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Opsonin-independent and -dependent Phagocytosis in the Ascidian *Halocynthia roretzi*: Galactose-specific Lectin and Complement C3 Function as Target-dependent Opsonins

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ABSTRACT—To clarify the molecular mechanisms of phagocytosis, we have been preparing monoclonal antibodies that inhibit phagocytosis by the hemocytes of the ascidian *Halocynthia roretzi*. A monoclonal antibody, RA5, inhibited the phagocytosis of non-treated sheep red blood cells (SRBCs) and yeast cells. It was demonstrated that the phagocytosis by the hemocytes was enhanced by pretreatment of target cells, SRBCs or yeast cells, with *H. roretzi* plasma. However, the RA5 antibody was unable to inhibit the phagocytosis of plasma-treated target cells. These results strongly suggest that the molecule recognized with the RA5 antibody is involved in the opsonin-independent phagocytosis. Western blot analysis showed that this antibody recognized a 200 kDa protein in *H. roretzi* hemocytes. On the other hand, flow cytometry analyses showed that a galactose-specific lectin (Gal-lectin) and complement C3 (AsC3), present in *H. roretzi* plasma, can bind to SRBCs and yeast cells, respectively, to enhance the phagocytosis of the respective target cells. Thus, *H. roretzi* hemocytes undergo opsonin-independent and -dependent phagocytosis, and Gal-lectin and AsC3 both function in the opsonin-dependent phagocytosis.

INTRODUCTION

Phagocytosis is an important host defense reaction against invading foreign organisms in both vertebrates and invertebrates (Marchalonis, 1977). We have been investigating defense cellular reactions, including phagocytosis, in a solitary ascidian, *H. roretzi*, which occupies a phylogenetical position between vertebrates and invertebrates (Azumi and Yokosawa, 1996). *H. roretzi* hemocytes undergo various defense reactions, such as phagocytosis (Ohtake *et al*., 1994), hemocyte aggregation (Takahashi *et al*., 1994), and a self and non-self recognition reaction (Fuke, 1980).

Monoclonal antibodies that inhibit the cellular defense reactions of *H. roretzi* hemocytes are useful for defining hemocyte membrane-bound molecules that are involved in these reactions. The monoclonal antibody A74 inhibits phagocytosis of foreign materials by *H. roretzi* hemocytes and also aggregation of the hemocytes (Takahashi *et al*., 1995). We purified the A74 antigen protein from *H. roretzi* hemocytes (Takahashi *et al*., 1995) and isolated a cDNA clone encoding the A74 protein with a molecular mass of 160 kDa (Takahashi *et al*., 1997): The A74 protein has two immunoreceptor tyrosine-based activation motifs (ITAMs), which have been proposed to play an important role in signal transduction through mammalian T-cell and B-cell antigen receptors and Fc receptors.

To investigate further molecular mechanisms of phagocytosis by *H. roretzi* hemocytes, we tried to prepare other monoclonal antibodies that inhibit the phagocytosis of sheep red blood cells (SRBCs) by *H. roretzi* hemocytes. One of them was a monoclonal antibody, RA5. The RA5 antibody inhibited the phagocytosis of SRBCs that had not been treated with *H. roretzi* plasma (non-treated SRBCs) but not that of SRBCs that had been previously treated with the plasma (plasma-treated SRBCs).

Ohtake *et al*. (1994) reported that the phagocytosis of non-treated SRBCs by *H. roretzi* hemocytes was enhanced by the presence of *H. roretzi* plasma, suggesting that there are opsonins in *H. roretzi* plasma. As one of the candidate opsonins, we isolated a galactose-specific lectin (Gal-lectin) from *H. roretzi* plasma (Yokosawa *et al*., 1982, 1986) and demonstrated that the phagocytosis of non-treated SRBCs

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by *H. roretzi* hemocytes was enhanced by the presence of this lectin (Abe *et al.*, 1999). Recently, on the other hand, it was found that complement C3 (AsC3) was detected in *H. roretzi* plasma and that the AsC3-depleted plasma was unable to enhance the phagocytosis of yeast cells by *H. roretzi* hemocytes (Nonaka *et al.*, 1999).

Previous investigations on phagocytosis by *H. roretzi* hemocytes have been carried out under different conditions using different target materials, which produced ambiguity about elucidation of the mechanisms of phagocytosis. In this paper, we first analyzed the phagocytosis against different target materials under the same conditions, and we obtained definitive evidence for opsonin-independent and -dependent phagocytosis by *H. roretzi* hemocytes, by using the RA5 antibody. In addition, with respect to the latter phagocytosis, we defined different roles of two opsonins, Gal-lectin and AsC3 found in *H. roretzi* plasma, in the phagocytosis. We demonstrated that the lectin functions as an opsonin mainly in the phagocytosis of SRBCs, while AsC3 functions mainly in that of yeast cells. Thus, Gal-lectin and AsC3 function as opsonins, dependent on the nature of target cells.

