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Fat body and hemocyte contribution to the antimicrobial peptide synthesis in *Calliphora vicina* R.-D. (Diptera: Calliphoridae) larvae

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Abstract Antimicrobial peptides accumulated in the hemolymph in response to infection are a key element of insect innate immunity. The involvement of the fat body and hemocytes in the antimicrobial peptide synthesis is widely acknowledged, although release of the peptides present in the hemolymph from the immune cells was not directly verified so far. Here, we studied the presence of antimicrobial peptides in the culture medium of fat body cells and hemocytes isolated from the blue blowfly *Calliphora vicina* using complex of liquid chromatography, mass spectrometry, and antimicrobial activity assays. Both fat body and hemocytes are shown to synthesize and release to culture medium defensin, cecropin, diptericins, and proline-rich peptides. The spectra of peptide antibiotics released by the fat body and hemocytes partially overlap. Thus, the results suggest that insect fat body and blood cells are capable of releasing mature antimicrobial peptides to the hemolymph. It is notable that the data obtained demonstrate dramatic difference in the functioning of insect antimicrobial peptides and their mammalian counterparts localized into blood cells' phagosomes where they exert their antibacterial activity.

Keywords Insect immunity · Antimicrobial peptides · Cell cultures · Fat body · Hemocytes

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Introduction

Peptide antibiotics are widely used in insects as well as other living organisms to fight microbial pathogens (see reviews Kokryakov 1999; Bulet and Stöcklin 2005). Blowflies from Calliphoridae family are particularly rich in antimicrobial peptides, which either freely circulate in the hemolymph (Dimarcq et al. 1988; Chernysh et al. 2000; Cerovský et al. 2010; Chernysh and Gordja 2011) or are associated with barrier epithelia (Cerovský et al. 2010; Kruglikova and Chernysh 2011).

The synthesis of antimicrobial peptides in flies by its nature is inducible: Normally low concentration in the hemolymph increases abruptly in case of damage. Surface structures of pathogens serve as an additional trigger of the immune response: A signal of invasion is transmitted to immunocompetent cells and invokes the expression of antimicrobial genes (Hoffmann and Reicchart 1997). The antimicrobial activity of the hemolymph is already enhanced noticeably within several hours following infestation (Hultmark 1993) and reaches its maximum about 24 h after the infestation event (Trenczec and Faye 1988).

The primary source of antimicrobial peptides in insect blood is reported to be the fat body, and hemocytes seem to be the second producer, which synthesizes peptide antibiotics and releases them into the hemolymph after integument damage or microbial invasion (Dimarcq et al. 1997; Christophides et al. 2002; Bartholomay et al. 2004; Irving et al. 2005; Johansson et al. 2005). This conclusion is supported mainly by detection of antimicrobial peptides' messenger RNA (mRNA) in fat body cells and hemocytes but not direct demonstration of the peptide secretion to the hemolymph. Release of unidentified antibacterial compounds from fat body cells' culture medium was directly demonstrated only in lepidopteran *Hyalophora cecropia* (Faye and Wyatt 1980). Similarly,

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embryonic cells of the flesh fly *Sarcophaga peregrina* were found to release antimicrobial peptides called sapecins (Yamada and Natori 1993). To our knowledge, there are no data in the literature demonstrating antimicrobial peptide secretion by insect hemocytes. Thus, the fact that fat body cells and hemocytes release mature antimicrobial peptides in the course of insect immune response needs experimental verification. Cell culture approach is indispensable from that standpoint.

The aim of this work was to study release of structurally characterized antimicrobial peptides by fat body cells and hemocytes of blowfly *Calliphora vicina* larvae using cell culture approach. The cellular composition of *C. vicina* hemolymph (Kind 2003, 2007, 2012; Tulin and Chaga 2003) as well as structure and in vivo production of antimicrobial peptides in the course of immune response (Chernysh et al. 2000, 2015; Chernysh and Gordja 2011) have been previously reported.

Materials and Methods

Insects The work was carried out on diapausing *C. vicina* larvae (originating from St. Petersburg, Russia) which had ceased feeding 2 wk before the experiments. Larval diapause was induced by keeping parental adults under short-day conditions (12L/12D) at a temperature of +20°C and rearing the hatched larvae in darkness with temperature decreasing from +20°C on the first d of their life to 12°C throughout the feeding period and then to +6°C during the period of expulsion of gut contents. Diapausing larvae were kept at +2°C.

Hemolymph collection and separation Prior to hemolymph collection, the larval body surface was sterilized with 70% ethanol, rinsed in distilled water, and lightly dried on filter paper. Hemolymph was collected through a puncture in the cuticle, into polypropylene tubes cooled to 0°C. The cellular fraction (hemocytes) was separated by centrifugation at 90×*g* for 5 min at a room temperature. The sediment was rinsed in saline and centrifugated again. The sedimented cells were resuspended in a fixed volume of the culture medium. The hemocytes' final concentration in culture medium was equal to $3.7 \pm 0.2 \times 10^6$ cells/mL in experiments with antimicrobial activity dynamics study (total cell culture volume 0.4 mL) and $8.8 \pm 0.6 \times 10^6$ cells/mL in experiments dedicated to antimicrobial peptides' identification (total cell culture volume 2 mL), correspondingly.

