

Toward a Defined Anti-*Leishmania* Vaccine Targeting Vector Antigens: Characterization of a Protective Salivary Protein

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Abstract

Leishmania parasites are transmitted to their vertebrate hosts by infected phlebotomine sand fly bites. Sand fly saliva is known to enhance *Leishmania* infection, while immunity to the saliva protects against infection as determined by coinoculation of parasites with vector salivary gland homogenates (SGHs) or by infected sand fly bites (Kamhawi, S., Y. Belkaid, G. Modi, E. Rowton, and D. Sacks. 2000. *Science*. 290:1351–1354). We have now characterized nine salivary proteins of *Phlebotomus papatasi*, the vector of *Leishmania major*. One of these salivary proteins, extracted from SDS gels and having an apparent mol wt of 15 kD, was able to protect vaccinated mice challenged with parasites plus SGH. A DNA vaccine containing the cDNA for the predominant 15-kD protein (named SP15) provided this same protection. Protection lasted at least 3 mo after immunization. The vaccine produced both intense humoral and delayed-type hypersensitivity (DTH) reactions. B cell-deficient mice immunized with the SP15 plasmid vaccine successfully controlled *Leishmania* infection when injected with *Leishmania* plus SGH. These results indicate that DTH response against saliva provides most or all of the protective effects of this vaccine and that salivary gland proteins or their cDNAs are viable vaccine targets against leishmaniasis.

Key words: DNA vaccine • salivary gland • sand fly • *Leishmania* • leishmaniasis

Introduction

The leishmaniasis are a group of diseases caused by protozoa of the genus *Leishmania* and affecting many millions of people worldwide. In humans, it manifests either as a cutaneous disease caused mainly by *Leishmania major*, *Leishmania tropica*, and *Leishmania mexicana*, as a mucocutaneous disease caused mainly by *Leishmania brasiliensis*, or as a visceral disease caused mainly by *Leishmania donovani* and *Leishmania chagasi*. All leishmanial diseases are transmitted to their vertebrate hosts by phlebotomine sand flies, which acquire the pathogen by feeding on infected hosts and transmit them by regurgitating the parasite at the site of a subsequent blood meal (1).

While obtaining a blood meal, sand flies salivate into the host's skin. This saliva contains anticlotting, antiplatelet, and vasodilatory compounds that increase the hemorrhagic

pool where sand flies feed (2, 3). Some of these components are additionally immunomodulatory. For example, the New World sand fly *Lutzomyia longipalpis* contains the 6.5-kD peptide, maxadilan, which is the most potent vasodilator known (4). Maxadilan additionally has immunosuppressive activities of its own (5), as do many persistent vasodilators such as prostaglandin E₂ (6–8) and calcitonin gene-related peptide (9). Old World sand flies (who share a common ancestor with New World sand flies before the separation of the present tectonic plates, or about the time of irradiation of mammals) do not have maxadilan, but instead use AMP and adenosine as vasodilators (10). Adenosine is also an immunomodulatory component, promoting the production of IL-10 and suppressing TNF- α and IL-12 in mice (11–13). Additionally, *Phlebotomus papatasi* salivary gland homogenate (SGH)* as well as adenosine was shown

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*Abbreviations used in this paper: DTH, delayed-type hypersensitivity; PVDF, polyvinylidene difluoride; SGH, salivary gland homogenate; wt, wild-type.

to downregulate the nitric oxide synthase gene in activated macrophages (14). Additionally, we previously reported that SGH induced IL-4 at the site of inoculation in mice, and that this effect was abrogated when IL-4 knockout mice were used (15). Perhaps due to this immunomodulatory effect of saliva, coinjection of *Leishmania* with as little as the equivalent of 0.25 pairs of vector SGH significantly enhances *Leishmania* infectivity (16, 17). Conversely, immunity to SGH or previous exposure to sand flies prevents leishmaniasis infectivity either by coinoculation of *Leishmania* with SGH (15) or by infected sand fly bites (18).

Although enhancement of *Leishmania* infectivity by SGH is probably due to the immunomodulatory components of sand fly saliva, explanation of the anti-*Leishmania* effect resulting from host immunization against salivary antigens is not straightforward. We previously suggested that the exacerbating effect of SGH was mediated by an early release of IL-4 at the inoculation site (15). Mice preexposed to SGH were protected against this early burst of IL-4 and from the exacerbatory effect of the saliva. Immunity in this system could be derived from neutralization by antibodies of yet unidentified *P. papatasi* salivary immunomodulator(s). Alternatively, immunity could derive from a delayed-type hypersensitivity (DTH) reaction at the site of the bite generated by a cellular response to salivary antigens injected by the fly (18, 19). This particular reaction could turn the feeding lesion and its surroundings into an inhospitable site for the establishment of *Leishmania* into the new host or alter the milieu priming the initial events of the host immune reaction to *Leishmania* (19). In a natural challenge model, mice preexposed to the bite of sand flies showed a significant increase of the release of IL-12 and IFN- γ at the site of the bite compared with naive mice (18). Although the two possibilities (antibodies and DTH) are not mutually exclusive, the first explanation cannot justify why previous exposure to *P. papatasi* SGH or bites confer immunity to *L. major* (17) if we consider that adenosine is the main immunosuppressor in *P. papatasi* saliva. Each of the theories requires distinct strategies for vaccine development: the immunomodulatory neutralization theory requires identification of the immunomodulator and its modification into an appropriate immunogen, while the DTH theory will make any protein in the saliva a fair target. We report here the complete sequence of nine major salivary proteins from the sand fly vector of *L. major*, *P. papatasi*, identify a candidate antigen that confers protection against *L. major* transmission in mice when coinjected with SGH, and investigate the role of antibodies and DTH in this vaccine model.

Materials and Methods

Sand Flies and Preparation of SGH. *P. papatasi*, Israeli strain, were reared at the Walter Reed Army Medical Research Institute, using as larval food a mixture of fermented rabbit feces and rabbit food (20). Adult sand flies were offered a cotton swab containing 20% sucrose and were used for dissection of salivary glands at 2–7 d after emergence. Salivary glands were stored in groups of 20 pairs in 20 μ l NaCl (150 mM) Hepes buffer (10

mM, pH 7.4). Salivary glands were disrupted by ultrasonication within 1.5-ml conical tubes. Tubes were centrifuged at 10,000 g for 2 min and the resultant supernatant used for the studies.

Mice. C57BL/6 mice were purchased from the Division of Cancer Treatment, National Cancer Institute. C56Bl/10 μ MTKO (B cell-deficient [B^{-/-}]) mice were obtained from Taconic Farms. Mice were maintained in the National Institute of Allergy and Infectious Diseases Animal Care Facility under pathogen-free conditions.

SDS-PAGE. Tris-glycine gels (16%), 1-mm thick, were used (Invitrogen). Gels were run with Tris-glycine buffer according to the manufacturer's instructions. To estimate the mol wt of the samples, SeeBlue™ markers from Invitrogen (myosin, BSA, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin, lysozyme, aprotinin, and insulin, chain B) were used. SGH were treated with equal parts of 2 \times SDS sample buffer (8% SDS in Tris-HCl buffer, 0.5 M, pH 6.8, 10% glycerol, and 1% bromophenol blue dye). 30 pairs of homogenized salivary glands per lane (\sim 30 μ g protein) were applied when visualization of the proteins bands stained with Coomassie blue was desired. For NH₂-terminal sequencing of the salivary proteins, 40 homogenized pairs of glands were electrophoresed and transferred to polyvinylidene difluoride (PVDF) membrane using 10 mM 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS), pH 11, 10% methanol as the transfer buffer on a Blot-Module for the XCell II Mini-Cell (Invitrogen). The membrane was stained with Coomassie blue in the absence of acetic acid. Stained bands were cut from the PVDF membrane and subjected to Edman degradation using a Procise sequencer (PerkinElmer).