MATERIALS AND METHODS

Hemocytes and plasma

Hemocytes and plasma of the solitary ascidian, *H. roretzi*, were prepared as described previously (Takahashi *et al.*, 1994), and the hemocytes were suspended in Ca^{2+}-, Mg^{2+}-free Herbst's artificial seawater (F-HASW; 450 mM NaCl, 9.4 mM KCl, 32 mM Na_2SO_4, 3.2 mM $NaHCO_3$, pH 7.6).

Preparation of monoclonal antibody RA5

Monoclonal antibody RA5 was prepared according to the method described previously (Takahashi *et al.*, 1995) except that the membrane fraction (0.1 mg protein) prepared from *H. roretzi* hemocytes was used as an antigen. In the screening of the hybridoma clone producing the RA5 antibody, hybridoma media were tested for their inhibitory activity against phagocytosis of non-treated SRBCs by *H. roretzi* hemocytes. The RA5 antibody was isolated by gel filtration from ammonium sulfate precipitates of ascites fluid, which had been produced by injecting RA5 hybridoma cells into pristane-primed Balb/c mice. The RA5 antibody was classified as IgM.

Preparation of polyclonal antibodies

Gal-lectin purified from *H. roretzi* plasma (Yokosawa *et al.*, 1982) was immunized to rabbits with Freund's complete adjuvant. Boost immunization of the lectin was carried out twice at two-week intervals. The blood was collected one week after the second booster and anti-lectin IgG was isolated by chromatography on protein A-Sepharose (Pharmacia). The polyclonal antibody to AsC3 was prepared as described previously (Nonaka *et al.*, 1999).

Preparation of Gal-lectin-depleted plasma and C3-depleted plasma

One hundred μl of acid-treated Sepharose (Yokosawa *et al.*, 1982) was added to 500 μl of *H. roretzi* plasma that had been diluted with 500 μl of phosphate-buffered saline (PBS), and the mixture was incubated at 4°C for 1 hr. After centrifugation, the resulting supernatant was used as a Gal-lectin-depleted plasma. The treatment with acid-treated Sepharose resulted in the depletion of Gal-lectin but not of AsC3 and an *N*-acetyl galactosamine-specific lectin present in *H. roretzi* plasma (Harada-Azumi *et al.*, 1987). To prepare an AsC3-depleted plasma, 500 μl of plasma was successively incubated at 4°C for 1 hr with 500 μl each of anti-AsC3 rabbit IgG (1 mg/ml in PBS) or non-immune rabbit IgG (1 mg/ml in PBS) as a control and then with 100 μl of protein A-Sepharose (a wet volume of 50 μl) (Sigma), followed by centrifugation to remove IgG and also antigen-antibody complex. Plasma that had been diluted twice with PBS and centrifuged was used as a positive control.

Assay for phagocytosis

H. roretzi plasma was appropriately diluted with normal Herbst's artificial seawater (N-HASW; 450 mM NaCl, 9.4 mM KCl, 48 mM $MgSO_4$, $CaCl_2$, 32 mM Na_2SO_4, 3.2 mM $NaHCO_3$, pH 7.6). SRBCs, yeast cells (W303D), and latex beads (5.7 μm in diameter, Sigma) were incubated at 20°C for 30 min with 5%, 25%, and 25% plasma, respectively, extensively washed with F-HASW, and then suspend in F-HASW. Ten-μl of plasma-treated target materials and non-treated materials (2×10^8 cells in the case of SRBCs, 1×10^8 cells in the case of yeast cells, and 1×10^8 particles in the case of latex beads) were mixed with 10 μl of *H. roretzi* hemocytes (5×10^6 cells) and the mixtures incubated at 20°C for 30 min in polypropylen test tubes. The phagocytosis was stopped by adding 10 mM diethyldithiocarbamete, a metal-chelating agent. After the hemocytes were transferred to a slide glass and stained with Nile blue dye, the phagocytic activity was measured under a Nikon phase contrast microscope. Approximately 200 hemocytes were inspected to check whether the hemocytes had ingested materials. A hemocyte that had ingested at least one material 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.

Effect of the RA5 antibody on phagocytosis

H. roretzi hemocytes were incubated with the RA5 antibody or control IgM in F-HASW at 20°C for 30 min. Non-treated materials (latex beads, SRBCs, and yeast cells) or plasma-treated materials (SRBCs and yeast cells) were added to the hemocyte suspension and the mixtures incubated at 20°C for 30 min. The phagocytic activity of hemocytes was measured by the method described above.

