Cloning and Expression of a *Leishmania donovani* Gene Instructed by a Peptide Isolated from Major Histocompatibility Complex Class II Molecules of Infected Macrophages

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Summary

The studies reported here describe the isolation of peptides from MHC class II molecules of murine macrophages infected with *Leishmania donovani*, and the use of the derived peptide sequences to rescue the pathogen peptide donor protein. The isolation of the peptides was carried out by comparing the RP HPLC profile of peptides extracted from infected macrophages with the peptides extracted from noninfected cells. Several distinct HPLC peaks unique to infected macrophages were sequenced. One of the peptides that was not homologous to any known protein was used to instruct the designing of an oligonucleotide sense primer that was used in combination with an oligo dT nucleotide (anti-sense primer) to amplify by PCR a DNA fragment from *L. donovani* cDNA. The amplified DNA fragment was cloned and used as a probe to screen a *L. donovani* cDNA library. The cloned gene (Ld peptide gene) has an open reading frame of 525 bp and has no homology with any known protein/gene sequence. Northern blot analyses indicated that the Ld peptide/gene is broadly distributed and expressed among species of the *Leishmania* genus, in both the amastigote and promastigote life cycle forms. Using the pGEX 2T vector, the gene was expressed and the relationship of the purified recombinant protein with *L. donovani* was confirmed using both antibody and T cell responses from immunized or infected animals. The gene encodes a 23-kD molecule (Ldp 23) associated with the cell surface of *L. donovani* promastigotes. In addition, T cells purified from the lymph nodes of BALB/c mice immunized with *L. donovani* or infected with *L. major*, and from CBA/J mice infected with *L. amazonensis* were stimulated to proliferate by the recombinant Ldp 23 and produced high levels of IFN-γ and no IL 4. This observation suggests that the Ldp 23 is an interesting parasite molecule for the studies concerning the host/parasite interaction because the Th1 pattern of cytokine response that it induces is correlated with resistance to *Leishmania* infections. These results clearly point to an alternative strategy for the purification of proteins useful for the development of both vaccines and immunological diagnostic tools not only against leishmaniasis but also for other diseases caused by intracellular pathogens.

The development of an immune response against intracellular pathogens is strictly dependent on the ability of the host cell to process and present selected pathogen peptides in conjunction with MHC class I or class II molecules. MHC class I molecules present peptides processed in the cytosol whereas MHC class II molecules present peptides processed in the endocytic vesicles (1–3). In general, the infection of a given cell by a virus results in processing and presentation of the pathogen peptide in the context of MHC class I molecules. In contrast, peptides of pathogens that have tropism for the endocytic vesicles, such as certain bacteria (e.g., *Mycobacterium*), fungi (e.g., *Paracoccidioides braziliensis*), and protozoa (e.g., *Leishmania*) are presented in the context of MHC class II molecules. The MHC class I molecules are expressed in virtually all cells. MHC class II molecules have a more restricted distribution and are expressed mainly by cells of the immune system including B cells, macrophages, and dendritic cells. Peptide antigens that are bound by MHC class I molecules are recognized by cytolytic CD8 T cells whereas peptides that interact with MHC class II molecules are recognized by CD4 T cells (4, 5). This molecular/cellular organization, in gen-

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eral, confers to the immune system a well-balanced distribution of physiological labor. Thus, class I molecules/CD8 T cells are involved with recognition and control of the reactivity to self molecules, to several viral antigens, and other cytosolically processed antigens. Class II molecules/CD4 T cells are usually associated with the recognition and control of foreign antigens (intracellular pathogens including viruses) that are targeted to the intracellular vesicles (4, 6). Both, class I and class II molecules have been characterized by x-ray crystallography (7–9). It is generally accepted that the peptides present in the groove of the MHC molecules are ultimately the structures responsible for the development not only of immunity to foreign antigens but also for the establishment of self tolerance by positive and negative selection in the thymus (6, 10). Several groups have recently isolated naturally processed peptides bound to MHC class I and class II molecules and characterized them by direct sequencing or by analogy to synthetic peptides (11–20). The class I binding peptides are usually 8–9 amino acids. MHC class II molecules, by contrast, derive their peptides from proteins that are degraded in cellular vesicles and are usually 13–17 amino acids long (21). These findings have important implications for the design and development of vaccines against a variety of intracellular pathogens because they provide precise information about the epitopes selected by the infected host cell. However, thus far in practically all reported studies, the peptides that have been isolated from MHC class II molecules were obtained from experimentally noninfected B cells. Most of these peptides are derived from self proteins or are from proteins present in the culture medium. The present study was therefore conducted to investigate the usefulness of this novel approach to isolate peptides from experimentally infected cells. The protozoa *Leishmania donovani* was chosen because this pathogen infects macrophages exclusively and resides inside the phagosome vesicles that comprise the cellular compartment where most of the association between foreign peptides and MHC class II molecules takes place. We demonstrate here that pathogen peptides can indeed be isolated from MHC class II molecules of parasite-infected macrophages and also that this approach can be used to rescue immunogenic and potentially useful proteins from intracellular pathogens.

Materials and Methods

Animals, Immunizations, and mAb. BALB/c mice (H-2^d) of both sexes were obtained from The Jackson Laboratory (Bar Harbor, ME) and were used at 8–14 wk of age. New Zealand white rabbits were from Milbrook Farm (Amherst, MA).

To generate anti-*L. donovani*–specific T cells, BALB/c mice were immunized in the rear foot pad with 5–10 × 10^6 promastigotes emulsified in complete Freünd's adjuvant (CFA)[1] (Difco Laboratories, Madison, MI) as described (22). The draining lymph nodes were excised 8 d after the immunization and the T cells were purified as described below. For the anti-recombinant protein antibody production, BALB/c mice were immunized in the rear foot pad with 5–10 μg of protein emulsified in CFA. The mice were boosted 7 d later with 5–10 μg of protein emulsified in incomplete Freünd's adjuvant (IFA) inoculated into the peritoneal cavity. The mice were bled 7 d after the second immunization. The protocol for the rabbit immunization was: one intramuscular (IM) injection of 25–30 μg of purified recombinant protein emulsified in CFA into each thigh on day one; one IM injection of 25–30 μg of purified protein emulsified in IFA into each shoulder on day 7; on day 15, 25–30 μg of the purified protein in PBS was injected into the subcutaneous tissue. The rabbit was bled 7 d after the last immunization.

