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Hemocyte Aggregation in the Solitary Ascidian *Halocynthia roretzi:* Plasma Factors, Magnesium Ion, and Met-Lys-Bradykinin Induce the Aggregation

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Abstract. Hemocytes of the ascidian *Halocynthia roretzi* undergo aggregation in hemolymph that has been collected from the body through the tunic. To investigate the mechanisms involved, we first established two methods of measuring hemocyte aggregation. In one method, hemocyte aggregation was quantified by its reduction of light scattering intensity as measured with a fluorescence spectrophotometer. In the other method, the increase of transmittance accompanying aggregation was measured with an ELISA reader. We found that ascidian plasma, Mg^{2+}, and Met-Lys-bradykinin can induce the hemocytes of *H. roretzi* to aggregate. The aggregation induced by any of these three substances was inhibited by EDTA, *N*-ethylmaleimide, and cytochalasin B. Lipopolysaccharide had little inducing effect. We also demonstrated that, when *H. roretzi* plasma was treated with trypsin, low molecular weight aggregation-inducing substances were produced. These results suggest that metal ions and peptide-like substances present in the hemolymph play essential roles in the progression of hemocyte aggregation of *H. roretzi.*

Introduction

Coagulation of body fluid (hemolymph) in some species of invertebrates is analogous to blood clotting in vertebrates (Boolootian and Giese, 1959; Young *et al.,* 1972; Barwig, 1985; Levin, 1985; Iwanaga, 1993). Coagulation is thought to prevent the loss of hemolymph from a wound, and it may also immobilize microorganisms that invade the body. In animals such as mollusks (Bayne, 1981), however, hemolymph coagulation does not occur, although the hemocytes do aggregate.

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We have been investigating the defense mechanisms of the solitary ascidian *Halocynthia roretzi,* in which hemocytes play an important role. The hemocytes contain two antimicrobial substances (Azumi *et al.,* 1990a, b) and a hemagglutinin that can agglutinate various bacteria (Azumi *et al.,* 1991a). The hemocytes also undergo several cellular defense reactions, such as phagocytosis (Fuke, 1979), a self and nonself recognition reaction (Fuke, 1980), and an enzyme release reaction (Azumi *et al.,* 1991b, 1993). Body fluid does not coagulate after the tunic of *H. roretzi* is injured, but the hemocytes do aggregate upon injury to the tunic or when hemolymph is collected through the tunic. This aggregation appears to be a cellular defense reaction that arrests bleeding. The mechanisms of hemocyte aggregation, however, have not yet been clarified.

Here we report two methods of measuring hemocyte aggregation of *H. roretzi* that have enabled us to characterize factors that are involved in the aggregation. We found that ascidian plasma, Mg^{2+}, and Met-Lys-bradykinin can induce the aggregation of ascidian hemocytes.

Materials and Methods

Hemolymph and hemocytes

Solitary ascidians, *H. roretzi,* were harvested in Mutsu Bay, Aomori Prefecture, Japan. The ascidians were chilled on ice for at least 30 min before collection of hemolymph to reduce the rate of aggregation, and the animals were kept on ice during the experiment. The tunic matrix was extensively washed with seawater, and a sample of hemolymph (1 ml) was collected from the space just beneath the epithelium at the tunic papilla through a 5-ml disposable plastic syringe with a 23-gauge needle. The total

hemolymph (50–100 ml) was also collected from individual animals: a cut was made in the tunic matrix, and the animals were allowed to bleed into 0.56 *M* NaCl containing 2.7 m*M* EDTA at pH 5.2 (EDTA solution). The EDTA and low pH prevented the hemocytes from aggregating. The volume of EDTA solution was equal to that of the original hemolymph. After centrifuging (800 × *g*, 10 min) this twofold-diluted hemolymph, the resulting pellet (hemocytes) obtained from each animal was gently washed with 5 ml of Ca^{2+}-, Mg^{2+}-free Herbst's artificial seawater (Ca^{2+}-, Mg^{2+}-free HASW; 450 m*M* NaCl, 9.4 m*M* KCl, 32 m*M* Na_2SO_4, and 3.2 m*M* $NaHCO_3$, pH 7.6), and was then suspended individually in Ca^{2+}-, Mg^{2+}-free HASW. The hemocytes in the individual suspension were counted and adjusted to about 5×10^6 cells/ml.

Chemicals

Bovine pancreatic trypsin, cytochalasin B, kyotorphin, dynorphin (1-8), formyl-Met-Leu-Phe, Arg-Gly-Asp-Ser (RGDS peptide), and ADP were obtained from Sigma Chemical Co. (St. Louis, Missouri). Lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 was purchased from Difco Laboratories (Detroit, Michigan). ACTH was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Peptide E and metorophinamide were from Peninsula Laboratories, Inc. (Belmont, California). Other biologically active peptides, such as Met-Lys-bradykinin, Met-enkephalin, and bradykinin potentiator C, were obtained from the Peptide Institute (Osaka, Japan).