Western blot analysis

H. roretzi hemocytes (4×10^6 cells) were disrupted in 100 μl of sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE) that contained 5 mM EDTA, 1 mM leupeptin, 2 mM diisopropylfluorophosphate and 5% 2-mercaptoethanol. The cell lysate was then boiled at 90°C for 5 min. Proteins were separated by SDS-PAGE on a 7.5% gel (Laemmli, 1970) and were electrophoretically transferred to a nitrocellulose membrane. The membrane was treated with blocking buffer containing PBS and 3% skimmed milk, and then incubated with 2 μg/ml RA5 antibody or control mouse IgM in the same buffer overnight at 4°C. After washing four times with the same buffer containing PBS, 3% skimmed milk and 0.05% Tween 20, the membrane was incubated with peroxidase-conjugated anti-mouse immunoglobulins (1:2000 dilution; DAKO) in PBS containing 3% skimmed milk for 2 hr at room temperature, and then washed again five times with PBS. Finally the membrane was treated with an ECL (enhanced chemiluminescence) kit (Amersham) and the bands were visualized with X-ray film.

Flow cytometry analysis

Binding of ascidian Gal-lectin or AsC3 to either SRBCs or yeast cells was analyzed by flow cytometry. Plasma-treated SRBCs (4×10^6 cells) were previously washed with PBS containing 0.1% gelatin, while plasma-treated yeast cells (2×10^6 cells) were washed with PBS containing 10 mM EDTA and 0.1% gelatin. The washed target cells were then incubated with 10 μl of anti-Gal-lectin rabbit antibody (0.94 mg/ml) or anti-AsC3 rabbit antibody (1 mg/ml) at 4°C for 30 min. After washing, the target cells were stained with 20 μl of 100 μg/ml fluorescein isothiocyanate (FITC)-conjugated swan anti-rabbit immunoglo-

bulins (DAKO) at 4°C for 30 min. After washing, they were subjected to analysis on a Becton FACSsort.

RESULTS

Effects of *H. roretzi* plasma on phagocytosis by *H. roretzi* hemocytes

It has been reported that phagocytosis of non-treated SRBCs by *H. roretzi* hemocytes was enhanced by the presence of *H. roretzi* plasma, but that the phagocytosis of non-treated latex beads was not enhanced by the presence of the plasma (Ohtake *et al*., 1994). First we investigated whether opsonins that are capable of binding to exogenous target cells and enhancing the phagocytosis are present in *H. roretzi* plasma. SRBCs, yeast cells and latex beads pretreated with 5%, 25% and 25% plasma, respectively, in N-HASW were extensively washed and were then subjected to phagocytosis by *H. roretzi* hemocytes (Fig. 1). *H. roretzi* plasma at concentrations more than 5% caused aggregation of SRBCs, which prevented them from ingesting by *H. roretzi* hemocytes: We checked only the effect of 5% plasma in the case of SRBCs. Under the conditions used, about 20% of the hemocytes can ingest at least one non-treated SRBC, yeast cell or latex bead. When plasma-treated SRBCs and yeast cells were used as target cells, phagocytic activity of *H. roretzi* hemocytes toward them was enhanced; about 40% of the hemocytes ingested several target cells. However, the phagocytic activity toward plasma-treated latex beads was not enhanced. In any cases, granular amoebocytes most actively phagocytose target materials, and vacuolated cells were the next. These results confirmed that *H. roretzi* plasma contains opsonins, which would be able to bind to SRBCs and yeast cells.

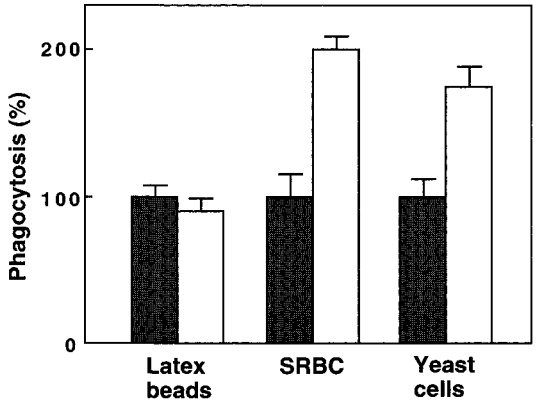

Fig. 1. Effects of plasma-treated target materials on phagocytosis by *H. roretzi* hemocytes. Phagocytosis of non-treated (closed bar) or plasma-treated (open bar) latex beads, SRBCs or yeast cells was measured. Amounts of plasma used were 5% in the case of SRBCs and 25% in both case of latex beads and yeast cells. The degree of phagocytosis of non-treated target material was defined as 100% in each case. Bars mean ±s.d.; n=3.

