Original Article

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Hemocytes and humoral factors in silkworm blood are cooperatively involved in sheep erythrocyte aggregation

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ABSTRACT: Sheep red blood cells (SRBCs) rapidly aggregated when injected into the blood (hemolymph) of living silkworms. SRBCs also rapidly aggregated when incubated with hemolymph *in vitro*. SRBCs did not aggregate when incubated with single hemolymph components, hemocytes and cell-free plasma separated by centrifugation, whereas incubation with the mixture of components induced SRBC aggregation, suggesting that both hemocytes and plasma are required for the reaction. Treatment of hemocytes with sodium azide inhibited SRBC aggregation. On the other hand, SRBCs pre-incubated with hemocytes aggregated in the plasma, even in the presence of sodium azide. SRBC aggregation was not observed when the SRBCs were physically separated from the hemocytes by a polycarbonate filter. These findings suggest that SRBCs are directly attacked by hemocytes and become sensitive to humoral factors that cause SRBC aggregation.

Keywords: Silkworm, SRBC, aggregation, innate immunity, hemocyte, humoral factor

1. Introduction

During evolution, multicellular organisms acquired immune systems to eliminate pathogenic invaders. Host immunity is classified as innate immunity or acquired immunity. In mammals, innate immunity acts as a primary defense against infectious agents and contributes to the successive activation of adaptive immunity. Invertebrates, on the other hand, lack antibody-producing organs and rely solely on innate immunity to eliminate foreign substances in the body (*1*). Therefore, investigations of defense mechanisms against pathogens in invertebrate animals are useful toward understanding how hosts overcome infectious diseases by innate immune responses.

Pattern recognition receptors (PRRs), such as peptidoglycan recognition proteins, beta-glucan recognition proteins, and lectins, trigger various defensive responses in innate immunity (*2-4*). These PRRs exist in immune cells or in plasma as soluble proteins; those that reside in immune cells comprise membrane bound forms and cytoplasmic soluble receptors. Binding of PRRs to foreign substances activates humoral and cellular responses. The molecular mechanisms of humoral immune reactions include antimicrobial peptide production (*5-7*) and melanization (*8-10*). Cellular immunity involves several types of hemocyte-dependent reactions, such as phagocytosis (*11*), nodulation (*12,13*), and encapsulation (*3,14*). Nodulation is a lectin-mediated reaction that entraps a large number of bacteria in a clot formed by aggregating hemocytes (*12,13*). Similar to nodulation, encapsulation is a reaction in which hemocytes surround larger substances such as parasites and latex beads (*14*). We recently demonstrated that an insect cytokine, paralytic peptide, in silkworms activates both humoral and cellular immune responses, leading to host resistance against bacterial infection (*15,16*). Coordinative regulation of the humoral and cellular immune systems seems to be critical for host animals to efficiently remove pathogens. Immune reactions in which humoral and cellular immunity act in concert, however, are rarely studied, and their regulatory mechanisms remain elusive.

In vitro sheep red blood cell (SRBC) aggregation tests are widely applied to study antigen-antibody reactions in mammals (*17*) and lectin-mediated defense reactions in the hemolymph of insects (*18*). Komano and Natori observed that SRBCs formed aggregates and were eventually hemolyzed when injected into the blood of the flesh fly, *Sarcophaga peregrina* (*18*). Moreover, Suzuki and colleagues reported that SRBCs pre-treated with trypsin and glutaraldehyde underwent aggregation mediated by unidentified lectin-like factors in the silkworm hemolymph (*19*). The roles of humoral and cellular immune factors in the recognition and removal of SRBCs in the insect body, however,

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are poorly understood. Here we show that hemocytes directly recognize and modify the surfaces of SRBCs, and then humoral factors induce SRBC aggregation in the silkworm hemolymph.

