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JB Reflections and Perspectives
Sadaaki Iwanaga: discovery of the lipopolysaccharide- and β-1,3-D-glucan-mediated proteolytic cascade and unique proteins in invertebrate immunity

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Horseshoe crab haemolymph contains a single type of cells, granular haemocytes, which are extremely sensitive to bacterial lipopolysaccharides (LPS) and lead to haemolymph coagulation. Sadaaki Iwanaga isolated protease zymogens from the haemocytes and reconstituted LPS and β-1,3-D-glucans-mediated haemolymph coagulation. This led to the first discovery of a proteolytic cascade triggered by pathogen-associated molecular patterns, an important milestone for studies on invertebrate innate immunity. Moreover, he separated components derived from haemocyte granules and haemolymph plasma, and consequently identified unique defense molecules, such as lectins, serpins, cystatins, antimicrobial substances and substrates for transglutaminase. Through steady and persistent studies on the horseshoe crab host defense system, he made great progress in the field. Now we know that LPS-induced haemocyte exocytosis leads not only to coagulation but also activates a sophisticated immune response network that coordinately induces pathogen recognition, elimination and wound healing.

Sadaaki Iwanaga attended the Graduate School of Pharmaceutical Sciences, Kyoto University as a graduate student in Tomoji Suzuki's lab, and received his PhD in 1960. He was a faculty member in Suzuki's lab at Kyoto University and at the Institute for Protein Research at Osaka University, and worked as a visiting investigator at Birger Brombeck's lab at the Karolinska Institute in Sweden. He was appointed professor at the Department of Biology of Kyushu University in December 1978. The first author of this review joined in his laboratory as one of its first undergraduate students in April 1979. At that time, there were no scientific instruments of value in the laboratory other than classic spectrophotometers and fraction collectors. Even after retirement from Kyushu University in March 1996, he has devoted himself to discuss the sciences with any researchers at the front line of domestic and international scientific meetings and to direct researchers belonging to the Chemo-Sero-Therapeutic Research Institute (Kaketsu-Ken) in Kumamoto (Fig. 1). Since his days as a graduate student at Kyoto University, he has published more than 450 scientific papers, contributing greatly to biochemical and structure–function studies of proteins and proteases involved in snake venoms (1), plasma kallikrein-kinin systems (2), mammalian blood coagulation (3) and invertebrate host defense systems (4–14). Here, we review his contribution to the host defense system of the horseshoe crab, with an emphasis on the importance of haemocytes and haemolymph plasma.

Extant horseshoe crabs comprise four species—*Limulus polyphemus*, *Tachypleus tridentatus*, *T. gigas* and *Carcinoscorpius rotundicauda*—each having a distinct geographic distribution. *Limulus polyphemus* is distributed along the east coast of North America, and the other three species are distributed throughout Southeast Asia. In Japan, *T. tridentatus* inhabits coastal areas of the northern part of Kyushu as well as the Inland Sea (Fig. 2). *T. tridentatus* is relatively long-lived; the embryo molts four times within the fertilized eggs, and after hatching it molts every year for 15 years to become a mature adult (15). When Iwanaga arrived at Kyushu University, he understood his good fortune to have a position at one of the best places to investigate the host defense system of the horseshoe crab.

Historical background of horseshoe crab studies

Haemolymph coagulation of *L. polyphemus* was described, perhaps for the first time, by William H. Howell of Johns Hopkins University in 1885 (16). Howell aptly concluded that there was a striking resemblance in the methods by which corpuscles unite to form clots in the blood of mammals and in *L. polyphemus*, suggesting that the formation of fibrous gels in the two cases may result from an essential similar series of changes. Early studies on haemolymph gelation and granular haemocytes were performed by Leo Loeb, and granular haemocytes were named as amebocytes

Fig. 1 Sadaaki Iwanaga.

