Minireview

Original involvement of antimicrobial peptides in mussel innate immunity

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Abstract Recently, the existence and extended diversity of antimicrobial peptides has been revealed in two mussel species. These molecules are classified into four groups according to common features of their primary structure: defensins, mytilins, myticins and mytimycin. In *Mytilus galloprovincialis*, gene structure reveals synthesis as precursors in circulating hemocytes. Synthesised even in absence of challenge, the precursors mature and the peptides are stored in granules as active forms. The different peptides are engaged in the destruction of bacteria inside phagocytes, before being released into hemolymph to participate in systemic responses. Such involvement in anti-infectious responses is unique, and apparently more related to those of mammalian phagocytes than to those of insects. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Antimicrobial peptide; Defensin; Mytilin; Myticin; Innate immunity; Mussel; Mollusc

1. Introduction

Even if suspected in the 1920s [1], invertebrate humoral immunity was totally absent from records until the early 1980s when the Swedish group of Hans Boman began describing antibacterial proteins and peptides in insects [2]. Now, more than 100 peptides have been purified from plants [3], invertebrates [4,5] and vertebrates [6], and these peptides appear to be one of the actors in innate immunity that have been conserved during evolution. Meanwhile, their involvements in anti-infectious processes are different according to species, cells and tissues. Many antimicrobial peptides are located in epithelia, either of plants, insects or mammals [7], where they prevent invasion by pathogens. Others may be especially abundant in circulating cells. In horseshoe crabs, the peptides are stored in hemocyte granules and released into the plasma upon stimulation by microbial substances, such are lipopolysaccharides and β-glucans [4,8]. In contrast, mammalian peptides accumulate in granulocytes and certain macrophages in order to exert their microbicidal activity on engulfed bacteria [9]. In insects, septic injury results in a rapid synthesis of antimicrobial peptides, principally in the fat body, which are released immediately into hemolymph where they participate in a systemic response [10].

Extensively studied in arthropods, antimicrobial peptides appeared as one of the components of antimicrobial host defence throughout evolution. Meanwhile, until recently, these peptides were unknown in other invertebrate phyla. Applying the same methodology used for isolating antimicrobial peptides from insects, several small, cationic, cysteine-rich peptides have been characterised from hemolymph of mussels, a bivalve mollusc.

2. Diversity and structural features of mussel peptides

These small cationic antimicrobial peptides are characterised by their high cysteine content and they have been organised into four groups according to shared features of their primary structure, mainly their consensus cysteine array (Fig. 1). The first group comprises the defensins, which show similarities with molecules of the arthropod defensin family [5]. Two defensins containing six cysteines have been characterised from the plasma of the blue mussel, *Mytilus edulis* [11] and a defensin-like peptide, named MGD1 for *Mytilus galloprovincialis* defensin-1, from the plasma and hemocytes of the Mediterranean mussel, *M. galloprovincialis* [12,13]. A second isoform, MGD2, has been isolated from a hemocyte cDNA library [13]. MGDs are original members of the arthropod defensin family because of the presence of two extra cysteines, eight instead of six. As determined by ^{1}H-nuclear magnetic resonance [14], MGD1 three-dimensional (3D) solution structure appeared highly constrained and consists mainly of the canonical CSαβ structural motif (Cys4–Cys25, Cys10–Cys33 and Cys14–Cys35 disulfide bonds) similar to that of arthropod defensins [15], the two extra cysteines (Cys21–Cys38) being engaged in an original fourth disulfide bond. Synthetic MGD1, correctly folded to form the four disulfide bonds, retains the antibacterial activity of native MGD1, suggesting that the hydroxylation of Trp28 observed in native MGD1 is not involved in the biological effect. The second group of molecules, the mytilins, consists of five isoforms (A, B, C, D and G1). Isoforms A and B were isolated from *M. edulis* plasma [11], and isoforms B, C, D and G1 from *M. galloprovincialis* hemocytes [16]. The third group of peptides includes the myticins A and B, that have been derived and characterised from hemocytes (isoforms A and B) and plasma (isoform A) of *M. galloprovincialis* [17]. Even if possessing a similar molecular weight and eight cysteines, my-