Effects of RA5 antibody on phagocytosis

Next, we prepared the monoclonal antibody RA5 on the basis of the inhibition of phagocytosis of non-treated SRBCs by *H. roretzi* hemocytes, and examined the effects of the RA5 antibody on phagocytosis of various target materials by *H. roretzi* hemocytes (Fig. 2). The RA5 antibody inhibited the phagocytosis of non-treated SRBCs and yeast cells, but not that of plasma-treated ones. On the other hand, the antibody inhibited the phagocytosis of neither non-treated nor plasma-treated latex beads. It was demonstrated that the RA5 antibody inhibited the phagocytosis of non-treated SRBCs in a concentration-dependent manner (Fig. 3A): The antibody showed almost 50% inhibition at a concentration of 20 μg/ml, while control mouse IgM did not inhibit the phagocytosis. On the other hand, the antibody scarcely inhibited the phagocytosis of plasma-treated SRBCs (Fig. 3B). These results led to the following conclusions: (1) When target materials are SRBCs and yeast cells, *H. roretzi* hemocytes undergo opsonin-independent and -dependent phagocytosis, and the RA5 antibody inhibits only opsonin-independent phagocytosis. (2) When target materials are latex beads, *H. roretzi* hemocytes undergo only opsonin-independent phagocytosis.

Western blot analysis showed that the RA5 antibody recognized mainly 200 kDa protein of *H. roretzi* hemocytes (Fig. 4). The antibody recognized weakly 180, 110, 80 and 60 kDa hemocyte proteins.

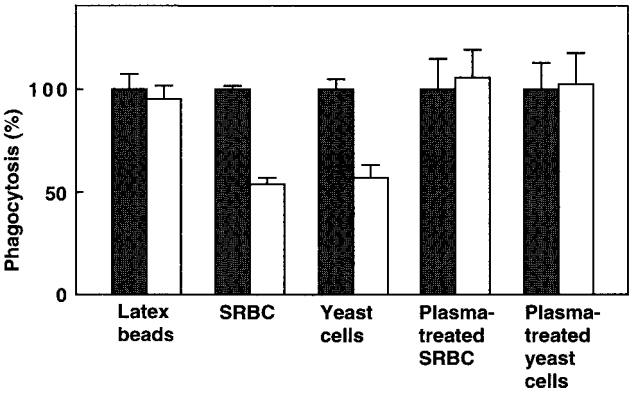

Fig. 2. Effect of the RA5 antibody on phagocytosis of various target materials by *H. roretzi* hemocytes. Phagocytosis of non-treated or plasma-treated target materials in the presence of the RA5 antibody (10 μg/ml) (open bar) or control IgM (10 μg/ml) (closed bar) was measured, and the degree of phagocytosis in the presence of control IgM was defined as 100% in each case. Bars mean ±s.d.; n=3.

Effects of Gal-lectin-depleted plasma and AsC3-depleted plasma on phagocytosis

To answer the question as to whether Gal-lectin and AsC3, present in *H. roretzi* plasma, both function as opsonins in the phagocytosis of SRBCs and yeast cells, we examined the enhancing activity of Gal-lectin-depleted plasma or AsC3-depleted plasma toward phagocytosis (Fig. 5). In the case of the phagocytosis of yeast cells (Fig. 5A), depletion of AsC3 from the plasma by treatment with anti-AsC3 antibody plus protein A-Sepharose resulted in complete loss of the enhancing activity of the plasma (Fig. 5A(e)), while depletion of Gal-lectin by acid-treated Sepharose (Fig. 5A(c)) or pretreatment

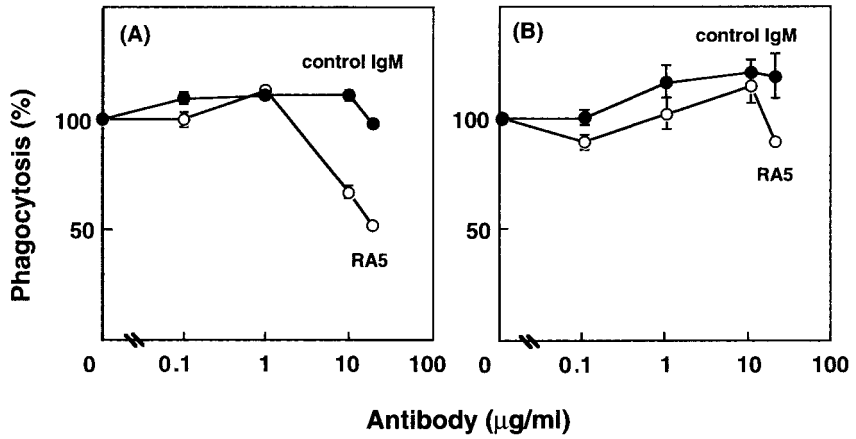

Fig. 3. Effect of the RA5 antibody on phagocytosis of SRBCs by *H. roretzi* hemocytes. The hemocytes were incubated with the RA5 antibody or control IgM in F-HASW at 20°C for 30 min. Non-treated SRBCs (A) or plasma-treated SRBCs (B) were added to the hemocytes suspension, and the mixture was incubated at 20°C for 30 min. The degree of phagocytosis observed in F-HASW, without the RA5 antibody or control IgM, was defined as 100%. Bars mean ±s.d.; n=3.

