INFECTION AND IMMUNITY, May 1988, p. 1180–1186
0019-9567/88/051180-07$02.00/0

Vol. 56, No. 5

Identification of Monomeric and Oligomeric Forms of a Major *Leishmania infantum* Antigen by Using Monoclonal Antibodies

KETTY P. SOTERIADOU,[1]* ATHINA K. TZINIA,[1] MARIA G. HADZIANTONIOU,[2] AND SOCRATES J. TZARTOS[1]

Laboratories of Biochemistry[1] and Parasitology,[2] Hellenic Pasteur Institute, 127 Vassilissis Sofias Avenue, Athens 11521, Greece

Received 16 September 1987/Accepted 19 January 1988

Ten monoclonal antibodies (MAbs) produced against isolated *Leishmania infantum* membranes were used as probes of *L. infantum* membrane antigens. Western blots of *L. infantum* membranes, sodium dodecyl sulfate solubilized and heated at 100°C before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, showed that all 10 MAbs recognized a band at 58 kilodaltons (kDa). However, when solubilized membranes were not heated, 2 of the 10 MAbs recognized, in addition to the 58-kDa band, bands of higher molecular weight. Limited digestion of heated or nonheated membranes showed that both groups of MAbs (i.e., not capable or capable of binding to the high-molecular-weight bands) recognized the same proteolytic digests. Hydrophilic forms of the above proteins, possessing proteolytic activity, were detected and isolated by gel filtration. Protein staining of the isolated monomer analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, under reducing and heating conditions, revealed incomplete reduction of the 58-kDa protein. The reduced form of the 58-kDa protein migrated at 63 to 65 kDa and was not recognized by the MAbs. These results suggest the existence of a monomeric and an oligomeric form of the 58-kDa antigen. The observed inhibition of *Leishmania* promastigote-macrophage binding caused by MAbs representative of the two groups (capable of oligomeric and/or monomeric antigen recognition) suggest that the 58-kDa monomer and oligomer play an important role in promastigote-macrophage interaction. We suggest that the 58-kDa *L. infantum* antigen is the major surface *Leishmania* antigen (p63) identified by others.

Leishmania donovani, the etiologic agent of kala-azar or visceral leishmaniasis, is the cause of significant mortality throughout the world. In its insect vector, the phlebotomus sandfly, the protozoon exists in the promastigote form. In the mammalian host, it is converted into the amastigote form and multiplies within the phagolysosomes of macrophages (30). The mechanism whereby the protozoon penetrates, survives, and multiplies in macrophages is not yet well understood. Some molecules of the parasite membrane play a crucial role in the binding and penetration of the parasite into the macrophage and its survival within the phagolysosomes (8).

It has been shown that monoclonal antibodies (MAbs) are useful tools for the diagnosis of leishmaniasis and for biochemical and immunopathological characterization of the parasite itself. Species-specific MAbs against *L. mexicana* and *L. braziliensis* (27–29) as well as the *L. tropica* complex and the *L. donovani* complex (11, 19–22) have been produced for immunodiagnosis and taxonomic classification of *Leishmania* species. Surface antigenic changes during differentiation in vitro of *L. mexicana* were identified by MAbs (17). Furthermore, MAbs were successfully used to protect BALB/c mice from infection with *L. mexicana* (1).

A major *Leishmania* surface glycoprotein of approximately 63,000 molecular weight, termed p63, has been identified by using kala-azar sera and human cutaneous leishmaniasis sera (26). p63 appears to be common among a great number of *Leishmania* species and is recognized by rabbit anti-*L. donovani* immune serum (10, 16, 18, 25). A p63 has been purified from *Leishmania mexicana amazonensis* by MAb affinity binding; this glycoprotein inhibits leishmania-macrophage binding (7). The involvement of the *L. mexicana mexicana* p63 in the attachment of *Leishmania* promastigotes to macrophages has also been reported (31). p63 was characterized as an integral membrane protein (14). A hydrophilic form was identified during its purification (5). It has also been demonstrated that p63 is a protease (15), designated as promastigote surface protease (3), and has a common membrane anchor with *Trypanosoma* variant surface glycoprotein (4). Promastigote surface protease was identified in seven different species of *Leishmania* including *L. infantum* (6).

In this report we describe the identification and partial purification of the monomeric and oligomeric forms of a predominant *L. infantum* protein of 58 kilodaltons (kDa) which possess proteolytic activity. The reduced form of this molecule, not recognized by the MAbs, migrated at 63 to 65 kDa. The observed in vitro inhibition of *L. infantum* promastigote-macrophage interaction caused by the MAbs recognizing the above proteins implies that the 58-kDa monomer and oligomer might be involved in the binding of promastigotes to macrophages and could subsequently be used for immunoprophylaxis. Current information on p63 and our results suggest that the 58-kDa protein identified in this study is the major *Leishmania* surface protein p63.

