

# The limulus clotting reaction

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Our biochemical studies on the hemolymph coagulation-complement system using limulus indicate that the circulating hemocytes contain at least four serine protease zymogens and one clottable protein, coagulogen, which constitute a cascade triggered by bacterial endotoxins and (1,3)- $\beta$ -D-glucan. We also found several antimicrobial substances, tachyplesin peptides and anti-lipopolysaccharide factor, in the hemocytes. These clotting factors and antimicrobial substances are released into the hemolymph in response to lipopolysaccharide, where they cooperate in immobilization and killing of invading microorganisms as a host defense.

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## Introduction

Limulus, the horseshoe crab, has an endotoxin [lipopolysaccharide (LPS)]-sensitive hemolymph coagulation cascade thought to be one defense system against invading microorganisms [1,2,3,4]. Gram-negative bacteria contain large amounts of LPS in the outer membrane. When limulus is infected by Gram-negative bacteria, such as *Vibrio cholerae*, hemocytes circulating in the hemolymph recognize the bacteria and responses, including cell adhesion, aggregation and degranulation, rapidly occur [2]. Following degranulation of the large granules in hemocytes, hemolymph coagulation is initiated by the granular components [3,4-7]. Several components associated with this coagulation reaction have been purified and reconstitution experiments using the purified clotting factors suggested to us a cascade-type of reaction [4], as shown in Fig. 1. In the presence of LPS, an LPS-sensitive serine protease zymogen, factor C, is autocatalytically activated. The active factor C then activates zymogen factor B to factor B, which subsequently activates proclotting enzyme to clotting enzyme. The resulting clotting enzyme converts soluble coagulogen, an invertebrate fibrinogen-like substance, to an insoluble coagulin gel. Like the mammalian coagulation and complement systems, this cascade reaction is propagated by limited proteolysis. On the other hand, the lysate from the hemocytes is known to respond to (1,3)- $\beta$ -D-glucan as well as to LPS. This (1,3)- $\beta$ -D-glucan-sensitive system also activates the proclotting enzyme through a glucan-sensitive factor, factor G, resulting in the formation of

coagulin gel [4]. Because (1,3)- $\beta$ -D-glucan is a major cell wall constituent of yeast and other fungi, this cascade reaction also seems to play an important role in the biological defense mechanism.

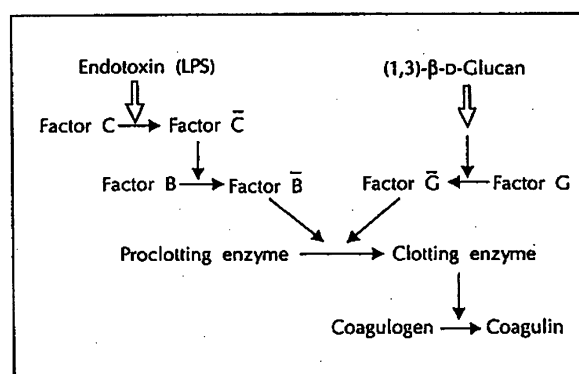


Fig. 1. Hemolymph coagulation cascade system in limulus (*Tachyplesus tridentatus*). See text for details.

## Function of limulus hemocyte

The limulus hemolymph contains two types of cells, granular hemocytes and cyanocytes. Based on cell morphology, there appears to be only one type of hemocyte in

## Abbreviations

EGF—epidermal growth factor; L—large; LDL—low density lipoprotein; LPS—lipopolysaccharide; S—small; TGase—transglutaminase.

the systemic circulation of the adult animal, the so-called granulocyte or amebocyte [2•,5,6,8•].

This cell is an oval, plate-shaped structure, 15–20  $\mu\text{m}$  in its longest dimension. Fig. 2 shows an electron micrograph of the hemocyte isolated from Japanese horseshoe crab, *Tachypleus tridentatus* [8•]. The cell contains numerous dense granules classed into two major types—large (L) and small (S) granules. The L granules contain at least 20 protein components including five clotting factors and one antimicrobial factor (anti-LPS factor), whereas the S granules contain exclusively an antimicrobial substance, named tachyplesin peptide, in addition to six major protein components. After treatment with LPS, the L granules are released more rapidly than the S granules, although almost all granules are finally exocytosed. This exocytosis is associated with clot formation, the process being complete within 90 seconds. The clot is softer than mammalian fibrin clot and contains coagulin gel generated from its precursor, coagulogen [9].

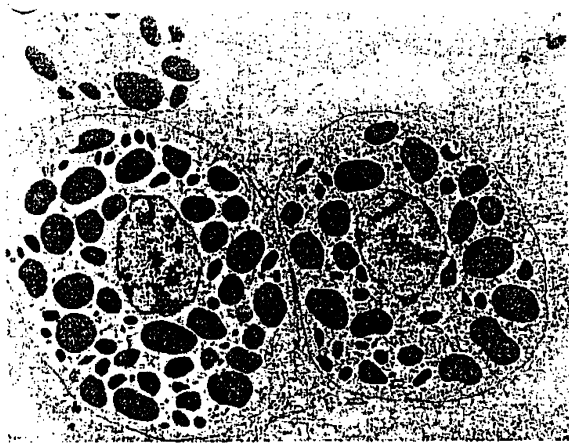


Fig. 2. An electron micrograph of granular hemocytes showing large and small granules.

