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Human T-Cell Activation by 14- and 18-Kilodalton Nuclear Proteins of *Leishmania infantum*

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Leishmanial antigens which stimulate T lymphocytes from primed individuals may be candidates for a vaccine. We recently found a significant concordance between the humoral response specific for two proteins from *Leishmania infantum* promastigotes, p14 and p18, and a positive leishmanin delayed-type hypersensitivity reaction, testifying to the occurrence of cell-mediated immunity. In this communication, we describe a partial characterization of these antigens and an in vitro analysis of their capacity to activate primed human T cells. We showed, by immunofluorescent staining and through analysis of subcellular fractions by Western immunoblotting, that in stationary-phase promastigotes, p14 and p18 were located only in the parasite nuclei; in the middle of the log phase, a transitory and only weak expression outside the nucleus was detected. We then showed that p14 and p18 antigens shared a common epitope(s). Finally, we analyzed the in vitro proliferation and interleukin-2 production induced by leishmanial proteins in human peripheral blood mononuclear cells from sensitized subjects. We showed that in some individuals who have been exposed to *L. infantum* the specific response to the whole lysate was mostly due to the nuclear antigens. We demonstrated directly the capacity of nitrocellulose-bound p14 and p18 to activate in vitro all of the tested primed peripheral blood mononuclear cells, which contrasted with a lack of stimulatory activity of other membrane-bound leishmanial proteins. Taken together, our results suggest that an antigenic determinant(s) dominant for some individuals might exist on both antigens.

Infection with parasitic protozoa of the genus *Leishmania* affects mammalian hosts, particularly humans, in the Old World and the New World. It causes a spectrum of disease, depending on the host and on the *Leishmania* species involved. In humans, the infection ranges from self-healing lesions to disseminated cutaneous disease or highly destructive mucosal lesions and from asymptomatic infection to fatal visceral dissemination, causing one of the world's major health problems. The development of vaccines is the essential aim of studies on leishmaniasis. Moreover, the research on immunopathology of parasitic infections has led to highly pertinent models for understanding several aspects of regulation in the immune system (25, 35). Infection with *Leishmania major*, an agent of human cutaneous leishmaniasis, is at present the best-documented example of the differential activation of the Th1 $CD4^+$ lymphocyte subset in resistant strains of inbred mice and the Th2 subset in susceptible strains (6, 7, 24, 33). In this model, the production of Th1-type cytokines (interleukin-2 [IL-2] and gamma interferon) and that of Th2-type cytokines (IL-4, IL-5, and IL-10) are mutually exclusive. However, the situation appears less well defined, even in the murine system, for infection with *L. donovani*, which is responsible for visceral disease. Indeed, the functional studies with inbred strains have failed to correlate the long-term outcome of the disease with differential expansion of the T-cell subsets (12). Recent data obtained in studies with humans demonstrate that Th1- and Th2-like responses coexist in patients cured of visceral leishmaniasis (11, 13–15). At present, there are no data indicating that an association between the expansion of one of the two subsets could lead either to visceral disease or to establishment of asymptomatic infection.

Among several factors, not all of which are known, determining the balance between T-cell subsets and the outcome of infection are the characteristics of leishmanial antigens. It was recently reported that some T-cell epitopes that are protective in the murine host did not elicit immune response in humans (28); this result emphasizes the importance of testing leishmanial antigens with human cells. In this work, we examine two *L. infantum* proteins of 14 kDa (p14) and 18 kDa (p18). A potential use of these antigens in the diagnosis and epidemiology of human visceral leishmaniasis was recently described (19): a Western immunoblot analysis of antibodies to *L. infantum* in patients with visceral leishmaniasis showed that p14 and/or p18 was recognized by virtually all tested patient sera. Recently, in an epidemiological survey carried out in a region in Southern France where *Leishmania* infection is endemic, we found that over 80% of asymptomatic individuals presenting positive leishmanin skin reaction had detectable antibodies against p14 and/or p18 (17). The leishmanin skin test is an indicator of delayed-type hypersensitivity (DTH); it is performed with a suspension of phenol-killed parasites, i.e., with total leishmanial antigens. We thus investigated whether this biological concordance between results of the skin test and of the Western blot (17) was merely fortuitous or whether p14 and/or p18 might contribute not only to humoral but also to cell-mediated specific immunity. First, we examined some of their characteristics in *L. infantum* promastigotes. Then we analyzed their potential to stimulate in vitro human T cells primed in vivo.

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MATERIALS AND METHODS

Parasites, media, and culture. *L. infantum* MON1 (MHOM/FR/81/LPN5) promastigotes were grown at 25°C in RPMI 1640 medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum. This will be referred to as complete culture medium. In each experiment, parasite growth was partially synchronized by incubation in the absence of fetal calf serum for 24 to 48 h. Cells were then seeded at 0.5×10^6 to 1×10^6 cells per ml in complete medium and allowed to proliferate. Cell counts were performed with a Malassez hemocytometer after immobilization of cells with 1% formaldehyde in phosphate-buffered saline (PBS). Promastigote growth curves showed typical mid-log, late log, and stationary phases at days 3 and 4, 5, and 7, respectively. Samples of parasites were collected and washed three times with PBS, and various protein preparations were obtained, as described below.

