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Research Article

Antimicrobial peptide induction in the haemolymph of the Mexican scorpion *Centruroides limpidus limpidus* in response to septic injury

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Abstract. Antimicrobial peptides (AMPs) are essential components of host defences against infectious microrganisms. In chelicerate organisms they have been implicated in three alternative defensive systems: one is defined by the immediate up-regulation of genes encoding AMPs, another is characterized by the inducible systemic release of AMPs from cellular reservoirs and the third alternative is the systemic constitutive production of AMPs. In this work we used a differential high-performance liquid chromatography and mass spectrometry approach to show that septic injury elicits an immune response in the haemolymph of the Mexican scorpion *Centruroides limpidus limpidus*. We isolated several haemolymph components, one of which was characterized extensively (amino acid sequence, disulphide pairing, cDNA and genomic clones) and demonstrated to be a novel member of the invertebrate defensin family and consequently named *C. limpidus limpidus* defensin-like peptide (Cll-dlp). This peptide accumulates in the haemolymph in response to septic injury, independently of transcriptional regulation.

Key words. Antimicrobial peptide; cysteine-stabilized α/β motif; defensin; haemolymph; innate immunity; scorpion.

Arthropods are the mostly widely spread phylum among living animals, comprising at least 75% of all extant metazoas and inhabiting an outstanding variety of ecological niches, where they cohabit with a huge diversity of potentially harmful microorganisms. This evolutionary success can be associated, in part, with their relatively simple but highly effective innate immune system [1, 2]. Their effectiveness relies primarily in the recognition of infectious non-self organisms and consequent activation of cellular and humoral responses leading to the clearance of foreign invaders [3–5]. Analysis of invertebrate immunity reveals striking similarities between their strategies of non-self recognition and the early immune responses of vertebrate organisms [6–8].

Antimicrobial peptides (AMPs) are a highly diverse category of gene-encoded molecules that critically participate in the clearance phenomenon by humoral processes, mainly due to their antibiotic properties, but also as effectors that can drive the whole process [9–12]. Apart from their diversity, AMPs share a few important structural characteristics, like the presence of several basic residues and the amphipathic character of the functional conformation of their polypeptide chains. Four families of AMPs have been recognized on the basis of structural

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similarities [9], namely: linear peptides that can adopt amphipathic α-helical structures in membrane environments; cysteine-rich peptides, closely packed by disulphide bridges; linear peptides with an unusually high content of certain amino acids (e. g. Pro, His or Trp), and relatively long polypeptides rich in Gly residues (a comprehensive list is available in the Antimicrobial Peptides Database at www.bbcm.univ.trieste.it/~tossi/pag1. htm).

Among cysteine-rich AMPs, the largest family corresponds to the invertebrate and plant defensins with the cysteine-stabilized α/β (CS-$\alpha\beta$) motif, which is defined by the presence of two disulphide bridges that join the consensus sequence…Cys_i-Xaa-Xaa-Xaa-Cys_{i+4}…Cys_j-Xaa-Cys_{j+2}…, through a Cys_i-Cys_j and Cys_{i+4}-Cys_{j+2} pairing [13, 14]. The CS-$\alpha\beta$ motif is a remarkably versatile framework shared by a variety of peptides with different functions, including the antimicrobial defensins from invertebrates [1, 15] and plants [16], the scorpion toxins, which are ion channel modulators [17], the sweet-tasting protein brazzein [18] and the rapeseed class of plant serine protease inhibitors [19]. Defensins containing the CS-$\alpha\beta$ motif have been found in three different phyla of invertebrates: arthropods [15], molluscs [20, 21] and nematodes [22–24]. At present, the most representative group comes from arthropods of the insect class, particularly from different orders of the subclass Neoptera.

From the best-characterized models of invertebrate immunity, in particular from the Diptera (Insecta) order [4, 5] and the Xiphosura (Merostomata) [3], two alternative mechanisms of systemic immune activation have been found. One is characterized by the inducible transcription of genes encoding AMPs in response to an immune challenge. The other is defined by the storage of AMPs in haemocyte granules which are either fused with phagocyted microbes or delivered to the haemocell after non-self recognition. Many insect orders appear to follow the first strategy [4, 5, 25–27], whereas some arachnids (the spider *Acanthoscurria gomesiana* [28, 29]), merostomatans (the horseshoe crab *Tachypleus tridentatus* [3]), malacostracans (the shrimp *Litopenaeus vannamei* [30, 31]) and molluscs (the edible mussels *Mytilus* spp. [32]) seem to adopt solely the second mechanism. However, at least in termites [33] and scorpions [34, 35], a third mechanism seems to be present, by which the AMPs are systemically constitutive and independent of an exogenous immune challenge. Intriguingly, the production of AMPs in the Ixodida (Arachnida) [36–39] and the relatively distant class Chromadorea (Nematoda) [40] are inducible by immune challenge.

In the subphylum Chelicerata the three different mechanisms of AMPs production were shown to exist [3, 28, 29, 34–39, 41]. Specifically regarding AMPs from scorpions, 9 different peptides have been documented, 5 were isolated from the venom of 4 different species of the Scorpionoidea superfamily [41] and 4 were found in the haemolymph of 2 Buthoidea species [34, 35]. In all the cases, AMPs appear as constitutive components. Four of such peptides present the consensus sequence of CS-$\alpha\beta$ motif.

In the present work, we describe the immune response caused by septic injury in the Mexican scorpion *Centruroides limpidus limpidus* (Buthidae, Scorpiones, Arachnida, Chelicerata). We isolated and characterized several components from the haemolymph of control and experimentally challenged organisms, using a combined differential high-performance liquid chromatography (HPLC) and mass spectrometry (MS) approach, as well as a semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). One of the inducible components was studied in detail, and shown to be a novel member of the invertebrate defensin family.

Material and methods

Animals, septic injury and haemolymph collection

Adult scorpions of the species *C. limpidus limpidus* were collected in the field (state of Guerrero, Mexico) and maintained in the laboratory with water and food (*Tenebrio* larvae and crickets) for several months. Experimental and control groups consisted of female animals (approximately 1 g body weight each), which were deprived of food (water ad libitum) for 2 weeks before experimentation. Scorpions can live perfectly well and healthily in captivity for up to month without solid food, but with water.