Western Blot Analysis. SDS-PAGE of *P. papatasi* SGH (50 salivary gland pairs) for Western blot analysis was done on 16% Tris-glycine gel containing a single long well (Invitrogen). After electrophoresis, salivary proteins were transferred to a nitrocellulose membrane using Tris-glycine buffer with 10% methanol as the transfer buffer on a Blot-Module for the XCell II Mini-Cell. The nitrocellulose membrane was then incubated overnight at 4°C with blocking buffer (Tris pH 8.0 plus 150 mM NaCl plus 5% non-fat milk). After the blocking step, the membrane was placed on a Mini-protein II multiscreen apparatus (Bio-Rad Laboratories) that allows testing of 16 different serum samples from a single blot. Sera from mice preexposed to SGH, from mice bitten by sand flies or from naive mice, were diluted 1:200 with blocking buffer and added individually to various channels on the multiscreen apparatus. The membrane was incubated with the different serum samples for 2 h at room temperature. Serum samples were removed from the channels of the multiscreen apparatus and washing buffer (Tris pH 8.0 plus 150 mM NaCl plus 0.1% Tween 20) was added three times. The membrane was then removed from the multiscreen device and washed again three times, 5 min per wash, with washing buffer. The membrane was then incubated with a 1:10,000 dilution of an anti-mouse IgG alkaline phosphatase-conjugated antibody for 1 h at room temperature. The membrane was washed three times with washing buffer as described above. Positive bands were visualized by adding alkaline phosphatase substrate (Promega) and the reaction stopped by washing the membrane three times with deionized water.

Salivary Gland cDNA Library Construction. *P. papatasi* salivary gland mRNA was isolated from 85 salivary gland pairs from adult females at days 1, 2, and 3 after emergence. Library construction was completed exactly as described previously (21).

Massive Sequencing of *P. papatasi* Salivary Gland cDNA Library. The *P. papatasi* salivary gland cDNA library was plated to \sim 200 plaques/plate (150-mm Petri dish). These procedures were also

completed as described previously (21).

DNA Vaccine Construction and Description of the VR1020 Vector. The gene coding for SP15 (from NH₂ terminus to stop codon) was amplified from *P. papatasi* SP15-specific cDNA by PCR using High-Fidelity platinum *Taq* polymerase (GIBCO BRL) and specific primers carrying BamHI restriction sites (forward SP15BHF 5'-TGCGGATCCGAAAATCCATCAAA-GAAG-3'; reverse SP15BHR 5'-ATTGGATCCTTATATAT-TGATTGTTTT-3'). The PCR product was immediately cloned into the TOPO TA cloning vector PCRII (Invitrogen) following the manufacturer's specifications. The ligation mixture was used to transform TOP10 cells (Invitrogen) and the cells were incubated overnight at 37°C. Eight colonies were picked and mixed with 10 µl of sterile water. 5 µl of each sample were transferred to Luria broth with ampicillin (100 µg/ml) and grown at 37°C. The other 5 µl were used as a template for a PCR reaction using two vector-specific primers from the PCRII vector to confirm the presence of the insert and for sequencing analysis. After visualization of the PCR product on a 1.1% agarose gel, we completely sequenced the eight PCR products as described above using a CEQ2000 DNA sequencing instrument (Beckman Coulter). We chose a sample that contained the sequence from the NH₂ terminus to stop codon of the SP15 gene including the incorporated BamHI sites. Cells containing the plasmid carrying the SP15 gene were grown overnight at 37°C on Luria broth with ampicillin (100 µg/ml), and plasmid isolation was performed using the Wizard Miniprep kit (Promega). The plasmid containing the SP15 gene with incorporated BamHI sites was digested with BamHI and then ligated with the BamHI predigested VR1020 DNA plasmid vector (VICAL). The VR1020 plasmid contains a kanamycin resistance gene, the human cytomegalovirus promoter, and the tissue plasminogen activator signal peptide upstream of the BamHI cloning site. The ligation reaction between the BamHI digested SP15 gene and the similarly digested VR1020 DNA vector was done overnight at 16°C to transform TOP10 cells (Invitrogen). Cells were incubated on a Luria broth kanamycin (30 µg/ml) plate overnight at 37°C. 32 colonies were picked and mixed with 10 µl of sterile water. 5 µl of each sample were transferred to Luria broth with kanamycin (100 µg/ml) and grown at 37°C. The other 5 µl were used as a template for a PCR reaction using two vector-specific primers from the VR1020 vector to confirm the presence of the insert and for sequencing analysis. After visualization of the PCR product on a 1.1% agarose gel, we proceeded to sequence completely four PCR products as described above using a CEQ2000 DNA sequencing instrument (Beckman Coulter). For the vaccine construct, we chose a sample that contained the sequence from the NH₂ terminus to the stop codon in the right orientation and in the correct open-reading frame after the tissue plasminogen activator signal peptide. Cells containing the SP15 gene on VR1020 were grown overnight at 37°C on Luria broth with kanamycin (100 µg/ml) and plasmid isolation was performed using the Wizard Miniprep kit. After plasmid isolation, the sample and control plasmids (VR1020 alone) were washed three times with ultrapure water using an Amicon-100 (Millipore). The concentrations of the samples were measured by UV absorbance, and they were stored at -70°C before immunization experiments.

DNA and Predicted Protein Sequence Analysis. DNA data derived from the mass sequencing project were analyzed by an in-house program written in VisualBASIC (Microsoft). This program removed vector and primer sequences from the raw sequence. Stripped sequences were compared with the NCBI non-redundant protein database using the program BlastX using the

BLOSUM-62 matrix (22). DNA sequences were clustered by blasting the database against itself with a preselected threshold cutoff, usually 10⁻¹⁰ (BlastN program) (22). Sequences from the same cluster were aligned using ClustalX (23). To find the cDNA sequences corresponding to the amino acid sequence obtained by Edman degradation of the proteins transferred to PVDF membranes from PAGE gels, we wrote a search program that checked these amino acid sequences against the three possible protein translations of each cDNA sequence obtained in the mass sequencing project. This was written using the same approach used in the BLOCKS (24) or Prosite (25) programs. Protein translations of the full-length clones were further processed to identify the predicted signal peptides using the Signal P program (26), available at <http://genome.cbs.dtu.dk/services/SignalP/>. Predicted signal peptide cleaved sites were compared with the NH₂ terminus sequence obtained from Edman degradation of *P. papatasi* salivary proteins. Estimation of isoelectric point and mol wt of translated protein was performed using the DNA STAR program (DNASTAR, Inc.). Full-length translated protein sequence information was compared with the nonredundant protein database of NCBI using the BLAST-P program (22) and searched for motifs by submitting each sequence to <http://www.motif.genome.ad.jp/>.