Antileishmanial human sera. Sera were obtained from patients with acute leishmaniasis, from healed subjects, and from asymptomatic individuals with a positive DTH test (all subjects lived in the area of endemicity [16] mentioned above), as indicated in figure legends. Sera from subjects with a negative DTH reaction were used as controls.

Antigen preparations. Washed parasites were resuspended at 2×10^8 to 3×10^8 cells per ml in 10 mM Tris buffer (pH 8) containing 250 mM sucrose, 2 mM $MgCl_2$, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1,000 U of aprotinin per ml. These preparations were either stored at −20°C or immediately lysed for 4 h at 4°C in 0.5% Triton X-100 and centrifuged for 15 min at 15,000 × *g*, unless indicated otherwise. The protein content in the pellets (insoluble fraction) and supernatants was measured with the MicroBCA protein assay reagent kit (Pierce) as specified by the manufacturer. In functional assays (primed human peripheral blood mononuclear cell [PBMC] proliferation and IL-2 production [see below]), the parasites used as stimulating antigens were lysed by 11 cycles of freeze-thaw procedure in distilled water containing protease inhibitors. Then the whole lysate was separated into soluble and sedimenting fractions by 15,000 × *g* centrifugation for 10 min at 4°C. Nitrocellulose-bound proteins used in the functional assays were prepared as follows (adapted from the procedure in reference 20). Electrophoretically fractionated p14 and p18 antigens transferred onto the nitrocellulose sheet were identified by the labeling (see below) of outer peripheral lanes. Horizontal strips of nitrocellulose bounding p14 or p18 and control strips were cut appropriately ($l \times h = [5 \times 1]$ mm^2 for microtiter plates, $[10 \times 1]$ mm^2 for 24-well plates) and washed quickly in a solution of 5,000 U of penicillin per ml and 5 mg of streptomycin per ml before being used for PBMC activation. Potential stimulatory activity of other nitrocellulose-bound proteins was tested on membrane sections of $l \times h = (2 \times 8)$ mm^2 for microtiter plates and (5×8) mm^2 for 24-well plates. The following sections of the membrane were tested: 21 to 28 kDa, 29 to 39 kDa, 40 to 62 kDa, and 63 to 102 kDa.

Nucleus preparations. Nuclei were prepared from stationary-phase promastigotes by the following method adapted from references 8 and 37. Washed cells (6×10^8) were lysed in 3 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–10 mM $MgCl_2$–2 mM dithiothreitol–250 mM sucrose buffer (nuclear homogenization buffer [NHB]) containing 0.5% Triton X-100 by 20 strokes in Dounce homogenizer and 10 passages through a 26-gauge needle. The lysate, in a final volume of 6 ml, was centrifuged for 20 min at 1,900 × *g*. The resulting pellet was washed in 6 ml of NHB, resuspended in 1 ml of the same buffer, and centrifuged at 100,000 × *g* for 90 min at 4°C over a 2 M sucrose cushion in NHB containing 0.5% Triton X-100. Cellular debris located at the NHB/sucrose interface were removed. The nuclear pellet was washed (for 15 min at 15,000 × *g*) in 50 mM HEPES–5 mM $MgCl_2$–2 mM dithiothreitol–0.1 mM EDTA containing 40% (vol/vol) glycerol and resuspended in 100 μl of the same buffer. The entire procedure was carried out on ice, and centrifugations were done at 4°C.

SDS-PAGE and Western blot analysis. Leishmanial antigens, prepared as described above, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14% polyacrylamide). The protein load per well is indicated in figure legends. The separated proteins were transferred onto nitrocellulose in 25 mM Tris–190 mM glycine–20% (vol/vol) methanol buffer at 15 V/cm for 30 min. The nitrocellulose strips were first saturated by incubation in PBS–1% (wt/vol) skim milk–2.5×10^{-4} M thimerosal (saturation buffer), incubated with sera diluted 1:10 in the same buffer for 4 h, and washed three times (5 min each) in PBS. Incubation of the nitrocellulose with the affinity-purified, Fc-specific, anti-human immunoglobulin G (IgG) peroxidase conjugate (Sigma) was carried out, at a 1:800 dilution in saturation buffer, for 2 h and was followed by three washes as above. Enzymatic activity was revealed with 1.5 mM diaminobenzidine–0.38 mM $CoCl_2$–0.03% H_2O_2 in PBS. The quality of the transfer of leishmanial proteins on the nitrocellulose was regularly checked and confirmed by concurrent gel staining with Coomassie blue and nitrocellulose membrane staining with Ponceau S.

Cross-blot assay. Anti-p14 and anti-p18 antibodies were immunopurified from human serum as follows. The insoluble cellular fraction of stationary promastigotes (corresponding to 10^8 cells per 6.4 cm of gel) was electrophoresed, transferred onto a nitrocellulose sheet, and incubated with the human serum, as described above. The sheet was washed, and the antibodies to the p14 and the p18 proteins were eluted from the corresponding horizontal strips with 70 μl of 200 mM glycine-HCl buffer (pH 2.8) for 1 min with vigorous shaking. The eluted antibodies were then neutralized with 4.5 μl of 1 M Tris, diluted in 400 μl of the saturation buffer, and used again in a Western blot experiment for immunological demonstration of proteins on a new nitrocellulose sheet.