2. Materials and Methods

2.1. *Insects*

Silkworm eggs (*B. mori*, Hu·Yo × Tukuba·Ne) were purchased from Ehime Sanshu (Ehime, Japan). Silkworm larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 27°C.

2.2. *Reagents*

SRBCs, beta-mercaptoethanol, and TC100 were purchased from Nippon Biotest Laboratories Inc., Nakalai Tesque, and AppliChem, respectively. Sodium azide and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Wako Pure Chemical Industries and Dojindo Laboratories, respectively, and dissolved in saline (0.9% NaCl) before use.

2.3. *Preparation of hemocytes and the plasma fraction*

Hemolymph was collected into a tube (containing 1.25% of beta-mercaptoethanol to avoid melanization) after cutting the abdominal legs of two silkworm larvae (day 2 of 5th instar). To obtain hemocytes and plasma fractions, the hemolymph was first centrifuged at 300 × g for 5 min. The precipitate was washed twice with phosphate-buffered saline (PBS, pH 8.0) and then suspended with insect cell culture medium TC100 containing 10% fetal bovine serum. The supernatant was further centrifuged at 9,000 × g for 5 min to obtain the plasma fraction.

2.4. *In vitro SRBCs aggregation assay*

A 2-mL sample of SRBCs was washed twice with 50 mL of PBS and suspended in 2 mL of PBS. A SRBC suspension (2 µL) was mixed with 100 µL of each sample in a microtiter plate (non-treated 96-well round bottom, Iwaki, Japan). In some experiments, 200 mM sodium azide was added to kill the hemocytes. After incubation at 27°C for 2 h, SRBC aggregation was visually judged based on the attachment of cells to the bottom of the wells.

2.5. *DAPI staining of hemocytes*

The SRBCs were incubated with silkworm hemolymph at 27°C for 1 h. A 100-µL aliquot of each sample was then mixed with 0.5 mL of 10 mg/mL DAPI and stained for 1 h. The remaining DAPI was washed in PBS three times. The morphology of cells and fluorescent nuclei was observed under an inverted fluorescence microscope (Leica, Tokyo, Japan).

3. Results

3.1. *SRBC aggregation depends on both hemocytes and humoral factors in the silkworm hemolymph*

Red-colored aggregates were observed in the silkworm hemolymph harvested 15 min after injecting the SRBCs (Figure 1B). We then tested whether SRBCs mixed with the silkworm hemolymph aggregated *in vitro*. When SRBCs suspended in PBS were applied to U-shaped wells and incubated for 2 h, the SRBCs precipitated to the center of the wells and appeared as spots (Figure 2A). In contrast, when the SRBCs were incubated with the silkworm hemolymph, they spread out and attached to the bottom of the wells to form aggregates (Figure 2B). Microscopic analysis revealed that when SRBCs were incubated alone in PBS, each cell remained separated from the other (Figure 2C), whereas those incubated in the hemolymph adhered together to form large clumps (Figure 2D). Based on the similar morphologic appearances of SRBC aggregates formed either *in vitro* or *in vivo*, further studies of the SRBC

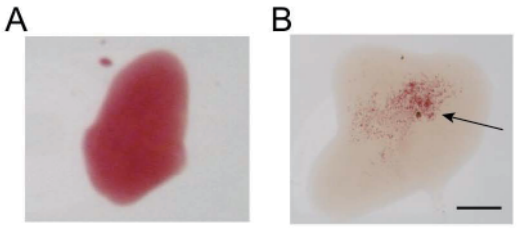

Figure 1. *In vivo* aggregation of SRBCs in the hemolymph of live silkworms. Silkworm hemolymph was collected immediately **(A)**, or 15 min **(B)** after injecting SRBCs into the hemolymph and was observed under a microscope. Arrow indicates SRBC clumps. Bar; 5 mm.

