Identification of an Immunodominant 32-Kilodalton Membrane Protein of *Leishmania donovani infantum* Promastigotes Suitable for Specific Diagnosis of Mediterranean Visceral Leishmaniasis

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Sera from 35 patients suffering from Mediterranean visceral leishmaniasis (caused by *Leishmania donovani infantum*) and 59 patients with various forms of cutaneous leishmaniasis prevalent in the sub-Mediterranean countries (caused by *Leishmania major*, *L. donovani infantum*, or *Leishmania tropica*) were tested by immunoblotting and enzyme-linked immunosorbent assay (ELISA) with both membrane and soluble antigens prepared from *L. donovani infantum* parasites. Control sera were from healthy children ($n = 41$), adults with nonleishmanial diseases ($n = 40$), and patients with Chagas' disease ($n = 12$). A P32 antigen present in the membrane preparation from *L. donovani infantum* parasites was recognized by 95% of serum specimens from patients with Mediterranean visceral leishmaniasis but not by serum specimens from patients with cutaneous leishmaniasis or sera from control individuals. An ELISA with electroeluted P32 antigen was found to have a specificity and sensitivity of 94% in the serodiagnosis of Mediterranean visceral leishmaniasis. Healthy children with asymptomatic *Leishmania* infection were seronegative for the P32 antigen by ELISA. These results suggest that antibodies to P32 antigen develop only in patients with visceral leishmaniasis and that the P32 ELISA may be useful in areas where the disease is endemic for discriminating between patients with this disease and those with other clinical conditions.

The leishmaniasis parasites are obligate intracellular protozoans which cause human cutaneous, mucocutaneous, or visceral leishmaniasis. In Tunisia and other North African countries, several forms of leishmaniasis coexist. Human cutaneous leishmaniasis (HCL) is caused by *Leishmania major*, *Leishmania tropica*, as well as a variant of *Leishmania infantum* (3). In opposition to HCL, Mediterranean visceral leishmaniasis (MVL), which is caused by *Leishmania donovani infantum*, is a severe and potentially lethal disease which needs a rapid and accurate diagnosis. The definitive diagnosis of leishmaniasis relies mainly on the demonstration of the presence of the parasite in a tissue sample and/or its isolation by culture as well as the detection by serological tests of specific antibodies to leishmanial antigens. The different methods of detection include indirect immunofluorescence (25), enzyme-linked immunosorbent assays (ELISAs) with whole parasites, crude extracts, and purified or recombinant antigens (2, 7, 13, 18), direct agglutination (14), or immunoblot analysis (4, 5, 11, 17, 26, 27). Some of these techniques suffer from a relative lack of specificity because of antigenic relatedness between *Leishmania* species and other microorganisms such as *Trypanosoma cruzi*, *Echinococcus granulosus*, *Toxoplasma gondii*, and the mycobacteria (4, 8, 26). In countries where such pathogens coexist with leishmanias, the results of serological tests may be difficult to interpret, since the different *Leishmania* species involved in the various clinical forms of the disease may share cross-reactive antigens. Hence, in areas where both zoonotic

cutaneous and visceral leishmaniases are endemic, a positive ELISA result could be due to an infection with either *L. major* or *L. donovani infantum*. The molecular basis of the cross-reactivity is not fully defined. Immunoblotting techniques have been used to define both species-specific and cross-reactive leishmania antigens (22, 26). Here we report on the identification by immunoblotting of an immunodominant P32 membrane antigen of *L. donovani infantum* promastigotes which allowed the development of a sensitive and specific ELISA for the diagnosis of MVL.

MATERIALS AND METHODS

Sera. A total of 187 serum specimens were analyzed in the study. These serum specimens were divided into eight groups.

(i) Group I. Thirty-five serum specimens were obtained before treatment from young Tunisian children suffering from MVL (age range, 1 to 3 years; median, 2.1 years). In all patients the diagnosis was confirmed by the presence of *Leishmania* amastigotes in Giemsa-stained bone marrow smears and/or culture in biphasic Nicolle-Novy-McNeal medium. For eight patients, we analyzed the sera obtained before (one sample), after, and during treatment (three samples).

(ii) Group II. Twenty-two serum specimens were obtained from patients who lived in the area of Sidi Bouzid, central Tunisia, where HCL is highly endemic and who suffered from the zoonotic form of HCL caused by *L. major*.