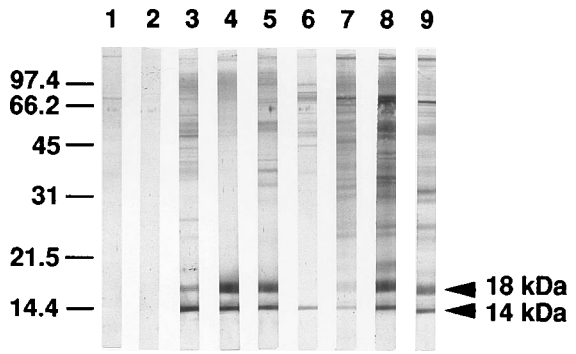

FIG. 1. Sera from subjects who have been exposed to *L. infantum* recognize p14 and/or p18 proteins on Western blot. Total proteins of *L. infantum* promastigotes were separated by SDS-PAGE (14% polyacrylamide) (55 μg of proteins per cm of gel) and transferred to nitrocellulose. Human sera, diluted 1:20, were incubated overnight with nitrocellulose strips. Sera were from leishmanin skin test-negative controls (lanes 1 and 2), leishmanin skin test-positive asymptomatic individuals (lanes 3 to 6), patients with healed leishmaniasis (lanes 7 and 8), and a patient with acute leishmaniasis (lane 9). Molecular mass markers are indicated.

PBMC. Peripheral blood was obtained from two patients healed of *L. infantum* infection (donors 2 and 3), from asymptomatic subjects with positive DTH tests and detectable anti-p14 and/or anti-p18 antibodies (donors 1, 4, and 5), and from leishmanin skin test-negative, control donors. PBMC were isolated by centrifugation of heparinized blood over lymphocyte separation medium (Eurobio, Les Ulis, France). The interface cells were washed three times in RPMI 1640 and resuspended at 1×10^6 to 1.5×10^6 cells per ml in complete culture medium. The cells were then seeded at 100 μl per well in flat-bottom culture plates for proliferation studies and at 1 ml per well in 24- or 48-well plates for IL-2 assays and were cultured at 37°C in a 5% CO_2, humidified atmosphere. Cell activation was initiated 16 to 24 h later, as described below.

Cell activation and proliferation assays. Cells were stimulated by (i) unfractionated promastigote antigens (15 μg/ml), (ii) insoluble (15 μg/ml) or (iii) soluble (15 μg/ml) antigen fractions prepared by freeze-thaw lysis and centrifugation, or (iv) electrophoretically fractionated nitrocellulose-bound antigens or were left unstimulated. Assays were carried out in triplicate in an incubation volume of 200 μl. Nitrocellulose fragments of the same surface excised after transfer from blank SDS-PAGE wells were used as controls. After 4 days of culture, cells were pulsed with 1 μCi of [^{3}H]thymidine per well, incubated for an additional 18 h, and harvested onto a fiberglass filter. Incorporated ^{3}H was measured by β counting. Data are represented as mean counts per minute ± standard deviation, in units of 10^3 cpm.

IL-2 assay. PBMC cultured in 1 ml in 24- or 48-well plates at 1×10^6 to 1.5×10^6 cells per well were activated as indicated above or left unstimulated and were incubated for an additional 36 to 42 h. The plates were stored at −20°C until cytokine production was measured. IL-2 levels in the supernatants were assayed by a sandwich enzyme-linked immunosorbent assay (ELISA), able to detect 10 pg of the cytokine per ml, as previously described (4).

Indirect immunofluorescence. Stationary-phase promastigotes were washed three times in ice-cold PBS, and 10 μl of a 10^6-cell-per-ml suspension was allowed to settle in 18-well immunofluorescence slides for 30 min at room temperature in a humidified atmosphere. Cells were fixed by incubation at 60°C and either stored at −20°C (up to 1 month) or used immediately. Parasites were incubated with control or tested sera at $1{:}10 \times 2^n$ ($n = 2$ to 4) dilutions for 30 min at 37°C and then washed twice in PBS and once in distilled water. Antibody fixation was revealed with fluorescein isothiocyanate-conjugated anti-human IgG (Fluoline G; Biomérieux), prepared at 1:100 in 0.01% Evans blue for counterstaining.

