The role of hemolymph coagulation in innate immunity

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Invertebrate animals, which lack adaptive immune systems, have developed defense systems that respond to common antigens on the surface of potential pathogens. Hemolymph coagulation is one such defense system in innate immunity. The discovery of lipopolysaccharide-sensitive and (1→3)-β-D-glucan-sensitive serine protease zymogens in horseshoe crab (limulus) hemocytes, both of which trigger the coagulation cascade, has exemplified how the animals detect and respond to foreign materials.

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Abbreviations

EGF	epidermal growth factor
LAL	limulus amebocyte lysate
LICI	limulus intracellular coagulation inhibitor
LPS	lipopolysaccharide

Introduction

Virtually all multicellular organisms possess various defense systems against invading microorganisms. These defense systems are essential for their survival and perpetuity. Invertebrates, which do not have immunoglobulins, have developed unique modalities to detect and respond to microbial surface antigens, such as lipopolysaccharide (LPS), peptideglycan and (1→3)-β-D-glucan. Because both invertebrates and vertebrate animals respond to these substances, it is likely that a system recognizing these epitopes emerged at a very early stage in the evolution of animal species. It is well known that components of the bacterial cell wall elicit various responses, depending on the species of animal or cell type. In invertebrates, hemolymph coagulation [1–3] and melanin formation are prominent as such responses [4–8]. In addition to these enzymatic cascades, a variety of antimicrobial substances and lectins also cooperate with the reactions for killing the invaders [9]. This review focuses on the role of hemolymph coagulation in innate immunity.

To date, two types of clotting mechanisms have been reported in invertebrate animals. One of these is found in crustaceans (lobster and crayfish) and insects (cockroach and grasshopper) [10–13] where a gel is formed through the polymerization of clottable protein(s), catalyzed by Ca^{2+}-dependent transglutaminase. The transglutaminase is released from the hemocytes or muscle cells through an unknown mechanism. The clottable proteins isolated from lobster and crayfish hemolymph plasmas have similar characteristics to each other [10,11]. They are large lipoglycoproteins consisting of dimers with about 200 kDa subunits. Their amino-terminal sequences suggest that these are vitellogenin-like proteins. [11,14]. In insects, another abundant plasma protein, lipophorin, seems to be cross-linked upon gelation [13,15]. The other type of coagulation is proceeded by a cascade-type reaction composed of serine protease zymogens as is mammalian blood coagulation system. In horseshoe crab (or limulus), two pathways that involve either a LPS-mediated or a (1→3)-β-D-glucan-mediated coagulation reaction have recently been established and all essential clotting factors are now disclosed [3,16••].