Fig. 2 Horseshoe crab (*T. tridentatus*) collected in Imatsu Bay, Fukuoka prefecture. Toshiyuki Miyata (left) and Sadaaki Iwanaga (right) in 1986.

by virtue of their amoeboid movement. Interestingly, Hideyo Noguchi at the University of Pennsylvania—who later gained fame from his studies on snake venoms and syphilology—in 1903 injected horse red blood cells into *L. polyphemus* and reported a strong haemagglutinating activity in haemolymph plasma (*17*). In 1956, Frederik Bang of Johns Hopkins University and the Marine Biological Laboratory in Woods Hole, published a landmark study on horseshoe crab haemolymph coagulation, finding that

L. polyphemus intravascular clotting was induced by an injection of a ubiquitous marine pathogen, *Vibrio* (*18*). In 1964, Jack Levin and Bang made the important discovery that coagulation occurs when exposed to bacterial endotoxin [lipopolysaccharide (LPS)]. Moreover, in 1968, they found that procoagulant activity and a clottable protein are located in granular haemocytes and not in haemolymph plasma. They immediately recognized the high sensitivity of LPS-mediated haemolymph coagulation and its applicability to haemocyte lysate, named *Limulus* amebocyte lysate (LAL), for assaying the bacterial endotoxin. These historical events have been described in detail in ref. (*16*). The clottable protein was named coagulogen by virtue of its functional similarity to mammalian fibrinogen.

Molecular mechanism of haemolymph coagulation

In the early 1970s, Makoto Niwa of Osaka City University Medical School displayed a micrograph for haemolymph clots to Iwanaga at Osaka University's Institute for Protein Research. Iwanaga was very impressed to find a fibrin-like structure of clots on the micrograph. He then started to purify coagulogen and to biochemically characterize the proteolytic conversion of coagulogen to coagulin, using a partially purified protease, the clotting enzyme. Shin Nakamura (now at Kyoto University) and Iwanaga published their results in 1976 (*5*). Fumio Shishikura and Koichi Sekiguchi also found that the antigenicity of the coagulogen of *L. polyphemus* is very different from those of the other Asian species, based on Ouchterlony double immunodiffusion. In 1977, Teh-Yung Liu of the FDA's Bureau of Biologics in Bethesda, reported the purification of coagulogen from *L. polyphemus* and the proteolytic conversion of coagulogen by the proclotting enzyme. Liu also showed that the activation of the proclotting enzyme depends on the presence of Ca^{2+} and LPS, and that the proclotting enzyme contains γ-carboxyglutamic acids, as does bovine prothrombin. Later, Iwanaga and his group clearly indicated that the proclotting enzyme that cleaves coagulogen is insensitive to LPS, requires no Ca^{2+} for its activity, and contains no γ-carboxyglutamic acid in the sequence (*19*, *20*).

After the LAL test for detecting LPS was established, Iwanaga developed a chromogenic peptide substrate for the clotting enzyme (*4*). He showed that the proclotting enzyme is not an LPS-sensitive protease zymogen and that another protease zymogen that is partially purified, named factor B—since the zymogen was present in 'fraction B' separated by heparin–agarose chromatography of haemocyte lysate—is involved in the proteolytic activation of the proclotting enzyme to the clotting enzyme. Upon further purification of fraction B by introducing dextran–sulphate column chromatography, an LPS-sensitive serine protease zymogen factor C was identified in 'fraction C' and characterized in detail (*21–23*). Takanori Nakamura (now at Kagawa University), Takashi Morita (who

recently retired from Meiji Pharmaceutical University) and Iwanaga highly purified three serine protease zymogens—the proclotting enzyme, factors B and C—and succeeded in reconstituting LPS-mediated haemolymph coagulation with the purified protease zymogens and coagulogen, thus establishing the cascade-type reaction (19, 21). Chromogenic substrates highly specific to each protease in the cascade were developed for the purification and characterization of the enzymes. They contributed the development of the second-generation LAL test, which is much more sensitive and quantitative than the original test, which is based on the turbidity of the lysate (4).

In 1980, Iwanaga was informed by Atsushi Kakinuma of Takeda Chemical Industries that a pyrogen-free preparation of antitumor β-1,3-D-glucans (BDG) gives a positive reaction against the LAL test at a concentration as low as $1.0\,\mu g/ml$ (24). Shortly thereafter, Iwanaga identified a new protease zymogen involved in BDG-mediated haemolymph coagulation, named factor G—so-called since the zymogen was present in the 'glucan-sensitive fraction' separated by heparin–agarose chromatography of haemocyte lysate—and reported that granular haemocytes contain two independent coagulation pathways, LPS- and BDG-mediated pathways, which respond to Gram-negative bacteria and fungi, respectively (25). However, another 10 years passed before factor G was obtained with high homogeneity, due to its instability during purification. Muta and Iwanaga reported that purified factor G is a heterodimeric serine protease zymogen with a large BDG recognition subunit and a small protease zymogen subunit (26, 27). The purified factor G was sensitive to as low as 1 ng/ml BDG.