RESULTS

Subcellular location of p14 and p18 proteins in stationary-phase promastigotes. Western blot analysis of the protein preparation from stationary-phase promastigotes typically shows several antigens, revealed by sera from treated patients and from subjects with a positive leishmanin skin test (Fig. 1) (17, 19). We observed, however, that some sera from asymp-

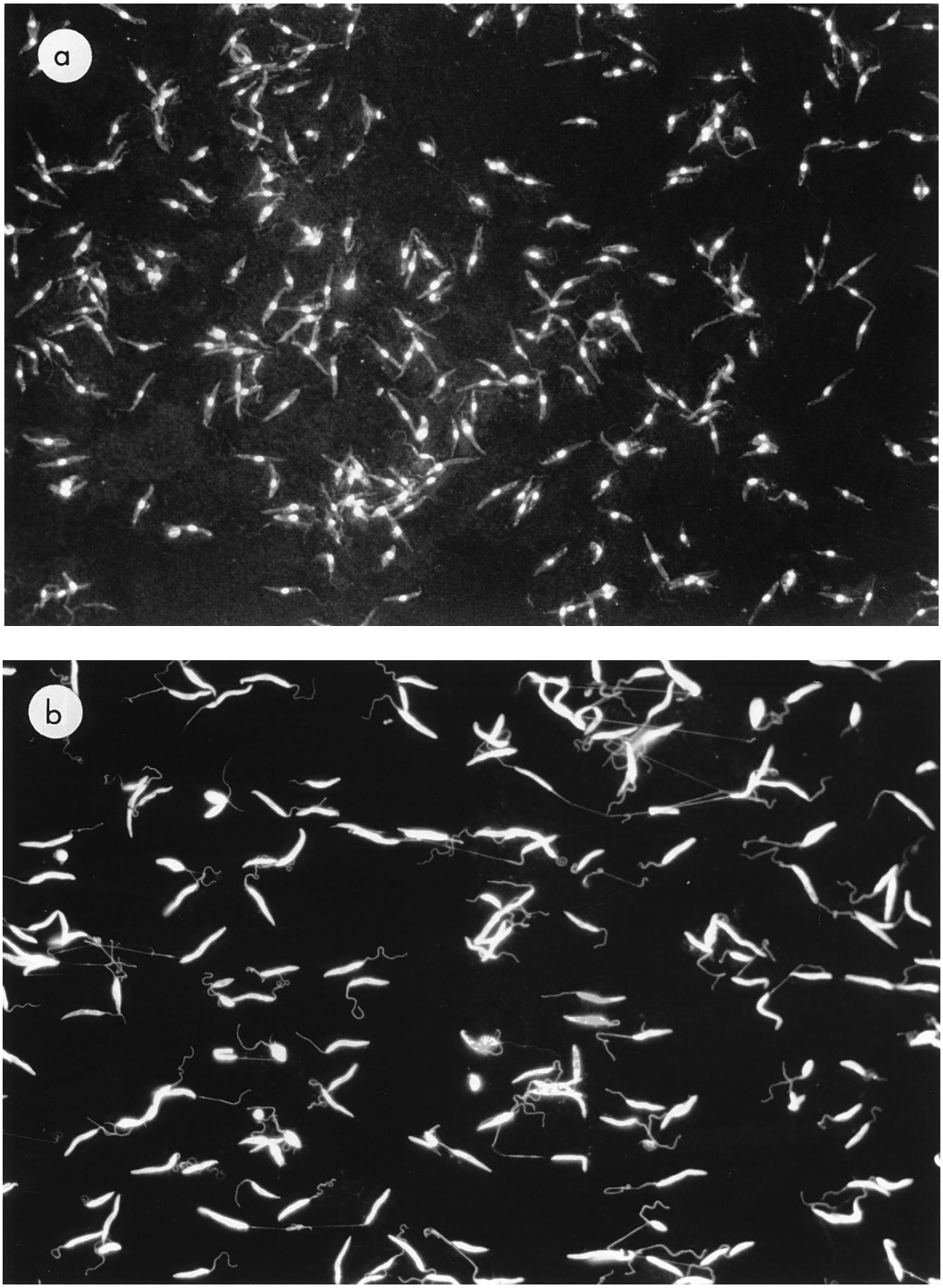

FIG. 2. Sera which detect mainly p14 and/or p18 antigens on Western blot stain promastigote nuclei in indirect immunofluorescence. (a) Fixed promastigotes were labeled with serum shown in Fig. 1, lane 4, which recognizes mainly p14 and p18 antigens, from a leishmanin skin test-positive asymptomatic individual and stained with fluorescein isothiocyanate-conjugated anti-human IgG. Red counterstaining was carried out with Evans blue; it allows one to visualize the promastigote outline. (b) Fixed promastigotes were labeled with serum shown in Fig. 1, lane 8, which recognizes multiple bands, from a patient with healed leishmaniasis and stained with fluorescein isothiocyanate-conjugated anti-human IgG. Magnification, ×400.

tomatic individuals exhibited a more restricted Western blot pattern, recognizing mainly p14 and/or p18 leishmanial proteins (Fig. 1, lanes 4 and 6). Interestingly, when analyzed by indirect immunofluorescence on whole parasites, these sera exhibited almost exclusively nuclear labeling (Fig. 2a); for comparison, a typical intense and homogenous IF staining of whole promastigotes, performed with a serum recognizing multiple bands on Western blot, is shown in Fig. 2b. This immunoflu-