## Limulus clotting factors

### Coagulogen

Among the clotting factors so far found in the hemocytes, the clottable protein, coagulogen, has a functional similarity with vertebrate fibrinogen and is known to play a central role in the limulus clotting reaction (Fig. 1). In the intact animal, a soluble precursor of this protein is absent from the plasma, but instead coagulogen is sequestered in the cytoplasmic L granules of the hemocytes. Granule exocytosis releases coagulogen into the external milieu. The extracellular clot is composed of fibers of the protein, named coagulin gel. The conversion to a gel is mediated by a clotting enzyme in the hemocytes. Coagulogen consists of a single basic polypeptide chain and has a calculated molecular mass of 19.7 kD ( $\pm 50$ ) [9–12]. This protein contains three segments, A chain, peptide C and B chain. The clotting enzyme cleaves the Arg–Gly and Arg–Thr linkages, both located at the amino-termi-

nal region. The Arg–Gly linkage cleaved by the clotting enzyme is the same type as that cleaved by  $\alpha$ -thrombin in the transformation of mammalian fibrinogen to fibrin. The amino acid sequences of four coagulogens isolated from the hemocytes of American (*Limulus polyphemus*), Japanese (*T. tridentatus*) and two Southeast Asian (*T. gigas* and *Carcinoscorpius rotundicauda*) horseshoe crabs have now been established [9,10]. In addition the cDNAs for two of them (*L. polyphemus* and *T. tridentatus*) have been cloned [11,12]. Upon gelation of all the coagulogens by a limulus clotting enzyme, a large peptide, peptide C, is released from the inner portion of the parent molecules. The resulting gel consists of an A chain (18 residues) and a B chain (129 residues), which are bridged by two disulfide linkages. The overall sequences of the four coagulogens, comprising 147 residues, have considerable homology, particularly in the A- and B-chain regions. Surprisingly, 73–92 per cent of the sequences in these regions contain the same amino acid residues in identical positions and homologies are found along the entire molecules. In addition, 16 half-cysteine residues (a cysteine residue linked to another cysteine residue by a disulfide bond) of the four are in the same positions in the sequences, suggesting that they are paired in the same fashion, and thus that the molecules have very similar conformations. In contrast, the sequence homology of the peptide C region is less than that of the A- and B-chain regions. The homologies of the peptide C region in four coagulogen species amount to approximately 43–57 per cent of the structure. It is of interest that coagulogen appears to contain an  $\alpha$ -helical region (comprising 14.9 per cent of the molecule) exclusively around the peptide C segment released by the clotting enzyme, suggesting that there is a marked conformational change in the transformation of coagulogen to coagulin gel. On the other hand, the  $\beta$ -sheet (comprising 24.6 per cent of the molecule), reverse-turn (comprising 29.7 per cent) and coiled (comprising 30.9 per cent) structures are commonly distributed in the B chain segment, suggesting that it is a  $\beta$ -type protein [9].

### Proclotting and clotting enzymes

A clotting enzyme that catalyzes the transformation of coagulogen to coagulin exists as its zymogen form in the hemocytes [13]. This proclotting enzyme is activated by both factor B and factor G (Fig. 1) and it is a single chain glycoprotein with an apparent molecular mass of 54 kD. Upon activation by factor B, it is converted to a two chain active form, clotting enzyme, composed of a light (L) chain and a heavy (H) chain, with molecular masses of 25 and 31 kD, respectively. The clotting enzyme cleaves two bonds in coagulogen to yield a coagulin gel. Thus, proclotting enzyme/clotting enzyme seems to be an invertebrate prothrombin/ $\alpha$ -thrombin counterpart, since both catalyze a final step for gelation. The proclotting enzyme is composed of 346 amino acid residues with a molecular mass of 38.194 kD (i.e. the molecular mass not including the carbohydrate moieties) (Table 1). The cleavage site associated with the zymogen activation is Arg–Ile (residues 98 and 99, respectively), the sequence of which links the carboxyl-terminal residue

of the L chain and the amino-terminal residue of the H chain. The H chain of the proclotting enzyme is composed of a typical structure of a serine protease. The locations of four disulfide linkages in the H chain are the same as those in prothrombin and clotting factor X [13]. The entire sequence of the H chain corresponding to the serine protease region shows a close similarity with that of human factor Xa (34.1 per cent homology). It contains also the His-Asp-Ser (residues 143, 199 and 297, respectively) triad known to be the catalytic triad of serine proteases. The substrate binding site corresponding to serine (residue 189) in chymotrypsin numbering, is aspartic acid, thereby indicating that this clotting enzyme has a typical trypsin-like specificity. The amino-terminal portion of the L chain consists of a novel disulfide-knotted structure. This structure forms a 'clip-like' shape consisting of a discrete domain, the amino terminal L chain portion of which shows sequence homology with a *Drosophila* protein, serine protease zester precursor. Although the significance of these results is still unknown, they may suggest that the 'clip-like' domain found in both limulus proclotting enzyme and factor B (described below) is one of the common structural elements in the serine protease zymogen of invertebrate animals. An immunohistochemical study utilizing anti-clotting enzyme antibody has demonstrated that the proclotting enzyme is present in the L granules in hemocytes [8•,13].

#### Factor B

The zymogen factor B is activated by factor C, which then, in turn, activates proclotting enzyme (Fig. 1). Factor B is a single chain glycoprotein with a molecular mass of 64 kD (Table 1). Upon activation of the zymogen factor B by active factor C, it is converted into active factor B, which is composed of an H chain (32 kD) and an L chain (25 kD), with the release of an activation peptide [14]. Recently, we succeeded in determining the cDNA sequence of zymogen factor B. It consists of 400 amino acid residues with a 23 residue signal sequence. The mature protein is thus composed of 377 amino acids with a calculated molecular mass of 40.57 kD. The entire amino acid sequence has a similarity with that of limulus proclotting enzyme. In particular, the sequence identity of the carboxyl terminal serine protease domains between factor B and proclotting enzyme is 43.9 per cent. Moreover, both proteins share a similar structure in the region comprising residues 1-60 of the amino-terminal end; they both contain six cysteine residues, suggesting that these proteins have arisen from a gene duplication.