Culture supernatants of MK-D6 hybridoma cells (American Type Culture Collection, Rockville, MD) were employed as the source for anti-MHC class II (H-2^d) monoclonal antibody. Protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Richmond, CA).

Tissue Culture and Cell Lines. The medium used for tissue culture was Bruffs supplemented with L-glutamine, 10% FCS (HyClone Laboratories Inc., Logan, UT), and gentamicin. The macrophage cell line P338D1 (H-2^d) was a gift of Dr. Martin Dorf (Harvard Medical School, Boston, MA). All cultures were performed at 37°C in 5% CO_2. Lymph node T cells were purified in an anti-mouse Ig column to remove the B cells (23) followed by a passage through a Sephadex G10 column to remove the macrophages (24). Mitomycin treated BALB/c mouse spleen mononuclear cells were used as antigen presenting cells (APC). To assay for in vitro antigen-specific T cell response, 2 × 10^5 antigen pulsed APC were cultured for 3 d with 10^5 purified T cells and proliferation was measured by [^{3}H]thymidine (DuPont-NEN, Boston, MA) incorporation.

Leishmania Maintenance and Macrophage Infection. *L. donovani* (1S strain), *L. amazonensis* (MHOM/BR/77/LTB0016), *L. major* (MHOM/IR/79/LRC-L251), and *L. pifanoi* (MHOM/VE/60/Ltrod) promastigotes were grown and maintained at 26°C in Schneider's medium containing 20% FCS and 50 μg/ml gentamicin. The amastigote forms of *L. amazonensis* were obtained by differential centrifugation of a "pus-like" foot pad lesion of a CBA/J mouse infected for 6 mo with this parasite. *L. pifanoi* amastigotes were obtained from axemic culture as previously reported (25). Infection of the macrophage cell line P388D1 with *L. donovani* promastigotes was carried out for an initial incubation of 6 h at room temperature. The cultures were then transferred to 37°C for additional incubation periods. At a ratio of 3–5 parasites per macrophage nearly 90% of the macrophages were infected after 24 h of incubation at 37°C.

Purification of MHC Class II-associated Peptides from P388D1 Macrophages Infected with L. donovani. Approximately 1.5 × 10^{10} *L. donovani*-infected or an equal number of noninfected P388D1 macrophages were used for each purification. The cells were harvested, washed with PBS and incubated for 30 min in cold lysis buffer (PBS, 1% Nonidet P40, 25 mM iodoacetamide, 0.04% sodium azide, 1 mM aprotonin, and 1 mM PMSF). The insoluble material was removed by centrifugation at 40,000 *g* for 1 h and the supernatant was recycled overnight at 4°C over a 5 ml anti-MHC class II molecules (H-2^d) Sepharose column (Protein G Sepharose column to which the monoclonal antibody MK-D6 had been bound). The column was washed with 50 ml of lysis buffer and then with 50 ml of PBS containing 0.5% octyl glucopyranoside detergent. Bound molecules were eluted from the column with 1M acetic acid in 0.2% NaCl. The MHC/peptide

[1] *Abbreviations used in this paper:* CFA, complete Freünd's adjuvant; GST, glutathione S-transferase; IFA, incomplete Freünd's adjuvant; IM, intramuscular; IPTG, isopropyl-β-thiogalactopyranoside; NGS, normal goat serum.

molecules were separated from the IgG (MK-D6 mAb) using a Centricon 100 filter unit (Amicon Division, W.R. Grace & Co., Beverly, MA). The peptides were then dissociated from the class II molecules by the addition of acetic acid to 2.5 M, followed by separation using a Centricon 10 filter unit. The resulting peptide preparation, present in the low molecular weight sample, was then dried using a speed vac concentrator (Savant Instrument Inc., Farmingdale, NY). The peptides were redissolved in 200 μl of 0.05% TFA and separated by reverse-phase high performance liquid chromatography (RP-HPLC) using a 2.1 mm × 25 cm Vydac C-18 column at a flow rate of 0.15 ml/min employing a 1–30% acetonitrile gradient (60 min) followed by 30–60% gradient (30 min) and the 60–80% gradient (90–110 min).

Sequences of I-A^d and I-E^d-associated Peptides. The RP-HPLC separations were analyzed by comparing the UV-absorption profiles from preparations of MHC class II-associated peptides from infected and noninfected macrophages. Selected peaks, unique to the infected macrophages, were sequenced by Edman degradation on a gas-phase protein sequencer (model 477; Applied Biosystems, Inc., Foster City, CA). Protein sequence and amino acid analysis were performed by the W. M. Keck Foundation, Biotechnology Resource Laboratory (Yale University, New Haven, CT). Amino acid sequences were searched for identity with proteins in the GenBank database using the GENPETP, PIR, and SWISSPROT programs.

PCR. A peptide-derived oligonucleotide (5′-GGAATTCC-CCInCAGCTInGTInTTCGAC-3′) containing an EcoRI restriction endonuclease site (underlined) and a poly-thymidine oligonucleotide (oligo dT, downstream primer) containing a XhoI restriction endonuclease site, were used to amplify by PCR a gene fragment from a *L. donovani* promastigote cDNA preparation. The reaction conditions were: one cycle of 3 min at 94°C immediately followed by 35 cycles of 1 min at 94°C, 1 min at 45°C and 1 min at 72°C. The *L. donovani* cDNA was prepared from 5 × 10^7 washed promastigote forms harvested at the log growth phase (3-d culture). The cDNA was obtained using an Invitrogen cDNA cycle™ kit (Invitrogen Co., San Diego, CA). Oligonucleotide primers were synthesized by the DNA Synthesis Laboratory, Department of Pathology (Yale University School of Medicine).