Fat body isolation Prior to fat body isolation, the larval body surface was sterilized with 70% ethanol, rinsed in distilled water, and lightly dried on filter paper. The posterior part of the body was cut away with scissors, and the fat body with associated tracheae, gut, and Malpighian tubules was taken out and placed into a petri dish filled with saline. The fat body was cleaned of tracheae, gut, and Malpighian tubules and, after preliminary rinsing, transferred to a plate well with culture medium in an amount of 400–500 μL per fat body of a single larva. The number of fat body cells per one larva (7870 ± 169) was accounted planimetrically on high-resolution photo scanner Epson V350 (Fig. 1).

Culturing conditions In the experiments on the dynamics of antimicrobial activity of fat body and hemocytes, cells were cultured on the MM insect medium containing lactalbumin hydrolysate and yeast extract (Mitsuhashi and Maramorosch 1964). In some cases, a transcription inhibitor actinomycin D (Reanal, Hungary) was introduced in the cell culture at a concentration of 1 μg per 1 mL of the medium to exclude antimicrobial compounds de novo synthesis. The culturing was done at a temperature of 25°C in the conditions of uniform shaking. The culture medium was sampled 30 min, 1, 3, 6, 9, 12, and 24 h after the start of culturing for the determination of the total antimicrobial activity.

In the experiments with subsequent extraction of antimicrobial peptides, lactalbumin hydrolysate and yeast extract were not included in the medium, i.e., modified Carlson's saline (Carlson et al. 1947) was used. The whole culture medium was collected and used for antimicrobial peptide extraction on the next d.

Cell culture sterilization To prevent contamination of the culture, antibiotics streptomycin and penicillin G (Biolot, Russia) were added to the medium in amounts of 150 μg and 150 U per 1 mL of the medium, respectively. The use of the antibiotics allows to effectively sterilize *C. vicina* cell culture and thus enables the testing of the immunocyte antimicrobial (anti-*Escherichia coli*, but not anti-*Micrococcus luteus*) activity without prior removal of penicillin and streptomycin. These antibiotics were replaced by meropenem (Sumitomo Pharmaceuticals, Japan) in experiments needed in the culture medium chromatographic fractionation in order to avoid overlapping of penicillin and some antimicrobial peptides' fractions (Fig. 2*A*). Meropenem possesses broad antibacterial activity spectrum comprising both gram-negative and gram-positive aerobic and anaerobic bacteria (Pitkin et al. 1997) and has no overlapping with *C. vicina* antimicrobial peptides on the chromatogram (Fig. 2*B*). Meropenem was added to the cell culture medium in final concentration of 2 μg per 1 mL of the medium.

Tests for viability of cultured cells The viability of fat body cells was estimated by adding trypan blue (Sigma-Aldrich). The dye was dissolved in saline to obtain a 0.2% concentration and poured into the medium in the ratio of 1:1. After 5 min, the fat body was washed of the dye and the number of stained (i.e., dead) cells was counted under binocular microscope.

Figure 1. Scan of *Calliphora vicina* larval fat body.

Solid-phase extraction of antimicrobial peptides from the culture medium The total moderately hydrophobic components were isolated from the culture medium by solid-phase extraction on Sep-Pak C18 cartridges (Waters, Milford, Massachusetts) with a sorbent that reversibly binds hydrophobic groups of peptides and polypeptides. The cartridge was washed in one direction with 5 mL of acetonitrile and 0.05% aqueous solution of trifluoroacetic acid. Then, an acidified sample and 5 mL of 0.05% aqueous solution of the acid were applied on the cartridge. The eluate represented an extract of hydrophilic compounds from the hemolymph. Hydrophobic substances were eluted using 0.05% aqueous solution of the acid with 50% of acetonitrile, and the resulting fraction was used in subsequent work. The organic solvent and water were removed from the resulting extract by vacuum drying; the lyophilizate was diluted in deionized water and fractionated chromatographically.

Chromatographic fractionation Chromatographic fractionation was carried out by means of reversed-phase HPLC on a Shimadzu chromatograph (Kyoto, Japan) with a Vydac C18 column (5 mm, 4.6 mm i.d. × 250 mm; Grace, Columbia, MD). The amount of tested material applied on the column was synthesized by 1.0×10^5 fat body cells and 1.4×10^7 hemocytes, correspondingly. Prior to application, the column was equilibrated with 0.1% solution of trifluoroacetic acid. Peptides were eluted with a linear gradient from 0 to 50% acetonitrile for 50 min using a flow rate of 1 mL/min. Detection was performed by light absorption at a wavelength of 214 nm; the eluate was fractionated in an automatic collector. The fractions were vacuum-dried; the lyophilizate was diluted in deionized water, and the quantity of antimicrobial components was estimated in each fraction.

