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Biochimica et Biophysica Acta 1500 (2000) 77–87
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Molecular characterization of a *Leishmania donovanii* cDNA clone with similarity to human 20S proteasome a-type subunit[1]

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Received 13 January 1999; received in revised form 21 June 1999; accepted 5 August 1999

Abstract

Using plasma from patients infected or previously infected with *Leishmania donovanii*, we isolated a *L. donovanii* cDNA clone with similarity to the proteasome a-type subunit from humans and other eukaryotes. The cDNA clone, designated LePa, was DNA sequenced and Northern blot analysis of *L. donovanii* poly(A^+)mRNA indicated the isolation of a full length cDNA clone with a transcript size of 1.9 kb. The expressed recombinant LePa fusion protein induced proliferation of peripheral blood mononuclear cells in one out of seven patients who had suffered from visceral leishmaniasis. Plasma from 16 out of 25 patients with visceral leishmaniasis and four out of 18 patients with cutaneous leishmaniasis contained IgG antibodies which reacted with the purified LePa fusion protein as evaluated in an ELISA. The LePa DNA sequence was inserted into an eukaryotic expression vector and Balb/c mice were vaccinated. DNA vaccination of Balb/c mice with LePa generated an initial significant reduction in lesion size after challenge. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: *Leishmania*; cDNA library; Proteasome a-subunit; DNA vaccination

1. Introduction

The leishmaniases are a group of parasitic diseases caused by *Leishmania* species. Depending largely on the species of parasite and the immune response of the host, the disease spectrum ranges from self-healing cutaneous leishmaniasis to fatal visceral leishmaniasis or kala azar.

The immune mechanisms operating during disease and cure of leishmaniasis have been extensively studied both in animal models and in human infection. T-cells play an important role in the development of immunity and cure [1]. The humoral response during visceral leishmaniasis consists primarily of an unspecific hyperinduction of IgM- and IgG-type antibodies, but a multitude of specific antibodies are induced against a variety of *Leishmania* promastigote and amastigote antigens [2–6].

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[1] The sequence data reported in this paper have been submitted to EMBL/GenBank and DDJB data libraries under accession No. AF088882.

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PII: S0925-4439(99)00086-1

Several *Leishmania* antigens activate T-cells from individuals who have recovered from leishmaniasis, indicating that T-cell clones recognizing epitopes on these proteins are expanded during natural infection [7–9]. These findings have supported the candidature of the antigens in vaccine development. A common feature of these antigens is that natural infection not only induces a T-cell response, but also activates specific B-cells, since specific IgG can be detected in patient plasma both in the acute phase before treatment and during convalescence [8].

In the search for antigens which can induce protective immune responses during *Leishmania* infection, we used a pre-selected high-titred pool of antibodies from visceral leishmaniasis patients with an ongoing or cured infection to select immunological relevant antigens from an amastigote *Leishmania donovanii* cDNA library. In this study, we report the cloning, expression and immunological properties of an antigen which has similarity to the human 20S proteasome a-type subunit. Its potential as a DNA vaccine candidate is also evaluated.

2. Materials and methods

2.1. Parasites

L. donovanii (strain Ldd MHOM/SD/00/1S-2D, clone Ldd 1SC12, abbreviated Ld2d) was kindly donated by Dr Dennis Dwyer (NIH, MD, USA). A Ld2d amastigote culture was used for cDNA library construction and production of antigens. *Leishmania major* (MHOM/IL/67/LRC-L437) was used for DNA vaccine experiments. *L. donovanii* Ld2d amastigotes were cultured as described by [10] whereas *L. donovanii* and *L. major* promastigotes were cultured at 26°C in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, 20 IU/ml of penicillin and 20 mM streptomycin.

2.2. Donors and separation of blood samples

Peripheral blood was collected by venipuncture into heparinized vacutainers (Becton Dickinson) and peripheral blood mononuclear cells (PBMC) and plasma were isolated after Lymphoprep (Nyegaard, Oslo, Norway) density gradient centrifugation and cryopreserved as described previously [11]. The donors were (1) Sudanese patients with parasitologically confirmed visceral leishmaniasis caused by *L. donovanii* and patients who had been treated for visceral leishmaniasis [12], (2) Sudanese individuals with active or healed cutaneous leishmaniasis caused by *L. major* [13] or donors with healed cutaneous leishmaniasis [14], (3) Sudanese control donors from an area not endemic for leishmaniasis [15], (4) Sudanese donors with malaria [16], (5) Danish control donors without known exposure to *Leishmania*.

