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Effects of plant lectins on cellular defense reactions of ascidian hemocytes

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Abstract. The effects of plant lectins on the three cellular defense reactions of hemocytes of the solitary ascidian, *Halocynthia roretzi* (hemocyte aggregation, phagocytosis, and an allogenic reaction), were investigated. Concanavalin A inhibited aggregation, while wheat germ agglutinin and ricin inhibited the allogenic reaction. Neither of the lectins showed inhibitory effects on phagocytosis, but ricin promoted phagocytosis. These effects of the lectins were diminished by the addition of sugars specific for the respective lectins. These results strongly suggest that different surface carbohydrates are involved in the recognition mechanisms of three *H. roretzi* cellular defense reactions.
Key words. Lectin; ascidian hemocyte; hemocyte aggregation; phagocytosis; allogenic reaction; *Halocynthia roretzi*.

Ascidians occupy a phylogenetic position between vertebrates and invertebrates. The mechanism of the ascidian defense system is thought to be different from that of the vertebrate immune system[1]. We have been investigating the defense mechanism of the solitary ascidian, *Halocynthia roretzi*, and have found that several humoral factors, such as lectins[2-4], antimicrobial substances[5] and an LPS-binding protein[6], are involved. As the cellular defense reactions, *H. roretzi* hemocytes undergo aggregation[7], phagocytosis[8], an allogenic recognition reaction (contact reaction)[9], and an enzyme release reaction[10]. Several cell populations were microscopically observed in *H. roretzi* hemolymph[11]: vacuolated or vesicular cells (59%), small granular amebocytes (30%), large granular amebocytes (11%), dense granular cells (<1%) and lymphoid cells (lymphocyte-like cells) (<1%). Vacuolated (or vesicular) cells play a major role in the contact reaction, small granular amebocytes actively phagocytose foreign materials, and almost all the cell populations undergo aggregation. We also succeeded in separating *H. roretzi* hemocytes into several groups, which were found to contain different defense substances and undergo different cellular reactions[12]. To understand the molecular mechanisms of the cellular defense reactions of *H. roretzi* hemocytes, it is necessary to define what kinds of membrane proteins are involved in the respective cellular reactions.

Plant lectins are useful tools for the analysis of surface glycoproteins on hemocytes. In this communication, we show that different plant lectins have inhibitory or promoting effects on the three cellular reactions of *H. roretzi* hemocytes, hemocyte aggregation, phagocytosis, and the allogenic reaction.

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Materials and methods

Plant lectins were purchased from Seikagaku Kogyo, Co. (Japan). Solitary ascidians, *H. roretzi*, type C, were harvested in Mutsu Bay (Japan). The hemocytes and plasma were collected as described previously[11].

Hemocyte aggregation was also measured as described previously[7]. Briefly, to a hemocyte suspension of *H. roretzi* in Ca^{2+}, Mg^{2+}-free Herbst's artificial seawater (F-HASW, 450 mM NaCl, 9.4 mM KCl, 32 mM Na_2SO_4, and 3.2 mM $NaHCO_3$, pH 7.6), 1×10^7 cells/ml in each well of a 96-well plate, a lectin solution in F-HASW was added, and the mixture was incubated at 4 °C for 30 min. Aggregation of hemocytes was induced by the addition of Mg^{2+} (2 mM) and the hemocytes were shaken gently at 20 °C for 10 min. The absorbance at 405 nm was measured before adding a stimulus [$(A_{405})_0$] and after standing for 1 h at 20 °C with the stimulus [$(A_{405})_{60}$]. The degree of aggregation was defined as the difference in the transmittance (T) calculated from A_{405} before and after incubation at 20 °C [$(T_{405})_{60} - (T_{405})_0$]. Percent aggregation was calculated by defining the degree of aggregation observed in the presence and absence of 2 mM $MgCl_2$ as 100% and 0%, respectively.

The measurement of phagocytosis was carried out as follows: sheep red blood cells (SRBC) were washed three times with phosphate buffered saline (PBS) by centrifugation ($800 \times g$, 5 min) and suspended in F-HASW to adjust to 0.5% (v/v). SRBC were shrunk, but did not undergo lysis. To 10 µl of the hemocyte suspension (5×10^6 cells/ml), 10 µl of the lectin solution in F-HASW was added. After incubation for 30 min at 20 °C, 10 µl of 0.5% SRBC was added and the mixture was again incubated for 1 h at 20 °C. Phagocytic activity against shrunk SRBC was measured under a Nikon

phase contrast microscope. Usually around 200 hemocytes were inspected to check whether phagocytosis of SRBC by the hemocytes would occur. The hemocyte that ingested at least one SRBC was counted as a positive one. The degree of phagocytosis was expressed as the ratio of the number of positive hemocytes to that of total hemocytes.

