Molecular and Antigenic Characterization of the *Leishmania (Viannia) panamensis* Kinetoplastid Membrane Protein-11

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The kinetoplastid membrane protein 11 (KMP-11) has been recently described in Leishmania (Leishmania) donovani *as a major component of the promastigote membrane. Two oligonucleotide primers were synthesized to PCR-amplify the entire coding region of New World* Leishmania *species. The* Leishmania (Viannia) panamensis *amplification product was cloned, sequenced and the putative amino acid sequence determined. A remarkably high degree of sequence homology was observed with the corresponding molecule of* L. (L) donovani *and* L. (L) infantum *(97% and 96%, respectively). Southern blot analysis showed that the KMP-11 locus is conformed by three copies of the gene. The* L. (V) panamensis *ORF was subsequently cloned in a high expression vector and the recombinant protein was induced and purified from* Escherichia coli *cultures. Immunoblot analysis showed that 80%, 77% and 100% sera from cutaneous, mucocutaneous and visceral leishmaniasis patients, respectively, recognized the recombinant KMP-11 protein. In a similar assay, 86% of asymptomatic* Leishmania-*infected individuals showed IgG antibodies against the rKMP-11. We propose that KMP-11 could be used as a serologic marker for infection and disease caused by* Leishmania *in America.*

Key words: New World leishmaniasis - *Leishmania (Viannia) panamensis* - kinetoplastid membrane protein-11 - antigenicity

The number of cases of American tegumentary leishmaniasis (ATL) has been estimated to be around 59,000 every year, and approximately 59 million people reside in areas where the ATL-causing *Leishmania* species are transmitted (Ashford et al. 1992). ATL caused by the species complex *Leishmania (Viannia) braziliensis* is endemic in large areas of Central and South America (Grimaldi et al. 1989). In Colombia, *L. (V) panamensis* is responsible for most of the ATL cases, including cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and disseminated cutaneous leishmaniasis (DCL) (Corredor et al. 1990, Colombian Health Ministry 1994, Velez et al. 1994).

Control of leishmaniasis in the American continent is particularly complicated due to the fast evolving variety of *Leishmania* parasites and the complexity in their epidemiological patterns (Grimaldi & Tesh 1993). Recent attempts to develop molecularly defined vaccines for leishmaniasis have focused on major parasite cell surface molecules, including the metalloproteinase GP63, the promastigote surface antigen complex (PSA) and the lipophosphoglycans (LPG) (McConville et al. 1987, Chang et al. 1990, Burns et al. 1991). For reasons such as its abundance on promastigotes and its important physiological role in parasite-macrophage interactions (Turco & Descoteaux 1992), the latter was considered an attractive vaccine candidate. In early works, it has been demonstrated that immunization with LPG might confer immunoprotectivity in mice (Handman & Mitchell 1985, McConville et al. 1987, Russell & Alexander 1988) and that peripheral blood mononuclear cells from leishmaniasis patients were strongly stimulated to proliferate in response to LPG (McConville et al. 1987, Jaffe et al. 1990, Mendoca et al. 1991). However, it could be recently shown that T-cell stimulation associated with protective immunity elicited in mice, was due to a tightly associated small protein in the LPG fraction which was recently renamed as the kinetoplastid membrane pro-

tein-11 (KMP-11) (Jardim et al. 1991, Stebeck et al. 1995). So far, KMP-11 has been cloned and partially characterized in *L.(L) donovani* (Jardim et al. 1995a,b) and the *L. (L) infantum* and *Trypanosoma brucei rhodesiense* counterparts have been recently reported (Stebeck et al. 1996, Berberich et al. 1997). To date, no homologous locus in any New World *Leishmania* species has been isolated and characterized. Here, we report the cloning, sequencing and bacterial expression of the recombinant *L.(V) panamensis* KMP-11 protein. In addition, in a detailed study with sera from patients suffering from different forms of New World leishmaniasis we provide evidence that specific antibodies against KMP-11 are produced during natural course of infection.