Measurement of hemocyte aggregation

Method A: The hemolymph, or a suspension of hemocytes in Ca^{2+}-, Mg^{2+}-free HASW (1 ml, about 5×10^6 cells/ml), was added to a cuvette that contained a stirring bar and had been placed in a Hitachi 650-60 fluorescence spectrophotometer equipped with a holder controlled thermostatically at 15°C. The hemocyte suspension was stirred gently to promote aggregation, and the hemocyte aggregation that occurred spontaneously, or that was induced by addition of 100 μl of an inducer, was monitored by light scattering with excitation at 380 nm and emission at 400 nm.

Method B: 90 μl of a suspension of hemocytes in Ca^{2+}-, Mg^{2+}-free HASW (1×10^7 cells/ml) was added to each well of a 96-well plate and incubated at 4°C for 30 min to allow the hemocytes to attach to the bottom. The plate was placed on a Bio-Rad 2550 ELISA reader, and the absorbance at 405 nm [$(A405)_0$] was measured. After 10 μl of inducer was added to each well, the plate was shaken gently at 20°C for 10 min to promote aggregation and was then kept standing at 20°C. After 50 min, the absorbance at 405 nm [$(A405)_{60}$] was again measured. The degree of aggregation was defined as the difference in the transmittance (T) calculated from $A405$ before and after incubation at 20°C [$(T405)_{60} - (T405)_0$]. Percent aggregation in the presence of each inducer was calculated on the assumption that the degrees of aggregation observed in the presence and absence of 2 m*M* $MgCl_2$ were defined as 100% and 0%, respectively. Experiments were performed in triplicate, and the mean was calculated.

Plasma treatment

We used various preparations of plasma as inducers of aggregation: *Intact plasma* was prepared by centrifuging the hemolymph exuded from a cut made in the tunic and collected into a solution lacking EDTA. *Reacted plasma* was prepared by centrifuging the hemolymph after its hemocytes had been completely aggregated by incubation with agitation for 30 min at 20°C. *Acid-* or *alkali-treated plasma* was exposed to acid (pH 1) or alkali (pH 11) for one hour at 4°C and neutralized. *Heat-treated plasma* was heated to 90°C for 15 min. And *dialyzed plasma* was placed in dialysis tubing (10,000 molecular weight cut-off) and incubated overnight, at 4°C, against Ca^{2+}-, Mg^{2+}-free HASW.

Low molecular weight substances were produced from plasma by treatment with trypsin as follows: Intact plasma or dialyzed plasma was treated with 0.1 mg/ml trypsin at 20°C for 4 h. The reaction mixture was filtered through a Centricut membrane (10,000 molecular weight cut-off) by centrifugation (2700 × *g*, 1 h); the filtrate contains the low molecular weight substances, but does not contain the trypsin (molecular weight, 23,000) and other high molecular weight substances present in the plasma. The inducing effect on hemocyte aggregation of the filtrate containing low molecular weight substances was tested.

Results

Aggregation of hemocytes in hemolymph

Whether the hemolymph of *H. roretzi* was collected by syringe and needle or through a cut made in the tunic, aggregation of the hemocytes was observed under the microscope. In the initial stage of aggregation, hemocytes containing many vacuoles with filament-like inclusions (hemocytes of type C named by Azumi *et al.*, 1993) make contact to form small homogeneous aggregates. At the same time, amoeba-like hemocytes (type A and B hemocytes) migrate toward and contact vacuolated cells containing several vacuoles of high density (type F hemocytes); this triggers and promotes the aggregation of the latter type F hemocytes themselves. Finally, several large heterogeneous aggregates including almost all types of hemocytes are formed. The aggregation reaction on a slide (*without shaking*) is complete within a 1-h incubation at

15°C; (the reaction takes place more rapidly *with shaking* and is then complete within about 10 min). The hemocytes seem to remain intact during an overnight incubation (the hemocytes lyse slightly during a much longer incubation).

The change in light scattering measured in a cuvette at 15°C (Fig. 1) occurs concomitantly with the aggregation observed microscopically; when we took up the hemocyte suspension at increasing time intervals and examined it microscopically, aggregate formation seemed to be roughly concurrent with the decrease of light scattering. The aggregation was strongly inhibited by the presence of 1 m*M* EDTA. Thus, the degree of aggregation can be quantified from the curve generated by this method (method A).

Ascidian specimens were placed on ice to reduce hemolymph circulation. When samples of hemolymph were collected with a syringe at increasing time intervals from the same location on the tunic (point A in Fig. 2), the rate of hemocyte aggregation measured at 15°C increased with time (upper panel of Fig. 2; the slopes at 58 min, 96 min, and 142 min became steeper in this order). Thus, hemocyte aggregation seems to be activated at the point on the tunic that was wounded by the syringe needle. Indeed, when hemocytes were collected from another point on the same animal (point B in Fig. 2) 234 min after the initial collection of hemocytes at point A, the rate of aggregation of hemocytes at point B was almost the same as that at point A at 0 min (lower panel of Fig. 2). But when the ascidian was put into seawater (15°C), allowing hemolymph circulation, the rate of aggregation of hemocytes at point B increased to that seen at point A at 142 min (data not shown). These results suggest that soluble factors inducing hemocyte aggregation are released from a tunic wound, or in the aggregation reaction, and circulate through the body cavity.

