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New Types of Clotting Factors and Defense Molecules Found in Horseshoe Crab Hemolymph: Their Structures and Functions[1]

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Invertebrate animals, which lack adaptive immune systems, have developed defense systems, so-called innate immunity, that respond to common antigens on the surface of potential pathogens. One such defense system is involved in the cellular responses of horseshoe crab hemocytes to invaders. Hemocytes contain two types, large (L) and small (S), of secretory granules, and the contents of these granules are released in response to invading microbes *via* exocytosis. Recent biochemical and immunological studies on the granular components of L- and S-granules demonstrated that the two types of granules selectively store granule-specific proteins participating in the host defense systems. L-Granules contain all the clotting factors essential for hemolymph coagulation, protease inhibitors including serpins and cystatin, and anti-lipopolysaccharide (LPS) factor and several tachylectins with LPS binding and bacterial agglutinating activities. On the other hand, S-granules contain various new cysteine-rich basic proteins with antimicrobial or bacterial agglutinating activities, such as tachyplesins, big defensin, tachycitin, and tachystatins. The co-localization of these proteins in the granules and their release into the hemolymph suggest that they serve synergistically to construct an effective host defense system against invaders. Here, the structures and functions of these new types of defense molecules found in the Japanese horseshoe crab (*Tachypleus tridentatus*) are reviewed.

Key words: (1,3)-β-D-glucans, horseshoe crab, host defense, innate immunity, lipopolysaccharides.

Invertebrate defense systems, so-called innate immunity, include hemolymph coagulation, melanization, cytolysis, cell agglutination, antimicrobial actions, and phagocytosis against pathogens. Among them, hemolymph coagulation and phenoloxidase-mediated melanization are induced by foreign substances, that result in the engulfment of invading microorganisms. These immobilized invaders are finally killed by antimicrobial substances released mainly from various hemocytes (*1*).

In spite of the absence of immunoglobulin itself, the invertebrate hemolymph is capable of responding with a high degree of specificity against invaders. For instance, hemolymph clotting factors, complements and many lectins found in hemolymph may serve recognitive roles as antibodies or opsonins. Other recognition molecules in innate immunity are found in the prophenoloxidase activating system of insects (*2*). Although biochemical studies on these molecules are currently in progress (*3*), the authors will focus here on the present state of our knowledge concerning the clotting factors and new defense molecules in the horseshoe crab.

1. The functional role of horseshoe crab hemolymph in innate immunity

In the horseshoe crab (Fig. 1), one of the major defense systems is carried by the hemolymph, which contains at least two types of hemocytes, granular and non-granular cells (*4*). Table I summarizes the proteins and peptides so far found in horseshoe crab hemocytes and the hemolymph plasma (*5-11*). These components, including clotting factors, protease inhibitors, antibacterial substances, lectins, and others, are closely associated with the host defense of this animal. The hemolymph plasma contains three major proteins: hemocyanin, limulin (lectin)/C-reactive proteins, and α_2-macroglobulin (*12, 13*). On the other hand, the granular cells/amebocytes comprise 99% of the total hemocytes, which are filled with two populations of secretory granules, named large (L) and small (S) granules (*14*). These can be easily distinguished on electron microscopy due to their different electron densities. The cells are highly sensitive to lipopolysaccharides (LPS), a major outer membrane component of Gram-negative bacteria, and respond by degranulating these granules after stimulation by LPS. This response is thought to be very important for host defense involving the engulfing and killing invading

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Abbreviations: L-granule, large granule; S-granule, small granule; LPS, lipopolysaccharides; LICI, limulus intracellular coagulation inhibitors; α_2M, α_2-macroglobulin; TGase, transglutaminase; DCA, dansylcadaverine; T., *Tachypleus*; L., *Limulus*; C., *Carcinoscorpius*.

Fig. 1. **A mating couple of the Japanese horseshoe crab (*Tachypleus tridentatus*), "Kabutogani (helmet crab)" in Japanese.**

microbes, in addition to preventing the leakage of hemolymph.

In 1975, Mürer *et al.* (*15*) reported the isolation of L-granules and showed that an extract of the granules was caused to gel by LPS. More recently, we succeeded in separately isolating L- and S-granules (*16*) (Fig. 2). The separation of these granules made it possible to identify various new granular components (Table I). The L-granules contain more than 20 proteins, the majority of which have molecular masses between 8 and 123 kDa (*16*). In contrast, the S-granules contain at least 6 proteins with molecular masses of less than 30 kDa, in addition to an antimicrobial peptide tachyplesin and its analogues. More recent studies on S-granules demonstrated that they store selectively not only tachyplesins but also tachycitin, tachystatins, and big defensin (also contained in L-granules), all of which show antimicrobial activity against Gram-negative and -positive bacteria, and fungi, as described later (*7, 17-19*).

Figure 3 shows the defense systems found in hemocytes of the horseshoe crab. When Gram-negative bacteria invade the hemolymph, the hemocytes detect LPS on their surface, and then release the contents of granules through rapid exocytosis. The released granular components include two biosensors of the coagulation reaction, factor C and factor G. These serine protease zymogens are autocatalytically activated by LPS and (1,3)-β-D-glucans, which are major cell wall components of Gram-negative bacteria and fungi, respectively. The activation of these two zymogens triggers the coagulation cascades resulting in the conversion of coagulogen to an insoluble coagulin gel. In parallel with the process of coagulin gel formation, various agglutinins/lectins that induce cell aggregation are released from L-granules (*4-10, 12*). Thus, the invaders in the hemolymph are engulfed and immobilized by the clot, and subsequently killed by antimicrobial substances that are released from the two types of granules.

2. LPS-mediated coagulation cascade

The LPS-mediated coagulation cascade involves three serine protease zymogens, factor C, factor B, and proclotting enzyme, and a clottable protein, coagulogen (Table I). Factor C (123 kDa) is a biosensor that responds to LPS (*20-25*). In the presence of LPS or synthetic lipid A analogs, it is autocatalytically converted to an active form, factor $\bar{C}$. Factor B (64 kDa) is then activated by factor $\bar{C}$ and, in turn, its active form (factor $\bar{B}$) converts proclotting enzyme to clotting enzyme (54 kDa) (*26-30*). The active clotting enzyme converts coagulogen to an insoluble coagulin gel (Fig. 3).

Figure 4 summarizes the gross structures of these new clotting factors. All the factors except for coagulogen are typical glycoproteins and differ from each other in molecular mass. The initiation factor, factor C sensitive to LPS, comprises one EGF-like domain, five short consensus repeats (SCR, also called CCP or the sushi domain) found mainly in mammalian complements, one C-type lectin domain, and a serine protease domain, the latter of which is located in the COOH-terminal portion (*25*). The finding of SCR in factor C makes it the first protein in invertebrates that has been discovered to contain this type of domain. The fact that this initiator of the clotting cascade contains SCR led us to speculate that coagulation and complement systems may have evolved from a common origin.

Both factor B and proclotting enzyme are similar in the domain structure to each other (Fig. 4) (*28-30*). In addition to the COOH-terminal protease domain, both clotting factors contain a "clip"-like domain (formerly called the "disulfide-knotted" domain) in the NH_2-terminal light chain, and this portion shows sequence similarity to the NH_2-terminal light chain of *Drosophila* proteins, namely serine protease easter and snake precursors. Both easter and snake proteins are indispensable for normal embryogenesis in flies. The presence of this type of domain in *Drosophila* strongly suggests the existence of a protease cascade similar to that in the horseshoe crab (*30*).

Recently, we also found that the folding pattern of the three disulfide bridges in the "clip" domain is identical to that of "big defensin" isolated from horseshoe crab hemocytes (described later) (*9*). Since the COOH-terminal ends of the "clip" domain in both factor B and clotting enzyme constitute the hinge region susceptible to protease attack, these "clip" domains may be released through the activation of the zymogens to act as antimicrobial agents. If this is the case, the cascade itself could produce antimicrobial substances during the activation. The system may have dual actions, that is, coagulation and the killing of invaders (Fig. 5) (*9*). Therefore, the possible release of defensin homologs from factor B and proclotting enzyme strongly suggests that this coagulation cascade is closely associated with host defense.