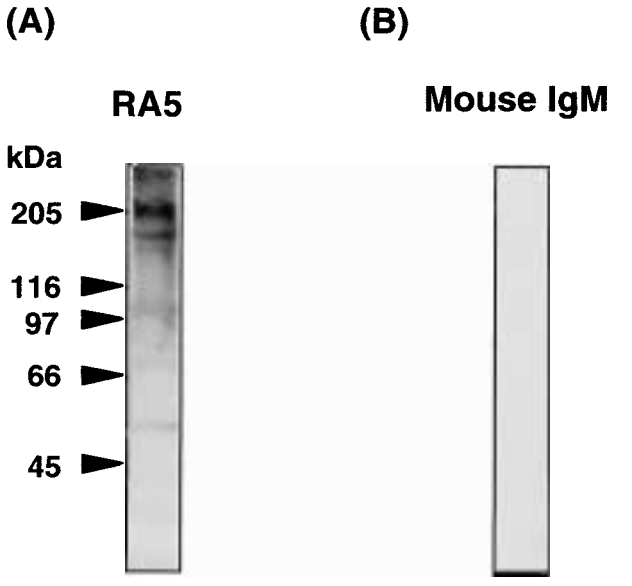

Fig. 4. Western blotting of *H. roretzi* hemocyte proteins with the RA5 antibody. The hemocytes were disrupted and subjected to SDS-PAGE, followed by Western blotting with the RA5 antibody (A) or control mouse IgM (B).

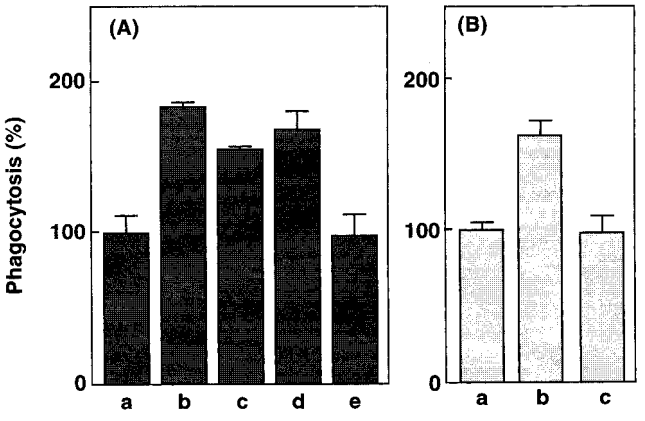

Fig. 5. Effects of control plasma and Gal-lectin-depleted and AsC3-depleted plasma on phagocytosis of yeast cells or SRBCs by *H. roretzi* hemocytes. Phagocytosis of non-treated or plasma-treated yeast cells (A) or SRBCs (B) was measured using the respective target cells pretreated with control plasma (b), Gal-lectin-depleted plasma (c), control IgG-treated plasma (d), or AsC3-depleted plasma (e). The degree of phagocytosis of non-treated target cells (a) was defined as 100% in each case. Bars mean ±s.d.; n=3.

with control rabbit IgG plus protein A-Sepharose (Fig. 5A(d)) resulted in a little or no loss of activity. On the other hand, in the case of the phagocytosis of SRBCs (Fig. 5B), Gal-lectin-depleted plasma lacked enhancing activity (Fig. 5B(c)). These results indicated that AsC3 enhanced the phagocytosis of yeast cells, while Gal-lectin enhanced the phagocytosis of SRBCs.

To determine whether AsC3 and Gal-lectin can actually bind to yeast cells and SRBCs, respectively, flow cytometry analyses of the target cells pretreated with AsC3-depleted or Gal-lectin-depleted plasma, as well as with control plasma, were performed using anti-AsC3 and anti-Gal-lectin antibodies (Fig. 6 and 7). In the flow cytometry analyses using anti-AsC3 antibody (Fig. 6), the population pattern of yeast cells pretreated with control plasma (Fig. 6A(b)) was shifted to the right in comparison with the case of non-treated yeast cells (Fig. 6A(a)), indicating that AsC3 derived from plasma is present in the control plasma-treated yeast cells. The population pattern of yeast cells pretreated with the Gal-lectin-depleted plasma (Fig. 6A(c)) was the same as that in the above case of control plasma, while that of yeast cells pretreated with the AsC3-depleted plasma (Fig. 6A(e)) was shifted to the left in comparison with that in the case of control IgG (Fig. 6A(d)). Analyses of SRBCs using anti-AsC3 antibody showed almost the same pattern in any case of pretreatment with plasma preparation, including control plasma (Fig. 6B). These results suggest that AsC3 can bind to yeast cells but not to SRBCs. On the other hand, in the analysis using anti-Gal-lectin antibody (Fig. 7), the pattern of SRBCs pretreated with