(iii) Group III. Twenty-two serum specimens were obtained from patients who lived in the districts of Bejà and Jendouba, northern Tunisia, and who suffered from the sporadic form of HCL caused by a variant of *L. donovani infantum*.

(iv) Group IV. Fifteen serum specimens were obtained from

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patients suffering from active anthroponotic HCL caused by *L. tropica* and living in Aleppo, Syria.

(v) **Group V.** Six serum specimens were obtained from healthy children with asymptomatic leishmanial infection. These children were detected during a systematic survey in an area in northern Tunisia (district of Medjez El Bab) where MVL is endemic, and their sera were found by indirect immunofluorescence and ELISA to react with leishmanial antigens at high titers. These children were apparently healthy and did not present with any clinical symptoms related to MVL.

(vi) **Group VI.** Thirty-five control serum specimens were obtained from healthy Tunisian children (age range, 3 to 10 years).

(vii) **Group VII.** Forty control serum specimens were obtained from adult Tunisian patients suffering from various diseases such as systemic lupus erythematosus ($n = 10$), toxoplasmosis ($n = 10$), hydatidosis ($n = 10$), and tuberculosis ($n = 10$).

(viii) **Group VIII.** Twelve serum specimens from South American patients suffering from Chagas' disease were provided by F. Veas, Orstom-Montpellier, FRANCE.

Parasite and antigen preparation. The antigens used in the study were prepared from an *L. donovani infantum* strain isolated from a Tunisian patient suffering from MVL (strain MHOM/TN/80/IPT1). Promastigotes were grown at 26°C in RPMI 1640 medium supplemented with 10% fetal calf serum and were harvested after four days, at the late log phase. The promastigotes were washed three times by centrifugation (3000 × g, 15 min) in 10 mM Tris–150 mM NaCl (pH 7.6; TBS) before being resuspended in 1 ml of TBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 2 mM EGTA [ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid], 2 μg of pepstatin per ml, 2.5 mM N-ethylmaleimide, and 0.127 IU of aprotinin per ml; the mixture was stored at −80°C until use.

Crude membrane antigens (MBAs) were prepared from 2 × 10^{10} promastigotes of *L. donovani infantum* parasites. Cell pellets were washed and resuspended in 10 mM Tris-HCl-2 mM EDTA (pH 8) and were kept on ice for 20 min. The cell suspension was subsequently made to 1 mM PMSF in the same buffer and was disrupted on ice in a Dounce homogenizer. The cell homogenate was then centrifuged at 10,000 × g for 30 min. Alternatively, *L. donovani infantum* membranes were further purified by ultracentrifugation (at 33,000 × g for 90 min at 4°C in a Beckman L7 Ultracentrifuge with 50 TI rotor [fixed angle]) on a 7 to 17% discontinuous sucrose gradient (10).

Soluble leishmania antigens (SLAs) were prepared from *L. donovani infantum* promastigotes as described by Melby et al. (24). The protein concentration was determinated by the protein assay described by Lowry et al. (23).

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 7 to 17% gradient gel with a 1% stacking gel as described by Laemmli (20). Approximately 400 μg of protein per slab was electrophoresed for 4 to 5 h at 30 mA per gel. Gels were stained with Coomassie brilliant blue (0.1%; wt/vol). Glycoconjugates were detected in polyacrylamide gels by periodic acid-Schiff staining (30).

Detection of the proteolytic activities of the separated parasite polypeptides was performed as described by Bouvier et al. (6) by using bovine serum albumin (BSA) incorporated within the polyacrylamide gel.

Proteins from unstained polyacrylamide gels were electrotransferred onto a 0.45-μm-pore-size nitrocellulose membrane (HAHY; Millipore, Watford, United Kingdom) by the procedure described by Towbin et al. (29); the membrane was then incubated with sera from patients with MVL (diluted 1/500) or HCL (1/100) or with control sera (1/100) and then with peroxidase-conjugated sheep antihuman immunoglobulin (Amersham International Inc., Buckinghamshire, United Kingdom) and was developed with diaminobenzidine–H_2O_2. Furthermore, the Gp63 antigen of *L. donovani infantum* was identified within MBA extracts by immunoblotting by using a mouse monoclonal antibody to Gp63 (a generous gift from Robert McMaster, Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada).