In the final step of the clotting cascade, coagulogen, a 175 amino acid single chain polypeptide, is converted to insoluble coagulin through limited proteolyses at two sites (Arg18-Thr19 and Arg46-Gly47), as shown in Fig. 4 (*31-38*). Excision of intermediate peptide C (Thr19-Arg46) results in the formation of the coagulin monomer, AB, consisting of the NH_2-terminal A chain (Ala1-Arg18) and the COOH-terminal B chain (Gly47-Phe175) covalently linked *via* two disulfide bridges. The coagulin monomer self-aggregates to form a gel-like substance composed of polymerized AB monomers. Although a transglutaminase has been isolated from hemocytes (*39, 40*), coagulin itself is not cross-linked as in the case of fibrin clot or clottable proteins (vitellogenin) of insects and crustaceans (*41, 42*). The exact mechanism underlying this coagulin gel formation is as yet unknown, but recent X-ray analysis of

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

Proteins and peptides	Mass (kDa)	Function/specificity	Localization	References
Coagulation factors				
Factor C	123	Serine protease	L-granule	*20-26*
Factor B	64	Serine protease	ND	*29, 30*
Factor G	110	Serine protease	L-granule	*44-47*
Proclotting enzyme	54	Serine protease	L-granule	*27, 282*
Coagulogen	20	Gelation	L-granule	*31-38*
Protease inhibitors				
LICI-1	48	Serpin/factor C	L-granule	*48*
LICI-2	42	Serpin/clotting enzyme	L-granule	*49*
LICI-3	53	Serpin/factor G	L-granule	*50*
Trypsin inhibitor	6.8	Kunitz-type	ND	*102*
LTI	16	New type	ND	*103*
LEBP-PI	12	New type	L-granule	*83*
Limulus cystatin	12.6	Cystatin family 2	L-granule	*101*
α_2-Macroglobulin	180	Complement	Plasma & L-granule	*97, 98, 104*
Antimicrobial substances				
Anti-LPS factor	12	GNB	L-granule	*51-53*
Tachyplesins	2.3	GNB, GPB, FN	S-granule	*56-64*
Polyphemusins	2.3	GNB, GPB, FN	S-granule	*60*
Big defensin	8.6	GNB, GPB, FN	L & S-granule	*19, 73*
Tachycitin	8.3	GNB, GPB, FN	S-granule	*17, 18*
Tachystatins	6.5	GNB, GPB, FN	S-granule	Unpublished
Factor D	42	GNB	L-granule	*76*
Lectins				
Tachylectin-1	27	LPS (KDO), LTA	L-granule	*78*
Tachylectin-2	27	GlcNAc, LTA	L-granule	*84*
Tachylectin-3	15	LPS (O-antigen)	L-granule	Unpublished
Tachylectin-4	470	LPS (O-antigen), LTA	ND	*95*
Tachylectin-5	380-440	*N*-Acetyl group	ND	Unpublished
Limunectin	54	PC	L-granule	*90*
18K-LAF	18	Hemocyte aggregation	L-granule	*89*
Limulin	300	HLA/PC, PE, SA, KDO	Plasma	*79-82, 105*
LCRP	300	PC, PE	Plasma	*81, 82, 105*
TCRP-1	300	PE	Plasma	Unpublished
TCRP-2	330	HLA/PE, SA	Plasma	Unpublished
TCRP-3	340	HLA/SA, KDO	Plasma	Unpublished
Polyphemin	ND	LTA, GlcNAc	Plasma	*106*
TTA	ND	SA, GlcNAc, GalNAc	Plasma	*85-87*
Liphemin	400-500	SA	Hemolymph	*92, 107, 108*
Carcinoscorpin	420	SA, KDO	Hemolymph	*91, 109, 110*
Others				
Transglutaminase	86	Cross-linking	Cytosol	*39, 40*
8.6 kDa protein	8.6	TGase substrate	L-granule	*39*
Pro-rich protein	80	TGase substrate	L-granule	Unpublished
Limulus kexin	70	Precursor processing	ND	*111*
L1	11	Unknown	L-granule	Unpublished
L4	11	Unknown	L-granule	Unpublished

LICI, *Limulus* intracellular coagulation inhibitor; LTI, *Limulus* trypsin inhibitor; LEBP-PI, *Limulus* endotoxin-binding protein-protease inhibitor; GNB, Gram-negative bacteria; GPB, Gram-positive bacteria; FN, fungus; LPS, lipopolysaccharide; LAF, *Limulus* 18-kDa agglutination-aggregation factor; KDO, 2-keto-3-deoxyoctonic acid; PC, phosphorylcholine; PE, phosphorylethanolamine; SA, sialic acid; TTA, *Tachypleus tridentatus* agglutinin; LCRP, *Limulus* C-reactive protein; TCRP, *Tachypleus* C-reactive protein; HLA, hemolytic activity; LTA, lipoteichoic acid; ND, not determined.

coagulogen from the Japanese horseshoe crab provided a structural basis for understanding of the polymerization mechanism (*38*).

A stereo view of the coagulogen monomer, showing the A chain, peptide C, and B chain, and the secondary structure is presented in Fig. 6 (*38*). The coagulogen monomer is an elongated molecule of approximate dimensions, 60×30×20 Å. The structure is mainly dominated by the β-strands in blue, labeled sequentially B1 to B6. The multiple coils and turns of the B chain are colored green. Short helical segments in orange, in the B chain are in the background. The mainly α-helical peptide C, in red, covers a reasonable part of the top surface. This characteristic helix-wheel is embraced like a piece of sugar in tongs formed by the B chain segments. The NH_2-terminal A chain, in violet, is connected to the B chain *via* two disulfide bridges colored yellow. The whole cysteines comprise 8 disulfide bridges. The peptide C helix covers an extended hydrophobic core, which becomes accessible upon the cleavage and release of peptide C. Thus, the uncovered core newly exposed in one molecule after the release of peptide C might interact with a hydrophobic edge of a second molecule, like a head to tail, to form a multimer, as shown in Fig. 7. The mechanism for polymerization based on the conformational structure is now being studied in detail.

During these studies, we found that the COOH-terminal half of the coagulogen molecule exhibits striking topological similarity to the neutrophin nerve growth factor (NGF),

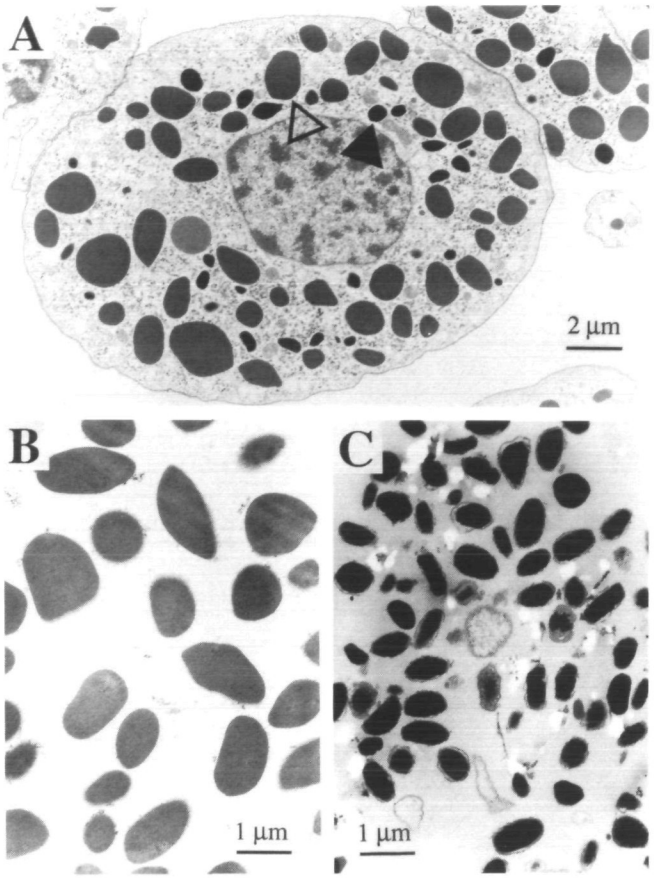

Fig. 2. **Electron micrographs of horseshoe crab hemocytes (A), and L- (B), and S- (C) granules isolated from the hemocytes (*16*).**

providing the first evidence for a neutrophin fold in invertebrates (*38*). The unusual large twist of the β-sheet found in coagulogen (Fig. 6) is also found in NGF. The 6 cysteines which link the two β-sheets oriented parallel to the molecular axis are strongly conserved and show the same disulfide linking pattern. Furthermore, sequence alignment based on topological equivalence showed significant sequence similarity of 21% in the topologically identical regions. Thus, coagulogen is assigned as a new member of the TGF β_2 superfamily (*43*).