As a result, the protease zymogens of the haemolymph coagulation were identified. This resulted in the first discovery of a proteolytic cascade induced by the pathogen-associated molecular patterns (PAMPs), LPS and BDG, in invertebrates as an important milestone for studies on invertebrate immunity. In summary, factor C secreted from granular haemocytes is autocatalytically activated in the presence of Gram-negative bacteria or LPS, and activated factor C activates coagulation factor B, which in turn converts the proclotting enzyme into the clotting enzyme. The clotting enzyme then promotes the proteolytic conversion of coagulogen to coagulin, releasing the activation peptide, and the resulting coagulin spontaneously forms an insoluble polymer. Alternatively, activated factor G in the presence of BDG triggers the activation of the proclotting enzyme to the clotting enzyme. In this manner, factors C and G independently serve to couple the recognition of LPS and BDG, respectively, and thus to form a physical barrier at the site of microbial invasion (Fig. 3).

Coagulogens derived from the four species were completely sequenced at protein level by Toshiyuki Miyata (now at the National Cardiovascular Center) and Iwanaga. The sequencing indicated that *T. tridentatus* and *C. rotundicauda* are phylogenetically more closely related than the other species, and that *L. polyphemus* is evolutionarily distant from the other three species (5). In the early 1980s, Miyata and Iwanaga

introduced cutting-edge technologies for molecular cloning and immediately completed the nucleotide sequence of coagulogen from *T. tridentatus* (28). Muta and Iwanaga cloned cDNAs for all the protease zymogens involved in the coagulation cascade to reveal their molecular architectures (20, 29–31). The proclotting enzyme and factor B harbor a unique common motif, named the clip-like domain, at their NH$_2$-terminal region (20, 29). Later, other groups identified homologous clip-like domains in the NH$_2$-terminal regions of many insect serine protease zymogens (10, 11). Factor C was a unique mosaic protein that contained a Cys-rich region, an epidermal growth factor-like domain, five Sushi domains (also designated as complement control protein modules or short consensus repeats), and a C-type lectin domain, in addition to a typical serine protease domain at the COOH-terminus (30). This was the first report that a Sushi domain found in mammalian complement-related proteins is involved in the invertebrate serine protease family. Factor G was a heterodimeric serine protease zymogen composed of two noncovalently associated subunits, α and β. The β subunit contains a serine protease domain, and the α subunit comprises three types of noncatalytic glycosidase-like modules: a single β-1,3-D-glucanase A1-like module, three tandem xylanase A-like modules, and two tandem xylanase Z-like modules (31).

During the purification of coagulation factors, the presence of their inhibitors in the haemocytes was suggested. Kawabata and Iwanaga then showed that the proteolytic cascade of haemolymph coagulation is regulated and scavenged by three types of serine protease inhibitors (serpins 1–3) with relatively high specificities for activated factor C, the clotting enzyme and the activated factor G, respectively (32–34). Interestingly, horseshoe crab serpins were more closely related to mammalian serpins than to insect serpins: for example, horseshoe crab serpin-1 versus human plasminogen activator inhibitor (40% sequence identity) and silkworm antichymotrypsin (27%) (32). Mammalian serpin–protease complexes are hypothesized to be rapidly cleared through a cell-surface receptor, which recognizes a hydrophobic consensus sequence that is selectively exposed on the complexed forms of serpins. This consensus sequence was conserved in horseshoe crab serpins in the corresponding region. Horseshoe crab serpins appear to prevent diffusion of the activated forms of coagulation factors by scavenging activated proteases that escape into the haemolymph from the surfaces of microbes at the site of injury, and thereby prevent unnecessary clot formation (Fig. 3).