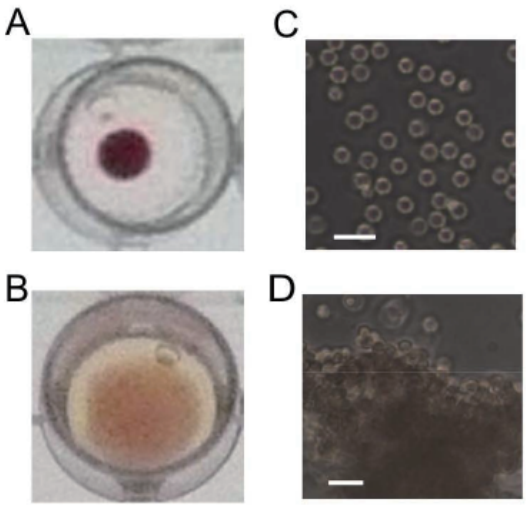

Figure 2. *In vitro* aggregation of SRBCs in the silkworm hemolymph. SRBCs in PBS **(A, C)** or silkworm hemolymph **(B, D)** were incubated at 27°C for 2 h in a microtiter plate. **A** and **B**; microscopic views of suspended samples of **C** and **D**, respectively. Bar; 10 µm.

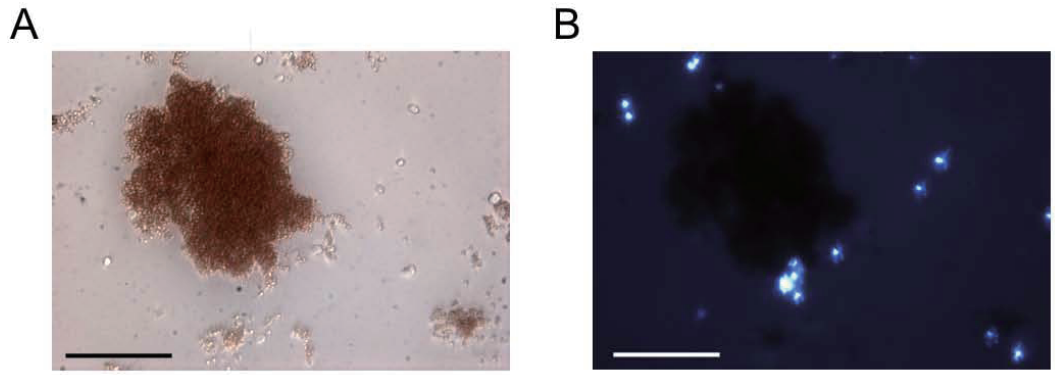

Figure 3. DAPI staining of hemocytes incorporated in clumps of SRBCs. Hemocytes were stained with DAPI. Clumps of SRBCs formed by incubation with silkworm hemolymph observed under a bright-field **(A)** or fluorescence **(B)** microscope. Bar; 100 μm.

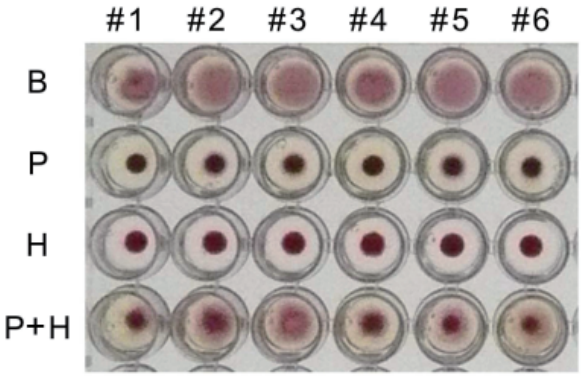

Figure 4. Aggregation of SRBCs depending on both hemocytes and humoral factors in the silkworm hemolymph. Hemolymph fractions from 6 independent silkworms were separated into hemocytes and plasma. Numbers indicate different batches of silkworms. SRBCs incubated with hemolymph **(B)**, plasma **(P)**, hemocytes **(H)**, and mixtures of plasma and hemocytes **(P + H)**.