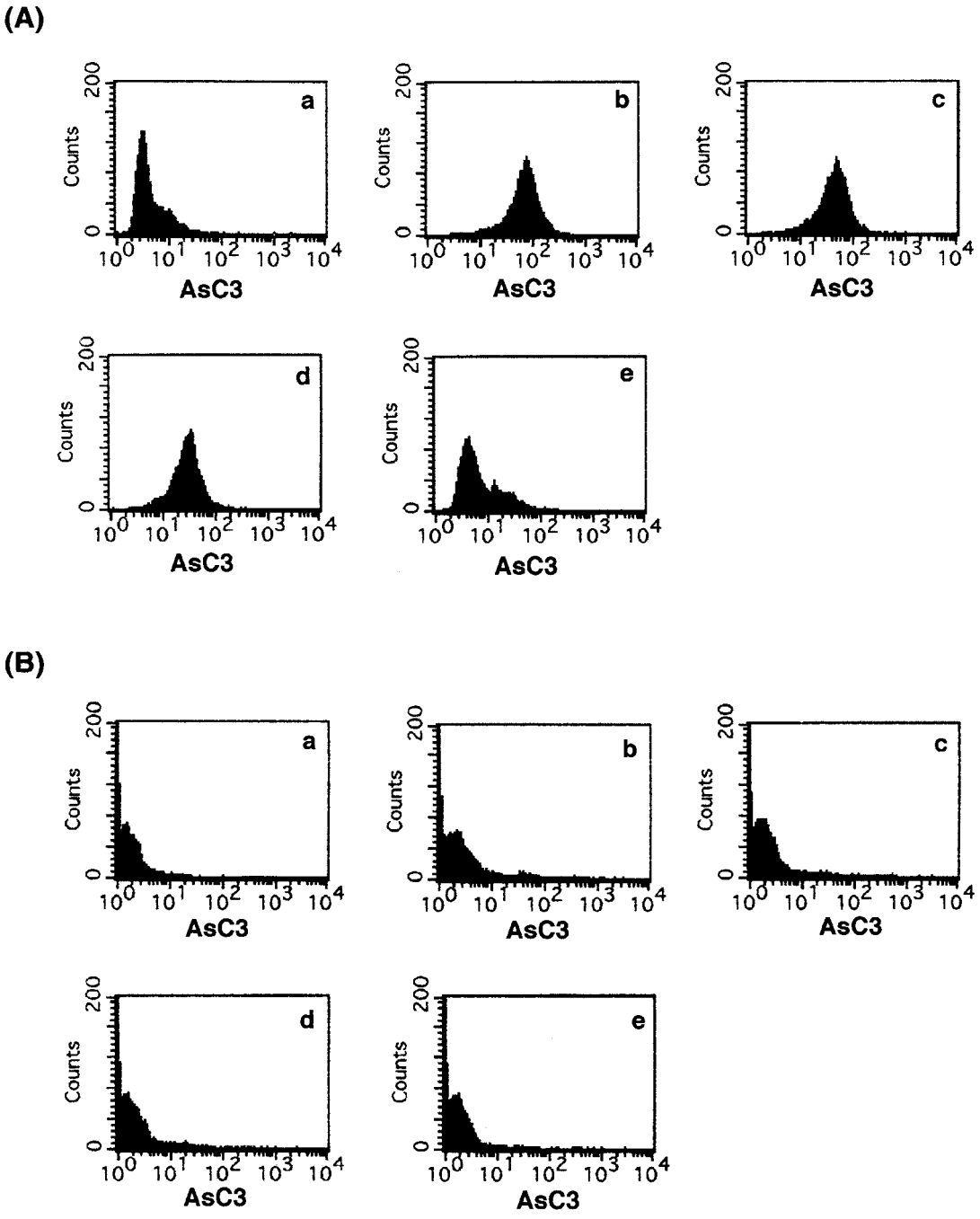

Fig. 6. Flow cytometry analyses of yeast cells and SRBCs using anti-AsC3 antibody. Yeast cells (A) or SRBCs (B) were pretreated with control plasma (b), Gal-lectin-depleted plasma (c), control IgG-treated plasma (d), or AsC3-depleted plasma (e), and were then subjected to flow cytometry using the anti-AsC3 antibody. In control (a), the respective target cells without plasma treatment were subjected to flow cytometry. Note that AsC3 binds to yeast cells only .

control plasma (Fig. 7A(b)) was shifted to the right in comparison with that in the case of non-treated SRBCs (Fig. 7A(a)), indicating that Gal-lectin derived from plasma is present in the control plasma-treated SRBCs. The pattern of SRBCs pretreated with the Gal-lectin-depleted plasma (Fig. 7A(c)) was shifted to the left in comparison with that in the above case of control plasma, which confirmed the above assumption that the lectin binds to SRBCs. In the analyses of yeast cells using anti-Gal-lectin antibody, the pattern of yeast cells remained almost unchanged in any case of pretreatment with plasma preparation, including control plasma (Fig. 7B). These results suggest that Gal-lectin can bind to SRBCs but not to yeast cells.