MATERIALS AND METHODS

Reagents. Hemoglobin, neopeptone, lactalbumin hydrolysate, Noble agar, and Freund adjuvant were purchased from Difco Laboratories (Detroit, Mich.); brain heart infusion was from Oxoid Ltd. (Basingstoke Heints, England); phenylmethylsulfonyl fluoride and Nonidet P-40 were from BDH Chemicals LTD (England); sodium dodecyl sulfate (SDS) was from Fluka AG (CH-9470 Buchs); Dulbecco modified Eagle medium was from Flow Laboratories (Irvine, Scotland); fetal calf serum was from Gibco Ltd. (Paisley, Scotland); and aminopterin, hypoxanthine, thymidine, EGTA (polyethyleneglycol-bis-*N*,*N*'-tetraacetic acid), EDTA, *N*-

* Corresponding author.

ethylmaleimide, pepstatin, aprotinin, iodoacetamide, Triton X-100, protein A, bovine serum albumin (BSA), and rabbit anti-mouse immunoglobulins were from Sigma Chemical Co. (St. Louis, Mo.). All other chemical products were purchased from E. Merck AG (Darmstadt, Federal Republic of Germany).

Leishmanias. The strain referred to as *L. infantum* in this study was isolated in Greece from a person with visceral leishmaniasis. The strain was typed according to its excreted factor serotype and according to electrophoretic mobilities of malate dehydrogenase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, as *Leishmania donovani infantum H* HOM-Gr78-L4 (34). *L. infantum* promastigotes were cultured at 27°C in a monophasic medium consisting of hemoglobin (1 g/liter), neopeptone (5 g/liter), brain heart infusion (5 g/liter), lactalbumin hydrolysate (3 g/liter), 14 mM glucose, and 1% human hemolyzed blood. Parasites were subcultured every 3 to 4 days (M. Hadziantoniou, Ph.D. thesis, Medical School University of Athens, Athens, Greece, 1987).

Preparation of *Leishmania* membranes. Membrane preparations of *L. infantum* promastigotes were obtained by disruption of the promastigotes, followed by subfractionation by differential centrifugation and then isolation on sucrose density gradients by the method of Dwyer (13). Washed cell pellets and isolated membranes were suspended in phosphate-buffered saline (PBS; Miles Laboratories, Inc., Elkhart, Ind.), and the cell suspension was made to 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 2 mM EGTA, 2.5 mM *N*-ethylmaleimide, 20 μg of pepstatin per ml, 2 U of aprotinin per ml, and 10 mM iodoacetamide and stored at −70°C until used.

Production of MAbs. BALB/c mice were injected with intact or detergent (2% SDS, 2% Triton X-100, or 2% sodium cholate)-solubilized promastigote membranes in incomplete Freund adjuvant. They subsequently received at least two inoculations at 4-week intervals and were boosted 4 days before sacrifice. Hybrid cells secreting MAbs were produced by fusing 3×10^7 cells of the nonproducer mouse myeloma S194/5.XXO.BU.1 (S194) cells with spleen cells isolated from the immunized mice (10^8) in 0.5 ml of 45% (vol/vol) polyethylene glycol 4000 (Merck). Fusions were carried out by the direct-cloning method of Tzartos (35), which is a modification of the classical cell fusion technique of Kohler and Milstein (23). The modification is based on the introduction of agar immediately after fusion of the cells. Fused cells were suspended in 0.25% agar in Dulbecco medium containing 20% heat-inactivated fetal calf serum, 2 mM glutamine, 50 μM 2-mercaptoethanol (2-ME), antibiotics, and hypoxanthine-aminopterin-thymidine (HAT medium). The cell suspension was distributed into 96-well flat-bottomed culture plates (40 μl/well) or 24-well plates (200 μl/well). HAT medium was then added on the solidified agar cell layer, and culture plates were incubated at 37°C in humidified air containing 10% CO_2. Cell colonies were visible 2 weeks after fusion, and the presence of antibody in the supernatants was assessed by a solid-phase radioimmunoassay. Colonies from positive wells were transferred to individual wells and then tested for antibody production. Positive colonies were cloned twice in agar to ensure homogeneity.

Solid-phase radioimmunoassay. A solid-phase radioimmunoassay was used to identify hybrids secreting MAbs to *L. infantum* (32). Briefly, *L. infantum* membranes diluted in bicarbonate buffer (pH 9.6) were plated in polyvinyl chloride flat-bottomed microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated overnight at 4°C. The microdilution plates were washed three times in wash buffer (PBS containing 0.05% Tween 20 and 0.02% NaN_3) and incubated for a minimum of 1 h at room temperature with PBS containing 3% BSA. After a further three washes in wash buffer, culture medium supernatants were added and incubated for 3 h at room temperature or overnight at 4°C. Immune mouse serum (diluted 1:100) and cell culture medium were used as positive and negative controls, respectively. The plates were then washed three times with wash buffer. Rabbit anti-mouse immunoglobulins (0.2 μl per well) were added to the wells and incubated for 2 h at room temperature, followed by a further three washings. ^{125}I-labeled protein A (10,000 cpm/well) was added and incubated for 1 h at room temperature. Finally, the plates were washed five times, and the radioactivity bound to each well was removed by SDS and measured in a Kontron MDA 312 multidetector radioimmunoassay analyzer.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 7.5 or 10% polyacrylamide gels by the method of Laemmli (24). Samples were SDS solubilized and either not heated or heated for 5 min at 100°C. When membranes were used, a clearing centrifugation was performed (10,000 rpm, 10 min, 4°C) on SDS-solubilized membranes before electrophoresis. Reduction, when indicated in the legends, was done with 5% 2-ME or 10 mM dithiothreitol. Gels were either stained with 0.2% Coomassie blue or dried and autoradiographed (for ^{125}I-labeled samples). A low-molecular-weight standard mixture (Pharmacia) was used for estimating the M_r.