Analysis of antimicrobial activity The bactericidal activity of the material was determined using a standard method of agar plates (Lambert et al. 1989) with a gram-negative bacillus *E. coli* D31 and a gram-positive coccus *M. luteus* CIP A270 (ATCC 4698) routinely used in insect antimicrobial peptide studies (Boman et al. 1978; Bulet et al. 1991; Cociancich et al. 1993; Boulanger et al. 2002). The bacterial cultures were obtained from the Institute of Molecular and Cellular Biology

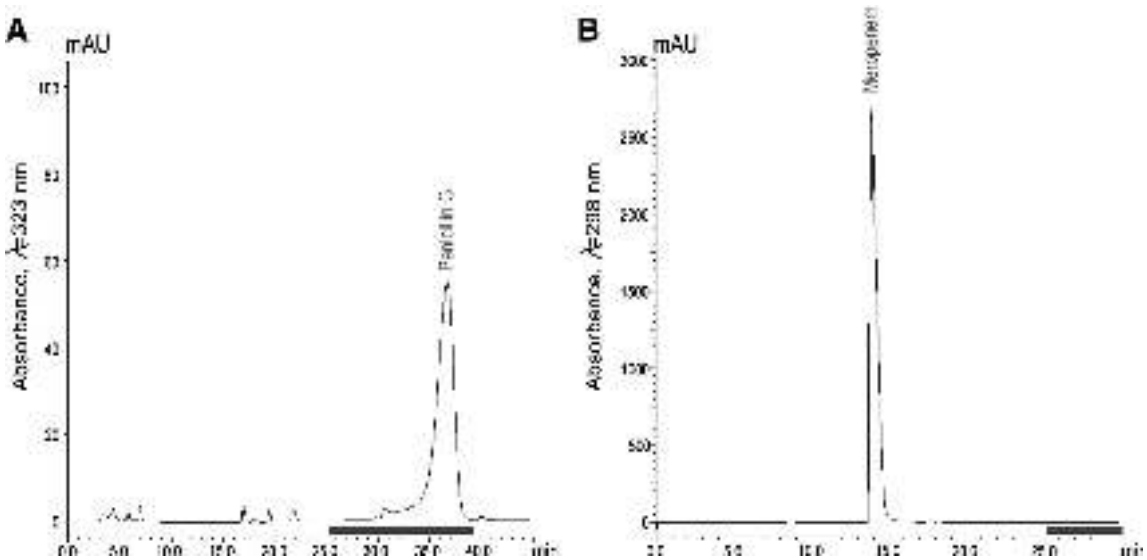

Figure 2. Chromatograms illustrating retention time of *C. vicina* antimicrobial peptide fractions (*bold line*) and antibiotics: –penicillin (*A*), meropenem (*B*).

(Strasbourg, France). *E. coli* D31 and *M. luteus* A270 genotypes are characterized at http://cgsc.biology.yale.edu/Strain.php?ID=10033 and http://www.ncbi.nlm.nih.gov/nuccore/CP001628, respectively.

Bacteria were introduced into the agar medium in the amount 5×10^5 cells per 1 mL of the medium; total volume of the medium placed in the petri dish (9 cm in diameter) was 7.5 mL. Aliquots of material applied directly onto the surface of an agar plate in a volume of 10 μL (for whole culture medium) and 5 μL (for chromatographic fractions). In the experiments on the dynamics of antimicrobial activity, the amount of tested substance applied on the agar medium was obtained from approximately 200 fat body cells or 3.7×10^4 hemocytes whereas in the experiments on fractionation of antimicrobial peptides, 1.5×10^4 or 2.3×10^6 cells, correspondingly.

After incubation at +37°C, the medium in petri dishes became opaque due to the emergence of observable bacterial colonies, except for the zones surrounding the applied samples where antimicrobial substances inhibited bacterial growth. As the area of such a zone is proportional to the content of bactericidal components in the sample, the described method was used for quantitative evaluation of the antimicrobial peptides in the medium. The activity of peptides against gram-negative bacteria was tested on *E. coli*, and that against gram-positive bacteria was tested on *M. luteus*.

Mass spectrometry The chromatographic fractions containing antibacterial materials were diluted in deionized water to 100 μL volume. The molecular masses of the materials were determined by the ESI method on a MaXis chromato-mass spectrometer (Bruker Daltonik, Bremen, Germany). Mass specters were registered using positive-ion mode in 50–1000 mass diapasons. Capillary voltage was established at 4500 V, and 500 V at the end of the capillary. Dry gas was applied at flow rate 4 L/min under temperature 180°C. In-source collision-induced dissociation (ISCID) was turned on, and collision voltages up to 200 V were used. Mass spectrograms were performed manually to take into account that all the peaks differ from the background signal.