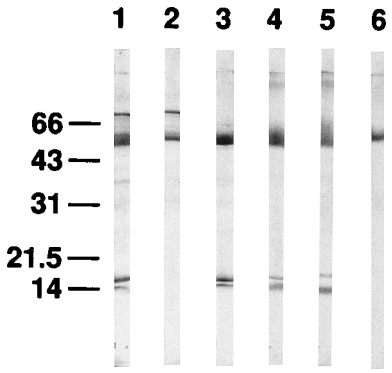

FIG. 3. Nuclei isolated from *L. infantum* stationary-phase promastigotes contain p14 and p18 antigens. Cellular fractions obtained in the process of preparation of nuclei, described in Materials and Methods, were analyzed by Western blotting. Lanes: 1, total parasite lysate of 2.5×10^6 cells; 2, supernatant from centrifugation at $1,900 \times g$ (2.5×10^6 cells); 3, corresponding pellet (2.5×10^6 cells); 4, pellet from ultracentrifugation over 2 M sucrose cushion at $100,000 \times g$ corresponding to nuclei of 10^7 cells; 5 and 6, pellets from centrifugation at $15,000 \times g$ of nuclei (lane 5; 5×10^7 cells) and of cellular debris located at the interface of sucrose/NHB (lane 6; 1.5×10^8 cells). Molecular mass markers are indicated.

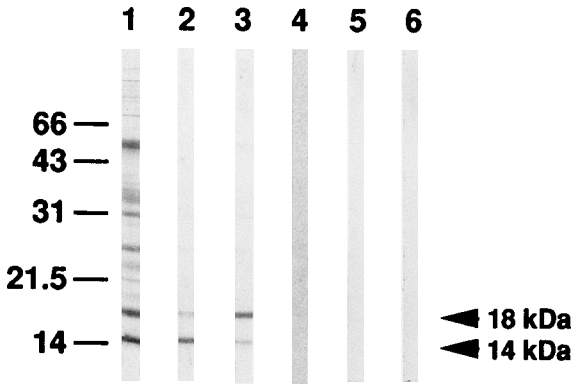

FIG. 4. p14 and p18 antigens share common epitopes. Anti-p14 and anti-p18 monospecific antibodies were immunopurified on nitrocellulose from the serum of the acutely ill patient (Fig. 1 lane 9), as described in Materials and Methods. Insoluble proteins were analyzed by Western blotting with the total serum (lane 1) and with anti-p14 (lane 2) and anti-p18 antibodies (lane 3). Control incubations in the absence of antigens (lane 4), of serum (lane 5), and of anti-IgG conjugate (lane 6) are shown. Molecular mass markers are indicated.

orescence pattern suggested that p14 and p18 antigens were located in parasite nuclei. Nuclear proteins were therefore prepared from stationary-phase promastigotes as described in Materials and Methods and analyzed by Western blotting. Figure 3 shows that, indeed, the nuclei isolated from the parasites contained p14 and p18 antigens (lanes 4 and 5), while no staining was detected in the remaining cellular fractions (lanes 2 and 6). An alternative procedure for nucleus preparation, modified from that in reference 22, gave the same result (not shown). We next confirmed that when the stationary-phase parasites were solubilized with 0.2% and up to 4% of Triton X-100, p14 and p18 proteins always remained in the pellet after $15,000 \times g$ centrifugation (not shown). Therefore, in what follows, Western blot analyses of p14 and p18 were carried out with pellets and supernatants of antigenic preparations obtained by solubilization of cells with 0.5% Triton X-100 and centrifugation at $15,000 \times g$. It should be added that disulfide bond disruption by 2-mercaptoethanol did not modify either the SDS-PAGE or the Western blot pattern, indicating a monomeric structure of p14 and p18.

Subcellular location of p14 and p18 proteins in logarithmically growing promastigotes. We next investigated whether p14 and p18 antigens were located exclusively in nuclei during all phases of parasite growth or whether a modulation of their expression and/or location could be detected. Preliminary experiments were carried out to determine the optimal protein load allowing a partial antigen quantification. For most tested sera, a 10-μg protein load of the insoluble fraction represented the threshold of p14 and p18 signal saturation and the ratio between the antigen detection and the signal saturation threshold was found to be 3:1 to 4:1 (data not shown). The expression of p14 and p18 during promastigote culture was then investigated, analyzing either a constant number of cells or a constant amount of proteins. We found that both antigens were again dominantly present in the insoluble fraction. Interestingly, both were also found in the solubilizable fractions but only on days 3 and 4 of the culture, corresponding to the mid-logarithmic growth phase (data not shown). These results indicate a transitory presence of p14 and p18 outside the nucleus in logarithmically growing promastigotes.

Antigenic relation between p14 and p18 proteins. Next, we carried out a cross-blot analysis of both proteins; monospecific anti-p14 and anti-p18 antibodies were obtained by immunopurification of human polyspecific, polyclonal antisera on nitrocellulose as described in Materials and Methods. Figure 4 shows that polyclonal antibodies directed against p14 antigen recognized p18 (lane 2) and, reciprocally, that antibodies directed against p18 antigen recognized p14 (lane 3), thus indicating that these proteins share a common epitope(s).

