The molecular basis of innate immunity in the horseshoe crab

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During the past two decades, the molecular structures and functions have been established for various defense molecules, using horseshoe crab (limulus) as a model animal. These defense molecules include clotting factors, proteinase inhibitors, lectins, antimicrobial peptides and other humoral factors found mainly in the hemolymph. These components of the cellular and humoral systems, which together comprise innate immunity, defend horseshoe crab effectively from invading microbes.

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Abbreviations

3D	three-dimensional
CRP	C-reactive protein
KDO	2-keto-3-deoxyoctonate
L-granule	large granule
LPS	lipopolysaccharide
LTA	lipoteichoic acid
SA	sialic acid
tCRP	*T. tridentatus* CRP
TL	tachylectin

Introduction

Invertebrate animals, which lack an adaptive immune system, have developed various defense systems that make-up their so-called 'innate immunity' and respond to common antigens on the surface of potential pathogens [1••]. These defense systems include hemolymph coagulation, melanization, complement activation, cell agglutination, antimicrobial action, active oxygen formation and phagocytic action. Among them, hemolymph coagulation, phenoloxidase-mediated melanization and cell agglutination are induced directly by foreign substances such as lipopolysaccharide (LPS), (1,3)-β-D-glucan, muramyl peptides, proteoglycans and lipoteichoic acid (LTA), resulting in the engulfment of invading microbes. The immobilized invaders are finally killed by antimicrobial substances released mainly from many types of hemocyte (Figure 1) [2,3,4••].

The invertebrate *Tachypleus tridentatus* (Japanese horseshoe crab), an anthropod, relies completely on innate immunity, employing a unique and very efficient host defense system [5•,6]. The hemolymph of horseshoe crab contains soluble defense molecules and large numbers of hemocytes (or amebocytes), which undergo degranulation on contact with pathogens. Granular hemocytes, which comprise 99% of the circulating hemocytes, are filled with two populations of secretory granules, named large (L)- and small (S)-granules [5•].

The L-granules selectively store more than 20 defense molecules with molecular masses mainly between 8 and 123 kDa, such as clotting factors, a clottable protein coagulogen, proteinase inhibitors, lectins and antimicrobial proteins. In contrast, the S-granules contain at least six proteins with molecular masses of less than 30 kDa, and large amounts of hairpin-like tachyplesin peptides (>10 mg per individual), tachystatins, tachycitins and big defensins, all of which show antimicrobial activities against Gram-negative and Gram-positive bacteria, and fungi (Table 1) [7].

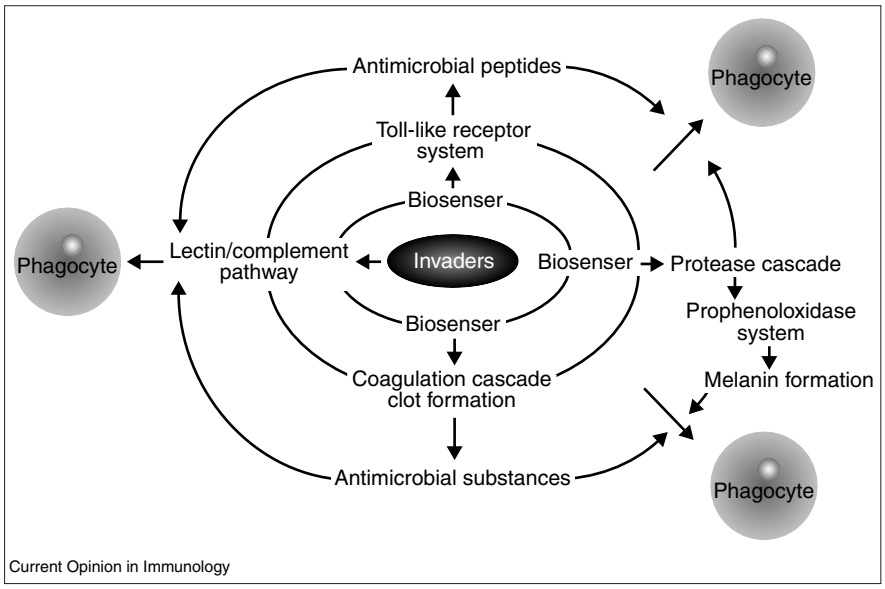
With colleagues, I have previously reviewed in detail the LPS- and 1,3-β-D-glucan-mediated coagulation cascade found in the *Tachypleus* hemocytes, and the biochemical characteristics of five clotting factors — factor C, factor G, factor B, proclotting enzyme and a clottable protein coagulogen — all of which participate in the cascade system [5•,6,7]. Furthermore, three hemocyte-derived serpins, called *Limulus* intracellular coagulation inhibitors, cystatins and Kunitz-type proteinase inhibitors, which also participate in regulation of the cascade reaction, have been described [8]. Here, I want to review mainly our current knowledge of novel antimicrobial peptides and bacterial agglutinins/lectins that are involved in the innate immunity of the horseshoe crab. Several reviews summarize recent developments and the state of this field before 1998 [5•,6–9].