Individuals from test groups were pricked with a fine needle soaked in a mixture of bacteria (*Escherichia coli* DH5α and *Bacillus subtilis*). Two control groups were either kept untreated (naïve scorpions) or pricked with a sterile needle. Haemolymph (25–35 µl per scorpion) was collected by a puncture between the second and third segments of the scorpion metasoma, and collected into ice-cooled plastic tubes. Haemolymph was extracted from control and infected groups (50 specimens each) at 6, 12, 24 and 48 h. To obtain sufficient polypeptide amounts for further biochemical characterization, haemolymph was collected in a separate experiment from 1000 scorpions (male and female individuals) 24 h after bacterial inoculation.

Haemolymph fractionation and peptide purification

The following procedure was adapted from Hetru and Bulet [42]. After collection, haemolymph was immediately centrifuged (Beckman OptimaTL ultracentrifuge) for 30 min at 4 °C and 30,000 *g* to remove cellular content. The supernatant was then diluted 1:1 with 0.10% trifluoroacetic acid (TFA) (v/v), kept in an ice-bath with agitation for 30 min to improve cationic extraction, and again centrifuged for 30 min at 4 °C and 30,000 *g*. This

acidic extract (indicated here as HCll) was loaded onto Sep-Pack Vac C18 cartridges (Waters Associates) equilibrated with 0.10% TFA (v/v). The first fraction collected was the washing with 0.10% TFA (v/v). Thereafter, cartridge elution was performed stepwise with solutions containing 50 and 100% acetonitrile in 0.10% TFA (v/v). The fraction eluted at 50% acetonitrile is indicated here as HCll-II. All fractions were freeze dried under vacuum (Speed Vac; Savant Instruments), except for those derived from the large infected group, which due to their quantity were lyophilized.

The three Sep-Pack fractions (washing, 50% and 100% acetonitrile) were reconstituted in MilliQ water, and applied on a semi-preparative reverse phase (RP) C18 column (10 × 250 mm, Vydac TM) equilibrated with 0.12% TFA (v/v) (solution A) and eluted with acetonitrile containing 0.10% TFA (v/v) (solution B). Elution was performed with a triphasic acetonitrile gradient (0–30% solution B for 15 min, 30–50% solution B for 20 min and 50–100% solution B for 15 min), at a flow rate of 2 ml/min. Active subfractions (see below) were further loaded onto an analytical C18 column (4.6 × 250 mm, Vydac TM) equilibrated with solution A, and eluted with a triphasic solution B gradient (0–25% for 12.5 min, 25–40% for 22.5 min and 40–100% for 15 min), at a flow rate of 1 ml/min. Final peptide purifications were performed on the same analytical C18 column, equilibrated with 15% solution B and eluted with a linear 15–40% gradient of solution B for 45 min, at a flow rate of 0.75 ml/min. All experiments were carried out using a Waters HPLC system model 600E coupled with a tuneable absorbance detector model 486. The column effluent was monitored by absorbance at 230 nm.

Antibacterial and haemolytic assays

Two Gram-positive (*B. subtilis* and *Staphylococcus aureus* ATCC 25923) and two Gram-negative (*E. coli* DH5α and *Klebsiella pneumoniae* ATCC 13883) strains were used for antibacterial assays. The HCll extract and fractions from Sep-Pak and RP-HPLC steps were qualitatively proven according to the classical growth inhibition assay in solid medium [43]. Briefly, circa 10 µg of total proteins resuspended in 10 µl of sterile ultra-pure water were placed in dishes over plates of Luria-Bertiani medium with agar containing 5×10^5 bacteria. Antibacterial activity was visualized as clear zones (no bacterial growth) after overnight incubation at 37 °C.

To determine minimal inhibitory concentration (MIC) values, pure peptides and reconstituted HPLC subfractions were tested in a liquid growth inhibition assay following an adapted microdilution protocol of the National Committee of Clinical and Laboratory Standards [44] performed in polypropylene plates with 96 wells (Corning). Briefly, 50 µl of sample (50 µg/ml final concentration) was mixed with 50 µl of Mueller-Hinton medium (Becton Dickinson), serial dilutions were performed and each well was inoculated with 5 µl of saline buffer containing 5×10^4 bacteria. The plates were incubated overnight at 37 °C.

Haemolytic activity was assayed as described in Torres-Larios et al. [45]. Freshly collected human blood was washed several times and aliquots of resuspended cells in phosphate buffer saline (PBS) were incubated with a final concentration of 100 µg/ml of total protein from HCll-II subfractions or pure peptides. Haemolysis was estimated by absorbance at 541 nm of the supernatant after 1 h incubation at 37 °C. Positive and negative controls were obtained using 1% Triton X-100 (v/v) and cells without treatment kept in the appropriate buffer, respectively.

Structural characterization

Amino acid composition of the native peptide was obtained after acid hydrolysis using a Beckman 6300 analyser, essentially as previously described [45]. Sequence analysis was performed using a Beckman L300 instrument on in situ alkylated native peptide samples (propionamide-cysteine derivatives obtained as described by the manufacturer) or a Procise 491 instrument (Applied Biosystems) on peptides generated from enzymatic hydrolysis of carboxamidomethylated or native species. Cystine-containing peptides were analysed according to Haniu et al. [46]. Mass analysis of native peptide or enzymatic digests was obtained using a Thermo Finnigan LCQ DUO electrospray ionisation-ion trap (ESI-IT) mass spectrometer or a MALDI-TOF mass spectrometer, as reported below.

Purified peptide samples were eventually treated with 10 mM dithiothreitol in 0.25 M Tris-HCl, pH 8, for 45 min at 55 °C, before alkylation, or directly alkylated with 55 mM iodoacetamide in 0.25 M Tris-HCl, 1.25 mM EDTA, 6 M guanidinium chloride, pH 7, for 30 min at 25 °C. Peptide products were freed from salt and reagent excess by passing the reaction mixture through an analytical C18 column (4.6 × 250 mm, Vydac TM), as previously reported [47], manually collected and dried for further characterization. Native or carboxamidomethylated peptide samples were digested with trypsin in 50 mM ammonium bicarbonate, pH 6.5, overnight. Tryptic digests were directly analysed by MALDI-TOF MS or resolved on a microbore C18 column (1 × 250 mm, Vydac TM) equilibrated with 5% solution B and eluted with a linear 5–40% gradient of solution B for 45 min, at a flow rate of 0.06 ml/min. Peptide fractions were manually collected for further characterization.