Gene Cloning and DNA Sequencing. The PCR amplified gene fragment was ligated into the pCR™ vector using the TA cloning system (Invitrogen). Transformants were selected in LB medium containing 100 μg/ml ampicillin and the plasmid DNA was isolated using the Wizard™ Minipreps DNA purification kit (Promega Co., Madison, WI). Insert DNA was released with the restriction enzymes EcoRI and XhoI (New England Biolabs, Beverly, MA), purified from an agarose gel electrophoresis and labeled with ^{32}P using random priming method (Megaprime Labeling Kit; Amersham Life Science, Buckinghamshire, England). This DNA fragment was used as a probe to screen a *L. donovani* promastigote cDNA library (26). The gene encoding the protein containing the MHC class II-associated peptide (Ld peptide/gene) was excised from the phagemid by in vivo excision using the Stratagene protocol. DNA sequencing was performed using the Sequenase version 2 system (DNA sequencing kit) in the presence or absence of 7-deaza-GTP (United States Biochemical, Cleveland, OH). Sequence analysis was performed using the University of Wisconsin Genetics Computer Group Programs and the GenBank and EMBL data bases of protein and DNA sequences.

Bacterial Expression and Purification of Recombinant Protein. PCR was used to subclone the cloned gene in frame into the expression vector pGEX 2T. Primers containing the appropriate restriction site enzymes, initiation and termination codons were: 5′-GGATCCATGGTCAAGTCCCACTACATCTGC-3′ for the upstream primer and 5′-GAATTCAGACCGGATAGAAAT-AAGCCAATGAAA-3′ for the downstream primer (restriction sites of BamHI and EcoRI are underlined, respectively). PCR conditions were as the indicated above for the amplification of the original peptide related DNA fragment. The template used was pBluescript plasmid containing the cloned gene from the cDNA library. Overexpression of the recombinant fusion protein was accomplished by growing the transformed *Escherichia coli* (DH5α) and inducing the *tac* promoter with 1 mM isopropyl-β-thiogalactopyranoside (IPTG) (Stratagene, La Jolla, CA). Cells were collected, centrifuged, and analyzed for the presence of the fusion protein by SDS-PAGE. Purification of the fusion protein could not be done by affinity chromatography because of its high insolubility. To circumvent this problem the protein was purified from a preparative SDS-PAGE electrophoresis. The band was visualized with 0.1 M KCl, cut and electroeluted from the gel followed by extensive dialysis against PBS and concentration on Centricon 10 filters.

Northern Blot Analysis. The RNA was prepared from 2 × 10^7 parasite cells using the Micro RNA isolation kit (Stratagene) accordingly to the company's recommended instruction. RNA was prepared from *L. donovani* promastigotes (logarithmic growth phase); from *L. major* promastigotes (logarithmic and stationary growth phases); from *L. amazonensis*, both promastigotes (logarithmic and stationary growth phases) and amastigotes purified from CBA/J-infected mice; and from *L. pifanoi*, both promastigotes (logarithmic and stationary growth phases) and amastigotes (from axenic culture medium). The hybridization was carried out at 45°C in the presence of 50% formamide, 5× Denhardt's solution, 0.1% SDS, 100 μg/ml single stranded salmon sperm DNA, and 5× SSPE using 0.45-μm Nytran membrane filters (Schleicher & Schuell, Keene, NH). The probe was the ^{32}P-labeled Ld peptide/gene as indicated above.

Western Blot Analysis. Approximately 2 × 10^6 *L. donovani* promastigotes (log phase) were lysed with SDS-PAGE loading buffer and separated under reducing conditions using a 15% polyacrylamide gel. The proteins were transferred onto 0.45-μM Immobilon-P transfer membrane (Millipore Co., Bedford, MA) using a wet-type electroblotter (Mini Trans-Blot Electrophoretic Transfer Cell; BioRad Life Science Division, Richmond, CA) for 2 h at 50 V. The membranes were blocked overnight at room temperature with PBS containing 3% normal goat serum (NGS), 0.2% Tween-20, and 0.05% sodium azide, followed by three washes with PBS. The blots were then incubated for 3–4 h at 4°C with several dilutions of anti-recombinant protein antisera (or control sera). The sera were previously absorbed 2× with nonviable desiccated *Mycobacterium tuberculosis* H-37 RA (Difco Laboratories) and were diluted in PBS containing 1% NGS and 5% powdered nonfat bovine milk (Carnation, Nestlé Food Company, Glendale, CA). The membranes were then washed with PBS, incubated for 1 h at room temperature with goat anti–rabbit IgG (or goat anti–mouse IgG) antibody conjugated with alkaline phosphatase (Promega), washed once with PBS and 2× with veronal buffer, pH 9.4. The reaction was visualized using the substrate mixture 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) according to the manufacturer's instructions.

Flow Cytometry. *L. donovani* promastigotes (log phase) (3 × 10^6/100 μl) were washed 3× with staining buffer (PBS containing 3% FCS and 0.1% sodium azide) and then incubated for 1 h at

4°C with either various dilutions of mouse anti-recombinant protein antisera or control sera. The parasites were washed 3× with staining buffer and incubated for 45 min at 4°C with FITC-conjugated rabbit anti–mouse IgG/IgM antibody (HyClone Laboratories Inc.). The promastigotes were washed 3× at 4°C with staining buffer and the intensity of fluorescence was analyzed (ungated) by flow cytometry using a FACScan (Becton Dickinson, Sunnyvale, CA).

Cytokine Analysis. The levels of IFN-γ and IL 4 were measured by ELISA as previously described (27–29) using specific anti IFN-γ and IL 4 mAbs (PharMingen, San Diego, CA).