Defense molecules found in horseshoe crab hemolymph

Table 1 summarizes the various proteins and peptides found to date in the hemocytes and hemolymph plasma. These components are closely associated with the host defense of this animal. Unlike mammalian blood plasma, the hemolymph plasma does not contain many different proteins but instead circulates three principal proteins: hemocyanin [10], C-reactive proteins (CRPs) [11••] and α2-macroglobulin [12]. The circulating hemocytes are extremely sensitive to LPS, and respond by degranulating the granular components after LPS-mediated stimulation, resulting in the formation of hemolymph clots [13•,14]. This response is thought to be very important for host defense that involves engulfing and killing invading microbes, in addition to preventing, by clot formation, the leakage of hemolymph, as described previously [5•].

A recent interesting finding is that the coagulation cascade of *T. tridentatus* is linked to the activation of the oxygen carrier hemocyanin functioning as a substitute for prophenoloxidase [15]. *Tachypleus* clotting enzyme, in addition to the active-site-masked (i.e. inactive) enzyme, transforms hemocyanin functionally to phenoloxidase, and the conversion reaches a plateau at 1:1 stoichiometry without proteolytic cleavage. Furthermore, this transformation of hemocyanin to phenoloxidase is also induced in the

Figure 1



The principal defense systems linked to phagocytosis in invertebrate animals. The innate immunity systems in arthropods include hemolymph coagulation [5•,37–40], melanization mediated by phenoloxidase [41,42], expression of antimicrobial peptides mediated by Toll-like receptors [43–47], and the lectin/complement pathway mediated by bacterial cell wall components such as LPS, peptidoglycan, muramyl peptide, (1, 3)-β-D-glucan and LTA [34••,41,48•,49,50]. The invaders detected by these systems are ultimately engulfed by phagocytes [51], such as macrophage-like, neutrophil-like and dendritic-like cells, internalized into phagosomes, processed and killed.

presence of tachyplesin — a major antimicrobial peptide with amphiphilic structure — although the detailed mechanism of these transformations is not known [16].

Antimicrobial polypeptides and peptides

Hemocytes of the horseshoe crab contain in their S-granules a family of arthropodous peptide antibiotics called the 'tachyplesin' family, as well as tachycitin and tachystatin, and in their L-granules an antibacterial protein called anti-LPS factor (Table 1). The S-granule-drived antimicrobial peptides bind to chitin but not to other polysaccharides, such as cellulose, mannan, xylan and laminarin [6].

Big defensin

In continuing studies on granular components, my co-workers and I have recently identified a novel defensin-like substance that is present in both L- and S-granules (Table 1) [17]. This substance strongly inhibits the growth of Gram-negative and -positive bacteria, and fungi such as *Candida albicans* (Table 2). The isolated substance, termed 'big defensin', comprises 79 amino acid residues of which the carboxy-terminal 37 residues have a sequence that is similar to those of mammalian neutrophil-derived defensins [18]. But big defensin is distinct from the mammalian defensins in size, the latter of which commonly have only 29–34 residues [19].