Statistical analyses The results are expressed in text as a mean ± SE. Continuous variables (antibiotic titers) and discrete parameters (cell survival rates) were compared by the non-parametric Mann-Whitney test in the Statistica 7.1 program (StaSoft Inc., Tulsa, OK).

Results

The dynamics of antimicrobial activity in the culture of immunocytes The culture medium per se, in the control variant, does not exhibit antimicrobial activity, although it contains exogenous antibiotics—streptomycin and penicillin G (Fig. 3). This occurs for two reasons: Firstly, the content of these antibiotics in the aliquot is relatively small (1.5 μg and 1.5 U, respectively); secondly, *E. coli* D31 strain has increased resistance to both streptomycin and penicillins like ampicillin (Boman et al. 1978). In the fat body culture, antimicrobial activity is first detected only 6 h after inoculation (Fig. 3*A*). Subsequently, the antimicrobial activity of the medium gradually increases and reaches its maximum after 24-h exposure (within the studied time interval). The addition of actinomycin D blocks the accumulation of insect antimicrobial components in the medium completely ($n_1 = 3$, $n_2 = 3$, $U = 0$, $p < 0.05$). Notably, this effect is caused by the inhibition of the synthesis of antimicrobials and not by the death of producer cells: Survival rates of fat body cells in the medium with actinomycin D (95.8%) do not differ from those in the control (93.7%) ($n_1 = 3$, $n_2 = 3$, $U = 3$, $p < 0.5$).

There is no antimicrobial activity found when the total hemocytes of one larva are cultivated in the same volume of the medium (400 μL). Increasing the number of hemocytes tenfold (i.e., using hemocytes from 10 larvae—1.5×10^6

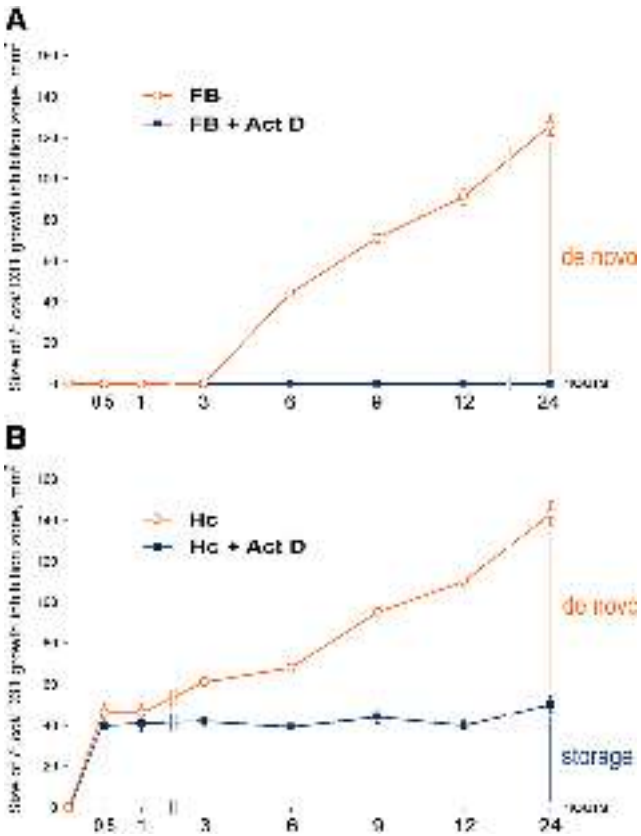

Figure 3. The dynamics of the antimicrobial activity of the fat body (*A*) and hemocytes (*B*) when cultured in the presence and absence of actinomycin D (1 μg/mL). The concentration of active components in aliquots of the culture medium corresponds to 200 fat body cells and 3.7×10^4 hemocytes. Control culture medium containing penicillin and streptomycin did not show antibacterial activity. The number of biological replicates at each point is 3.

cells—per 400 μL of the medium) enables the detection of antimicrobial components (Fig. 3*B*). The antimicrobial activity of hemocytes grows in two steps, in contrast to its gradual increase in the fat body. In the first step (30-min exposure), the titer of antimicrobial components goes up abruptly and then remains unchanged for few hours. After 3 h of incubation, the antimicrobial activity of the medium starts increasing gradually and reaches its maximum by 24 h. The effect of added actinomycin is manifested in the total blockade of the second step, during which a gradual increase in the titer of antimicrobial substances would be observed otherwise ($n_1 = 3$, $n_2 = 3$, $U = 0$, $p < 0.05$). However, the earlier rise of antimicrobial activity occurs regardless of the presence of the transcription inhibitor in the medium.

The qualitative composition of antimicrobial substances released by immunocytes The results of the chromatographic mapping of the culture medium of the fat body are shown in Fig. 4 and those for hemocytes in Fig. 5. Under given conditions, antimicrobial components were eluted from the column in two steps: on the 14th–16th min (fractions F14–F16) and on the 24th–38th min (fractions F24–F38) of fractionation.