Results

Peptides Extracted from MHC Class II Molecules of L. donovani-infected P388D1 Macrophages Display a Distinct HPLC Profile. To ascertain that in vitro infection of macrophages would load their MHC class II molecules with parasite peptides, initial experiments were carried out to test the ability of the *L. donovani*-infected macrophage cell line P388D1 to present parasite antigens to *L. donovani*-specific T cells. This macrophage cell line was chosen because it has the same H-2 haplotype as the BALB/c mouse, which is a strain of mouse moderately susceptible to *L. donovani* infection and selected to conduct the in vivo experiments. Using a proportion of 3–5 parasites per cell and an initial incubation at room temperature for 4–6 h followed by 37°C for 24–48 h, close to 90% of the macrophages were infected. The level of MHC class II molecules expression as determined by FACS analysis, indicated that infection did not cause an effect on the levels of MHC class II expression when compared to noninfected control cells (not shown). To test the ability of the *L. donovani*-infected P388D1 cells to present parasite antigens, macrophages were infected as indicated above and incubated at 37°C for either 24, 48, or 72 h. At each of these time points the nonadherent cells and free parasites were washed out and the adherent cells were mechanically dislodged, washed, and fixed with paraformaldehyde. These cells were then used as APCs for purified lymph node T cells from BALB/c mice immunized with *L. donovani* promastigotes emulsified in complete Freünd's adjuvant. Results in Fig. 1 indicate that *L. donovani*-infected P388D1 macrophages process parasite antigens and that optimal presentation occurs after 48 h of infection. No stimulation of the T cells by the noninfected macrophages was observed. To isolate the MHC class II-associated *L. donovani* peptides, P388D1 macrophages were infected for 48 h and the MHC class II molecules were affinity purified as indicated in Material and Methods. Peptides were then obtained by acid elution and filtration over a Centricon 10 membrane and fractionated by RP-HPLC on a C18 column. Noninfected P388D1 cells were similarly processed to serve as a background control for endogenous MHC class II-associated peptides. Fig. 2 shows a representative experiment; four distinct peaks that are present only in the material isolated from infected macrophages are indicated.

Amino Acid Sequence of Peptides Isolated from MHC Class II of Infected P388D1 Macrophages. Out of three independent peptide extraction, 25 distinct HPLC peptide peaks were isolated from *L. donovani*-infected macrophages and were subjected to protein sequence analysis using automated Edman degradation. In practically all determinations no assignment could be made for the first position. Also, in most cases the definition of the amino acid residue of the next 10–15 positions was usually based on the quantitative dominance of one residue over others. Using this approach, the sequences obtained for several peptides showed the presence of 3–6 different residues in many of the 10–15 sequence cycles analyzed for each determination. Similar observations were recently reported for peptides isolated from MHC class I molecules of *Listeria monocytogenes*-infected macrophages (30) and reflects a mixture of peptides. In addition, sequences could not be obtained for some peaks

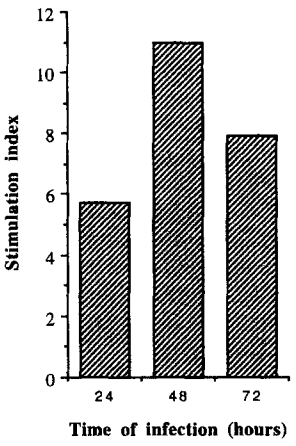

Figure 1. Presentation of parasite epitopes to specific T cells by P338D1 macrophages infected with *L. donovani*. P388D1 macrophages were infected with *L. donovani* promastigotes for an initial period of 6 h at 26°C and then incubated at 37°C for 24, 48, and 72 h. At each of these time points the nonadherent macrophages and free parasites were removed and the adherent cells were mechanically dislodged, washed, and fixed with paraformaldehyde. These cells were then washed and used as APCs (2×10^5/well) for a purified T cell preparation (10^5 cells/well) obtained from lymph nodes of BALB/c mice immunized with *L. donovani* promastigotes. Stimulation index was calculated by dividing the CPM obtained for the cells cultured in the presence of infected P388D1 macrophages by the CPM obtained for the cells cultured in the presence of noninfected macrophages but submitted to the same conditions of the infected macrophages.

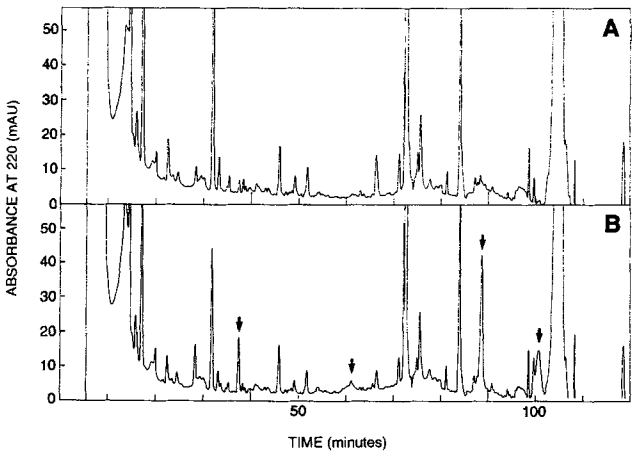

Figure 2. HPLC profile of peptides isolated from purified MHC class II molecules from P388D1 macrophages. Peptides were isolated from noninfected (*A*) or from *L. donovani* infected (*B*) P388D1 macrophages and submitted to RP-HPLC in a Vydac C-18 column. The arrows in *B* indicate peptide peaks present only in the infected macrophage preparation.

(e.g., the peak at ~90 min in Fig. 2) because the peptides were blocked. Notwithstanding, three peptide sequences were determined (Table 1). The sequence data base analysis revealed that one of the peptides was highly homologous to glyceraldehyde-3-phosphate dehydrogenase of various species. Another peptide had homology with elongation factor of several species, including leishmania. The third sequence was not clearly related to any known proteins.

Cloning and Sequencing the L. donovani Gene (Ld Peptide/Gene) That Encodes the Peptide Donor Protein. In order to retrieve the *L. donovani* protein that was processed into a peptide associated with the MHC class II molecules of infected macrophages, the peptide sequence of uncertain origin was chosen (Table 1) to guide the strategy to clone the corresponding parasite gene. A DNA fragment was initially amplified from *L. donovani* promastigotes cDNA by PCR. The sense primer was a peptide based oligonucleotide (see Materials and Methods). The bases were selected following the preferential codon usage of *L. donovani* (31). Inosine was used for the residues of positions 4, 6, and 7 because of the low codon usage assurance for the corresponding amino acids. In addition, the carboxyl-terminal L-glutamic acid was not included for the design of the primer. The antisense primer was oligo dT. PCR was carried out for 35 cycles and the products were analyzed by gel electrophoresis. Only one band of ~300 bp was obtained (Fig. 3). This fragment was cloned and its sequence confirmed the sequence of the peptide-based primer including the glutamic acid codon, deliberately not included in the primer sequence. The 300-bp insert was isolated and used as a probe to screen an *L. donovani* cDNA library. An ~650-bp cDNA gene (Ld peptide/gene) was cloned and sequenced (Fig. 4). The sequence data indicated complete homology with the original 300-bp PCR fragment. A 525-bp open reading frame containing an ATG codon that follows the last four bases of the spliced leader sequence and three stop codons adjacent to the poly A tail was identified. This frame also codes the carboxyl-terminal sequence (KVFDE) of the purified MHC class II associated peptide. The sequence analysis of the deduced protein sequence revealed one potential glycosylation site (Asn-Cys-Ser) at positions 68–70. The search for homology of the Ld peptide/gene with known sequences