2.3. Leishmania donovanii soluble antigens

Promastigotes and amastigotes were sonicated for use in PBMC and ELISA as described previously [17].

2.4. Selection of plasma for antibody screening

Plasma from donors exposed to *Leishmania* were tested in ELISA measuring antibodies against *L. donovanii* sonicates from promastigotes or amastigotes, respectively. The assays were performed as described earlier [17]. Plasma from 13 patients with visceral leishmaniasis and five individuals cured from visceral leishmaniasis was selected, pooled and stored at 4°C until further use.

2.5. Construction and screening of amastigote cDNA library

Total RNA was isolated from axenic amastigotes by the method described in [18], utilizing the single step acid guanidinium thiocyanate procedure. Poly-(A^+)mRNA was isolated by loading total RNA onto an oligo(dT) column according to the protocol provided by the manufacturer (Life Technologies). The amastigote cDNA library was constructed in the λZAP Express vector system according to the manual provided by the manufacturer (Stratagene). Standard protocols [19] and protocols supplied by the manufacturer (Stratagene) were used for screening the amastigote cDNA library. The filters were probed with the absorbed antibody solution diluted 1/1000 in blocking buffer and processed as described for Western blots. Antibody-reactive cDNA clones appearing on both set of filters were selected and

then in vivo excised from the λZAP Express vector to form the pBK-CMV phagemid.

2.6. Northern blot analysis

Amastigote and promastigote poly(A^+)mRNA were purified as described above. Three μg of promastigote poly(A^+)mRNA and 0.5 μg of amastigote poly(A^+)mRNA were separated in a standard denaturing MOPS-formaldehyde agarose gel [19]. The DNA probe was generated by PCR, amplifying a DNA fragment positioned in the open reading frame of the cloned cDNA and subsequently purified by Qiaquick (Qiagen). Labelling of the probe with dCTP ([α-^{32}P]dCTP, 9.25 MBq, Amersham) was done using the Megaprime system (Amersham) following the manufacturer's protocol. The blot was processed as described by [19].

2.7. Subcloning of LePa into pGEX-4T3 and pcDNA 3.1 vectors and purification of fusion protein

To obtain fusion protein for immunological assays, the cDNA insert from the isolated pBK-CMV phagemid was subcloned into the GST fusion protein vector pGEX-4T3 (Pharmacia). The plasmid was subsequently transformed into *Escherichia coli* BL-21 cells (Pharmacia) for high level fusion protein expression. Induction and purification was done as described in [20]. pGEX-4T3 vector without insert was treated as described above and the eluate from the column was used as a control in the PBMC assay (see below). In order to ligate LePa cDNA into the pcDNA vector for immunization of mice, the LePa cDNA insert was PCR amplified using primers containing restriction sites. The pcDNA vector and PCR generated insert were ligated after digestion with restriction enzymes.

2.8. SDS-PAGE and Western blotting

All SDS-PAGE mini-gels for monitoring induction, solubility and purification of fusion proteins for Western blots were performed on Novex minigel electrophoresis equipment (Xcell II, Novex) using standard Laemmli buffers [21]. After electrophoresis, SDS-PAGE gels were incubated 10 min in transfer buffer and processed as described by the manufacturer (Novex). Blotting of proteins onto nitrocellulose membranes was performed for 2 h at 30 V constant voltage. After blotting, nitrocellulose membranes were washed and subsequently blocked and processed as described in [22]. Western blots were incubated with a promastigote antigen-absorbed serum pool from the original selected screening donors and controls or mice sera. Blots were then washed and incubated for 1 h at room temperature with the relevant IgG alkaline phosphatase-conjugated antibody. Development of the blot was done using standard techniques.

2.9. PBMC assays

On the day of assay PBMC were thawed, washed and counted [11]. Wells of round-bottomed microtitre plates received 0.66×10^6 cells/ml of PBMC in 150 μl of RPMI 1640 supplemented with 15% heat-inactivated pooled normal human serum, 58.4 μg/ml L-glutamine, 20 IU/ml penicillin, and 20 μg/ml streptomycin (Gibco, Paisley, UK). Twenty μl of antigen solution or 20 μl of medium was added to test cultures and control cultures, respectively. The recombinant LePa and control GST solutions were diluted in RPMI 1640 and used at a final concentration of 1–20 μg/ml. A sonicate of *L. donovanii* promastigotes was used at a final protein concentration of 7 μg/ml. Purified protein derivative of tuberculin (PPD; Statens Seruminstitut, Copenhagen, Denmark) was used at a final concentration of 12 μg/ml. After incubation for 6 days at 37°C in a humidified atmosphere containing 5% CO_2 the cells were pulsed with 20 μl/well of [^{3}H]thymidine (New England Nuclear, Boston, MA, USA) (1.85 Mbq/well). The cells were harvested onto glass fibre filters on day 7 and the incorporation of [^{3}H]thymidine into DNA was determined by a Matrix counter (Packard, Greve, Denmark).