Differential MALDI-TOF MS analysis

Samples of cell-free haemolymph and the HCll-II fraction were obtained as described above and used for MS analysis following the addition of 200 pmol fragment

(1–17) from adreonocorticotropic hormone (ACTH), used as internal standard. The analysis of cell-free haemolymph (30 µl) was conducted on six independent samples, each obtained from a single individual. Three control (sterile injured) and three 24-h post-infection samples were investigated, all presenting a similar protein content as determined with the Bio-Rad Protein Assay. These samples were filtered through sterilized 0.45-µm filters before mass spectrometric analysis. The HCll-II fractions were obtained from a pool of haemolymph from about 30 scorpions in each sample (three control and three infected), normalized to contain the same amount of protein.

Differential MALDI-TOF MS analyses were performed using a Voyager-DE PRO mass spectrometer (Applied Biosystems). Samples (1 µl) were loaded on the instrument target, using the dried-droplet technique and α-cyano-4-hydroxycinnamic acid [5 mg/ml in 50% acetonitrile, 5% formic acid (v/v)] as matrix, as previously reported [47]. Spectra were acquired either in reflectron or linear mode with delayed extraction. Spectra were calibrated either by external or internal calibration using the molecular ions from angiotensin I, ACTH (18–39), ACTH (7–38) and bovine insulin. Data are reported as average masses.

Genomic and cDNA cloning

To prevent excessive cuticle debris and to avoid venom gland contamination, total RNA was obtained only from the first six tergites of the scorpion opisthosoma. Total RNA was isolated from infected *C. limpidus limpidus* scorpions (females of same weight), after 12 h of infection, using the Promega TotalRNA isolation system. First-strand synthesis was achieved with SuperScriptII Moloney murine leukaemia virus (M-MLV) RT (Gibco-BRL) in the presence of RNase inhibitor (Roche Diagnostics), using poly$(T)_{22}$NN as primer. The PCR was performed with Vent DNA polymerase (New England Biolabs), using first-strand cDNA as template and 25-mer degenerated oligonucleotide (5′-GCNTGYCARTTYTG-GWSYTGYAAY-3′) and poly$(T)_{22}$NN as primers. The cloning and sequencing were obtained using PCR products purified in a Centricon 100 column (Millipore), following the manufacturer's instructions and then ligated into the *Eco*RV site of the pKS plasmid (Stratagene). This construct was used to transform *E. coli* DH5α cells. Plasmid DNA was isolated from clones carrying the insert and sequenced from both strands in an Applied Biosystems 3100 apparatus, as described by the manufacturer. The 5′ cDNA sequence was obtained by the rapid amplification of cDNA ends (RACE) method using 5′RACE system version 2.0 (Invitrogen). Briefly, first-strand cDNA was synthesized from total RNA using a gene-specific primer (5′-TTATTGACACTGGCAATATTT-3′) and M-MLV RT. A homopolymeric tail was then added to the 3′ end of the cDNA using TdT and dCTP. PCR amplification was accomplished using Taq DNA polymerase, an anchor oligonucleotide provided in the kit as sense primer and a nested gene-specific antisense primer (5′-TTTAT-ACTGTATTCCCCAGCAATA-3′). The 5′RACE products were cloned into an appropriate vector for subsequent characterization as described above.

The genomic DNA was obtained from scorpion females with the DNeasy mini kit (Qiagen). Two specific oligonucleotides were used as primers for PCR amplification (sense, 5′-ATGAAAGCAATCGTTGTTCTT-3′; antisense, 5′-GGCAATATTTATACTGTATTC-3′), using Taq DNA polymerase. Products visualized in agarose gel electrophoresis were purified with a QIAquick gel extraction kit (Qiagen) and cloned into the pGEM vector (Promega). Sequencing was determined as described above.

Semiquantitative RT-PCR analysis

Gene expression was analysed by RT-PCR. Total RNA extraction and first-strand cDNA synthesis from two naïve and two bacteria-challenged scorpions were performed as described above, except that random decamers (Ambion) were used as primers. Equal amounts of each cDNA were used in the subsequent PCR, carried out with Taq DNA polymerase and two specific primers (sense, 5′-CATGACAACCGTGGAAGG-3′; antisense, 5′-TTATT GACACTGGCAATATTT-3′), which resulted in an expected 117-base pair product. The primers for the housekeeping gene of 18S rRNA were used according to the QuantumRNA Universal 18S Internal Standards Kit (Ambion). PCR samples were performed for 26, 29, 32 and 35 cycles; the products stained with ethidium bromide were analysed in 2% agarose gel electrophoresis.

Similarity searches and sequence analysis

The sequences obtained in this work were deposited at SWISS-PROT (P83738) and GenBank (AY520534) databases. Similarity searches were performed using BLAST (www.ncbi.nlm.nih.gov/BLAST) and FASTA 3 (www.ebi.ac.uk/fasta33) against non-redundant databases, using BLOSUM 62, PAM 70 or PAM 120 weight matrices. Sequence alignments were performed with CLUSTAL_X [48]. The signal peptide was predicted using the SignalP server (www.cbs.dtu.dk/services/SignalP).

Results

Antibacterial activity in fractions of *C. limpidus limpidus* haemolymph

Earlier work conducted with haemolymph of two Old World scorpions, *Leiurus quinquestriatus hebraeus* [34] and *Androctonus australis* [35] showed the presence of constitutive antibacterial peptides. This motivated our re-

search with haemolymph of the New World species *C. limpidus limpidus*. At the starting point of this work, 500 naive individuals were used for haemolymph extraction and analysis. The animals were kept and fed in captivity for several months. Five groups of individuals were separated containing 50–250 individuals each. The haemolymph from each group was treated independently, as reported in Materials and methods. Each fraction from the C18 cartridge extraction was tested for antibacterial activity, using a solid-medium assay [43]. In all cases, only the HCll-II fraction contained antibacterial activity. These fractions were further separated on a semi-preparative RP-HPLC column, from which three positive subfractions were found and called HCll-II.10, -II.11 and -II.12 (fig. 1, table 1). The chromatographic profiles obtained with haemolymph of the different scorpion groups was quite reproducible, except for the subfaction labelled with an asterisk in figure 1 (subfraction II.11). The relative concentration of this subfraction was not constant when compared with various haemolymph samples from other scorpion groups (data not shown).