Apparently, the substance responsible for the antimicrobial activity of fractions F14–F16 is meropenem. Such conclusion is drawn from three facts: First, the retention time of this antimicrobial activity corresponds well to meropenem behavior in the same chromatographic conditions (Fig. 1); secondly, this antimicrobial activity was observed both in experimental (Figs. 4*B* and 5*B*) and control (Figs. 4*A* and 5*A*) samples; thirdly, no antimicrobial activity was observed in these fractions following addition of other antibiotics, namely penicillin and streptomycin, in the immunocyte culture medium (data not shown). An interesting observation was made concerning meropenem: In the presence of the fat body or hemocytes (Figs. 4*B* and 5*B*), its antimicrobial activity was higher than in the control variants where this antibiotic was incubated alone (Figs. 4*A* and 5*A*). For example, in the experiment with hemocytes, meropenem activity against *E. coli* noticeably increased (Fig. 5*B*). In the experiment with the fat body, where the final concentration of meropenem applied on the chromatographic column was an order of magnitude greater, this antibiotic exhibited anti-gram-positive, in addition to anti-gram-negative, activity (Fig. 4*B*). Although meropenem is indeed reported to be active against both *E. coli* and *M. luteus* (Caicedo et al. 2014), our results show that *E. coli* D31 appears to be more sensitive to meropenem than *M. luteus* A270 (Figs. 4 and 5). We have no explanation for the elevated activity of meropenem in the *C. vicina* immunocyte culture, which is a promising topic for further research.

Fractions F24–F38 contain antimicrobials produced by insect's immunocompetent cells, which is clear from the fact that the corresponding fractions of the cell-free medium (control) show no antimicrobial activity. Anti-gram-positive activity of the culture medium from both the fat body and hemocytes is confined to a single group of fractions that exert bactericidal effect on *M. luteus*, namely F26–F33 with a maximum in F28. Anti-gram-negative activity of the culture medium from the fat body is found in two groups of fractions: F24–F27 (with a maximum in F26) and F30–F37 (with a maximum in F31). Hemocytes have a third group of fractions with anti-*E. coli* activity, besides these two: F28–F29, with maximal activity in the F28 fraction.

In order to confirm the occurrence of antimicrobial peptide synthesis in fat body cells and hemocytes, medium's chromatographic fractions with the highest antimicrobial activity were analyzed by mass spectrometry. The molecular weights obtained for the most "active" fractions were compared with weights of the already known antimicrobial peptides from infected *C. vicina* larvae (Table 1).

Antimicrobials from the fraction F26 are partly identified. One of the compounds was found to be novel proline-rich peptide with molecular mass 4441.5 Da. The peptide sequence present in Table 1 footnote is deduced from *C. vicina* mRNA database; its calculated molecular mass fully complies to the mass spectrometrically detected value. It belongs to the proline-rich peptide family of insect antimicrobial peptides (AMPs) and demonstrates strong sequence similarity with domesticin AMP from *Musca domestica* (Tang et al. 2014). The peptide was detected in the fraction F26 of both fat body and hemocyte cultures. The anti-gram-positive activity of the fraction F28 is presumably exerted by a defensin with a molecular weight of 4032 Da. Besides that, this fraction contains proline-rich peptide with a weight of 2987 Da in the hemocyte culture. The fraction F31 of the culture medium appears to contain all the four known diptericins, their molecular weights being 8886, 8914, 9000, and 9029 Da. In contrast to diptericins from the fat body, those from hemocytes are represented by the only isoform with a weight of 9000 Da. The proline-rich peptide weighing 2987 Da is found in this fraction too. The main «active» component of the fraction F33 is a cecropin with a molecular mass of 4156 Da. It is produced both by the fat body and by hemocytes as well. It is notable that all the antimicrobial peptides in the mass spectrogram represented multiply charged ions; the number of charges ranged from 3 to 13.

Discussion

This study confirms that the antimicrobial peptide complex, which was isolated by earlier researchers from the hemolymph of infected *C. vicina* larvae, is in fact synthesized by the fat body and hemocytes. The fat body is shown to produce defensin (4032 Da), four diptericins (8886, 8914, 9000, and 9029 Da), and one cecropin