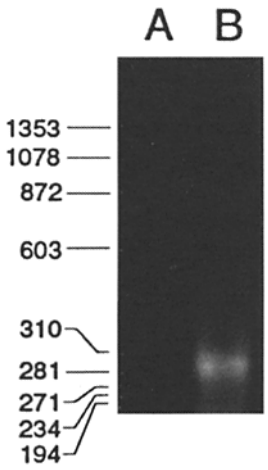

Figure 3. PCR amplification of a *L. donovani* cDNA fragment encoding the peptide isolated from the MHC class II molecules. *L. donovani* promastigote cDNA was used as template and the sense primer was a peptide-based deoxyribo oligonucleotide and the anti-sense primer was a poly-thymidine oligonucleotide. Amplification was performed as described in Materials and Methods and the PCR product was analyzed by agarose (1.5%) gel electrophoresis. Lane *A*, control (no template added to the reaction); lane *B*, PCR amplified product. Numbers on the left side indicate the molecular weights of the markers in base pairs.

was carried out using the FASTA program for both nucleotide and peptide revealed no significant homology.

Expression of the Ld Peptide/Gene in Leishmania Organisms. To ascertain that the Ld peptide/gene is expressed in the *Leishmania* organisms, a Northern blot analysis was performed using RNA prepared from different promastigote growth phases (logarithmic and stationary) and from the amastigote form of these parasites. The reason to investigate the mRNA expression in all the leishmania growth phases and forms is based on the infection pattern of these parasites: the amastigote is the form adopted by these parasites in the vertebrate host; and for some leishmanias (e.g., *L. major*) the stationary growth phase of the promastigote form is correlated with high infectivity (32). RNA was prepared from: logarithmic growth phase of *L. donovani* promastigotes; from logarithmic and stationary growth phases of *L. major* promastigotes; and from promastigotes (logarithmic and stationary growth phases) and amastigotes of *L. amazonensis* and *L. pifanoi*. Fig. 5 shows that one single RNA band of 680 bp was observed for all growth phases and forms of all tested *Leishmania*. This result is consistent with the corresponding gene size (525 bp) and with the MW of the expressed protein (see section below) and points to the ubiquitous distribution and expression of this gene within the genus *Leishmania*.

Table 1. *Amino Acid Sequence Analysis of Purified MHC Class II-associated Peptides from L. donovani-infected P388D1 Macrophages*

	Sequencing cycle																
Peptide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Homology
1	X	G	V	N	G	F	G	R	I	G	R	L	V	T	R	A	Glyceraldehyde-3-phosphate dehydrogenase
2	X	N	I	V	V	I	G	H	V	D	S	G	K	S	T	X	Elongation factor
3	X	Q	X	P	Q	L (K)	V	F	D	E	X	X					Unknown

X indicates that no amino acid could be assigned for that position. Remaining letters stand for the amino acid code. In parenthesis is an alternative amino acid for position 6 of peptide 3.

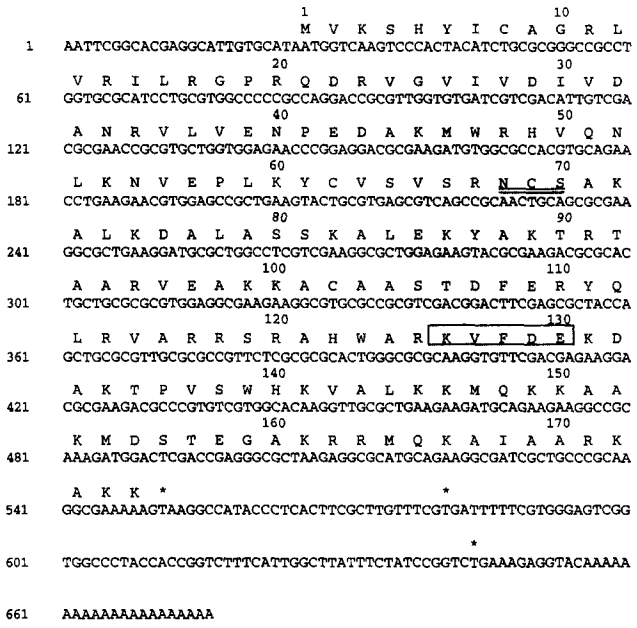

Figure 4. Nucleotide sequence and deduced amino acid sequence of the Ld peptide/gene cDNA. Numbers on the right refer to the nucleotide positions and the numbers above the protein sequence refer to the amino acid positions. The sequence of the last presumptive four bases of the 3′ end of the spliced leader DNA sequence is underlined. Putative translation initiation is indicated as amino acid position 1 (*Met*), which corresponds to the first ATG codon after the spliced leader sequence. Potential glycosylation site (*Asn-Cys-Ser*) is double underlined. Three stop codons preceding the poly A tail are marked by asterisks. The protein sequence that matches the five carboxy-terminal amino acids (KVLDE) of the peptide isolated from the MHC class-II molecules of *L. donovani*-infected macrophages is indicated in the boxed residues. These data are available under GenBank/EMBL accession number X86551LDP23CSPR.

Identification and Purification of the L. donovani Peptide Donor Protein. The recombinant *L. donovani* peptide donor protein was produced in *E. coli* transformed with the pGEX 2T expression vector in which the Ld peptide/gene was subcloned in frame. A glutathione-S-transferase fusion protein of 43–44 kD was produced (Fig. 6 *A*) therefore indicating a leishmanial protein of approximately 18 kD, as glutathione S-transferase (GST) has a molecular weight of 26 kD. However the fusion protein was very insoluble and therefore could not be purified by affinity chromatography using a glutathione column. The use of low concentrations of detergents like SDS, sarcosyl, deoxycholate, and octylglucopyranoside during the extraction steps was efficient to solubilize the fusion protein but unfortunately prevented its binding to the glutathione column (not shown). Other maneuvers, such as the growth of the *E. coli* and incubation and induction of the *tac* promoter with IPTG at 33°C, did not improve the protein solubility (not shown). However, the purification was achieved by preparative SDS-PAGE. Approximately 500 μg of purified protein was obtained (Fig. 6 *B*). Attempts to further purify the leishmanial protein by cleaving it out from the fusion protein GST with thrombin were unsuccessful (not shown).