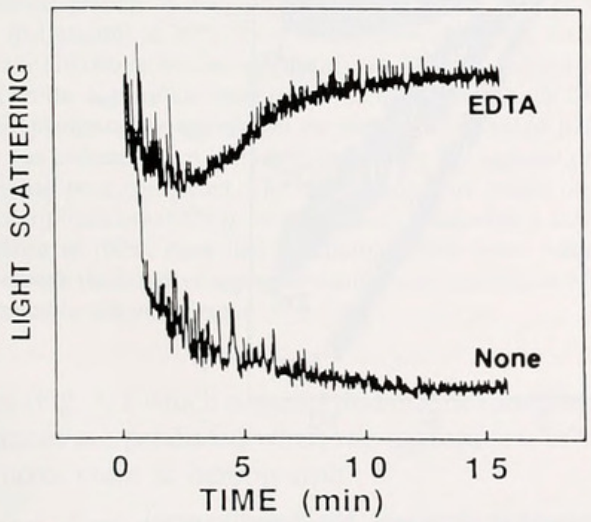

Figure 1. Effect of EDTA on aggregation of hemocytes in hemolymph of *Halocynthia roretzi*. The aggregation was measured at 15°C by method A. The concentration of EDTA used was 1 m*M*. The extent of light scattering is shown on the ordinate in arbitrary units.

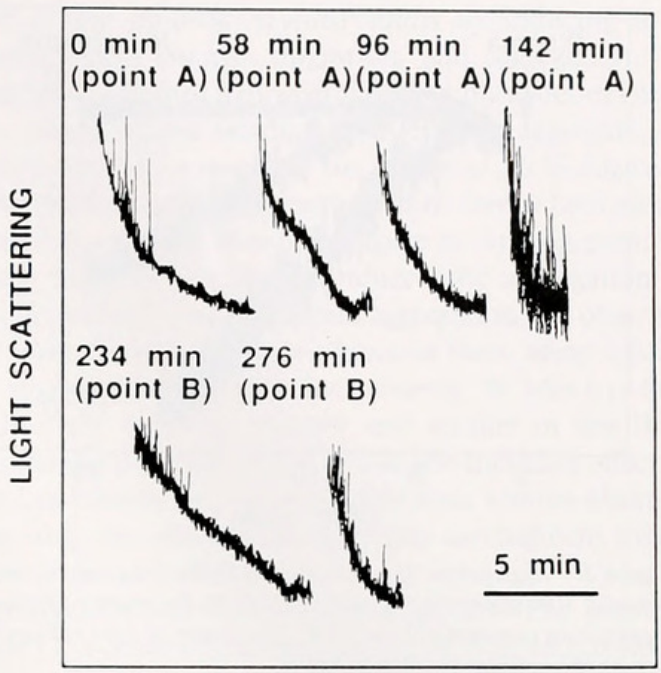

Figure 2. Activation of hemocyte aggregation in *Halocynthia roretzi*. Point A represents the same point on the tunic through which the hemolymph was repeatedly taken, and point B represents a different point on the tunic. The time between the initial collection of hemolymph (0 min) and subsequent collections is indicated above each record. The bar represents 5 min. The extent of light scattering is shown on the ordinate in arbitrary units.

Inducing effects of plasma and metal ions on aggregation of hemocytes

Isolated hemocytes of *H. roretzi* that had been collected from the hemolymph, washed, and resuspended in Ca^{2+}-, Mg^{2+}-free HASW exhibited a lower degree of aggregation than whole hemolymph. Moreover, hemocyte aggregation was stimulated by the addition of ascidian plasma (the final concentration was 10%), and the plasma-induced aggregation was dependent on temperature (Fig. 3). Thus, the plasma of *H. roretzi* seems to contain factors that can induce (or activate) the aggregation of hemocytes.

Because the aggregation, whether in hemolymph or in Ca^{2+}-, Mg^{2+}-free HASW supplemented with plasma, was inhibited by EDTA, a metal chelating agent, we next investigated the effects of metal ions on the aggregation. Magnesium ions ($MgCl_2$ or $MgSO_4$) induce the aggregation of hemocytes in a concentration-dependent manner (Fig. 4a). The aggregation reached a plateau at concentrations higher than 2 m*M* (aggregation was linear in the range of 0–2 m*M* Mg^{2+}). Of the other ions tested, 2 m*M* Mn^{2+}, Zn^{2+}, and Co^{2+} had inducing effects as strong as those of Mg^{2+}, whereas Ca^{2+} and Ni^{2+} had little effect (Fig. 4b). We usually measured the effects of Mg^{2+} on aggregation of hemocytes *with shaking* and detected its