Evolutionary meaning of the coagulation cascade of the horseshoe crab

At a site of vascular injury, the mammalian coagulation system acts locally on the cell membrane phospholipid surface in cooperation with Ca^{2+}. In an analogous fashion, the haemolymph coagulation in the horseshoe crab is restricted to the surfaces of
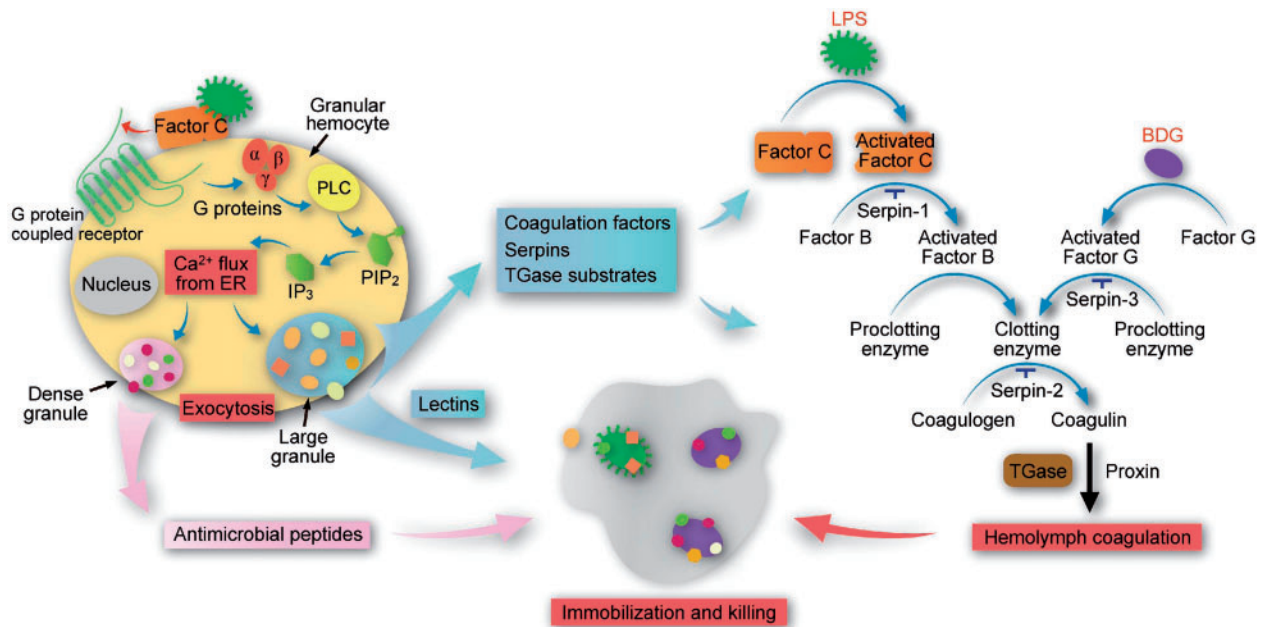
Fig. 3 LPS-activated immune response network of the horseshoe crab. PLC, phospholipase C; PIP$_2$, phosphatidylinositol-4,5-biphosphate; IP$_3$, inositol-1,4,5-triphosphate; ER, endoplasmic reticulum.

invading pathogens, such as Gram-negative bacteria and fungi. This mechanistic similarity between the coagulation cascades of vertebrates and those of horseshoe crabs may lead to the erroneous assumption of a common evolutionary origin. The three-dimensional structure of coagulogen was determined in collaboration with Robert Huber and Wolfram Bode of the Max Plank Institute, and indicated no structural similarity or evolutionary relatedness of coagulogen to fibrinogen (*35*). The crystal structure of coagulogen exhibited a striking topological similarity to the neurotrophin nerve growth factor, providing the first evidence for a neurotrophin fold in invertebrates.

A protease cascade in *Drosophila* is well characterized as the morphogenetic cascade for determining embryonic dorsal–ventral polarity, leading to the production of a Toll 1igand spätzle. The spätzle is postulated to belong to the nerve growth factor family and possesses a cystine knot motif similar to that of coagulogen (*35*, *36*). In addition, a clip-like domain located in the NH$_2$-terminal region of horseshoe crab coagulation factor B and the proclotting enzyme was identified in the proteins snake and easter of the *Drosophila* toll pathway (*10*, *11*, *36*). The structural similarity between coagulogen and spätzle, as well as that between the clip-like domain containing serine protease zymogens participating in the two cascades, together suggest that the two functionally distinct cascades may have a common evolutionary origin. Now we know that the *Drosophila* toll pathway also controls resistance to fungal and Gram-positive bacterial infections by producing antimicrobial peptides as an innate immune system in the adult (*37*). The identity of the *Drosophila* protease cascade is now being intensely explored. It has been found that many of the serine protease zymogens involved in the cascade also harbor the clip-like domain. Thus, the

horseshoe crab coagulation cascade has provided a most extensively and comprehensively characterized model of the invertebrate innate immune system.