#### Factor C

As shown in Fig. 1, factor C is an initial activator of the clotting cascade triggered by LPS [15]. The zymogen factor C is a glycoprotein with a calculated molecular mass of 123 kD. It consists of an H chain (80 kD) and an L chain (43 kD) [16•]. Factor C is converted autocatalytically to an activated form, designated factor C (123 kD), in the presence of LPS. Upon activation, a single cleavage of the Phe-Ile bond in the L chain occurs [15], resulting in the accumulation of two new

fragments, a B chain (34 kD) and an A chain (8.5 kD). A diisopropylfluorophosphate-sensitive active site is localized in the B chain of factor C. The zymogen factor C consists of 994 residues (Table 1) with a calculated molecular mass of 109.648 kD (i.e. the molecular mass not including the carbohydrate moieties). In the H and A chains, there are several interesting amino acid sequences [16•]. First, factor C has five repeating units ('Sushi' domain or short consensus repeat) of about 60 amino acid residues each, which are found in many proteins participating in the mammalian complement system. Factor C is the first example of a protein that has the complement-like structure in invertebrates. Second, an epidermal growth factor (EGF)-like domain is present in the H chain. Proteins containing this type of domain include several blood coagulation factors, complement factors and some receptors, including low density lipoprotein (LDL) receptor. Third, a lectin-like domain is found in between the third and fourth 'Sushi' domains. This region is composed of about 120 amino acids and shows homology with so called C-type lectins. This type of domain is also found in proteins whose functions are not considered only to bind the carbohydrate moiety. Two other characteristic sequences are also found in the H chain. One is a cysteine-rich region, which is located in the amino-terminal portion of the H chain. Eleven cysteine residues, out of 56, are clustered in this region. The other is in the carboxyl-terminal portion of the H chain, in which 13 out of 48 residues are proline. This region has homology with the connecting region of mammalian coagulation factor XII. Its significance, however, remains unknown. The B-chain portion of factor C is composed of a typical serine protease, with an active site triad [17•]. The B chain shows the strongest homology with human  $\alpha$ -thrombin (36.7 per cent). Immunohistochemical observation with electron microscopy utilizing anti-factor C antibody demonstrates that factor C is localized in the L granules in the cell [8•].

#### Relationship between limulus clotting factors and mammalian complement and clotting factors

To our knowledge, the only proteases with Sushi domains are the mammalian complement factors C1r, C1s, C2 and factor B, and limulus factor C. Moreover, the only proteases that have both Sushi and EGF domains include C1r, C1s and limulus factor C, all of which are associated with the initiation of the cascade reaction. 'Selectins', including three membrane proteins, endothelial leukocyte adhesion molecule, lymph node homing receptor and granule membrane protein 140, have been reported to contain a lectin-like domain, an EGF-like domain and several 'Sushi' domains. The similarity in domain composition between factor C and these proteins is of interest, because these proteins all seem to be associated with biological defense systems. On the other hand, the fact that factor C is structurally related to mammalian complement factors indicates an evolutionary relationship between the two systems. Factor C is a newly-discovered type of serine protease zymogen, a 'coagulation-complement factor', which may play an important role both in hemostasis and defense mecha-

**Table 1.** Components closely associated with the biological defense system in limulus (*Tachypleus tridentatus*).

| Component                   | Molecular mass (kD)<br>(number of residues) | Carbohydrate<br>content | Active form                            | Function                               | Intracellular<br>localization | References                    |
|-----------------------------|---------------------------------------------|-------------------------|----------------------------------------|----------------------------------------|-------------------------------|-------------------------------|
| Coagulogen                  | 19.7<br>(175)                               | None                    | Coagulin<br>(two chains)               | Gelation                               | L granule                     | [8**,9-12]                    |
| Factor C                    | 123<br>(994)                                | 9%                      | Factor $\bar{C}$<br>(three chains)     | Serine protease<br>(LPS-sensitive)     | L granule                     | [4,8**,15,16**,17*,<br>35-37] |
| Factor B                    | 64<br>(400)                                 | Present                 | Factor $\bar{B}$<br>(two chains)       | Serine protease                        | Unknown                       | [14]                          |
| Proclotting enzyme          | 54<br>(346)                                 | 11%                     | Clotting enzyme<br>(two chains)        | Serine protease                        | L granule                     | [13]                          |
| Factor G                    | 110                                         | Present                 | Factor $\bar{G}$<br>(two subunits)     | Serine protease                        | Unknown                       | [34]                          |
| Factor D                    | 42<br>(386)                                 | —                       | Unknown                                | Pseudo serine protease                 | Unknown                       | unpublished data              |
| Transglutaminase<br>(TGase) | 86<br>(764)                                 | None                    | TGase<br>(Ca <sup>2+</sup> -dependent) | Cross-link                             | Cytosol                       | [38**,39**]                   |
| Anti-LPS factor             | 11.6<br>(102)                               | None                    |                                        | Antimicrobial                          | L granule                     | [4,18,20,23]                  |
| Tachyplesin                 | 2.263<br>(17)                               | None                    |                                        | Antimicrobial                          | S granule                     | [21,25,40]                    |
| Polyphemusin                | 2.453<br>(18)                               | None                    |                                        | Antimicrobial                          | S granule                     | [22]                          |
| Factor C inhibitor          | 43<br>(394)                                 | Present                 |                                        | Anticoagulant                          | Unknown                       | unpublished data              |
| Trypsin inhibitor           | 6.8<br>(61)                                 | None                    |                                        | Protease inhibitor<br>(Aprotinin-type) | Unknown                       | [41]                          |
| L-6                         | 27<br>(221)                                 | None                    |                                        | LPS-binding                            | L granule                     | unpublished data              |
| 8.6 kD protein              | 8.671<br>(81)                               | None                    |                                        | TGase substrate                        | Unknown                       | [38**,39**]                   |
| Pro-rich protein            | 80                                          | ?                       |                                        | TGase substrate                        | L granule                     | [38**,39**]                   |

L, large; S, small. Unpublished data from the author's laboratory

nisms. In considering the structural similarity of initiators of the two systems, the limulus coagulation and mammalian complement systems may have evolved from a common origin. Concerning the evolution of host defense systems in the animal kingdom, this is consistent with a possible common evolutionary origin of the coagulation and complement cascades.