Components of *C. limpidus limpidus* haemolymph involved in the septic injury response

The unexpected finding that the subfraction indicated with an asterisk in figure 1 was variable within different haemolymph batches prompted us to examine if these differences could be due to extrinsic factors, rather than to

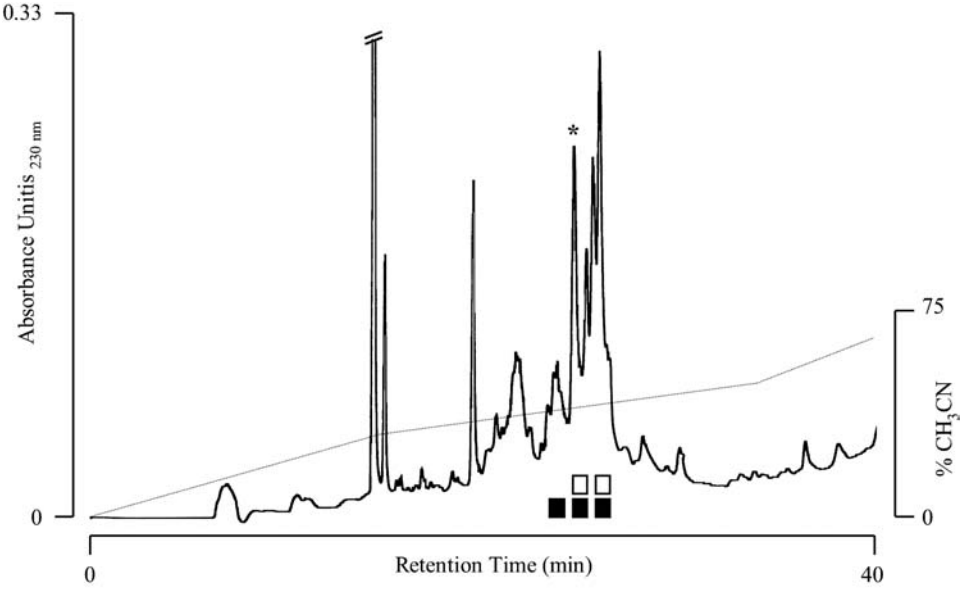

Figure 1. Chromatographic separation of HCll-II. One milligram of total protein from the HCll-II fraction was loaded on a C18 semi-preparative column and eluted with the acetonitrile gradient displayed (broken line). Squares under the chromatogram profile indicate subfractions with antibacterial activity in a solid-medium assay against *E. coli* (empty) or *B. subtilis* (closed). Asterisk indicates a variable subfraction called HCll-II.11 (see text for details).

Table 1. Antibacterial assays.

	E. coli		*K. pneumoniae*		*B. subtilis*		*S. aureus*	
	solid	liquid	solid	liquid	solid	liquid	solid	liquid
HCll-II	+	NT	+	NT	+	NT	+	NT
Cll-dlp	–	> 50	–	> 50	–	> 50	–	> 50
HCll-II.12a	+	50–25	NT	NT	+	NT	NT	> 50
HCll-II.12b	+	50–25	NT	NT	+	NT	NT	> 50
12a + 12b	+	50–25	NT	NT	+	NT	NT	NT
Cll-dlp + 12a + 12b	NT	20*	NT	20*	+	20*	NT	20*

Solid-medium assay [43]: +, presence of inhibition zones; –, no inhibition.
Liquid growth inhibition assay [44], concentrations are given in µg/ml: MICs are expressed as intervals between the minimum concentration at which no growth was observed and the maximum at which bacterial growth was still present.
NT, not tested.
* No serial dilutions were performed.

constitutive determinants. The idea was that a mechanism of innate defence could be associated with these variations, depending on the environmental conditions used to maintain and feed the scorpions. To test this hypothesis, we selected three independent groups of 350 female scorpions of the same weight (around 1 g), kept starved for 2 weeks to avoid feeding interferences, and injected with the bacteria described in Materials and methods. Haemolymph collected from control and test groups was analysed using the same protocol described above. Samples were separated by a stepwise elution on the C18 cartridge and the respective HCll-II fractions (normalized to contain 1 mg of total protein) were loaded onto a C18 semi-preparative column. The results indicated that the animals challenged with bacteria injections showed a higher relative content of the subfraction eluting at 24 min (data not shown). This increment was visible from 6 to 24 h after injection, but returned to the basal level after 48 h. The highest value was obtained at 24 h after injection. This suggested that septic injury could elicit an increment of the relative abundance of this subfraction in the haemolymph. The remaining components showed comparable relative concentrations, independent of the treatment applied. The control groups did not show the inducible increment of this specific subfaction in the same time intervals. These experiments were performed on three independent groups, with duplicate analysis of each.

The corresponding subfractions of HCll-II from the control and bacterial-challenged group of scorpions were separated by analytical RP-HPLC. The chromatographic profile of the injured animals showed comparable subcomponents, except for the one shown with an asterisk in figure 2A, which increased from 6 to 12 and to 24 h after infection (highest level), decreasing to normal levels at 48 h after injury. This component was purified to homogeneity after a third RP-HPLC step (inset in fig. 2A), and its molecular mass was estimated by ESI-IT MS, showing a monoisotopic signal at 3816.5 atomic mass units. This peptide corresponded to 0.018% of the total protein content for haemolymph extracts of challenged scorpions. On the basis of our chromatographic and MS determinations we estimated that induction by septic injury would double or triple the relative concentration of this component in the circulating haemolymph. To proceed with its chemical characterization, the haemolymph of 1000 infected scorpions was obtained and the corresponding peptide isolated as described above. About 180 µg was used for final characterization. This peptide was named Cll-dlp, meaning *C. limpidus limpidus* defensin-like peptide.

Amino acid sequence and disulphide bridges of Cll-dlp

The polypeptide sequence was obtained by direct Edman degradation of the native species (first 31 amino acids) and mass mapping measurement and sequencing of overlapping fragments generated following tryptic digestion of a reduced and alkylated peptide sample (fig. 2C). The sequence obtained is consistent with the amino acid composition determined by acid hydrolysis (data not shown). The peptide presented six cysteine residues, all involved in disulphide bridges, as determined by ESI-IT MS and MALDI-TOF MS analysis of native and carboxamidomethylated peptide samples, following alkylation in reducing or non-reducing denaturing conditions (data not shown).