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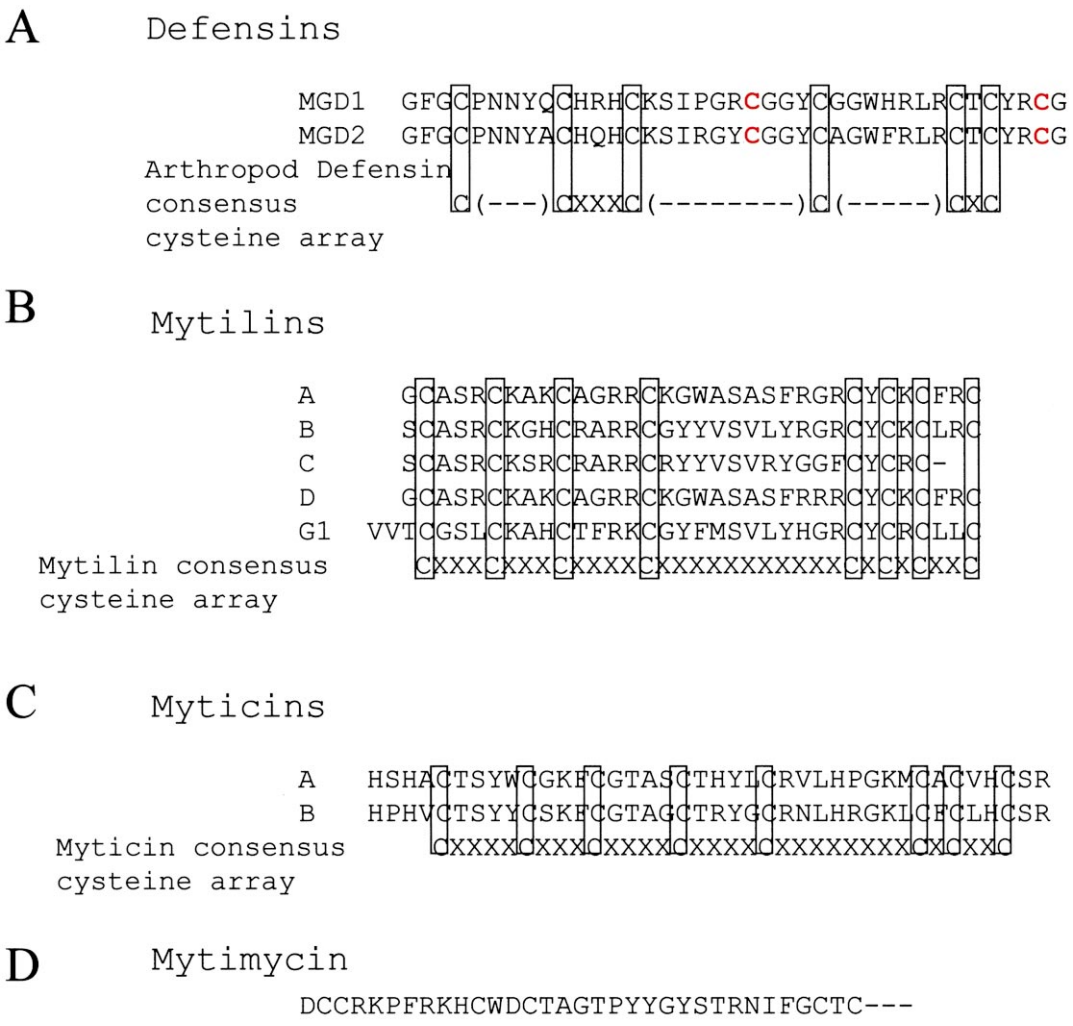

Fig. 1. Diversity of the mussel antimicrobial peptide families: defensins (A), mytilins (B), myticins (C) and mytimycin (D). Cysteines are boxed revealing the consensus cysteine arrays. The two extra cysteines of MGDs are in red.

tilins and myticins are not related to any known molecule. In addition, an anti-fungal peptide of 6.5 kDa containing 12 cysteines, mytimycin, has been partially characterised from *M. edulis* plasma [11].