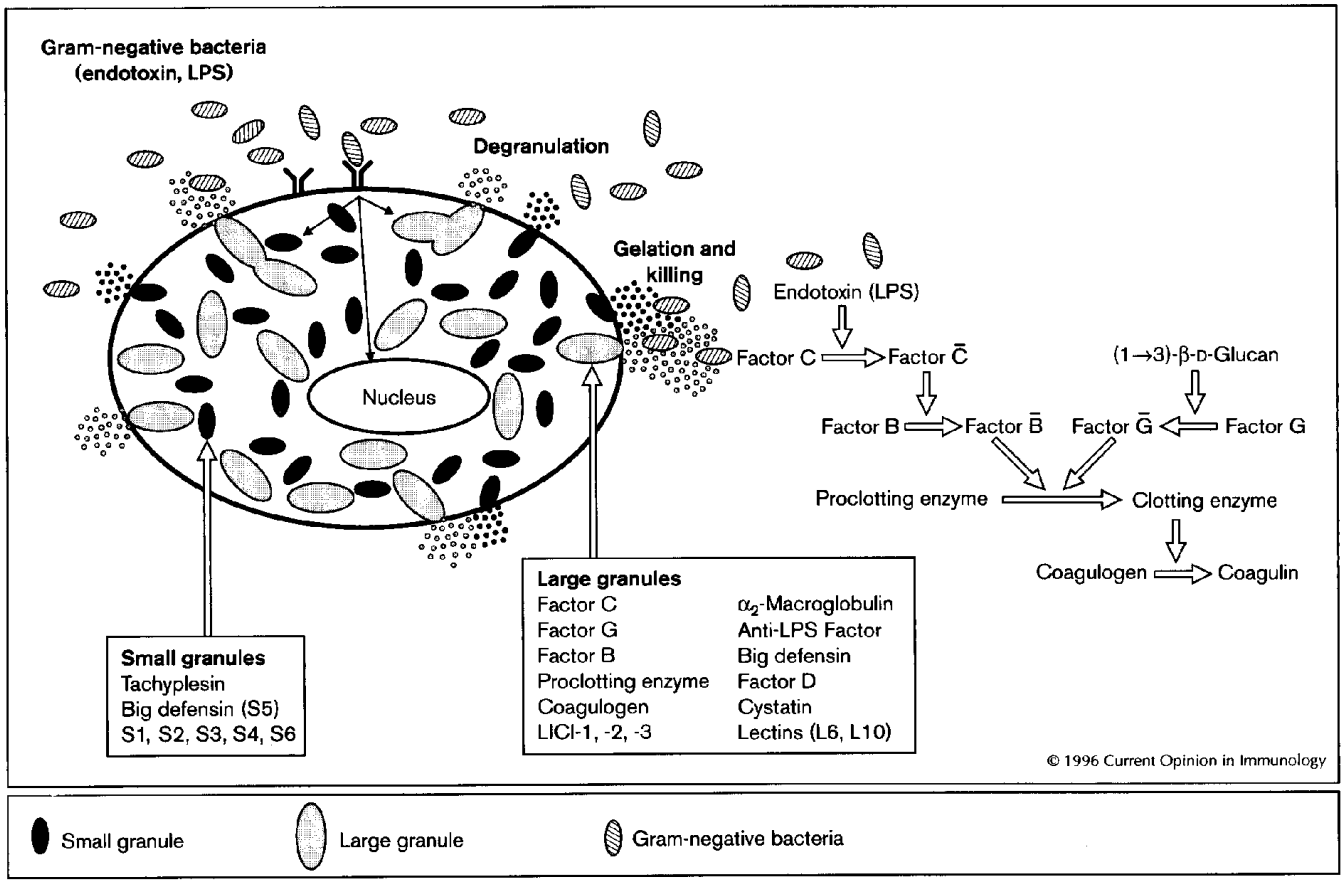
Exocytosis of hemocytes mediated by lipopolysaccharide

Hemocytes in the hemolymph of the horseshoe crab play a major role in the defense system of this animal. In contrast to hemocytes of insects or other invertebrates, a single type of hemocyte circulates in horseshoe crab hemolymph [17]. The hemocyte, which is also called granulocyte or amebocyte, contains two types of secretory granules: large granules and small, but dense, granules [17]. This cell is extremely sensitive to bacterial endotoxins (e.g. LPS). When Gram-negative bacteria invade the hemolymph, the hemocyte detects LPS molecules on their surface, and then releases the contents of the granules by rapid exocytosis (Fig. 1). The released granular components include two biosensors of the coagulation reaction, factor C and factor G [16••,18]. 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 (Fig. 1). The invaders in the hemolymph are thus engulfed or immobilized by the clot. They are subsequently cell-agglutinated and killed by various lectins and antimicrobial substances that are also released from the granules [3,19•].

Coagulation mediated by lipopolysaccharide

The LPS-mediated coagulation cascade involves three serine protease zymogens (factor C, factor B, and proclotting enzyme) and a clottable protein, coagulogen (Fig. 1 and Fig. 2). Factor C (123 kDa) is a biosensor that responds to LPS [18]. In the presence of LPS or synthetic lipid A analogs, it is autocatalytically activated to an active form, factor $\overline{C}$, which is composed of three chains held together by disulfide bonds: H chain (80 kDa), A chain (7.9 kDa) and B chain (34 kDa). Factor B (64 kDa) is then activated by factor $\overline{C}$ and, in turn, its active form (factor $\overline{B}$) activates

Figure 1



Defense systems in horseshoe crab hemocytes. The hemocyte detects LPS on Gram-negative bacteria and initiates exocytosis of the large and small granules. The coagulation 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 lectins [45,46] and subsequently killed by antibacterial substances, such as tachyplesins, anti-LPS factor, and big defensin [19•,28•]. The large granules also contain proteinase inhibitors, such as α_2-macroglobulin [47] and cystatin [48], and an azurocidin-like pseudo-serine protease with antimicrobial activities, factor D (S Kawabata *et al.*, personal communication).

proclotting enzyme to clotting enzyme (54 kDa) [20,21]. The active clotting enzyme converts coagulogen to an insoluble coagulin gel [2].