3. (1,3)-β-D-Glucan-mediated coagulation cascade

As is well known, the LPS-mediated clotting system is the principle of the so-called *Limulus* test for the endotoxin assay. Because of its high sensitivity and convenience, this test is being widely utilized to detect and quantitate trace amounts of a contaminating pyrogen or LPS in the clinical field. However, during the diagnostic application of the *Limulus* test, it was pointed out that positive reactions were often observed in some patients' plasma even in the absence of LPS. Since some of these patients were suffering from a fungal infection, or were undergoing hemodialysis, this pseudopositive reaction had been suspected to be caused, at least in part, by glucans. In 1981, we and others found the presence of a possible protease zymogen sensitive to β-glucan in a hemocyte lysate (*44, 45*). Since then, the purification of this protein, named factor G, had been hampered by its instability. However, it was recently isolated in a pure form, which allowed the characterization of this novel zymogen.

The purified factor G zymogen is autocatalytically activated in the presence of (1,3)-β-D-glucan (*46*). The resulting active factor $\bar{G}$ activates proclotting enzyme directly, which is linked with the coagulin gel formation (Fig. 3). As factor G is colocalized in L-granules together with components participating in the LPS-mediated coagulation cascade, it can be released into the hemolymph upon cell activation. This β-glucan-mediated coagulation pathway could be activated on the surface of fungi.

Factor G is a heterodimer composed of two subunits, α and β, associated through non-covalent bonds (Fig. 4) (*46*). As the two subunits are derived from separate genes, they are translated independently and assembled in hemocytes (*47*). Subunit β is a serine protease zymogen with a short 15 amino acid NH_2-terminal extension. The serine protease domain is most homologous to factor B (40.5% amino acid identity) and proclotting enzyme (37.7% identity), suggesting that these three had a common origin. On the other hand, subunit α shows a unique mosaic structure. The NH_2-terminal region contains a bacterial β-(1,3) glucanase-like sequence. The COOH-terminal region has two tandem repeats, each of which shows sequence similarity with that found in xylanase Z. In the middle part of the molecule, there are three tandem-repeat structures. This type of tandem-repeat has been found in xylanase A, *Rarobacter* protease I, and ricin B chain (*47*).

In both subunits, an Arg15-Ile16 bond in β and an Arg150-Glu151 bond in α are cleaved in the autocatalytic activation process (*46*). However, none of the horseshoe crab clotting factors with trypsin-like specificity, or trypsin itself, activates zymogen factor G, suggesting that the cleavage site in subunit β is somehow masked by subunit α. Therefore, the binding of β-glucan to subunit α appears to expose the activation site of subunit β, which is hindered in the zymogen form, thus allowing autocatalytic activation through a bimolecular interaction between subunit βs. We suppose that some interaction of subunit α with (1,3)-β-D-glucan may induce a specific conformational change, resulting in the activation of subunit β. The purified factor G is also activated by other various glucans containing (1,3)-β linkages from different origins, but not by LPS, sulfatides, or cholesterol sulfates (*46*). The most effective activators are linear (1,3)-β-D-glucans, such as curdlan and paramylon. As little as 1 ng of curdlan significantly activates zymogen factor G (Fig. 8). Branching of the linear chain with (1,4)- or (1,6)-β-linkages appears to reduce the factor G activating activity. Shorter oligosaccharides containing two to seven glucose units do not activate factor G at all. Kinetic studies on the β-glucan-dose dependency of the factor G activation revealed a bell-shaped curve: activation is inhibited at higher concentrations of β-glucan. Under the optimum conditions, the molar ratio of factor G and β-glucan is constant, indicating that the activation of factor G occurs through an intermolecular interaction between each factor G molecule bound to β-glucan.

4. Regulation of the LPS- and (1,3)-β-D-glucan-mediated coagulation cascade

To date, three types of serpins have been isolated from horseshoe crab hemocytes, named limulus intracellular coagulation inhibitors, LICI-1, LICI-2, and LICI-3 (*48-*

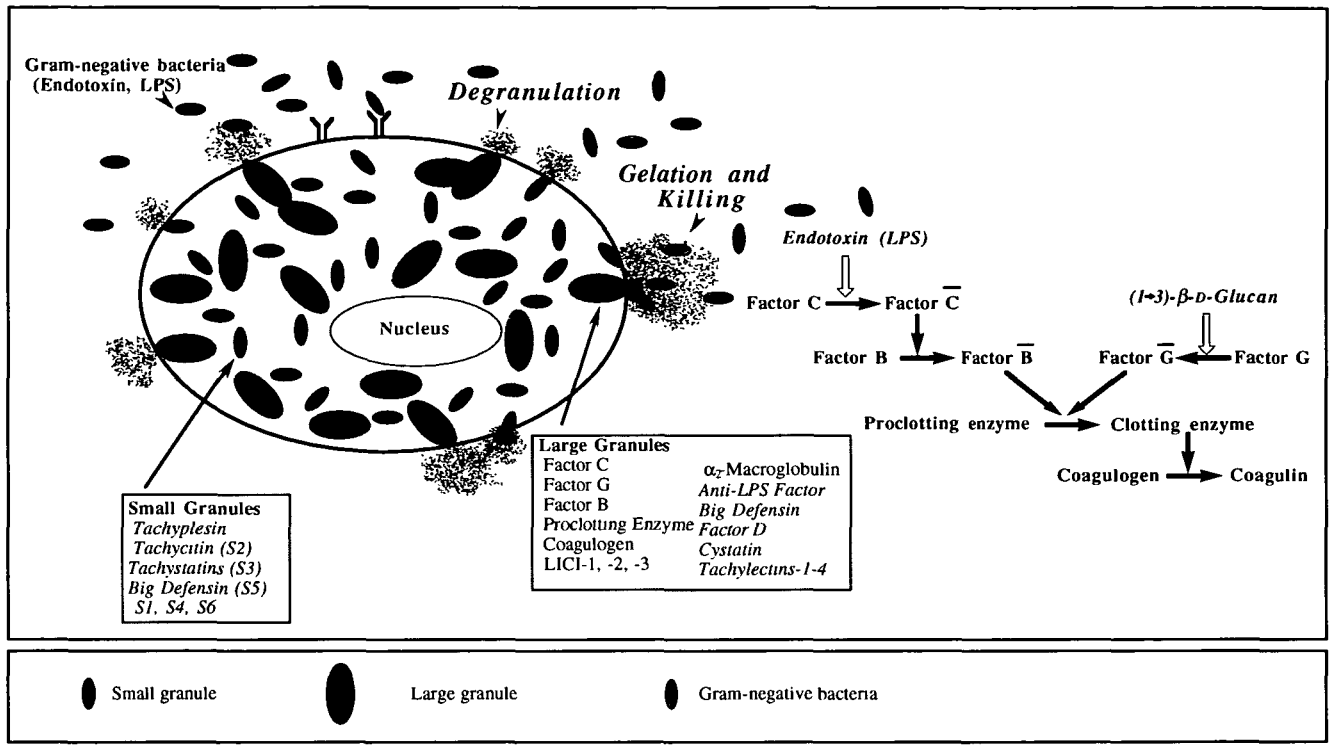

Fig. 3. **Defense systems in horseshoe crab hemocytes.** The hemocytes detect LPS on Gram-negative bacteria, and initiate exocytosis of the L- and S-granules. The clotting factors thus released are activated by LPS or (1,3)-β-D-glucan on the pathogens, which results in hemolymph coagulation. Thus, the pathogens are cell-agglutinated by various lectins and subsequently killed by antibacterial substances, such as tachyplesins, anti-LPS factor, big defensin, tachycitin, and tachystatins. The L-granules also contain protease inhibitors, such as serpins (LICI-1, -2, and -3), α_2-macroglobulin, and cystatin, and an azurocidin-like pseudoserine protease with antibacterial activity, named factor D.

50). All LICIs belong to the serpin family and form stable complexes with target serine proteases. Of these serpins, LICI-1 specifically inhibits factor C̄, while both LICI-2 and LICI-3 inhibit factor C̄, and factor Ḡ and the clotting enzyme activities. LICI-2 inhibits the clotting enzyme more strongly, and LICI-3 favors factor Ḡ more than other enzymes. All the inhibitors are stored in L-granules and are exocytosed upon activation of the cells. Thus, these inhibitors are likely to act to prevent diffusion of the active clotting factors, which may cause unnecessary clot formation. They may also function as scavengers of the proteases that have escaped into the hemolymph from the site of injury. It is of interest that one of the antimicrobial substances, big defensin (described later), is copurified during the purification of LICI-1 and that it interacts specifically only with LICI-1, *i.e.* not with LICI-2 or LICI-3 (*50*). Thus, the interaction may be of physiological importance in the neutralization or intercellular sorting of big defensin.