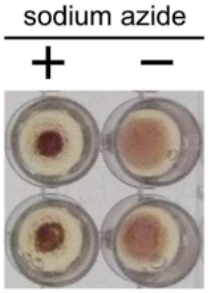

Figure 5. Inhibition of SRBC aggregation in the silkworm hemolymph by sodium azide. SRBCs incubated with hemolymph in the presence (+) or absence (−) of 200 mM sodium azide were observed from the top.

aggregation reaction were performed *in vitro* using isolated hemolymph.

Encapsulation and nodulation are hemocyte-dependent aggregation reactions of foreign substances in insects (*13,14*). In these reactions, hemocytes surround non-self substances to form clots. To test whether hemocytes were incorporated into the clumps of SRBCs under our experimental conditions, we used DAPI to stain the hemocyte nuclei. Analysis under fluorescence microscopy revealed only a few DAPI-positive cells in the SRBC clumps that formed in the hemolymph (Figure 3). Therefore, we assumed that the aggregation of SRBCs induced by silkworm hemolymph was distinct from other cellular responses, such as encapsulation and nodulation in which hemocytes are present in the clots.

We then separated hemocytes and plasma from silkworm hemolymph by centrifugation to test their abilities to aggregate SRBCs *in vitro*. SRBCs incubated with either hemocytes or plasma alone did not aggregate, while those incubated in a mixture of hemocytes and plasma did aggregate (Figure 4). These results suggest that the aggregation of SRBCs in the silkworm hemolymph requires both hemocytes and plasma factors.

3.2. *Requirement of direct interaction of hemocytes and SRBCs for the aggregation reaction*

We further examined whether hemocyte viability was necessary for the SRBC aggregation. SRBC aggregation in the hemolymph was inhibited by the addition of sodium azide, a cytotoxic agent (Figure 5). When cells were stained with trypan blue, the addition of sodium azide reduced the ratio of trypan blue-negative cells from 100% to less than 10% (data not shown). These results indicate that viable hemocytes are required for the SRBC aggregation induced by silkworm hemolymph.

To elucidate the mechanism of the SRBC aggregation reactions mediated by both hemocytes and humoral factors, we tested the effect of pre-incubation of SRBCs with isolated hemocytes. When SRBCs were incubated with hemocytes prior to the addition of silkworm plasma, SRBC aggregation proceeded even when sodium azide was added. In contrast, SRBCs that were not pre-incubated with hemocytes did not form aggregates in the plasma (Figure 6). These findings suggest that SRBCs are initially attacked by hemocytes in the hemolymph and made vulnerable to humoral factors in plasma that induce the SRBC aggregation process.

We then tested whether direct interaction with hemocytes was needed for the SRBC aggregation.

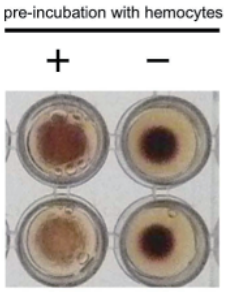

Figure 6. The effect of pre-incubation of SRBCs with hemocytes on SRBC aggregation in the presence of sodium azide. SRBCs pre-incubated with (+) or without (−) hemocytes were mixed with sodium azide-containing plasma, and incubated at 27°C for 2 h.

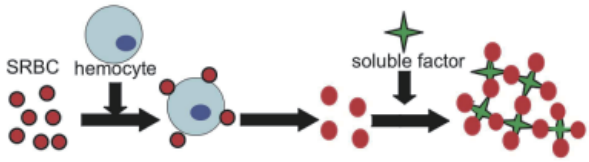

Figure 7. Proposed mechanism of SRBC aggregation in silkworm hemolymph.

Incubation of SRBCs in the plasma fraction that were physically separated from the hemocytes using a polycarbonate membrane filter that was permeable to soluble proteins did not lead to SRBC aggregation (data not shown). This result suggests that direct contact of hemocytes with SRBCs is required for SRBC aggregation in silkworm hemolymph.