Parasite Preparation and Intradermal Inoculation. *L. major* clone V1 (MHOM/IL/80/Friedlin) was cultured in 199 medium with 20% heat-inactivated fetal bovine serum (HyClone), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 40 mM Hepes, 0.1 mM adenine (in 50 mM Hepes), 5 mg/ml hemin (in 50% triethanolamine), and 1 mg/ml 6-biotin (M199/S). Infective-stage metacyclic promastigotes of *L. major* were isolated from stationary cultures (4–5 d old) by negative selection using peanut agglutinin (Vector Laboratories). 500 metacyclic promastigotes with or without 0.5 pair of SGH were inoculated intradermally into the left ear dermis using a 27-gauge needle in a volume of ~5 µl. The evolution of the lesion was monitored by measuring the diameter of the induration of the ear lesion with a direct-reading Vernier caliper (Thomas Scientific). Parasite titrations were performed as described previously (17, 27).

Vaccination. SGH (30 salivary gland pairs) was separated by SDS-PAGE and stained with Coomassie blue as described above. The gel was divided into three groups containing proteins in the range of 200 to 50 kD (fraction A, ~11 µg), from 49 to 20 kD (fraction B, ~9 µg), and below 20 kD (fraction C, ~10 µg). A control piece of acrylamide from the gel was used (fraction E). The gel was cut and transferred to a 1.5-ml tube and washed three times with sterile PBS (pH 6.8) plus 150 mM NaCl. The bands were then triturated using a plastic pestle until the preparation could be resuspended in 500 µl of sterile phosphate-saline buffer using a 27-gauge needle to give a solution of ~0.02 µg/µl. Immunization with SDS-PAGE fractions was performed by the injection of 10 µl of either fraction A, B, C, or E into the right ear of each mouse, followed by a boost 2 wk later. For genetic immunization, mice were inoculated in the right ear with 5–10 µg of the plasmid encoding for SP15 or control DNA (empty vector) suspended in 5 µl of PBS. Each group was boosted 2 wk later using the same regimen. Mice were bled and the presence of antibodies assessed for each individual mouse by Western blot as described above.

Analysis of the Inflammatory Response in the Ear Dermis. The left ears of mice vaccinated (in the right ear) or not with the plasmid vector were exposed to the bite of sand flies as described previously (19). 24 h after the sand fly exposure, seven mice per group were killed and the left ear collected. Each ear was processed in-

dividually. The cells in the inflammatory ear dermis were recovered as described previously (15).

Immunolabeling for Flow Cytometry Analysis. The dermal cells were incubated with 10% normal mouse serum in PBS containing 0.1% BSA, 0.01% NaN_3 before being incubated with anti-Fc receptor antibody (2.4G2; BD PharMingen). The double staining was done by using directly conjugated antibodies incubated simultaneously. The dermal inflammatory cells were identified by characteristic size forward scatter (FSC) and granularity side scatter (SSC) combined with two-color analysis, as described previously (28).

Statistical Analysis. Statistical tests were performed with SigmaStat (Jandel Software). Because most comparisons derived from data with nonhomogeneous variances, Kruskal-Wallis analysis of variance (ANOVA) on ranks was performed and multiple comparisons were done by the Dunn method. Dual comparisons were made with the Mann-Whitney rank sum test. All data from parasite numbers were log transformed before conducting statistical tests.

Results

The salivary gland of *P. papatasi* is a sac-like structure consisting of a unicellular epithelium layer surrounding a large lumen (29). After a blood meal, the gland total protein content decreases to half or less from its $\sim 1 \mu\text{g}$ value (30). Accordingly, most of the protein from the fly SGH must be destined for secretion. Indeed, SDS-PAGE of SGH reveals a low complexity composition consisting of ~ 12 major bands varying from 10–100 kD (Fig. 1 A). Sera from individual mice inoculated with SGH or exposed to sand fly bites recognized antigens that colocalized with one or more of these major bands when detected by Western blot assays (Fig. 1 B). Nonexposed controls or preimmune sera did not react to *P. papatasi* salivary antigens (Fig. 1 B).

To characterize the primary structure of the main proteins of *P. papatasi* SGH, we transferred SDS-PAGE gels to PVDF membranes, and estimated the NH_2 -terminal sequence of each cut band by Edman degradation. Of 12 bands, with the exception of one that is probably blocked by a pyroglutamyl residue (NH_2 -terminal sequence QXXX), 11 bands yielded information as shown in Fig. 2. The NH_2 -terminal sequences were used to screen a 3-frame translation of a cDNA database originating from ~ 600 DNA sequences obtained from randomly picked clones from a unidirectionally cloned salivary gland cDNA library from *P. papatasi*. Matching clones were subsequently fully sequenced using custom primers. The proteins in Fig. 2 are named according to their mobility in nonreducing SDS-PAGE. A brief description of the clones is enclosed in Table I, with their NCBI accession nos., best match to the NCBI NR protein database, predicted mol wt, and motifs found. All proteins had a predicted signal secretory sequence (26), and their site of cleavage was confirmed by Edman degradation. All proteins were basic, with a pI ranging from 8.28 to 9.43. Note that we obtained the same NH_2 -terminal sequence for two SDS-PAGE bands, located at 32 and 64 kD; we presume the heavier is a homodimer. Additionally, the same NH_2 -terminal sequence was obtained from the SDS-

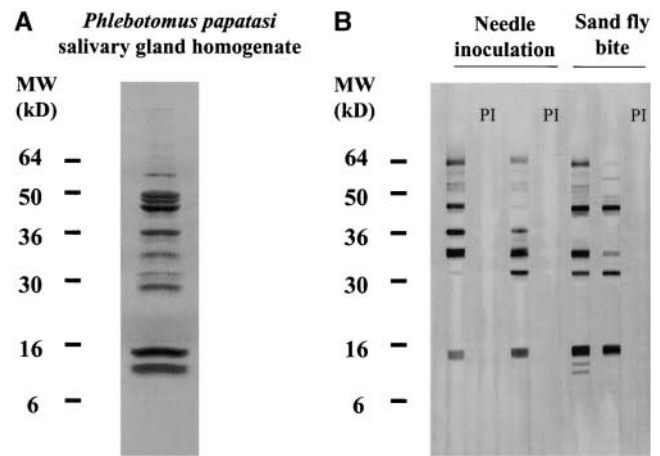


Figure 1. SDS-PAGE and Western blots of SGH of *P. papatasi*. (A) Coomassie blue-stained PVDF membrane after gel transfer of 40 homogenized pairs of glands. The numbers represent the position of the mol wt markers. (B) Western blots of the gels with mouse sera obtained from mice immunized by intradermal inoculation of SGH (needle inoculation) or the bite of uninfected sand flies (sand fly bite) with their preimmunization (PI) controls.

PAGE bands located at 42 and 46 kD, the difference possibly arising from posttranslational modification.

SGH of *P. papatasi* is known to enhance infection of *L. major* when injected in naive mice (16, 17). Previous immunization of mice with SGH or by exposure to noninfected sand flies not only prevents the enhancing effect of SGH but also actually protects mice from *Leishmania* when coinoculated with saliva or by bites of infected sand flies (15, 18). To investigate which fractions from saliva produce the most effective protection when mice are subsequently challenged with parasites and SGH, we separated SGH by SDS-PAGE, divided the gel into three groups containing proteins in the range of 200 to 40 kD, from 39 to 20 kD, and below 20 kD. A control group receiving gel without proteins was also run, together with noninjected controls. After intradermal immunization in the ear, mice were challenged in the contralateral ear with parasites and SGH. As shown before, naive mice inoculated with SGH plus parasites had significantly increased lesion size at 5.5 wk postinoculation, when compared with naive mice inoculated with parasites only. Mice vaccinated intradermally with the lower mol wt fractions (fraction C), and challenged with both parasites and SGH had the best protection in this assay (Fig. 3 A; $P < 0.05$). Parasite load at 4.5 wk postinoculation were also lower in fraction C-vaccinated mice ($P < 0.05$; Fig. 3 B). A strong antibody response was obtained when mice were inoculated with the smaller protein size region of the gel (Fig. 4). Within this group, a stronger reaction was mounted against an antigen identical to or comigrating with a protein of 15 kD, here named SP15 protein (PpSP15), as identified in Fig. 2 and Table I.