Fig. 3 summarizes the gross structures of limulus clotting factors so far described. Like mammalian clotting factors, factor C, factor B and proclotting enzyme are typical serine protease zymogens related to the trypsin family. They have structural domains in their amino-terminal portions that are different from those of mammalian clotting factors. There is no Gla domain, no kringle domain and no

finger domain. Moreover, mammalian clotting factors do not have a 'Sushi' domain, whereas factor C contains several 'Sushi' domains, as do complement factors, and the proclotting enzyme and factor B contain a 'clip-like' domain in the amino-terminal portions. On the other hand, the coagulogen molecule is much smaller than vertebrate fibrinogen. Thus, all the limulus intracellular clotting factors are thought to be a separate entity from the vertebrate clotting factors, although there is an intracellular clotting cascade, which is similar to that of mammals.

### Antimicrobial substances

In 1982, a protein component that inhibits the coagulation cascade was found in the hemocyte lysate from Japanese (*T. tridentatus*) and American horseshoe crabs (*L. polyphemus*) and named anti-LPS factor [18,19]. The

purified protein specifically inhibits the LPS-mediated activation of limulus factor C and has a strong antibacterial effect, especially on the growth of Gram-negative R-type bacteria. Furthermore, it has hemolytic activity on the red blood cells sensitized with LPS and cytolytic activity on LPS-sensitized polymorphonuclear leukocytes, mononuclear cells and human leukemia cells [20]. The whole amino acid sequence of anti-LPS factor isolated from *T. tridentatus* was determined in 1986 [18]. In our continued study of the limulus clotting system, we found also a new cationic peptide, named tachyplesin, in the hemocyte debris, which binds with LPS and displays antimicrobial activity against Gram-negative bacteria [21].

Tachyplesin I is a cationic peptide first isolated from acid extracts of Japanese horseshoe crab (*T. tridentatus*) hemocyte debris. This peptide inhibits the LPS-mediated activation of factor C in a similar manner to that of anti-LPS factor. Afterwards, two peptide analogs of tachyplesin

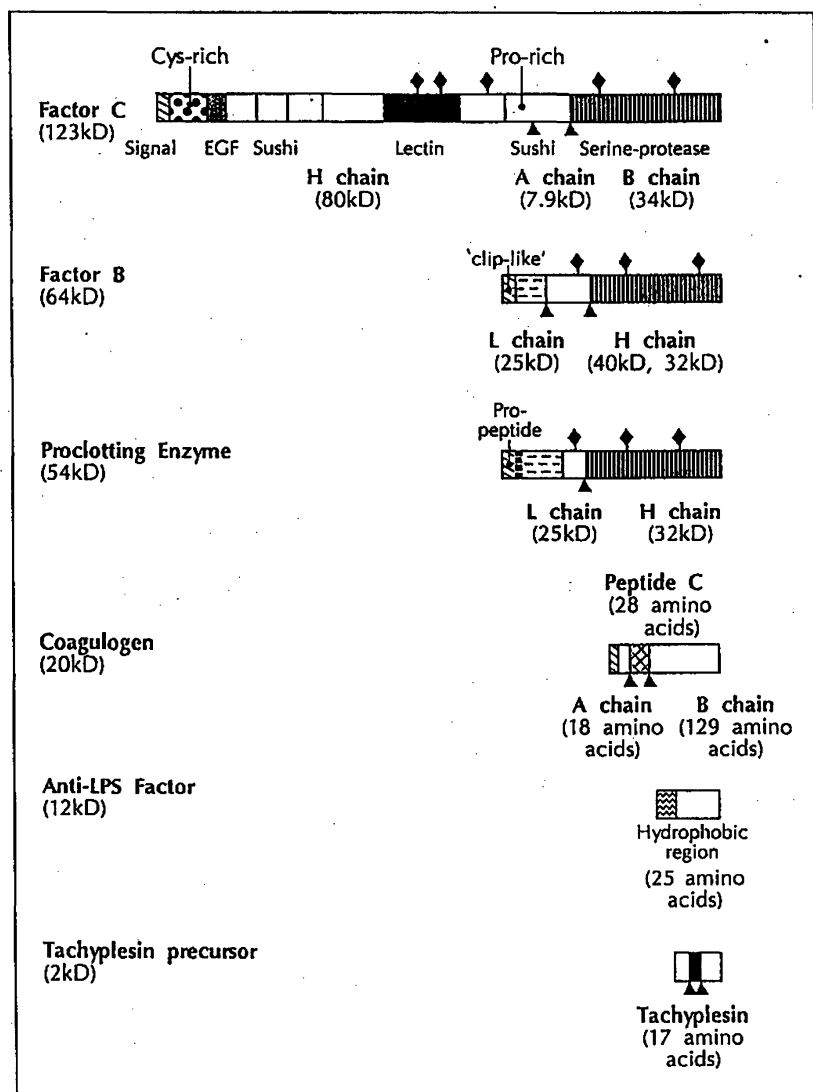


Fig. 3. The domain structures of limulus clotting factors and antimicrobial substances. The arrowheads indicate the cleavage sites for zymogen activation. The potential carbohydrate attachment sites are indicated with closed diamonds.

I, named tachyplesin II and III, have been isolated from the hemocytes of two Southeast Asian horseshoe crabs, *T. gigas* and *C. rotundicauda* [22,23]. Moreover, in the hemocyte debris of American *L. polyphemus*, two tachyplesin analogs, named polyphemusins I and II, have also been found [23]. The primary structures of the tachyplesin family are shown in Fig. 4. They are highly basic polypeptides composed of 17 or 18 residues with arginine  $\alpha$ -amide as the carboxyl-terminal end. Tachyplesin shows a characteristic structure with three tandem repeats of a tetrapeptide sequence, namely hydrophobic amino acid-Cys-hydrophobic amino acid-Arg [21]. Tachyplesin I takes on a fairly rigid conformation constrained by two disulfide bridges and adopts a conformation consisting of an anti-parallel  $\beta$ -sheet (residues 3-8 and 11-16) connected by a  $\beta$ -turn (residues 8-11). In this planar conformation, five bulky hydrophobic side groups are localized in one side of the plane and six cationic side groups are distributed at the 'tail' part of the molecule (residues 1-5 and 14-17). This arrangement is presumed to be closely associated with the bactericidal activity [24•].