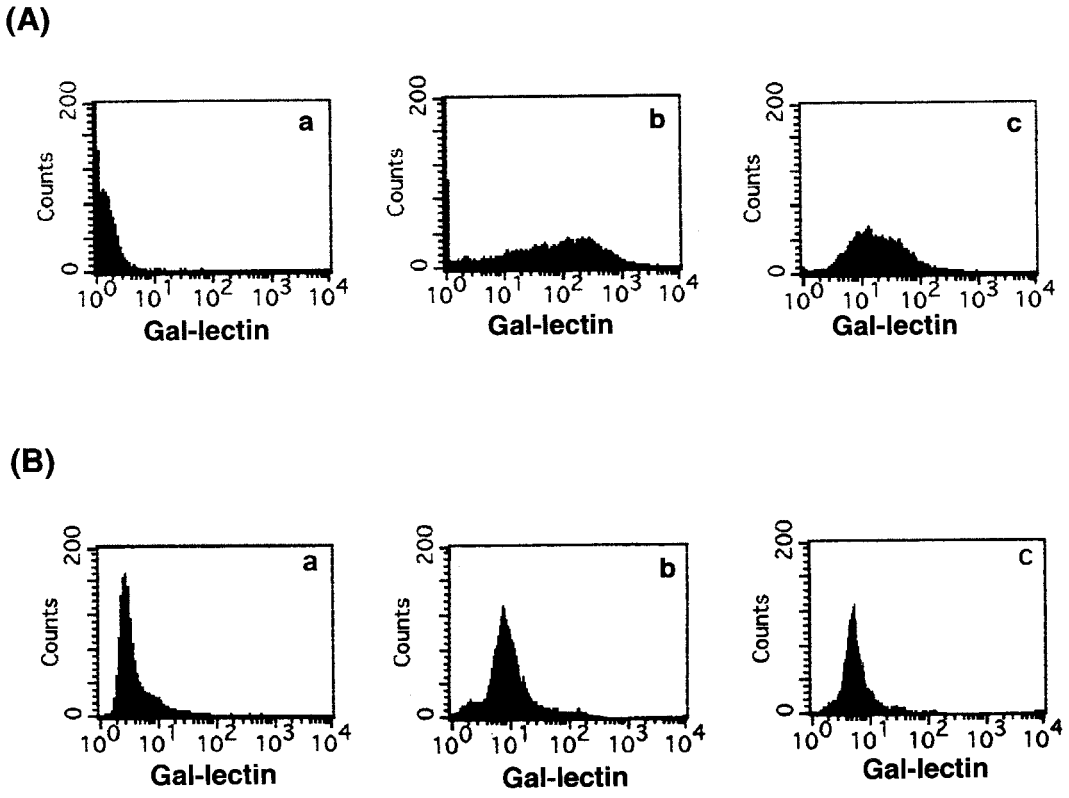

Fig. 7. Flow cytometry analyses of SRBCs and yeast cells using anti-Gal-lectin antibody. SRBCs (A) or yeast cells (B) were pretreated with control plasma (b) or Gal-lectin-depleted plasma (c), and were then subjected to flow cytometry using the anti-Gal-lectin antibody. In control (a), the respective target cells without plasma treatment were subjected to flow cytometry. Note that Gal-lectin binds to SRBCs only.

DISCUSSION

Hemocytes of *H. roretzi* undergo phagocytosis of SRBCs, yeast cells, latex beads and *Escherichia coli* in vitro (Ohtake *et al.*, 1994; Takahashi *et al.*, 1995; Azumi and Yokosawa, 1996). To determine the molecules that are involved in the phagocytosis by *H. roretzi* hemocytes, we previously prepared the monoclonal antibody A74, which strongly inhibited the phagocytosis of non-treated SRBCs and recognized the 160 kDa antigen protein (Takahashi *et al.*, 1995). Whether the A74 antibody would inhibit the phagocytosis of plasma-treated target materials has not been investigated. In this study, we prepared another monoclonal antibody, RA5, and found that the RA5 antibody inhibited the phagocytosis of non-treated SRBCs or yeast cells but not that of plasma-treated SRBCs or yeast cells. In the case of latex beads as a target, the RA5 antibody did not inhibit the phagocytosis toward them with or without plasma pretreatment. Thus, the molecule recognized with the RA5 antibody is involved in opsonin-independent phagocytosis. The RA5 antibody recognized mainly a 200 kDa hemocyte protein, which can be thought to interact with surface molecules present in SRBCs and yeast cells but not with latex beads. Hemocyte proteins with molecular masses of 180, 110, 80 and 60 kDa were weakly recognized with the RA5 antibody. These proteins might be degradation products of the 200 kDa protein or proteins that shared the same epitopes with the 200 kDa protein. In mammalian macrophages, the mannose-receptor with a molecular mass of about 180 kDa has been reported to function in opsonin-independent phagocytosis (Sung *et al.*, 1983), which is required for the presence of divalent cations (Lennartz *et al.*, 1987). In *H. roretzi* hemocytes, magnesium ions can enhance the phagocytosis of non-treated SRBCs (unpublished data). Thus, it is possible that the RA5 antigen in *H. roretzi* hemocytes might be an ascidian homolog of the mannose-receptor in mammalian macrophages.