Allogenic recognition reaction (contact reaction) was measured as described below. Hemocytes of individual ascidians were washed with F-HASW. After the hemocyte suspensions (1×10^7 cells/ml) were incubated with lectins for 30 min at 20 °C, two hemocyte suspensions derived from two different individuals were mixed, 10% *H. roretzi* plasma was added to accelerate the reaction, and then the mixture was incubated for 1 h at 20 °C. In the contact reaction, in which allogenic hemocytes contacted each other, both allogenic hemocytes underwent lysis, and the color of the mixed hemocyte suspension became red. Two hours after incubation, the hemocyte suspension was centrifuged (1500 × g, 5 min, 4 °C), and the absorbance at 490 nm of the resulting supernatant was measured as an indication of the progression of the contact reaction.

Antagonistic effects of sugars against the effects of lectins were examined by adding various sugars (10 mM) to the hemocyte suspension prior to incubation with lectins. The experiment with lectin alone or together with sugars was performed in duplicate and repeated at least three times, and the mean value was calculated from the data of duplicate measurements.

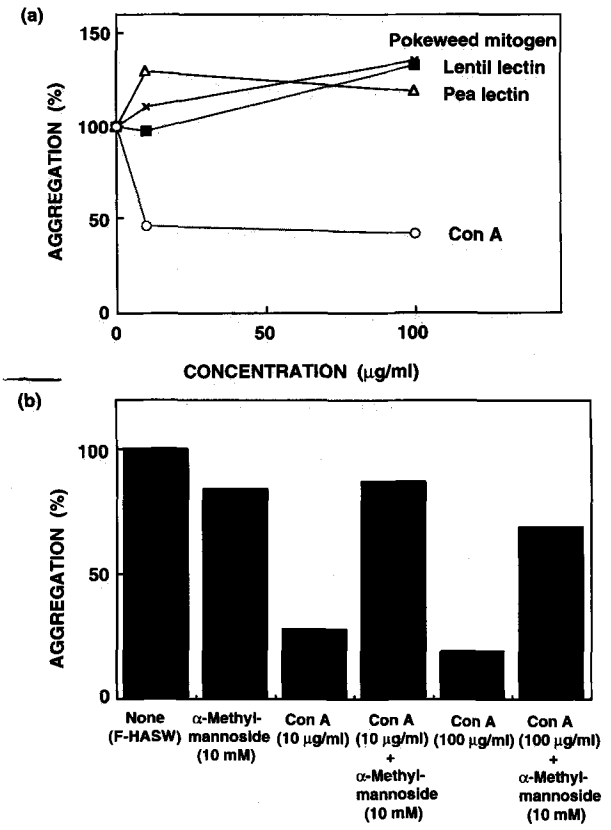

Figure 1. Effects of plant lectins on ascidian hemocyte aggregation. (*a*) Concanavalin A (Con A) (○) inhibited the aggregation in a concentration-dependent manner. Lentil lectin (■), pea lectin (△), and pokeweed mitogen (×) had weak stimulatory effects. (*b*) The inhibitory effect of Con A at concentrations of 10 and 100 μg/ml was weakened by the presence of 10 mM α-methyl-mannoside. Each point represents the mean value calculated from the data of duplicate measurements.

Results and discussion

We investigated whether plant lectins had inhibitory or promoting effects on three cellular reactions of *H. roretzi* hemocytes, hemocyte aggregation, phagocytosis, and the contact reaction.

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. Thus, the hemocyte aggregation appears to be a cellular reaction that arrests bleeding. We established the method of measuring hemocyte aggregation and found that Mg^{2+} can induce the aggregation of washed hemocytes in vitro[7]. The Mg^{2+}-induced hemocyte aggregation reaction of *H. roretzi* was strongly inhibited by concanavalin A (Con A) even at a concentration of 10 μg/ml (fig. 1a), whereas other lectins, including lentil lectin, pea lectin, and pokewood mitogen, showed little inhibitory effect. The inhibitory effect of Con A was weakened by the presence of 10 mM α-methyl-mannoside, a sugar specific for Con A (fig. 1b). Other sugars had little antagonistic effect (data not shown). It should be noted that the above three lectins, which had no inhibitory effect, as well as wheat germ agglutinin (WGA) and ricin (data not shown), slightly accelerated the aggregation (the mechanisms of this remain unknown). Taking into account that pea and lentil lectins also recognize mannose and/or glucose residues, these results suggest that at least two different glycoproteins containing mannose and/or glucose residues function in the cell-to-cell interaction in hemocyte aggregation of *H. roretzi*.