2.10. rLePa ELISA

The rLePa and GST were expressed in *E. coli*, purified as described above and the ELISA procedure described in [17] was essentially followed.

2.11. Immunization and DNA vaccine experiments

For generation of antibodies for use in Western

blots four Balb/c mice were immunized intradermally in the tail with 80 μg of pcDNA-LePa in 50 μl of saline a total of 3 times with 2 week intervals. Mice were bled 2 weeks after the last immunization. For DNA vaccination groups of six female Balb/c mice were injected with pcDNA 3.1 (control plasmid) or pcDNA 3.1 containing the LePa cDNA insert. Each mouse received 50 μg of plasmid in 50 μl saline in each tibialis anterior muscle (100 μg total). Mice were boosted with the same amount of DNA at 3 weeks and 6 weeks post priming. Mice were challenged with 10^6 *L. major* promastigotes in the rump at 2 weeks after the last immunization. The lesion size was measured weekly using a calliper. Parasite loads in the lesions and in the spleen were determined by microscopy and by a limiting dilution assay [23], respectively.

2.12. DNA sequencing and analysis

DNA sequencing was performed using an automated ABI 377 sequencing system and the AmpliCycle Sequencing Kit (Perkin Elmer). DNA sequence analysis was performed using DNAstar computer software (Madison, WI, USA), while sequence homology analysis was performed by the programmes offered in the Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group (Madison, WI, USA) available at Biobase (Aarhus, Denmark).

2.13. Calculations and statistical analysis

PBMC and ELISA tests were done in duplicate. In the ELISA a positive plasma pool was used as a reference. The results were calculated as arbitrary ELISA units $(OD_{sample}-OD_{background})/(OD_{reference\ plasma}-OD_{background})\times 100\%$ to account for day-to-day variation. The cutoff level was determined as the mean ELISA units of the plasma from non-endemic controls plus 2 S.D.s. Because of the skewed distributions of the data the Kruskal-Wallis one way analysis of variance on ranks was applied for multiple comparisons and if significant differences were found, Dunn's method was used to identify significant differences between groups. All P values less than 0.05 were considered significant. All calculations were performed using SigmaStat version 2 software (Jandel Scientific). In the DNA vaccination experiment, a two way repeated measures analysis of variance on two factors was used to analyse the data. All P values less than 0.05 were considered significant. All calculations were performed using SigmaStat version 2 software (Jandel Scientific).

In the PBMC assay, the response to an antigen was expressed as the increment in kilocounts per minute (kCPM). A positive response was defined as stimulatory index (SI) >2.0 and increment of >0.3 kCPM.

3. Results

3.1. Screening of cDNA library

Plasma from 18 donors with active or cured visceral leishmaniasis was used to screen the *L. donovanii* amastigote cDNA library. From a total of 2×10^6 recombinant clones, 54 clones were isolated and insert size evaluated by PCR. A number of clones were selected and pBK-CMV phagemids were generated by in vivo excision. One clone, which reacted strongly with the visceral leishmaniasis plasma pool, was selected for further study.