Our data revealed that mussel antimicrobial peptides are produced in hemocytes and stored in hemocyte granules [13,16,17]. cDNAs were isolated from hemocyte mRNAs and revealed that defensins, mytilins and myticins are synthesised as 81, 103 and 96 amino acid precursors, respectively [13,16,17]. Consequently, the peptides are processed from precursors to active peptides within the hemocytes, an observation similar to that done in the Japanese horseshoe crab, *Tachypleus tridentatus* [18], and in the shrimp, *Penaeus vannamei* [19].

cDNAs of MGD1 and 2, mytilin B and myticins A and B revealed that all initial translation products share the same structural features with: (i) an N-terminal canonical signal peptide, followed by (ii) the sequence corresponding to the mature peptide and (iii) a C-terminal extension rich in anionic residues. While the N-terminal pre-segment is presumed to be a signal sequence for translocation to the lumen of the rough endoplasmic reticulum, the functional significance of the C-terminal extension is unknown. This region might interact with the active peptide: (i) to neutralise positive charges of the peptide, allowing suitable proteolytic processing and/or addressing to a particular hemocyte compartment, or (ii) to protect the producing cells from eventual cytotoxic effects. Similar C-terminal extension has been observed in other antimicrobial peptide precursors, like the tachyplesin precursor from *T. tridentatus* [18].

3. Biological activity of mussel peptides

Such a diversity of antimicrobial peptides is questionable. One possible explanation results from establishment of an activity spectrum of the different molecules [11,12,16,17]. Defensins and myticins are essentially active against Gram-positive bacteria, including some pathogens for marine invertebrates [20,21], and are much less active against Gram-negative bacteria or fungi. Mytimycin is strictly anti-fungal. Mytilins display a wider spectrum of activity according to isoforms. Whereas B, C and D isoforms are toxic for both Gram-positive and Gram-negative bacteria, mytilin G1 displays activities only on Gram-positive bacteria. Mytilin B and C isoforms share a high degree of homology in their primary structure. However, they possess different activities, especially against the fungus *Fusarium oxysporum*, pathogen for marine crustaceans [22,23], and against the Gram-negative bacteria