Western blotting (immunoblotting). Isolated *L. infantum* membranes were electrophoretically transferred from SDS-polyacrylamide gels to nitrocellulose paper by the procedure first described by Towbin et al. (33). The nitrocellulose strips were incubated for 1 h at 40°C in PBS containing 3% BSA and then overnight at 4°C in nonimmune mouse serum, culture medium, or hybridoma culture medium supernatants. Control mouse sera were diluted 1:100 in wash buffer (0.5% BSA in PBS). The strips were then washed three times and incubated with rabbit anti-mouse immunoglobulins for 3 h at room temperature. After three more washings, the strips were incubated with ^{125}I-protein A for 1 h at room temperature. The strips were washed at least four times, air dried, and exposed to autoradiography at −70°C with Kodak X-Omat S film.

Limited digestion. *L. infantum* membranes were incubated at room temperature for 1 h in the presence of 0.1 μg of *Staphylococcus aureus* V8 protease per well (10). The resulting digests were analyzed first by SDS-PAGE and then by Western blotting with the anti-*L. infantum* MAbs as probes.

Iodination. Lactoperoxidase-catalyzed iodination was used (12).

Gel filtration. Hydrophilic forms of the studied antigens in PBS were loaded on a Superose 12 TM column and analyzed by fast-protein liquid chromatography. The column was equilibrated with PBS (pH 7.4), and the samples were chromatographed at a flow rate of 0.25 ml/min. Fractions (0.5 ml) were collected and subjected to SDS-PAGE followed by protein staining and/or analyzed by Western blotting. In some experiments where a small amount of ^{125}I-labeled material was added to the sample, the eluants were counted to detect any radioactivity present.

***L. infantum* infection of mouse macrophages.** The mouse macrophage cell line J774G8 was cultured on cover slips placed in petri dishes. The cells (10^6/ml) were allowed to

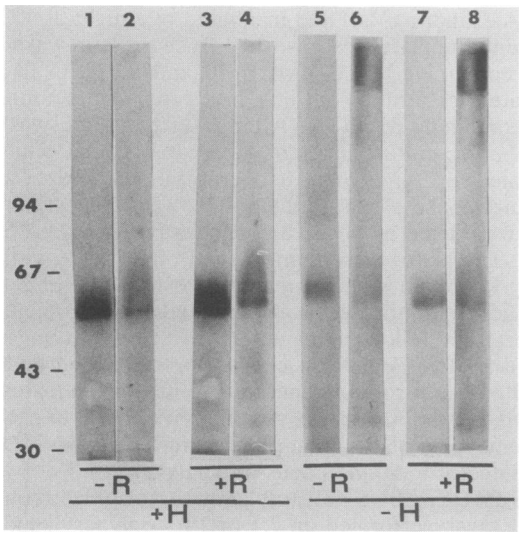

FIG. 1. Autoradiographs of *L. infantum* membranes identified by Western blotting with MAbs to *L. infantum* as probes. SDS-PAGE (7.5% polyacrylamide) was performed under reducing (+R), or nonreducing (−R) conditions. Membranes were either heated at 100°C (+H), or not heated (−H). MAb LD16 recognized only a 58-kDa band under all conditions (lanes 1, 3, 5, and 7). LD20 recognized a 58-kDa band when membranes were heated (lanes 2 and 4) and, in addition to the 58-kDa band, a band of approximately 200 kDa when membranes were not heated (lanes 6 and 8). LD23 and six other MAbs gave the same recognition pattern as LD16, and LD24 gave the same pattern as LD20.

adhere at 37°C in an atmosphere of 10% CO_2 for a minimum of 1 h. The cover slips were then washed to remove nonadherent cells, and *L. infantum* promastigotes, either nontreated or treated with MAbs, were added at a ratio of 10 parasites per cell. After 1 h of incubation the cover slips were washed vigorously with medium, fixed in methanol, and stained with Giemsa. The number of cells infected was determined by counting 500 cells in a Giemsa-stained culture (9). Before infection of macrophage monolayers, parasites were incubated with different dilutions of MAbs or medium for 1 h at room temperature. The parasites were then washed, suspended in RPMI 1640 medium with 10% fetal calf serum, and added to the macrophages.

Immunoglobulin subclass typing. Immunoglobulin subclass typing of the MAbs was determined by double immunodiffusion against mouse immunoglobulin subclass-specific sera in 1% Noble agar dissolved in PBS.

RESULTS

Spleen cells of BALB/c mice immunized with isolated *L. infantum* membranes (either intact or solubilized) were fused with S194 mouse myeloma cells. Ten stable hybridomas, producing MAbs to promastigote membrane antigens, were produced by the direct-cloning method (35) and detected by a solid-phase radioimmunoassay with intact, isolated membranes. The homogeneity of the hybridomas was insured by recloning them twice in 0.25% agar. All 10 MAbs produced were immunoglobulin M molecules.