5. New defense molecules (antimicrobial substances) in hemolymph

5.1. Anti-LPS factor and tachyplesins. Anti-LPS factor (102 amino acid residues) and tachyplesins (17 residues for tachyplesin I) or polyphemusins (18 residues for polyphemusin I) were initially identified as defense molecules in hemocytes, both of which neutralize a variety of LPS activities (*51-64*). Anti-LPS factor is located in L-granules, together with several clotting factors and protease inhibitors, whereas peptide tachyplesins are exclusively concentrated in S-granules (Table I). In particular, the concentration of tachyplesins in hemocytes is extremely high, approximately 10 mg being present in the hemolymph of an individual horseshoe crab. Anti-LPS factor exhibits growth inhibitory activity against some strains of Gram-negative bacteria, probably through its ability of binding to the lipid A portion of LPS (*53*). On the other hand, tachyplesin significantly inhibits the growth of Gram-negative and Gram-positive bacteria and fungi. Tachyplesin increases the K^+ permeability of bacteria, such as *Staphylococcus aureus* and *Escherichia coli*, through a similar mechanism to that of gramicidin S, a peptide forming an amphiphilic structure analogous to tachyplesin (*61*).

The crystal structure of anti-LPS factor comprises a single domain consisting of three α-helices packed against a four-stranded β-sheet to form a wedge-shaped molecule with a striking charge distribution and amphipathicity (*62*). The binding site for LPS probably involves the extended amphipathic loop, which represents an LPS-binding motif shared by two mammalian proteins, LPS-binding protein (*65*) and bactericidal/permeability-increasing protein (*66*). As seen in NMR experiments, tachyplesin takes on a fairly rigid conformation constrained by two disulfide bridges and adopts a conformation consisting of an anti-parallel β-sheet connected by a β-turn with an amphipathic structure, which is presumed to be closely associated with the bactericidal activity (*63, 64*).

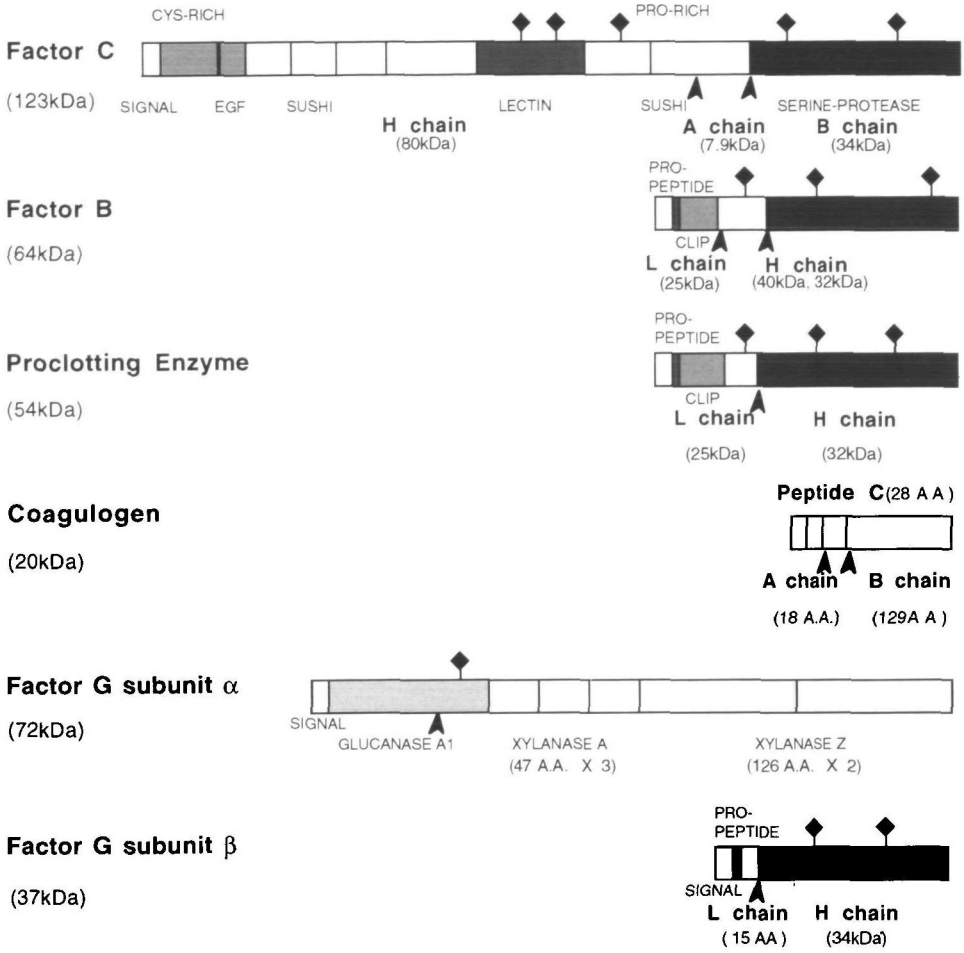

Fig. 4. **Domain structures of horseshoe crab clotting factors.** Coagulogen is a much smaller protein than the mammalian homolog, fibrinogen. The other clotting factors contain various unique domain structures, in addition to a serine protease domain located in the COOH-terminal portion. Arrowheads indicate cleavage sites for zymogen activations. The potential carbohydrate attachment sites are indicated by closed diamonds.

5.2. Big defensin. An antimicrobial substance of 8 kDa, named big defensin, was recently found in hemocyte granules (*19*). Big defensin consists of a total of 79 amino acid residues, its COOH-terminal region composed of 37 amino acids resembling that of mammalian defensins (*67*). Big defensin, however, is distinct from the mammalian defensins in molecular size, the latter of which have 29–34 amino acid residues in common (*67, 68*). The disulfide motif in the defensin-like domain of big defensin is identical to that of β-defensin from bovine neutrophils, but not to that of classical defensins. 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. Insect defensins isolated from various species, such as *Phormia terranovae* (*69*), *Sarcophaga peregrina* (*70, 71*), and *Holotrichia diompalia* (*72*), are cationic 34 to 43 residue peptides, all containing six cysteines involved in three intramolecular disulfide bridges. Therefore, the overall structure of big defensin is unique in the NH_2-terminal extension.

Big defensin shows a highly amphipathic nature, high hydrophobicity in the NH_2-terminal region, and hydrophilicity with clustering of cationic residues in the COOH-terminal region. This amphipathic character may be closely related to its biological activity, as many amphipathic peptides and proteins exhibit cytolytic and antibacterial activities. Big defensin shows strong antibacterial activity against not only Gram-negative and Gram-positive bacteria, but also fungi. This broad antimicrobial spectrum of big defensin is also a characteristic of the mammalian and insect defensins. A noteworthy characteristic of big defensin is that there is a functional difference between the NH_2-terminal portion comprising residues 1 to 37 and the COOH-terminal portion comprising residues 38 to 79. Although intact big defensin exhibits antibacterial activity against both *Salmonella typhimurium* and *Staphylococcus*

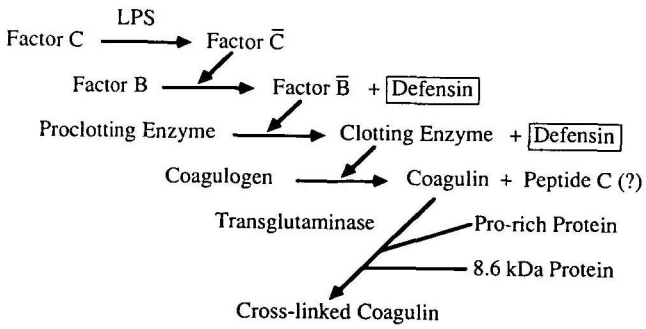

Fig. 5. **Dual actions of factor B and proclotting enzyme during the clotting cascade reaction.** The defensin-like domains, also named "clip"-domains, located in the NH_2-terminal portions of both clotting factors may be released during the cascade reaction and may act as antimicrobial substances for host defense (*28*).

