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PHAGOCYTOSIS IN THE COLONIAL ASCIDIAN *Botryllus schlosseri*

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□**Abstract—Phagocytosis by *Botryllus schlosseri* hemocytes is influenced by temperature, pH, concentration, and physicochemical properties of the test particles and requires Ca^{2+} or Mg^{2+} ions to occur. Phagocytes recognize glucosyl or mannosyl residues on the surface of yeast cells, and a respiratory burst is associated with phagocytosis, as indicated by increased superoxide production. Factors that enhance phagocytosis of yeast, sheep red blood cells, and latex beads and reduce the uptake of yeast and sheep erythrocytes are present in the plasma.**

□**Keywords—Ascidians; *Botryllus*; Phagocytosis.**

Nomenclature

BP	blood plasma
EGTA	ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid
FSW	filtered sea water
ISO	isotonic salt solution
NBT	nitroblue tetrazolium
SRBC	sheep red blood cells

Introduction

Phagocytosis is a fundamental defense mechanism that multicellular animals probably inherited from unicellular ancestors in which it played a role in food collection. It is a two-step process as non-self particles must adhere to phagocytes before being ingested and subsequently digested.

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In vertebrates, phagocytosis is enhanced by complement-derived opsonins and antimicrobial antibodies that coat foreign invaders and are recognized by Fc receptors on the macrophage surface (1–3). In addition, opsonin- and antibody-independent phagocytosis of microorganisms, mediated by lectin–sugar interactions, has been reported (4,5): lectins, expressed on the microbial surface, can recognize sugars on macrophage plasma membranes (6,7); conversely, macrophage membranes can bear lectin-like receptors for sugars of bacterial and yeast cell walls (8). Mannose and β-1,3 glucan receptors have been identified on mammalian macrophages (9,10); the presence of the latter kind of receptor has recently been indirectly proved in teleostean macrophages (11). Vertebrate phagocytosis is also associated with a respiratory burst, namely, a series of reactions leading to increased production and excretion of highly reactive, toxic oxygen metabolites, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, which are involved in killing bacteria and parasites (12).

Invertebrate phagocytosis shows some similarities with its vertebrate counterpart. Phagocytes have been identified in all of the major invertebrate phyla (13): they are characterized by abundant lysosomal granules rich in hydrolytic enzymes (14–18). Opsonizing effects of humoral components have been reported in molluscs (19–21), anne-

lids (22), arthropods (23,24), echinoderms (25), and tunicates (26,27), but opsonin-independent phagocytosis has also been described (28–30). A β-1,3 glucan receptor on phagocytic hemocytes of the snail *Biomphalaria glabrata,* involved in the uptake of yeast, has been suggested by Fryer et al. (20); physical factors influencing phagocytosis have been studied in the same species (31). The production of reactive oxygen derivatives during phagocytosis has been demonstrated in arthropods (32), molluscs (33–38), and echinoderms (25).

From an evolutionary viewpoint, ascidians are related to vertebrates (39), and studying their defense strategies is of particular interest with respect to the vertebrate immune system. Macrophage-like cells bearing large vacuoles filled with cell debris and devoid of amoeboid activity have been described in the blood of all ascidian species (40,41), and amoebocytes of the hyaline/microgranular type are reported to be involved in phagocytosis (42,43). Up to now, however, except for recent work by Kelly et al. (27) using a solitary species, scanty data are available on phagocytosis in ascidians, the physical and humoral factors affecting the process, the possible association with respiratory bursts, the nature of receptors on phagocytes, the cell types involved, and their mutual relationships.

We present results on phagocytosis in the colonial ascidian *Botryllus schlosseri* (using yeast cells, zymosan, sheep erythrocytes, latex beads, and *E. coli* cells as test particles). These results show its independence of humoral opsonizing factors, suggesting a direct cell–particle interaction which, in the case of yeast, is mediated by glucosyl- or mannosyl-specific membrane lectins, and its association with a respiratory burst as evidenced by superoxide anion production. In addition, data presented here show that three cell types are directly involved in phagocytosis: hyaline amoebocytes, macrophage-like cells, and signet-ring cells. These data confirm the close relationship between these hemocyte types, previously suggested by the study of the distribution patterns of some hydrolytic and oxidative enzymes among *Botryllus* hemocytes (44).

Materials and Methods

Ascidians

Colonies of *B. schlosseri* from the Venetian lagoon were cultivated in our laboratory, attached to 5 × 5 cm glass slides immersed in aquaria, and fed Liquifry Marine (Liquifry Co., Dorking, U.K.) and algae.

Collection of Hemocytes

Blood was obtained by puncturing, with fine tungsten needles, the marginal vessels of colonies previously rinsed in filtered sea water (FSW) containing 10 mM L-cysteine, pH 7.0, to prevent clotting. Hemocytes were washed by centrifugation at 780 × g for 15 min, and the pellets were resuspended in FSW, pH 7.0, to a final concentration of 8–10 × 10^6 cells/mL.