Identification and characterization of membrane antigens recognized by anti-*L. infantum* MAbs. The identification of membrane antigens by the 10 MAbs was carried out by Western blotting followed by autoradiography. Blots of SDS-solubilized *L. infantum* membranes, heated at 100°C (with or without 2-ME) before analysis by SDS-PAGE, showed that all 10 *L. infantum* MAbs recognized a band at 58 kDa. However, when non-heat-treated solubilized membranes (in the presence or absence of 2-ME) were used, the above MAbs gave two distinct recognition patterns: eight of the MAbs still recognized the 58-kDa band (e.g., LD16 and LD23) whereas two (MAbs LD20 and LD24) recognized, in addition to the 58-kDa band, bands of higher molecular mass (approximately 200 kDa) (Fig. 1). Occasionally, when membranes were not heated before analysis by SDS-PAGE, multiple intermediate-molecular-size bands were detected which may represent proteolytic breakdown products of the 200-kDa band. The presence of 2-ME in the sample buffer did not affect the migration of the high-molecular-mass bands. Therefore, when SDS-solubilized membranes were not heat treated, MAbs LD20 and LD24 recognized, in addition to the 58-kDa band, bands of high molecular mass, whereas when solubilized membranes were heated at 100°C these MAbs recognized only the 58-kDa band.

Autoradiographs of ^{125}I-labeled isolated membranes subjected to SDS-PAGE showed that a 58-kDa polypeptide was mainly labeled. The ^{125}I-labeled polypeptide comigrated with the 58-kDa polypeptide detected on blots by the MAbs (Fig. 2).

The possibility that the 58-kDa polypeptide detected by MAbs LD16 and LD23 is identical to the 58-kDa polypeptide detected by LD20 and LD24 was investigated by peptide mapping. Limited digestion of *L. infantum* membranes with *S. aureus* V8 protease, followed by SDS-PAGE analysis of the digests and Western blotting, showed that all four MAbs (LD16, LD20, LD23, and LD24) recognized the same digests (Fig. 3). Thus, they recognized the same 58-kDa polypeptide. The above results strongly suggest the existence of a monomeric and an oligomeric form of the 58-kDa polypeptide.

Identification, partial purification, and proteolytic activity of hydrophilic forms of the 58-kDa monomer and oligomer. Evidence for the generation of hydrophilic forms of the 58-kDa monomer and oligomer was obtained by the following experiments. Membranes were dialyzed against PBS for 4 h and then centrifuged. Both the pellet and the supernatant were analyzed by Western blotting with MAbs LD16 and LD20 (i.e., representatives of the two groups of MAbs). Although the MAbs recognized both the 58-kDa monomer and oligomer in the pellet, significant fractions of both proteins were also detected in the supernatant (Fig. 4). Thus,

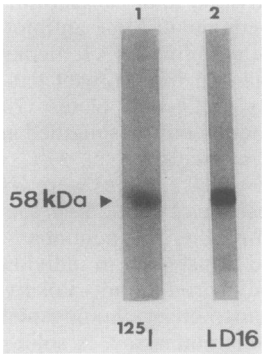

FIG. 2. Autoradiograph of ^{125}I-labeled *L. infantum* membranes. The ^{125}I-labeled 58-kDa antigen (lane 1) comigrated with the 58-kDa antigen detected on blots by MAb LD16 (lane 2). SDS-PAGE was performed with 7.5% polyacrylamide gels.

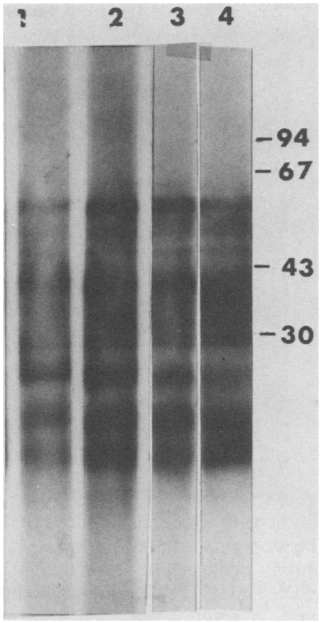

FIG. 3. *L. infantum* membranes submitted to limited digestion with *S. aureus* V8 protease. The digests were reduced and heated before SDS-PAGE (10% polyacrylamide) and then analyzed by Western blotting. MAbs LD16, LD23, LD20, and LD24 recognized the same proteolytic digests (lanes 1, 2, 3, and 4, respectively).

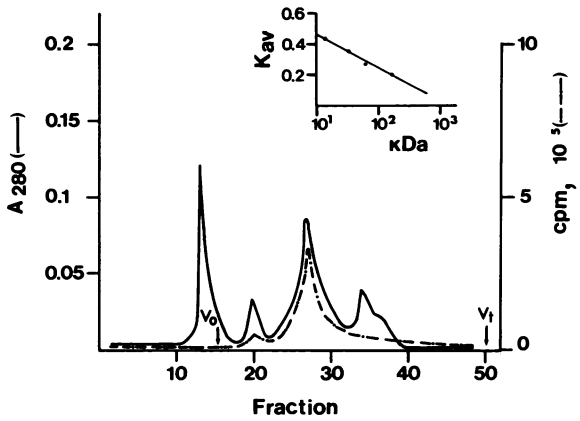

FIG. 5. Partial purification of the hydrophilic 58-kDa monomer and oligomer by gel filtration. A Superose-TM fast-protein liquid chromatography column (Pharmacia) was equilibrated with PBS (pH 7.4) and calibrated with an immunoglobulin G MAb (150 kDa), BSA (67 kDa), ovalbumin (43 kDa), and RNase A (13.7 kDa). The column then was loaded with hydrophilic forms of the studied antigens (supernatant), and fractions of 0.5 ml were collected. Absorbance at 280 nm revealed four peaks (——). The molecular masses of the second and third peak were approximately 200 and 60 kDa, respectively. V_t is the total volume and V_0 is the void volume of the column. When the column was loaded with a small amount of iodinated supernatant and fractions were measured for radioactivity, a major radioactive peak was detected coinciding with the peak of 60 kDa (–·–). The inset shows the partition coefficient (K_{av}) versus size.

we have detected hydrophilic forms of the proteins. Radioiodinated supernatant (obtained by dialyzing the membranes against PBS) subjected to SDS-PAGE gave the same autoradiography pattern as the ^{125}I-labeled membranes (data not shown).