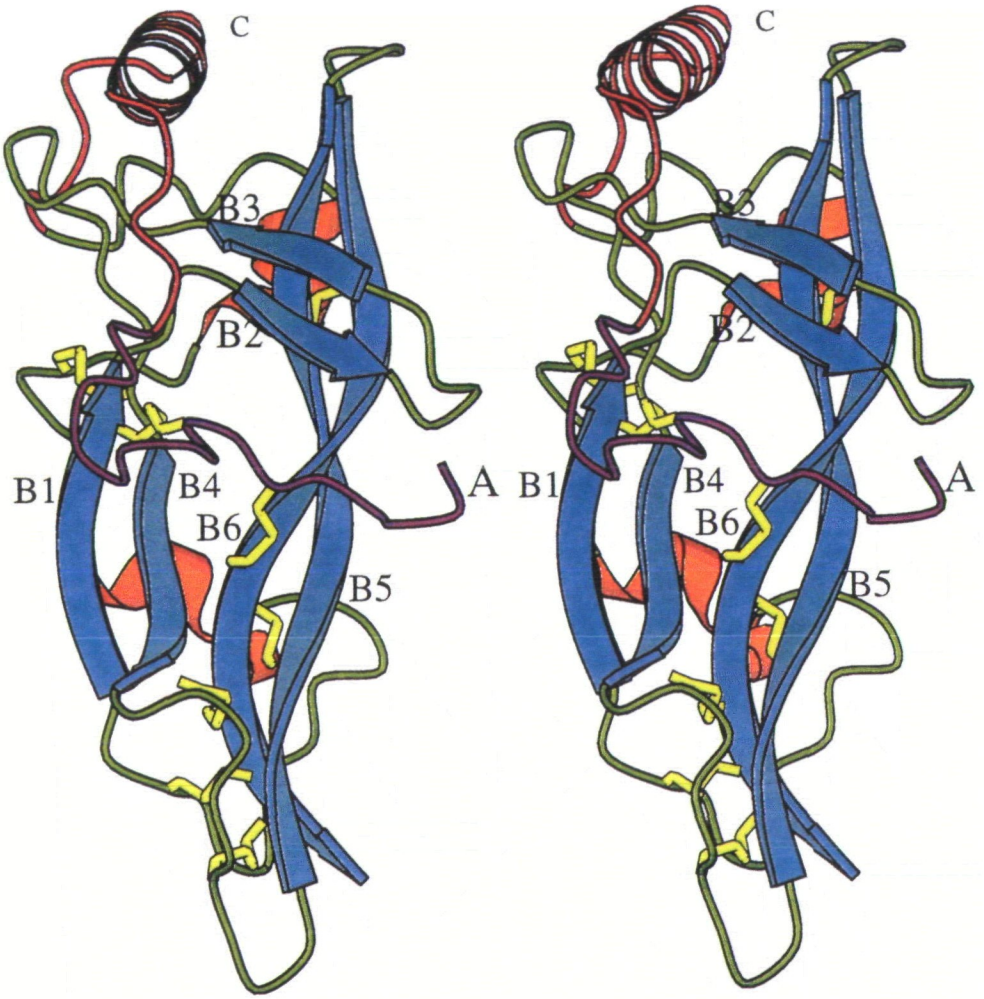

Fig. 6. **Stereo view of a coagulogen monomer showing the A chain, peptide C and B chain, and the secondary structure (*38*).** The structure is dominated by the β-strands (blue, labeled sequentially B1 to B6), and multiple coils and turns (green) of the B chain. The main α-helical peptide C (red), which is released upon cleavage, covers a reasonable part of the surface at the top. The NH_2-terminal A chain is connected to the B chain by two disulfide bridges (yellow). The whole cysteine-rich structure possesses eight disulfide bridges.

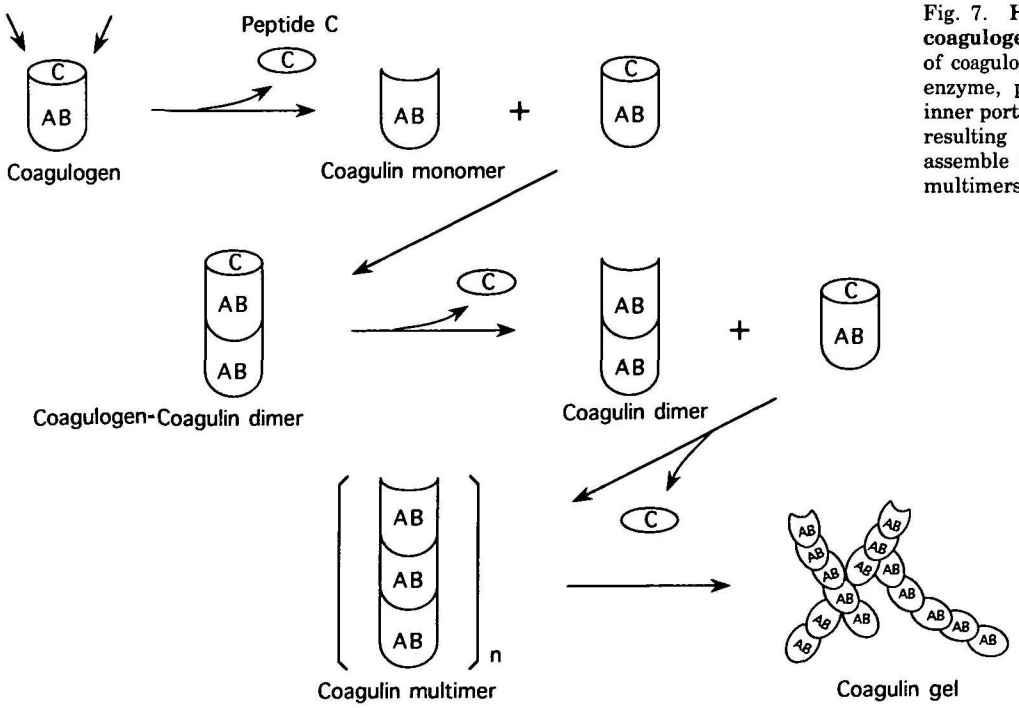

Fig. 7. **Hypothetical mechanism of coagulogen gel formation.** Upon gelation of coagulogen by a horseshoe crab clotting enzyme, peptide C is released from the inner portion of the parent molecules. The resulting coagulin monomer may self-assemble to form the dimer, trimer, and multimers (*38, 112*).

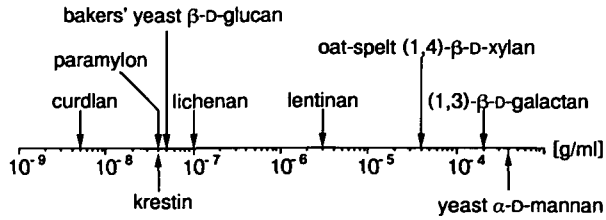

Fig. 8. **Concentrations of various glucans required for the activation of factor G zymogen.** The most effective activators so far examined are linear (1,3)-β-D-glucans, such as curdlan and paramylon (*46*).

aureus, the NH_2-terminal hydrophobic fragment is more effective than the COOH-terminal defensin fragment against Gram-positive bacteria. In contrast, the COOH-terminal fragment displays more potent activity than the NH_2-terminal hydrophobic fragment against Gram-negative bacteria, suggesting a kind of chimera molecule. Therefore, big defensin may prove to represent a new class of defensin family possessing two functional domains with different antibacterial activities.

Big defensin exhibits no hemolytic activity against sheep erythrocytes but it shows erythrocyte-agglutinating activity, so-called LPS-binding activity, with sheep erythrocytes sensitized with *Salmonella minnesota* Re-LPS. However, the NH_2- and COOH-terminal fragments, in addition to *S*-alkylated big defensin, exhibit weak LPS-binding activity, indicating that the native conformation of the entire molecule is required for its binding with LPS. Big defensin was shown to be localized in both L- and S-granules by immunoblotting and immuno-gold staining analyses involving anti-big defensin antiserum, but isoforms of big defensin may exist in L- and S-granules (*73*).

5.3. Tachycitin. As reported previously, S-granules, in addition to tachyplesins and big defensin, contain at least six cysteine-rich basic proteins, designated numerically as S1 to S6, based on their retention times on reversed phase HPLC (*16*). Among these cysteine-rich proteins, S2, now named tachycitin, was recently characterized (*17*). Tachycitin has a molecular weight of 8,363, and exhibits broad antibacterial activity against Gram-negative and Gram-positive bacteria, and fungi, but the IC_{50} values (50% inhibitory concentration) are 5 to 70-fold higher than those of big defensin. Tachycitin exhibits significant binding ability as to an LPS-immobilized nitrocellulose membrane, and it agglutinates *E. coli* and *Enterococcus hirae*.