3.2. DNA sequence analysis

The sequence of the cloned cDNA insert is shown in Fig. 1. The sequence contained a 1821 bp insert with an open reading frame of 753 bp found in the 5′ end of the sequence. A putative methionine start codon was identified at nucleotide position 69 in the open reading frame. No additional start codons (ATG) were found upstream from the putative start codon, and the nucleotide at position −3 relative to the start codon was an A as predicted for 95% of eukaryotic mRNA sequences [24]. However, the nucleotide at position +4 was different from the expected purine base (G). The insert showed a poly(A) sequence at the 3′ terminus indicating the poly-(A^+)mRNA origin. However, a poly(A) adenylation signal sequence (AATAAA) was not identified. The translated open reading frame indicated expression of a putative protein with a molecular mass of approx. 27.2 kDa (250 amino acids) and a calculated p*I* of 7.7. The open reading frame showed a 49.2%

```
   1 caaagtgcgactctcatcgactttctgattctctttccgttcttatcctttttcttctag

  61 acagcaccATGAACCGCGCAGGCTTCGACAAGTACATTACCGTCTTCAGCCCTGAGGGCT
   1         M  N  R  A  G  F  D  K  Y  I  T  V  F  S  P  E  G  S

 121 CGCTGTACCAGGTGGAGTACGCATTCAAAGCCGTCACCTACCCTGGCTTGCTCACCATTG
  19   L  Y  Q  V  E  Y  A  F  K  A  V  T  Y  P  G  L  L  T  I  A

 181 CCATTCGCTGCAAGGATGCCGTTCTGGTCGCAACGCAGCACGTGATTCCCGACCGCCTCA
  39   I  R  C  K  D  A  V  L  V  A  T  Q  H  V  I  P  D  R  L  M

 241 TGCGCCCTGACTCGGTGACGGCGCTCTACGAGGTCACTCCGAGCATCGGCTGCTGCATGA
  59   R  P  D  S  V  T  A  L  Y  E  V  T  P  S  I  G  C  C  M  T

 301 CCGGTCGCGCCCCCGACGGGCGCGCGCTAGTGCAGCGCGCGCGAGAGGAAGCGTCGGATT
  79   G  R  A  P  D  G  R  A  L  V  Q  R  A  R  E  E  A  S  D  Y

 361 ACCACTACCGCTACGGTGTGCAGATTCCAATCGCTGTGCTGGCGAAGCGCATGGGCGACA
  99   H  Y  R  Y  G  V  Q  I  P  I  A  V  L  A  K  R  M  G  D  K

 421 AGGCCCAGGTGCGCACGCAACAGGCCGGCCTGCGGCCGATGGGTGTGGTGAGCACCTTCA
 119   A  Q  V  R  T  Q  Q  A  G  L  R  P  M  G  V  V  S  T  F  I

 481 TCGGCATGGATCAGAGTGACCAGGATGGCTCGCTGAAGCCGCAGATTTACACCGTCGACC
 139   G  M  D  Q  S  D  Q  D  G  S  L  K  P  Q  I  Y  T  V  D  P

 541 CGGCCGGCTGGACCGGTGGGCACATTGCATGCGCTGTTGGCAAGAAGCAGGTGGAGGCGA
 159   A  G  W  T  G  G  H  I  A  C  A  V  G  K  K  Q  V  E  A  M

 601 TGGCATTCTTGGAGAAGCGCCAGAAGAGCACCAAGTTTGACGAGCTGACGCAGAAGGAGG
 179   A  F  L  E  K  R  Q  K  S  T  K  F  D  E  L  T  Q  K  E  A

 661 CCGCGATGATCGCGCTGGCGGCGCTGCAGAGCGCGATCGGCACGGCTGTCAAAGCGAAAG
 199   A  M  I  A  L  A  A  L  Q  S  A  I  G  T  A  V  K  A  K  E

 721 AGGTGGAGGTGGGCCGCTGCACAGCCGCCAACCCGGCCTTTCAGCGTGTGCCGAATAGCG
 219   V  E  V  G  R  C  T  A  A  N  P  A  F  Q  R  V  P  N  S  E

 781 AGGTTGAGGAGTGGCTGACCGCCGTGGCCGAGGCGGATTAAgcggtgcgcgggcgacaac
 239   V  E  E  W  L  T  A  V  A  E  A  D  *

 841 ttagaacttcgagcaccgcaccgatttctgttcctttggcgcttttcctctccttgccct
 901 tcccctcctcacctgatctaccgcggcgcgtgcaatcgcatccgctttctctctattcgc
 961 actggaggctgctcagcgccgtgggcggcccacggcgcaaggggacgtgtgcaggtccca
1021 cgctggttcacgcggctggcgctcttgtactgcatgtccgtagcaaagacaggaaggaag
1081 gcacaccatggctgctctgttttcctgttcatgtgaagtgctcagaggaggtgttccacg
1141 catgcggacacgcaccctagcaccttccttgaaagaagcggaggggtggctgacgtacga
1210 tttgcctgagctgtgtctccgtcttatgtttattctccttccaccttctttttctttgg
1261 ggttcgtcctggtctttgtctatcttcttcatgtgtgcggttgtggttgctgagagcgcg
1321 ctgacaggcgcgcacggttttacgtgtgaaggctgtggcgagcccacacgagacgtggaa
1381 aagtgacgaccgcgcatcctcatggatcgctgtctgcgggggcctcgagtgtcggccccg
1441 ctgcgtgggtgcagcaaagggagagctagaaggcactgccggcgaggaggcgggtgcaat
1501 gaaaaggagctggaggcgagggatgggtacaggcaagagacgctgcttttccgttgcctg
1561 ttaaccggatcgcggtacgagcgagaggagggcggaggagacgaagaggtcgataccaag
1621 aagcacggcggcactgctgtgccaagcacatgtggcgcccacacgcctgccagctgcacc
1681 gtcgcccccctccctccctccctctcgcactttggcagcaccccttcgttcgcacttctt
1741 aattgttcgttctacggttggttggactactattctatgaatgcacatgttgaagaagaa
1801 acaaaaaaaaaaaaaaaaaa
```