All the three zymogens contain a serine protease domain at their carboxyl terminus. Their amino-terminal end, however, shows interesting structures, indicating a mosaic protein probably derived from exon shuffling. The H chain of factor C, which binds LPS, contains five 'sushi' domains, an epidermal growth factor (EGF)-like domain, and a C-type lectin like domain [22]. The finding of 'sushi' (also called SCR or CCP) domains in factor C made it the first protein in invertebrates that has been discovered to have this type of domain. The fact that this initiator of the horseshoe crab clotting cascade contains 'sushi' domains that are found mainly in mammalian complement factors, led us to speculate that both coagulation and mammalian complement systems may have evolved from a common origin.

The amino-terminal L chains of factor B and proclotting enzyme contain a small compact domain with three disulfide bonds, called 'clip' domain (formerly called 'disulfide-knotted' domain) [23,24]. The 'clip' domain has been found in the *Drosophila* snake and easter protease precursors [25]. Both easter and snake proteins are indispensable for the normal embryonic developments in flies [26,27]. The presence of this type of domain in *Drosophila* strongly suggests the existence of a protease cascade system similar to that of the horseshoe crab. The folding pattern of the three disulfide bridges in the 'clip' domain is identical to that of 'big defensin', which was recently identified as the third antimicrobial protein in the horseshoe crab hemocytes (Fig. 3) [28•]. As the carboxy-terminal end of the 'clip' domain in proclotting enzyme constitutes a hinge region susceptible to protease attack [23], the 'clip' domain(s) might be released during the activation of the zymogens to work as antimicrobial substances. If this is the case, the coagulation cascade

itself could produce antimicrobial substances during the activation. The system may have dual actions: coagulation and the killing of invading microorganisms.

Coagulogen is one of the major proteins in the large granules of the hemocytes [29]. Coagulogens from four species of horseshoe crab are all basic proteins composed of 175 amino acids, with the molecular mass of 20 kDa [2]. Upon activation by clotting enzyme, two peptidyl bonds are cleaved, resulting in the conversion into an insoluble coagulin gel consisting of A and B chains connected by disulfide bonds, and, thus, releasing a peptide of 28 residues, peptide C. Although a transglutaminase has been isolated from the hemocytes [30,31], the coagulin gel is not cross-linked as in fibrin clot or clottable proteins of insects. The exact mechanism of this gelation is as yet unknown, but the crystallization of coagulogen has been accomplished recently. Thus, the X-ray crystallography would provide insights into the elucidation of molecular mechanism of the gelation.