The cDNA encoding tachyplesin precursors has been cloned and sequenced [25]. The tachyplesin precursors consist of 77 amino acids with 23 residues in a pre-

segment and two types of mRNA corresponding to the isopeptides of tachyplesins I and II have been identified. Both precursors contain a putative signal peptide, a processing peptide sequence and a carboxyl-terminal amidation signal 'Gly-Lys-Arg' connected to the mature tachyplesin peptide. Moreover, an unusual acidic amino acid cluster with Asp-Glu-Asp-Glu-Asp-Asp-Glu-Glu-COOH is present in the carboxyl-terminal portions of both precursors. This acidic region might interact with a cationic part of the tachyplesin peptide to stabilize a conformational structure of the precursor for proteolytic degradation. The subcellular localization of tachyplesin in the hemocytes proves to be in the S granules, as determined immunohistochemically [25].

Tachyplesin displays potent antimicrobial activity against several strains of microorganisms. It is effective against not only rough and smooth strains of Gram-negative but also Gram-positive organisms, such as *Staphylococcus* species and fungi. In the presence of tachyplesin, at a concentration of 3.5  $\mu\text{g/ml}$ , *Salmonella* strains irreversibly lose viability. Therefore, it seems likely that tachyplesin acts as an antimicrobial peptide for the defense of horseshoe crabs against microbial infections [22,26].

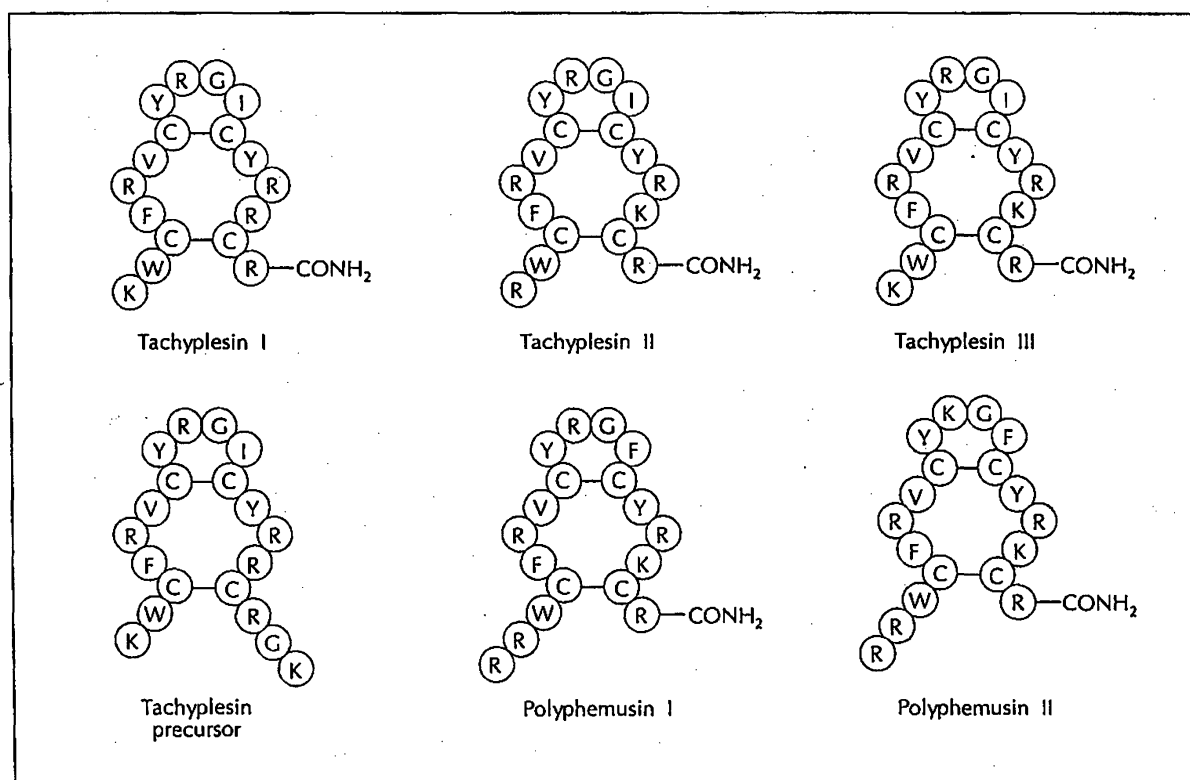


Fig. 4. Primary structures of six kinds of tachyplesins and their analogs isolated from four species of horseshoe crabs. Tachyplesin III was isolated from *Tachyplesus gigas*, tachyplesin I from *T. tridentatus*, *T. gigas* and *Carcinoscorpius rotundicauda*, and tachyplesin II from *T. tridentatus*. Polyphemusins I and polyphemusins II were isolated from *Limulus polyphemus*. Amino acids are represented by the one letter code. The  $\alpha$ -carboxyl group of the carboxyl-terminal arginine (R) is amidated.