Cll-dlp disulphide bridge pairing was investigated by a combined mass spectrometric-Edman degradation approach. Native Cll-dlp was digested with trypsin and the resulting peptide mixture was directly analysed by MALDI-TOF MS, producing the spectrum shown in figure 2B. A series of signals were assigned to S-S-bridged peptides on the basis of their unique mass values and their disappearance following incubation with dithiothreitol. The signal at 3475.6 m/z was associated with a three-peptide cluster involving fragments (1–14), (18–27) and (28–32) (theoretical value: 3475.9 m/z) linked by three disulphides. The remaining peaks were assigned to S-S-containing peptides originating from the mentioned above one, following non-specific hydrolysis at Trp5. In fact, the signal at 1897.6 m/z was interpreted as arising from peptides (1–5) and (18–27) linked by the disulphide Cys2-Cys21 (theoretical value: 1897.4 m/z). Similarly, the signal at 1595.7 m/z was associated with a complementary peptide cluster involving fragments (6–14) and (28–32) (theoretical value: 1596.1 m/z) linked by two disulphides. The final S-S assignment was obtained by direct Edman degradation of these latter Cys-containing peptides, following chromatographic purification and identification of the PTH-cystine at the expected degradation cycles [46]. This analysis led to the expected sequence in the case of the component with $(M+H)^+$ at 1897.6 m/z, with a straightforward identification of the disulphide Cys2-Cys21. Similarly, the peptide at 1595.7 m/z, associated with the peptide cluster (6–14) + (28–32) showed the presence of PTH-cystine at the second and the sixth cycle during Edman degradation, thus demonstrating the occurrence of the disulphides Cys7-Cys29 and Cys11-Cys31. On the basis of these results, the determined Cll-dlp disulphide pairing (fig. 2C) was equivalent to that already reported for other invertebrate defensins (CS-$\alpha\beta$ motif signature pairing). Moreover, BLAST and FASTA 3 searches grouped the sequence of Cll-dlp with other invertebrate defensins and CS-$\alpha\beta$ motif-containing peptides (see below).

Cll-dlp systemic liberation in response to septic injury

To present an additional semi-quantitave measurement of this phenomenon, haemolymph samples from control animals and septic-injured scorpions were also analysed by

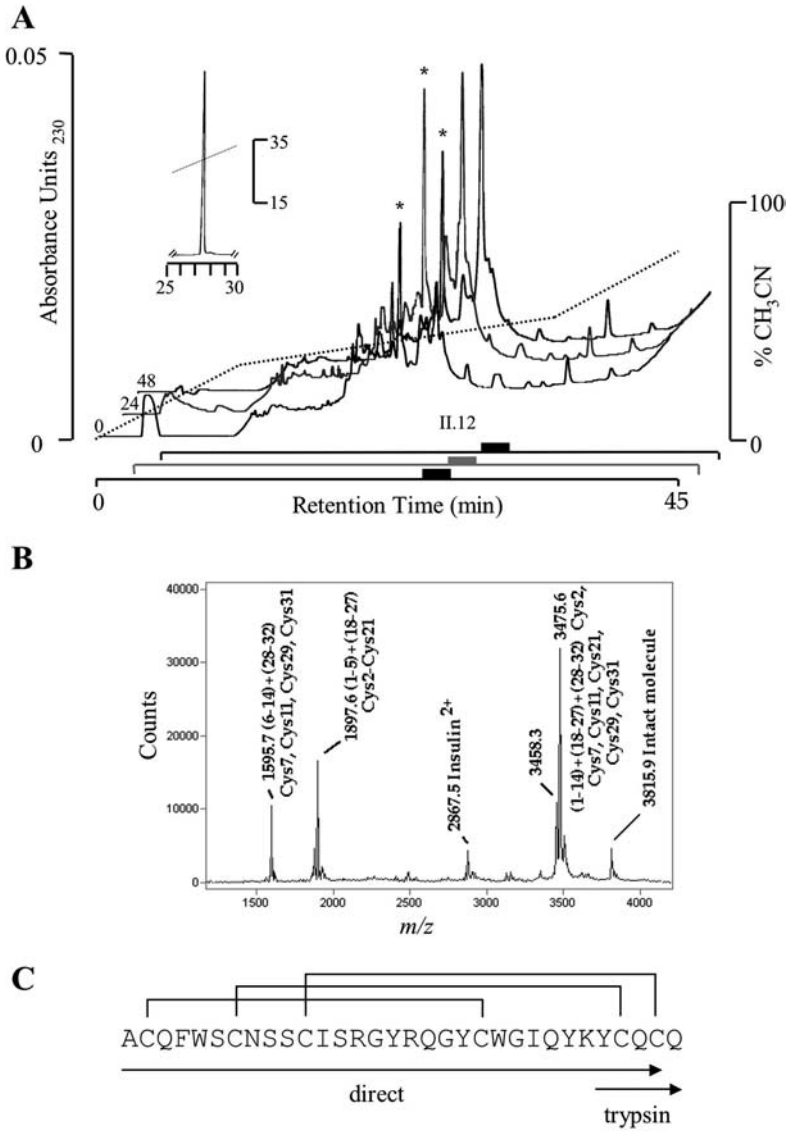

Figure 2. Purification and characterization of Cll-dlp. (*A*) The variable subfraction (HCll-II.11) in semi-preparative HPLC from naive (0) and bacterial-challenged scorpions after 24 (24) and 48 (48) h were further fractionated on an analytical C18 column eluted with the acetonitrile gradient displayed (broken line). The retention times over which the components HCll-II.12a and HCll-II.12b elute are indicated by solid rectangles. The inset shows the final purification of Cll-dlp from pooled peaks indicated with an asterisk in the main figure. (*B*) MALDI-TOF mass spectrum of native Cll-dlp following tryptic hydrolysis. Signals corresponding to the disulphide-bridged peptides are reported. Each signal was assigned to the corresponding peptide pair or triplet on the basis of its mass values, peptide sequence and enzyme specificity. The cysteine residues involved in S-S bonds are indicated. (*C*) Amino acid sequence of Cll-dlp as determined by Edman degradation and MS analysis. The fragments considered to assemble the full sequence are indicated below. Disulphide pairing is also displayed. The Cll-dlp sequence is in the SWISSPROT database under accession number P83738.

