

## Characterization of a Defensin from the Sand Fly *Phlebotomus duboscqi* Induced by Challenge with Bacteria or the Protozoan Parasite *Leishmania major*

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**Antimicrobial peptides are major components of the innate immune response of epithelial cells. In insect vectors, these peptides may play a role in the control of gut pathogens. We have analyzed antimicrobial peptides produced by the sand fly *Phlebotomus duboscqi*, after challenge by injected bacteria or feeding with bacteria or the protozoan parasite *Leishmania major*. A new hemolymph peptide with antimicrobial activity was identified and shown to be a member of the insect defensin family. Interestingly, this defensin exhibits an antiparasitic activity against the promastigote forms of *L. major*, which reside normally within the sand fly midgut. *P. duboscqi* defensin could be induced by both hemolymph or gut infections. Defensin mRNA was induced following infection by wild-type *L. major*, and this induction was much less following infections with *L. major* knockout mutants that survive poorly in sand flies, due to specific deficiencies in abundant cell surface glycoconjugates containing phosphoglycans (including lipophosphoglycan). The ability of gut pathogens to induce gut as well as fat body expression of defensin raises the possibility that this antimicrobial peptide might play a key role in the development of parasitic infections.**

AQ: A  
 AQ: B

**Fn1** Epithelial tissues are regularly faced with pathogens, and the gut epithelium is one of the main sites of interaction between host and pathogens in the digestive tract. An efficient immune response is therefore required to protect the epithelium and to distinguish pathogenic from commensal or symbiotic microbes. Antimicrobial peptides (AMPs) have been shown to play an important role in this local defense. AMPs such as defensins are widely distributed and have been identified as components of the local immune response of the gut in vertebrates (2, 3, 22, 35) and in invertebrates (21, 23, 39). Animal defensins are cationic peptides approximately 4 kDa in size, active against a wide range of bacteria and fungi (22).

AQ: C

In hematophagous insects, which act as vectors of parasitic diseases such as malaria, leishmaniasis, and trypanosomiasis, the gut represents a key tissue for parasite development (18). Midgut immunity has been shown to play a major role in the control of parasitic infections, and it is possible that different molecules (AMPs, NO, AND H<sub>2</sub>O<sub>2</sub>) secreted in the anterior part of the digestive tract might participate in the control of the infection (5, 15, 21, 37). We have evaluated further the possible role of AMPs during parasitic infections with the sand fly *Phlebotomus-Leishmania* model. Leishmaniasis are human and animal diseases caused by *Leishmania* spp. parasites. Par-

asites develop as amastigotes in the macrophages of the vertebrate host. After a sand fly bite, they transform into promastigotes in the digestive tracts of the vectors and undergo extensive morphological and biochemical changes. Lipophosphoglycans (LPGs), the major surface antigens of promastigotes, play an important role in the survival of the parasite within the sand fly midgut (31).

The present study investigates the possible role of AMPs in *Leishmania*-infected sand flies. We describe the isolation of a new member of the defensin family that is induced by bacteria or *Leishmania* and which has significant antileishmanial activity on the promastigote forms of the parasite. We also show that defensin mRNA is strongly induced by wild-type (WT) *Leishmania major* and that this induction is greatly reduced following infection with *L. major* mutants unable to survive well within the sand fly, due to the disruption of pathways leading to the synthesis of abundant surface glycoconjugates such as LPG (26).

### MATERIALS AND METHODS

**Insect infections.** Sand flies (*Phlebotomus duboscqi* Senegal strain, obtained from R. Killick-Kendrick, Imperial College at Silwood Park, Ascot, United Kingdom) were reared under standard conditions at 26 to 28°C.

For systemic infections with bacteria, male and female insects were pricked in the thorax with a thin needle dipped in a diluted mixture of *Erwinia carotovora* subsp. *carotovora* (a phytopathogenic gram-negative bacterium that uses flies as vectors).

For natural infections (per os) with bacteria, an overnight diluted culture of *E. c. carotovora* was overlaid on an apple slice. Insects were left in contact with the bacterium-infected food for 24 h before their hemolymph or their gut was

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collected. After the different protocols of infection, insects were maintained at 28°C.

For *Leishmania* infections, we used amastigotes of *L. major* (LV561, MHOM/IL/67/Jericho-II) and promastigotes of three lines of *L. major* (LV39 clone 5, MRHO/SU/59/P): the WT and mutants defective either in LPG1 (*lpg1*<sup>-</sup>) or LPG2 (*lpg2*<sup>-</sup>) through elimination of the respective genes by homologous gene replacement. The *lpg1*<sup>-</sup> mutant lacks a putative galactosylfuranose responsible for the formation of the LPG core but is otherwise normal in the expression of surface glycoconjugates (30, 34), while the *lpg2*<sup>-</sup> mutant is defective in the synthesis of all phosphoglycans (11, 25, 36). Promastigotes were grown at 25°C in 199 medium supplemented with 10% heat-inactivated fetal bovine serum and 25 mM HEPES (pH 7.4). Transfectants were maintained under the selective pressure of 15 µg/ml hygromycin B (*lpg2*<sup>-</sup>) or hygromycin and 20 µM puromycin (*lpg1*<sup>-</sup>). For infective blood meals, promastigotes were washed by centrifugation, counted, and diluted in phosphate-buffered saline (PBS). The amastigotes were produced in BALB/c mice injected subcutaneously in the rump (10<sup>6</sup> parasites per mouse). Lesions were monitored weekly; 10 weeks postinfection, the mice were sacrificed, dissected lesions were macerated in sterile saline, and amastigotes were counted on Giemsa-stained smears.