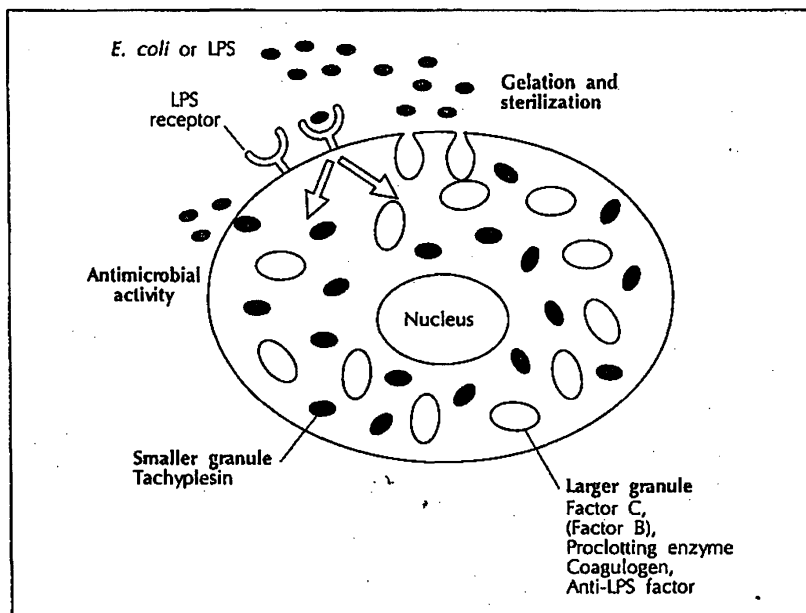


Fig. 5. This figure illustrates how the horseshoe crab hemocyte functions against invading microorganisms. Bacterial endotoxin (lipopolysaccharide (LPS)) first contacts with an LPS receptor/LPS-binding protein, which is probably located in the plasma membrane of the cell. Through this contact, some cellular reactions induce the fusion of granules and the plasma membrane, resulting in dispersal of the components of the large granules into the plasma. This exocytosis activates the intracellular clotting system and the clot generated during activation immobilizes the invaders. The peptide tachyplesins and anti-LPS factor released from small and large granules, respectively, act as the bactericidal substances.

### Humoral factors in hemolymph plasma

Although I have not mentioned plasma components in this review, there are many humoral factors, such as proteinase inhibitors [2•],  $\alpha_2$ -macroglobulin [27], lectins [2•], C-reactive protein and polyphemins [2•], all of which are important in antimicrobial defense. Furthermore, Liu and his colleagues have also reported several endotoxin-binding proteins [28–31, 32•] and a cell adhesion protein found in the limulus hemocytes [33•]. Although the exact functions of these substances are not known at present, they may act in concert with other components to serve as the biological defense of this animal. However, we still know less about the cell biology and physiology of horseshoe crab hemocytes in comparison with mammalian blood cells.

### Conclusion

From all the results described above, we speculate that bacterial endotoxin first contacts with an LPS receptor or LPS-binding protein, probably present in plasma membrane of the hemocyte, and activates factor C located in the granules; the resulting active factor C induces the activation of the intracellular clotting system (Fig. 5). The  $\beta$ -glucan-mediated pathway may also lead to activation of this system [34]. The clot generated during the activation encapsulates and immobilizes the Gram-negative bacteria and the peptide tachyplesin and anti-LPS factor released from S and L granules, respectively, act as the bactericidal substances. Therefore, this intracellular clotting system may have a crucial role in host defense against invading microorganisms.

### References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. RAVINDRANATH MH: Hemocytes in Hemolymph Coagulation of Arthropods. *Biol Rev* 1980, 55:139–170.
2. ARMSTRONG PB: Cellular and Humoral Immunity in the Horseshoe Crab. In *Limulus polyphemus. Immunology of Insects and Other Arthropods*. Edited by Gupta AP. Boca Raton: CRC Press; 1991:3–17.  
This is an up-to-date review of research into the functions of horseshoe crab hemolymph.
3. SÖDERHÄLL K, CERENIUS L: Crustacean Immunity. *Annual Rev Fish Diseases* 1992, 1:3–23.  
This review summarizes recent progress in the *in vitro* handling of crayfish hemocytes and the prophenoloxidase-activating system and associated factors important to crustacean immunity.
4. IWANAGA S, MORITA T, MIYATA T, NAKAMURA T, AKETAGAWA J: The Hemolymph Coagulation System in Invertebrate Animals. *J Protein Chem* 1986, 5:225–268.
5. ORNBERG RL, REESE TS: Beginning of Exocytosis Captured by Rapid-freezing of *Limulus* Amebocytes. *J Cell Biol* 1981, 90:40–54.
6. ARMSTRONG PB, RICKLES FR: Endotoxin-induced Degranulation of the *Limulus* Amebocyte. *Exp Cell Res* 1982, 140:15–24.
7. BRANDIN ER, PISTOLE TG: Presence of Microorganisms in the Hemolymph of the Horseshoe Crab, *Limulus polyphemus*. *Appl Environ Microbiol* 1985, 49:718–723.
8. TOH Y, MIZUTANI A, TOKUNAGA F, MUTA T, IWANAGA S: Structure of Hemocytes of the Japanese Horseshoe Crab *Tachy-*

*pleus tridentatus*. Fine Structure, Morphological Changes During Coagulation and Localization of Clotting Factors and Antimicrobial Substances. *Cell Tissue Res* 1991, 266:137-147.

This paper describes the structure of hemocytes in the normal state during hemolymph coagulation and the intracellular localization of three clotting factors and two antimicrobial substances in *T. tridentatus* hemocytes. The data are discussed in relation to the self-defense mechanisms of limulus.