To test the role of the SP15 gel region in conferring resistance to leishmaniasis in mice, we vaccinated mice with the SP15 band obtained from SDS-PAGE or with an acrylamide control injection and challenged these animals with

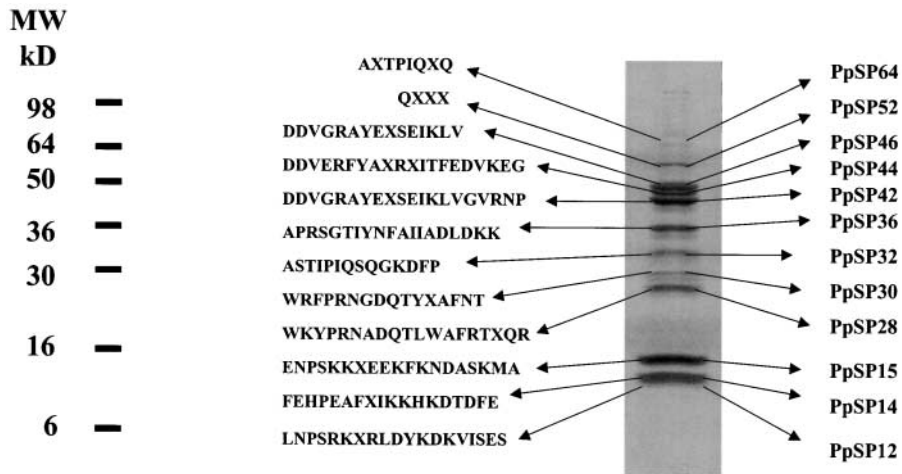


Figure 2. SDS-PAGE of 20 homogenized pairs of salivary glands from *P. papatasi*. Left side of figure, indication of the NH₂-terminal sequence found for each band by Edman degradation; right side of figure, clone identification name. Numbers indicate the position of the mol wt marker in the same gel.

Table I. Salivary Proteins from *Phlebotomus papatasi*

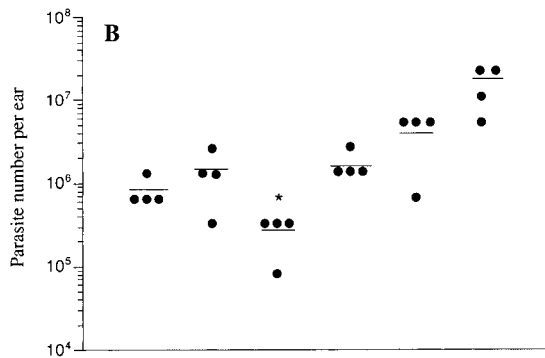
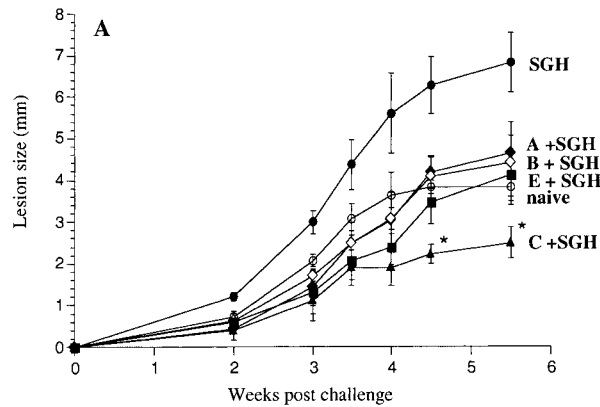
Salivary protein ^a (accession number)	NCBI best match ^b	kD ^c (predicted)	Motifs ^d (http://www.motif.genome.ad.jp/)
PpSP64 (AF335490)	Flagelliform silk protein, (<i>Nephila clavipes</i>), (AF027972), E = 0.043	24.4	(PROSITE) ATP/GTP binding motif (GNVDSGKT)
PpSP52	N.D.	N.D.	N.D.
PpSP46 (AF335491)	Yellow protein, (<i>Lutzomyia longipalpis</i>), (AF132518), E = 10 ⁻⁹⁷	44.4	(PRODOM) Protein major royal jelly, bee-milk glycoprotein, yellow
PpSP44 (AF335492)	Yellow protein, (<i>Lutzomyia longipalpis</i>), (AF132518), E = 10 ⁻¹⁰¹	43.6	(PRODOM) Protein major royal jelly, bee-milk glycoprotein, yellow (PRINTS) Rhosospin-like GPCR superfamily signature
PpSP42 (AF335491)	Yellow protein, (<i>Lutzomyia longipalpis</i>), (AF132518), E = 10 ⁻⁹⁷	44.4	(PRODOM) Protein major royal jelly, bee-milk glycoprotein, yellow
PpSP36 (AF261768)	Salivary apyrase, (<i>Phlebotomus papatasi</i>), (AF261768)	35.8	(PRINTS) Rhosospin-like GPCR superfamily signature
PpSP32 (AF335490)	Flagelliform silk protein, (<i>Nephila clavipes</i>), (AF027972), E = 0.043	24.4	(PROSITE) ATP/GTP binding motif (GNVDSGKT)
PpSP30 (AF335489)	D7 protein, (<i>Aedes aegypti</i>), (P18153), E = 10 ⁻⁰⁵	27.8	(PRINTS) transforming protein P21 RAS signature
PpSP28 (AF335488)	D7 protein, (<i>Aedes aegypti</i>), (P18153), E = 20 ⁻⁰⁵	27.2	(PRINTS) Rhosospin-like GPCR superfamily signature
PpSP15 (AF335487)	SL1 protein, (<i>Lutzomyia longipalpis</i>), (AF132517), E = 60 ⁻¹²	14.5	(PRINTS) Rhosospin-like GPCR superfamily signature
PpSP14 (AF335486)	SL1 protein, (<i>Lutzomyia longipalpis</i>), (AF132517), E = 10 ⁻¹⁸	14.7	None
PpSP12 (AF335485)	SL1 protein, (<i>Lutzomyia longipalpis</i>), (AF132517), E = 20 ⁻¹³	13.8	None

^aName and NCBI accession number for salivary protein cDNA.

^bBest match of the salivary protein to the NR protein database from NCBI, as accessed by Blast-P program.

^cPredicted molecular weight of the mature protein.

^dMotifs found on indicated salivary protein.



Immunization	A	B	C	E
SGH	+	+	+	+

Figure 3. Effect of mouse immunization with fractions of SGH separated by SDS-PAGE. Effect on (A) the lesion size and (B) the number of parasites 4.5 wk postchallenge after *L. major* infection. Naive mice were inoculated intradermally with 500 *L. major* promastigotes alone (○) or in the presence of 0.5 pairs of SGH (●). Mice previously vaccinated on the right ear (2-wk intervals) with acrylamide alone, E (■) or mol wt range from 200 to 40 kD, A (◆); 39 to 20 kD, B (◇); or 19 to 3 kD, C (▲) were challenged in the left ear 2 wk after the last immunization with 500 *L. major* promastigote in the presence of 0.5 pairs of SGH. The above symbols and bars represent the mean induration in mm ± SE (five mice per group) or the mean number of parasite per ear ± SE (four mice per group). (*) indicate significance at $P < 0.05$ when treatment curve (C) was compared with the acrylamide control (E).