Purified Recombinant Protein Induces Anti-L. donovani Antibody Response in Mice and Rabbits. In order to evaluate the immunogenicity of the recombinant leishmanial protein and to investigate its expression in the parasites, mice and rabbits were immunized with the GST-fusion protein in CFA. Sera were prepared and the anti-leishmania Ab response was measured by Western blot analysis and by FACScan. In both cases *L. donovani* promastigotes were used as antigen. Fig. 7 shows that the rabbit anti-recombinant protein antiserum detects a single protein of 23 kD (Ldp 23) in the leishmania crude extract antigen preparation. No bands were observed when an anti-GST antiserum was used (not shown). Moreover, the FACScan analysis (Fig. 8) shows that the Ab induced by the recombinant Ldp 23 reacts with intact live *L. donovani* promastigotes, thus pointing to a cell surface expression of this molecule on these organisms.

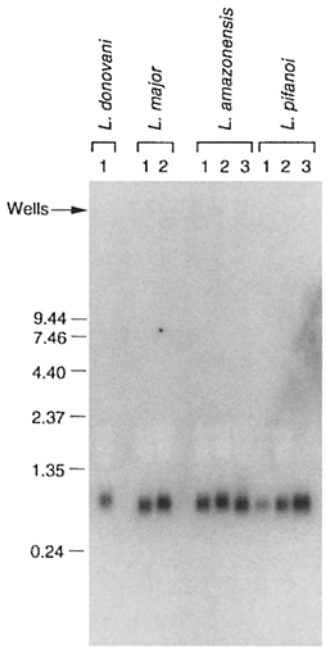

Figure 5. Expression of the Ld peptide/gene. Total RNA was prepared from *L. donovani* promastigotes, *L. major*, *L. amazonensis*, and *L. pifanoi* and used for Northern blot analysis. Blot was hybridized with ^{32}P-labeled Ld peptide/gene cleaved from pBluescript plasmid. 1, 2, and 3 refer to RNA obtained from promastigotes at the logarithmic growth phase, promastigotes at the stationary growth phase and from amastigote forms, respectively. Numbers on the left side indicate the molecular weights of the markers in base pairs.

Leishmania-specific Lymph Node T Cells Recognize the Recombinant Ldp 23. To test the responsiveness of T cells to

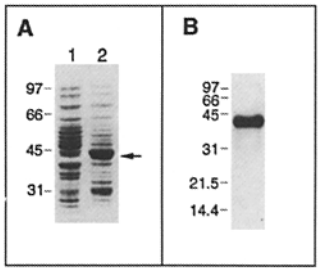

Figure 6. Expression of the Ld peptide/gene as a recombinant GST fusion protein. (*A*) *E. coli* (DH 5α) transformed with the expression vector pGEX 2T containing the Ld peptide/gene was grown in LB medium and the *tac* promoter was induced with IPTG for 3 h. The cells were pelleted, resuspended in loading buffer and submitted to SDS-PAGE (10%) under reducing conditions. The gel was stained with Coomassie blue. Lane *1*, *E. coli* transformed with pGEX 2T, noninduced; lane *2*, *E. coli* transformed with pGEX 2T, IPTG induced. Arrow indicates the recombinant fusion protein. (*B*) Transformed *E. coli* was induced and lysed as in *A* and submitted to a preparative SDS-PAGE. The band corresponding to the over expressed recombinant fusion protein was identified by KCl, cut out, electroeluted from the gel strip, dialyzed against PBS, and then submitted to analytical SDS-PAGE (12%). Numbers on the left side indicate the molecular weights of the markers.

the Ldp 23 protein, two sets of experiments were performed. Initially, BALB/c mice were immunized with promastigote forms of *L. donovani* in CFA. T cells were purified from draining lymph nodes 8 d after the immunization and were stimulated to proliferate with mitomicin-treated mononuclear spleen cells (APC) pulsed with the purified recombinant fusion protein. Fig. 9 shows that leishmania-specific T cells proliferate well and in a dose response manner to recombinant Ldp 23. No response was observed when purified GST was added instead of the recombinant fusion protein nor lymph node T cells from mice immunized with CFA alone were stimulated to proliferate in the presence of the leishmanial fusion protein (not shown). The recognition of the recombinant Ldp 23 by leishmania-specific T cells was also tested using two murine models of leishmaniasis, the *L. major* highly susceptible BALB/c mice and the *L. amazonensis* susceptible CBA/J mice (33). These models were selected to investigate the cytokine pattern induced by Ldp 23. In the mouse model of leishmaniasis, resistance is associated with Th 1 cytokines whereas susceptibility is linked to Th 2 responses (34). Lymph node cells were obtained 3 wk after the initiation of infection of BALB/c mice with *L. major* and the ability of these cells to recognize the recombinant Ldp 23 was measured by proliferation and by the production of the cytokines IFN-γ and IL-4. Ldp 23 did stimulate these cells to proliferate (not shown) and induced typical Th 1 type of cytokine response as indicated by the production of high levels of IFN-γ and no IL-4 (Fig. 10). Stimulation of these cells with a leishmania crude lysate yielded a mixed Th cytokine profile. Exactly the same pattern of cytokine production was obtained from the CBA/J mice infected with *L. amazonensis* (not shown). These results clearly indicate that Ldp 23 is a powerful and selective activator of Th 1 cytokines by mouse cells.