The supernatant containing soluble forms of the 58-kDa monomer and oligomer was analyzed by gel filtration on a Superose-12TM column by fast-protein liquid chromatography. Absorbance at 280 nm revealed four major peaks (Fig. 5). The contents of each peak were analyzed by SDS-PAGE. Gels were either stained for protein or analyzed by Western blotting. Oligomeric forms of the 58-kDa protein were detected by the MAbs in the second peak (~200 kDa). The 58-kDa protein was detected in the third peak (~60 kDa) (Fig. 6a). Staining for protein gave the same results (Fig. 6b). The isolated 58-kDa protein was analyzed by SDS-PAGE and stained for protein. The addition of 5% 2-ME or 10 mM dithiothreitol and alkylation of the reduced disulfide bonds revealed, in addition to the 58-kDa band, a band of 63 to 65 kDa (Fig. 7). However, the reduced form is not recognized by the MAbs, since it was not detectable on blots under reducing conditions (Fig. 1, lanes 3 and 4). The presence of the 58-kDa protein in the third fast-protein liquid chroma-

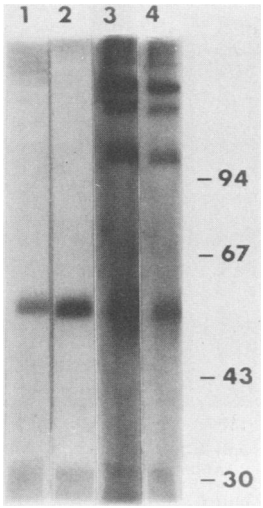

FIG. 4. Generation of hydrophilic forms of the 58-kDa monomer and oligomer. *L. infantum* membranes were dialyzed against PBS for 4 h and then centrifuged. Both the pellet and the supernatant were analyzed by Western blotting. The antigens recognized by MAbs LD16 and LD20 were detected both in the supernatant (lanes 1 and 3, respectively) and the pellet (lanes 2 and 4, respectively).

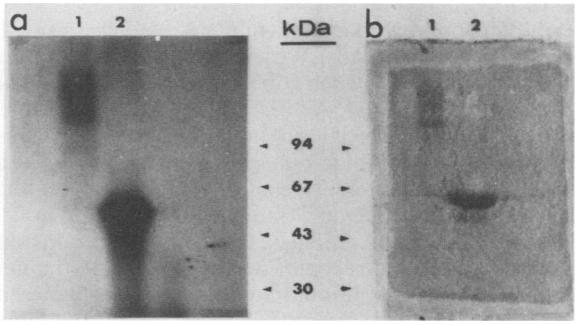

FIG. 6. Detection of the 58-kDa monomer and oligomer partially isolated by gel filtration as described in the legend to Fig. 5. Fractions of the second and third peak (lanes 1 and 2, respectively) were pooled, concentrated by ultrafiltration, and analyzed by SDS-PAGE (7.5% polyacrylamide). Samples were neither heated nor reduced. (a) Western blotting analysis. The blots were probed with a mixture of MAbs (LD16 and LD20). (b) Coomassie blue staining.

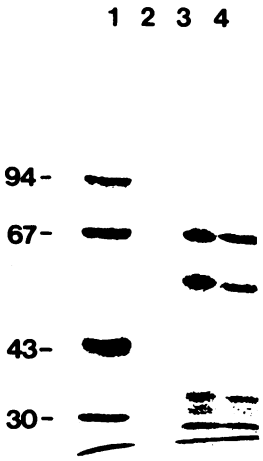

FIG. 7. SDS-PAGE analysis and Coomassie blue staining of the purified 58-kDa antigen. SDS-PAGE (7.5% polyacrylamide) was performed under reducing conditions. The 58-kDa antigen reduced either with 5% 2-ME (lane 3) or 10 mM dithiothreitol (30 min at 37°C and alkylated with 10 mM iodoacetamide; lane 4). Other lanes contained molecular mass markers in kDa (lane 1) and SDS-PAGE sample buffer (lane 2). Occasionally degradation products were detected (bottom of lanes 3 and 4).

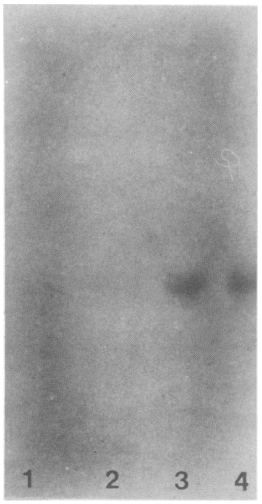

FIG. 8. Proteolytic activity of partially purified 58-kDa monomer and oligomer. The contents of the second and third fast-protein liquid chromatography peaks were preincubated with BSA for 4 h and analyzed by SDS-PAGE (7.5% polyacrylamide). Samples were heated and reduced. A reference standard comprised only of an equal quantity of BSA was run on the same gel (lanes 3 and 4). The lack of the BSA band is due to the digestion of BSA by the 58-kDa monomer (lane 1) and the 58-kDa oligomer (lane 2).

tography peak was confirmed by the analysis of ^{125}I-labeled supernatant (whose major labeled antigen was the 58-kDa band); the major peak comigrated with the third peak of the unlabeled supernatant (Fig. 5).