Identification of defense molecules in haemocytes

Iwanaga also isolated many antimicrobial substances from both haemocytes and haemolymph of the horseshoe crab. During the separation of the coagulation factors, a potent anticoagulant protein with LPS binding activity, named the anti-LPS factor, was found in the haemocyte lysate of *T. tridentatus* and *L. polyphemus* (*38*, *39*). The anti-LPS factor strongly inhibited the LPS-mediated activation of the coagulation cascade, and exhibited an antibacterial effect on the growth of various Gram-negative bacteria. Takanori Nakamura and Iwanaga discovered another potent anticoagulant peptide with LPS binding activity, named tachyplesin, in an acid extract of haemocyte debris (*40*). The ^{1}H NMR analysis of tachyplesin showed an amphipathic structure of an anti-parallel β-sheet, possibly associated with bactericidal activity (*41*). Several tachyplesin homologues, named polyphemusins, were also isolated from *L. polyphemus* by the acid-extraction method (*42*). Thereafter, the acid extraction of basic peptides from haemocyte debris became a standard methodology for purifying horseshoe crab antimicrobial peptides. Iwanaga then reported the synthesis of tachyplesin as a precursor protein with an acidic extension peptide and that tachyplesin is localized in the dense granules rather than in the large granules in the haemocytes (*43*).

In 1985, Copeland and Levin examined the morphology and fine structure of the granular haemocyte of *L. polyphemus*, and found that the haemocyte contains two types of granules: large and dense ones. Yoshihiro

Toh (who recently retired from Kyushu University) and Iwanaga, using immunocytochemical analysis, revealed an interesting characteristic of the haemocyte granules of *T. tridentatus*: the large granules contain coagulogen, proclotting enzyme, and factor C, whereas the dense granules contain only tachyplesin (*20*, *30*, *44*). Moreover, Muta and Iwanaga separated the two types of granules by continuous sucrose density gradient centrifugation and purified their protein components by reverse-phase high-performance liquid chromatography (*45*). The information about partial amino acid sequences and amino acid compositions of the isolated components became indispensable for screening unknown proteins or peptides involved in horseshoe crab immunity. Kawabata and Iwanaga newly identified several kinds of cysteine-rich peptides with antimicrobial activity and chitin-binding activity in the dense granules, including big defensin, tachycitin and tachystatins (*46*–*48*). For example, the NH$_2$-terminal domain of big defensin possessed more potent antimicrobial activity against Gram-positive bacteria than did the COOH-terminal domain. In contrast, the COOH-terminal domain homologous to mammalian β-defensins displayed more potent antimicrobial activity than did the NH$_2$-terminal domain against Gram-negative bacteria, suggesting a new hybrid-type sub-class within the defensin family that possesses two discrete functional domains with different antimicrobial activities.

During these studies, Kawabata and Iwanaga also found four types of lectins in the large granules, including tachylectins-1, -2, -3 and -4 (*49*–*52*). Although each tachylectin recognized PAMPs, their carbohydrate ligand specificities differed (*53*). Tachylectin-1 interacted with 2-keto-3-deoxyoctonate on the surface of Gram-negative bacteria, whereas tachylectin-2 bound to GlcNAc or GalNAc and recognized lipoteichoic acids of Gram-positive bacteria. In contrast, tachylectin-3 specifically recognized a certain sugar moiety on O-antigens of S-type LPS from several Gram-negative bacteria, such as *Escherichia coli* 0111:B4. Tachylectin-4 also recognized the O-antigen of *E. coli* 0111:B4 and showed ligand specificity for colitose (3-deoxy-L-fucose). In addition, fibrinogen homologues of the horseshoe crab, named tachylectins-5 and -5B, were newly found in haemolymph plasma as acetyl-group-recognizing lectins (*54*). Tachylectin-5A possessed extraordinarily strong haemagglutinating activity against all types of human erythrocytes (minimum agglutinating concentration ∼4 ng/ml). The extraordinarily strong activity and extremely high concentration of tachylectin-5A in the plasma (∼10 μg/ml) indicates that it plays a major role in the recognition and agglutination of invading pathogens. Furthermore, in 1996, a complement-related protein in plasma, α$_2$-macroglobulin, was sequenced in collaboration with Peter Armstrong *et al.* (*55*). Iwanaga also found three types of C-reactive proteins, including a number of isoproteins, all of which exhibited functional and structural diversity (*56*).