Carrying out experiments with the same antigen stock at different times after its preparation, we observed that p18 seemed to undergo a much faster degradation than p14 while stored at −20°C, as judged by noticeable fading of the p18 signal on Western blot. The causes of this phenomenon remain unclear: an autocatalytic activity or a p18-specific, cold-resistant catalytic activity can be evoked, since this phenomenon was slowed in the presence of protease inhibitors in the storage buffer and it did not occur when parasites were lysed directly in the electrophoresis sample buffer containing 3% SDS. We also found that p18 underwent a faster degradation than p14 at high pH. These observations will probably be explained as the role played by p14 and p18 proteins in the maturation of promastigotes becomes elucidated.

Activation of human primed T lymphocytes, induced in vitro by p14 and p18 proteins. Activation experiments were carried out on PBMC from five primed donors, described in Materials and Methods, and three negative controls. The complete proliferation data from the five tested subjects who have been exposed to *L. infantum* are compiled in Table 1 and presented as mean counts per minute ± standard deviation, in units of 10^3 cpm. First, the capacity of nitrocellulose-bound p14 and p18 to activate human primed PBMC was demonstrated: proliferation induced by p14 alone (donors 2, 3, 4, and 5) and p18 alone (donors 3, 4, and 5) or p14 plus p18 (donor 1) was, as expected, modest compared with that induced by phytohemagglutinin (PHA) at 1 μg/ml but was systematically higher than that of the control (blank nitrocellulose) for all primed PBMC which we examined. Next, the response to the insoluble fraction of leishmanial antigens (15 μg/ml), containing p14 and p18, was also analyzed and was compared with that induced by the soluble fraction (15 μg/ml) and by the unfractionated preparation (15 μg/ml). The data show that the response to the insoluble antigens was much stronger than that to the soluble

TABLE 1. Proliferation of human PBMC from subjects exposed to *L. infantum*[a]

Activator	Proliferation of PBMC (mean cpm ± SD) (10^3) from donor:				
	1	2	3	4	5
No activation	3.0 ± 0.6	1.1 ± 0.1	1.4 ± 0.4	0.1 ± 0.01	0.6 ± 0.2
Control nitrocellulose	5.2 ± 4.6	2.1 ± 0.1	3.1 ± 1.6	1.6 ± 0.7	4.0 ± 0.6
Nitrocellulose-bound p14	28.3 ± 4.2	15.4 ± 4.9	5.9 ± 2.6	4.0 ± 1.0	5.9 ± 0.5
Nitrocellulose-bound p18		ND[b]	4.6 ± 2.2	2.6 ± 0.4	8.2 ± 1.9
Insoluble fraction (15 μg/ml)	38.4 ± 2.8	ND	22.6 ± 0.3	ND	1.4 ± 0.1
Soluble fraction (15 μg/ml)	ND	ND	6.9 ± 0.4	5.1 ± 0.6	10.6 ± 3.8
Total lysate (15 μg/ml)	21.4 ± 6.8	14.3 ± 2.8	23.0 ± 2.7	2.0 ± 0.5	11.6 ± 5.8
PHA (1 μg/ml)	45.6 ± 9.1	66.1 ± 4.7	44.1 ± 2.0	191.8 ± 31.5	139.9 ± 13.3

[a] Control donors were subjects who had not been exposed to *L. infantum*; no proliferation was induced by leishmanial antigens; PHA-induced proliferation, expressed as mean counts per minute ± standard deviation, in units of 10^3 cpm, was 41.6 ± 8.7, 74.2 ± 11.7, and 91.7 ± 7.4 for three different donors.
[b] ND, not determined.

fraction for one donor (donor 3) while it was much lower for another donor (donor 5). The stimulatory activity of other membrane-bound proteins was examined in some experiments (donors 3, 4, and 5 [data not shown]). We found that only one donor (donor 3) responded significantly and that this response was only to antigens bound on nitrocellulose between 29 and 39 kDa. No proliferation was induced by other membrane-bound proteins (the sections of the membrane tested were 21 to 28 kDa, 29 to 39 kDa, 40 to 62 kDa, and 63 to 102 kDa). The quality of the transfer on the nitrocellulose was, of course, checked by gel staining with Coomassie blue and membrane staining with Ponceau S. In all experiments, PBMC obtained from negative controls (as assessed by negative skin test and Western blot pattern) were included and were shown to respond to stimulation by PHA (see the footnote to Table 1) but not to stimulation by the tested preparations of leishmanial antigens.

In a second series of experiments, PBMC from two positive donors (donors 2 and 5) and of two control individuals were examined in IL-2 assays. Figure 5 shows that the IL-2 production elicited by the whole promastigote lysate and by nitrocellulose-bound p14 (donors 2 and 5) and p18 (donor 5) was again small but significant compared with control stimulation (no activation or blank membrane). Control PBMC obtained from negative individuals did not respond to stimulation by leishmanial antigens. The IL-2 response to PHA of all tested donors is described in the legend to Fig. 5.

DISCUSSION

In this article, we report on a partial parasitological and antigenic characterization of two proteins from *L. infantum* promastigotes, p14 and p18, and on an in vitro analysis of their potential to activate in vivo primed human T cells.