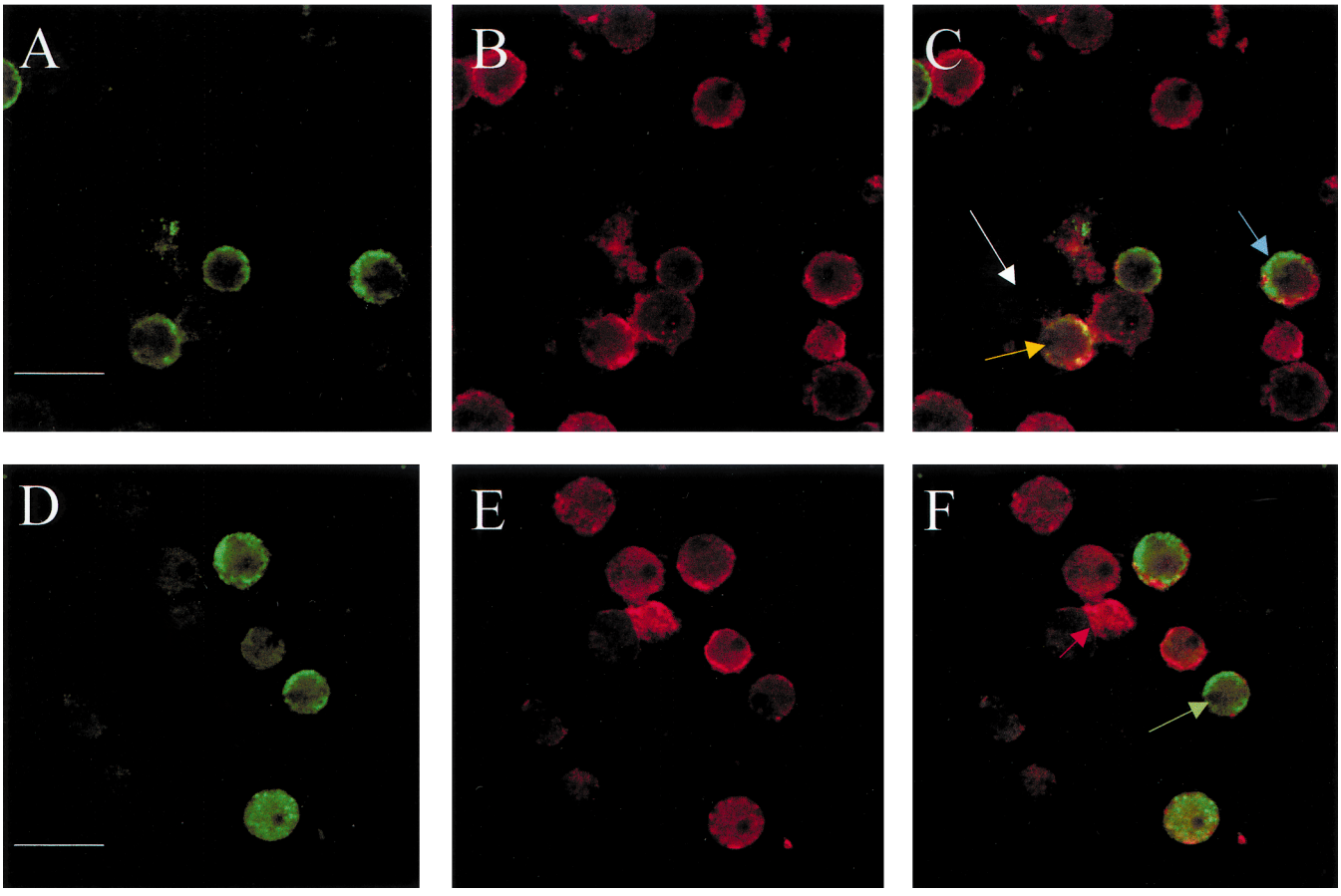

Fig. 2. Confocal microscopy imaging of defensins (green) and mytilins (red) double immune labelling in mussel hemocytes. Hemocytes were fixed, centrifuged onto slides and subjected to immune histochemical detection of defensins (A, D) and mytilins (B, E) using FITC-labelled and Texas red-labelled secondary antibody, respectively. C, F are the merged confocal images of A and B and D and E, respectively. Defensin immune reactivity was detected as small intensely fluorescent particles distributed throughout the cytoplasm (A and D). Mytilin immune reactivity was also detected throughout the cytoplasm, the labelling being more intense at the cell periphery (B and E). Hemocytes might be positive either for defensins (green arrow) or for mytilins (red arrow) but both immune reactivities often appeared within the same cells. Merged confocal microscope images suggested that defensins and mytilins were packed in different (blue arrow) or in the same (yellow arrow) cell compartments. Note that some hemocytes did not stain positively for defensins or for mytilins (C, white arrow). Scale bar: 25 μm.

Vibrio splendidus, pathogen for bivalve molluscs [24]. In fact, the different peptide families and isoforms possess complementary properties that might permit an increase in antimicrobial capabilities of mussels.

All peptides exert bactericidal effects, but important differences in kinetics have been observed. When the different peptides were incubated with *M. luteus*, more than 2 and 6 h were necessary for mytilin D and myticin A or mytilin G1, respectively, to kill all bacteria, whereas only a few minutes in the presence of either mytilins A, B, C or MGD1 were sufficient. Such a rapid bactericidal activity resembles the action of defensins from the fleshfly, *Phormia terranovae*. In this insect, defensins disrupt within seconds the permeability barrier of the cytoplasmic membrane of Gram-positive bacteria, resulting in partial depolarisation, decrease in cytoplasmic ATP, inhibition of respiration and rapidly ensuing death [25] perhaps by apoptosis.

4. Location and ontogeny of expression

Hemocytes are the main site for precursor production of defensins, mytilins and myticins, as confirmed by Northern blot analysis. Nevertheless, among mussel tissues, mantle, labial palps, gills and digestive gland showed a faint band with the same mobility as that of hemocytes [13,16,17]. In fact, these bands are probably derived from insignificant numbers of contaminant hemocytes in the dissected tissues as demonstrated by in situ hybridisation. Indeed, numerous hemocytes expressing these genes were found throughout mussel body, both in sinuses or infiltrating tissues, particularly in epithelia in contact with the environment [26]. Moreover, in situ hybridisation revealed a differential distribution of hemocytes expressing these different genes. Whereas numerous defensin-expressing cells were shown to infiltrate the digestive tubule epithelia, mytilin- and myticin-expressing cells are much less or not represented in these epithelia, respectively. In addition, mytilin- and myticin-expressing cells are well represented in gills, where no defensin positive cells were observed. These data suggest that: (i) different genes are expressed in different hemocyte sub-types and (ii) different peptides are transported by their respective expressing hemocytes to different areas of the mussel. Consequently, and in addition to the increase of antimicrobial capabilities (isoforms with complementary antimicrobial properties), another biological significance of the peptide diversity could result from their involvement in different regions of the mussel body.