Tachycitin consists of 73 amino acid residues including ten half-cystines, and the precursor has an NH_2-terminal signal sequence of 22 residues. Tachyplesin and big defensin are synthesized as preproproteins with a propeptide on the COOH-terminal and NH_2-terminal sides of the mature protein, respectively, whereas tachycitin has no propeptide. The COOH-terminal threonine of tachycitin is amidated, as found for tachyplesin (*17*). The NH_2-terminal 28 residues of tachycitin show sequence homology to a part of the chitin-binding regions found in antifungal chitin-binding peptides, chitin-binding lectins, and chitinases, all of which have been isolated from plants. Tachycitin shows specific binding to chitin but does not bind to polysaccharides, such as chitosan, cellulose, mannan, xylan, and laminarin. In addition, tachycitin does not bind to chito-oligo-agarose immobilized with tri- to pentasaccharides, suggesting that the binding requires longer units of *N*-acetyl-D-glucosamine, or a specific conformation of the polysaccharide (*17, 18*). Tachycitin may represent a new class of the chitin-binding protein family in animals.

5.4. Tachystatins. An S-granule-derived component, S3, now named tachystatin, was found to be a single chain peptide of 6.5 kDa on Tricine-SDS-PAGE (*18*). Ultracentrifugal analysis revealed tachystatin to be present in the monomer form in solution. Tachystatin inhibits the growth of both Gram-negative and Gram-positive bacteria, and fungi, with a bacterial agglutinating property (Kawabata, S. *et al.*, unpublished results). Two isopeptides of tachystatin have been found, both consisting of 44 amino acid residues including three disulfide bonds with no *N*-linked sugar. One of them contains Tyr at the COOH-terminal end (M_r=5,055.8), and the other contains Phe at the corresponding site (M_r=5,039.8). A cDNA sequence coding for tachystatin comprises an NH_2-terminal signal sequence followed by the mature peptide without a pro or extension peptide (Kawabata, S. *et al.*, unpublished results).

Tachystatin, as well as tachycitin, also binds to chitin and, therefore, the chitin-binding property may be a common feature of the S-granular components. Chitin is a cell wall component of fungi, and chitin-binding proteins identified in plants as antibacterial substances, lectins and chitinases, are considered to be defense molecules that kill fungal pathogens when recognizing cell wall component(s), perturbing the plasma membrane, or hydrolyzing the polysaccharides on the cell wall (*74*). In *Candida albicans*, the attenuated virulence of chitin-deficient mutants has been reported (*75*). Furthermore, chitin is the major structural component of the arthropod exoskeleton. The physiological significance of the chitin-binding activity of the S-granular components found in hemocytes is unknown, but they probably recognize chitin exposed at the site of a lesion. Thus, they appear to serve not only as antibacterial molecules against invading microbes but also in wound healing, which may stimulate and accelerate the biosynthesis of chitin at the site of injury.

5.5. Factor D. Factor D is a new antimicrobial glycoprotein of 43 kDa, which was copurified during the isolation of horseshoe crab serpins from hemocyte lysates. The antimicrobial activity of this protein against Gram-negative bacteria is greater than that against Gram-positive bacteria (*76*). Factor D consists of 394 amino acid residues including a signal sequence. The established sequence indicates that the COOH-terminal domain of 262 residues exhibits a significant sequence homology with the catalytic domains of serine proteases, in particular with that of human tissue plasminogen activator (32% identity), except for an interesting amino acid substitution. A substitution of Ser in the active site triad to Gly has occurred in the serine protease-like domain of factor D, in spite of the conservation of the other two catalytic residues (His and Asp), indicating no endogenous proteolytic activity of factor D. In fact, the isolated factor D did not show any amidase activity toward the synthetic peptide substrates tested.

Factor D is present in L-granules of hemocytes. Human neutrophils are also known to contain serine proteases (cathepsin G and elastase) and a serine protease homologue (azurocidin) in azurophil granules, a specialized lysosome of the cells, in addition to defensins (*77*). They possess

antimicrobial activity, and hence are referred to as "serprocidins," a family of serine proteases and serine protease homologues with antimicrobial activity. Azurocidin also shows no demonstrable protease activity, as might be predicted from the two amino acid substitutions, His to Ser and Ser to Gly, in the catalytic triad of the molecule. Factor D, in this sense, may belong to the serprocidins. However, factor D has a unique NH_2-terminal extension of 114 residues and shows weak homology with a part of the interleukin 6 receptor, suggesting another important physiological activity of factor D.

6. Various lectins found in hemolymph

In Table I, the various lectins thus far isolated from horseshoe crab hemolymph are summarized. They include a sialic acid-lectin, called limulin, polyphemin, carcinoscorpin and others. In the hemolymph of the Japanese horseshoe crab, at least five lectins, now called the tachylectin family, have been identified, four of which are localized in L-granules. These lectins are thought to be involved, directly or indirectly, in the host defense mechanism.

6.1. Tachylectin-1 (previously named L6). Tachylectin-1 was recently purified from hemocytes by affinity chromatography on LPS-immobilized-agarose (*78*). This lectin also binds to agarose itself and it is eluted with high concentrations (0.5-1 M) of monosaccharides, such as glucose, mannose, and galactose. However, it shows no binding activity as to *p*-nitrophenyl derivatives of monosaccharides, including Gal, Man, or GlcNAc, at low concentration (10 μM) on equilibration dialysis. On the other hand, tachylectin-1 exhibits LPS-binding potential and agglutinates sheep erythrocytes coated with LPS, and this activity is inhibited by the addition of free LPS, suggesting that tachylectin-1 recognizes the oligosaccharide portion of LPS. However, it apparently has no hemagglutinin activity against sheep and rabbit red blood cells, or the human A, B, and O types of red blood cells.

Tachylectin-1 shows antibacterial activity toward Gram-negative but not Gram-positive bacteria. It also exhibits more effective agglutinating activity toward Gram-negative than Gram-positive ones. Limulin, a sialic acid-binding lectin present in the hemolymph plasma of the American horseshoe crab (*L. polyphemus*), reacts directly with bacterial coat oligosaccharides in a Ca^{2+}-dependent manner to initiate a humoral defense system, including agglutination of the microbes (*79-81*). This property seems similar to that of tachylectin-1, but the amino acid sequences of limulin and tachylectin-1 (*82*), in addition to their molecular weights and localizations, are different from each other. Tachylectin-1 is a novel type of lectin located exclusively in L-granules of hemocytes.

Tachylectin-1 is a single-chain protein consisting of 221 amino acids with no *N*-linked sugar chain and contains three intrachain disulfide bonds, and a free Cys residue. The calculated molecular weight of 24,383 is lower than that estimated by SDS-PAGE on a 15% gel. Based on a hydropathy plot, tachylectin-1 is primarily a hydrophilic protein and contains one atom of zinc. Tachylectin-1 is rich in Trp (9 residues) and positively-charged amino acids (27 residues: 11 Lys, 10 Arg, and 6 His), as compared to negatively-charged amino acids (16 residues: 14 Asp and 2 Glu). The isoelectric point was calculated to be 9.69. Moreover, an outstanding structural feature of tachylectin-1 is that it consists of six tandem repeats, each comprising 33-38 amino acids with 32-61% internal sequence identities. Two short consensus sequences, -GXWXQIXGXLK- and -GVNSNDXIY-, are highly conserved in each repeat. It is also of interest that three disulfide small loops consisting of five amino acid residues are present in every two repeats. Tachylectin-1 shows no significant sequence similarity with other known proteins, including various animal and plant lectins and LPS-binding proteins, such as *Limulus* endotoxin-binding protein-protease inhibitor (*83*) and mammalian plasma LPS-binding proteins (*65*).

Quite recently, we succeeded, in collaboration with Professor W. Bode's group of Max-Planck-Institute (Martinsried), in establishing the crystal structure of tachylectin-1. The secondary structure is mainly dominated by six β-sheets, each of which corresponds to a tandem repeat. It looks like a six-bladed β-propeller structure. Surprisingly, this structure exhibits striking topological similarity to those of sialidases and integrin subunit α, which have a six- and seven-bladed β-propeller structures, respectively, although there is no significant sequence identity between them (Beisel, H.-G. *et al.*, unpublished).