*P. dubosqi* females, 6 to 8 days old, were fed through a chick skin membrane with heat-inactivated rabbit blood containing 5 × 10<sup>6</sup> logarithmic-phase promastigotes or 1 × 10<sup>6</sup> amastigotes per ml. Blood-engorged females were separated, maintained at 25°C on 50% sucrose, and dissected 4, 8 and 10 days after the infective blood meal. The location and intensity of gut infections were evaluated under a light microscope as described previously (10).

To collect hemolymph from each insect, PBS was injected into the insect thorax with a fine capillary with the Nanoject system (Drummond Scientific). Hemolymph was aspirated and transferred into a 0.1% trifluoroacetic acid (TFA) solution to prevent protease degradation.

**Isolation and structural characterization of defensin.** Sand fly hemolymph (from 200 to 400 insects, according to the experiment) was subjected to reverse phase-high performance liquid chromatography (RP-HPLC) on a narrow Aquapore OD RP300 C<sub>18</sub> column (220 × 2.1 mm; Brownlee), with a linear gradient of 2 to 60% acetonitrile (ACN) in acidified water (0.05% TFA) over 90 min at a flow rate of 0.2 ml/min at 35°C. The column effluent was monitored by absorbance at 214 nm and fractions were hand collected. After evaporation under vacuum (SpeedVac; Savant), fractions were reconstituted in MilliQ water (Millipore). An equivalent of 40 insects per 2 µl was tested for antimicrobial activity by a solid growth inhibition zone assay as previously described (16). HPLC fractions with activity against gram-positive bacteria (*Micrococcus luteus*) were further purified to homogeneity on a microbore Aquapore RP 300 C<sub>8</sub> column (1 by 100 mm; Brownlee) with linear biphasic gradients of ACN in acidified water over 60 min at a flow rate of 80 µl/min. The column effluent was monitored by absorbance at 214 nm at 35°C. The purity of the active fraction was controlled between each chromatography by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS).

**Reduction and S-pyridylethylation of defensin.** The purified active peptide was subjected to reduction with dithiothreitol and alkylation with 4-vinylpyridine in the presence of guanidinium hydrochloride by the procedure described previously (8). The S-pyridylethylated peptide was concentrated under vacuum, analyzed by MALDI-TOF MS, and subjected to primary structure determination.

**MALDI-TOF MS analysis.** MS measurements were performed on a Bruker Daltonique (Bremen, Germany) BIFLEX III mass spectrometer in a positive linear mode with an external calibration. Samples were prepared according to the sandwich method (19).

For MS analysis of gut infected naturally either with bacteria or with *Leishmania*, two separate experiments with two different protocols were performed. For bacterial infections, one gut was analyzed individually 24 h after infection and compared to the gut of an uninfected insect. For parasitic infections, a pool of 10 guts was dissected 4 days after the blood meal, lyophilized, resuspended in 5 µl of 1% TFA, and sonicated for 5 min. An aliquot was analyzed in triplicate by MS. The induction of defensin in *Leishmania*-infected sand flies was compared to a pool of 10 insects fed an uninfected blood meal.

For MS analysis of hemolymph, the hemolymph of pathogen-infected sand flies was collected in 50 µl of PBS and dried under vacuum (SpeedVac; Savant). Samples were reconstituted in 80 µl of 1% TFA, concentrated with C<sub>18</sub> ZipTip (Millipore), and eluted with 4 µl of 80% ACN. An aliquot of the eluate (0.5 µl) was analyzed by MS.

**Microsequence analysis of defensin.** Automated Edman degradation of the purified sand fly defensin and detection of the phenylthiohydantoin derivatives were carried out with a pulse liquid automatic sequenator (model 473A; Applied Biosystems, Inc.).

**RNA isolation and cDNA determination.** Total RNA was collected from whole bodies of immune activated or noninoculated (control) insects with TRI reagent (Molecular Research Center) following the manufacturer's instructions. Total RNA was quantified with a Biophotometer (Eppendorf, Germany), and 1 µg of total RNA was reverse transcribed as described previously (24) with the primer 5'-CGGGCAGTGAGCGCAACGT<sub>14</sub>-3'. Degenerate PCR was done with the RT primer and a forward degenerate primer (5'-GGNCAYGCIGCNTGYGCI GC-3') designed according to the amino acid sequence obtained by Edman degradation. PCR conditions were 95°C (3 min), and 30 cycles of 95°C (10 s), 50°C (10 s), and 72°C (30 s), followed by a 5-min extension period at 72°C on an Indy Cycler (Idaho Technologies, ●●●, Oreg.). PCR products were size fractionated on a 1.2% low-melting-point agarose gel and visualized on a BioDoc gel documentation system (UVP, Upland, Calif.). Bands of the predicted size were excised, placed at 65°C to liquefy the fragment, and cloned directly into a P-GEM-T vector (Promega, Madison Wis.) following the manufacturer's instructions. Blue-white screening of XLI-Blue cells (Stratagene) was used to identify potential transformants. These colonies were grown overnight in 5 ml of Luria-Bertani medium with 5 µl ampicillin (100 µg/µl) and purified using the Wizard Plus Miniprep DNA Purification System (Promega). Sequencing of these clones was done with an ABI 310 sequencer (Applied Biosystems, Inc.) using Big Dye chemistry. Sequences were compared with known sequences in the National Center for Biotechnology Information database. We designed specific primers for the defensin clone based on the sequences obtained from the degenerate primer PCR. Full-length cDNA sequences were obtained with the Marathon cDNA synthesis kit (Clontech) with our specific primers and the flanking primers obtained with the kit, as described previously (24). PCR amplification, cloning, transformation, and sequencing were done as described above.