9. SRIMAL S, MIYATA T, KAWABATA S, IWANAGA S: The Complete Amino Acid Sequence of Coagulogen Isolated from Southeast Asian Horseshoe Crab, *Carciniscorpius rotundicauda*. *J Biochem (Tokyo)* 1985, 98:305-318.
  10. MIYATA T, HIRANAGA M, UMEZU M, IWANAGA S: Amino Acid Sequence of the Coagulogen from *Limulus polyphemus* Hemocytes. *J Biol Chem* 1984, 259:8924-8933.
  11. MIYATA T, MATSUMOTO H, HATTORI M, SAKAKI Y, IWANAGA S: Two Types of Coagulogen mRNAs Found in Horseshoe Crab (*Tachypleus tridentatus*) Hemocytes: Molecular Cloning and Nucleotide Sequences. *J Biochem (Tokyo)* 1986, 100:213-220.
  12. CHENG SM, SUZUKI A, ZON G, LIU TY: Characterization of a Complementary Deoxyribonucleic Acid for the Coagulogen of *Limulus polyphemus*. *Bichim Biophys Acta* 1986, 868:1-8.
  13. MUTA T, HASHIMOTO R, MIYATA T, NISHIMURA H, TOH Y, IWANAGA S: Proclotting Enzyme from Horseshoe Crab Hemocytes: cDNA Cloning, Disulfide Locations, and Subcellular Localization. *J Biol Chem* 1990, 265:22426-22433.
  14. NAKAMURA T, HORIUCHI T, MORITA T, IWANAGA S: Purification and Properties of Intracellular Clotting Factor, Factor B, from Horseshoe Crab (*Tachypleus tridentatus*) Hemocytes. *J Biochem (Tokyo)* 1986, 99:847-857.
  15. TOKUNAGA F, MIYATA T, NAKAMURA T, MORITA T, KUMA K, MIYATA T, IWANAGA S: Lipopolysaccharide-sensitive Serine-protease Zymogen (Factor C) of Horseshoe Crab Hemocytes: Identification and Alignment of Proteolytic Fragment Produced during the Activation Show that it is a Novel Type of Serine Protease. *Eur J Biochem* 1987, 167:405-416.
  16. MUTA T, MIYATA T, MISUMI Y, TOKUNAGA F, NAKAMURA T, TOH Y, IKEYARA Y, IWANAGA S: Limulus Factor C: An Endotoxin Sensitive Serine Protease Zymogen with a Mosaic Structure of Complement-like, Epidermal Growth Factor-like, and Lectin-like Domains. *J Biol Chem* 1991, 266:6554-6561.
- This report describes the entire amino acid sequence of factor C, which initiates the clotting cascade system in the limulus hemolymph. Factor C, a newly discovered type of serine protease zymogen, is a 'coagulation-complement factor' that may play an important role in both hemostasis and host defense mechanisms.
17. TOKUNAGA F, NAKAJIMA H, IWANAGA S: Purification and Characterization of Lipopolysaccharide-sensitive Serine Protease Zymogen (Factor C) Isolated from *Limulus polyphemus* Hemocytes: A Newly Identified Intracellular Zymogen Activated by  $\alpha$ -chymotrypsin, not by Trypsin. *J Biochem (Tokyo)* 1991, 109:150-157.
- The authors demonstrate that zymogen factor C, which is sensitive to LPS, exists in American *L. polyphemus* hemocytes and that its immunological and biochemical properties are indistinguishable from those of factor C isolated from Japanese *T. tridentatus* hemocytes. This serine protease zymogen is unique in that it is activated by  $\alpha$ -chymotrypsin, but not by trypsin.
18. AKETAGAWA J, MIYATA T, OHTSUBO S, NAKAMURA T, HAYASHIDA H, MIYATA T, IWANAGA S: Primary Structure of *Limulus* Anti-coagulant Anti-lipopolysaccharide Factor. *J Biol Chem* 1986, 261:7375-7365.
  19. SMITH RH, PISTOLE TG: Bactericidal Activity of Granules Isolated from Amebocytes of the Horseshoe Crab, *Limulus polyphemus*. *J Invertebr Pathol* 1985, 45:272-279.
  20. OHASHI K, NIWA M, NAKAMURA T, MORITA T, IWANAGA S: Anti-LPS Factor in the Horseshoe Crab, *Tachypleus tridentatus* Its Hemolytic Activity on the Red Blood Cell Sensitized with Lipopolysaccharide. *FEBS Lett* 1984, 176:207-210.
  21. NAKAMURA T, FURUNAKA H, MIYATA T, TOKUNAGA F, MUTA T, IWANAGA S, NIWA M, TAKAO T, SHIMONISHI Y: Tachyplesin, a Class of Antimicrobial Peptide from Hemocytes of the Horseshoe Crab, (*Tachypleus tridentatus*). *J Biol Chem* 1988, 263:16709-16713.
  22. MIYATA T, TOKUNAGA F, YONEA Y, YOSHIKAWA K, IWANAGA S, NIWA M, TAKAO T, SHIMONISHI Y: Antimicrobial Peptides Isolated from Horseshoe Crab Hemocytes, Tachyplesin II, and Polyphemusins I and II: Chemical Structures and Biological Activity. *J Biochem (Tokyo)* 1989, 106:663-668.
  23. MUTA T, MIYATA T, TOKUNAGA F, NAKAMURA T, IWANAGA S: Primary Structure of Anti-lipopolysaccharide Factor from American Horseshoe Crab, *Limulus polyphemus*. *J Biochem (Tokyo)* 1987, 101:1321-1330.
  24. YONEZAWA A, KUWAHARA J, FUJII N, SUGIURA Y: Binding of Tachyplesin I to DNA Revealed by Footprinting Analysis: Significant Contribution of Secondary Structure to DNA Binding and Implication for Biological Action. *Biochemistry* 1992, 31:2998-3004.
- This is the first report of the interaction of DNA with tachyplesin, which contains a unique antiparallel  $\beta$ -sheet structure.
25. SHIGENAGA T, MUTA T, TOH Y, TOKUNAGA F, IWANAGA S: Antimicrobial Tachyplesin Peptide Precursor: cDNA Cloning and Cellular Localization in Horseshoe Crab (*Tachypleus tridentatus*). *J Biol Chem* 1990, 265:21350-21354.
  26. AKAJI K, FUJII N, TOKUNAGA F, MIYATA T, IWANAGA S, YAJIMA H: Studies on Peptides CLXVIII: Syntheses of Three Peptides Isolated from Horseshoe Crab Hemocytes, Tachyplesin I, Tachyplesin II, and Polyphemusin I. *Chem Pharm Bull* 1989, 37:2661-2664.
  27. ARMSTRONG PB, QUIGLEY JP, RICKLES FR: The *Limulus* Blood Cell Secretes  $\alpha_2$ -macroglobulin When Activated. *Biol Bull* 1990, 178:137-143.
  28. NGUYEN NY, SUZUKI A, CHENG SM, ZON G, LIU TY: The Amino Acid Sequence of *Limulus* C-reactive Protein. *J Biol Chem* 1986, 261:10450-10455.
  29. LIANG SM, SAKMAR TP, LIU TY: Studies on *Limulus* Amebocyte Lysate III: Purification of an Endotoxin-binding Protein from *Limulus* Amebocyte Membranes. *J Biol Chem* 1980, 255:5586-5590.
  30. LIANG SM, LIANG CM, LIU TY: Studies on *Limulus* Amebocytes. Isolation and Identification of a Membrane-bound Protein Activator of Cyclic Nucleotide Phosphodiesterase from *Limulus* Amebocytes. *J Biol Chem* 1981, 256:4968-4972.
  31. COPELAND DE, LEVIN J: The Fine Structure of the Amebocyte in the Blood of *Limulus polyphemus*. I: Morphology of the Normal Cell. *Biol Bull* 1985, 169:449-457.

