients.

The second approach was based on two-dimensional (NEPHGE and SDS-PAGE) separation of the antigenic fraction and subsequent LC-MS/MS analysis of immunodominant spots purified from Coomassie blue-stained gels. The 10 spots at 30 to 36 and 50 kDa analyzed in this way allowed the unambiguous identification of five Leishmania products and confirmed the power of this approach. First, the two-dimensional separation increased the resolution of the polypeptide content of the bands within the pH 5.85 to 7.6 and 30- to 36-kDa ranges of the study, making possible the differentiation of at least 14 immunogenic polypeptide spots. Second, the abundant antigens detected on and picked from the Coomassie blue-stained gels were analyzed by LC-MS/MS, a sensitive and powerful method that uses mass spectra to assign the polypep-

| Spot | pH at end of migration | Apparent molecular mass (kDa) | Protein (organism) | Hits (molecular mass [kDa]/pI) | Swiss-Prot database accession no. | Probability-based Mowse score\(^a\) | % Sequence coverage |
|------|-----------------------|-------------------------------|--------------------|-------------------------------|---------------------------------|-------------------------------------|------------------|
| a    | 7.4                   | 32                            | Elongation factor 1α (L. donovani) | 49.097/9.03                   | Q95VF2                          | 140                                  | 8                |
| b    | 7.3                   | 32                            | Elongation factor 1α (L. donovani) | 49.097/9.03                   | Q95VF2                          | 315                                  | 23               |
| c    | 7.3                   | 31.5                          | Elongation factor 1α (L. donovani) | 49.097/9.03                   | Q95VF2                          | 145                                  | 9                |
| d    | 6.95                  | 34                            | Guanine nucleotide binding protein beta subunit-like protein (activated protein kinase C receptor homolog LACK) (L. donovani) | 34.351/6.05                   | Q27434                          | 195                                  | 21               |
| e    | 6.7                   | 33                            | Probable ubiquinol-cytochrome c reductase (EC 1.10.2.2) Rieske iron-sulfur protein precursor (T. brucei) | 33.634/6.03                   | Q27785                          | 155                                  | 17               |
| f    | 6.8                   | 31                            | Guanine nucleotide binding protein beta subunit-like protein (activated protein kinase C receptor homolog LACK) (L. donovani) | 34.351/6.05                   | Q27434                          | 160                                  | 20               |
| g    | 6.9                   | 50                            | Elongation factor 1α (L. donovani) | 49.097/9.03                   | Q95VF2                          | 90                                   | 21               |
| h\(_1\) | 7.45              | 50                            | Elongation factor 1α (L. donovani) | 49.097/9.03                   | Q95VF2                          | 725                                  | 53               |
| h\(_2\) | 7.5                | 50                            | Elongation factor 1α (L. donovani) | 49.097/9.03                   | Q95VF2                          | 550                                  | 34               |
| h\(_3\) | 7.6                | 50                            | Elongation factor 1α (L. donovani) | 49.097/9.03                   | Q95VF2                          | 300                                  | 22               |

\(^a\) The Mowse score is calculated as \(-\log P\), where \(P\) is the probability that the observed match is a random event.

FIG. 6. Alignment of the peptides matching the sequence of \(L.\) donovani elongation factor 1α promastigotes (Swiss-Prot database accession no. Q95VF2). Underlined sequences indicate the matching peptides identified for spots a, b, and c. Sequences in boldface and italic indicate the matching peptides for spots g, h\(_1\), h\(_2\), and h\(_3\).
tide content of each spot to proteins deposited in data banks. The significance of the hits was evaluated by the use of various statistical parameters, such as the Mowse score and the percent sequence coverage, which allowed us to consider the identification to be unambiguous. The Mowse scores of the first hit for each polypeptide was so significant and so divergent from the range of scores of the following hits that one could confidently consider the first hit as significant. Furthermore, several peptides generated from the various spots (usually 4 to 19 of 6 to 29 residues) matched these hits, resulting in a significant range of sequence coverage (8 to 53%). Indeed, the last parameter is influenced by any kind of modification to any of the residues of the peptides. Modifications alter the mass of the peptide, therefore causing the relative scores to deviate, which allowed identification of the peptides and, consequently, their match. Furthermore, the approach is powerful enough to detect overlapping polypeptides whenever this occurs. In our study, two such spots (spots d and f) were observed that redundantly and concomitantly identified two antigens that are already known to be products that are up-regulated in the promastigote stage: the LACK antigen and a member of the aldehyde reductase family (6, 25, 55).