In the case of phagocytosis of SRBC by *H. roretzi* hemocytes, ricin (RCA 60) had a promoting activity at a concentration of 5 μg/ml (fig. 2a). Other lectins, including WGA, Con A, RCA 120, lentil lectin and peanut lectin, had little inhibitory or promoting effect on phagocytosis (data not shown). In the presence of lactose, a sugar specific for ricin, the promoting activity of ricin was undetectable (fig. 2b). It can be inferred that ricin acts as an opsonin which is capable of enhancing the interaction between hemocytes and SRBC. We previously found that a galactose-specific lectin in *H. roretzi* plasma enhanced phagocytosis of SRBC by *H. roretzi* hemocytes (unpubl.). Ricin, like plasma galactose-specific lectin, may bind to both SRBC and the hemocytes to promote the interaction between them.

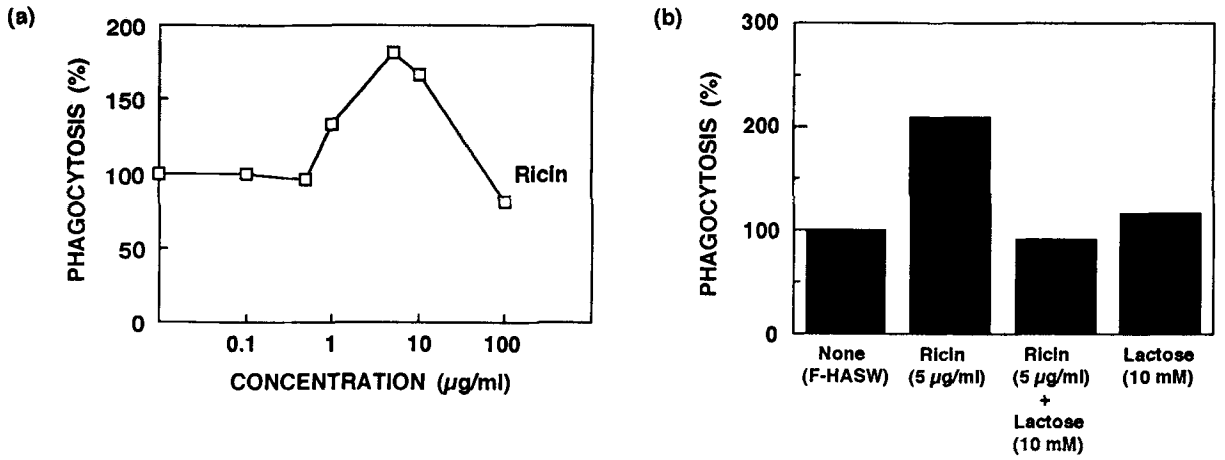

Figure 2. Promoting effect of ricin on phagocytosis of SRBC by ascidian hemocytes. (*a*) Ricin had the largest promoting effect at 5 μg/ml. (*b*) The promoting effect of ricin (5 μg/ml) was inhibited by 10 mM lactose. The degree of phagocytosis in the absence of ricin was defined as 100%. Each point represents the mean value calculated from the data of duplicate measurements.