MATERIALS AND METHODS

Parasites - Promastigotes of *L. (V) panamensis* (MHOM/PA/71/LS94), *L.(V) panamensis* (MHOM/CO/87/UA140), *L. (V) guyanensis* (MHOM/BR/75/M4147), *L. (L) major* (MHOM/MA/81/LEM265), *L. (V) braziliensis* (MHOM/CO/88/UA301), *L. (V) colombiensis* (IHAR/CO/85/CL500), and *L. (L) infantum* (MHOM/FR/78/LEM75), were grown at 26ºC in Schneider's Drosophila Medium (Sigma, USA) supplemented with 15% heat-inactivated fetal bovine serum and antibiotics (streptomycin 0.01 mg/ml, penicillin 10 U/ml). Promastigotes were grown to late logaritmic phase, harvested, washed three times with PBS and resuspended at 5×10^7/ml.

Monoclonal and polyclonal antibodies - The L98 monoclonal antibody (mAb), originally raised against the *L. (L) donovani* LPG molecule (Tolson et al. 1989), but further described to recognize a carboxy-terminal epitope on the tightly associated KMP-11 molecule (Jardim et al. 1991, 1995a), was used to demonstrate the presence of the recombinant KMP-11. This mAb detects the 11 kDa protein in all kinetoplastid parasites so far tested (Stebeck et al. 1995). A rabbit polyclonal serum recognizing the recombinant *L. (L) infantum* KMP-11 (Berberich et al. 1997) was also used to detect the recombinant *L (V) panamensis* KMP-11 protein.

Study subjects - Twenty six patients with parasitological diagnosis of leishmaniasis were studied. The presence of *Leishmania* parasites in patients was determined by direct microscopic examination and culture methods. Indirect immunofluorescence microscopy using mAbs capable to distinguish between species-specific LPG epitopes (McMahon-Pratt & David 1981) was applied in order to confirm the identity of *Leishmania* parasites. Twenty four patients had ATL caused by *L. (V) panamensis* or *L. (V) braziliensis* (15 CL and 9

MCL) and live in recognized endemic areas for leishmaniasis (departments of Antioquia, Tolima, Caldas and Cundinamarca, Colombia). Two patients had visceral leishmaniasis (VL) caused by *L. (L) chagasi/L.(L) infantum* and live in San Andres de Sotavento, an endemic region for VL at the Colombian Carribean Coast. ATL patients were treated as recommended by the WHO with Glucantime, 20 mg of antimony/kg daily intramuscularly (20 days for CL patients and 28 days for MCL patients) (WHO 1990). The two VL patients were treated with liposomal amphotericin B, 2 mg/kg intravenously for 21 days. A second group consisted of seven asymptomatic *Leishmania*-infected individuals that live in endemic areas for leishmaniasis and show a positive Montenegro skin test (MST). These individuals were clinically evaluated to detect any scar or current leishmaniasis compatible lesion. As negative controls, nine healthy individuals which have never lived or visited endemic areas for leishmaniasis and have a negative MST were included. Serum samples were collected from these individuals in sterile vacutainer blood collection tubes (Becton, Dickinson and Company, Rutherford, NJ, USA) and frozen at -20ºC until use.

Polymerase chain reactions (PCR) - Genomic DNA was isolated as described previously (Medina-Acosta & Cross 1993). To amplify the entire coding region of the KMP-11 protein from the genomic DNA, the following oligonucleotide primers were used: forward, (CB1): 5" ATGGCCACCACGTACGAGGAG 3" and reverse, (CB2): 5" GGACGGGTACTGCGCAGCC TT 3". The primer sequences were based on the published KMP-11 coding region from *L. (L) donovani* (Jardim et al. 1995b). A "DNA thermal cycler 480" and "Amplitaq kit" (Perking-Elmer Cetus Instruments, New Jersey, USA) were used according to manufacturers instructions. Reactions mixtures included 25 pmol of each primer, 200 mM of each dNTP and 1.5 mM $MgCl_2$. The following PCR protocol was performed: (a) denaturation at 94ºC for 1 min; (b) annealing at 58ºC for 1 min; and (c) extension at 72ºC for 1 min; 30 cycles.