Fig. 1. DNA and deduced amino acid sequence of LePa. The deduced coding region (residues 1–250) is indicated by single-letter amino acid abbreviations together with uppercase base designations. Stop codon is indicated by an asterisk.

```
LePa     1 ...MNRAGFDKYITVFSPEGSLYQVEYAFKAVTYPGLLTIAIRCKDAVLV  47
Hpas     1 MSRGSSAGFDRHITIFSPEGRLYQVEYAFKAINQGGLTSVAVRGKDCAVI  50

LePa    48 ATQHVIPDRLMRPDSVTALYEVTPSIGCCMTGRAPDGRALVQRAREEASD  97
Hpas    51 VTQKKVPDKLLDSSTVTHLFKITENIGCVMTGMTADSRSQVQRARYEAAN 100

LePa    98 YHYRYGVQIPIAVLAKRMGDKAQVRTQQAGLRPMGVVSTFIGMDQSDQDG 147
Hpas   101 WKYKYGYEIPVDMLCKRIADISQVYTQNAEMRPLGCCMILIGIDEEQ... 147

LePa   148 SLKPQIYTVDPAGWTGGHIACAVGKKQVEAMAFLEKRQKSTKFDELTQKE 197
Hpas   148 ..GPQVYKCDPAGYYCGFKATAAGVKQTESTSFLEKKVKK.KFD.WTFEQ 193

LePa   198 AAMIALAALQSAIGTAVKAKEVEVGRCTAANPAFQRVPNSEVEEWLTAVA 247
Hpas   194 TVETAITCLSTVLSIDFKPSEIEVGVVTVENPKFRILTEAEIDAHLVALA 243

LePa   248 EAD
Hpas   244 ERD
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Fig. 2. Protein sequence comparison between LePa (residues 1–250) and human proteasome a-subunit (Hpas; residues 1–246 [25]), showing 45.6% identity. Identical residues are shown with dark background.

DNA sequence similarity to the human 20S proteasome a-type subunit [25], a multi-substrate catalytic proteinase complex [26,27]. A Swiss-Prot protein database search showed 45.7% identity with the a-type subunit at the protein level. Fig. 2 shows the protein sequence comparison between the *Leishmania* sequence and human 20S proteasome a-type subunit.

3.3. Northern blot analysis

A labelled nucleotide probe spanning the first 712 nucleotides of the open reading frame hybridized to a transcript of approx. 1.9 kb from both the amastigote and promastigote stage (Fig. 3). The highest expression of the transcript, as indicated by the intensity of the signal, was seen in the promastigote stage as expected due to the higher concentration of loaded promastigote poly(A^+)mRNA.

3.4. GST fusion protein reactivity (Western blot, PBMC and ELISA)

The cDNA sequence encompassing the open reading frame was inserted into pGEX and recombinant LePa (rLePa) was produced. In a Western blot analysis using crude *E. coli* induced and non-induced lysate, anti-GST antibodies reacted with GST alone and with rLePa (Fig. 4A, lanes 2 and 4). Plasma from visceral leishmaniasis patients reacted with rLePa but not with GST (Fig. 4B, lanes 2 and 4). Plasma from Danish controls did not react with GST or rLePa (Fig. 4C). rLePa was then used in proliferation assays and ELISA.

The rLePa fusion protein induced proliferation in PBMC cultures from one of seven individuals previously infected with *L. donovanii* (Table 1). PBMC isolates from four individuals previously infected with *L. major* or four Danish controls did not respond to rLePa (data not shown). rLePa was used as solid phase ligand in an ELISA (Fig. 5). Antibodies to the fusion protein were detected in plasma from 16 of 25 visceral leishmaniasis patients and in

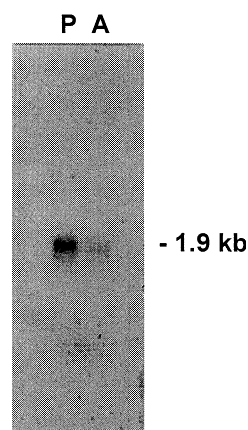

Fig. 3. Northern blot analysis of LePa. Three μg of poly(A^+)mRNA from promastigotes (P) and 0.5 μg of poly(A^+)mRNA from amastigotes (A) were separated on a formaldehyde-agarose gel. After transfer onto nitrocellulose, the blot was hybridized with a probe encompassing the open reading frame of LePa. The size of the transcript is indicated on the right.

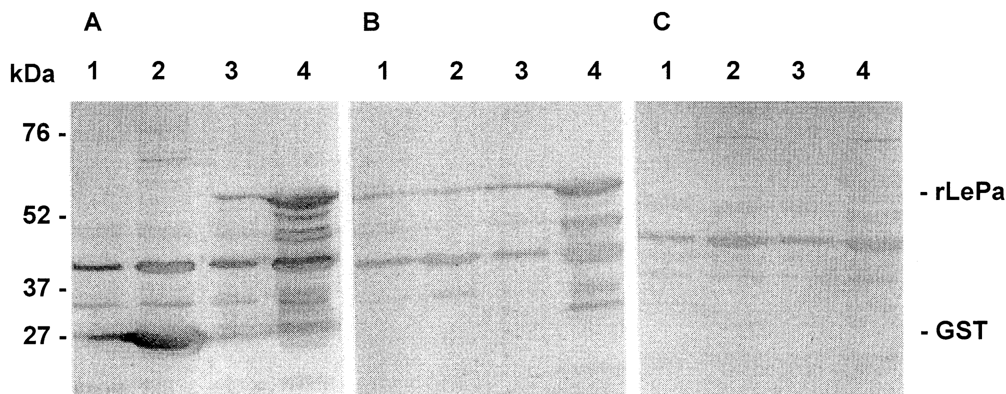