**Production and purification of the recombinant sand fly defensin.** The peptide was produced in *Saccharomyces cerevisiae* according to a previously described protocol (20). Briefly, 12 liters of supernatant from recombinant yeast culture was successively purified by RP-HPLC, cation-exchange chromatography, and finally RP-HPLC on a preparative column to get the purified recombinant defensin. Confirmation of the integrity and purity of the recombinant peptide was followed by MALDI-TOF MS.

**Liquid growth inhibition assay.** The activity spectrum (MIC) of sand fly defensin (concentration range, 0.2 µM to 100 µM) was determined for bacteria and fungi by liquid growth inhibition assays (16). The strains used were from private and public collections (33). *Aedes aegypti* defensin A was used as an internal control to validate the reproducibility of our assay and tested on three representative pathogens: *Staphylococcus aureus*, *Fusarium culmorum*, and *Candida glabrata*. The assays were done twice.

**Antiparasitic assay of *Leishmania*.** Briefly, 2.5 × 10<sup>5</sup>/ml promastigote forms (insect stages) of *L. major* (strains LEM771 and MHOM/YE/84) were incubated at 27°C with or without peptide dilution (highest concentration of defensin tested, 122 µM) in a 96-well microtiter plate (7). The test was run twice in duplicate (two- and threefold serial peptide dilutions). After 72 h of incubation, Alamar blue was added, plates were read with a microplate fluorometer system (Spectramax Gemini; Molecular Devices), and values of drug concentrations inhibiting 50% of fluorescence development (IC<sub>50</sub>) were calculated (29).

**Quantitative PCR on whole sand flies.** For estimation of mRNA abundance, we used real-time quantitative PCR (Q-PCR) methods. We first amplified and purified PCR products for sand fly defensin and actin sequences. We then established standard curves for actin and defensin, using serial dilutions (1 ng to 10<sup>-10</sup> ng) of the purified cDNAs. The regression line for defensin has an R<sup>2</sup> value of 0.99288, an efficiency of 0.81, and parameters for M of -0.258 and for B of 1.997. The regression line for actin has an R<sup>2</sup> value of 0.99969, an efficiency of 0.85, and parameters for M of -0.266 and B of 2.274. Subsequently, cDNAs from our experimental samples were tested under the same conditions along with our standards, and relative amounts of defensin were determined. Reverse transcription was done as described above with 1 µg of total RNA from whole bodies of naïve, bacteria-inoculated, and bacteria- or parasite-exposed insects. Samples were run on a Bio-Rad iCycler and a Corbett Rotor-Gene machine under the following conditions: 95°C (2 min); and 40 cycles of 95°C (30 s), 62°C (30 s), and 72°C (1 min). PCR reagents were similar to those for regular PCR with the addition of 1 µl of a 1/10,000 dilution of Sybr-Green I (Sigma) to measure the amounts of double-stranded DNA produced in the reaction and 2.5 µl of a 1/1,000 dilution of fluorescein to control for background fluorescence. The amount of defensin or actin was calculated from the standard curves we had generated, and the relative amount of defensin produced per unit of actin was calculated for each sample. Q-PCR was done on samples collected from different batches of immune-stimulated or naïve insects. Each analysis was done at least five times.

AQ: D

AQ: E

AQ: F

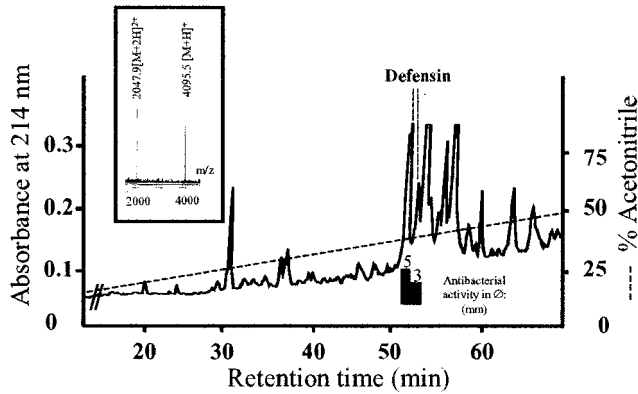


FIG. 1. RP-HPLC of hemolymph of *P. duboscqi* adult insects (around 400 insects) inoculated with *E. carotovora* subsp. *carotovora*. The antimicrobial activity of defensin, detected by a solid growth inhibition zone assay, is expressed by black bars, representing the level of activity in millimeters against *M. luteus*. Elution was performed with a gradient of ACN (dotted line), and the absorbance was measured at 214 nm (solid line). The inset shows the spectrum obtained by MALDI-TOF MS of the fraction with activity against gram-positive organisms, corresponding to the sand fly defensin.