Southern blot analysis - 3 mg of *L. (V) panamensis* promastigote genomic DNA were digested with restriction enzymes (Sal I, Nco I, Xho I, EcoR I, Hind III and BamH I), separated on a 0.8% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Int., UK) and subsequently hybridized with an [a-^{32}P]dATP random-primed-labelled probe consisting of the 273 bp PCR fragment of the KMP-11 coding region. Hybridization was done in 6X SSC (1X SSC is 0.15 M NaCl, 15 mM Sodium-Citrate, pH 7.0), 0.1% SDS, 250 mg/ml hering sperm DNA and 50% formamide, at

42°C overnight. After hybridization, membranes were washed with (a) 2X SSC, 0.1% SDS; (b) 1X SSC, 0.05% SDS and (c) 0.1X SSC, 0.02% SDS, each washing step for 30 min at 42°C. Membranes were then exposed to an autoradiographic film for 96 hr.

Cloning and sequencing - The 273 bp PCR fragment was blunt-end cloned in the EcoR V site of pBluescript (Stratagene, La Jolla, CA) and eight different recombinant clones were selected for restriction mapping to detect full-length inserts. Two of these clones were subsequently selected (clones pBx-KMP-4 and pBx-KMP-5) for DNA sequencing on double-stranded DNA by the dideoxy chain termination method (Sanger et al. 1977), using both comercial T7 and T3 primers or the CB1/CB2 primer pair and the "sequenase kit" (US Biochemical Corp., Cleveland, OH, USA). The analysis of DNA sequence was done using the University of Wisconsin Genetics Computer Group Program (Devereux et al. 1984).

Subcloning, expression and purification of the recombinant protein - The pBx-KMP-5 insert was subcloned directionally into the BamH I/Hind III site of the expression plasmid pQE30 (Quiagen GMBH, Hilden, Germany). Induction of expression (2 mM IPTG) and protein purification was performed following the instructions of the manufacturer. Purification of proteins was performed under denaturing conditions in 8 M Urea and passage of the bacterial extract through a Ni-NTA affinity column. Purified protein was subsequently dialysed against 1X PBS. The expression and purification process of the recombinant protein was monitored by SDS-PAGE and coomassie blue staining.

Western blotting - The purified recombinant protein was separated by 15% SDS-PAGE and electrotransferred to nitrocellulose membranes (Schleicher & Schöll Inc., Keene, NH, USA) using the MiniTrans blot system (BioRad Labs, Hercules, CA, USA). Protein transfer was monitored by Ponceau-S (Sigma Co., St Louis, USA) staining before blocking the filters in 5% blotto in PBS (Blocking solution) at 4°C overnight. The filters were then incubated with monoclonal (1:500), polyclonal (1:2000) or human (1:100) serum diluted in blocking solution with 0.5% tween-20 for 2 hr. To detect specific antibodies in human sera, the membranes were incubated successively with biotin-labelled anti-human IgG (Sigma Co., St Louis, USA) for 1 hr, streptavidin (BioRad Labs, Hercules, CA, USA) for 1 hr, and alkaline phosphatase-labelled biotin (BioRad Labs, Hercules, USA) for 1 hr. The immune reactions were visualized by incubating the membranes in bromochloroindolylphosphate-nitro blue tetrazolium

(BCIP-NBT) for 30 min. For monoclonal and polyclonal sera, a peroxidase-labelled anti-mouse IgG and a alkaline phosphatase-labelled anti-rabbit IgG were used. Colour development was done with diaminobenzidine (DAB) and BCIP-NBT respectively.

RESULTS

Locus characterization and sequence of open reading frame - By using the CB1/CB2 primer pair, which sequence was based on the *L. (L) donovani* KMP-11 open reading frame (Jardim et al. 1995b), we amplified a 273-bp fragment in all species tested (Fig. 1) including *L. (V) panamensis*, *L. (V) braziliensis* and *L. (V) guyanensis*, the most important species causing CL and MCL in Colombia (Corredor et al. 1990, Colombian Health Ministry 1994). These results confirm, at the genomic level, the high degree of conservation for this gene between Old and New World *Leishmania* species as it was expected in view of the immunological cross-reactivity of the protein in various kinetoplastid species when using the L98 and L157 mAbs originally raised against the LPG (Tolson et al. 1994).
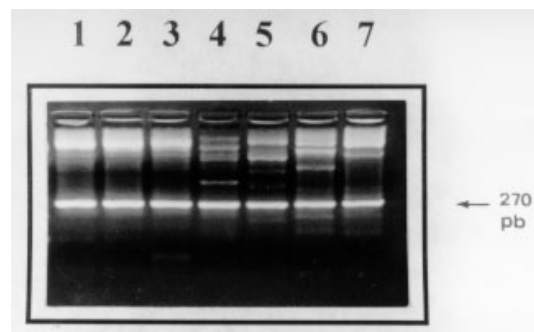