Fig. 4. Western blot analysis of antibody reactivity against bacterial lysates containing GST or LePa. (A) Anti-GST antibodies; (B) plasma from visceral leishmaniasis patients; (C) Danish plasma controls. The antigen was: lanes: 1, GST culture before IPTG induction; 2, GST culture after IPTG induction; 3, rLePa culture before IPTG induction; 4, rLePa culture after IPTG induction. Molecular mass markers are indicated.

four of 18 patients with cutaneous leishmaniasis. Plasma from Sudanese controls did not show any antibody reactivity to rLePa. A significant difference was found between the three patient groups ($P < 0.05$).

3.5. DNA vaccination

The LePa open reading frame was ligated into the pcDNA3.1 eukaryotic expression vector. In order to verify the integrity of the construct and its ability to express protein, four Balb/c mice were immunized 3 times intradermally in the tail. The antibodies were tested in a Western blot and were shown to react with the rLePa, whereas serum from mice immunized with empty pcDNA3.1 did not react with the rLePa (Fig. 6). To test the efficacy of DNA vaccination with LePa DNA, groups of six mice were vaccinated 3 times intramuscularly and challenged with 10^6 *L. major* promastigotes 2 weeks after the last DNA vaccination. DNA plasmid without insert was used as a control. As shown in Fig. 7, DNA vaccination of mice with pcDNA-LePa reduced the lesion size up to 7 weeks post infection as compared to the controls. However, at week 9 post infection, the lesion size of pcDNA-LePa vaccinated mice was identical to that of the controls. When data

Table 1
Proliferative response using PPD, crude *L. donovanii* promastigote sonicate (LDS) and rLePa in PBMC from individuals previously infected with *L. donovanii*

Antigen	Donor						
	1	2	3	4	5	6	7
PPD	2.6[a]	10.7	2.3	2.8	14.2	1.7	25.4
LDS	9.2	16.0	3.6	3.5	6.4	0.7	21.7
rLePa	0	2.9	0.2	0.2	1.4	0.1	0.5
Control[b]	0	0	0.1	0	0	0	0.3

A positive proliferative response was defined as SI > 2.0 with increment of > 0.3 kCPM.
[a]The incorporation of [^{3}H]thymidine into unstimulated cultures, which was > 0.4 kCPM for all donors, was subtracted.
[b]Control antigen was column eluate without rLePa.

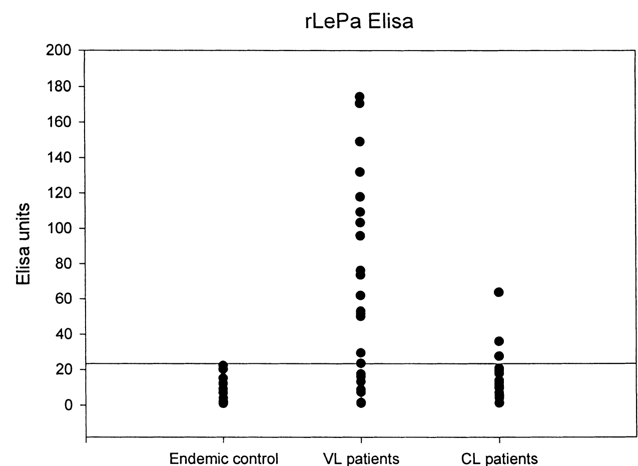

Fig. 5. Antibody reactivity (units) in plasma evaluated by ELISA using individual plasma samples from three different groups. The groups were endemic controls from the Sudan, visceral leishmaniasis (VL) patients from the Sudan and cutaneous leishmaniasis (CL) patients from the Sudan, respectively. The horizontal line indicates the cutoff value.

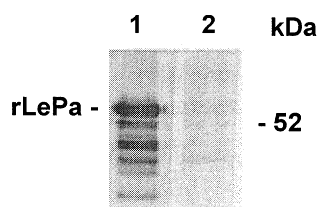

Fig. 6. Western blot analysis of antibody reactivity against purified rLePa. Lanes: 1, serum from mice immunized with pcDNA-LePa; 2, serum from mice immunized with empty pcDNA. Molecular mass marker is indicated.

were statistically analysed, a significant difference ($P<0.01$) was found between the two experimental groups.

The mice were sacrificed at week 10 post infection and the lesions were evaluated for the presence of parasites, but no difference was found between the number of parasites in the pcDNA-LePa vaccinated mice and the controls (data not shown). However, one of the pcDNA-LePa vaccinated mice had no visible amastigotes in the lesion and no promastigotes could be grown from the spleen (data not shown).

4. Discussion