Notably, the disulfide motif in big defensin is identical to that of β-defensins from bovine neutrophils but not to that of classical defensins such as rat NP-2 defensin [18]. Furthermore, the structural organization of big defensin differs markedly from those of insect defensins, not only in disulfide bridge locations but also in molecular size [20]. As discussed further in [5•], a type of defensin-like domain, named the 'clip' domain, is also found in the amino-terminal portions of horseshoe crab clotting factors, factors B and D, and proclotting enzyme; this domain is likely to be released during the hemolymph coagulation cascade.

Tachystatin

A novel tachystatin family exhibits a broad spectrum of antimicrobial activity against Gram-negative and -positive bacteria, and fungi [21]. Of these tachystatins, tachystatin C is the most effective (Table 2). Tachystatin A is homologous to tachystatin B, but tachystatin C has no significant sequence similarity with tachystatins A and B. Both tachystatins A and B show sequence similarity to ω-agatoxin-IVA and its analogs of funnel web spider venom — a potent blocker of voltage-dependent calcium channels [21]. However, they exhibit no blocking activity, which differs from ω-agatoxin in functional activity. Tachystatin C also shows sequence similarity to several insecticidal neurotoxins of spider venom [21]. As the horseshoe crab is a close relative of the spider, tachystatins and spider neurotoxins may have evolved from a common ancestral peptide with adaptive functions.

Tachycitin

Tachycitin consists of 73 amino acid residues containing five disulfide bonds with no *N*-linked sugar [22]. The antimicrobial activity of tachycitin is not so strong by itself (Table 2); however, tachycitin synergistically enhances the antimicrobial activity of big defensin. The concentration of big defensin needed to reduce the growth of Gram-negative bacteria to 50% (i.e. half-maximal inhibitory concentration [IC_{50}]) is decreased to one-fiftieth of the normal value in the presence of a small amount of tachycitin. As shown in Figure 2, the three-dimensional (3D) structure of tachycitin is largely divided into the amino- and the carboxy-terminal domains [23]. In the latter, the structure

Table 1

Defense molecules found in hemocytes and hemolymph plasma of the horseshoe crab.

Proteins and peptides	Mass (kDa)	Function/specificity	Localization
Coagulation factors			
Factor C	123	Serine protease	L-granule
Factor B	64	Serine protease	L-granule
Factor G	110	Serine protease	L-granule
Proclotting enzyme	54	Serine protease	L-granule
Coagulogen	20	Gelation	L-granule
Protease inhibitors			
LICI-1	48	Serpin/factor-C	L-granule
LICI-2	42	Serpin/clotting enzyme	L-granule
LICI-3	53	Serpin/factor-G	L-granule
Trypsin inhibitor	6.8	Kunitz type	ND
LTI	16	New type	ND
LEBP-PI	12	New type	L-granule
Limulus cystatin	12.6	Cystatin family 2	L-granule
α2-Macroglobulin	180	Complement	Plasma and L-granule
Chymotrypsin inhibitor	10	ND	Plasma
Antimicrobial substances			
Anti-LPS factor	12	GNB	L-granule
Tachyplesins	2.3	GNB, GPB, FN	S-granule
Polyphemusins	2.3	GNB, GPB, FN	S-granule
Big defensin	8.6	GNB, GPB, FN	L- and S-granules
Tachycitin	8.3	GNB, GPB, FN	S-granule
Tachystatins	6.5	GNB, GPB, FN	S-granule
Factor D	42	GNB	L-granule
Lectins			
TL-1	27	LPS (KDO), LTA	L-granule
TL-2	27	GlcNAc, LTA	L-granule
TL-3	15	LPS (*O*-antigen)	L-granule
TL-4	470	LPS (*O*-antigen), LTA	ND
TL-5	380–440	*N*-acetyl group	Plasma
Limunectin	54	PC	L-granule
18K-LAF	18	Hemocyte aggregation	L-granule
Limulin	300	HLA/PC, PE, SA, KDO	Plasma
LCRP	300	PC, PE	Plasma
tCRP-1	300	PE	Plasma
tCRP-2	330	HLA/PE, SA	Plasma
tCRP-3	340	HLA/SA, KDO	Plasma
Polyphemin	ND	LTA, GlcNAc	Plasma
TTA	ND	SA, GlcNAc, GalNAc	Plasma
Liphemin	400–500	SA	Hemolymph
Carcinoscorpin	420	SA, KDO	Hemolymph
GBP	40	Gal	Hemolymph
PAP	40	Protein A	Hemolymph
Others			
Transglutaminase	86	Crosslinking	Cytosol
8.6 kDa protein	8.6	TGase substrate	L-granule
Pro-rich protein	80	TGase substrate	L-granule
Limulus kexin	70	Precursor processing	ND
Hemocyanin	3600	O_2 transporter (PO activity)	Plasma
Toll-like receptor (tToll)	110	ND	Hemocyte
L1	11	Unknown	L-granule
L4	11	Unknown	L-granule