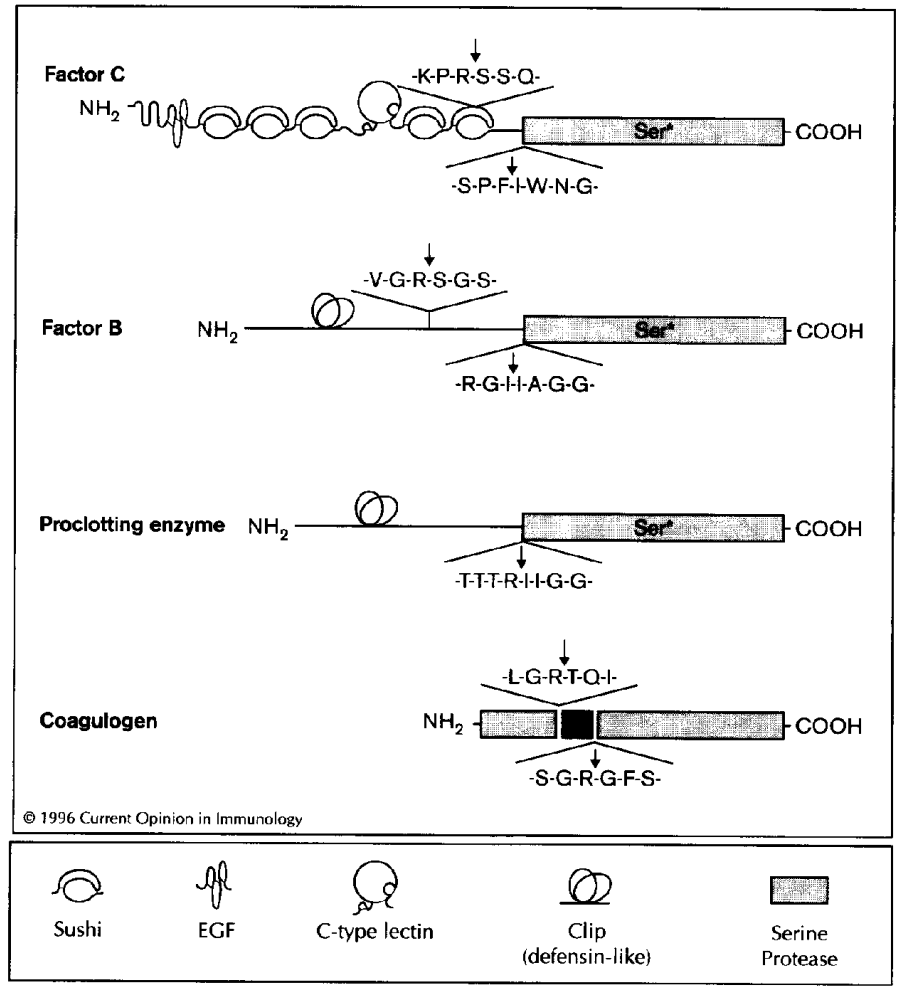
Activation of all the factors is accompanied by limited proteolyses. Coagulogen and proclotting enzyme are activated by the cleavage(s) of Arg–X bonds (Fig. 2). In contrast, the autocatalytic activation site in factor C is Phe–Ile, although factor $\overline{C}$ shows trypsin-like substrate specificity against small peptidyl substrates. Furthermore, the activation site in factor B is Ile–Ile, again emphasizing unique substrate specificity of factor $\overline{C}$.

Coagulation mediated by (1→3)-β-D-glucan

The LPS-mediated coagulation system is the principle of the so-called limulus test or limulus amebocyte lysate (LAL) test. Because of its high sensitivity and convenience, this test is now being widely utilized to detect and quantitate trace amounts of contaminating pyrogen or LPS in the clinical field [32]. However, during the diagnostic application of the limulus test, it was pointed out that positive reactions are often observed in some patients' plasma even in the absence of LPS. As some of those patients were suffering from a fungus infection, or were undergoing hemodialysis, this pseudopositive reaction had

Figure 2

Gross structures of limulus coagulation factors. Coagulogen is a much smaller protein than mammalian homolog, fibrinogen. The other factors contain various unique domain structures, in addition to a serine protease domain. Arrowheads indicate the cleavage site(s) (one-letter amino acid code) resulting from the activation.



been suspected to be caused, at least in part, by glucans. In 1981, we and others [33,34] found the presence of a protease zymogen that was sensitive to β-glucan in the hemocyte lysate. Since then, the purification of this protein, factor G, had been hampered by its instability. However, it was recently isolated as a pure zymogen, which allowed the characterization of this novel zymogen [16••,35••].