To test whether the 58-kDa oligomer and/or monomer has proteolytic activity, the partially purified (by gel filtration) hydrophylic proteins were incubated with BSA for 4 h at room temperature before SDS-PAGE. Coomassie blue staining of the gel showed that at the migration distance of BSA very little or no protein was visualized in the lanes where BSA had been preincubated with the content of the third peak (which included the 58-kDa monomer) and with the content of the second peak (which included the 58-kDa oligomer) (Fig. 8). The 58-kDa monomer and oligomer are not visible in Fig. 8 (lanes 1 and 2, respectively), because the protein concentration used was below the sensitivity of Coomassie blue staining. The protease inhibitor iodoacetamide completely inhibited the proteolytic activity (data not shown).

Inhibition of parasite attachment to macrophages caused by the anti-*L. infantum* MAbs. We examined the in vitro interference with parasite penetration into mouse macrophages by the MAbs (Fig. 9). MAbs LD16 and LD23 (capable of monomeric antigen recognition) inhibited about 30% parasite attachment to mouse macrophages, and MAbs LD20 and LD24 (capable of monomeric and oligomeric antigen recognition) exhibited about 50% inhibition. Anti-tubulin MAb (kindly provided by P. Libery, Hellenic Pasteur Institute) and anti-acetylcholine receptor MAbs 13 and 136 (immunoglobulin M molecules [35]) did not affect promastigote binding to macrophages (Fig. 9). The effect of the anti-*L. infantum* MAbs seems to be specific, since treatment of macrophages with these MAbs before infection had no effect, nor did these MAbs affect promastigote mobility. Therefore, the surface antigens recognized by these MAbs may play an important role in parasite-macrophage interaction.

DISCUSSION

This study was aimed at identifying and characterizing *L. infantum* membrane antigens of potential pathophysiological significance by the use of MAbs. We identified monomeric and oligomeric forms of a 58-kDa *L. infantum* antigen. Hydrophilic forms of the 58-kDa monomer and oligomer, possessing proteolytic activity, were detected and partially purified. Reduction of the 58-kDa purified monomer revealed a 63- to 65-kDa reduced form not recognized by the MAbs.

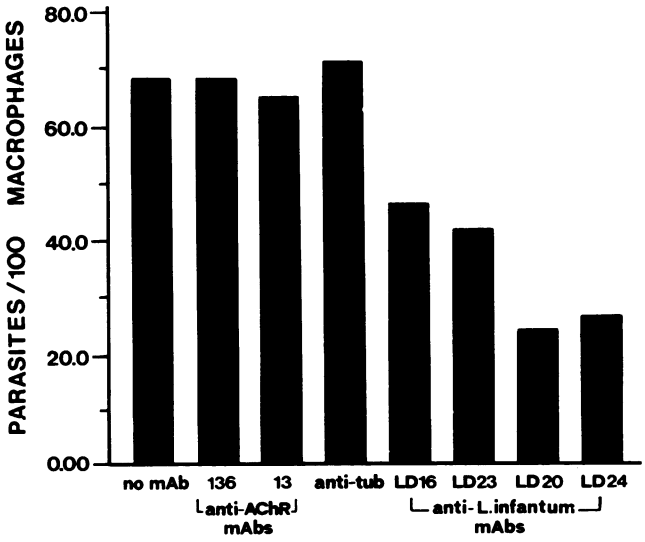

FIG. 9. Effect of anti-*L. infantum* MAbs on the attachment of promastigotes to mouse macrophage cell line J774G8. The anti-*L. infantum* MAbs LD16 and LD23 inhibited about 30% parasite attachment, and LD20 and LD24 inhibited about 50% parasite attachment. Anti-acetylcholine receptor (AChR) MAbs (immunoglobulin M) and the anti-tubulin MAb (anti-tub) used as negative controls had no effect on the binding of *L. infantum* to cells.

The 58-kDa oligomer appears to be a heat-modifiable *Leishmania* membrane antigen. Heating probably induces changes in antigen-SDS interaction. This phenomenon is not unique to the 58-kDa *L. infantum* antigen, since it has been demonstrated by SDS-PAGE that certain envelope proteins of *Bordetella pertussis* are heat modifiable (2). Heating of oligomers in SDS may result in dissociating them, and when analyzed by SDS-PAGE they migrate as monomers (2). The 58-kDa oligomer does not appear to be formed by disulfide bonds, since reduction by 2-ME without heat treatment does not affect its migration. Hence, the 58-kDa oligomer is comprised of noncovalently associated subunits. MAbs LD20 and LD24 seem to have a higher affinity for the oligomer than for the 58-kDa monomer, since on blots the high-molecular-weight bands appear to be more intense. It thus seems probable that the oligomeric conformations favor binding of MAbs LD20 and LD24 to the corresponding epitopes. MAbs LD16 and LD23 bind under all conditions to the 58-kDa monomer. Hence, their corresponding epitopes may be on the sites of attachment of the 58-kDa subunits. The MAbs used in this study do not bind to the reduced linearized form. Maintenance of the tertiary structure of the 58-kDa antigen seems to be necessary for MAb binding to it.