4. Discussion

Here we describe a novel defense mechanism involving sequential contributions of hemocytes and soluble factors in the hemolymph plasma that were required for SRBC aggregation in silkworm hemolymph (Figure 7). This finding provides the first evidence that foreign substances are directly attacked by insect hemocytes, followed by clot formation induced by humoral factors.

Both cellular and humoral immunity are involved in the process of nodulation. Sato and colleagues reported that lectins in the hemolymph of silkworms bind to foreign substances, which are surrounded by an assembly of hemocytes to form nodules (*12*,*20*). Here we report that hemocytes first attacked the foreign substances, SRBCs, and then humoral factors aggregated the SRBCs. Furthermore, we showed that hemocytes were not incorporated into the clump of SRBCs formed in the hemolymph. Based on these findings, we consider that SRBC aggregation involving hemocytes and plasma factors is distinct from other self-defense reactions, such as nodulation and encapsulation.

Suzuki and Natori reported that lectin-like proteins in silkworm hemolymph aggregate trypsin- and glutaraldehyde-treated SRBCs (*19*). We assume that the factors in the silkworm plasma that cooperate with hemocytes might be similar to the lectin-like proteins reported by Suzuki. Moreover, we found that direct interactions of SRBCs with hemocytes are necessary for the silkworm hemolymph to induce SRBC aggregation. Thus, the hemocyte attack on SRBCs might correspond to the trypsin and glutaraldehyde treatment of SRBCs required for the silkworm lectin-like proteins to induce SRBC aggregation (*19*).

Hemocytes circulating in silkworm hemolymph are classified into five subtypes, prohemocytes, plasmatocytes, granulocytes, spherulocytes, and oenocytoids (*21*,*22*). Among them, granulocytes are one of the major cell types responsible for defensive responses, and they attach to small foreign substances, such as SRBCs, by elongating their filopodia (*23*).

Thus, we speculate that granulocytes are a plausible candidate hemocyte subtype that directly interacts with SRBCs to form aggregates. Recently, Nakahara and colleagues established a fluorescence activated cell sorting-based method to separate each subtype of silkworm hemocyte from the hemolymph (*22*). Application of such methods will allow for the identification of the hemocyte subtypes involved in the aggregation of foreign substances.

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References

1. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002; 20:197-216.
2. Basbous N, Coste F, Leone P, Vincentelli R, Royet J, Kellenberger C, Roussel A. The *Drosophila* peptidoglycan-recognition protein LF interacts with peptidoglycan-recognition protein LC to downregulate the Imd pathway. EMBO Rep. 2011; 12:327-333.
3. Yang J, Wang L, Zhang H, Qiu L, Wang H, Song L. C-Type Lectin in *Chlamys farreri* (CfLec-1) mediating immunerecognition andopsonization. PLoS One. 2011; 6: e17089.
4. Goodridge HS, Wolf AJ, Underhill DM. Beta-glucan recognition by the innate immune system. Immunol Rev. 2009; 230:38-50.
5. Boman HG, Faye I, Gudmundsson GH, Lee JY, Lidholm DA. Cell-free immunity in Cecropia. A model system for antibacterial proteins. Eur J Biochem. 1991; 201:23-31.
6. Natori S, Shiraishi H, Hori S, Kobayashi A. The roles of *Sarcophaga* defense molecules in immunity and metamorphosis. Dev Comp Immunol. 1999; 23:317-328.
7. Okada M, Natori S. Purification and characterization of an antibacterial protein from haemolymph of *Sarcophaga peregrina* (flesh-fly) larvae. Biochem J.