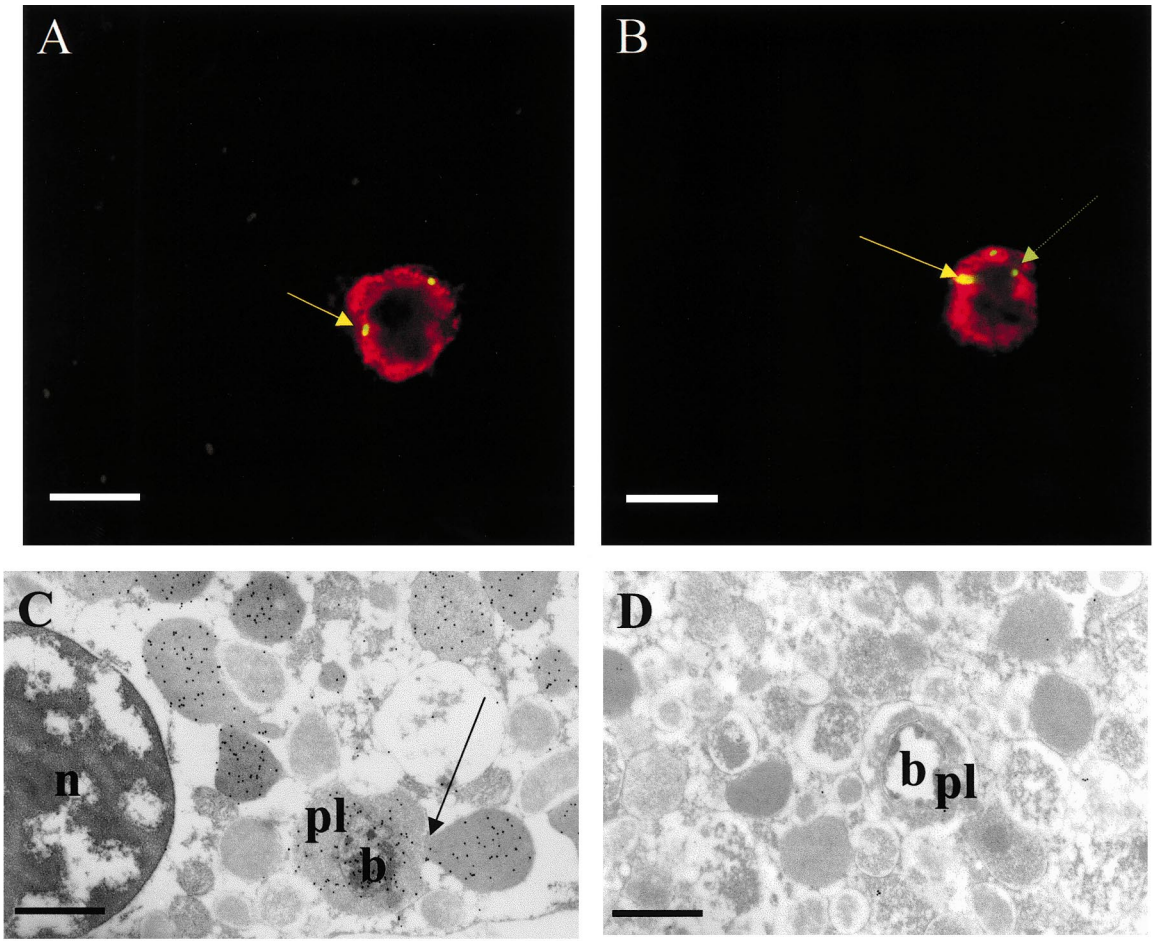

Fig. 3. Dual localisation of peptides and phagocytosed bacteria. Hemocytes were incubated for 20 min in the presence of FITC-labelled *Vibrio alginolyticus*, then fixed and subjected to immune cytochemical detection of mytilins using a Texas red-labelled secondary antibody. Analysis by confocal microscopy showed that mytilin-containing hemocytes were capable of phagocytosis (B, green arrow). Furthermore, in numerous cases, mytilin immune reactivity was co-localised with bacteria in cellular organelles (A and B, yellow arrow). Scale bar: 10 μm. (C and D) Hemocytes were first incubated 20 min with bacteria and then submitted to the immune cytochemical detection of mytilins (C) and defensins (D) using a 15 nm gold particle-conjugated secondary antibody. After phagocytosis, the co-localisation of bacteria and mytilins appeared in phagolysosomes (C). Exocytosis of some mytilin-containing granules into phagosomes suggested a probable fusion of the two organelles (C, arrow). No defensin labelling was observed in phagocytosing cells (D). b, bacteria; n, nucleus; pl, phagolysosome. Scale bar = 1 μm.