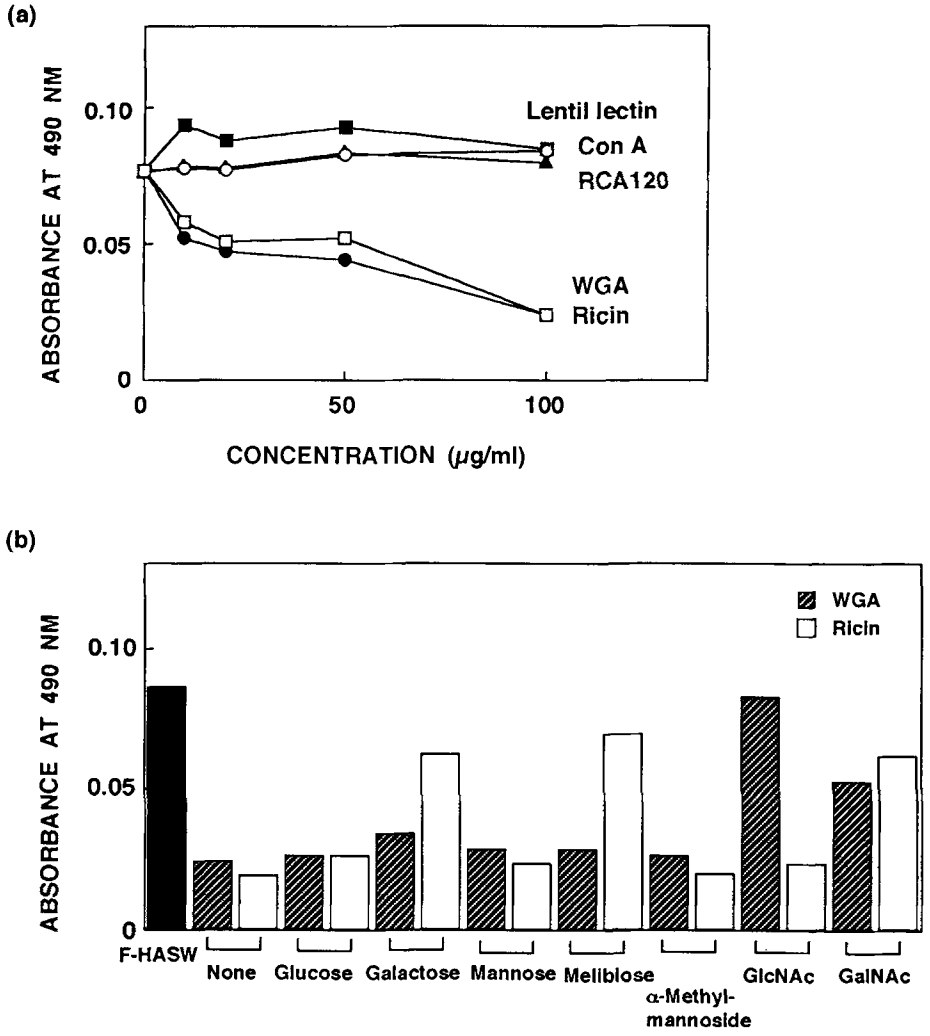

Figure 3. Effects of plant lectins on ascidian hemocyte allogenic reaction. (*a*) WGA (●) and ricin (□) inhibited the allogenic reaction in a concentration-dependent manner. Con A (○), lentil lectin (■), and RCA 120 (▲) had little effect. (*b*) The inhibitory effects of WGA and ricin at a concentration of 10 μg/ml were diminished by the presence of sugars (10 mM) specific for WGA and ricin. Each point represents the mean value calculated from the data of duplicate measurements.

In the contact reaction, we found that WGA and ricin inhibited the reaction (fig. 3a). Other lectins, including RCA 120, lentil lectin, pea lectin, and pokewood mitogen, as well as Con A, which showed an inhibitory effect on hemocyte aggregation, had little effect. In the contact reaction, allogenic hemocytes contact each other, lysis of both allogenic hemocytes occurs, and the color of the mixed hemocyte suspension becomes red. The red substance remains unknown. We found that the lysate of vacuolated cells, a major cell type of *H. roretzi* hemocytes, contained unknown low molecular weight substances (with a molecular weight of less than 10,000), which turned to red outside the hemocytes (at neutral pH). It is possible that allogenic vacuolated cells undergo lysis to release red substances in the contact reaction. The absorption maximum of the supernatants obtained after the contact reaction was 500 nm. Thus, we usually measured the absorbance at 490 nm, which reflected the degree of the contact reaction. We also found that the addition of 10% *H. roretzi* plasma accelerated this reaction (data not shown); in the absence of plasma, the allogenic reactions occurred, but the degree of lysis of hemocytes was weak. Both inhibitory effects of WGA and ricin were diminished by the presence of the respective specific sugars, *N*-acetylglucosamine (GlcNAc) for WGA, and galactose, melibiose and *N*-acetylgalactosamine (GalNAc) for ricin, respectively (fig. 3b). The reason why GalNAc blocked the inhibition of the contact reaction by WGA remains unknown. These results suggest that glycoproteins on the surface of hemocytes, which contain GlcNAc, galactose and/or GalNAc, participate in the allogenic recognition of *H. roretzi* hemocytes. Whether WGA and ricin recognize identical membrane molecules remains unproven. Both WGA and ricin also inhibited the contact reaction in the absence of *H. roretzi* plasma, which confirms the above result that both lectins actually interact with the hemocytes.

In conclusion, surface carbohydrates that are recognized by Con A play an essential role in hemocyte aggregation in *H. roretzi*, while those that are recognized by WGA and ricin function in its allogenic contact reaction. Of the lectins used, none inhibit phagocytosis by *H. roretzi* hemocytes. These results strongly suggest that different glycoproteins are involved in the individual cellular defense reactions of *H. roretzi* and that different cell recognition mechanisms work in these reactions. Isolation and characterization of glycoproteins capable of interacting with the respective lectins are necessary to define this.

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