1983; 211:727-734.
8. An C, Budd A, Kanost MR, Michel K. Characterization of a regulatory unit that controls melanization and affects longevity of mosquitoes. Cell Mol Life Sci. 2011; 68:1929-1939.
9. Kan H, Kim CH, Kwon HM, Park JW, Roh KB, Lee H, Park BJ, Zhang R, Zhang J, Söderhäll K, Ha NC, Lee BL. Molecular control of phenoloxidase-induced melanin synthesis in an insect. J Biol Chem. 2008; 283:25316-25323.
10. Ishii K, Hamamoto H, Imamura K, Adachi T, Shoji M, Nakayama K, Sekimizu K. *Porphyromonas gingivalis* peptidoglycans induce excessive activation of the innate immune system in silkworm larvae. J Biol Chem. 2010; 285:33338-33347.
11. Hashimoto Y, Tabuchi Y, Sakurai K, Kutsuna M, Kurokawa K, Awasaki T, Sekimizu K, Nakanishi Y, Shiratsuchi A. Identification of lipoteichoic acid as a ligand for draper in the phagocytosis of *Staphylococcus aureus* by *Drosophila* hemocytes. J Immunol. 2009; 183:7451-7460.
12. Watanabe A, Miyazawa S, Kitami M, Tabunoki H, Ueda K, Sato R. Characterization of a novel C-type lectin, *Bombyx mori* multibinding protein, from the *B. mori* hemolymph: Mechanism of wide-range microorganism recognition and role in immunity. J Immunol. 2006; 177:4594-4604.
13. Koizumi N, Morozumi A, Imamura M, Tanaka E, Iwahana H, Sato R. Lipopolysaccharide-binding proteins and their involvement in the bacterial clearance from the hemolymph of the silkworm *Bombyx mori*. Eur J Biochem. 1997; 248:217-224.
14. Ling E, Yu XQ. Cellular encapsulation and melanization are enhanced by immulectins, pattern recognition receptors from the tobacco hornworm *Manduca sexta*. Dev Comp Immunol. 2006; 30:289-299.
15. Ishii K, Hamamoto H, Kamimura M, Sekimizu K. Activation of the silkworm cytokine by bacterial and fungal cell wall components *via* a reactive oxygen species-triggered mechanism. J Biol Chem. 2008; 283:2185-2191.
16. Ishii K, Hamamoto H, Kamimura M, Nakamura Y, Noda H, Imamura K, Mita K, Sekimizu K. Insect cytokine paralytic peptide (PP) induces cellular and humoral immune responses in the silkworm *Bombyx mori*. J Biol Chem. 2010; 285:28635-28642.
17. Licence ST, Binns RM. Analysis of a sheep anti-pig T lymphoblast serum with specificity for E rosette-forming lymphocytes. Vet Immunol Immunopathol. 1984; 7:255-273.
18. Komano H, Natori S. Participation of *Sarcophaga peregrina* humoral lectin in the lysis of sheep red blood cells injected into the abdominal cavity of larvae. Dev Comp Immunol. 1985; 9:31-40.
19. Suzuki T, Natori S. Identification of a protein having hemagglutinating activity in the hemolymph of the silkworm, *Bombyx mori*. J Biochem. 1983; 93:583-590.
20. Koizumi N, Imai Y, Morozumi A, Imamura M, Kadotani T, Yaoi K, Iwahana H, Sato R. Lipopolysaccharide-binding protein of *Bombyx mori* participates in a hemocyte-mediated defense reaction against gram-negative bacteria. J Insect Physiol. 1999; 45:853-859.
21. Beaulaton J. Hemocytes and hemocytopoiesis in Silkworms. Biochimie. 1979; 61:157-164.
22. Nakahara Y, Shimura S, Ueno C, Kanamori Y, Mita K, Kiuchi M, Kamimura M. Purification and characterization of silkworm hemocytes by flow cytometry. Dev Comp Immunol. 2009; 33:439-448.
23. Wago H. Humoral factors promoting the adhesive properties of the granular cells and plasmatocytes of the silkworm, *Bombyx mori*, and their possible role in the initial cellular reactions to foreignness. Cell Immunol. 1980; 54:155-169.

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