AQ: N

AQ: G

**Nucleotide sequence accession number.** The peptide sequence reported in this manuscript has been submitted to SWISS-PROT under accession number P83404 (*P. duboscqi* defensin).

RESULTS

**Characterization of *Phlebotomus* defensin.** To isolate and identify the AMPs present in sand flies, male and female insects were inoculated with a diluted mixture of the bacteria *E. carotovora* subsp. *carotovora*. Twenty-four hours postimmunization, insect hemolymph was collected and subjected to RP-HPLC purification. Screening of the HPLC fractions revealed the presence of two fractions with activity against gram-positive bacteria (Fig. 1). After MALDI TOF MS analysis and successive RP-HPLCs, the two fractions were found to contain the same molecule. This peptide had a measured molecular mass of 4095.5 MH<sup>+</sup>. Reduction and alkylation of the molecule revealed the presence of six cysteine residues engaged in three internal disulfide bridges. After Edman degradation, a primary structure of 40 amino acid residues was identified with the following sequence: ATCDLLSAFGVGHAAACAHCIGHYRGGYCNSKAVCTCRR. The calculated molecular mass of this sequence (4095.7 MH<sup>+</sup>) was in perfect agreement with the measured molecular mass (4095.5 MH<sup>+</sup>). Data bank searching revealed that this peptide corresponds to a defensin. No additional molecule with antimicrobial activity was detected in HPLC fractions of hemolymph (Fig. 1) or crude immunized insect extract (data not shown).

F1

The complete cDNA sequence was obtained by 5' rapid amplification of cDNA ends-PCR on the cDNA isolated from the RNA extracted from bacteria-inoculated insects, as described in Materials and Methods. The sand fly defensin cDNA contained 480 bp (Fig. 2). This comprised a 5' untranslated region (5'UTR) of 69 bp, a coding region of 294 bp, a stop codon (TAA), a 100-bp 3'UTR containing a polyadenylation consensus sequence (AATAAAA) at position 73 to 78 of the 3'UTR, and a poly(A) tail. The 294-bp coding region encoded a 98-amino-acid prepropeptide. This is made up of a 50-amino-

F2

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CGCCCGGGCAGGCTTCACACTCTTACAATTAATCGTGAAAACACCGCTCACCTTAACTCACTCACA 69
M R T F L V T F V L V V V V G V I S
ATG CGT ACG TTC TTG GTA ACA TTT GTC CTT GTC GTT GPT GPT GGA GTT ATC AGT 123
A Y P S N P V E V E A E D F D A Q D
GCT TAT CCG TCC AAT CCT GTC GAA GTT GAA GCT GAA GAT TTT GAC GCC CAA GAT 177
F D L Q T F Q D T F Y E V F Q V H S
CCT GAT CTA CAA ACT TTC CAA GAT ACT TTC TAT GAG TGA CCC CAG GTT CAT TCA 231
R Q K R A T C D L L S A F G V G H A
CGC CAA AAA CGA GCC ACT TGC GAT CTA CTT AGT GCT TTT GGA GTT GCT CAT GCT 285
A C A A H C I G H G Y R G G Y C N S
GCT TGT GCT GCC CAT TGT ATC GGA CAC GGA TAC AGA GGA GGA TAT TGC AAC TCA 339
K A V C T C R R *
AAG GCT GTC TGC ACC TGC AGG AGA TAA 366
ACAATTCAGC AATTAGGAAA ATCTTCTATG GCATTTAACAAC ATCCTTA ATTTTCGGAA
ATAGTTTTTA TGAATAAA ATAATTACTT CAGATTCTTAAAA AAAAAAAAAA
    
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FIG. 2. The nucleotide and deduced amino acid sequences of *P. duboscqi* defensin cDNA. Amino acids are presented as single-letter codes above each codon. The sequence coding for the 40-amino-acid mature peptide is shown in boldface type. The stop codon (TAA) is indicated by an asterisk, and the putative polyadenylation consensus signal (AATAAAA) is underlined.

acid region containing the signal peptide (residues 1 to 29) and a propeptide region (residues 30 to 58). This pre-proregion terminated with the characteristic dipeptide KR cleavage site found in many defensin sequences isolated from the order Diptera.

The sequence of the mature peptide was aligned with selected sequences of insect defensins available in GenBank. The sand fly defensin aligns closely to other members of the insect defensins, especially those from Diptera (Fig. 3). The *P. duboscqi* defensin shares 79 and 76% identity with mosquito defensins from *A. aegypti* and *Anopheles gambiae*, respectively, but has a unique arginine residue at the C-terminal part of the molecule. Less identity was shared with the stable fly, *Stomoxys calcitrans* (60%), *Glossina morsitans* (37.5%) (suborder Brachycera), and the bug *Rhodnius prolixus* (55%) (Hemiptera).

F3

**Induction of defensin in insect midgut and in hemolymph during different protocols of infections.** MS analysis of sand fly gut tissue revealed that defensin peptide was induced after per os infection with the bacteria *E. carotovora* subsp. *carotovora* (Fig. 4A). Since sand flies are natural vectors for protozoan parasites of the genus *Leishmania*, we examined sand flies infected per os with *L. major* by MS and found that the defensin was induced in the gut (Fig. 4B) and the hemolymph as well (Table 1). This induction was specifically caused by the pathogen, since the peptide was not found in either naive insects or in insects after an uninfected blood meal. Defensin synthesis peaked at 24 h post per os infection with bacteria and at day 4 when infection was performed with *Leishmania*.