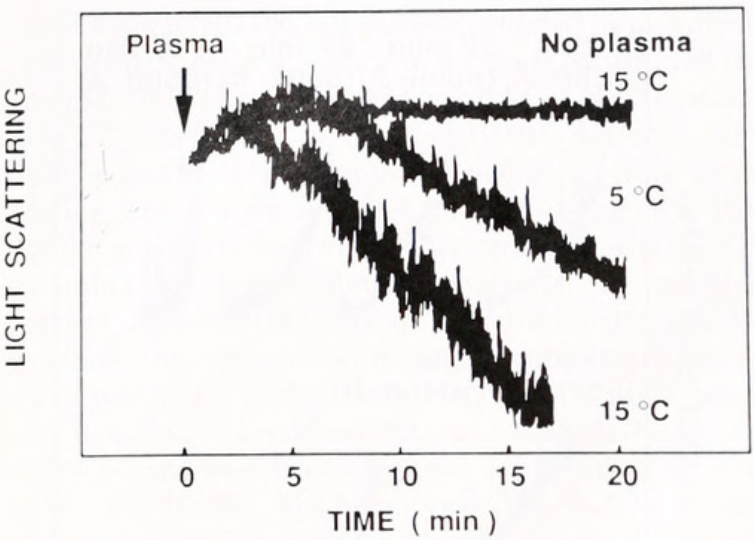

Figure 3. Aggregation of hemocytes of *Halocynthia roretzi* in the presence of 10% plasma at 5°C and at 15°C. In the absence of plasma, no aggregation occurred even at 15°C. The extent of light scattering is shown on the ordinate in arbitrary units.

inducing effects. *Without shaking,* however, Mg^{2+} had little inducing effect in contrast to the case of plasma; *i.e.,* the inducing effects of plasma were detectable irrespective of shaking.

Effects of various preparations of plasma on hemocyte aggregation

We developed method A to analyze the aggregation of *H. roretzi* hemocytes. This method is useful for tracing the progress of the reaction, but is too time-consuming for investigating the effects of various substances on aggregation. Therefore, we developed another quantitative method (B) in which an ELISA reader is used to measure the increased transmittance that should occur as hemocyte aggregates form. In fact, the transmission was increased at a rate consistent with the formation of aggregates detected microscopically. By this method, we confirmed that both plasma and Mg^{2+} induced the aggregation of *H. roretzi* hemocytes.

To characterize the plasma factor that can induce the aggregation of *H. roretzi* hemocytes, we used method B to examine the effects of various preparations of plasma (10%) on the aggregation. The plasma factor was stable under acidic and alkaline conditions and resistant to heat treatment; its molecular weight was less than 10,000 because dialyzed plasma had little inducing activity (Fig. 5a). In fact, the filtrate (containing low molecular weight substances) obtained from intact plasma by filtration through a Centricut filter (10,000 molecular weight cut-off) had inducing activity (control experiment in Fig. 5b), whereas that obtained from dialyzed plasma had little activity (second bar in Fig. 5b). The filtrate from trypsin-treated intact plasma had strong inducing activity (third bar in Fig. 5b). But the filtrate from trypsin-treated dialyzed plasma also had some inducing activity (fourth bar in Fig. 5b), suggesting that low molecular weight peptide-like inducing substances had been produced from plasma proteins by treatment with trypsin. Furthermore, the reacted plasma showed stronger effects than the intact