Fig. 1: PCR amplification of the KMP-11 gene in differents species of *Leishmania* using the CB1/CB2 primers. 1: *L. (V) panamensis* (LS94), 2: *L. (V) panamensis* (UA140), 3: *L. (V) braziliensis* (UA301), 4: *L. (V) guyanensis* (M4147), 5: *L. (V) colombiensis* (CL500), 6: *L. (L) major* (LEM265), 7: *L. (L) infantum* (LEM75). The amplification product of about 273 bp is indicated.

The KMP-11 gene copy number of *L. (V) panamensis* was determined by southern blot analysis using a variety of restriction endonucleases, including Sal I, which contains a cleavage site within the KMP-11 ORF, and others for which cleavage sites were not found within the gene (Nco I, Xho I, EcoR I, Hind III and BamH I). As shown in Fig. 2A, the Sal I restriction digests produced three DNA fragments that hybridized with the 273 bp KMP-11 PCR product used as probe, while the Xho I, EcoR I, Hind III and BamH I digests only revealed a single hybridizing DNA fragment. Three

labelled bands were equally observed in the Nco I digested DNA representing fragments of 1.9, 3.0 and 7.6 Kb, all different in size compared with the Sal I fragments of 1.4, 1.8 and 2.2 Kb, thus indicating Nco I cleavage sites in intergenic regions. These results suggest that the KMP-11 locus consists of three gene copies separated by variable intergenic regions, although the presence in multiple genomic sites cannot be excluded. However, in view of the described genomic arrangement of the *L. (L) donovani* and *L. (L) infantum* KMP-11 genes (Jardim et al. 1995b, Berberich et al. 1997), the *L. (V) panamensis* counterparts should be probably organized in a similiar way as suggested in Fig. 2B. Differences in the genomic organization of the KMP-11 locus in *Leishmania* are only found in the lengh of the intergenic regions between each gene copy with variations from one species to another. In contrast to this *Leishmania*-specific genomic arrangement of the KMP-11 locus, the *T. b. rhodesiense* KMP-11 like gene is transcribed from a single copy gene (Stebeck et al. 1996).
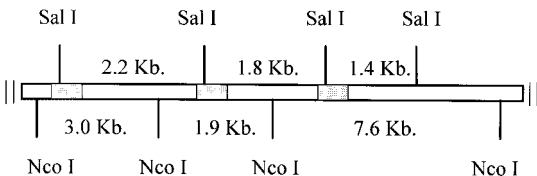
A
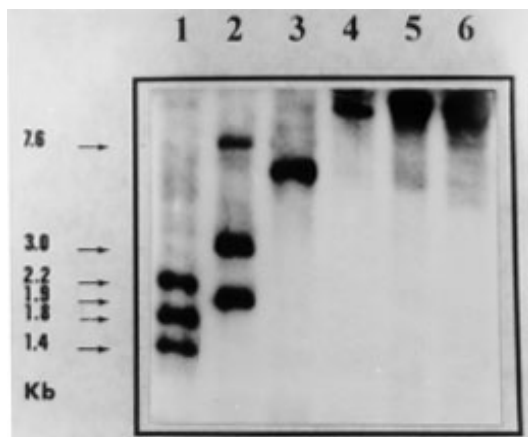
B

Fig. 2: genomic organization of the *Leishmania (V) panamensis* KMP-11 gene locus. A: southern blot analysis of restriction enzyme digested genomic DNA using as probe a 273 bp-fragment of the KMP-11 coding region. Restriction enzymes: 1: Sal I, 2: Nco I, 3: Xho I, 4: EcoR I, 5: Hind III and 6: BamH I. The sizes of the labelled DNA fragments are indicated. B: schematic representation of the possible genomic organization of the KMP-11 locus. Shaded boxes indicate the position of the coding regions of the KMP-11 locus. The restriction sites and length of DNA fragments are indicated.