L. major in the presence and absence of SGH. Results indicated that vaccination with the SP15 band greatly affected disease manifestation as measured by lesion size, which were significantly smaller ($P < 0.05$) than those of mice vaccinated but not receiving SGH at the time of *Leishmania* inoculation or of acrylamide-vaccinated mice receiving parasites plus SGH (Fig. 5 A). The parasite load at 9-wk postinoculation was smaller on the SP15-vaccinated mice challenged with parasites and SGH, but only borderline so ($P = 0.056$) when compared with the acrylamide-vaccinated controls (Fig. 5 B).

To further confirm whether immunity against SP15 could protect mice when parasites are coinjected with SGH, we constructed a DNA vaccine using the *P. papatasi* salivary SP15 gene. The SP15 cDNA is 522 bp in length and codes for a protein of 142 amino acids including the signal peptide. The mature protein, as predicted by the Sig-

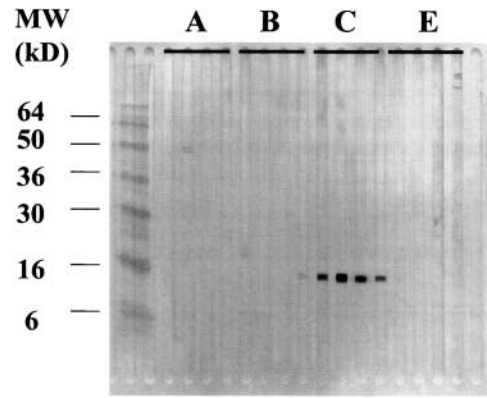


Figure 4. Western blots of SGH of *P. papatasi* reacted with sera from mice immunized with polyacrylamide fractions separating the SGH by SDS-PAGE. Divisions were a high (A), medium (B), and low (C) mol wt region, as detailed in the legend to Fig. 3, or from control mice immunized with polyacrylamide (E), as shown.

nal P program and confirmed by Edman degradation, has 14,494.81 dalton. Accordingly, the SP15 gene coding for the mature protein was inserted downstream and in frame with the signal peptide of tissue plasminogen activator present in the VR1020 vector. This construct is named SP15-Pl. Control plasmids, consisting of VR1020 plasmid alone, were used to immunize control mice. This control plasmid is named CTL-Pl. Mice immunized with SP15-Pl, but not those immunized with the CTL-Pl, produced antibodies recognizing a single band in Western blots of SGH, at the position of SP15 in the gel (Fig. 6 A). Additionally, to determine whether the SP15 vaccination would induce a DTH response in mice after the bite of uninfected sand flies, we exposed naive, SP15-Pl-immunized, and plasmid control-immunized mice to sand fly bites. Results indicate that mice vaccinated with SP15-Pl developed an intense DTH response 24 h after exposure to sand flies (Fig. 6 B), comparable to the DTH observed after saliva inoculation in sensitized mice (data not shown) and as shown previously (18). This reaction is characterized by swelling and a massive cell infiltrate consisting primarily of neutrophils, eosinophils, and macrophages (Fig. 6 B). This experiment indicates that the plasmid vaccine is producing both serum and cellular immunity reactions.

We investigated the effects of immunization with SP15-Pl on the development of *L. major* infection coinjected or not with SGH. The intradermal coinoculation of 500 *L. major* with 0.5 pair of SGH had a dramatic effect on the pathology in naive mice or mice vaccinated with CTL-Pl, consistent with previous results (15, 18; Fig. 7). The lesions developed more rapidly and presented an ulcerative pathology compared with controls inoculated without saliva. In contrast, mice previously vaccinated with SP15-Pl and challenged with parasite plus SGH developed a minor and nonulcerative pathology. The lesions were significantly smaller not only compared with controls (naive or CTL-Pl) inoculated with saliva but also compared with mice inoculated with the parasite alone (data not shown). The indura-

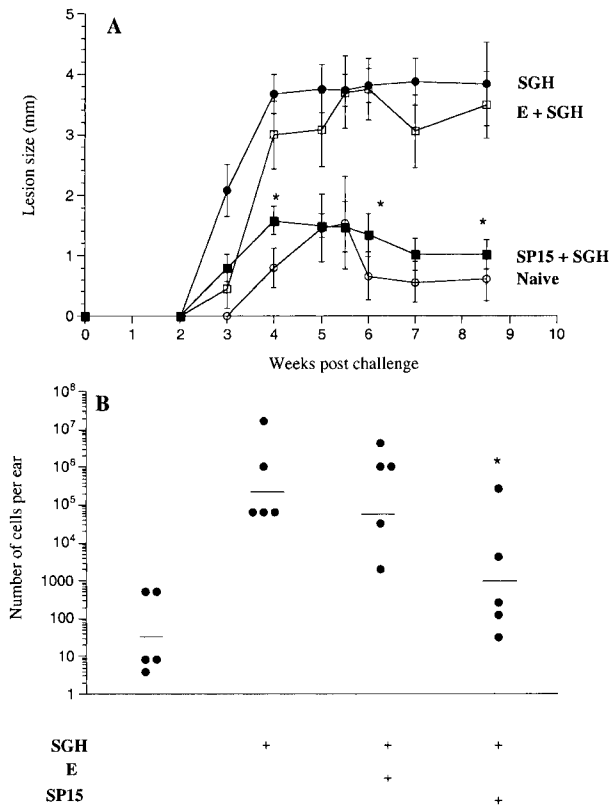


Figure 5. Effect of mouse immunization with band SP15 from SDS-PAGE gels separating the SGH of *P. papatasi* on the lesion size (A) and the number of parasites 9 wk postchallenge (B) after *L. major* infection. Naive mice were inoculated intradermally with 500 *L. major* promastigotes alone (○) or in the presence of 0.5 pairs of SGH (●). Mice previously vaccinated on the right ear (2-wk intervals) with acrylamide alone, E (□) or band SP15 (■) were challenged in the left ear 2 wk after the last immunization with 500 *L. major* promastigotes in the presence of 0.5 pairs of SGH. The above symbols and bars represent mean induration in mm ± SE (five mice per group) or mean number of parasites per ear ± SE (five mice per group). (*) indicate significance at $P < 0.05$ when the treatment curve was compared with the acrylamide control.

tion in the SP15-PI-vaccinated mice resolved as early as 6 wk postchallenge, while in the CTL-PI-immunized mice inoculated with saliva, the large ulcerative, necrotic lesions were maintained. Similarly, the intradermal coinoculation of 500 *L. major* with 0.5 pair of SGH enhanced the parasite load in naive and CTL-PI vaccinated C57BL/6 mice compared with their respective controls, inoculated with the parasite alone (Fig. 7).

To evaluate the persistence of the immunity induced by the DNA vaccine, the animals were challenged or not with SGH 12 wk, rather than 2 wk, after the last boosting. The protection was comparable to that achieved when the challenge was performed 2 wk postvaccination, with a significant reduction of both lesion size and parasite number (Fig. 8).