Except for elongation factor 1α, all antigens identified had an expected molecular mass that fit within the range of 30 to 36 kDa. Furthermore, different spots were shown to correspond to the same antigen, an indication of the likely presence of isoforms within the preparation. For elongation factor 1α, there was a discrepancy between the expected molecular mass (49 kDa) and the actual one observed on the gels at 32 kDa. We could confirm the presence in our preparation of elongation factor 1α at the expected size by analyzing four 49- to 50-kDa spots (spots g, h₁, h₂, and h₃) which strongly reacted with the sera from MVL patients. The matching peptides of spots a, b, and c could be aligned only to the N-terminal part of the sequence of the elongation factor 1α protein, which argued in favor of the existence of truncated forms of the elongation factor 1α protein in the promastigote membrane preparations. Consultation of the L. major-specific gene database actually identified genes coding for truncated, N-terminal forms of elongation factor 1α, which confirmed our hypothesis. It is noteworthy that elongation factor 1α of L. donovani was recently described as a Leishmania virulence factor. This factor is able to activate tyrosine phosphatase-1, which contains the Src homology 2 domain, leading to deactivation of the infected macrophages. The 49-kDa elongation factor 1α Leishmania protein was shown to diffuse into the cytosol of infected macrophages, where it exerts this activity (38). To our knowledge, no information has been provided as to whether the naturally truncated elongation factor 1α products could also act as virulence factors and whether they could be expressed in a stage-specific manner. However, our results show that they are at least present at the late growth phase of the promastigote stage.

Drawbacks of the two-dimensional approach could relate to the number of two-dimensional gels that needed to be run to resolve the entire content of the fraction of interest, as well as to the need to use various pH ranges of separation by using different ampholyte ratios. As Coomassie blue staining of gels limits the analysis to only a small number of antigens, the use of the more sensitive fluorescent labeling of the gels could improve the analysis (49). Finally, identification of the peptides generated by both approaches could be hampered by the incomplete data available in the common databases generated by the ongoing Leishmania genome projects. Alternatively, one could use locally tailored resources by extraction of data from Leishmania genome project web pages. Using this alternative, we could identify matches to peptides P2 and P3 on Leishmania Friedlin Genome Project sequences, while the NCBI database search did not yield significant results.

The antigens identified in the present report correspond to evolutionarily conserved proteins encountered in cells as part of multicomponent complexes that could be considered pan-antigens (a term coined earlier by Requena and coauthors [52]). That study identified antigens known to be integral components of the mitochondrial membranes, such as mitochondrial carrier proteins, NADH-cytochrome b₄ reductase, and the ubiquinol-cytochrome c reductase (EC 1.10.2.2) Reiske iron-sulfur precursor. The other proteins are tightly associated with multicomponent complexes. Indeed, Gonzalez-Aseguinolaza et al. (17) showed that the LACK P36, although it is structurally not membrane associated, is organized within large complexes which copurify with membranes. This is not discordant with our results, as treatment of our membrane preparation at pH 11 only partially liberated a P36 product.

Elongation factor 1α is actually described as being a highly abundant protein in eukaryotic cells, having a molecular mass of 49 to 50 kDa (greater than 0.4% of the amount of total protein) (27), which interacts with the cytoskeleton by binding and bundling actin filaments and microtubules and which is associated with the endoplasmic reticulum membrane by phosphatidylinositol (20, 27, 29, 63). Therefore, if such interactions hold true for Leishmania parasites, it would account for the association of the different forms of elongation factor 1α with our membrane preparations.