Purification of P32 antigen from *L. donovani infantum* membranes. The 32-kDa protein of *L. donovani infantum* was purified by electroelution from the gel after SDS-PAGE. Two vertical strips were excised from both sides of the gel and were stained with Coomassie blue to localize the P32 antigen; then, horizontal strip containing the relevant antigen was cut out from the gel and placed into a dialysis bag filled with 2 ml of phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M phosphate [pH 7]). Electroelution was performed at 120 V for 1 h at 4°C, and the eluted polypeptides were extensively dialyzed against PBS.

ELISA. ELISA with polystyrene plates coated with P32 antigen (1.25 μg/ml) or SLA or MBA extracts (10 μg/ml) was performed by standard methods (16).

Statistical analysis. The cutoff values of the ELISAs with the P32 antigen or SLA or MBA were defined as the mean optical density (MOD) plus two standard deviations (2SDs) of the values for the control group (group VI). The sensitivity, specificity, and accuracy of the positive prediction, as well as the accuracy of the negative prediction, the efficiency of prediction, and the error of prediction, were calculated as described elsewhere (9, 12).

RESULTS

Immunoblot analysis of *L. donovani infantum* antigens reacting with sera from patients with MVL and HCL. Soluble extracts or membrane antigens of *L. donovani infantum* run on an SDS-polyacrylamide gel and stained with Coomassie blue showed more than 50 components (Fig. 1). The most prominent protein of MBA migrated as a diffuse band at 56 to 64 kDa. This antigen was periodic acid-Schiff stain positive, displayed proteolytic activity against BSA, and reacted by immunoblotting with a mouse monoclonal antibody to Gp63 (data not shown). This antigen was therefore identified as being the major surface glycoprotein Gp63 of *L. donovani infantum*.

Twenty-three serum specimens from patients with MVL (group I) were tested by immunoblotting with MBA of *L. donovani infantum*; each serum specimen reacted variably with 4 to 17 antigenic components ranging in molecular mass from 10 to 80 kDa. Five serum specimens reacted with fewer than 5 polypeptides and five serum specimens reacted with more than 10 polypeptides. Six immunodominant regions were observed in the immunoblots (Table 1). The major reactive component was a 32-kDa polypeptide which was recognized by 95% (22 of 23) of serum specimens from patients with MVL (Fig. 2, lanes 1 to 10). Interestingly, the majority (21 of 23) of serum specimens from patients with HVL did not react with the major surface glycoprotein Gp63. This region appeared as a clearly unreactive band in the immunoblot with the MBA preparation (Fig. 2, arrow).

Twenty serum specimens from patients with HCL caused by *L. major* (group II) were also tested by immunoblotting with the crude membrane preparation of *L. donovani infantum*. The
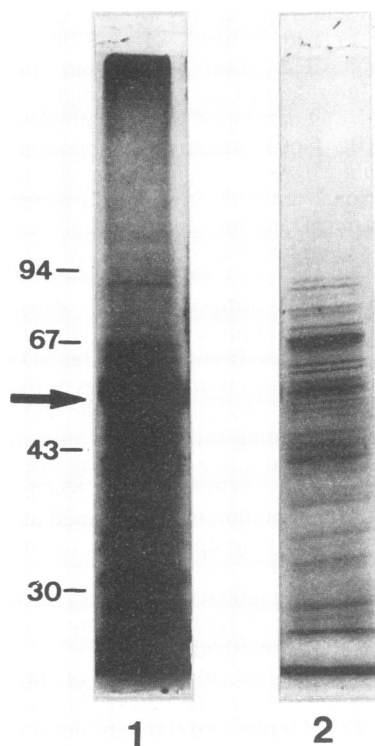
FIG. 1. MBAs (lane 1) and SLAs (lane 2) extracted form *L. donovani infantum* promastigotes resolved by SDS-PAGE and stained with Coomassie blue. Arrow, the major surface protease Gp63.

major reactive antigens were Gp63 ($n = 18$; 90%) and the low-molecular-mass antigens (10 to 20 kDa) ($n = 19$; 95%). However, the 32-kDa polypeptide of *L. donovani infantum* membranes revealed by the sera from patients with HVL was found to be constantly unreactive (Fig. 2, lanes 11 to 15).