6.2. Tachylectin-2 (previously named L10). An L-granule component, named tachylectin-2, has been proved to be a lectin with hemagglutinating activity against human A-type erythrocytes and with specificity for GlcNAc (Table I). At least three isoproteins have been isolated (*84*). The molecular differences between the isoproteins must be due to a few amino acid replacements and a β-shift at -Asn-Gly- bonds in the molecules. Under the assay conditions used, the minimum concentration of tachylectin-2 required for the agglutination of trypsin-treated human A-type erythrocytes is half of that for wheat germ agglutinin. Furthermore, tachylectin-2 exhibits an agglutinating activity against *Staphylococcus saprophyticus* KD and the agglutination is inhibited in the presence of GlcNAc. There is little or no inhibitory effect on the growth of Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*S. aureus*).

Tachylectin-2 is a single-chain protein consisting of 236 amino acids with no cysteine, and no *N*-linked or *O*-linked sugar chains. The deduced sequence exhibits no significant sequence similarity with other known proteins, including various animal lectins. A horseshoe crab agglutinin from the same species has been named *T. tridentatus* agglutinin (*85-87*), but it has clearly different characteristics from tachylectin-2: its hemagglutinating activity is inhibited by *N*-acetylglycine and *N*-acetylglutamic acid, which do not have any effect on the activity of tachylectin-2. The other horseshoe crab hemagglutinin from *C. rotundicauda* (*88*) has an apparent $M_r = 24{,}000$, similar to that of tachylectin-2, but its amino acid composition is clearly distinct from that of tachylectin-2. Furthermore, agglutinins from the American horseshoe crab, *L. polyphemus*, *Limulus* 18-kDa agglutinin-aggregation factor (*89*) and limunectin (*90*), have also been reported but their primary sequences are not structurally related to that of tachylectin-2.

The most intriguing feature of the amino acid sequence of tachylectin-2 is the presence of internal homology which consists of five tandem repeats of 47 amino acids. The sequence identities between the repeats range from 49 to 68%. There has to be at least two sugar binding sites in these repeats for hemagglutinating activity expression, since tachylectin-2 is present as a monomeric protein in

solution, as deduced from the data on ultracentrifugal analysis. This is also a unique characteristic of tachylectin-2, compared to other horseshoe crab agglutinins so far purified, such as limulin from *L. polyphemus* (*79-82*), carcinoscorpin from *C. rotundicauda* (*91*), and sialic acid-specific binding lectin from *T. tridentatus* (*85-87, 92*). These lectins are present in the hemolymph as high molecular mass oligomers of 460, 420, and 533 kDa, respectively. Tachylectin-2, as well as tachylectin-1, has also been crystallized, and its structure is now being analyzed.

Wheat germ agglutinin (*93*), *T. tridentatus* agglutinin (*86*), and *Solanum tuberosum* agglutinin (*94*) have been shown spectrophotometrically or fluorometrically to contain tryptophan residue(s) in their sugar binding sites. Similar to these agglutinins, the addition of a specific sugar, GlcNAc, to a sample of tachylectin-2 also causes a blue shift of the emission spectrum, indicating the presence of tryptophan residue(s) in the sugar binding sites. The sequences containing tryptophan in the polypeptide of tachylectin-2, DNW and IGXGGW, are completely conserved in the COOH-terminal region of each repeat, suggesting that they probably participate to some extent in the formation of sugar binding sites. Several sugars containing an *N*-acetyl group inhibit the hemagglutinating activity of tachylectin-2, but *N*-acetylneuraminic acid and *N*-acetylmuramic acid do not. In addition, *N*-acetylallolactosamine (Galβ1-6GlcNAc) exhibits inhibitory activity but its isomer, *N*-acetyllactosamine (Galβ1-4GlcNAc) has no effect. Therefore, tachylectin-2 seems to recognize not only the *N*-acetyl group but also other parts of the ligands.

6.3. Tachylectin-4. We have newly identified a horseshoe crab hemocyte-derived lectin, named tachylectin-4 (*95*). It exhibits more potent hemagglutinating activity against human A-type erythrocytes than that of tachylectin-2. The purified tachylectin-4 is an oligomeric glycoprotein of 470 kDa, and is composed of subunits of 30 and 31.5 kDa. Ca^{2+} at 10 mM enhances the hemagglutinating activity 4-fold, and the activity is inhibited by EDTA and *o*-phenanthroline. L-Fucose and *N*-acetylneuraminic acid at 100 mM completely inhibit the activity of tachylectin-4. The activity is also inhibited more strongly by bacterial S-type LPS, but not by R-type LPS lacking O-antigen. The most effective S-type LPS is from *E. coli* O111:B4, and the minimum concentration required for inhibiting agglutination of human A-type erythrocytes (0.1 μg/ml) is 160-fold lower than that of S-type LPS from *S. minnesota*. Therefore, colitose (3-deoxy-L-fucose), a unique sugar present in the O-antigen of *E. coli* O111:B4, with structural similarity to L-fucose, is the most probable candidate for a specific ligand of tachylectin-4.

A cDNA coding for tachylectin-4 has been isolated from a hemocyte cDNA library. The open reading frame of the 1,344-base-pair cDNA codes for the mature protein with 232 amino acids. There is no significant sequence similarity to any other known LPS-binding lectins, whereas tachylectin-4 is homologous to the NH_2-terminal domain with unknown functions of *Xenopus laevis* pentraxin 1 (*96*).

7. Other components associated with host defense

7.1. α_2-Macroglobulin. Proteins of the α_2-macroglobulin (α_2M) family are abundant components of the plasma of mammals and arthropods, comprising about 3% of the total plasma protein in man and being the third most abundant protein in the plasma of the American horseshoe crab, *L. polyphemus*. α_2M is present not only in the plasma but also in the hemocytes of horseshoe crab (*12, 13*).

Limulus α_2M shows many of the typical characteristics of mammalian α_2Ms, including the presence of an internal thiol ester, reactivity with a diversity of endopeptidases, a unique proteinase trapping mechanism, and reactivity with the mammalian α_2M receptor. Additionally, *Limulus* α_2M mediates the clearance of proteases from the plasma (*97*). A cDNA encoding *Limulus* α_2M has an NH_2-terminal signal sequence of 25 amino acid residues and a mature protein of 1,482 residues (*98*). The entire amino acid sequence is homologous with those of mammalian α_2Ms (28-29% identity) and contains common features found in mammalian α_2Ms, a bait region, an internal thiol-ester site, and a receptor-binding domain. However, the NH_2-terminal portion (positions 24-105) exhibits no sequence similarity with those of mammalian α_2Ms, and it is structurally related to that of the human complement factor C8γ chain, consistent with a role for *Limulus* α_2M in host defense. Component sugar analysis of *Limulus* α_2M revealed the existence of a complex-type of oligosaccharide chains similar to those of mammalian α_2Ms. However, unlike mammalian α_2Ms no sialic acid is detectable in *Limulus* α_2M and it contains approximately three mol/mol of *N*-acetylgalactosamine, suggesting the presence of *O*-linked sugar chains, which have not been found in mammalian α_2Ms.

Expression of the α_2M was detected in hemocytes, but not in hepatopancreas, heart, stomach, intestine, coxal gland, brain, or skeletal muscle. Furthermore, immunoblotting of hemocytes with antiserum against α_2M indicated the presence of the α_2M in L-granules. Trypsin-treated *Limulus* α_2M, but not the native α_2M, displaced methylamine-treated [^{125}I]-human α_2M from the human α_2M-receptor with a K_d of 30 nM, suggesting the conservation of the proteinase clearance mechanism in the mammalian and arthropod evolutionary lineages.

7.2. Cysteine protease inhibitor (cystatin). The cystatin superfamily comprises a group of inhibitors for cysteine proteases that are widely distributed in mammalian tissues and body fluids. Cystatin may play important roles not only in the protection of cells from unfavorable proteolysis by intracellular and external cysteine proteases but also in biological defense systems against invaders. In invertebrates, two cystatins exhibiting sequence similarity to family 2 have been isolated and characterized, sarcocystatin A from the hemolymph of *S. peregrina* larvae (*99*) and a cystatin-like protein from *Drosophila melanogaster* (*100*). While the physiological roles of these cystatins are unclear, sarcocystatin A may participate in the morphogenesis of larval and adult structures.