F4

T1

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Nematocera suborder
Phlebotomus duboscqi ATCDLLS----AFQVGHAAACAHCIGHYRGGYCNSKAVCTCRR
Aedes aegypti A ATCDLLS----GFSVGDSSACAARCIARGNRGGYCNSKAVCVCRN
Anopheles gambiae ATCDLAE----GFGVSSLLCAARCIARRRGGYCNSKAVCVCRN
Brachycera suborder
Stomoxys calcitrans ATCDLLS----MNVNHSACAARCLLLGKSSGRCNDDAVCVCRK
Glossina morsitans VTCN-----IGEWCVARHNSKSKSKSCYC-SRQVICYCN
Hemiptera order,
Rhodnius prolixus A ATCDLFSFRSKVWTPNHAAACAARCLLRNRRGRC-KGTICHCRK
    
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FIG. 3. Multisequence alignment of *P. duboscqi* defensin with representative defensins of different insect vectors. Defensins of Dipteran insects from the suborders Nematocera and Brachycera and insects from the order Hemiptera are shown. Gaps are introduced to optimize the alignment.

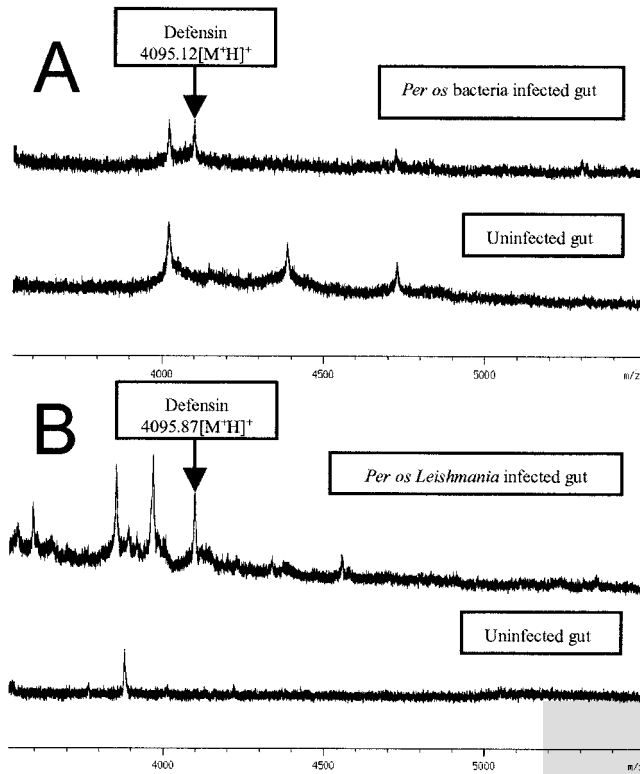


FIG. 4. MS analysis of sand fly defensin induction in gut tissue (A) 24 h after per os infection with bacteria (*E. carotovora* subsp. *carotovora*) and (B) 4 days after per os infection with *L. major*. The results shown (A and B) represent data from two separate experiments. For bacterial infections, defensin was detected in individual sand flies (10 insects tested for each protocol); for parasitic infections, defensin was detected in a pool of 10 midguts (three pools for each protocol).

**In vitro activity of recombinant defensin on various pathogens.** Recombinant sand fly defensin was highly effective (MIC <10  $\mu$ M) against filamentous fungi, especially *Trichoderma viride*, *F. culmorum*, and *Fusarium oxysporum*, and on yeast, especially *Candida albicans* and to a lower extent, *S. cerevisiae*. The *P. duboscqi* defensin was more active against yeast than mosquito defensins were (Table 2) (39). The antiparasitic activity of defensin on the promastigotes (insect forms) of *Leishmania* spp. was tested. *P. duboscqi* defensin was active against promastigotes of *L. major* (MHOM/YE/84) (IC<sub>50</sub>, 68 to 85  $\mu$ M) (Table 3).

TABLE 1. Presence of defensin in crude sand fly hemolymph measured by MALDI-TOF MS after different protocols of infection<sup>a</sup>

Protocol	Day 1	Day 4	Day 10
None	–	ND	ND
Pricked with <i>E. carotovora</i> subsp.	+++	ND	ND
p.o. infection with <i>E. carotovora</i> subsp.	++	+	–
Negative blood meal	–	–	ND
<i>L. major</i>	–	+	–
Mutant <i>lpg1</i> <sup>–</sup>	–	+	–
Mutant <i>lpg2</i> <sup>–</sup>	–	+	–

<sup>a</sup> p.o., per os; ND, no data.