Twenty-two serum specimens from patients with HCL caused by *L. donovani infantum* (group III) and 15 serum specimens from patients with HCL caused by *L. tropica* (group IV) were also tested. None of them recognized the Gp63 or the 32-kDa antigen of the *L. donovani infantum* membranes. A faint reactivity was found with low-molecular-mass antigens (less than 20 kDa) (Fig. 2, lanes 16 to 22).

Control sera from healthy children and patients with diseases other than leishmaniasis (groups VI and VII, respec-

TABLE 1. Frequency of recognition of major *L. donovani infantum* antigens by sera from patients with HVL and LCZ on immunoblotting

Antigen (kDa)	% Recognition by sera from[a]:	
	Patients with HVL ($n = 23$)	Patients with ZCL ($n = 20$)
80	48	
74	52	30
68	52	
56–64[b]	10	90
54	75	
32	95	0
<20	74	80

[a] HVL, human visceral leishmaniasis; ZCL, zoonotic cutaneous leishmaniasis.
[b] This major fraction corresponded to Gp63.

tively) were negative or faintly stained some polypeptides in crude membrane preparations (Fig. 2, lanes 23 to 27).

When *L. donovani infantum* SLAs were immunoblotted with sera from patients with MVL, several reactive bands were identified; they ranged in molecular mass from 12 to 130 kDa. The most intensely stained band was a doublet of 74 and 80 kDa ($n = 17$; 74%). The other reactive bands were P12, P80, P45/49, P130, P23, P32, and P28 (Fig. 3). Control sera from healthy children were unreactive except for a faintly staining 60-kDa polypeptide.

Characterization of antibody response to P32 antigen of *L. infantum*. Immunoblot analysis with MBAs extracted from *L. donovani infantum* parasites allowed a clear discrimination between sera from patients with MVL and HCL caused by *L. major* (Fig. 2). Sera from patients with HCL caused by *L. major* had a prominent and constant reactivity against the Gp63 glycoprotein but did not react with the P32 antigen, in contrast to the sera from patients with MVL caused by *L. major*, which strongly reacted with the P32 antigen but not with Gp63. SLA preparations of *L. donovani infantum* were found to be much less discriminatory. Actually, the P32 membrane antigen of *L. donovani infantum* appeared to be immunodominant since it induced a strong humoral response in 95% of patients with MVL. These antibodies were always of the immunoglobulin G class, and no specific immunoglobulin M antibodies were detected (data not shown). Immunoglobulin G antibody levels decreased slightly after treatment but were still detectable in eight patients 6 months after they were cured, while no antibodies to the P32 antigen were detected by immunoblotting in the sera of two patients 2 years and 8 years after they were cured, respectively. Sera from patients with the various forms of cutaneous leishmaniasis and 75 control serum specimens (groups VI and VII, respectively) were found to be constantly unreactive with the P32 antigen.

The fact that the P32 antigen was most readily detectable by immunoblotting in the MBA extracts of *L. donovani infantum* than in the SLA preparations suggested that it was likely a membrane-associated antigen and not a soluble antigen that contaminated the membrane preparation. In fact, we obtained essentially the same results when we performed immunoblot analysis with sera from patients with HVL with *L. donovani infantum* membranes which were further purified by ultracentrifugation on a discontinuous sucrose gradient.

The P32 antigen appeared to be a minor component of the membrane preparation, as judged by Coomassie blue staining of SDS-polyacrylamide gels. The polypeptide was not stained by the Schiff reagent (periodic acid-Schiff stain negative) and had no proteolytic activity with BSA (data not shown). It had the same molecular mass under reducing and nonreducing conditions. Finally, the P32 antigen was equally detected in membranes extracted from *L. donovani infantum* promastigotes harvested at the exponential or the late plateau phase of growth, suggesting that this antigen was not expressed only at a given growth phase of the promastigote.

Specific diagnosis of HVL by ELISA with P32 antigen. Immunoblot analysis of membrane preparations of *L. donovani infantum* suggested that the P32 antigen could be a suitable antigen for use in the serodiagnosis of MVL. This point was further analyzed by the more sensitive ELISA with SLA, MBA, or the P32 antigen electroeluted from the gel after SDS-PAGE.