FN, fungus; GBP, galactose-binding protein; GNB, Gram-negative bacteria; GPB, Gram-positive bacteria; HLA, hemolytic activity; LAF *Limulus* 18-kDa agglutination-aggregation factor; LCRP, *Limulus* C-reactive protein; LEBP-PI, *Limulus* endotoxin-binding protein-protease inhibitor; LICI, *Limulus* intracellular coagulation inhibitor; LTI, *Limulus* trypsin inhibitor; ND, not determined; PAP, protein A binding protein; PC, phosphorylcholine; PE, phosphorylethanolamine; PO, phenoloxidase; TTA, *T. tridentatus* agglutinin.

adopts a hairpin loop with a two-stranded β sheet, which mimics the structure of the chitin-binding site of hevein — an antifungal peptide isolated from the rubber tree *Havea basiliensis* [23] — although there is no sequence similarity.

Table 2

Antimicrobial activities (IC_{50} μg/ml)* of the horseshoe crab hemocyte-derived proteins and peptides.

Protein/peptide	*E. coli K12*	*Staphylococcus aureus 2O9P*	*C. albicans M9*	*Picia pastoris*
Tachyplesin	< 2.5	0.3	0.2	0.1
Polyphemusin	6.3	6.3	6.3	ND
Big defensin	2.5	< 2.5	20	42
Tachystatin A	25	4.2	3.0	0.5
Tachystatin B	NI	7.4	3.0	0.1
Tachystatin C	1.2	0.8	0.9	0.3
Tachycitin	33	56	52	41
Anti-LPS factor	> 20	1.3	ND	ND
Factor D	36	NI	ND	ND
Limulus cystatin	86	> 100	ND	ND

*Half-maximal inhibitory concentration. ND, not determined; NI, no inhibition at 100 μg/ml.

As well as tachycitin, tachyplesin, which has strong chitin-binding ability, contains a similar hairpin loop of a two-stranded β sheet (Figure 2) [5•]. In both tachycitin and tachyplesin, therefore, the hydrophobic residues that are clustered on the one face of their β-hairpin loops probably function as the chitin-binding sites (Figure 2). Chitin is a component of the cell wall of fungi and is also a principal structural component of arthropod exoskeletons, such as the helmet. Thus, the antimicrobial substances released from hemocytes probably recognize chitin exposed at the site of a lesion. They appear to function not only as antibacterial molecules against invading microbes but also in wound healing, where they may stimulate and accelerate biosynthesis of chitin at the sites of injury.

Tachylectins and their related bacterial agglutinins

The innate immune system of the horseshoe crab recognizes invading pathogens not only through biosensers of clotting factors C and G, but also through a combinatorial method using lectins with different specificities for carbohydrates exposed on pathogens [9]. Five types of lectins, named tachylectin (TL)-1 to -5, and several bacterial agglutinins have been identified recently in circulating hemocytes and hemolymph plasma [5•,6]. These TLs may function synergistically to accomplish an effective host defense against invading microbes and foreign substances.

Tachylectin-1

The hemocyte-derived TL-1 interacts with Gram-negative bacteria probably through 2-keto-3-deoxyoctonate (KDO), one of the constituents of LPS [24]. Neither TL-1 nor the antibacterial protein anti-LPS factor [5•] exhibits hemagglutinating activity, but TL-1 causes the agglutination of sheep erythrocytes coated with LPS. TL-1 is a single-chain protein consisting of 221 amino acid residues with no *N*-linked sugar chain, and contains three intrachain disulfide bonds and a free cysteine residue.