The involvement of the antigens identified in the present report in the humoral immune response triggered during visceral leishmaniasis has been illustrated only for LACK (31) and the aldehyde reductase homologue (25). In the first case, the recombinant L. major LACK protein was shown to react consistently with sera from patients with MVL (31), allowing confirmation of the L. infantum protein identified here as a potent seroreactive antigen during the disease. LACK was also previously reported to elicit immune responses and cytokine secretion in human or experimental mouse leishmaniasis (8, 17, 31). The L. donovani homologue to the L. major P100/11E protein (a probable aldehyde reductase; Swiss-Prot database accession no. P22045) was previously shown to have limited humoral and cellular antigenic properties in Sudanese visceral leishmaniasis patients (25), and because we tested an MVL serum pool in this study, the consistent response of MVL patients to this antigen still needs to be established. The description of elongation factor 1α, its truncated N-terminal parts, and mitochondrial proteins as potent antibody inducers during visceral leishmaniasis is a novel finding. Interestingly, a protein homologous to elongation factor 1α was found to be suitable for the antibody-based diagnosis of hymenopteran parasitism (60). Other elongation factors like elongation factor 2 or elongation factor 1βδ were also shown to be associated with the elicitation of cellular immune responses in leishmaniasis patients (47) or allergic manifestations in patients with
cystic echinococcosis due to Echinococcus granulosus (32, 42), respectively.

Using proteomic methods, we have successfully identified some of the antigens included in the immunodominant 30- to 36-kDa fraction that is specifically involved in the humoral immune response during MVL. The superiority of 2DE analysis followed by LC-MS/MS analysis over microsequencing analysis of gel-purified bands is stressed. Work is in progress in order to assess the reactivities of recombinant forms of the proteins identified with a large set of individual serum samples from patients with MVL and to develop sensitive and specific assays for diagnosis of this disease on the basis of such products used either separately or in various combinations.

ACKNOWLEDGMENTS

This work received financial support from the Tunisian State Secretariat for Scientific Research and Technology (SERST). It was also supported in part by the NIH-NIAID-MERC Program (grant N01-A1-45183), the DGXII European Union INCO-MED Programme (grant I C18/CT97/0252), and the Special Programme of UNDP/World Bank/World Health Organization, TDR (RSG grant 890 266) and FAG (grant A 11032).

This work was performed at the Laboratoire d’Immunologie, Virologie et Génétique Moléculaire (LAF301) (World Health Organization Collaborating Center for Research and Training in Leishmaniasis), Institut Pasteur de Tunis, Tunis-Belvedere, Tunisia.

REFERENCES

1. Anonymous. 1990. Control of leishmaniasis. Report of a WHO expert committee. WHO Tech. Rep. Ser., 703:1–13.

2. Badaro, R., D. Bensoussan, M. C. Eulalio, F. Frere, S. Cunha, E. M. Netto, D. Pedral-Sampaio, C. Madureira, J. M. Burns, R. L. Houghton, J. R. David, and S. G. Reed. 1996. rK39: a cloned antigen of Leishmania chagasi that detects specific antibody in American visceral leishmaniasis. J. Infect. Dis. 173:758–761.

3. Badaro, R., S. G. Reed, A. Barral, G. Orte, and T. C. Jones. 1986. Evaluation of the micro enzyme-linked immunosorbent assay (ELISA) for antibodies in American visceral leishmaniasis; antigen selection for detection of infection-specific responses. Am. J. Trop. Med. Hyg. 35:72–78.

4. Badaro, R., S. G. Reed, and E. M. Carvalho. 1983. Immunofluorescent antibody test in American visceral leishmaniasis; specificity and sensitivity of different morphological forms of two Leishmania species. Am. J. Trop. Med. Hyg. 32:480–484.