In mammals, the blood coagulation cascade culminates in the proteolytic conversion of soluble fibrinogen into insoluble fibrin. The resulting fibrin fibrils are further stabilized by intermolecular ε-(γ-glutamyl) lysine cross-linking by the plasma transglutaminase (TGase) factor XIIIa. In crustaceans, haemolymph coagulation depends directly on the TGase-mediated cross-linking of a specific clotting protein (a vitellogenin-related protein) without prior proteolytic cleavage. Fuminori Tokunaga (now at Osaka University) and Iwanaga purified and characterized TGase from the haemocyte of *T. tridentatus*; TGase was not present in the plasma and was ordinarily restricted to the cytoplasm of the haemocyte (*57*). The resulting data suggested that coagulogen is not a substrate for TGase and that coagulin or polymerized coagulin is cross-linked with one or more unknown substances. Two protein substrates for TGase, a proline-rich protein and an 8.6 kDa protein, were also identified, and they were localized in the large granules of the haemocyte. Then, Tokunaga and Iwanaga published the nucleotide sequence of TGase in collaboration with Akitada Ichinose (now at Yamagata University) and Earl Davie of the University of Washington; this was the first information about the nucleotide sequences of invertebrate TGases (*58*).

The present state and perspectives on horseshoe crab immunity

After Iwanaga's retirement from Kyushu University in March 1996, studies on the horseshoe crab immune system have continued by Kawabata and his colleagues, as detailed in a recent review (*59*). The crystal structural analyses of tachylectin-2 and -5A in collaboration with Huber and Bode clearly pointed out the importance of multivalency for achieving high specificity and high affinity to PAMPs (*60*, *61*). The NMR analyses in collaboration with Keiichi Kawano at Hokkaido University revealed the unique solution structures of horseshoe crab antimicrobial peptides (*62*). The prophenoloxidase activation system is an important part of innate immunity in crustaceans and insects, where it acts to detect and kill invading pathogens as well as to synthesize melanin for wound healing and encapsulation of pathogens. In 2000, the clotting enzyme or activated coagulation factor B was found to convert haemocyanin to phenoloxidase by forming a 1:1 complex. In addition, tachyplesin interacted with haemocyanin and also induced its intrinsic phenoloxidase activity. In 2004, an LPS sensor on the haemocyte surface was finally identified; factor C also existed on the haemocyte surface as a biosensor for LPS (*63*). Upon activation by LPS, factor C initiated haemocyte exocytosis via a G-protein-dependent exocytic pathway that was dependent on the proteolytic activity of factor C (Fig. 3). Factor C interacted with LPS via its NH$_2$-terminal Cys-rich domain. The binding parameter, however, could not explain the extraordinarily high sensitivity of the haemolymph for LPS as low as ∼10^{-13} g/ml (∼10^{-14} M). In 2005, an enhancing mechanism of LPS sensitivity of the haemolymph was solved. Interestingly, tachyplesin in the exocytosed fluid acted as a secondary secretagogue, thereby

dramatically enhancing the sensitivity of the haemocyte to LPS.

Through steady and persistent studies on the horseshoe crab host defense system, Iwanaga has made great strides in the field, catapulting horseshoe crabs into the limelight not as a living fossil but as a comprehensively characterized precedent model for invertebrate innate immunity. Now we know that LPS-induced haemocyte exocytosis in the horseshoe crab leads not only to coagulation but also activates a sophisticated innate immune response network that coordinately induces pathogen recognition, prophenoloxidase activation and TGase-dependent wound healing.

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Conflict of interest
None declared.

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