The interesting feature of these proteins which prompted our study was the finding of a significant correlation between the humoral response elicited by these proteins and a positive leishmanin DTH reaction, testifying to the occurrence of cell-mediated immunity. Sera from patients with classic visceral leishmaniasis (active or healed) contain antibodies, shown to exhibit high specificity for *L infantum*, directed against p14 and p18 (19). Furthermore, anti-p14 and/or anti-p18 antibodies are quite systematically detectable in sera of asymptomatic individuals who live in areas where *L. infantum* infection is endemic and have a positive leishmanin skin test (17). Finally, the Western blot profile rich in antigenic bands revealed by sera from acutely ill patients (compatible with high levels of parasite-specific antibodies developed early in disease) contrasts with the detection predominantly of p14 and p18 by sera from asymptomatic individuals (18). Taken together, these observations suggested that the humoral response elicited by p14 and p18 is strong and long lasting. We thus attempted to determine whether these proteins might also contribute to the cell-mediated immunity.

First, we studied the subcellular location of p14 and p18 antigens and investigated whether their expression varied as a function of the parasite growth in vitro. Indeed, the transformation of log-phase promastigotes into stationary phase correlates with events occurring naturally during maturation of the parasite: it was demonstrated that promastigotes logarithmically growing in the culture medium mimic the procyclic stage of development inside the sand fly gut, while the station-

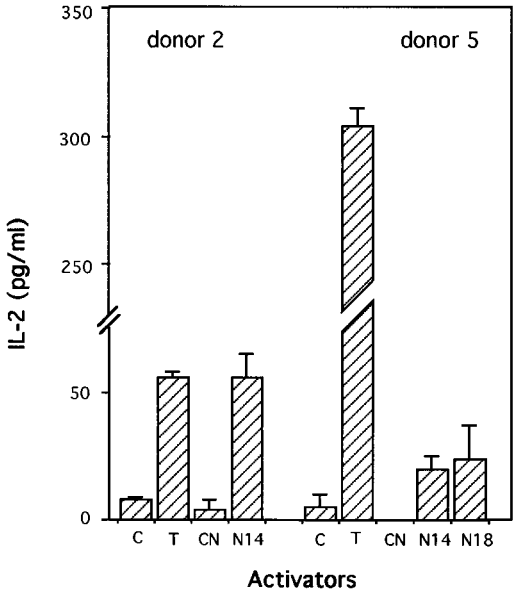

FIG. 5. Nitrocellulose-bound p14 and p18 proteins stimulate IL-2 production by primed human T lymphocytes. PBMC were from donor 2 (healed patient) and donor 5 (asymptomatic, leishmanin DTH-test-positive subject). Cells (10^5 cells per well) were stimulated by nitrocellulose-bound p14 (N14; donors 2 and 5) or p18 (N18; donor 5) or by total promastigote lysate (T; 15 μg/ml). Control incubations were carried out in the absence of activators (C) or in the presence of a blank nitrocellulose fragment (CN). IL-2 production was measured by ELISA after 36 h (donor 2) or 46 h (donor 5) of cell stimulation. The level of PHA-induced IL-2 was 800 ± 48 pg/ml in donor 2 (PHA at 20 μg/ml) and 3.90 ± 0.13 ng/ml in donor 5 (PHA at 75 μg/ml). No IL-2 production was induced by leishmanial antigens in PBMC of two tested control subjects who were not exposed to *L. infantum*; the PHA-induced (50 μg/ml) IL-2 levels in these donors were 4.13 ± 0.6 and 1.14 ± 0.5 ng/ml.

ary phase of the culture corresponds to the metacyclic, infective form of the parasite (30–32). We showed, by immunofluorescent staining and through a Western blot study of subcellular fractions, that in the stationary phase of promastigote growth, p14 and p18 proteins were located only in the parasite nuclei. In the logarithmically growing parasites, p14 and p18 were still present predominantly in the nuclei, but their transitory expression out of the nucleus, in the middle of the log phase, was detected. We also found that despite a 1.5-fold reduction of total protein synthesis as promastigotes approach the metacyclic growth stage (our results, data not shown; 1), the percentage of p14 and p18 expressed by the parasites increased (data not shown). These results indicate that the expression of p14 and p18 proteins was modulated while promastigotes proliferated in vitro and suggest that it might be regulated developmentally during natural maturation in the phlebotomine vector.

The common location of the p14 and p18 antigens and their concurrent evolution during promastigote culture prompted us to inquire whether they could be antigenically related. We found that immunopurified antibodies directed against p14 (or against p18) reacted with nitrocellulose-bound p18 (or p14), pointing to a likely common epitope(s). Further characteristics of p14 and p18 proteins in the promastigote and amastigote stages of *L. infantum*, the mechanisms of their intracellular trafficking, and a possible relationship between p14 and the recently cloned histone H3 of *L. infantum* (36) are at present under investigation in our laboratory.

Next, we analyzed the in vitro activation of human primed PBMC. We studied cell proliferation induced by leishmanial proteins in five positive donors and IL-2 production in two positive donors.