An outstanding structural feature of TL-1 is its six tandem repeats. Gel filtration chromatography indicates that TL-1 exists as a monomer in solution. The 3D structure of TL-1 is mainly dominated by six β sheets, each of which corresponds to the six tandem repeats. It looks like a six-bladed propeller structure (Figure 2). A zinc atom is located in the center and coordinated with three aspartate residues, two serine residues and one crystal water. The center of TL-1 is also clustered with six lysine residues contained in the six repeated structures and forms a hole that may interact with trisaccharide containing KDO (for more details see [9]).

Interestingly, an isolectin named TL-P, which has a similar sequence to that of TL-1, has been found in the perivitelline fluid of the horseshoe crab [25] (perivitelline fluid has an important role during embryogenesis). Moreover, tectonin, a homologue of TL-1 with unknown functions, has been isolated from the myxomycete *Physarum polycephalum* and shows 33% sequence identity to TL-1 [26]. This myxomycete in its plasmodial form feeds on bacteria and organic detritus by phagocytosis. Tectonin is located on the plasmodial surface and might therefore recognize the LPS of Gram-negative bacteria for phagocytosis, as well as have affinity for several saccharides for non-self recognition.

Tachylectin-2

The unique lectin TL-2 has hemagglutinating activity against human A-type erythrocytes [27]. TL-2 binds specifically to GlcNAc with a dissociation constant of 0.05 mM, and detailed sugar-binding analyses indicate that the acetamide group at the C-2 position and a free OH group at the C-4 position of GlcNAc are required for recognition by TL-2.

The most interesting feature of the sequence of TL-2, which comprises 236 amino acids, are five tandem repeats of 47 amino acids. TL-2 contains neither cysteine nor *N*- or *O*-linked sugars, and is present as a monomer in solution, as judged by ultracentrifugal analysis [27]. The X-ray structure of TL-2 in complex with GlcNAc has been solved at 2.0 Å resolution (Figure 2) [28•]. TL-2 is a five-bladed β-propeller structure and the single chain is organized in five β sheets, arranged in consecutive order with five-fold symmetry around a central tunnel.

The structure contains five equivalent binding sites, with virtually identical occupancy and geometry in the crystal. The GlcNAc points its acetamide group to the bottom of the binding pocket and the C-6 OH group points outside into solvent. TL-2 shows virtually no change in main or side-chain conformation on binding of GlcNAc. The root mean-square deviation between free TL-2 and ligand-bound TL-2 is only 0.18 Å, suggesting that there is no cooperativity for ligand binding.

The nature of the binding pocket explains the strict specificity of TL-2 toward GlcNAc. The exoskeleton of the horseshoe crab contains the ubiquitous polysaccharide chitin, which comprises β-1,4-linked GlcNAc. TL-2 shows no significant binding affinity for chitin, however, because there is no free 4-OH group in the β-1,4-linked GlcNAc units except for the non-reducing end. The mechanism of self/non-self distinction is reinforced by a distance of two individual binding sites of 25 Å and 40 Å, respectively, which seems to prevent multiple binding to distant terminal GlcNAc units of chitin. Therefore, the high valency of five binding sites of the single TL-2 molecule suggests the recognition of GlcNAc units on pathogens with a fairly high ligand density. Multiple binding of the five binding-sites to the repetitive structures on pathogens will generate very tight interactions [29].

Tachylectin-3

TL-3 exhibits hemagglutinating activity specifically against human A-type erythrocytes [30]. The hemagglutinating activity is equivalent to that of TL-2, but the activity is not inhibited by D-GlcNAc or D-GalNAc. Interestingly, the hemagglutinating activity is completely inhibited by a synthetic pentasaccharide of blood group A antigen and more strongly inhibited by *S*-type LPS from several Gram-negative bacteria at concentration ranges of 5–10 ng/ml, but not by the corresponding *R*-type LPS, indicating the high specificity of TL-3 for *O*-antigens. TL-3, which contains 123 amino acids, consists of two repeating sequences and is present as a dimer in solution [31].