In addition, we demonstrated the presence of mytilin B messenger and peptide in enterocytes [26]. In a previous study, we reported the presence of defensin-like molecules in the intestinal epithelium and their location in granular structures contained in enterocytes [13]. Similar location of antimicrobial peptides expression in epithelial cells of gastrointestinal tracts has been reported in insects [27,28] and mammals, where they were observed in Paneth cells [29]. Our data suggested that a similar mechanism of local mucosal defence against microorganisms also exists in mussel intestine.

Knowledge of immune ontogeny is of particular importance as bivalve larvae are susceptible to bacterial infections, especially by marine *Vibrio*s, which are pathogenic for larval stages in several bivalve molluscs, including mussels [30]. Although phagocytic cells and certain enzymes associated with adult hemocytes are present in larval mussels and oysters [31], bivalve larvae are not fully competent to fight infections. In previous studies, we demonstrated that most of the mytilins are active against marine *Vibrio*s [16]. Consequently, larval susceptibility to *Vibrio*s could be linked to the lack of expression of particular antimicrobial peptides in early developmental stages. Indeed, mRNAs encoded by *mytilin B* and *MGD2* genes were undetectable from eggs to settlement, the expression of both genes starting during (*mytilin B*) or 1 week after (*MGD2*) metamorphosis [32]. During metamorphosis, drastic morphological changes occurred in molluscs, during which larval organs disappeared and relative sizes of permanent organs and their orientation were changed [33]. Our results suggested that the maturation of immune functions also occurred during metamorphosis.

5. Localisation of defensins and mytilins in hemocytes

Even if both stored in granulocytes, defensins and mytilins are observed in different subcellular organelles. As revealed by ultrastructural immunocytochemistry, defensins are located: (i) in vesicles of granulocytes containing small granules, and (ii) in large granules of granulocytes containing large granules [13]. Using the same techniques, mytilin labelling was exclusively observed in granulocytes exhibiting large granules and particularly in the large multivesicular structures [16]. Consequently, defensins and mytilins seemed to be partially distrib-

uted in the same hemocyte sub-population. Using confocal microscopy [26], we observed that 37% of hemocytes only stained positively for mytilins and 16% only for defensins, 32% contained both reactivity among which 21% exhibit a co-localisation in the same structures (Fig. 2). Finally, 15% of the hemocytes were not labelled. Such a localisation of both peptides within the same cells, packed in different or in the same cell compartments, was confirmed by electron microscopy observations [26].

6. Gene organisation

Three defensin cDNAs, sharing a high degree of homology, have been described: (i) a cDNA fragment corresponding to *MGD1* obtained by reverse transcription-PCR, (i) the cDNA corresponding to *MGD2a* obtained by screening a hemocyte cDNA library, and (i) a cDNA fragment corresponding to *MGD2b* obtained by 5′ rapid amplification of cDNA ends-PCR [32]. In addition, two different myticin cDNAs were obtained by screening the hemocyte cDNA library [17]. Since these different cDNAs were obtained from mRNA prepared from hemocytes of numerous mussels, it was not clear whether the isoforms are expressed in the same mussel. Southern blot analysis suggested that defensin and myticin genes are present as a single copy in the genome [17,32]. Consequently, differences in cDNA sequences probably resulted from genetic polymorphism. For *mytilin B* gene, Southern blot analysis suggested that at least two genes exist in the genome [32], probably encoding mytilin B and one of the numerous mytilin isoforms.

MGD2b and *mytilin B* genes have been cloned and sequenced, revealing that they both contain three large introns representing 83 and 75% of the nucleotides, respectively [32]. Moreover, the two genes are organised similarly and exons encode precursor domains that fulfil different functions. The second intron of both genes contains the cDNA sequence encoding the signal sequence required to address the precursor to the lumen of the endoplasmic reticulum, and the third and largest exon contains, in both genes, the sequence encoding the mature peptide. According to this particular structure, we hypothesised that the different domains of pre-existing genes could have been combined (by recombination) to create new genes able to fulfil new functions.