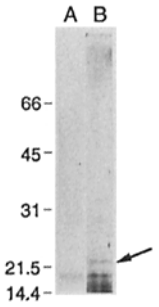

Figure 7. Western blot analysis of *L. donovani* promastigote antigens. Parasites (2 × 10^6) grown in Schneider's medium for 3 d (log phase) were washed with PBS, lysed with SDS-PAGE loading buffer, and submitted to electrophoresis under reducing conditions in a 15% gel. The proteins were transferred to Immobilon-P membranes and incubated with a 1/200 dilution of preimmune rabbit serum (lane *A*) or with the same dilution of anti-fusion protein rabbit antiserum (lane *B*). Reaction was detected using a phosphatase conjugated goat anti-mouse immunoglobulins. Arrow points to a molecule detected by the specific antibody. Numbers on the left side indicate the molecular weights of the markers.

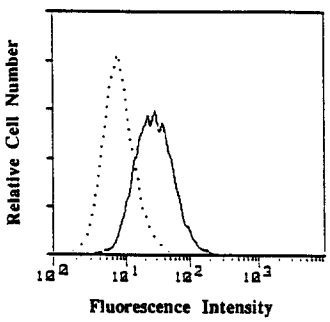

Figure 8. Surface expression of Ldp 23 on *L. donovani* promastigotes. Indirect immunofluorescence was performed with live *L. donovani* promastigotes using preimmune mouse serum (*dotted line*) or mouse anti-GST-Ldp 23 antiserum (*solid line*). Both sera were diluted at 1/100. Parasites were washed with staining buffer and incubated with FITC conjugated goat anti-mouse immunoglobulin antibody. Fluorescence intensity was analyzed by FACScan.

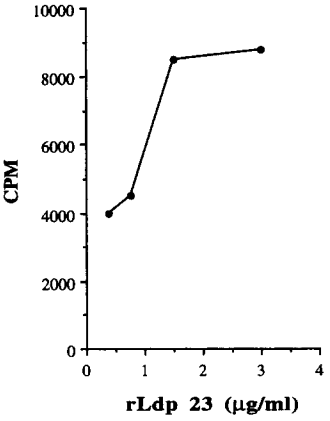

Figure 9. Stimulation of leishmania-specific T cells to proliferate by Ldp 23. T cells (10^5/well) were purified from lymph nodes of BALB/c mice immunized in the foot pad with *L. donovani* promastigotes in CFA and were cultured with various concentrations of the purified recombinant Ldp 23 in the presence of 2 × 10^5 mitomycin C treated normal BALB/c spleen mononuclear cells as APC. Proliferation of the T cells was measured at 72 h of culture. Values are expressed as cpm and represent the mean of [^{3}H]TdR incorporation of triplicate cultures. Background cpm of cells (T cells + APC) cultured in the presence of medium alone was 1291.

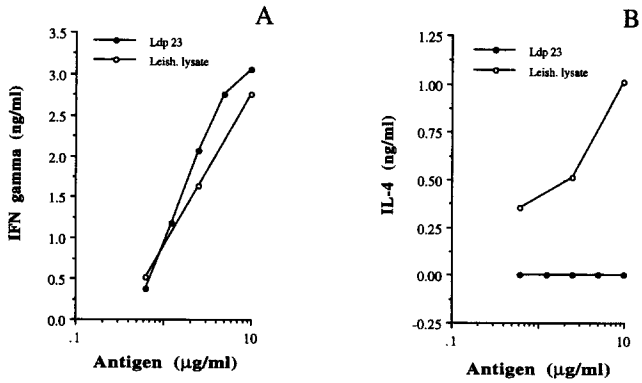

Figure 10. Ldp 23-induced cytokine production by lymph node cells of *L. major*-infected BALB/c mice. Cells (2.5 × 10^6) obtained from the draining popliteal lymph node of BALB/c mice infected in the foot pad with *L. major* 3 wk previously, were cultured for 72 h in the presence of recombinant Ldp 23 or leishmania lysate. Culture supernatants were collected and assayed by ELISA for the production of IFN-γ (*A*) and IL-4 (*B*). No cytokine could be detected in supernatants of nonstimulated cells.

Discussion

The isolation and characterization of peptides associated with MHC molecules has been a focus of intensive research over the past 4–5 yr. These studies clearly have defined new and solid concepts in the biology and molecular events of antigen processing, presentation, and recognition. However, much of this progress was made possible because of the continuous advancement in methodologies of increasing sensitivity, specificity and reproducibility for the isolation and characterization of minute amounts of peptides (35). The present investigation utilized these new concepts and tools as an approach to define novel protein

molecules involved in the host-pathogen interaction. The studies reported here describe both the isolation of peptides from MHC class II molecules of the macrophage cell line P388D1 infected with *L. donovani*, and the use of the derived peptide sequence to rescue the parasite's peptide donor protein. *Leishmania* parasites were chosen because they are strictly intracellular pathogens residing exclusively within the late endosomal compartment of macrophages, professional APCs of MHC class II-associated peptides. In addition, T cells primarily of the CD4+ phenotype appear to play a major role in the host defense against these parasites (34, 36–39). The infection and processing of parasite antigens by the P388D1 macrophages did not change the level of the class II molecules expression but it clearly modified the HPLC pattern of the peptides extracted from these molecules. Several distinct HPLC peaks were consistently present on the peptide preparation obtained from the infected macrophages and absent in the preparations obtained from noninfected cells. These results suggest that these peaks contain parasite peptides that were processed and presented by the infected macrophages. However it is also possible that the infected macrophages would abnormally process self peptides that could too be present in the unique HPLC peaks. Indeed, peptide sequences that support this possibility were obtained from these peaks (see below). These two possibilities are however not mutually exclusive. Unfortunately, in several cases the peptide amino acid sequence could not be determined because of the limitations of the protein sequencer system used in these studies that has a sensitivity in the order of nanomoles of peptide, a concentration that was only rarely obtained for the peptides isolated from the infected macrophages. This problem should be easily solved in future studies by the use of the recent developed technologies like the Laser Desorption Mass Spectrometry (LDMS) or the combination of microcapillary RP-HPLC separation coupled with electrospray ionization tandem mass spectrometry (35). The sensitivity of these methodologies is in the order of a few fentomoles of peptides. Despite the methodological limitation, three peptides could be sequenced from the infected macrophage preparation. One of these peptides had sequence homology with elongation factor of several origins including *Leishmania*, and another peptide was highly homologous to glyceraldehyde-3-phosphate dehydrogenase of various species. These two peptides were not considered of interest because of their ubiquitous distribution. The third peptide did not appear to be homologous to any known protein and consequently was employed to instruct the rescue of a possible parasite donor protein. The peptide sequence was initially used to instruct the design of an oligonucleotide sense primer which was used in combination with an oligo dT nucleotide (anti-sense primer) to amplify by PCR a DNA fragment from *L. donovani* cDNA. The DNA fragment amplified (~300 bp) was cloned and subsequently sequenced which unequivocally confirmed the sequence of the peptide based primer including the carboxy-terminal glutamic acid codon that was not deliberately incorporated in the primer sequence. Using this cloned DNA fragment as probe the corresponding *L. donovani* gene (Ld peptide/ a gene) was successfully cloned from a *L. donovani* cDNA library. The cloned Ld peptide/gene has an open reading frame of 525 bp and has no homology with any other gene sequence so far reported. In addition the gene is apparently broadly distributed and expressed among the *Leishmania* genus because its corresponding mRNA transcript is present not only in *L. donovani* but also in other species of this genus in both amastigote and promastigote forms of the parasite cell cycle.