The optimal dilution of the serum specimen was determined by serially diluting representative serum specimens from each group by using microplates coated with the relevant antigen. A dilution of 1/500 was found to be appropriate and was used throughout the study.
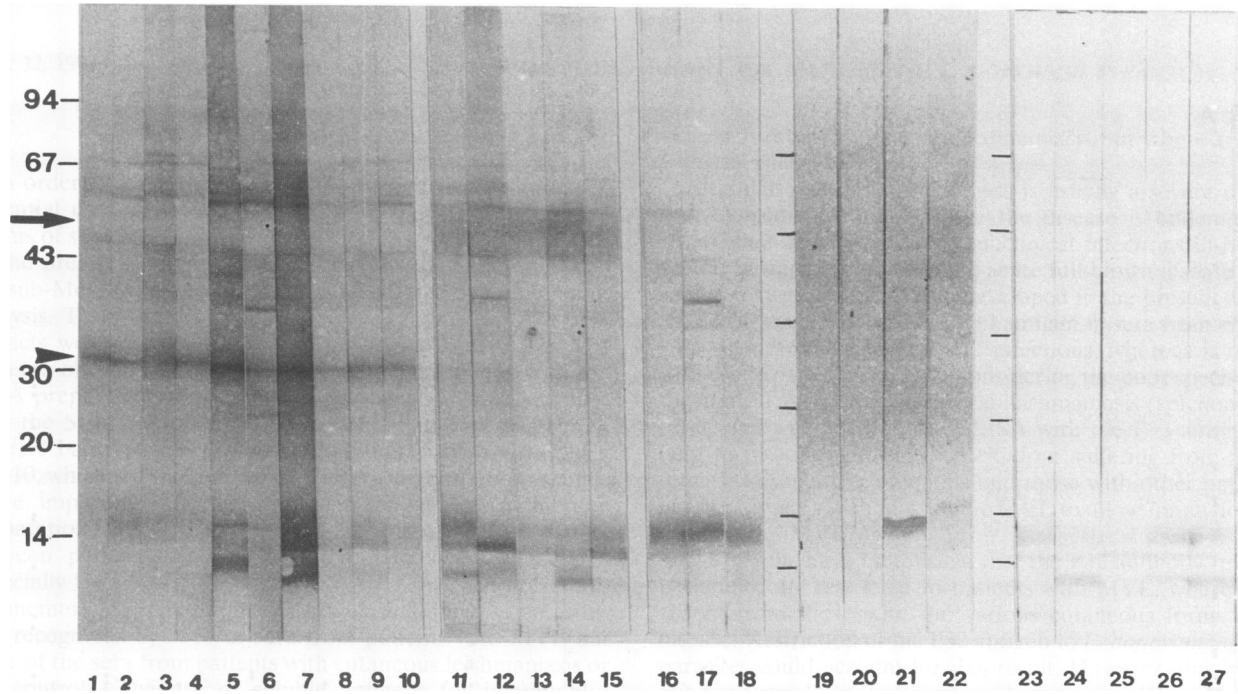
FIG. 2. MBAs extracted from *L. donovani infantum* promastigotes and immunoblotted with human sera. Sera were obtained from patients with MVL caused by *L. donovani infantum* (lanes 1 to 10), zoonotic cutaneous leishmaniasis caused by *L. major* (lanes 11 to 15), sporadic cutaneous leishmaniasis caused by *L. donovani infantum* (lanes 16 to 18), anthroponotic cutaneous leishmaniasis caused by *L. tropica* (lanes 16 to 22), and healthy children (lanes 23 to 27). Arrow, the major surface protease Gp63; arrowhead, P32 antigen.

The absorbance values of the ELISA with the P32 antigen obtained with sera from the various groups are shown in Fig. 4A. Sera from patients with MVL strongly reacted with the P32 antigen (lane 1), in contrast to sera from patients with the various forms of cutaneous leishmaniasis (lanes 2 to 4). Control sera from healthy children or from patients with various bacterial or parasitic diseases showed only weak reac-
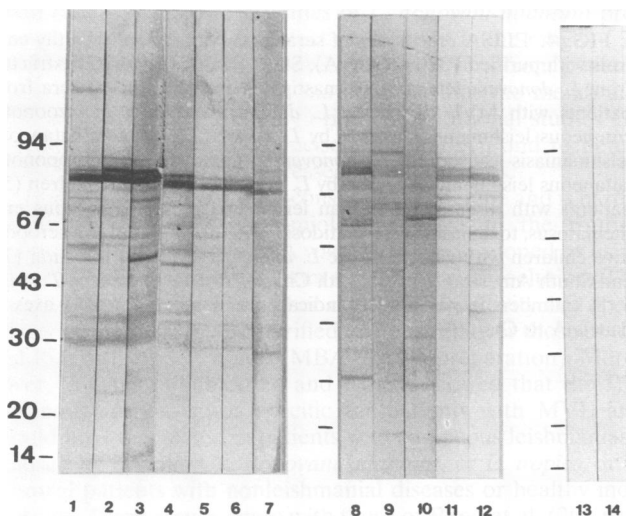