In this study we used antibody-based screening of a cDNA library to identify a novel *Leishmania* antigen with similarity to the human 20S proteasome a-type subunit. The *L. donovanii* antigen, LePa, is immunogenic during natural infections in humans and a LePa DNA vaccine induced an initial reduction in the lesion size of mice challenged with *L. major*.

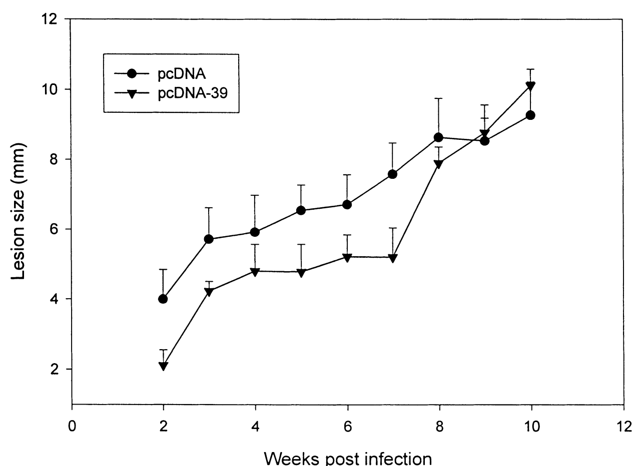

Fig. 7. DNA vaccination of Balb/c mice with LePa DNA. Values represent mean lesion diameter (±S.E.M.) for the two groups (six mice/group) following challenge with *L. major* promastigotes.

During the initial DNA sequencing of isolated cDNA clones, several of these clones showed similarity to already known sequences. More than 20% of all the isolated cDNA clones showed various degrees of similarity to kinesin-like proteins and heat shock proteins [4].

DNA sequence analysis and Northern blot analysis indicated that a full length poly(A^+)mRNA was isolated, and only one transcript of the expected size was present. In the Northern blot we used less amastigote poly(A^+)mRNA than promastigote poly(A^+)mRNA due to the time consuming task of producing amastigotes in large amounts. However, a transcript of similar size was also observed in the amastigote stage. By aligning the derived amino acid sequence to human proteasome a-subunit, the putative *Leishmania* methionine start codon also aligned at the start codon of the human homologue, indicating the isolation of a full length poly(A^+)mRNA.

DNA and protein database search showed that the translated protein was novel to *L. donovanii*, but showing similarity to the human 20S proteasome a-type subunit and other proteasome homologues. The a-type subunit is part of the multicatalytic proteinase complex as described in [25]. In vivo, the 20S proteasome has been shown to degrade abnormal proteins, e.g. misfolded proteins induced by heat shock, and function as a regulatory protein by degrading active enzymes [28]. Recently, a 20S proteasome was reported isolated from *Trypanosoma brucei* [29], but no DNA sequence data were given. Thus, this is the first report on isolation and sequencing of a 20S proteasome subunit from *Leishmania* and trypanosomatid parasites in general.

The immunogenicity of the isolated LePa cDNA clone was confirmed in a Western blot by the reactivity of GST-LePa with the same plasma pool from visceral leishmaniasis patients as used in the screening process. In the Western blot analysis, several antibody reactive bands were visible in the crude GST-LePa antigen fraction that was incubated with the human leishmaniasis plasma pool. These bands are probably breakdown products of the GST-LePa.

That these bands are not contaminating bands is indicated by the lack of bands above the expected molecular mass of GST-LePa.

rLePa induced proliferation in two of seven PBMC cultures from patients who had suffered from visceral leishmaniasis, indicating that the donors had expanded T-cell clones recognizing LePa after exposure to the parasite. The low percentage of responders might be due to the fact that the antigen was produced in *E. coli*. In several studies it has been shown that recombinant *E. coli* fusion proteins have been found to be less effective in activating T-cells than the native molecules or constructs made in yeast [30–32]. However, the immunogenicity of LePa during natural infection in humans was also demonstrated by the fact that antibodies to rLePa were found in more than 50% of patients with visceral leishmaniasis caused by *L. donovanii*. This percentage is comparable to the prevalence of anti-gp63 antibodies in patients with visceral leishmaniasis [17].