MALDI-TOF MS procedures (fig. 3). Two kinds of samples were used in these experiments: cell-free haemolymph extracts (fig. 3A) and fraction HCll-II (fig. 3B). Consistently, the MS spectra of cell-free haemolymph showed the same set of components in samples from both control and bacterial-challenged groups; however, a clear induction of at least four components with a molecular mass ranging from 3100 to 4100 m/z was registered in experimentally infected scorpions (compare lower and upper panels in fig. 3A). As a result of a stepwise purification, fractions HCll-II from control and septic-injured animals seemed to be enriched in the component with the molecular mass at 3816.9 m/z. This species showed a clear apparent increase in samples from septic-injured scorpions (fig. 3B). The weaker signals occurring in the spectra were not considered to be statistically significant (experiments done in triplicate). Important to note is that the component Cll-dlp with an $(M+H)^+$ signal at 3816.9 m/z was the most conspicuously inducible.

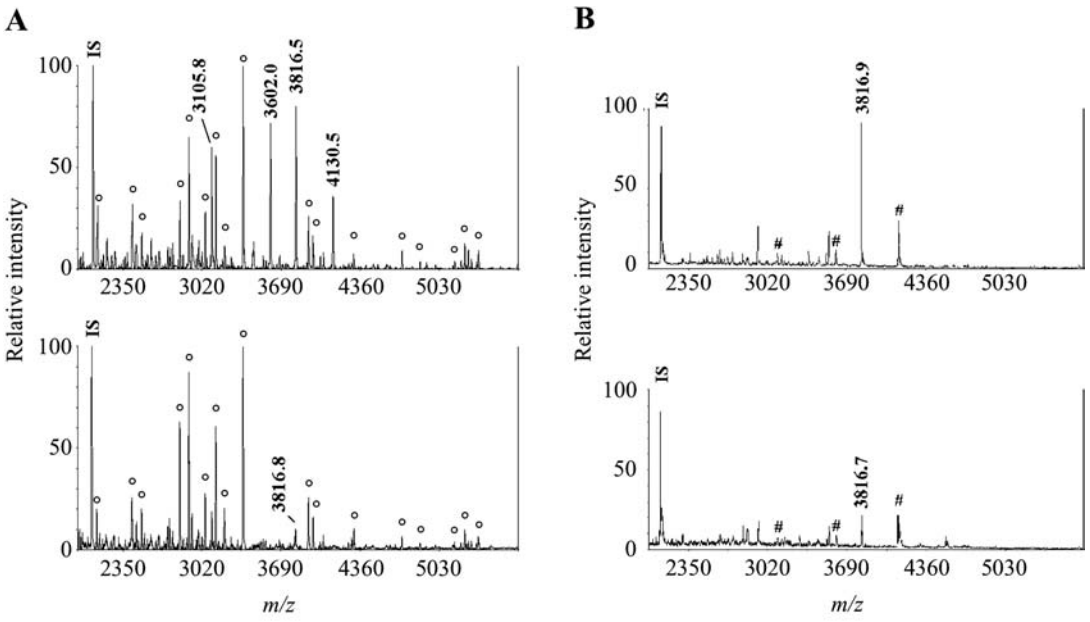

Figure 3. Differential MALDI-TOF MS analysis. (*A*) The cell-free haemolymph was collected from a single-bacterial challenged scorpion (upper panel) or sterile injured (lower panel), to which with the ACTH (1–17) internal standard was added and analysed as reported in Materials and methods. Circles indicate molecular components common to both samples, whereas indicated m/z values correspond to the signals variable between both kinds of samples. (*B*) The fraction HCll-II was collected from the stepwise purification of challenged (upper panel) or sterile-injured (lower panel) scorpions, to which with the internal standard was added, and analysed as mentioned above. Indicated with # are the peptides with $(M+H)^+$ at 3105.8, 3602.0 and 4130.5 m/z, already reported in (*A*). All analyses were performed in duplicate for three independent sterile-injured or bacterial-challenged scorpion samples; all samples were prepared in parallel under the same experimental conditions. IS means the internal standard.

Cll-dlp gene structure and mRNA expression after septic injury

The apparent increase in Cll-dlp in response to septic injury could be associated with its de novo synthesis (as in some insects, nematodes and arachnids [4, 5, 25–27, 36–40]), or to the release of pre-synthesized and stored peptide in special reservoirs, which could liberate the peptide upon stimulation (as in mussels, shrimps and some chelicerates [3, 28–32]). To solve this question, we cloned the gene from cDNA and studied the expression of mRNA in response to septic injury.

The cDNA sequence encoding mature Cll-dlp was obtained by classical 3′ and 5′RACE protocols. This clone consisted of 363 base pairs (bp), comprising 61 at the 5′ untranslated region, a putative 24-residue-long signal peptide, the full mature sequence for Cll-dlp followed by a stop codon and 131 bp at the 3′ untranslated region, including a putative polyadenylation signal (fig. 4). The genomic sequence was determined by PCR and cloning. The sequence overlaps with cDNA from position –72 to position +88. This clone included a single phase I intron of 128 bp with canonical splicing sites splitting an Ala codon within the putative signal peptide region (fig. 4). Determination of the nucleotide sequence provided the information needed to synthesize the appropriate oligonucleotides for mRNA expression analysis. RT-PCR assays were performed with mRNA isolated from naive and bacterial-challenged scorpions (10 h post-infection), using specific primers for Cll-dlp and the housekeeping rRNA of ribosomal subunit 18S (see Materials and methods). No significant differences in relative abundance of the Cll-dlp transcript were observed between these two conditions (data not shown).

Cooperative antibacterial effect of Cll-dlp

The HCll-II subfraction from which Cll-dlp was isolated (asterisk in fig. 1), showed antibacterial activity against *E. coli* and *B. subtilis* in solid-medium qualitative assays (table 1). However, for the pure peptide Cll-dlp, only marginal activity was observed at relatively high concentrations (maximum concentration tested was 70 µg/ml, due to scarcity of sample). Nevertheless, almost one-half of the Cll-dlp-containing HCll-II subfraction consisted of two other major components: peptides HCll-II.12a and HCll-II.12b, which coelute in the HPLC conditions used (labelled HCll-II.12 in fig. 2A). These two peptides were isolated in homogeneous form after a third HPLC step and are now being analysed. These components were tested for their antibacterial activity: the MIC values against *E. coli* were 50–25 µg/ml (table 1). When a mixture of HCll-II.12a and HCll-II.12b, at a ratio of 1:1, was prepared, the measured MIC was practically the same. Thus, apparently no cooperative antibacterial effect existed for these peptide species. This situation was quite different when a pep-