Although the A74 antibody inhibited opsonin-independent phagocytosis by *H. roretzi* hemocytes as the RA5 antibody did, the respective antigen molecules are thought to be different from each other on the basis of the following reasons. First, the former antibody recognized 160 kDa protein (Takahashi *et al.*, 1995), while the latter recognized mainly 200 kDa hemocyte protein. Second, the former antibody inhibited both phagocytosis by and aggregation of *H. roretzi* hemocytes (Takahashi *et al.*, 1995), while the latter inhibited only phagocytosis.

With respect to the opsonin-dependent phagocytosis, it has been reported that the body fluids of ascidians strongly enhance phagocytosis by hemocytes of the respective ascidians (Kelly *et al.*, 1993; Ballarin *et al.*, 1994; Ohtake *et al.*, 1994). Gal-lectin and AsC3 both are candidate opsonic factors in *H. roretzi* plasma. Lectins are widely distributed in invertebrate hemolymph and are involved directly in internal defense mechanisms (Vasta *et al.*, 1994). We already

isolated two C-type lectins from *H. roretzi* hemolymph, designated Gal-lectin (Yokosawa *et al*., 1982) and an *N*-acetyl-galactosamine-specific lectin (Harada-Azumi *et al*., 1987). *H. roretzi* plasma contains a large amount of the former lectin and a smaller amount of the latter lectin. We have already demonstrated that purified Gal-lectin had an enhancing activity toward the phagocytosis of non-treated SRBCs by *H. roretzi* hemocytes (Abe *et al*., 1999), suggesting that Gal-lectin is one of the candidate opsonins in *H. roretzi* plasma. Recently, we found that AsC3 and the complement activation system exist in *H. roretzi* hemolymph and that the AsC3 can bind to yeast cells to enhance the phagocytosis by *H. roretzi* hemocytes (Nonaka *et al*., 1999), strongly suggesting that AsC3 is another candidate opsonin in *H. roretzi* plasma.

We investigated whether Gal-lectin and AsC3 both function as opsonins in phagocytosis of SRBCs and yeast cells by *H. roretzi* hemocytes. When yeast cells were used as target cells, the plasma at a concentration more than 10% can enhance the phagocytosis. AsC3 was detectable on the surface of yeast cells previously treated with 25% control plasma, under the conditions where phagocytosis was strongly enhanced. The results using AsC3-depleted plasma confirmed this. Under the same conditions, on the other hand, Gal-lectin was undetectable on the surface of yeast cells. Thus, the level of AsC3 bound to yeast cells is strongly correlated with the ability of plasma to enhance the phagocytosis of yeast cells. When SRBCs were used as target cells, on the other hand, the plasma enhanced the phagocytosis even at a concentration as low as 5%, under the conditions where Gal-lectin was detectable on the surface of SRBCs. It can be inferred that the amount of Gal-lectin in 5% plasma is enough to enhance the phagocytosis. The results using the Gal-lectin-depleted plasma confirmed this. In this experiment, we used acid-treated Sepharose to deplete Gal-lectin from the plasma in place of the anti-Gal-lectin antibody because an antibody at a rather high concentration that is required for immunodepletion is not available at present. In addition, the effects of the AsC3-depleted plasma and control IgG-treated plasma on the phagocytosis of SRBCs have not been demonstrated because the use of Sepharose coupled with protein A for the immunodepletion of AsC3 or control IgG also depleted Gal-lectin from the plasma. Thus, we conclude that AsC3 and Gal-lectin function as opsonins mainly in the phagocytosis of yeast cells and SRBCs, respectively. It can be inferred that the difference in the target cells between them is due to the difference in the nature of the cell surface molecules recognized by the respective opsonins. In addition, *H. roretzi* hemocytes are expected to express the putative receptors, to which the respective opsonins, AsC3 protein and Gal-lectin, can bind.

In conclusion, *H. roretzi* hemocytes undergo phagocytosis in opsonin-independent and -dependent manners. The putative molecule recognized with the RA5 antibody, together with the A74 protein, plays a role in opsonin-independent phagocytosis, while Gal-lectin and AsC3 function as opsonins in phagocytosis, dependent on the nature of the target cells. Further investigation of the structures of the RA5 antigen and the putative opsonin receptors is necessary for an understanding of the mechanisms underlying phagocytosis by *H. roretzi* hemocytes.

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