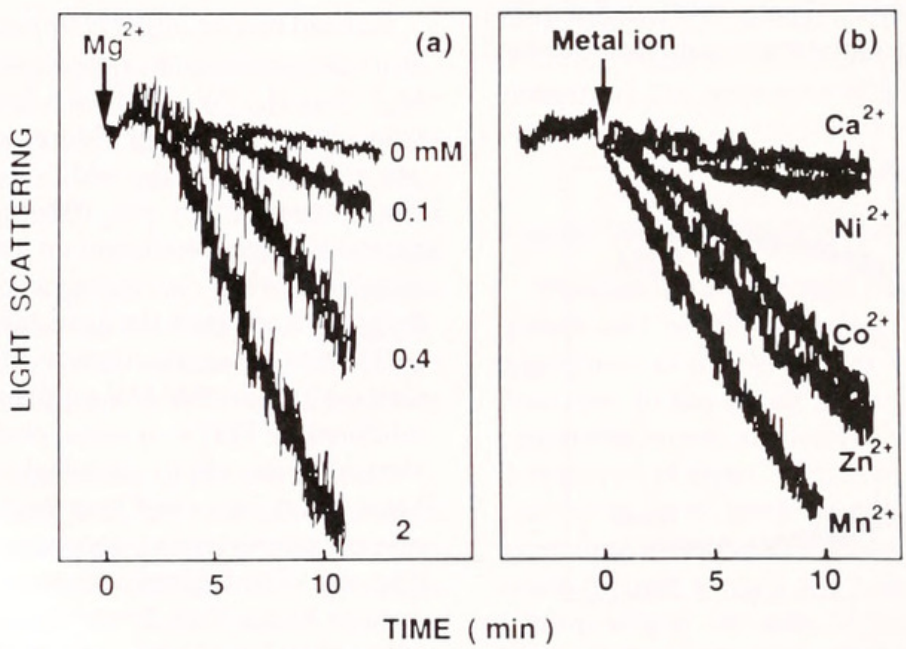

Figure 4. Aggregation of hemocytes of *Halocynthia roretzi* induced by metal ions. The respective metal ion was added to the hemocyte suspension in the Ca^{2+}-, Mg^{2+}-free HASW. (a) The extent of stimulation by Mg^{2+} was dependent on its concentration. (b) Mn^{2+}, Zn^{2+}, and Co^{2+} had an inducing effect, whereas Ca^{2+} and Ni^{2+} had little inducing effect. The concentration of metal ion used was 2 m*M*. The extent of light scattering is shown on the ordinate in arbitrary units.

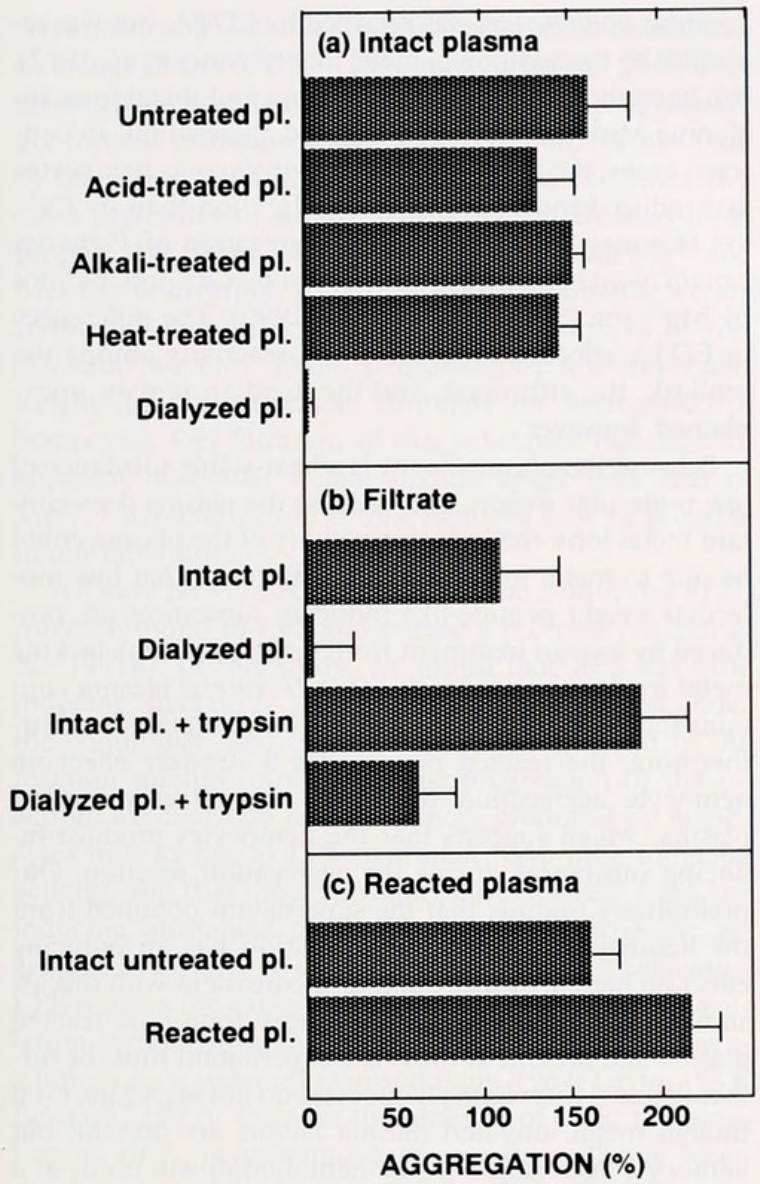

Figure 5. Effects of various preparations of plasma on the aggregation of hemocytes of *Halocynthia roretzi.* (a) Intact plasma was previously treated with acid, alkali, or heat (90°C), or was dialyzed, and effects of these preparations (10%) on the aggregation were measured by method B. (b) Intact plasma or dialyzed plasma was treated with or without trypsin (0.1 mg/ml) at 20°C for 4 h and filtered through a Centricut membrane (10,000 molecular weight cut-off). The effects of the resulting filtrates on the aggregation were measured by method B. (c) The effect of reacted plasma on the aggregation was measured by method B. Reacted plasma was collected from hemolymph in which the aggregation of hemocytes had been completed. The results shown are means of relative values of triplicate tests (±SD); the aggregation induced by 2 m*M* $MgCl_2$ was defined as 100%. Note that an approximately linear relationship exists between the extent of aggregation and the concentration of plasma factor tested in this experiment.

plasma (Fig. 5c), which suggests that one or more inducing substances are produced when the aggregation of hemocytes takes place in hemolymph.