7. Involvement in defence

Subjecting mussels to bacterial challenge approached the participation of antimicrobial peptides in anti-infectious processes. Defensins and mytilin B messenger concentrations in circulating hemocytes did not increase after bacterial challenge [32], but mytilin-containing hemocytes migrated and accumulated around infectious sites in the first hours following bacteria injection [16]. This argued in favour of a local participation of mytilins in antimicrobial responses. Meanwhile, the local participation of mytilins can be: (i) extracellular after exocytosis of peptides into the immediate hemocyte environment or (ii) intracellular after engulfment of bacteria. To distinguish between these two possibilities, hemocytes were confronted with bacteria in vitro. Observations on both confocal and electron microscopy revealed that: (i) mytilin-containing granulocytes were able to phagocytose bacteria, (ii) engulfed bacteria are first localised in phagosome-like structures and then (iii) bacteria and mytilins co-localised in phagolysosome-like organelles (Fig. 3). Moreover, exocytosis of mytilin-containing granules into phagosomes was observed suggesting that the co-localisation of bacteria and mytilins occurred after fusion of phagosomes with mytilin-containing granules [26].

As defensin immune reactivity was never detected in phagocytic cells (Fig. 3), the granulocyte sub-type containing only mytilins seems to be the only one involved in phagocytosis of bacteria. Furthermore, confocal microscopy observations demonstrated that a sub-population of circulating hemocytes contained both defensins and mytilins [26]. The role of this latter hemocyte sub-type remains to be elucidated. Bacterial challenge triggered an increase of plasma-associated defensin and mytilin concentrations 24 h later and provided evidences for hemocyte origin of the released peptides, i.e. exocytosis figures of mytilin-containing granules and shifting of defensin immune reactivity towards the plasma membrane [13,16]. The granulocyte sub-type containing both peptides could be involved in these simultaneous plasmatic increases. Co-localisation of defensins and mytilins in the same granules, revealed by both confocal and electron microscopy, supports the hypothesis that both peptides are simultaneously released by exocytosis. Consequently, we hypothesised that the two granulocyte sub-types, one containing only mytilins and the other containing both defensins and mytilins, are probably involved at different stages of the anti-infectious response. The first sub-type would be involved in an early phase response by migrating towards infectious sites and phagocytosing microorganisms and the second sub-type would be involved at a later stage by releasing the peptides that trigger systemic response.

8. An original model

Consequently, we propose that the involvement of antimicrobial peptides in mussels constitutes an original model of anti-infectious defence in invertebrates based on: (i) molecular structures (cysteine array and 3D) similar to that of insect defensins, (ii) constitutive expression in circulating hemocytes and storage in different granules of different hemocytes, and (iii) two routes of involvement, immediate intracellular and later as released in plasma. In contrast, a different system operates in insects, where the expression of antimicrobial peptide encoding genes is undetectable in non-challenged animals but immediately triggered by bacterial injection, showing similarities with that of the mammalian acute phase response genes [34]. The horseshoe crab model is also different, even if hemocytes also produce and store antimicrobial peptides in hemocyte granular organelles. In this case, upon stimulation by microbial substances, such as lipopolysaccharides and β-glucans, hemocytes degranulate and release into the immediate cell environment a series of substances involved in immune defence, including antimicrobial peptides [4]. Even if sharing the same name, human defensins possess totally different amino acid sequences and molecular structures. In fact, the mussel model exhibits functional similarities with that reported for human neutrophil α-defensin peptides (HNP), in which HNP1–4 are stored in azurophil granules that discharge their contents into microbe-containing phagosomes through a process of phagosome–granule fusion [35]. HNP do not appear to be massively secreted. Meanwhile, increased plasma levels of

human defensins have been reported in patients with septicaemia or bacterial meningitis [36] arguing in favour of a putative systemic antimicrobial response of these peptides, as observed in mussels.

Recent data have highlighted similarities between pathogen recognition and signalling pathways of innate immunity in arthropods and mammals [37]. Here, we describe a new invertebrate model of antimicrobial peptide involvement in mollusc that is closely related to that of mammals, but mediated by molecules structurally related to those of insects. Our present knowledge accumulated from different phyla suggests that vertebrate innate immunity is a patchwork of ancestral mechanisms still present in more primitive animals.

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