Having shown that immunization with SP15-PI induces both antibody and DTH (Fig. 6) responses, we evaluated the contribution of antibodies versus cellular immunity in the anti-*Leishmania* protective effect. $B^{-/-}$ mice were im-

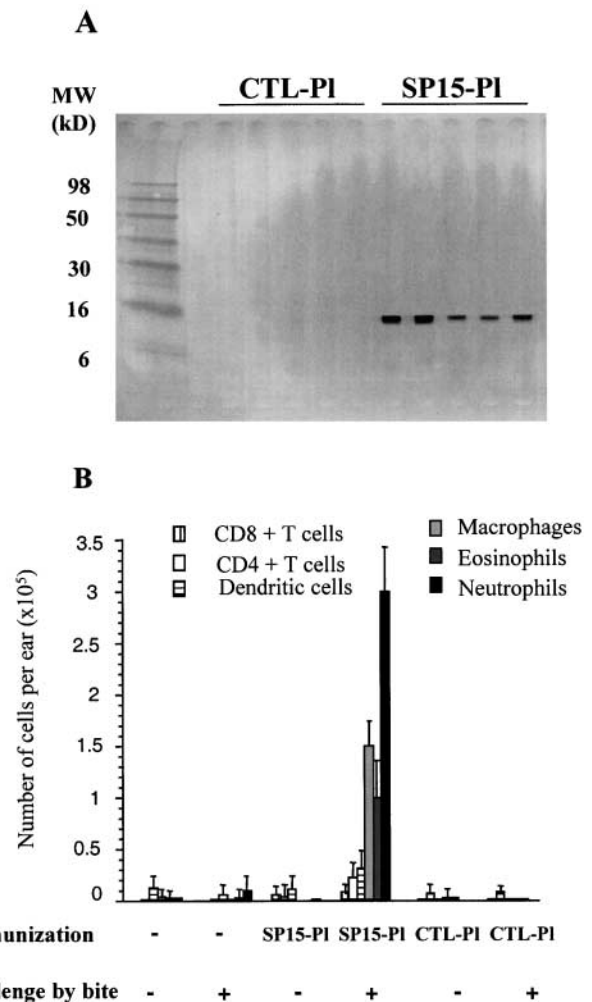


Figure 6. Mouse humoral and cellular immunities are generated by injection of the VR1020 plasmid containing the SP15 sequence (SP15-PI). (A) Western blots of salivary homogenates of *P. papatasi* reacted against sera of mice immunized twice in the right ear (2-wk interval) with 5 μ g of the SP15-PI or control VR1020 plasmid (CTL-PI). (B) DTH reaction induced by sand fly bites on mice vaccinated with SP15-PI. The left ears of naive mice, mice immunized with SP15-PI, or control CTL-PI mice were exposed to the bite of 10 sand flies. 24 h later, the mice were killed, and the inflammatory cells (neutrophils, eosinophils, macrophages, dendritic cells, CD4⁺, and CD8⁺ T cells) present in the dermis were analyzed. The symbols and bars represent the mean number of cellular subsets per ear ± SE; seven mice per group.

munized with SP15-PI and CTL-PI with the expectation that this model animal would provide only a cellular response to SP15. Indeed, similarly to the wild-type (wt) controls, only $B^{-/-}$ mice vaccinated with SP15-PI developed an intense DTH response when challenged with 500 *L. major* promastigotes in the presence of 0.5 pairs of SGH (Fig. 9 B). As expected, no antibodies from the $B^{-/-}$ mice were detected by Western blots (Fig. 9 A), although antibodies were produced by the control wt mice (B10 mice). When challenged with parasites and SGH, vaccinated $B^{-/-}$ mice had significantly lower pathology at 5.5 wk postinoculation than did mice vaccinated with the control plasmid ($P = 0.008$; Fig. 10 A). When compared with the CTL-

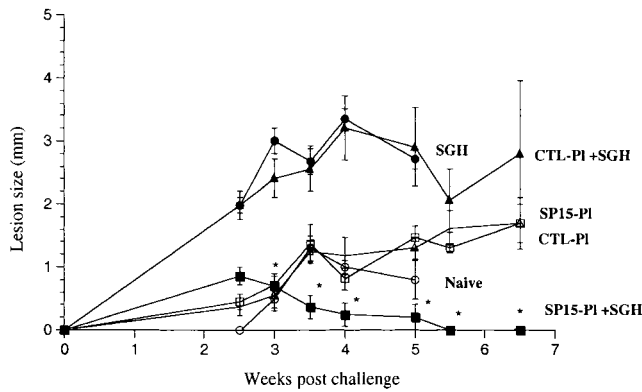


Figure 7. Effect of mouse immunization with SP15-PI on the lesion size caused by *L. major* infection. Naive mice were inoculated intradermally with 500 *L. major* promastigotes alone (○) or in the presence of 0.5 pairs of SGH (●). Mice previously vaccinated on the right ear (2-wk intervals) with SP15-PI (□, ■) or CTL-PI (△, ▲) were challenged in the left ear 2 wk after the last immunization with 500 *L. major* promastigotes in the presence (■, ▲) or not (□, △) of 0.5 pairs of SGH. The symbols and bars represent the mean induration in mm ± 1 SE; five mice per group. (*) indicate significance at $P < 0.05$ when the treatment curve was compared with the control (CTL-PI + SGH).

PI-vaccinated mice ($P < 0.05$), parasite loads at 5.5 wk postinoculation were significantly lower in mice vaccinated with SP15-PI and inoculated with parasites plus SGH, irrespective of whether the mice were B cell deficient (Fig. 10 B). Comparisons of the parasite loads between $B^{-/-}$ and wt mice indicated that $B^{-/-}$ mice had smaller parasite loads than the wt controls ($P = 0.006$).

Discussion

Having proposed that immunity to sand fly saliva may confer protection to subsequent *Leishmania* infection (15, 18), we proceeded to (a) characterize the main proteins in the salivary glands of the sand fly *P. papatasi* that may serve as antigens; (b) identify a protein band from SDS-PAGE that was effective in providing protection against coinoculation of *L. major* plus SGH; (c) construct a DNA vaccine containing the coding information of the main protein co-eluting with the gel band described in b; and (d) characterize the role of cell-mediated immunity in the action of the DNA vaccine.

Two strategies are possible in selecting vaccine candidates from the salivary glands of sand flies. It is known that sand fly SGH enhances *Leishmania* transmission when coinoculated with parasites in naive mice but not in mice vaccinated with SGH or pre-exposed to sand fly bites (16–18). It is also known that sand fly saliva has immunomodulatory components (5, 14). In particular, *P. papatasi* SGH induce the release of IL-4 at the site of inoculation (15). If one considers that the effect of saliva in enhancing *Leishmania* infection is due to these immunomodulatory substances, then salivary gland-induced immunity could be due to the neutralization of such compounds. For example, in a previous work, the release of IL-4 was neutralized by antibodies