Limulus (L)-cystatin isolated from the Japanese horseshoe crab is a single-chain protein consisting of 114 amino acids of 12.6 kDa, which exhibits significant sequence similarity with members of family 2 cystatins (*101*), such as bovine colostrum cystatin (33%) and human cystatin S (31%). L-Cystatin inhibits the amidolytic activity of papain by forming a noncovalent 1:1 complex with an equilibrium constant (K_i) of 0.08 nM. It also inhibits cathepsin L (K_i = 0.17 nM) and ficin (K_i = 0.52 nM), but not argingipain (a

bacterial cysteine protease) or calpains. Northern blotting revealed the expression of the mRNA in hemocytes, but the expression is negligible in heart, hepatopancreas, intestine, stomach, and muscle. Immunoblotting revealed its localization in L-granules of hemocytes. Furthermore, L-cystatin exhibits an antimicrobial activity against Gram-negative bacteria, which is much stronger than that of chicken egg white cystatin. These data suggest that the L-granule-derived cystatin serves synergistically to provide effective defense against invading microbes, together with other defense molecules that are released in response to external stimuli.

7.3. Transglutaminase. It is well known that in the mammalian clotting system, the fibrin clot generated *via* the cascade reaction is finally cross-linked to form a huge fibrin network, in addition to being cross-linked to other plasma proteins, such as soluble fibronectin, α_2M, and α_2-plasmin inhibitor. This final step is catalyzed by plasma transglutaminase (TGase), factor XIIIa, and the cross-linking of fibrin itself and with other proteins is essential for normal hemostasis and wound healing. It is, therefore, expected that in the horseshoe crab clotting system a TGase might participate in the cross-linking of the coagulin gel and/or in the immobilization against invading microorganisms.

Horseshoe crab TGase isolated from hemocytes has an apparent molecular mass of 86 kDa and mammalian-type II (cytosolic) TGase-like enzymatic properties (*39*). The activity is Ca^{2+}-dependent and is inhibited by primary amines, EDTA and SH-reagents. However, the TGase is not inhibited by GTP, ATP, or CTP, unlike some mammalian TGases. In a similar manner to guinea pig liver TGase, horseshoe crab TGase does not exist as a zymogen form, in contrast to plasma factor XIII.

Two cDNAs for horseshoe crab TGase have been cloned: they differ only in three amino acid residues, suggesting the existence of isoforms (*40*). Both cDNAs encode the mature TGase of 764 residues. The mRNA of TGase is observed in hemocytes, hepatopancreas, and stomach, but not in heart, intestine, or muscle. Horseshoe crab TGase shows significant sequence identity with members of the mammalian TGase family in the catalytic region, as follows: guinea pig liver TGase (32.7%), human factor XIII a subunit (36.9%), human keratinocyte TGase (39.9%), and human erythrocyte band 4.2 (23.7%). In addition to the catalytic domain, horseshoe crab TGase has a unique NH_2-terminal cationic 60 residue extension with no homology to other mammalian TGases or any other known proteins. The genealogical tree of known TGases suggests that horseshoe crab (invertebrate) TGase and mammalian (vertebrate) TGases diverged at the oldest root.

Although two potential TGase substrates have been found in hemocyte lysates (*39*), the physiological function of these substrates is not clear. No dansylcadaverine (DCA) is incorporated into hemocyte membrane proteins, hemolymph plasma proteins, or coagulogen. When a dialyzed hemocyte lysate was incubated in the presence of Ca^{2+}, dithiothreitol, and DCA, two major proteins were found to incorporate DCA through the endogenous TGase activity in the lysate. Rapid (<5 min) incorporation of DCA was observed for 10 and 80 kDa bands, and slow incorporation (<60 min) was observed for a 20 kDa protein. Of these protein substrates, the 80 kDa protein contains a large number of proline residues, amounting to about 22% of the total amino acids. It is, thus, called the proline-rich protein (Kawabata, S. *et al.*, unpublished results). On the other hand, the 10 kDa protein, which was also identified as an intracellular substrate for TGase, consists of 81 amino acid residues with a calculated molecular weight of 8,671, named the 8.6 kDa protein (*39*). It is a Cys-rich protein consisting of 14 half-cystine residues. The 8.6 kDa protein is readily intermolecularly cross-linked by TGase, forming multimers as large as pentamers. Significant sequence similarity was observed between the 8.6 kDa protein and fulvocin C, a bacteriocin isolated from *Myxococcus fulvus*, which consists of a total of 45 amino acid residues. However, the 8.6 kDa protein does not show any bactericidal activity, such as against *E. coli* K12.

Both the proline-rich and 8.6 kDa proteins are major proteins in L-granules of hemocytes, whereas horseshoe

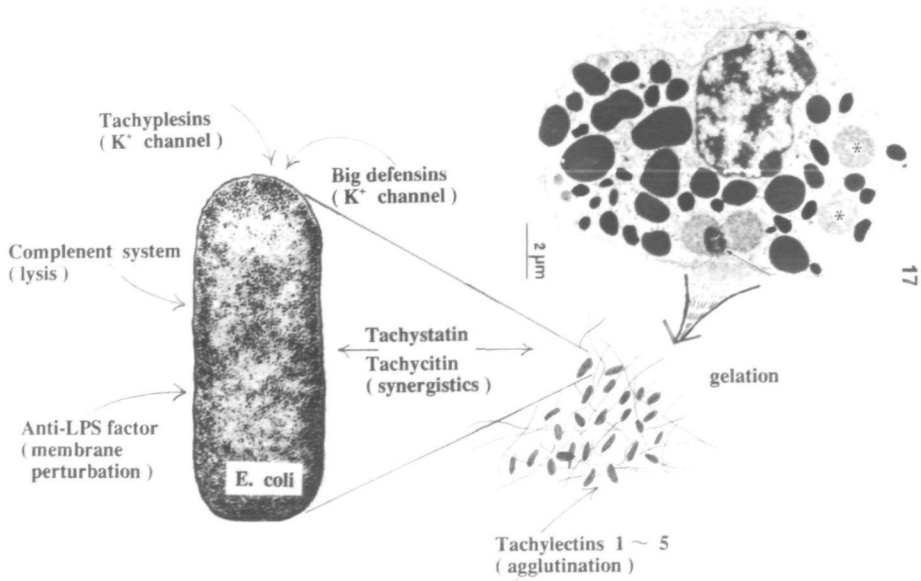

Fig. 9. **Working hypothesis for the functional roles of horseshoe crab hemocytes in host defense.** When pathogens invade the hemolymph, the hemocytes detect the invaders, and then release many defense molecules stored in their L- and S-granules. The clotting cascade, in parallel with cell agglutination mediated by various lectins, may occur initially for immobilization of the invaders into the gel. Then, the invaders may be killed by antimicrobial proteins (anti-LPS factor and complement-like proteins), and polypeptides (big defensin, tachycitin, and tachystatins) and peptides (tachyplesins).

crab TGase is of the cytosolic type. Therefore, there is no possibility that the TGase and these potential substrates could interact with each other. If such a TGase is secreted, as is the case of factor XIII, they would come in contact on degranulation by the hemocytes. TGase may catalyze the cross-linking of coagulin or microbial cell walls with other proteins.

8. Conclusion

The blue blood of the horseshoe crab contains a sophisticated defense system very sensitive to pathogens or foreign materials. The hemocytes circulating in the hemolymph detect trace amounts of LPS molecules on the invading microorganisms and respond quickly by releasing the granular components into the external milieu (Fig. 9). The coagulation system composed of three serine protease zymogens, factor C, factor B, and proclotting enzyme, and a clottable protein, coagulogen, is activated by LPS to form insoluble coagulin gel. The coagulation system also responds to β-(1,3)-glucan through the activation of a unique heterodimeric serine protease zymogen, factor G. The pathogens are, thus, engulfed in the gel and subsequently killed by antimicrobial substances with various specificities, which are also released from the cells. Although the hemolymph coagulation reaction in horseshoe crabs is well known, it is not the only defense mechanism of this animal. Many agglutinins, protease inhibitors, and antimicrobial proteins and peptides are present in either the hemolymph plasma or hemocytes. The hemolymph plasma also exhibits cytolytic activity against foreign cells. These cellular and humoral defense systems covering the innate immunity, in concert, defend the horseshoe crab from invading foreign organisms. Such a defense system has allowed the horseshoe crab to survive for more than 200 million years on the earth. Horseshoe crabs are often called "living fossils." However, they are not fossils, they are living.

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