FIG. 3. SLAs extracted from *L. donovani infantum* promastigotes and immunoblotted with human sera. Sera were obtained from patients with MVL caused by *L. donovani infantum* (lanes 1 to 7), zoonotic cutaneous leishmaniasis caused by *L. major* (lanes 8 to 12), and healthy children (lanes 13 to 14).

tivities (lanes 5 and 6). Interestingly, sera from six children with asymptomatic leishmanial infection were also unreactive with the P32 antigen (lane 7). However, significant reactivities were obtained with sera from patients with Chagas' disease (lane 8).

The MOD was 0.577 ± 0.083 (mean + standard error) for sera from patients with MVL and 0.105 ± 0.005 for sera from healthy controls. The cutoff value for reactivity with the P32 antigen, defined as the MOD + 2SDs of the value for the control group (group VI), was 0.161. This cutoff value allowed us to estimate the sensitivity and specificity of the ELISA with the electroeluted P32 antigen. A specificity of 94.3% and a sensitivity of 94.3% were obtained.

When ELISA was performed with SLA (Fig. 4B) the MOD for each group was higher (cutoff OD = 0.344). A significant reactivity was observed with some sera from patients with HCL caused by *L. major* (lane 2). This cross-reactivity was even more prominent when MBA was used as the antigen (Fig. 4C) and extended to some of the sera from patients with HCL caused by *L. infantum* (lane 3) (cutoff OD = 0.349). The sensitivities of the tests with P32, SLA, and MBA antigens were 94.3, 71, and 83.5%, respectively, whereas the specificities under the same conditions were found to be 94.3, 91.4, and 88.5%, respectively.