The nucleotide sequence of the PCR-amplified coding region of KMP-11 *L. (V) panamensis* gene is shown in Fig. 3 and is compared with the published *L. (L) infantum* and *L. (L) donovani* sequences (Jardim et al. 1995b, Berberich et al. 1997). A remarkably high level of sequence homology was observed between the aligned sequences (97.1%, 96.3% and 82% for *L. (L) infantum, L. (L) donovani* and *T. b. rhodesiense*, respectively). The analysis of the deduced amino acid sequence predicts a putative *L. (V) panamensis* KMP-11 protein with a molecular mass of 11,087 Da and a calculated PI of 6.1. Only three amino acid changes were observed with respect to the sequences of the *L. (L) infantum* and *L. (L) donovani* KMP-11 protein. The amino acid subtitutions were conservative in polarity.

Subcloning and bacteria expression - The 273 bp PCR fragment of the *L. (V) panamensis* KMP-11 coding region was introduced in frame into the polylinker of the bacterial expression vector pQE30 (Quiagen, Hilden, Germany). Induction of recombinant protein expression by IPTG resulted in high-level expression of the His-tagged KMP-11 with a molar mass of 14 kDa, slightly higher than the native protein due to the additional 6-His-tag and polylinker sequences. Purification of the recombinant protein was performed by affinity chromatography on Ni-NTA resin and the fractions were analyzed by SDS/PAGE. The results are shown in Fig. 4A. Subsequently, the purified protein was transferred to nitrocellulose membranes for western blot analysis. As shown in Fig. 4B, the rKMP-11 protein was strongly recognized by sera from VL patients known to contain high levels of anti-*Leishmania* antibodies (Sacks et al. 1993). A strong cross-reactivity was observed with a rabbit polyclonal anti-serum originally raised against the *L. (L) infantum* rKMP-11 protein (Berberich et al. 1997). In the same way, the L98 mAb recognized specifically the 14 kDa recombinant protein, confirming the identity of the rKMP-11 protein.

We also observed a second minor immunoreactive band of 28 kDa in some western blots when using the polyclonal rabbit serum or sera from some leishmaniasis patients (Fig. 4B). That this band represents a dimeric form of the rKMP-11 due to its tendency to aggregate *in vitro* (Jardim et al. 1995, Berberich et al. 1997) was subsequently confirmed by using an antibody that specifically recognizes the His-tail in His-tagged proteins (data not shown). The lack of immunoreactivity of the 28 KDa dimeric form in an immunoblot hybridized with the L98 mAb could be explained by the different accessibility between the monomeric and dimeric form.

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                                                           i
          M   A   T   T   Y   E   E   F   S   A   K   L   D   R   L   D   E   17
L.pan     ATGGCCACCACGTACGAGGAGTTTTCGGCGAAGCTGGACCGCCTGGATGA  50
L.inf-b   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .G. . .
L.don     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .C .

              id
          E   F   N   K   K   M   Q   E   Q   N   A   K   F   F   A   D    33
L.pan     GGAGTTCAACAAGAAGATGCAGGAGCAGAACGCCAAGTTCTTTGCGGACA  100
L.inf-b   . . . . . . . . . .G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
L.don     . . . . . . . . . .G . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                                                      d
          K   P   D   E   S   T   L   S   P   E   M   K   E   H   Y   E   K    50
L.pan     AGCCGGATGAGTCGACGCTGTCGCCCGAGATGAAGGAGCACTACGAAAAG  150
L.inf-b   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .G . . .
L.don     . . . . . . . . . . . . . . . . . . . . . . . . . .GA . . . . . . . . . .G . . .

           F   E   R   M   I   K   E   H   T   E   K   F   N   K   K   M   H   67
L.pan     TTCGAGCGCATGATCAAGGAGCACACGGAGAAGTTCAACAAGAAGATGCA  200
L.inf-b   . . . . . . . . . . . . . . . . . . . . . . . .A . . . . . . . . . . . . . . . . . . .
L.don     . . . . . . . . . . . . . . . . .A . . . .A . . . . . . . . . . . . . . . . . . .