Inducing effects of Met-Lys-bradykinin on hemocyte aggregation

Stefano *et al.* (1989a, b) have reported that Met-enkephalin triggers inflammatory responses from the hemocytes of the mollusk *Mytilus edulis* by inducing their morphological change, migration, and aggregation. To investigate whether neuropeptides, such as Met-enkephalin, would induce ascidian hemocytes to aggregate, we used method B to examine the effects of 26 biologically active peptides on the aggregation of *H. roretzi* hemocytes. Typical results are shown in Figure 6. Among peptides tested, Met-Lys-bradykinin induced the aggregation as strongly as Mg^{2+} (the maximum aggregation was observed at 2 μM). Despite the equivalence of these assay results, the aggregates formed in the presence of Met-Lys-bradykinin are larger in number and smaller in size than those in the presence of Mg^{2+}, and the inducing effect of Met-Lys-bradykinin was detectable even *without shaking,* suggesting that different intracellular mechanisms might underlie the two effects.

Bradykinin was a moderate stimulant. The peptides (1 μM) Lys-bradykinin, substance P, ACTH, and oxytocin also had moderate inducing activity similar to that of bradykinin. Supporting the possibility that the presence of endogenous bradykinin-like peptides may induce hemocyte aggregation, we obtained the interesting result that bradykinin potentiator C (1 μM), a kininase inhibitor, had a moderate inducing activity similar to that seen with bradykinin. This inhibitor may potentiate the activity of an endogenous peptide-like inducer by blocking inactivating proteases. On the other hand, Met-enkephalin, an inducer of hemocyte aggregation in *M. edulis,* had little effect on the aggregation of ascidian hemocytes. The following 19 peptides (1 μM) also had little inducing activity; Leu-enkephalin, α- and γ-endorphins, α- and β-neoendorphins, angiotensin I and II, mastoparan, LHRH, CGRP, somatostatin, neurotensin, physalaemin, sub-

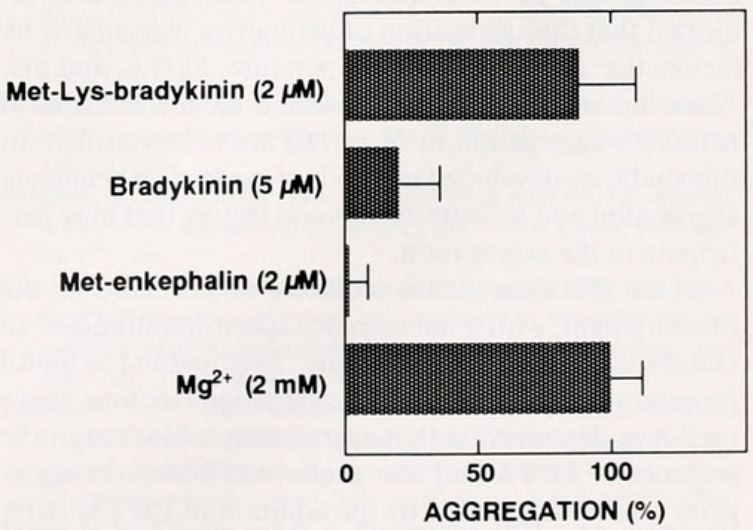

Figure 6. The effects of biologically active peptides on the aggregation of washed hemocytes of *Halocynthia roretzi.* The aggregation was measured by method B in Ca^{2+}-, Mg^{2+}-free HASW. Inducers used were Met-Lys-bradykinin (2 μM), bradykinin (5 μM), Met-enkephalin (2 μM), and Mg^{2+} (2 mM). The results shown are means of triplicate tests (±SD).

stance K, kyotorphin, dynorphin (1-8), peptide E, metorophinamide, and formyl-Met-Leu-Phe. In addition, ADP (1 m*M*), Arg-Gly-Asp-Ser (RGDS peptide) (0.001–1 m*M*), and LPS (1–100 μg/ml) also had little effects on the aggregation.

Inhibition of aggregation of hemocytes

Using method B, we examined the effects of several reagents on the hemocyte aggregation induced by 2 m*M* Mg^{2+}, 10% plasma, and 2.5 μM Met-Lys-bradykinin. *N*-Ethylmaleimide (0.5 m*M*) completely inhibited the hemocyte aggregation induced by any of the inducers. EDTA (2 m*M*) and cytochalasin B (0.01 mg/ml) also strongly inhibited aggregation (about 80 and 60% inhibitions were observed with EDTA and cytochalasin B, respectively). Because the hemocyte aggregations induced by Met-Lys-bradykinin and Mg^{2+} are both inhibited by EDTA, we propose that the aggregation reaction consists of several stages, and that Met-Lys-bradykinin functions at a stage prior to an EDTA-sensitive event.