Tachylectin-4

TL-4, which contains 232 amino acids, is an oligomeric glycoprotein of 470 kDa [32]. It has more potent hemagglutinating activity against human A-type erythrocytes than has TL-2 or TL-3. Although L-fucose and *N*-acetylneuraminic acid at 100 mM completely inhibit the activity of TL-4, the activity is more strongly inhibited by bacterial *S*-type LPS than by *R*-type LPS lacking *O*-antigen. The minimum concentration of *S*-type LPS required for inhibiting the agglutination of human A-type erythrocytes is 160-fold lower than that of *S*-type LPS from *Salmonella minnesota*. Therefore, colitose (3-deoxy-L-fucose) — a unique sugar present in the *O*-antigen of *Escherichia coli* O111: B4 with structural similarity to L-fucose — is the most probable candidate for a specific ligand of TL-4.

Tachylectin-5

Tachylectins-5A and -5B, which were identified recently in hemolymph plasma, show the strongest bacterial agglutinating activity among the five types of tachylectins, and exhibit broad specificity for substances containing *N*-acetyl groups [33]. They cause the agglutination of all types of human erythocytes, indicating that the primary recognition substance is not a blood group antigen. Their hemagglutinating activities are inhibited by the metal-ion chelator EDTA (5 mM), and this inhibition is neutralized by adding an excess amount of calcium chloride. Both lectins specifically recognize acetyl-group-containing substances including noncarbohydrates; the acetyl group only is required, and is sufficient for recognition. They also strongly agglutinate Gram-negative and Gram-positive bacteria. TL-5A is synthesized in heart and intestine, whereas TL-5B is synthesized only in hemocytes [33]. The overall sequence identity between the 269 amino acid residues of TL-5A and the 289 residues of TL-5B is 45%. They have significant sequence similarity with the carboxy-terminal fibrinogen-like globular domain. Interestingly, a collagenous domain found in ficolins is missing in the corresponding regions of TL-5A and -5B. Because filcolin, like mannan-binding lectin, is known to participate in a novel lectin pathway of the complement system [34••,35], TL-5A and -5B — which are structural homologs of ficolin — may also trigger the activation of the horseshoe crab complement system.

The X-ray crystal structure of TL-5A in complex with GlcNAc has been solved recently by multiple isomorphous replacement (Figure 2). It is an ellipsoidal molecule with overall dimensions of roughly $34 \times 36 \times 53$ Å, and is subdivided into three distinct but interacting domains. In more detailed studies of the three distinct domains, the calcium-binding site and the acetyl-group-binding pocket have been elucidated [36]. In addition to these results, a systematic computer search for sequence homologies revealed that TL-5A is structurally related to the γ fragment of fibrinogen (root mean-square deviation 1.09 Å overall), thus verifying our recent prediction made on the basis of amino acid sequence similarity [33]. On the basis of electron microscopy images, TL-5A forms a three- or four-bladed propeller structure, whereas TL-5B forms a two-bladed structure. If the ligand-binding site (or sites) are located in each blade, then TL-5A and -5B could have more avidity for ligands with a high density on pathogens. Their polyvalent bindings of acetyl groups must be a key factor in binding to invading microorganisms, and they function as non-self-recognizing proteins.

C-reactive proteins

The hemolymph of horseshoe crabs contains another class of bacterial agglutinins that are structurally related to mammalian CRP [11••]. CRP was first recognized in human plasma as a non-immunoglobulin that precipitated with C-polysaccharides derived from the cell wall of *Streptococcus pneunomiae* [11••]. Limulin, a sialic-acid