                            id
          E   H   S   E   H   F   K   H   K   F   A   E   L   L   E   Q    83
L.pan     CGAGCACTCGGAGCACTTCAAGCACAAGTTCGCTGAGCTGCTTGAGCAGC  250
L.inf-b   . . . . . . . . . . . . . . . . . . .G . . . . . . . .C . . . . . . . .G . . . . . .
L.don     . . . . . . . . . . . . . . . . . . .G . . . . . . . .C . . . . . . . .C . . . . . .

          Q   K   A   A   Q   Y   P   S          91
L.pan     AGAAGGCTGCGCAGTACCCGTCC          273
L.inf-b   . . . . . . . . . . . . . . . . .A . . .
L.don     . . . . . . . . . . . . . . . . . . . .
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Fig. 3: sequence of the PCR-amplified *Leishmania (V) panamensis* KMP-11 coding region. The sequence is compared with the coding region of *Leishmania (L) infantum* (gene B) (Berberich et al. 1997) and *Leishmania (L) donovani* KMP-11 gene (Jardim et al. 1995b). Dots indicate identical nucleotides and differences in the sequence are shown by the corresponding letters. Amino acid differences with regard to the *L. (L) infantum* and *L. (L) donovani* KMP-11 are indicated (*i* and *d* respectively). Underlined nucleotides at the 5' and 3' ends represent the sequences of the CB1 and CB2 oligonucleotide primers used to PCR-amplify the coding region of *L. (V) panamensis* gene.

Antigenicity - The ability of the KMP-11 protein to induce proliferation of primed murine and human T-lymphocytes has been widely documented (Handman & Mitchell 1985, McConville et al. 1987, Russell & Alexander 1988, Jaffe et al. 1990, Jardim et al. 1991, Mendoca et al. 1991, Tolson et al. 1994). The B-cell antigenic potential has been also recently reported in dogs naturally infected with *L. (L) infantum* (Berberich et al. 1997). To date, a systematic study about the presence of specific anti-KMP-11 anti-bodies in humans suffering from different forms of leishmaniasis has not been carried out. Therefore, in order to analyze the antigenicity of KMP-11 during New World *Leishmania* infections in humans, the re-

combinant protein was assayed by western blots using sera obtained from CL, MCL and VL patients. In a similiar way, asymptomatic ("resistant") *Leishmania*-infected persons were tested.

The Table shows that the rKMP-11 protein was recognized by 80% (12/15) and 77% (6/7) of sera from CL and MCL patients, respectively, infected with *L. (V) panamensis*. A high degree of variability in the levels of anti-KMP-11 antibodies was observed in these groups of patients. However, sera from several CL and MCL patients showed a very strong reactivity, evidencing that the protein may act as a potent human B-cell immunogen. Both (100%) of the human VL sera obtained from *L. (L) chagasi*-infected persons showed very strong
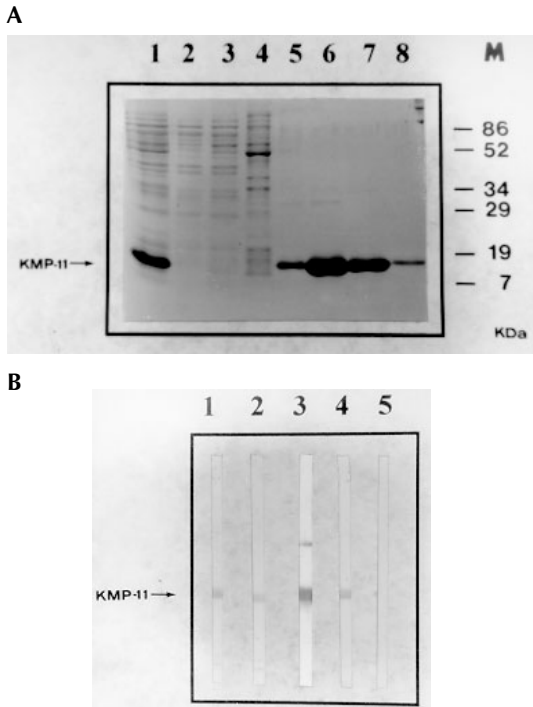
A