Discussion

We have been studying the roles of defense factors in the hemolymph of *H. roretzi* for about 10 years and have never observed clot formation, even at room temperature. In contrast, hemocyte aggregation has always been detectable. Moreover, when a part of the tunic is removed by cutting so that the muscle is exposed to seawater, the hemocytes seem to migrate to the area of injured tunic and to form aggregates. We therefore assumed that *H. roretzi* lacks the so-called coagulation system, which functions in maintaining hemostasis, and that instead the hemocyte aggregation system plays a role in stopping bleeding. Our previous qualitative observations also indicated that the aggregation of hemocytes is regulated by factors that are sensitive to temperature, EDTA, and pH. These factors must be characterized if the mechanisms of hemocyte aggregation in *H. roretzi* are to be clarified. In this study, we developed methods of measuring hemocyte aggregation and investigated several factors that may participate in the aggregation.

Of the two quantitative methods, one is based on the measurement, with a fluorescence spectrophotometer, of platelet aggregation in mammals. This method is useful for monitoring the kinetics of aggregation reaction, so we used it to demonstrate that aggregation is blocked in the presence of EDTA and that preformed hemocyte aggregates can be dissociated by the addition of EDTA. After dissociation by EDTA, however, the hemocytes never reaggregated, even upon addition of excess Mg^{2+}. Therefore, dissociation of hemocyte aggregates by EDTA is irreversible; the precise mechanism remains unclear. In contrast to our findings with *H. roretzi*, hemocyte aggregation in *Limulus polyphemus* was retarded by EDTA, but was reversible by the addition of metal ions (Kenney *et al.*, 1972). We have shown that ascidian plasma and metal ions, including Mg^{2+} but not Ca^{2+}, induced aggregation. In contrast, again, the aggregation of *L. polyphemus* hemocytes was induced more efficiently by Mg^{2+} ion than by Ca^{2+} ion (Kenney *et al.*, 1972), and aggregation of *Pomacea canaliculata* hemocytes was induced by Ca^{2+} ion, but not by Mg^{2+} ion (Shozawa and Suto, 1990). The differences in EDTA effects and in metal ion reactivity among the mollusk, the arthropod, and the ascidian remain unexplained, however.

Because the plasma factor is a heat-stable substance of low molecular weight, and because the plasma does contain metal ions, the inducing activity of the plasma could be due to metal ions. However, the result that low molecular weight peptide-like inducing substances are produced by trypsin treatment from dialyzed plasma lacking metal ions supports the idea that *H. roretzi* plasma contains inducing substances in addition to metal ions. Furthermore, the reacted plasma has a stronger effect on hemocyte aggregation than does the intact untreated plasma, which suggests that the hemocytes produce inducing substances during the aggregation reaction. Our preliminary finding, that the supernatant obtained from the hemocyte lysate by centrifugation has an inducing effect on hemocyte aggregation, is consistent with this assumption. The inducing substances found in reacted plasma and present in the lysate supernatant must be further defined. Circulating hemocytes do not aggregate, even though metal ions and plasma factors are present; but hemocyte aggregation in the hemolymph will occur at a restricted site on the tunic that has been damaged with a needle. This observation suggests that specific aggregation activation mechanisms, as yet unidentified, occur in *H. roretzi*.

Met-enkephalin has been reported to induce the aggregation of hemocytes of the mollusk *M. edulis*, and a Met-enkephalin-like substance has been demonstrated in hemolymph of this species (Stefano *et al.*, 1989a, b). These studies suggest that bioactive peptides such as Met-enkephalin may regulate hemocyte aggregation in the hemolymph of other animals. In the case of the ascidians, Met-Lys-bradykinin is the strongest inducer of hemocyte aggregation among the peptides tested, whereas Met-enkephalin has little effect. Bradykinin and Lys-bradykinin have weaker activity, thus the methionyl residue of Met-Lys-bradykinin may be necessary for the full expression of its inducing activity. These results suggest that a Met-Lys-bradykinin-like substance may be one of the candidates for a natural inducers. Our observations on the production of peptide-like inducing substances by trypsin treatment are consistent with this assumption. In addition, inducing effects of both plasma and Met-Lys-bradykinin

were detectable without shaking (under these conditions, inducing effects of Mg^{2+} were undetectable). The above observations, together with the fact that smaller aggregates are formed in the presence of Met-Lys-bradykinin than in the presence of Mg^{2+} and plasma (in the latter two cases aggregates of similar sizes are formed), lead us to propose that plasma contains factors other than Mg^{2+} and Met-Lys-bradykinin. In our current experiments, we are trying to isolate natural inducers from plasma of *H. roretzi,* and we have found a heat-stable, low molecular weight substance that can stimulate the aggregation of hemocytes. Gel filtration of this substance indicates an apparent molecular weight slightly larger than that of Mg^{2+}. Characterization of this factor is now in progress in our laboratory.