Membrane proteins may aggregate and so appear as larger oligomers. However, nonspecific aggregation of the 58-kDa protein is unlikely for the following reasons. (i) Gel filtration chromatography repeatedly showed that the oligomeric forms were eluted in a symmetrical peak of approximately 200 kDa. This was independent of the protein concentration used. (ii) The oligomeric forms of the 58-kDa protein were also obtained in the presence of the nonionic detergents Triton X-114 and Triton X-100, which were used for their purification (K. P. Soteriadou, manuscript in preparation). This was performed by a modification of the phase separation method in Triton X-114 and its substitution by Triton X-100 on a DEAE-cellulose column and subsequent purification of the eluted protein on a Mono-Q column (5). Therefore, the appearance of the ~200-kDa oligomeric form seems to be independent of the conditions used (no detergent in Fig. 5; SDS in Fig. 1; Triton X-114 and X-100). (iii) In a random aggregation of the 58-kDa monomers all or most of the anti-58-kDa MAbs would be expected to bind to the aggregates because masking or denaturation of antigenic sites would be random. Thus each site would always be available in at least a fraction of the molecules. However, we repeatedly found that only 2 MAbs of the 10 used bound to the high-molecular-weight bands, suggesting a specific assembly of the monomers. (iv) The existence of a 58-kDa oligomeric form of *L. infantum* antigen is compatible with the data presented on *L. mexicana amazonensis* (7), suggesting a polymeric form of the p63 (six subunits) and the presence of p63 dimers in *L. major* LEM513 (4).

Sufficient experimental evidence supports the identity of the 58-kDa antigen (identified in this study) as p63 (3–6, 7, 10, 14, 15, 18, 25, 26). This includes the following. (i) The 58-kDa antigen is recognized by all of our MAbs, implying that this protein is very immunogenic and abundant on the surface of *Leishmania* species. Similarly, p63 is considered as the major component of *Leishmania* membrane conserved among species (5, 10, 18, 26). (ii) Membrane iodination results in labeling of only the 58-kDa antigen, which is in accordance with published data concerning the predominant labeling of p63 in many *Leishmania* species including *L. infantum* (3, 5, 6, 10, 14, 18, 25). (iii) The shift observed in the migration of the 58-kDa antigen under reduced conditions of SDS-PAGE is similar to the well-documented shift of p63 in some *Leishmania* species (5, 10, 18). The presence of the 58-kDa form in the reduced gels is possibly due to incomplete reduction of internal sulfhydryl groups of the 58-kDa antigen. (iv) Hydrophilic forms of the 58-kDa antigen were detected and partially purified. This characteristic of solubility of the 58-kDa antigen is similar to the generation of a hydrophilic p63 during its purification (3). The higher amounts of hydrophilic 58-kDa antigen obtained, compared with those obtained by Bouvier et al. (5), may be due to the existence of more endogenous lipases in our *L. infantum* preparations. (v) *L. infantum* p63 is a protease (6). We also demonstrated that the 58-kDa monomer and oligomer possess proteolytic activity. Finally, the anti-58-kDa MAbs recognized the p63 from *L. major* LEM513 (5), kindly provided by C. Bordier (Université de Lausanne).

The 58-kDa monomer and oligomer identified in this study, which are homologous to p63, play an important role in parasite-macrophage binding as observed by the 30 and 50% inhibition, respectively, of parasite attachment to mouse macrophages with the corresponding MAbs. Therefore, further study of these antigens may be of pathophysiological interest.

ACKNOWLEDGMENTS

We are grateful to C. Serie and G. Cohen for their valuable suggestions and encouragement during the course of this work and C. Bordier (Université de Lausanne) for supplying p63 from *L. major* LEM513 and for valuable discussions. We also thank A. Garifallou for providing the *L. infantum* strain and A. Efthimiadis, M. Patrikoussis, and A. Kokla for technical assistance.

This work was supported by the Greek General Secretariat of Research and Technology and the French Delegation Generale à la Recherche Scientifique et Technique.

LITERATURE CITED

1. **Anderson, S., J. R. David, and D. McMahon-Pratt.** 1983. In vivo protection against *Leishmania mexicana* mediated by monoclonal antibodies. J. Immunol. **131**:1616–1618.
2. **Armstrong, S. K., and C. D. Parker.** 1986. Heat-modifiable envelope proteins of *Bordetella pertussis*. Infect. Immun. **54**: 109–117.
3. **Bordier, C.** 1987. The promastigote surface protease of *Leishmania*. Parasitol. Today **3**:151–153.
4. **Bordier, C., R. J. Etges, J. Ward, M. J. Turner, and M. L. Cardoso de Almeida.** 1986. *Leishmania* and *Trypanosoma* surface glycoproteins have a common glycophospholipid membrane anchor. Proc. Natl. Acad. Sci. USA **83**:5988–5991.
5. **Bouvier, J., R. J. Etges, and C. Bordier.** 1985. Identification and purification of membrane and soluble forms of the major surface protein of *Leishmania* promastigotes. J. Biol. Chem. **260**:15504–15509.
6. **Bouvier, J., R. Etges, and C. Bordier.** 1987. Identification of the promastigote surface protease in seven species of *Leishmania*. Mol. Biochem. Parasitol. **24**:73–79.
7. **Chang, C. S., and K. P. Chang.** 1986. Monoclonal antibody affinity purification of a *Leishmania* membrane glycoprotein and its inhibition of *Leishmania*- macrophage binding. Proc. Natl. Acad. Sci. USA **83**:100–104.
8. **Chang, K. P.** 1983. Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. Int. Rev. Cytol. Suppl. **14**:267–302.
9. **Chang, K. P., and D. Fong.** 1983. Cytopathology of parasitic disease. CIBA Found. Symp. **99**:113–137.
10. **Colomer-Gould, V., L. G. Quintao, J. Keithly, and N. Nogueira.** 1985. A common major antigen on amastigotes and promastigotes of *Leishmania* species. J. Exp. Med. **162**:902–916.
11. **de Ibarra, A. A. L., J. G. Howard, and D. Snary.** 1982. Monoclonal antibodies to *Leishmania tropica major*: specificities and antigen location. Parasitology **85**:523–531.
12. **Dissous, C., C. Dissous, and A. Capron.** 1981. Isolation and