B

Fig. 4: *Escherichia coli* expression and western blot analysis of *Leishmania. (V) panamensis* KMP-11 protein. A: SDS-PAGE analysis of the expression and purification of the His-tagged recombinant KMP-11 protein. At each purification step, an aliquot was electrophoresed and the gel stained with coomasie blue. 1: supernatant phase of sonicated bacterial culture, 2: column flowthrough, 3 and 4 : washing fractions, 5, 6, 7 and 8: elution fractions, M: molecular weight marker. B: western blot of KMP-11 using serum of two VL patients (1, 2), rabbit polyclonal serum raised against the *L. (L) infantum* rKMP-11 (3), L98 mAb (4) and negative control (no primary antibody was added) (5). The immunoreactive band corresponding to a 14 kDa protein is indicated.

immunoreactivity against the heterologous KMP-11 (Table) from *L. (V) panamensis*, consistent with the high levels of anti-*Leishmania* antibodies observed in these patients (Sacks et al. 1993). In addition, the immunological cross-reactivity between these VL sera and the *L. (V) panamensis* protein confirms the high level of conservation of KMP-11 between species causing either CL, MCL or VL.

The potent B-cell immunogenic role of KMP-11 was also demonstrated when assaying sera from asymptomatic *Leishmania*-infected persons in western blots. A total of 86% (6/7) of these sera were seropositive for KMP-11, although, in general, the immunoreactivity was weak compared with the titers observed in most of the CL, MCL and VL sera. The low level of anti-*Leishmania* antibodies in these asymptomatic infections is consistent with the predominant Th1-like cellular immune reponse found in autohealing CL and patients

TABLE

Western blot reactivity of human sera against *Leishmania (V) panamensis* KMP-11. The immunoreactivity was semi-quantitatively evaluated and according to the intensity of the signals marked with one to four crosses. The minus sign means no reactivity. In the first group, 15 CL sera (CB 1-15), 9 MCL sera (W 1-9) and 2 VL sera (VL 2-3) were tested. In the second group 7 sera from asymptomatic MST positive individuals (R 1-7) and 9 sera from healthy MST negative individuals (CB 45-50, ESTBLC 1-3) were also tested

Reactivity		Reactivity	
Group 1			
Cutaneous leishmaniasis		Mucocutaneous leishmaniasis	
CB1	+++	W1 (3106)	+
CB2	+	W2 (1872)	+
CB3	++	W3 (2129)	++
CB4	+	W4 (1702)	-
CB5	+	W5 (3114)	-
CB6	++	W6 (2105)	+
CB7	-	W7 (2326)	+++
CB8	+	W8 (2534)	+
CB9	-	W9 (1874)	+++
CB10	+		
CB11	+		
CB12	++	Visceral leishmaniasis	
CB13	+		
CB14	++	VL2	++++
CB15	+	VL3	++++
Group 2			
Asymptomatic MST positive		Healthy MST negative	
R1	+	CB45	-
R2	-	CB46	-
R3	+	CB47	-
R4	+	CB48	-
R5	+	CB49	-
R6	+	CB50	-
R7	+	ESTBLC1	-
		ESTBLC2	-
		ESTBLC3	-

without clinical symptoms of leishmaniasis (Carvalho et al. 1995). The negative control group (non-*Leishmania* infected persons) in contrast, did not show any reactivity against KMP-11 indicating lack of natural IgG antibodies against this protein.

DISCUSSION

Recent reports on the cloning and molecular characterization of the KMP-11 protein in the Old World *Leishmania* species *L. (L) donovani* and *L. (L) infantum* (Jardim et al. 1995b, Berberich et al. 1997) and in the *Trypanosoma* species *T. b. rhodesiense* (Stebeck et al. 1996) revealed similiar