The proliferative responses induced by the unfractionated preparation of leishmanial antigens, by its soluble fraction, and by antigens sedimenting at 15,000 $\times$ *g* were measured. The finding that the insoluble proteins induced a strong proliferation, comparable to that induced by whole lysate in some primed individuals (donors 1 and 3), is important, since in most published reports the stimulatory activity of the soluble fraction only was examined. We also performed a (rough) quantitation of the transferred proteins by Ponceau S staining of the membrane after simultaneous transfer of the whole lysate at various dilutions and of molecular weight standards at known concentrations (results not shown). We estimated that p14 and p18 each account for 0.05 to 1% of the total transferred proteins and thus might represent up to 4% of the insoluble proteins. In these functional experiments, insoluble and soluble fractions were obtained after freeze-thaw lysis. Therefore, the separation is less complete than after Triton X-100 cell lysis, since freeze-thaw disruption of parasites is rarely perfect, even after 11 cycles. We found in control experiments that some proteins, including p14 and p18, could be detected by Western blot in both the pellet and the supernatant after the freeze-thaw disruption, in contrast with Triton X-100 cell lysis. These results indicate that the response to the whole lysate was, in fact, due to the nuclear antigens in some individuals.

We demonstrated directly that p14 and p18 in vitro had the capacity to activate PBMC from sensitized individuals. Cell proliferation was specifically stimulated by nitrocellulose-bound p14 and p18 in all tested positive donors, and IL-2 production was found in two donors. It should not be astonishing that activation induced by p14 and p18, which stimulate their specific memory cells, was relatively low compared with that induced by PHA, a potent polyclonal activator. The respective contributions of p14 and p18 to PBMC activation seemed equivalent. Even if the values were slightly different for different donors, the differences did not appear significant, and, obviously, we cannot say whether these variations might arise from individual major histocompatibility complex antigens and/or from the T-cell repertory.

The capacity of membrane-bound p14 and p18 to activate all of the tested human PBMC sensitized in vivo contrasts with a lack of stimulatory activity of other membrane-bound proteins. Among three donors examined (donors 3, 4, and 5), we found that only one (donor 3) responded significantly and then only to the antigen(s) bound on nitrocellulose between 29 and 39 kDa. No proliferation was induced by other membrane-bound proteins. Taken together, our results suggest that an antigenic determinant(s), dominant in some individuals, might exist on both proteins.

Data presented in this paper do not permit us to determine whether the in vitro p14- and p18-induced T-cell responses are protective in vivo. Specifically induced proliferation and IL-2 production in primed PBMC point to a possible existence of a p14- and p18-specific Th1-type response; the presence of humoral immunity provides indirect evidence for in vivo expansion of the Th2 subset. Thus, p14- and p18-specific Th1- and Th2-type responses could coexist in human visceral leishmaniasis. The role and significance of the humoral response in leishmaniasis remain unknown. As the parasite induces an abundant production of antibodies, it is possible that the protective cell-mediated immunity becomes less efficient (2), perhaps as a result of a competition between T- and B-cell repertoires. A study of a cutaneous leishmanial infection in a murine model suggested that the presence of B lymphocytes but not of antibodies was required for the generation of an effector T-cell population which, however, seemed unrelated to delayed hypersensitivity (34). Alternatively, it is possible to imagine that coexistence of Th1-like and Th2-like responses might contribute to the balance established between the human host and its persistent *Leishmania* parasite, with the Th2 subset preventing the Th1 population from undergoing immoderate expansion.

Specific antigens that stimulate T lymphocytes from individuals with immunity to leishmaniasis are of particular interest, since they may be potential candidates for a vaccine. Only a few have been characterized. The properties of the major surface protease gp63 and of lipophosphoglycan are by far the best described (3, 5, 26). It has been shown not only that gp63 was a powerful stimulator of proliferation and of IFNγ production in T cells from cured patients but also that the native gp63 could effectively induce T-cell responses by in vitro immunization of normal PBMC (27). Some host-protective T-cell epitopes of gp63 were identified in mice (9, 38) and in humans (28). T-cell-activating properties of lipophosphoglycan first remained a subject of debate. Then a protein complex which copurified with lipophosphoglycan, the lipophosphoglycan-associated protein, was shown to account for in vitro T-cell activation in murine (10) and human (14, 29) systems. Leishmanial gp30 and gp42 antigens were identified by Western blotting with patient sera and were found to stimulate in vitro human lymphocytes (23). Recently, a 36-kDa protein, expressed in promastigote and amastigote stages of *L. major*, was cloned (21); it was selected by screening of an expression library of *L. major* for the identification of major histocompatibility complex class II-associated antigens and was recognized by a protective murine Th1 clone. A 24-kDa recombinant portion of this antigen was used together with IL-12 to protect susceptible BALB/c mice (21). Interesting features were demonstrated by a previous immunoblotting analysis of T-cell activation by fractionated leishmanial antigens (20). The authors found that specific T-cell responses were elicited by a very

large number of leishmanial proteins in immune (healed) individuals whereas PBMC of patients with nonhealing lesions responded to a very limited number of antigens. No obvious immunodominant antigen was found in this quite extensive analysis. The control and resolution of infection in humans might necessitate sensitization to several antigens, including p14 and/or p18. Experiments are in progress to further characterize both proteins and to determine their potential immunoprotective capacities.

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