Figure 2

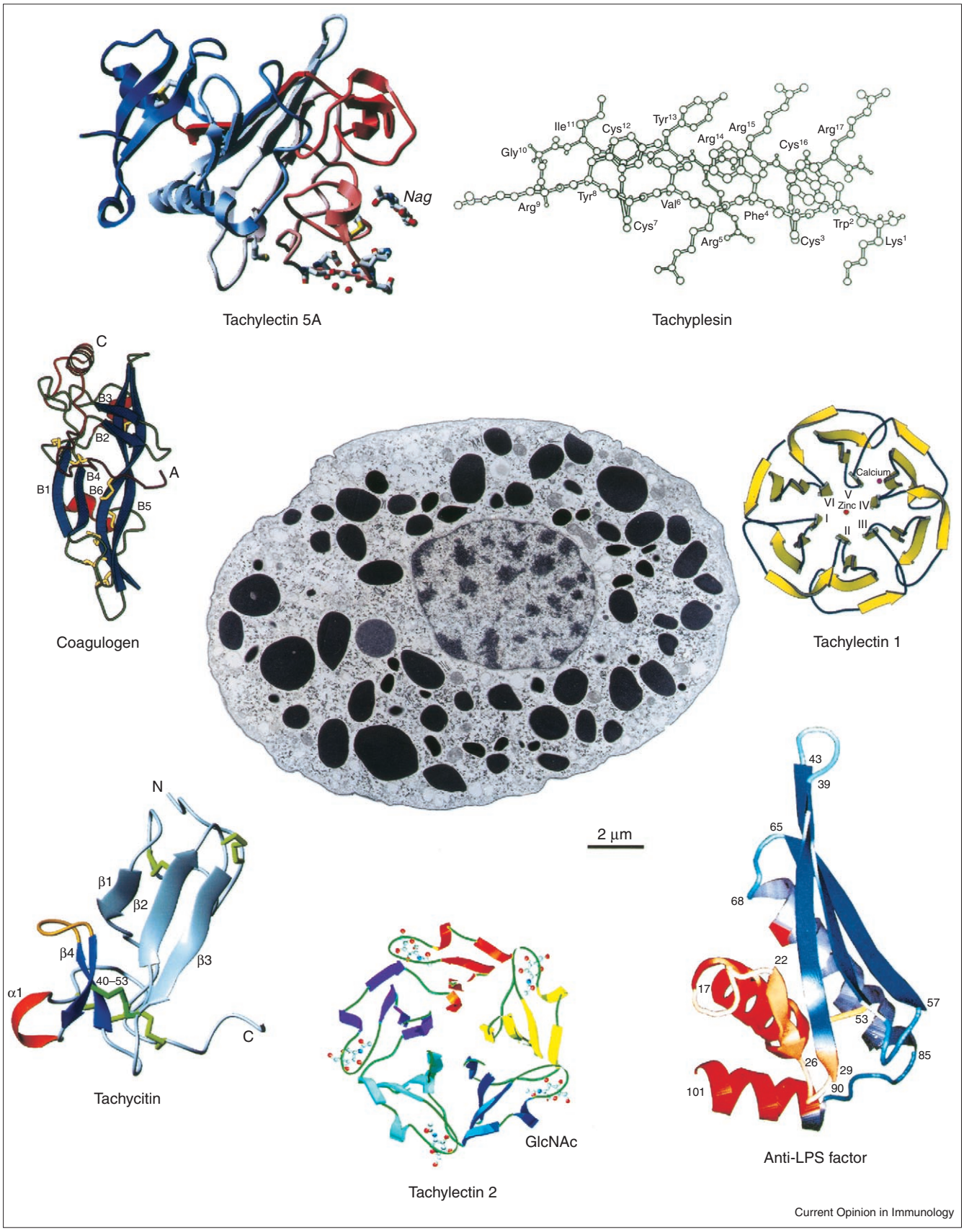

Nag
Tachylectin 5A
Ile11
Cys12
Tyr13
Arg15
Arg14
Arg17
Cys16
Gly10
Arg9
Tyr8
Val6
Phe4
Trp2
Cys7
Arg5
Cys3
Lys1
Tachyplesin
C
B3
B2
B4
A
B1
B6
B5
Coagulogen
2 μm
Calcium
VI
V
Zinc
IV
I
II
III
Tachylectin 1
N
β1
β2
β4
β3
40–53
α1
C
Tachycitin
GlcNAc
Tachylectin 2
43
39
65
68
22
17
57
53
26
29
85
101
90
Anti-LPS factor
Current Opinion in Immunology

Figure 2 legend

The 3D structures of the main defense molecules isolated from horseshoe crab *T. tridentatus* hemocytes and hemolymph plasma. A cross-section of a granular hemocyte under electron microscopy is also shown, in the center. Coagulogen, tachylectins-1, and -2, and anti-LPS factor are stored in L-granules [5•]. The antimicrobial peptides tachyplesin [7,8,52] and tachycitin are located in S-granules [17]. Tachylectin 5A is mainly found in hemolymph plasma [33].