TABLE 2. Antimicrobial activity of recombinant defensins from *P. duboscqi*<sup>a</sup>

Microorganism	Recombinant defensin ( $\mu$ M)		
	<i>Phlebotomus</i> defensin	<i>Aedes</i> defensin C	<i>Anopheles</i> defensin
Gram-positive bacteria			
<i>S. aureus</i>	0.78–1.56	1.56–3.12	0.4–0.75
Filamentous fungi			
<i>Aspergillus fumigatus</i>	12.5–25	ND	NA
<i>Beauveria bassiana</i>	NA	ND	NA
<i>F. culmorum</i>	1.56–3.12	50–100	3–6
<i>F. oxysporum</i>	3.12–6.25	ND	1.5–3
<i>Neurospora crassa</i>	6.25–12.5	ND	3–6
<i>T. viride</i>	3.12–6.25	ND	ND
<i>Trichophyton mentagrophytes</i>	25–50	ND	ND
Yeast			
<i>Candida albicans</i>	6.25–12.5	ND	NA
<i>C. glabrata</i>	NA	NA	NA
<i>S. cerevisiae</i>	25–50	ND	NA
Parasites			
<i>L. major</i> promastigote forms	68–85 <sup>b</sup>		

<sup>a</sup> Activity of recombinant defensins against bacteria, fungi, yeast, and parasites. The activity of sand fly defensin was compared to those from *A. aegypti* and *A. gambiae* (39). Values of inhibition of gram-positive bacteria, fungi, and yeast are MICs, given in micrometers. The highest MIC concentration tested was 100  $\mu$ M. NA, no activity; ND, no data.

<sup>b</sup> Activity of *P. duboscqi* against parasites was measured by an Alamar blue assay. Defensin was incubated at various concentrations (highest concentration tested, 122  $\mu$ M). Values shown are IC<sub>50</sub>s, in micrometers.

AQ: O

**Induction of defensin mRNA by bacteria introduced by different routes.**

First, defensin mRNA levels were assessed after bacterial infections by Q-PCR, with actin as a control mRNA. Whole bodies of naïve sand flies showed a very low baseline of defensin mRNA, which was arbitrarily given a relative expression level of 1 (Fig. 5). Flies pricked with bacteria (systemic infection) demonstrated a 490-fold increase in defensin transcripts compared with naïve insects, while flies infected per os with bacteria demonstrated a 32-fold increase in defensin transcripts. These data indicate that the injection of bacteria into the hemolymph induced higher levels of defensin mRNA than did the ingestion of bacteria.

F5

**Induction of defensin mRNA by Leishmania.**

We then checked the kinetics of induction of the sand fly defensin following infection with *L. major*. In the natural infectious cycle, after biting an infected host sand flies are initially infected by the amastigote form of the parasite, and the parasite differentiates to the infective promastigote form during the first day and replicates in this form thereafter. During the first few days within the fly, the blood meal containing parasites is enclosed by the midgut peritrophic membrane, which disperses after a few days. At that time, parasites bind to the midgut wall through specific interactions involving the abundant surface glycolipid LPG (31), which are required for survival. We measured defensin mRNA expression at day 1 (differentiation from amastigotes to promastigotes), day 4 (rapid multiplication), and day 10 (late-stage) infections.

We first analyzed defensin mRNAs in whole-fly preparations by Q-PCR methods with amastigote-induced parasitic infections. Relatively little defensin mRNA was induced until day

T2

AQ: P

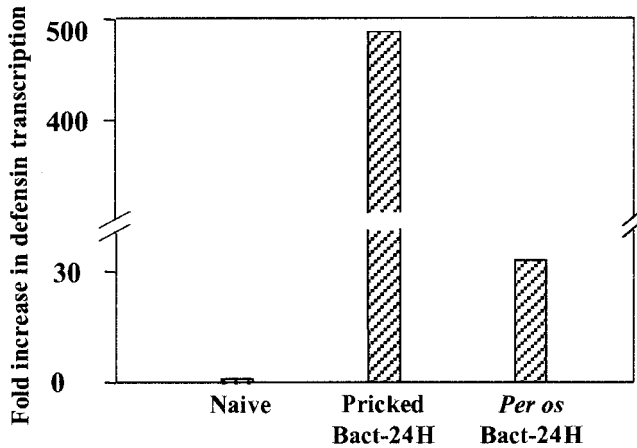


FIG. 5. Assessment of defensin transcription in whole bodies of *P. dubosqi* with Q-PCR after bacterial infections. Naïve (uninfected insects), bacterium-pricked insects at 24 h (Bact-24H) and per os bacterium-infected insects at 24 h are the three protocols studied. The values determined with naïve insects were arbitrarily given a value of 1, and other treatments are expressed as a difference in transcript numbers. Each bar represents an average of six assessments by Q-PCR.

10, when a fourfold increase was found. At this time, parasites were abundant and in the promastigote form. The induction was specific, since a parasite-negative blood meal did not induce defensin transcription (Fig. 6A).

We next analyzed defensin mRNA levels after infection with promastigotes. In these experiments, defensin mRNA induction was induced strongly at day 4 and day 10 (23 and 12 fold, respectively). Given that amastigotes differentiate rapidly to promastigotes, we were surprised that promastigote infections gave rise to a stronger level of induction; this phenomenon may warrant additional study in the future.

**Role of LPG and related phosphoglycans in defensin expression.** The abundant promastigote surface glycoconjugate of *L. major* LPG was demonstrated to have a major role in parasite survival in sand flies (31), and we asked whether this surface antigen might modulate defensin induction. For these studies, we used two *L. major* mutants that were constructed in WT strain LV39 clone 5: *lpg1*<sup>-</sup>, which specifically lacks LPG, and *lpg2*<sup>-</sup>, which lacks LPG as well as all related phosphoglycans.