Despite the limited accuracy of the amino acid sequence determined for the MHC class II-associated peptides and the consequent need to use a degenerate primer for PCR, the sequence of the cloned gene matches with the five carboxy-terminal residues of the original peptide sequence (KVFDE). It is important to emphasize that a sequence of only seven amino acids was used to design the primer for the PCR and that the carboxyl-terminal glutamic acid of the peptide sequence was not used to design the primer. The peptide sequence lies in the positions 126–130 of the 175 amino acids of the deduced protein sequence. It is interesting to note that coincidentally this is an area of the protein in which a very high antigenic index is deduced using the algorithm described by Jameson and Wolf (1988) to predict antigenic epitopes. However circumstantial, this information is consistent with the fact that this very epitope was processed and presented by *L. donovani*-infected P388D1 macrophages.

Once the Ld peptide/gene was cloned and sequenced its expression as a fusion protein was achieved using the pGEX 2T expression vector and the purified GST-fusion protein was used to investigate the antigenic relation of the recombinant protein with *L. donovani*. This relationship was demonstrated using both antibody and T cell responses. First, mice and rabbits immunized with the fusion protein developed antibodies that recognized a 23-kD molecule (Ldp 23) on the cell surface of *L. donovani* promastigotes. The small molecular mass difference between the recombinant leishmanial protein and the Ldp 23 might be due to post translational modifications such as glycosylation of the native protein (one glycosylation site at the amino acid positions 68–70 is present in this molecule as is indicated by its deduced protein sequence). The surface expression of Ldp 23 is of much interest because several leishmania surface proteins are buried beneath the glycoinositolphospholipids and lipophosphoglycans (LPG) glycocalyx coat present in these parasites (40). However, recent reports (40–42) described a novel hydrophilic protein called gene B protein, that is exposed on the cell surface of *L. major* promastigotes. This protein targets to the parasite's surface apparently by binding to LPG via an unusual amino acid repeat that is related to the peptidoglycan binding domain of protein A from *Staphylococcus aureus*. Similarly to the gene B protein, the deduced protein structure of Ldp 23 indicates a high hydrophilicity and high content in Lys residues. However, whether or not Ldp 23 also targets to the parasite's surface via binding to LPG remains to be investigated.

At the T cell level, the relationship of Ldp 23 with *L. donovani* was initially carried out using specific T cells purified from the lymph node of mice immunized with *L. donovani*. These cells were specifically stimulated to proliferate by Ldp 23-pulsed APC, thus confirming its leishmanial origin. Moreover, strong evidence indicates that Ldp 23 is preferentially involved in the activation of the Th 1 pattern of cytokine response which pattern is correlated with resistance to infection with *Leishmania* parasites (34, 44–46). This observation is particularly interesting in that Ldp 23 is selectively activating Th 1 clones even from the highly susceptible BALB/c mice which, as is well documented, have a strong genetic bias towards the development of the susceptible Th 2 type of response (43). A similar selective induction of Th 1 cytokines in human peripheral blood mononuclear cells, has been recently reported for another *Leishmania* recombinant antigen (44).

The cloning of Ld peptide/gene and expression of the corresponding protein constitute interesting findings for the studies concerning vaccine development to leishmaniasis. This possibility is supported by the facts that the Ld peptide/gene is broadly distributed among the *Leishmania* genus; it is expressed in both amastigote and promastigote forms (logarithmic and stationary phases) of all species tested; that the corresponding Ldp 23 is localized on the cell surface of the parasite is particularly attractive for vaccine development because this molecule can be a good target for immunological intervention on the parasite/host cell interaction. In addition Ldp 23 appears to induce preferentially Th 1 clones. The expansion of these studies and the analysis the Ldp 23 immunogenic epitopes for both humans and mice are in progress.

The results presented here support the idea that MHC class II molecules-associated peptides can be used to rescue a pathogen protein that is recognized and processed by the APCs. The virtue of this strategy is that it goes directly from the recognized peptide to the pathogen gene and from there to the peptide donor protein. Therefore the selection of a pathogen protein that might be involved in the host/pathogen interaction is achieved without the need of T cell clones and a battery of many different synthetic peptides. One limitation of this approach is however the chance that the rescued protein may not be relevant to the host/pathogen interaction, because there is no previous knowledge of its participation in the infectious process and also because of pathogen's vast antigenic universe that can be processed and presented by the APCs. Nevertheless, the use of good experimental animal models can be helpful in this context. For instance, in the leishmania system, a large number of mouse strains are relatively resistant to the infection with *L. donovani*. In contrast, *L. amazonensis* and *L. major* cause severe diseases in many strains of mice (37, 45). Therefore the infection of macrophages with *L. donovani* may be an interesting source of peptides to rescue cross reactive proteins that could be potentially useful for vaccine development not only against this parasite but also to other members of the *Leishmania* genus. Indeed, good protection of BALB/c mice against *L. major* infection has been achieved with a 72-kD protein from *L. donovani* (46).

Finally these results clearly point to a feasible alternative strategy for the purification of useful reagents for the development of both vaccines and immunological diagnostic tools not only against *Leishmania* infection but also for other diseases caused by intracellular pathogens.

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