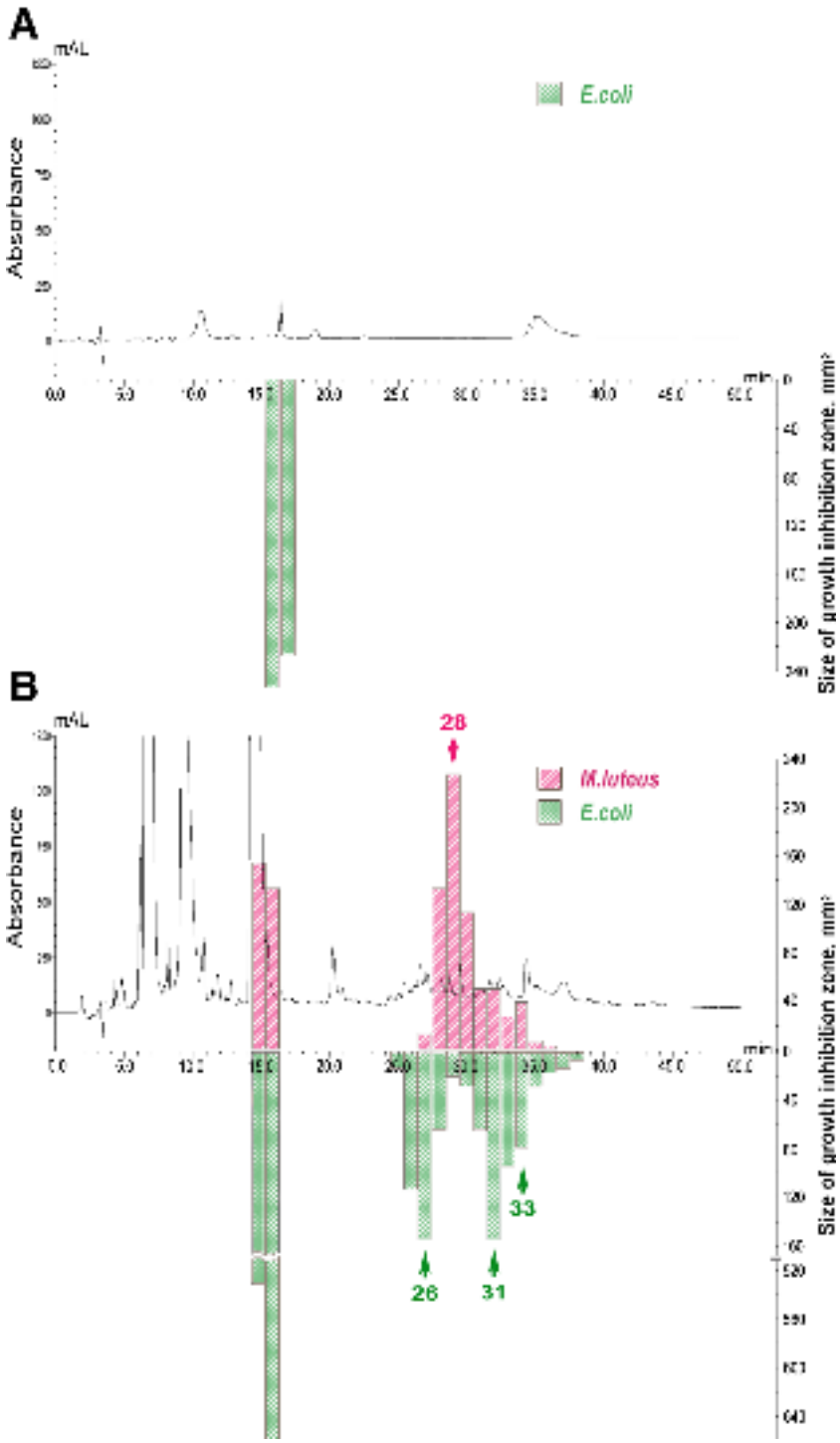

Figure 4. Chromatographic fractionation of the cell-free culture medium with meropenem (*A*) and from the fat body culture (*B*). Histogram columns show the areas of growth inhibition zones for two tested bacteria: *M. luteus* A270 (above the *x*-axis) and *E. coli* D31 (below the *x*-axis). *Arrows* indicate «active» fractions analyzed by mass spectrometry. The concentration of active components correspond to 1.0×10^5 fat body cells on the column and 1.5×10^4 fat body cells on the agar plate. Control culture medium containing meropenem did not show anti-*M. luteus* activity.

(4156 Da). The antimicrobial peptides released by hemocytes are composed of at least one defensin (4032 Da), one diptericin (9000 Da), and one cecropin (4156 Da) coinciding with that from the fat body as well as one proline-rich peptide (2987 Da) specific to hemocytes. Besides that, both the fat body and hemocytes produce proline-rich peptide with molecular mass of 4441.5 Da.