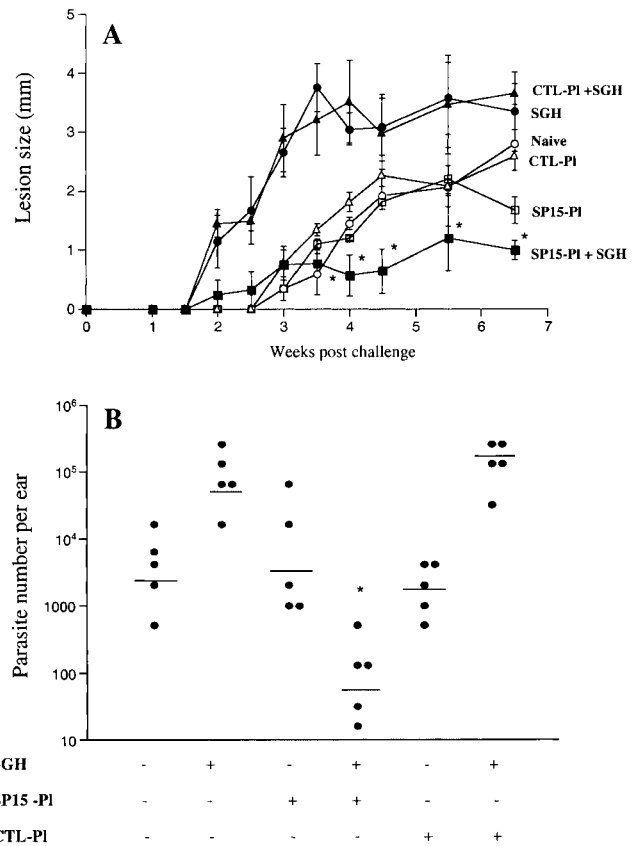
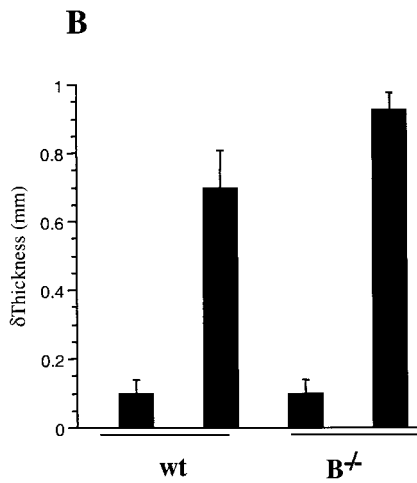
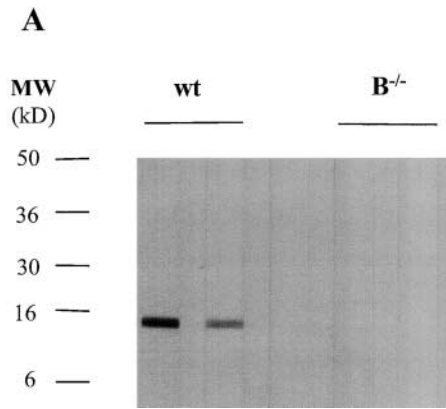


Figure 8. Long-term effect of mouse immunization with SP15-PI on *L. major* infection. (A) Effect on lesion size. Naive mice were inoculated intradermally with 500 *L. major* promastigotes alone (○) or in the presence of 0.5 pairs of SGH (●). Mice previously vaccinated on the right ear (2-wk intervals) with SP15-PI (□, ■) or CTL-PI (△, ▲) were challenged in the left ear 12 wk after the last immunization with 500 *L. major* promastigotes in the presence (■, ▲) or not (□, △) of 0.5 pairs of SGH. (B) Effect on parasite number at 6.5 wk postchallenge. The symbols and bars represent the mean induration in mm ± 1 SE; five mice per group. (*) indicate significance at $P < 0.05$ when the treatment curve was compared with the control (CTL-PI + SGH).

against SGH in presensitized mice, a result correlating with protection against infection (15). Alternatively, immunity against any salivary antigen could modify the site of parasite inoculation in a manner that would disrupt successful parasite transmission. For example, in a sand fly challenge experiment, the level of IFN- γ and IL-12 was significantly increased in the mouse skin after sand fly reexposure (18). While the first hypothesis limits the vaccine candidates to the immunomodulatory salivary components, the second makes all salivary proteins possible vaccine targets.

Whereas SGH of New World sand flies of the genus *Lutzomyia* contain the vasodilatory and immunomodulatory peptide maxadilan (4, 5), Old World sand flies of the genus *Phlebotomus* have adenosine and AMP as their main vasodilator (10), and lack maxadilan. Studies in progress in our laboratory indicate that a mixture of adenosine and AMP is as powerful in enhancing *Leishmania* infection as SGH of *P. papatasi* (unpublished data). Adenosine, but not maxadilan, was also shown to inhibit nitric oxide expres-



SGH + + + +
SP15-PI + + + +
CTL-PI + + + +

Figure 9. Humoral response and DTH reaction on B^{-/-} and wt mice after vaccination with SP15-PI. Western blots showing antibody reactivity of wt but not B^{-/-} mice against *P. papatasi* salivary homogenates (A). Measurements indicated the millimetric difference between the ear challenged with SGH and the noninoculated ear, on B^{-/-} and wt C57BL/10 mice vaccinated with SP15-PI (B). Mice were immunized twice in the right ear (2-wk intervals) or not with 5 μg of SP15-PI or CTL-PI and challenged in the left ear 2 wk after the last immunization with 500 *L. major* promastigotes in the presence of 0.5 pairs of SGH. 24 h after inoculation, the ear thickness was measured and the difference between the ear thickness before challenge and 24 h after challenge was computed. Symbols and bars represent mean thickness in mm ± SE; five mice per group.

sion in activated macrophages (14). Because exposure to *P. papatasi* bites or to needle inoculation of *P. papatasi* SGH confer protection to subsequent inoculation of parasites plus SGH (15, 18), we hypothesize that this immunity is not due to neutralization of salivary immunomodulators—such as adenosine—but rather to the modification of the parasite inoculation site due to a cell-mediated reaction with salivary gland antigens. Accordingly, any salivary gland protein is a potential vaccine candidate to *Phlebotomus*-transmitted *Leishmania*.

A combination of random sequencing of a salivary gland cDNA library with NH₂-terminal sequencing of salivary

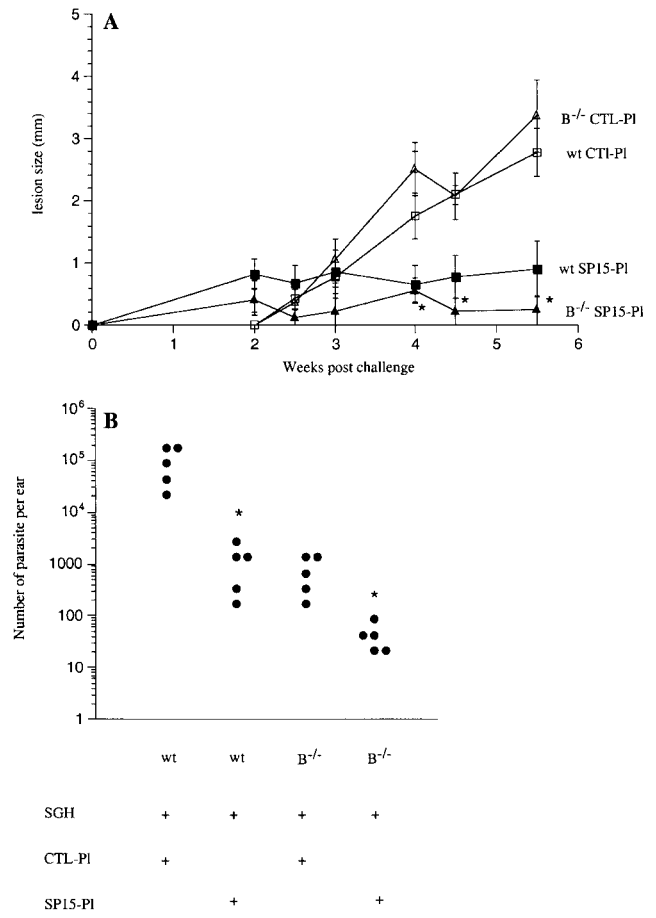


Figure 10. Role of DTH in mouse immunization with SP15-PI on subsequent *L. major* infection. B^{-/-} mice (Δ, ▲) and their controls (C57BL/10; wt; □, ■), were immunized twice in the right ear (2-wk interval) with the VR1020 plasmid with (filled symbols) or without (open symbols), the SP15 sequence and challenged 2 wk later in the left ear with *L. major* promastigotes in combination with 0.5 pairs of homogenized *P. papatasi* salivary glands. (A) Lesion size progression. (B) Parasite numbers recovered from the lesion at 5.5 wk. Each number and bar represents the average ± SE of five mice. (*) indicates significance at *P* < 0.05 when the number of parasites on the SP15-PI group was compared with the controls of the same mouse group.