structural features of this kinetoplastid-specific protein, although differences in the genomic arrangement and in the pattern of distribution were found that may influence its function. The present study was carried out to analyze the sequence and gene locus organization of KMP-11 in the New World *Leishmania* species *L. (V) panamensis*, the prevalent etiological agent of cases of CL and MCL in Colombia (Corredor et al. 1990, Colombian Health Ministry 1994). Here, we provide evidence that the sequence of KMP-11 is highly conserved (> 95% homolgy) in all *Leishmania* species so far analyzed as well as the genomic organization consisting of three gene copies, regardless of the epidemiological pattern and the clinical symptoms of leishmaniasis they cause. The *T. b. rhodesiense* counterpart of this protein, in contrast, shows 18% divergence with respect to *Leishmania* and the gene is transcribed from a single gene copy (Stebeck et al. 1996). Immunofluorescence studies also showed that the *L. (L) donovani* (Stebeck et al. 1995), the *L. (L) infantum* (C Berberich, manuscript in preparation) and the *T. b. rhodesiense* (Stebeck et al. 1996) KMP-11 exhibit different fluorescence signals pointing to strict species-specific differences in the distribution of this protein.

The biological function of this protein is still far from being clear. Recently, it has been speculated that the monomethylarginine in the *L. (L) donovani* KMP-11 sequence on position 45 might be involved in parasite-defense mechanisms (Jardim et al. 1995a) after partial degradation of the protein in the parasitophorus vacuole. This modified arginine residue is known as a competitive inhibitor of nitric oxide synthetase, a central enzyme involved in generation of the anti-*Leishmania* radical nitric oxide within activated murine macrophages (Liew et al. 1990). It was proposed that within the strongly hydrolytic conditions of the phagolysosome, the KMP-11 protein would be released from the parasite and degraded to amino acids by proteolytic enzymes. Subsequently the inhibitory monomethylarginine would contribute to the enzyme inhibition and parasite survival (Jardim et al. 1995a). However, in the *L. (V) panamensis* sequence as well in the *L.(L) infantum* (Berberich et al. 1997) and *T. b. rhodesiense* (Stebeck et al. 1996) molecules, this arginine was substituted by a lysine and should therefore not account for such a speculative function. Since the consistent co-purification of a minor component together with the full-length *L. (L) donovani* KMP-11 indicates a specific proteolytic event and, since major post-translational modifications including O-glycosylation and N-methylation of this protein seem to occur (Jardim et al. 1995a), we cannot, however, exclude that a monomethylarginine residue from another position might be responsible for such an effect. Both, the exact pattern of post-translational modifications and a potential inhibitory role of putative monomethylarginines in the KMP-11 molecule remain to be determined. So far, what can be assumed is, that the high level of sequence conservation of KMP-11 together with the wide distribution throughout kinetoplastid parasites, suggest an essential role of this protein in the parasite biology. Protein modeling analysis and detailed CD-experiments (Jardim et al. 1995b, M Fuertes & C Berberich, manuscript in preparation) suggest that KMP-11 has a highly amphipatic character with an unusually high degree of alpha-helical structure sharing important thermodynamic features with the class of apolipoproteins (Ryan et al. 1993). It has been therefore hypothezised, that the KMP-11 protein could be involved in the stabilization of the parasite membrane. However, recent findings of a strict developmentally regulated and stage-specific expression together with a defined cellular localization mainly around the flagellum and flagellar pocket in *L. (L) infantum* and *T. b. rhodesiense* (Stebeck et al. 1996, C Berberich, manuscript in preparation) suggests additional functions.

The detailed antigenicity analysis of the recombinant KMP-11 of *L. (V) panamensis* in human CL, MCL and VL sera suggests that this protein acts as a potent B-cell immunogen during natural course of disease. Our results show that 82% (27/33) of *Leishmania*-infected humans (symptomatic or asymptomatic) contain detectable levels of anti-KMP-11 IgG antibodies (Table). In general, a high degree of variability in the anti-KMP-11 antibody level was observed in CL and MCL sera and a stronger immunoreactivity is associated with symptomatic infections when compared with the asymptomatic form. Therefore, the high antigenicity of KMP-11 may point to a potential serological marker for diagnosis of infection and disease. However, in view of the immunological cross-reactivity observed against the *T. cruzi* counterpart of KMP-11 (Stebeck et al. 1995) it may be necessary to determine specific epitopes in KMP-11 capable to distinguish between leishmaniasis and Chagas disease. Future studies will also concentrate on the T-cell antigenicity in all forms of human and mouse leishmaniasis in order to evaluate KMP-11 as a possible vaccine candidate.

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