When the cutoff value of the ELISA was defined as the MOD + 2SD obtained with sera from patients with clinical conditions other than MVL (groups II to VII), the specificities of the ELISAs with SLA or MBA decreased from 91.4 to 80.6% and 88.5 to 65.9%, respectively, while the specificities of the ELISA with purified P32 antigen were still high (90.3%), as were the values of the accuracy of positive prediction (73.4, 43.9, and 35.3%, respectively, for P32, SLA, and MBA).
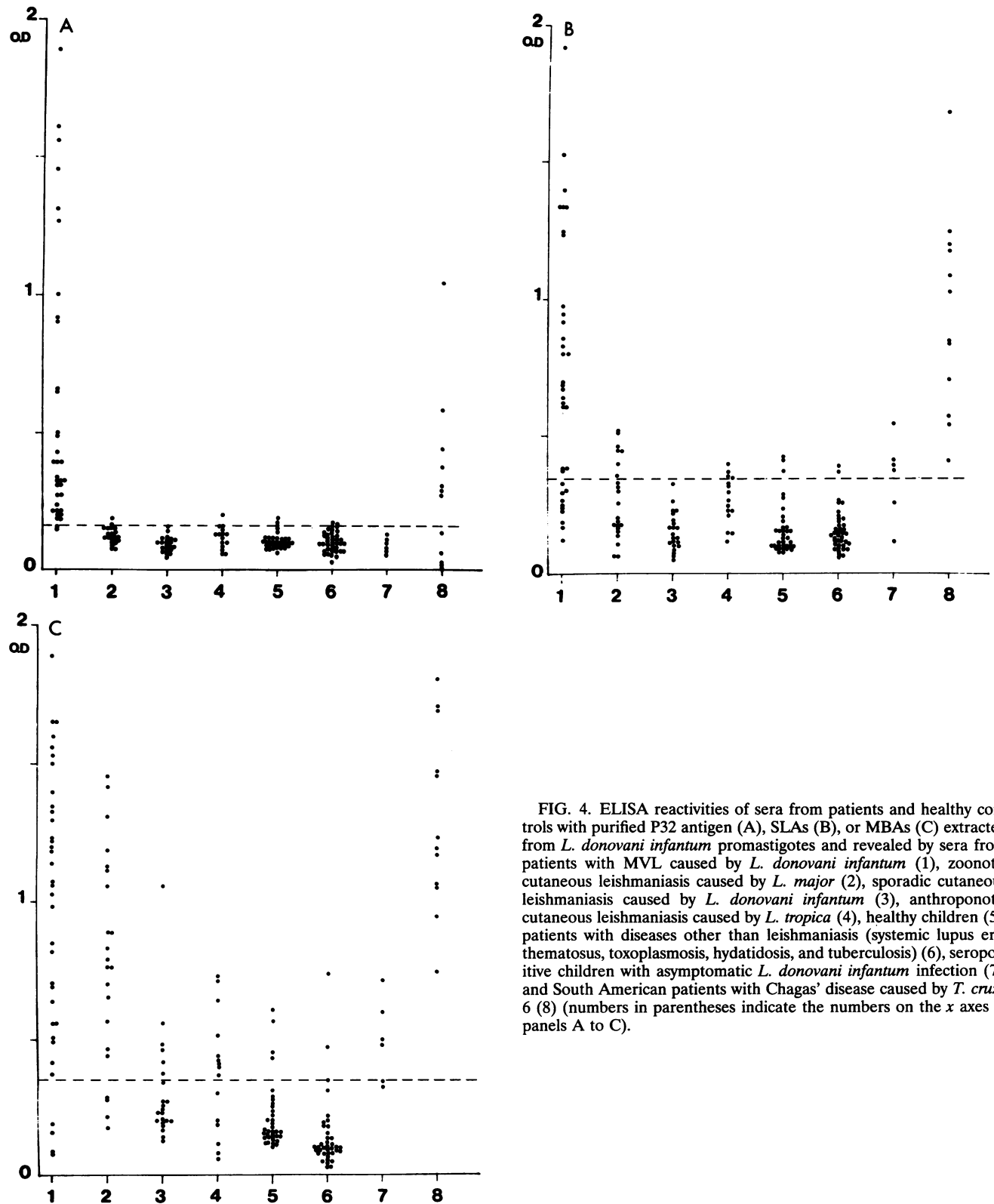
FIG. 4. ELISA reactivities of sera from patients and healthy controls with purified P32 antigen (A), SLAs (B), or MBAs (C) extracted from *L. donovani infantum* promastigotes and revealed by sera from patients with MVL caused by *L. donovani infantum* (1), zoonotic cutaneous leishmaniasis caused by *L. major* (2), sporadic cutaneous leishmaniasis caused by *L. donovani infantum* (3), anthroponotic cutaneous leishmaniasis caused by *L. tropica* (4), healthy children (5), patients with diseases other than leishmaniasis (systemic lupus erythematosus, toxoplasmosis, hydatidosis, and tuberculosis) (6), seropositive children with asymptomatic *L. donovani infantum* infection (7), and South American patients with Chagas' disease caused by *T. cruzii* 6 (8) (numbers in parentheses indicate the numbers on the *x* axes in panels A to C).