32. MINETTI CASA, LIN Y, CISLO T, LIU TY: Purification and Characterization of an Endotoxin-binding Protein with Protease Inhibitory Activity from *Limulus* Amebocytes. *J Biol Chem* 1991, 266:20773-20780.

This paper describes the purification and cDNA cloning of an endotoxin-binding protein from limulus hemocytes. The mature form of this protein comprises 105 residues with a pI of 9.62. It exists in the secretory granules of the hemocytes where enzymes and substrates for the clotting cascade reside.

33. LIU T, LIN Y, CISLO T, MINETTI CASA, BABA JMK, LIU TY: Limunectin: A Phosphocholine-binding Protein from *Limulus* Amebocytes with Adhesion-promoting Properties. *J Biol Chem* 1991, 266:14813-14821.

The cDNA sequence encoding limunectin predicts a protein of 54 kD with an unusual structural feature: it consists almost entirely of 10 contiguous segments, 45 amino acids in length, with extensive homology. Like mammalian vitronectin, it binds to bacterial cells, fixed hemocytes and a number of extracellular matrix proteins.

34. MORITA T, TANAKA S, NAKAMURA T, IWANAGA S: A New (1-3)- $\beta$ -D-glucan-mediated Coagulation Pathway Found in *Limulus* Amebocytes C. *FEBS Lett* 1981, 129:318-321.

35. URAGOH K, SUEISHI K, NAKAMURA T, IWANAGA S: A Novel Immunohistochemical Method for *in Vivo* Detection of Endotoxin Using Horseshoe Crab Factor C. *J Histochem Cytochem* 1988, 36:1275-1283.

36. NAKAMURA T, TOKUNAGA F, MORITA T, IWANAGA S, KUSOMOTO S, SHIBA T, KOBAYASHI T, INOUE K: Intracellular Serine-Protease Zymogen, Factor C, from Horseshoe Crab Hemocytes: Its Activation by Synthetic Lipid A Analogues and Acidic Phospholipids. *Eur J Biochem* 1988, 176:89-94.

37. NAKAMURA T, TOKUNAGA F, MORITA T, IWANAGA S: Interaction between Lipopolysaccharide and Intracellular Serine Protease Zymogen, Factor C, from Horseshoe Crab (*Tachypleus tridentatus*) Hemocytes. *J Biochem (Tokyo)* 1988, 103:370-374.

38. TOKUNAGA F, YAMADA M, MIYATA T, DING YL, HIRANAGA-KAWABATA M, MUTA T, IWANAGA S, ICHINOSE A, DAVIE EW: Limulus Hemocyte Transglutaminase: Its Purification, Characterization, and Identification of the Intracellular Substrates. *J Biol Chem* 1993, in press.

This is the first report that characterizes invertebrate transglutaminase (TGase) and its natural substrates. This enzyme demonstrates mammalian type II TGase-like properties. The TGase activity is  $\text{Ca}^{2+}$ -dependent and is inhibited by primary amines, EDTA and SH-reagents. Two major potential substrates for TGase are identified in the hemocyte lysate. One is a cysteine-rich protein consisting of 81 amino acid residues and the other is a proline-rich protein of 80 kD.

39. TOKUNAGA F, MUTA T, IWANAGA S, ICHINOSE A, DAVIE EW: Limulus Hemocyte Transglutaminase: cDNA Cloning, Amino Acid Sequence, and Tissue Localization. *J Biol Chem* 1993, in press.

The cloned cDNA for limulus TGase consists of 2884 base pairs. An open reading frame of 2292 base pairs encodes the mature protein, which comprises 764 residues and has two molecular masses of 87.021 and 87.110 kD, due to two different cDNA clones. Limulus TGase has a unique amino terminal cationic extension of 60 residues with no homology with the amino termini of mammalian TGases.

40. KAWANO K, YONEYA T, MIYATA T, YOSHIKAWA K, TOKUNAGA F, TERADA Y, IWANAGA S: Antimicrobial Peptide, Tachyplesin I, Isolated from Hemocytes of the Horseshoe Crab (*Tachypleus tridentatus*). *J Biol Chem* 1990, 265:15365-15367.

41. NAKAMURA T, HIRAI T, TOKUNAGA F, KAWABATA S, IWANAGA S: Purification and Amino Acid Sequence of Kunitz-type Protease Inhibitor Found in the Hemocytes of the Horseshoe Crab (*Tachypleus tridentatus*). *J Biochem (Tokyo)* 1987, 101:1297-1306.

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