characterization of surface antigen from *Schistosoma mansoni schistosomula*. Mol. Biochem. Parasitol. **3**:215–225.

13. **Dwyer, D. M.** 1980. Isolation and characterization of surface from *Leishmania donovani* promastigotes. J. Protozool. **27**:176–182.
14. **Etges, R., J. Bouvier, and C. Bordier.** 1985. The major surface protein of *Leishmania* promastigotes is anchored in the membrane by a myristic acid-labeled phospholipid. EMBO J. **5**:597–602.
15. **Etges, R., J. Bouvier, and C. Bordier.** 1986. The major surface protein of *Leishmania* promastigotes is a protease. J. Biol. Chem. **261**:9098–9101.
16. **Etges, R. J., J. Bouvier, R. Hoffman, and C. Bordier.** 1985. Evidence that the major surface proteins of three *Leishmania* species are structurally related. Mol. Biochem. Parasitol. **14**: 141–149.
17. **Fong, D., and K. P. Chang.** 1982. Surface antigenic change during differentiation of a parasitic protozoan, *Leishmania mexicana*: Identification by monoclonal antibodies. Proc. Natl. Acad. Sci. USA **79**:7366–7370.
18. **Gardiner, P. R., C. L. Jaffe, and D. M. Dwyer.** 1984. Identification of cross-reactive promastigote cell surface antigens of some leishmanial stocks by ^{125}I-labeling and immunoprecipitation. Infect. Immun. **43**:637–643.
19. **Greenblatt, C. L., G. M. Slutzky, L. de Ibarra, and D. Snary.** 1983. Monoclonal antibodies for serotyping *Leishmania* strains. J. Clin. Microbiol. **18**:191–193.
20. **Handman, E., and R. E. Hocking.** 1982. Stage specific, strain-specific and cross reactive antigens of *Leishmania* species identified by monoclonal antibodies. Infect. Immun. **37**:28–33.
21. **Jaffe, C. L., E. Bennett, G. Jr. Grimaldi, and D. McMahon-Pratt.** 1984. Production and characterization of species-specific monoclonal antibodies against *Leishmania donovani* for immunodiagnosis. J. Immunol. **133**:440–447.
22. **Jaffe, C. L., and D. McMahon-Pratt.** 1983. Monoclonal antibodies specific for *Leishmania tropica*. I. Characterization of antigens associated with stage and species-specific determinants. J. Immunol. **131**:1987–1993.
23. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
25. **Lemesre, J. L., F. S. Rizvi, D. Afchain, M. Sadigursky, A. Capron, and F. Santoro.** 1985. Subspecies-specific surface antigens of promastigotes of the *Leishmania donovani* complex. Infect. Immun. **50**:136–141.
26. **Lepay, D., A. Nogueira, and Z. Cohn.** 1983. Surface antigens of *Leishmania donovani* promastigotes. J. Exp. Med. **157**:1562–1572.
27. **McMahon-Pratt, D., E. Bennett, and J. R. David.** 1982. Monoclonal antibodies that distinguish subspecies of *Leishmania braziliensis*. J. Immunol. **129**:926–927.
28. **McMahon-Pratt, D., E. Bennett, G. Grimaldi, and C. L. Jaffe.** 1985. Subspecies and species-specific antigens of *Leishmania mexicana* characterized by monoclonal antibodies. J. Immunol. **134**:1935–1940.
29. **McMahon-Pratt, D., and J. R. David.** 1981. Monoclonal antibodies that distinguish between New World species of *Leishmania*. Nature (London) **291**:581–583.
30. **Pearson, R. D., D. A. Wheeler, L. H. Harrison, and H. D. Kay.** 1983. The immunobiology of leishmaniasis. Rev. Infect. Dis. **5**:907–927.
31. **Russell, D. G., and H. Wilhelm.** 1986. The involvement of the major surface glycoprotein (gp63) of *Leishmania* promastigotes in attachment to macrophage. J. Immunol. **136**:2613–2620.
32. **Stocker, J. W., and C. H. Heusser.** 1979. Methods for binding cells to plastic: application to a solid-phase radioimmunoassay for cell-surface antigens. J. Immunol. Methods **26**:87–95.
33. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
34. **Tzamouranis, N., L. F. Schnur, A. Garifallou, E. Pateraki, and C. Serie.** 1984. Leishmaniasis in Greece. I. Isolation and identification of the parasite causing human and canine visceral leishmaniasis. Ann. Trop. Med. Parasitol. **78**:363–368.
35. **Tzartos, S. J.** 1984. Monoclonal antibodies as probes of the acetylcholine receptor and myasthenia gravis. Trends Biochem. Sci. **9**:63–67.