Figure 4. Nucleotide sequences encoding Cll-dlp. The cDNA encoding Cll-dlp was obtained with standard 3′ and 5′RACE protocols, molecular cloning and sequencing. Specific primers flanking the cDNA of the mature region (indicated by forward and reverse arrows) were used to amplify a partial genomic clone. Asterisks above the sequence indicate the overlap between the cDNA and genomic clones. Exons are in uppercase letters. Splice donor and acceptor sites are grey shaded. The polyadenylation signal is double underlined. The translated amino acid sequence is below the nucleotide sequence and the putative signal peptide is on italics. Numbers on the right correspond to the full cDNA clone taking the first nucleotide of mature sequence as position +1. The sequence from the cDNA clone is available in the GenBank database under accession number AY520534.

tide mixture was prepared including Cll-dlp. Equal amounts of pure components (Cll-dlp, HCll-II.12a and HCll-II.12b) were mixed and assayed in a liquid growth inhibition assay. The rationale for choosing this proportion of components was based on the fact that it resembles the one present in the semi-preparative HPLC subfraction from which the Cll-dlp was obtained. When a total peptide content of 20 µg/ml was tested, the *E. coli* growth was null. We extended these results by testing the same mixture with other bacterial strains and found that this preparation was active at the same concentration against *B. subtilis*, *K. pneumoniae* and *S. aureus*. Thus, an apparently cooperative antibacterial activity existed between constitutive (HCll-II.12a and 12b peptides) and inducible (Cll-dlp) haemolymph components.

Because other AMPs isolated from scorpion [41] have been reported to have an intrinsic haemolytic activity, we needed to verify if any of the peptides we isolated could have similar activities on erythrocytes. The effect of all the fractions purified, including the cell-free haemolymph, did not show any haemolytic activity in human erythrocytes, assayed in the conditions described in Materials and methods.

Discussion

As described in the Materials and methods and results, this work required the use of several thousand scorpions. The handling of live dangerous animals to obtain the haemolymph was not a trivial task. Nevertheless, the purification of the haemolymph soluble peptide fractions was relatively simple, comprising a centrifugation followed by a reverse-phase extraction step on Sep-Pack cartridges and a couple of HPLC separations. The complete covalent structure of Cll-dlp was successfully obtained. Regarding its biological activity, Cll-dlp seemed to be a poor antibacterial agent; however, we were able to demonstrate a cooperative effect between this peptide and the constitutive components HCll-II.12a and 12b. After incubation with the mixture, no bacterial growth was recovered for four bacterial strains (table 1). This situation is similar to that already reported for mammalian antibacterial peptides (some of which are immune inducible) and lysozyme (which is constitutive) [49]. Cll-dlp and the other peptides shown to be present in subfraction HCll-II.11 (fig.1, labelled with asterisk) were also assayed for possible membrane lytic activity on hu-

man erythrocytes and ovarian insect cells obtained from *Spodoptera frugiperda* (cell line Sf9); in neither cases was disruptive activity recorded (data not shown). Taking together these results suggest that Cll-dlp is preferentially active against bacterial cells, but whether Cll-dlp acts like other invertebrate defensins, which display a membrane lytic activity [50–52], remains unclear. Further experiments are needed to explore if Cll-dlp follows a similar mode of action.

Comparison of the amino acid sequence of Cll-dlp with known invertebrate defensins

The Cll-dlp sequence resembles the known scaffold of the invertebrate defensins and most scorpion toxins [17]. Several authors have proposed a close relationship between the primary structure of some scorpion toxins and invertebrate defensins [53, 54]. The results of BLAST and FASTA 3 searches grouped the Cll-dlp sequence with both kinds of peptide, although with low scores. When all available sequences of short chain scorpion toxins (121 to date) and invertebrate defensins (a total of 60) were taken into consideration for analysis, the alignment obtained with CLUSTAL_X [48] confidently clustered the Cll-dlp sequence within defensins, whereas all the scorpion-venom-derived peptides were grouped with toxins (not shown). In figure 5, the Cll-dlp sequence is aligned with other invertebrate defensins; this figure clearly shows that the only fully conserved residues are cysteines. Two main defensin groups can be identified on the basis of this alignment. The largest one includes defensins from different orders of the subclass Neoptera (Diptera, Coleoptera, Hemiptera and Hymenoptera), although the defensins from lepidoterans are more diverse. A second group, often referred to as ancestral, contains defensins from phylogenetically distant invertebrates (mussels, arachnids and the dragonfly *Aeschnea cyanea*) [1, 15, 20, 21, 34–36, 39]. This analysis reveals that the Cll-dlp sequence is quite distant from both groups, but remains closer to the defensins than to the scorpion toxins.