Since previous studies had been performed with the vector *Phlebotomus papatasi*, we first verified that these mutants behaved similarly in *P. dubosqi* (Fig. 6C). At day 4 following promastigote infection, the WT and *lpg1*<sup>-</sup> developed similarly, since both produced very high infection rates (100% of flies) with a majority of moderate infections. On the other hand, *lpg2*<sup>-</sup> infections were found in 67% of the females and were less intense at day 4. At days 8 and 10, heavy infections in the WT strain with massive colonization of the thoracic midgut and stomodeal valve highly predominant (87% at day 8 and 100% at day 10). In *lpg1*<sup>-</sup>, the intensity of infections decreased with time. Heavy infections were found in 40% of flies at day 8 and in 7% at day 10. At day 10, few parasites reached the stomodeal valve (in 47% of infected females), but infections were less intensive than in the WT. For *lpg2*<sup>-</sup>, the infections were all negative at days 8 and 10.

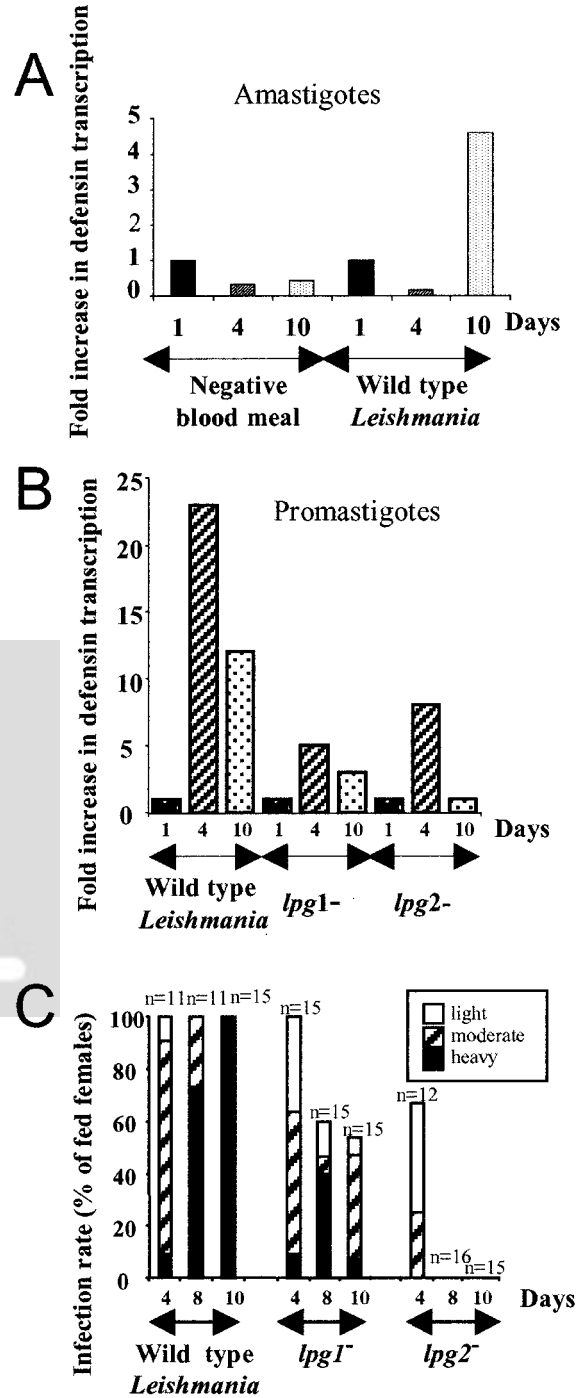


FIG. 6. (A) Defensin transcription in whole bodies of *P. dubosqi* after ingestion of a negative blood meal or amastigote forms of *L. major* (LV561). (B) Defensin transcription in whole bodies of *P. dubosqi* after *Leishmania* infection with promastigote forms of WT (LV39) and two mutants deficient in the LPG surface molecule (*lpg1*<sup>-</sup> and *lpg2*<sup>-</sup>). (C) Rates and intensities of infections in *P. dubosqi* females presented in panel B. Infections were classified into three categories: light (<100 parasites/gut), moderate (100 to 1,000 parasites/gut), heavy (>1,000 parasites/gut).

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These data correspond well with the previous observations with *P. papatasi* of this parasite-vector model (36). For *lpg1<sup>-</sup>* infections, small differences were seen from those observed by Sacks et al. (31), as in *P. papatasi*, *lpg1<sup>-</sup>* infections were maintained up to the time of peritrophic membrane dispersal and defecation (day 4) but were then lost, whereas with *P. duboscqi*, *lpg1<sup>-</sup>* infections persisted at a low level through day 10 (Fig. 6C). This observation seems to be related to a difference in vector competence of these two sand fly species, as *P. duboscqi* supports development of various *Leishmania* species, whereas *P. papatasi* is only susceptible to *L. major* with terminally exposed galactose residues on LPGs (28). For both species, *lpg2<sup>-</sup>* parasites were rapidly lost at all times studied.