5. Ben Salah, A., R. Ben-Ismail, F. Amri, S. Chih, F. Ben Rezig, H. Kharrat, H. Hadhri, M. Hassouna, and K. Dellagi. 2000. Investigation of the spread of human visceral leishmaniasis in central Tunisia. Trans. R. Soc. Trop. Med. Hyg. 94:382–386.

6. Ben Salah, A., S. Soder, M. Wiesgigl, J. Heukeshoven, C. Gelhaus, E. Krause, J. Clos, and I. Bruchhau. 2003. Developmentally induced changes of the proteome in the protozoan parasite Leishmania donovani. Proteomics 3:1811–1829.

7. Berneaman, A., X. Rolland, and G. Broun. 1988. Humoral response in Leishmania infantum clinical infections. Ann. Inst. Pasteur Immunol. 139:267–278.

8. Bourregey, E., G. Prevot, J. Gordon, R. Pradinaud, H. Hasagawa, G. Milton, and P. Lamois. 2002. LACK-specific CD4+ T cells that induce gamma interferon production in patients with localized cutaneous leishmaniasis during an early stage of infection. Infect. Immun. 70:3112–3119.

9. Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.

10. Bray, S. R. 1976. Immunodiagnosis of leishmaniasis, p. 66–76. In S. Cohen and E. H. Sadun (ed.), Manual of immunology of parasitic infections. Blackwell Scientific Publications, London, United Kingdom.

11. Burns, J. M., Jr., W. G. Shreffler, D. R. Benson, H. W. Ghalib, R. Badaro, and S. G. Reed. 1993. Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. Proc. Natl. Acad. Sci. USA 90:775–779.

12. Chaluy, J. D., and A. D. M. Bryceon. 1983. Quantification of amastigotes of Leishmania donovani in smears of splenic aspirates from patients with visceral leishmaniasis. Am. J. Trop. Med. Hyg. 22:473–479.

13. Evans, T. G., E. C. Krug, M. E. Wilson, A. W. Vasconcelos, J. E. De Alencar, and R. D. Pearson. 1989. Evaluation of antibody responses in American visceral leishmaniasis by ELISA and immunoblot. Mem. Inst. Oswaldo Cruz 84:157–166.
distinguishes between ongoing and previous *L. donovani* infection. APMIS 101:642–646.

41. Okong'o-Odera, E. A., A. Wamachi, J. M. Kagai, J. A. Kurtzhals, J. I. Gilhure, A. S. Hey, J. B. Were, D., K. Koche, E. S. Mitena, and A. Kharazmi. 1993. Field application of an ELISA using redefined *Leishmania* antigen for the detection of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 87:423–424.

42. Ortona, E., P. Marguttii, S. Vaccari, R. Rigano, E. Profumo, B. Buttari, A. Chersi, A. Taggi, and A. Siracusano. 2001. Elongation factor 1 beta/delta of Echinococcus granulosus and allergic manifestations in human cystic echinococcosis. Clin. Exp. Immunol. 125:110–116.

43. Ouelhazi, L., M. Filali, A. Decendit, J. C. Chenieux, and M. Rideau. 1993. Differential protein accumulation in zeatin-and 2,4-dtreated cells of Catharanthus roseus. Correlation with indole alkaloid biosynthesis. Plant Physiol. 31:421–431.

44. Pearson, R. D., and A. deq Ueiroz Souza. 1996. Clinical spectrum of leishmaniasis. Clin. Infect. Dis. 22:1–13.

45. Pfaff, E., M. Muesgay, H. O. Bohn, G. E. Schulz, and H. Schaller. 1982. Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. EMBO J. 1:869–874.

46. Priest, J. W., and S. L. Hajduk. 1996. In vitro import of the Rieske iron-sulphur proteins by trypanosome mitochondria. J. Biol. Chem. 271:20060–20069.

47. Probst, P., E. Stromberg, H. W. Ghalib, M. Mozol, R. Badaro, S. G. Reed, and J. R. Webb. 2001. Identification and characterization of T cell-stimulating antigens from *Leishmania* by CD4 T cell expression cloning. J. Immunol. 166:498–505.