Intriguingly, we isolated functional immunocompetent cells from *intact* larvae. Both the fat body and hemocytes release antimicrobial peptides in vitro without any additional external stimulus (e.g., without addition of bacteria or bacterial surface structures into the medium) necessary for in vivo immune response induction. In other words, the functioning of intact larvae's immunocytes is different in vivo and in vitro. The titer of

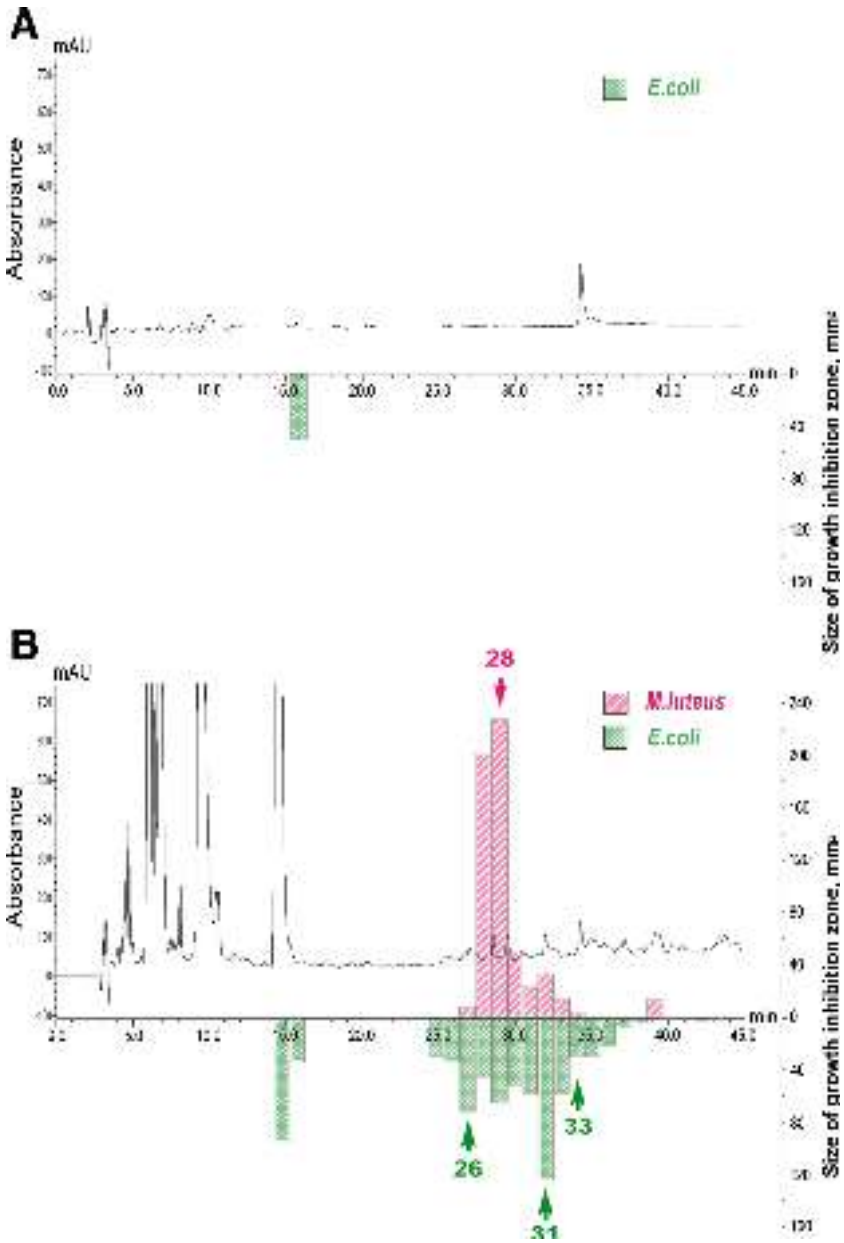

Figure 5. Chromatographic fractionation of the culture medium from the cell-free culture medium with meropenem (*A*) and from hemocyte culture (*B*). Histogram columns show the areas of growth inhibition zones for two tested bacteria: *M. luteus* A270 (above the *x*-axis) and *E. coli* D31 (below the *x*-axis). *Arrows* indicate «active» fractions analyzed by mass spectrometry. The concentration of active components corresponds to 1.4×10^7 hemocytes on the column and 2.3×10^6 hemocytes on the agar plate. Control culture medium containing meropenem did not show anti-*M. luteus* activity.

antimicrobial peptides in the plasma of intact larvae is extremely low, suggesting that antimicrobial peptides are not synthesized in vivo (Yakovlev 2011). Presumably, in our experimental work, isolation of immunocompetent cells and their transfer to a culture medium (which is a non-natural environment to them) have served as triggers of antimicrobial activity.

As the above results of in vitro experiments show, the fat body seems to be the primary producer of peptide antibiotics in the organism of the blue blowfly larva. In particular, the contribution to the antimicrobial activity of the larval fat body in vitro exceeds that of total hemocytes by an order of magnitude. Actinomycin D, being a transcription inhibitor, blocks the accumulation of antibiotics in the fat body culture completely, which means that all antimicrobial peptides are synthesized by the fat body de novo. The role of the fat body in the anti-infectious immunity of *C. vicina* larvae seems to consist in forming the systemic humoral defense against pathogenic microbes. Under normal conditions (in an intact larva), the fat body either produces no antimicrobial peptides at all or releases them into plasma in trace amounts only. Damage to the integument (or transfer of the fat body to culture medium, like in this work) results in a several-fold increase in the antimicrobial activity of the fat body and extensive release of antimicrobial peptides. Such a principle of functioning of insect antimicrobial peptides stands in stark contrast to that in mammals, whose peptide antimicrobials are localized into intracellular phagosomes where they exert their antibacterial activity (Ganz et al. 1985).