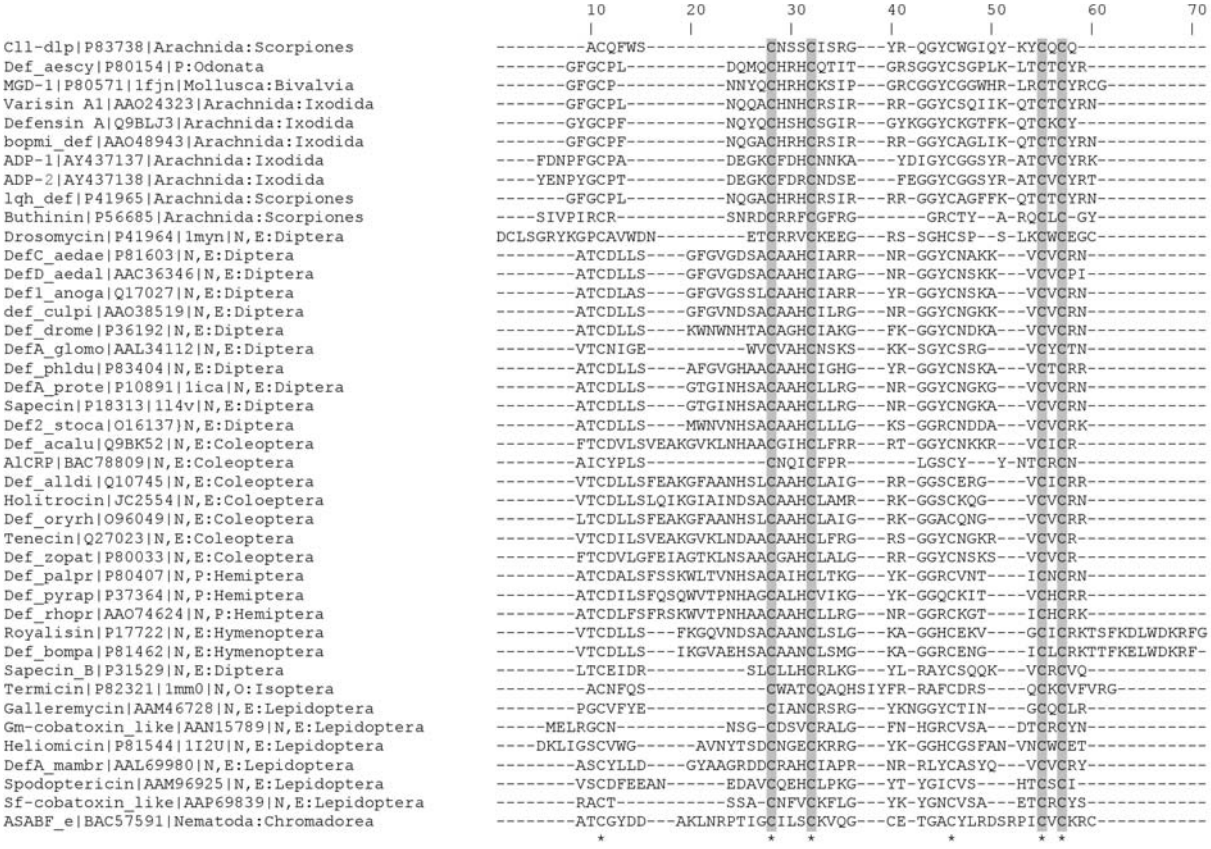

Figure 5. Multiple sequence alignment of some invertebrate defensins. Amino acid sequences of invertebrate defensins were retrieved from public databases and aligned with CLUSTAL_X [48]. Fully conserved amino acid residues are indicated by asterisks (*) below the alignment. Cys residues of the CS-$\alpha\beta$ motif signature sequence are grey shaded. Names, accession numbers and sources are indicated in the left column. In defensins from the class Insecta, the subclass (N, Neoptera; P, Palaeoptera), the infraclass (E, Endopterygota; O, Orthopteroidea; P, Paraneoptera) and the order are indicated. In defensins from other arthropods, only the class and order are indicated. For defensins from organisms of other phyla (molluscs and nematodes), the phylum and the class are indicated. Vertical lines on the right indicate the two main groups of invertebrate defensins: ancestral and classical.

Genomic organization of the Cll-dlp gene and the evolution of the CS-$\alpha\beta$ structural family

The structure of the gene coding for Cll-dlp, found in this work, seems similar to those of scorpion toxins. There is a signal peptide, interrupted by a short intron of variable length, followed by the sequence coding for the mature peptide and ending with a stop codon. Our results are consistent with the suggestion given by Froy and Gurevitz [55] for the gene encoding a defensin obtained from the North African scorpion *L. quinquestriatus hebraeus*, although these authors did not report any sequence or give any database reference useful to compare experimental results.

When comparing scorpion toxins and defensins with the CS-$\alpha\beta$ motif, due to their low sequence similarity (only cysteines were conserved at identical positions), it is difficult to make any valuable evolutionary predictions about the two types of peptides. However, taking into account the Cll-dlp gene organization and the common conservation of the CS-$\alpha\beta$ signature among the two peptide groups, there does seem to be an evolutionary relationship between these two classes of peptide. The very wide phylogenetic distribution of defensins suggests that they might be the ancestors of the CS-$\alpha\beta$ motif-containing peptide family.

Defensin gene organization is highly variable both at the genomic and transcript levels. Apart from scorpion defensins, all the other cloned transcripts for defensins contain a pro-sequence [55, 56]. The pro-segment of the sequence in other arthropods is situated at the N-terminal side of the mature peptide, whereas in molluscs and nematodes the pro-segment is at the C-terminal side. Unfortunately, there is a reduced number of known genomic clones available for defensins, and yet they show great variability [55–58]. The tick defensin genes contain two introns, one in the signal peptide, the other at the pro-sequence; mussels have a single intron interrupting the signal peptide, and for some dipterans and lepidopterans an intron is situated at the pro-sequence. For *Drosophila* and other insects there are no introns. Such variations are hard to reconcile with a divergent evolutionary process, although they could be achieved as a result of exon-shuffling of the mature region, as recently proposed by Froy and Gurevitz [55].

Inducible liberation of Cll-dlp in the context of invertebrate immune systems

Two previous reports on scorpion defensins [34, 35] showed that there is no difference in the level of these peptides when comparing the control with challenged animals. In both cases, the time elapsed between infection and haemolymph extraction was 1 week. These results could be contradictory to our report here. However, in our opinion this is not the case because different time intervals were used for the experiments. We performed a kinetic investigation with shorter time intervals. At 24 h, the amount of Cll-dlp was maximal, and this value decreased after 48 h (figs. 2 A, 3). Thus, if measured 1 week later we would expect to find the same basal peptide level, as already reported for the other scorpion defensins. Our results on the relative amount of Cll-ldp transcripts (mRNA measured by RT-PCR) also support the idea that this peptide is not synthesized in response to septic injury, but is, rather, liberated from a still uncharacterized cell reservoir. In this way, the systemic accumulation of Cll-dlp is similar to that already reported for AMPs from horseshoe crab [3], mussels [32], shrimps [30, 31] and spiders [28, 29]. The phylogenetic variety of organisms whose innate immune responses depend on the systemic release of AMPs, independently of transcriptional regulation, suggests that this strategy is ancestral to that followed by recent insect orders [3, 5, 32]. In the latter, the AMPs are often transcriptionally inducible. However, there are other arachnids of the order Ixodida [36–39] as well as the distant nematodes Chromadorea [40], in which the response is also via transcriptional activation of AMP genes. Thus, the documented cases show two distinct strategies for immune activation in invertebrates, on the one hand, the liberation of AMPs, prestored in haemocytes, and on the other, the transcriptional activation of AMP genes. However, still not clear is whether an evolutionary relationship exists between the two strategies. Additional studies with phylogenetically diverse organisms are needed before a better and clearer idea of the evolutionary history of innate immune systems can be fully comprehended.

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