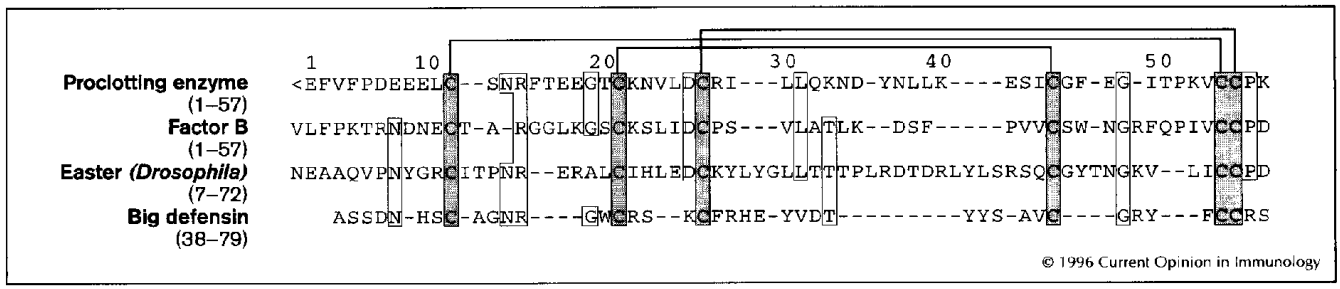
The purified factor G zymogen is autocatalytically activated in the presence of (1→3)-β-D-glucan, without any other proteins [16••]. The resulting active factor $\overline{G}$ activates proclotting enzyme directly, which links with the coagulin gel formation. As factor G colocalizes in the large granules together with components participating in the LPS-mediated coagulation cascade (Y Takaki, T Muta, N Seki, S Iwanaga, unpublished data), it can be released into hemolymph upon cell activation. This β-glucan-mediated coagulation pathway is activated on the surface of fungi.

Factor G is a physical heterodimer composed of a 72 kDa subunit α and a 37 kDa subunit β, associated with non-covalent bonds (Fig. 4) [16••]. As these two subunits derive from separate genes [35••], they are translated independently and assembled in the cell. Subunit β is a serine protease zymogen with a short, 15 amino acid, amino-terminal extension [35••]. The serine protease domain is most homologous to factor B (40.5% amino acid identity) and proclotting enzyme (37.7% amino acid identity), suggesting that these three had a common origin. On the other hand, subunit α shows a unique mosaic structure. The amino-terminal region contains a bacterial β-(1,3) glucanase-like sequence [36]. The carboxy-terminal region has two tandem repeats, each of which shows sequence similarity with that found in xylanase Z [37]. The catalytic domain of xylanase Z, is not, however, contained within these regions. In the middle part of the molecule, there are three tandem-repeat structures. This type of tandem-repeat is found in xylanase A [38], Rarobacter protease I [39], β-(1,3)-glucanase [40], and ricin B chain [41]. In all of these proteins, this structure is responsible for the carbohydrate binding. Recently, subunit α expressed by a baculovirus system was shown to have the ability to bind to β-glucan in the absence of subunit β (N Seki *et al.*, unpublished data).

The purified factor G is also activated by various other glucans containing (1→3)-β linkages from different origins, but not by LPS, sulfatides, and cholesterol sulfates [16••]. The most effective activators that have been examined are linear (1→3)-β-D-glucans, such as curdlan and paramylon. As little as 1 ng of curdlan significantly activates the zymogen factor G. Branching of the linear chain with (1→4)-β 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 factor G activation show a bell-shaped curve: activation is inhibited at higher concentrations of β-glucan. At the optimum condition, 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.

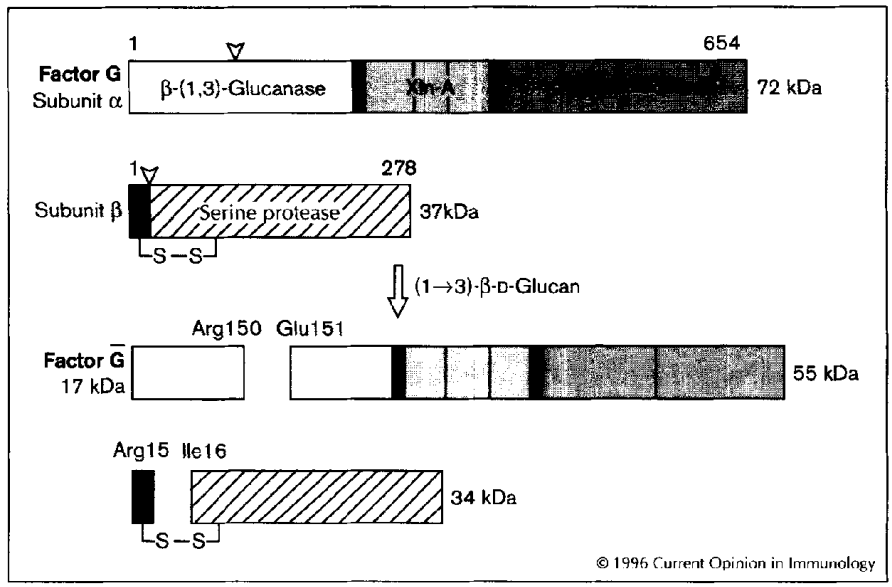
In the presence of β-glucan, factor G is involved in complex processes, including autoactivation and autoinactivation [16••]. Upon incubation with β-glucan, the emerged amidase activity increases up to 20 minutes, and then gradually decreases. During the activation process, an Arg15–Ile16 bond in subunit β and an Arg150–Glu151 bond in β-(1,3) glucanase-like domain in subunit α are cleaved. This results in the conversion of the 37 kDa subunit β to a 34 kDa fragment, and the 72 kDa subunit α to a 55 kDa and a 17 kDa fragment (Fig. 4). These chain conversions are completed within 20 minutes, and then the 55 kDa fragment is further degraded to a 46 kDa fragment, which seems to be responsible for the reduction of the activity. This fact suggests that the activity of subunit β is tightly regulated by subunit