gland proteins separated by unidimensional SDS-PAGE and transferred to PVDF membranes led to identification and full primary sequence characterization of the majority of the proteins visible by Coomassie blue staining of the gels. Except for the apyrase enzyme (PpSP36; reference 21), all identified proteins have unknown function. PpSP12, PpSP14, and PpSP15 are similar to a protein (SL1) present in the saliva of *L. longipalpis*. PpSP28 and PpSP30 belong to the odorant-binding superfamily (31) and may have a function in binding pharmacologic mediators of hemostasis, as do the tick histamine-binding proteins (32) or lipocalins of *Rhodnius prolixus* (33, 34). PpSP32 is similar to a silk protein from *Nephila clavipes*. PpSP42 and PpSP44 are similar to the Yellow-B protein from *Drosophila melanogaster* with unknown function and to the major royal jelly protein from *Apis mellifera*. Because *P. papatasi* produces an intense DTH reaction and this reaction enhances its feed-

ing success (19), it may be possible that some or several of these molecules have been selected for inducing this immune reaction in their hosts.

In this study, we used self-healing C57BL/6 mice, a relevant model of *L. major* vaccination on subsequent *Leishmania* infection. This system provides a challenge model that more closely reproduces the pathology and immunity associated with natural infection (15, 19). When used as an immunogen, the SDS-PAGE band containing SP15 was efficient in providing immunity against leishmaniasis when parasites were coinoculated with SGH. After DNA vaccination with SP15, mice produced high titer of anti SP15 antibodies and developed a strong DTH in response to sand fly bites, comparable with the one described previously using salivary extract as immunogen. Mice vaccinated with the SP15 plasmid and challenged with parasites plus SGH were efficiently protected against the manifestations of the disease at the site of the inoculation. The protection achieved using SP15 DNA vaccination was long lasting. The fact that both the pathology and the number of parasites were significantly reduced compared not only with appropriate controls but also with mice inoculated with the parasite alone suggests that the observed protection resulted not only from a neutralization of the SGH effect(s) but had an additional effect on the ulterior parasite survival. SP15 is one of the salivary antigens recognized by people naturally exposed in endemic areas of *L. major* (unpublished data). Additionally, this molecule is in the range of the candidates able to induce a strong DTH in response to SGH in the experimental mouse model (19). Together, these results validate the use of SP15 as a candidate vaccine against *L. major* infection.

The fact that B^{-/-} mice that lack B lymphocytes and cannot produce antibodies were also efficiently protected when vaccinated with the SP15-Pl plasmid and challenged with *L. major* plus SGH indicates that the cellular response to SP15 is sufficient to confer protection against *L. major*. We cannot exclude, however, the participation of antibodies on the protective effect of the vaccine on the wt mice. Additionally, B^{-/-} mice had fewer parasites and less pathology than did wt mice. This is in accordance with the effective immunity of B^{-/-} mice against *L. donovani* (35), and with anti-parasite IgG-enhancing parasite uptake by macrophages (36). These results suggest, as proposed before (18, 19), that the DTH response to sand fly antigens is responsible for the anti-*Leishmania* immunity conferred by sand fly homogenate or sand fly bite immunization.

Several nonexclusive mechanisms may explain the efficiency of the anti-SP15 vaccine against *Leishmania major* infection, in the context of the DTH reaction that it induces, as follows: we recently described (37) that the infection by *L. major* is characterized by an initial silent phase lasting 4–5 wk, at which time a large dermal load of parasites is established. This silent-growth phase is characterized by the absence of lesion formation, or any overt histopathologic changes in the site. We propose that the inoculation of parasites in the context of DTH response may not favor the

establishment of the homeostatic state necessary for the optimal growth of the parasite. The presence at the site of the DTH of IFN- γ or other inflammatory cytokine may induce a direct initial killing of the parasite. The DTH at the site may act as an adjuvant of the anti-*Leishmania* response. The anti-*Leishmania* lymphocytes will be primed in the context of a proinflammatory milieu leading to the potential generation of a stronger Th1 response. The mechanism by which the SP15 DNA vaccine and the sand fly bite protection occur could be similar. Indeed, we showed a rapid increase of the level of IFN- γ and IL-12 at the site of the rechallenge in mice presensitized by the bite of an uninfected vector (18). Finally, it is possible that lymphocytes attracted to the DTH site contain large amounts of adenosine deaminase (38) that could degrade salivary adenosine to inosine. We think this hypothesis unlikely because these lymphocytes should be attracted maximally several hours after saliva injection—when adenosine might have diluted out of the site—and because the product of adenosine deaminase, inosine, also has potent immunomodulatory properties (39).

We used SGH coinoculated with parasites as a surrogate for sand fly transmission. Validation of this method has recently been provided in which immunity to *P. papatasi* sand fly bites was found effective against sand fly transmission of *L. major* parasites (18). Additionally, we show that mouse immunity to *P. papatasi* antigens is similarly acquired by sand fly bite or SGH inoculation (Fig. 1), and that vaccination with SP15-Pl induces a DTH response after sand fly bites (Fig. 6). Unfortunately, the rearing of infected sand flies for challenge studies is not presently an easily reproducible methodology. Perhaps vaccine effectiveness studies for *L. major* and other *Leishmania*/vector associations could be conducted more appropriately with vaccinated rodents or dogs in endemic zones, after defining vaccine candidates by needle challenge of parasites co-inoculated or not with SGH.

There exist presently a number of vaccine candidates to prevent leishmaniasis, based on the GP63 (40), *Leishmania* homologue of receptors for activated C kinase (LACK) (41, 42), and promastigote surface antigen (PSA)-2 (43) antigens. None of these studies takes into account the dramatic effects of saliva. No study includes parasite challenge in the presence of vector saliva. Therefore, the possibility remains that even in the most efficient vaccination protocol described (42, 44), the adjunction of the saliva will counteract the efficiency of the vaccine. The use of an antisaliva vaccine, alone or in combination with anti-*Leishmania* vaccine, may allow the host exposed to the bite of infected sand flies to develop minor or no pathology and a strong anti-*Leishmania* immunity.

Although SP15 appeared to be the best vaccine candidate after our SDS-PAGE fractionation of SGH, it is possible that other salivary proteins could confer similar protection in the same mice, or that other proteins could be more effective in mice of different immunogenetic background. Indeed, we observed previously that different HPLC frac-

tions from SGH produced strong DTH response in mice of either BALB/c or C57BL/6 backgrounds (19). Most salivary proteins of *P. papatasi* are now available to test these hypotheses. It should also be noted that there is the possibility of using the recombinant salivary proteins described in this paper for epidemiologic investigations of the effect of human natural immunity against these immunogens and its impact on disease outcome, as is being done with *L. longipalpis*-transmitted leishmaniasis in Brazil (45)

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