Four patients with cutaneous leishmaniasis caused by *L. major* had antibodies to rLePa indicating that there is an immunological cross-reactivity between LePa from *L. major* and *L. donovanii*. This notion is supported by the fact that PCR amplification of the open reading frame of LePa from six *L. donovanii* isolates and two *L. major* isolates revealed a single band of the same molecular mass (data not shown). A high degree of similarity between the two *Leishmania* species is also to be expected given the similarity between the human protein and LePa, and the fact that proteasomes play a pivotal role in the metabolism in various higher organisms [28].

The DNA vaccination studies showed a significant protective effect in mice receiving the LePa DNA vaccine. Mice vaccinated with LePa DNA had smaller lesions than controls during the first 7 weeks of the experiment, thereafter lesion sizes were comparable between the two groups. After 10 weeks, the parasite loads in the two groups were also comparable. This suggests that DNA vaccination with LePa DNA induced immunological memory and that protective immunological effector mechanisms were activated at challenge, but they were not sufficient to keep the infection from progressing. Balc/c mice are notoriously difficult to protect from *L. major* infection, especially when the challenge dose is as high as 10^6 parasites as used in our study.

In studies by Gurunathan et al. [33], it was shown that DNA vaccination of Balb/c mice with LACK DNA and challenged with 10^5 promastigotes was able to significantly decrease the foot pad swelling as long as week 20 post infection. In addition, the parasite load in the draining lymph node was reduced by a factor of 100. In our studies, however, we did not see a significant difference between the number of parasites in lesions of pcDNA-LePa vaccinated mice and controls. In a recent study by Sjölander et al. [34], DNA vaccination with the soluble and membrane bound form of PSA-2 was able to reduce the lesion size in C3H/He mice. Walker et al. [35] were able to obtain a 30% protection in Balb/c mice when vaccinated with the cell surface glycoprotein gp63. However, it is important to point out that caution has to be taken when comparing different mouse strains and different challenge doses.

In conclusion, the vaccination results obtained by LePa DNA should not exclude LePa as a potential DNA vaccine candidate. A recent study [36] has shown that injection of immunostimulatory CpG oligonucleotides in Balb/c mice followed by *L. major* challenge was able to shift the initial Th2 response towards a Th1 response. Although the LePa DNA vaccine was unable to protect Balb/c mice against a *Leishmania* challenge infection, such immunostimulatory sequences added together with the DNA vaccine or incorporated into the plasmid construct could be a way to induce a protective immune response using less protective antigens such as LePa. Studies in this direction are currently underway.

Acknowledgements

We thank Anne Corfitz and Gitte Kristensen for excellent technical assistance. This work was supported by the Danish International Development Agency (Danida), the Danish Research Council for Development Research (RUF) and the Danish Medical Research Council (SSVF).

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