Figure 3



Sequence alignment of 'clip' domains and 'big defensin'. 'Clip' domains found in limulus coagulation factors and *Drosophila* serine protease zymogens show an identical disulfide-folding pattern to that of an antibacterial protein, 'big defensin'. The disulfide linkages have been determined in proclotting enzyme [23] and big defensin [28•]. Conserved residues among more than three proteins are boxed, and six cysteines conserved in all the proteins are shadowed.

Figure 4



Structural change during the activation of zymogen factor G. The zymogen factor G, which has been recently found in limulus hemocytes, consists of a heterodimeric protein. Subunit β contains a serine protease domain and subunit α has (1→3)-β-D-glucan-binding ability. Upon incubation with (1→3)-β-D-glucan, the zymogen factor G is converted to the active factor $\overline{G}$ with limited proteolyses in both subunits.

α. In both subunits, the carboxy-terminal sides of Arg residues are cleaved, which is consistent with the substrate specificity of factor $\overline{G}$. However, none of horseshoe crab coagulation factors with trypsin-like specificity, or trypsin itself, activates factor G, suggesting that the cleavage site in subunit β is somehow masked by subunit α.

The biochemical evidence described above suggests the following hypothesis on the activation of zymogen factor G: binding of β-glycan to subunit α exposes the activation site of subunit β, which is hindered in the zymogen form, thus allowing autocatalytic activation through bimolecular interaction between subunit βs. The active subunit β then quickly hydrolyzes the Arg150–Glu151 bond in subunit α. After this, another site in the same subunit is cleaved, which causes the inactivation of the protease activity.

Regulation of the coagulation cascade

To date, three types of serpin-type serine protease inhibitors have been isolated from the horseshoe crab hemocytes: limulus intracellular coagulation inhibitor (LICI)-1, LICI-2, and LICI-3 [42•,43•,44]. All LICIs belong to serpin family and form stable complexes with target serine proteases. Of these serpins, LICI-1 specifically inhibits factor $\overline{C}$, whereas both LICI-2 and LICI-3 inhibit factor $\overline{C}$, factor $\overline{G}$, and the clotting enzyme activities. LICI-2 inhibits the clotting enzyme more strongly, and LICI-3 favors factor $\overline{G}$ more than the other enzymes. All inhibitors are stored in the large granules and are exocytosed upon activation of the cells. Thus, these inhibitors are likely to work to prevent diffusion of the active coagulation factors, which may cause unnecessary clot formation.

Conclusions

Innate immunity requires 'adequately specific' biosensors; they should react with various epitopes that consist of slightly different structures on variety of pathogens, but must distinguish 'self' and 'non-self' epitopes. Examples of such specificity can be found in the activation of the horseshoe crab biosensors, factor C and factor G. Overall mechanisms of this coagulation system are now well understood as the last, long-hunted, coagulation factor, factor G, has now been isolated and characterized. As this coagulation is initiated by their pathogens, it plays important roles not only in hemostasis, but also in the defense to immobilize invading microorganisms. In contrast to these coagulation mechanisms, the cellular mechanisms detecting and responding to foreign materials are poorly understood. These responses must be initiated through 'pattern recognition' by specific protein(s) on the membrane. There is no doubt that the elucidation of such mechanisms will be one of the major focuses of this field in the next few years.

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