DISCUSSION

In order to identify antigens from *L. donovani infantum* of potential use in the development of a specific and sensitive means of serodiagnosis of MVL, sera from patients with MVL or the prevalent clinical forms of cutaneous leishmaniasis in the sub-Mediterranean countries were studied by immunoblot analysis. Two different preparations of *L. donovani infantum* extracts were used for this purpose: MBAs and SLAs. The patterns of reactivity of individual serum specimens with the MBA preparation were found to be less complex than those with the SLA preparation because of the limited number of reactive bands in the former preparation, usually between 5 and 10, which allowed for the easy interpretation of the results. More important was the fact that the use of membrane preparations allowed for a clear means of discriminating between patients with MVL and cutaneous leishmaniasis, especially HCL caused by *L. major*. A P32 antigen present in the membrane preparations of *L. donovani infantum* parasites was recognized by 95% of sera from patients with MVL but none of the sera from patients with cutaneous leishmaniasis or the controls. The use of soluble antigens for immunoblot analysis was clearly less discriminatory. Surprisingly, Gp63, the major surface antigen of *Leishmania* parasites, was found to be the immunodominant antigen of *L. major* in sera from patients with zoonotic cutaneous leishmaniasis, whereas it did not induce any significant antibody response in the sera of patients with MVL. These results extend previous studies in identifying parasite antigens that are potentially useful for the specific immunodiagnosis of the disease (7, 19, 27). Strict comparisons between our results and other results reported in the literature are rather difficult because of the variability in the techniques and the use of different strains and antigens. However, it appears likely that the immunodominant P32 membrane-associated antigen characterized in the present study corresponds to the P32-35 antigens reported by other investigators, who used whole parasite extracts (11, 26). However, studies with radioiodinated membranes of *L. donovani infantum* promastigotes have failed to detect any protein in the range of 30 to 35 kDa that was immunoprecipitable by sera from patients with MVL (21). This result may suggest that the membrane P32 antigen is not externally oriented. Additional studies are needed to localize more precisely this antigen and to determine whether it is also expressed by amastigotes.

Immunoblotting is a time-consuming technique and is hardly applicable for mass epidemiologic surveys in the routine laboratory. A simpler ELISA was therefore developed by using the P32 antigen extracted from the gel by electroelution. The results demonstrate the higher degree of sensitivity and specificity of the ELISA with purified P32 antigen over those of the ELISA with crude extracts (MBA or SLA preparation). Moreover, both immunoblotting and ELISA showed that the P32 antibody response was specific for patients with MVL and could not be detected in patients with cutaneous leishmaniasis caused by *L. major*, *L. donovani infantum*, or *L. tropica* or in control patients with nonleishmanial diseases or healthy individuals. These results agree with those of Reed et al. (26), who stressed the potential use of the 32- to 36-kDa region for serologic distinction between infection with *L. donovani chagasi* and *L. mexicana amazonensis*. Interestingly, 58% of serum specimens from South American patients with Chagas' disease (trypanosomiasis) reacted with the P32 antigen of *L. donovani infantum*. Therefore, the P32 antigen appears to be a good and reliable marker for MVL in the countries around the Mediterranean (North Africa and the Near and Middle East) where visceral leishmaniasis is endemic and coexists with the various clinical forms of cutaneous leishmaniasis but where trypanosomiasis is absent.

Although visceral leishmaniasis is usually a severe disease, several studies in areas where the disease is endemic have shown that asymptomatic or subclinical infections in humans are far more common than the acute full-blown disease (1). A salient feature of the ELISA developed in the present study is its lack of reactivity with the P32 antigen in sera from children with asymptomatic leishmanial infections, whereas it reacted with crude parasite extracts. Considering the poor specificity of the clinical symptoms of visceral leishmaniasis (splenomegaly, fever, and weight loss), the ELISA with the P32 antigen will help to discriminate between children suffering from asymptomatic leishmanial infections and those with other pathological conditions which may mimic MVL to allow for an accurate diagnosis of MVL.

Our results have established that the P32 antibody response is significantly restricted to patients with MVL, whereas it is absent from those with the various cutaneous forms of the disease. Restriction of the P32 antigen to *L. donovani infantum* parasites could account for this result. However, this hypothesis was formally excluded since a homologous P32 membrane-associated antigen could be detected in *L. major* promastigotes which strongly reacted with the sera from patients with MVL but only weakly with sera from patients with HCL caused by *L. major* (group II), indicating that P32 is an immunodominant antigen only in patients with the disseminated disease.

Several nonexclusive mechanisms may explain the strikingly different immunogenicities of the P32 antigen and Gp63 in the sera of patients with MVL and HCL, respectively. (i) P32 is selectively expressed in the promastigote stage of *L. major* but in both the amastigote and the promastigote stages of *L. donovani infantum* parasites, whereas the opposite would be true for Gp63. (ii) B cells or B-cell subsets induced in the cutaneous lesions could be different from the cells induced in lymph nodes and spleens by *L. donovani infantum* in patients with the disseminated form of disease. (iii) T-cell help may involve different T-cell subsets in patients with the cutaneous or disseminated forms of the disease (15, 28). Additional studies on the P32 antibody responses in the murine model of experimental leishmaniasis are in progress in order to unravel these issues.

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