Comparisons of promastigote infections of *P. duboscqi* by WT, *lpg1<sup>-</sup>*, and *lpg2<sup>-</sup>* *L. major* showed that the mutant parasites resembled the WT in showing maximum defensin mRNA expression on day 4 and detectable transcript on day 10, but overall the levels were 4- to 10-fold lower than with the WT (Fig. 6B). While these data could be taken as evidence for a role for LPG, this conclusion must be tempered by the fact that these mutants also showed greatly reduced numbers in infected flies at most of the time points studied (Fig. 6B versus C). Perhaps the best evidence specifically associating LPG with defensin induction involves comparisons of the *lpg1<sup>-</sup>* parasite against the WT at day 4. Quantitation of parasite numbers (Fig. 6C) shows an average of  $580 \pm 160$  versus  $480 \pm 480$  parasites/fly (values are averages  $\pm$  standard errors; in experiments with 10 or 11 flies, respectively) for WT and the *lpg1<sup>-</sup>* mutants. We believe this minor difference in parasite number is unlikely to account for the  $\sim$ 5-fold drop in defensin expression in the *lpg1<sup>-</sup>* mutant, and thus these data suggest that LPG may be an important factor.

## DISCUSSION

In insect vectors, it has been suggested that immune peptides might play a role in limiting parasitic infections (4, 6) and might help explain vector competence (5). Bloodsucking members of the order Diptera that carry pathogenic trypanosomatids (*Trypanosoma*, *Leishmania*) are good models to study the role of epithelia in local immune response and the implication of AMPs in such a process, since these parasites do not develop in insect hemolymph. In these models, AMP synthesis cannot be attributed to the tissue damage occurring during the migration of parasite from the gut to the hemolymph, as occurs in mosquitoes infected with *Plasmodia* or filarial worms. Although the presence of AMPs in sand flies was already suspected during a bacterial systemic infection (27), this represents the first report of the induction of AMPs in sand flies during the development of *Leishmania*. Using biochemical and molecular approaches, we identified a single AMP, a new member of the widespread defensin family that is expressed in response to various pathogen infections. This defensin is detected in the gut tissue and in the hemolymph during oral infection by pathogens. Defensins are AMPs present in animal and plant kingdoms, suggesting a major role in the innate immunity of organisms. In invertebrates, defensins have been found in all insect vectors studied so far (6, 23, 24, 39). Consequently, their role during parasitic infections appeared particularly interesting to analyze.

Sand fly defensin exhibits a broad spectrum of activity

against pathogens such as bacteria, yeast, and filamentous fungi. Most interestingly, *P. duboscqi* defensin showed a significant antiparasitic activity. This is the first time that an AMP is described with an antiparasitic activity in a natural host-parasite system. Most insect AMPs that have been studied for possible antiparasitic activity were tested in heterologous systems: frog magainin and giant silk moth cecropin with *Plasmodium* (13), *Drosophila* cecropin with *Trypanosoma cruzi* (12), insect defensins with *Plasmodium* (32), *Drosophila* cecropin with *Leishmania* (1), spider gomesin with *Leishmania* (33), *Phormia* dipterin with *Trypanosoma* spp. (14). The only natural system tested so far was *Plasmodium*-mosquito, but results were not as conclusive. Indeed, one study (24) revealed that before an infective blood meal with malaria, bacteria-challenged *Aedes* exhibits a lower prevalence of malaria oocysts, which suggests that the upregulation of immune peptides due to the bacterial infection might affect parasite development. A second study showed a very marginal effect of gambicin, an AMP isolated from an *A. gambiae* cell line, on a *Plasmodium* ookinete in vitro (38).

Flagellate parasites and bacteria present in the digestive tract induce AMP secretion locally (gut) and systemically (hemolymph) in *Phlebotomus*, *Drosophila*, and *Glossina*, even though no parasites are present in the hemolymph (4, 6, 14). Conversely, experimental systemic infection of the hemolymph with bacteria was shown to induce a local immune response with synthesis of AMPs in the insect midgut (21, 23). Therefore, the gut tissue and the fat body (the major site of peptide synthesis in insect hemolymph) seem to be two essential organs in immunity. Immune signals such as NO or H<sub>2</sub>O<sub>2</sub> might be used for communication signals between these two organs to protect the whole insect from lethal infections (14, 15).

The discovery of a new AMP for an important protozoan pathogen in the insect vector raises the possibility that it plays an important role during parasitic infections. The sand fly defensin was active against the promastigote stage of *L. major* normally found in the insect midgut, and parasite infection led to its induction at the mRNA and protein levels in a manner related to specific parasite glycoconjugates such as LPG, as well as to the intensity of infection. Although we were not able to quantify defensin secretion in the gut, we clearly detected its presence by MS analysis of gut tissue of insects infected per os with bacteria or with *Leishmania*. We are thus tempted to speculate that AMPs may participate in a subtle control of parasite population in sand fly midgut, perhaps in combination together with digestive enzymes and the peritrophic matrix. This observation is in perfect agreement with previous studies that also established that the gut epithelium participates in the control of infections by secreting AMPs (5, 21, 23, 37, 38). Our preliminary estimates suggest that the active concentration of sand fly defensin in vitro on promastigotes is in the range normally found in insects. Indeed, in *Drosophila*, seven AMPs have been identified (17), and the concentration of these AMPs ranges from 1  $\mu$ M for defensin to 100  $\mu$ M for drosomycin (9).

This study underlines the complexity of the interaction of insect vectors with the parasites they harbor and transmit to the vertebrate host. In the continuous and urgent search for new strategies to control major human parasitic diseases, AMPs such as *P. duboscqi* defensin might include engineering

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transgenic insects to express AMPs and reduce parasite transmission (12).

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