Table 1. Molecular masses found in active HPLC fractions isolated from fat body and hemocyte culture media in comparison with the masses of known *C. vicina* AMPs

HPLC fraction no.	Major molecular masses found in the fat body culture medium, Da	Major molecular masses found in the hemocyte culture medium, Da	Known AMP characteristics (Chernysh et al. 2015)		Peptide family
			Molecular masses, Da	AA sequence	
24–25	n/a	n/a			
26[a]	2481.5 4457.5 3292.8 4524.4	3953.2 4027.1	No masses corresponding to known *C. vicina* AMPs		
	3953.2 4539.4	4441.5			
	4159.2 4782.3	4457.5			
	4188.2 4856.7	4478.4			
	4204.2 4871.7	4555.5			
	4422.4 5149.5	4782.4			
	4441.5 6493.9	6493.9			
27	n/a	n/a			
28	3045.5 4189.2 3148.7 4204.2	2986.7 3371.9	2987.0	FVDRNRIPRSNNGPKIPIISNP (N-terminus)	Proline-rich peptide
	3239.7 4228.9	3670.2			
	3371.9 4446.0	4031.9			
	3549.7 4545.1	4129.9			
	3563.9 4554.3 3582.0 4603.4	4189.2 4446.1	4032.0	ATCDLLSGTGANHSACAAHCLLRGNR-GGYCNGKAVCVCRN	Defensin
	3768.1 4620.4	8707.6			
	4031.8	9413.1			
	4129.9				
29–30	n/a	n/a			
31	3629.9 8999.6 3645.9 9014.6	2986.8 8999.7	2987.0	See fraction no. 28	Proline-rich peptide
	3883.1 9029.5 3998.1 9044.5	9036.8 9067.8	8886.2	DSKPLNLVLPKEEPPNNPQTYGGGGGS-RKDDFDVVLQGAQEV... (N-terminus)	Diptericins
	4024.1 9167.7	9482.0			
	8886.5 8913.5		8999.4		
	8929.5				
32	n/a	n/a			
33	2885.7 4228.4 3865.1 4269.4	3853.5 3883.6	4156.0	GWLKKIGKKIGRVGQHTRDATIQGLAV-AQQAANVAATAR	Cecropin
	3903.0 4291.4	4155.4			
	4155.4 4306.3	4291.7			
	4177.4 4327.3	5348.0			
	4191.4 4497.4				
	4213.4 4541.6				
34–38	n/a	n/a			

n/a not analyzed

[a] The fraction 26 isolated from both fat body and hemocyte culture media contains compound with molecular mass 4441.5 Da. Search of *C. vicina* mRNA database revealed peptide with the same molecular mass having amino acid sequence as follows: SRDARPVQPRFNPPPPKRERPIIYDAPIRRPGPKTMYA. The peptide belongs to proline-rich peptide family and demonstrates 73% similarity with known insect AMP domesticin isolated from another fly species *Musca domestica*

That being said, the link between antimicrobial peptides and blood cells may not have been completely lost in insects. Our results suggest that hemocytes are also capable of synthesizing and releasing antimicrobial peptides. Like the fat body, hemocytes can produce bactericidal molecules de novo: Blockade of transcription leads to a reduction of their antimicrobial activity. On the other hand, hemocytes are able to release a part of antimicrobials rapidly, soon after treatment, regardless of the presence of transcription inhibitors. Hypothetically, it may be explained by the ability of intact larvae's hemocytes to store antimicrobial peptides (or to retain mRNAs encoding these peptides) in the cytoplasm.

The biological significance of the releasing of antimicrobial peptides by hemocytes is open to discussion. Notably, the total antimicrobial activity of larval hemocytes is far less than that of the fat body cells. It is therefore unlikely that hemocytes would serve as a functional backup of the fat body in the large-scale systemic immune response. The function of the antimicrobial peptides released by hemocytes is more likely to eliminate the pathogen at the earlier stages of the immune response, namely in the first hours after infestation, when the fat body has not yet started to emit its antimicrobial peptides into the plasma.

Conclusions

Here, we estimate an ability of *C. vicina* larvae's fat body and hemocytes to synthesize and release antimicrobial peptides in vitro. The fat body is shown to produce one defensin, four diptericins, one cecropin, and one proline-rich peptide. The antimicrobial peptides released by hemocytes are composed of at least one defensin, one diptericin, one cecropin, and one proline-rich peptide coinciding with that from the fat body as well as one proline-rich peptide specific to hemocytes. The major part of antimicrobial materials originates from fat body cells with minor contribution of hemocytes.

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