48. Quijada, L., J. M. Requena, M. Soto, and C. Alonso. 1998. Analysis of the antigenic properties of the *L. infantum* Hsp70: design of synthetic peptides for specific serodiagnosis of human leishmaniasis. Immunol. Lett. 65:169–174.

49. Rabilloud, T., J. M. Strub, S. Luche, A. Van Dorselaer, and J. Lunardi. 2001. A comparison between Sypro Ruby and ruthenium II Tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. Proteomics 1:699–704.

50. Ramagl, L. S., and L. U. Rodriguez. 1985. Quantification of microgram amounts of proteins in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis 6:559–563.

51. Reed, S. G., R. Badaro, and R. M. Lloyd. 1987. Identification of specific and cross-reactive antigens of *Leishmania donovani* chagasi by human infection sera. J. Immunol. 138:1596–1601.

52. Requena, J. M., C. Alonso, and M. Soto. 2000. Evolutionarily conserved proteins as prominent immunogens during *Leishmania* infections. Parasitol. Today 16:246–250.

53. Rolland, L., V. Zilberfarb, A. Furtado, and M. Gentilini. 1994. Identification of a 94-kilodalton antigen on *Leishmania* promastigote forms and its specific recognition in human and canine visceral leishmaniasis. Parasite Immunol. 16:599–608.

54. Rolland-Burger, L., X. Rolland, C. W. Grieve, and L. Monjour. 1991. Immunoblot analysis of the humoral immune response to *Leishmania donovani* infantum polypeptides in human visceral leishmaniasis. J. Clin. Microbiol. 29:1429–1435.

55. Samaras, N., and T. W. Spithill. 1989. The developmentally regulated P100/11E gene of *Leishmania major* shows homology to a superfamily of reductase genes. J. Biol. Chem. 264:4251–4254.

56. Shreffler, W. J., J. M. Burns, Jr., R. Badaro, H. W. Ghalib, L. L. Button, W. R. McMaster, and S. G. Reed. 1993. Antibody responses of visceral leishmaniasis patients to gp63, a major surface glycoprotein of *Leishmania* species. J. Infect. Dis. 167:426–430.

57. Soto, M., J. M., Requena, L. C Gomez, L. Navarete, and C. Alonso. 1992. Molecular characterization of a *Leishmania donovani* infantum antigen identified as histone H2A. Eur. J. Biochem. 205:211–216.

58. Soto, M., J. M. Requena, L. Quijada, M. Garcia, F. Guzman, M. E. Patarroyo, and C. Alonso. 1995. Mapping of the linear antigenic determinants from the *Leishmania infantum* histone H2A recognized by sera from dogs with leishmaniasis. Immunol. Lett. 48:209–214.

59. Soto, M., J. M. Requena, L. Quijada, M. J. Perez, C. G. Nieto, F. Guzman, M. E. Patarroyo, and C. Alonso. 1999. Antigenicity of the *Leishmania infantum* histones H2B and H4 during canine visceralcutaneous leishmaniasis. Clin. Exp. Immunol. 115:342–349.

60. Stuart, M. K. 1998. An antibody diagnostic for hymenopteran parasitism is specific for a homologue of elongation factor-1 alpha. Arch. Insect Biochem. Physiol. 39:1–8.

61. Toubourzki, F., A. El Gaid, H. Louzir, R. Ben Ismail, R. Kammoun, and K. Delligi. 1994. Identification of an immunodominant 32-kilodalton membrane protein of *Leishmania donovani* infantum promastigotes suitable for specific diagnosis of Mediterranean visceral leishmaniasis. J. Clin. Microbiol. 32:2474–2480.

62. Voller, A., D. E. Bidwell, and A. Bartlett. 1976. Enzyme immunoassays in diagnostic medicine. Theory and practice. Bull. W. H. O. 53:55–65.

63. Yang, F. M.emma, M. Warren, S. Dharmawardhane, and J. Condeelis. 1990. Identification of an actin-binding protein from Dictyostelium as elongation factor 1a. Nature 347:494–496.