We have previously reported that the hemocytes of *H. roretzi* respond to LPS and release a protease (Azumi *et al.,* 1991b). In this study, we found that LPS has little inducing effect on *H. roretzi* hemocyte aggregation under the conditions used. Because LPS was included in the reaction mixture throughout the progression of the reaction, we conclude that substances produced by hemocytes in response to LPS treatment have little inducing activity, and that those substances are different from the inducing substances produced by hemocytes in the aggregation reaction. In the horseshoe crab, *L. polyphemus,* LPS triggers the exocytosis of components of the coagulation system from amebocytes and initiates the coagulation cascade system (Levin and Bang, 1964; Levin, 1985; Iwanaga, 1993). Even in the absence of LPS, the amebocytes of *L. polyphemus* aggregate immediately when the hemolymph is removed from the animal (Levin, 1985). As previously noted, there are also differences in EDTA action and metal ion reactivity between *L. polyphemus* and *H. roretzi.* However, as in *H. roretzi,* hemocyte aggregation is prevented by *N*-ethylmaleimide in *L. polyphemus* (Bryan *et al.,* 1964), indicating that the mechanisms of hemocyte aggregation in the two species may have some similarities.

Acknowledgments

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Literature Cited

Azumi, K., H. Yokosawa, and S. Ishii. 1990a. Halocyamines: novel antimicrobial tetrapeptide-like substances isolated from the hemocytes of the solitary ascidian *Halocynthia roretzi. Biochemistry* **29:** 159–165.

Azumi, K., M. Yoshimizu, S. Suzuki, Y. Ezura, and H. Yokosawa. 1990b. Inhibitory effect of halocyamine, an antimicrobial substance from ascidian hemocytes, on the growth of fish viruses and marine bacteria. *Experientia* **46:** 1066–1068.

Azumi, K., S. Ozeki, H. Yokosawa, and S. Ishii. 1991a. A novel lipopolysaccharide-binding hemagglutinin isolated from hemocytes of the solitary ascidian, *Halocynthia roretzi:* it can agglutinate bacteria. *Dev. Comp. Immunol.* **15:** 9–16.

Azumi, K., H. Yokosawa, and S. Ishii. 1991b. Lipopolysaccharide induces release of a metallo-protease from hemocytes of the ascidian, *Halocynthia roretzi. Dev. Comp. Immunol.* **15:** 1–7.

Azumi, K., N. Satoh, and H. Yokosawa. 1993. Functional and structural characterization of hemocytes of the solitary ascidian, *Halocynthia roretzi. J. Exp. Zool.* **265:** 309–316.

Barwig, B. 1985. Isolation and characterization of plasma coagulogen (PC) of the cockroach *Leucophaea maderae* (Blattaria). *J. Comp. Physiol. B.* **155:** 135–143.

Bayne, C. J. 1981. Gastropod cells *in vitro.* Pp. 297–334 in *Advances in Cell Culture,* Vol. 1, K. Maramorosch, ed. Academic Press, New York.

Boolootian, R. A., and A. C. Giese. 1959. Clotting of echinoderm coelomic fluid. *J. Exp. Zool.* **140:** 207–229.

Bryan, F. T., C. W. Robinson, Jr., C. F. Gilbert, and R. D. Langdell. 1964. *N*-Ethylmaleimide inhibition of horseshoe crab hemocyte aggregation. *Science* **144:** 1147–1148.

Fuke, M. T. 1979. Studies on the coelomic cells of some Japanese ascidians. *Bull. Mar. Biol. St. Asamushi, Tohoku Univ.* **16:** 143–159.

Fuke, M. T. 1980. "Contact reactions" between xenogenic or allogenic coelomic cells of solitary ascidians. *Biol. Bull.* **158:** 304–315.

Iwanaga, S. 1993. The limulus clotting reaction. *Curr. Opin. Immunol.* **5:** 74–82.

Kenny, D. M., F. A. Belamarich, and D. Shepro. 1972. Aggregation of horseshoe crab (*Limulus polyphemus*) amebocytes and reversible inhibition of aggregation by EDTA. *Biol. Bull.* **143:** 548–567.

Levin, J. 1985. The role of amebocytes in the blood coagulation mechanism of the horseshoe crab *Limulus polyphemus.* Pp. 145–163 in *Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology,* W. D. Cohen, ed. Alan R. Liss, New York.

Levin, J., and F. B. Bang. 1964. The role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull. Johns Hopkins Hosp.* **115:** 265–274.

Shozawa, A., and C. Suto. 1990. Hemocytes of *Pomacea canaliculata:* I. Reversible aggregation induced by Ca^{2+}. *Dev. Comp. Immunol.* **14:** 175–184.

Stefano, G. B., M. K. Leung, X. Zhao, and B. Scharrer. 1989a. Evidence for the involvement of opioid neuropeptides in the adherence and migration of immunocompetent invertebrate hemocytes. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 626–630.

Stefano, G. B., P. Cadet, and B. Scharrer. 1989b. Stimulatory effects of opioid neuropeptides on locomotory activity and conformational changes in invertebrate and human immunocytes: evidence for a subtype of δ receptor. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 6307–6311.

Young, N. S., J. Levin, and R. A. Prendergast. 1972. An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. *J. Clinic. Invest.* **51:** 1790–1797.