(SA)- and phosphorylethanolamine-binding hemagglutinin in the hemolymph plasma of *Limulus polyphemus*, is a hemolytic CRP [9]. Three types of CRPs in the plasma of *T. tridentatus* have been purified on the basis of their different affinities to fetuin–agarose and phosphorylethanolamine–agarose. [11••]. These CRPs are named *T. tridentatus* CRP (tCRP)-1, tCRP-2 and tCRP-3 and each of these proteins consists of several isoforms. tCRP-2 and tCRP-3, but not tCRP-1, cause the agglutination of mammalian erythrocytes.

Of the three CRPs, tCRP-1 is the most abundant and exhibits the highest affinity to the phosphorylethanolamine–protein conjugate but lacks both sialic-acid-binding and hemolytic activities. tCRP-1 binds to both fetuin–agarose and phosphorylethanolamine–agarose, and exhibits Ca^{2+}-dependent hemolytic and sialic-acid-binding activities. Furthermore, tCRP-2 exhibits a higher affinity to colominic acid, a bacterial polysialic acid, than to sialic acid. By contrast, tCRP-3 shows stronger hemolytic, sialic-acid-binding and hemagglutinating activities than those of tCRP-2, but has no affinity for phosphorylethanolamine–agarose or colominic acid. tCRP-3 therefore represents a novel hemolytic CRP in that it lacks a common characteristic of CRPs — that is, a binding affinity for phosphorylethanolamine–agarose.

Twenty-two clones of tCRPs with different amino acid sequences have been identified [11••]. These tCRP clones possess high molecular diversity. Only tCRP-3 contains a hydrophobic nonapeptide sequence that appears in the transmembrane domain of an MHC class I heavy chain of rainbow trout, suggesting the importance of the hydrophobic patch to the hemolytic activity of tCRP-3. The structural and functional diversities of tCRPs provide a good model for study of properties of innate immunity in invertebrates, which survive without the benefit of acquired immunity.

Conclusions

The defense molecules actively at work in innate immunity are structurally and functionally very similar even between horseshoe crab and vertebrate animals. Innate immunity requires adequately specific biosensors that can react with various epitopes consisting of slightly different structures on a variety of pathogens, but must also distinguish between 'self' and 'non-self' epitopes. Examples of such specificity can be found in the activation of the horseshoe crab clotting factors C and G, both of which are sensitive to specific major bacterial cell wall components, such as LPS and (1,3)-β-D-glucan. The clotting system initiated by pathogens is important not only in hemostasis, but also in immobilizing invaders as part of the defense system. In addition, the circulating hemocytes recognize invading pathogens through a combinatorial method using many lectins with different specificities for carbohydrates exposed on pathogens. These clotting factors and lectins are stored mainly in L-granules in the circulating hemocytes. By contrast, antimicrobial substances including tachyplesins, tachycitin, tachystatins and big defensin, which kill invaders either directly or synergistically, are stored in S-granules. In response to foreign organisms, the contents of both granule populations are released into hemolymph through exocytosis, where they work together to immobilize and kill invaders. The hemolymph plasma containing CRPs and α_2-macroglobulin also exhibits cytolytic and opsonic activities against foreign cells, similar to the mammalian complement system. These cellular and humoral defense systems, which together comprise innate immunity, effectively defend the horseshoe crab from invading microbes.

Such a sophisticated defense system has allowed the horseshoe crab to survive for more than 200 million years. As mentioned here, much research has been conducted on the horseshoe crab hemocyte during the past two decades. However, there is as yet little knowledge as to the mechanisms of LPS-mediated degranulation of hemocytes. Further research is needed to characterize the phenomenon, specify the signaling pathway that is linked to the degranulation and examine the structural and functional aspects of the receptor molecules that recognize 'non-self' epitopes.

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- •• of outstanding interest

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