Hemocyte Cultures

Culture chambers were made by gluing teflon rings (15 mm internal diameter, 1 mm thick) on siliconized glass slides. Hemocyte suspensions (50 μL) were placed in the center of each chamber, and washed coverslips were laid over the teflon rings smeared with vaseline and gently pressed down to touch the drop of cell suspension. The culture slides were kept upside down for 30 min to allow cells to settle and adhere to the coverslips.

Test Particles: Yeast, Zymosan, Sheep Erythrocytes, Latex Beads and E. coli *Cells*

Ordinary baker's yeast, living or autoclaved (15 min at 120°C) cells, was thoroughly washed in FSW and used in most of our assays. Autoclaved yeast was FITC-labelled according to Hed (45). Sheep erythrocytes (SRBC) were also used as viable or formalinized (according to Abdul—Salam and Michelson (31)) cells. Zymosan and latex beads (1 and 3 μm diameter) were purchased from Sigma. Fluorescent *Escherchia coli* cells were part of the Orpegen Phagotest kit (Orpegen, Heidelberg, Germany). The number of test particles was adjusted to particle:hemocyte ratios of 1:10, 1:1 and 10:1.

Phagocytosis Assay

After adhesion of hemocytes to the coverslips, debris-containing FSW was discharged and replaced with equal volumes of test-particle suspension in FSW. Cultures were kept upside-down for 15, 30, and 60 min at 25°C. Viability, assessed by trypan blue exclusion, exceeded 95% after 2 h of incubation. At the end of the incubation period, hemocyte monolayers were washed by dipping the coverslips several times in a large volume (100 mL) of FSW, fixed in a solution of 1% glutaraldehyde and 1% sucrose in FSW at 4°C for 30 min, and stained with 10% Giemsa for 10 min. When fluorescent yeast or bacteria were used, noningested particles were quenched by quick immersion of coverslips in a solution of 2 mg/mL trypan blue and 2 mg/mL crystal violet in 0.02 M citrate buffer, pH 4.4, containing 33 mg/mL NaCl (modified after Hed (45)); the cells were then fixed in glutaraldehyde as described above. In both cases, the coverslips were finally mounted on glass slides with an aqueous medium (Acquovitrex, Carlo Erba).

Phagocytic Index

A Leitz Dialux 22 microscope, equipped with fluorescence apparatus (450–490 nm filter), was used to observe the cells. Hemocytes were counted at a magnification of 1250×, at least 200 cells per coverslip in 10 fields. The results are expressed as the phagocytic index, i.e., the percentage of hemocytes containing ingested particles (46). In tests with fluorescent yeast, the number of fluorescent particles was also recorded, both at different yeast concentrations and at different incubation times.

In order to identify the different hemocyte types involved in phagocytosis at different times, yeast was supplied for 5 min and monolayers were fixed after 15, 30, and 60 min of incubation, stained, and observed as described above. Hemocytes were classified according to their morphology.

Effects of Temperature and pH on Yeast Ingestion

Assays were carried out at 10, 19, 25, and 37°C. pH dependence was measured by incubation of hemocytes with yeast at 25°C in 20 mM Tris, 0.5 M NaCl plus 20 mM $CaCl_2$, adjusted at pH 8.5, 8.0, 7.5, and 7.0, and in 20 mM Bis-Tris plus 0.5 M NaCl and 20 mM $CaCl_2$, adjusted at pH 7.0, 6.5, 6.0, and 5.5.

Ca^{2+} and Mg^{2+} Dependence

Yeast cells and hemocytes (10:1 ratio) were incubated for 60 min in isotonic salt solution (ISO: 20 mM Tris, 0.5 M NaCl) (47) containing $CaCl_2$ or $MgCl_2$ at concentrations of 5, 10, and 20 mM and 0.1, 0.5, or 1.0 mM ethylene glycol-bis(β-

aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA). The pH of all of the solutions was adjusted to 7.0.

Effects of Sugars and Sugar Derivatives

The specificity of yeast recognition by phagocytes was studied with sugar inhibition experiments according to Engstad and Robertsen (11) and Fryer et al. (20). The following sugars and sugar derivatives were tested: D-ribose, D-fructose, D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, methyl-α-D-glucopyranoside, D-galactose, L-galactose, D-galactosamine, D-mannose, D-mannosamine, methyl-α-D-mannopyranoside, D-mannitol, D-sorbitol, lactose, sucrose, polygalacturonic acid, chitosan, laminarin, mannan, methylcellulose, and powdered *Ciona intestinalis* tunic. They were diluted in FSW at concentrations of 0.5 and 1%, and yeast cells were suspended in the solutions thus obtained before being incubated with hemocytes (yeast: hemocyte ratio = 10:1) for 60 min at 25°C.

Superoxide Assay

The nitroblue tetrazolium (NBT) method of Park et al. (49), modified according to Shozawa et al. (35), was followed to reveal superoxide anion production during phagocytosis. Yeast or zymosan particles, ten times more abundant than hemocytes, were used in the assay. They were added to FSW containing 0.2% NBT in which monolayers were incubated for 60 min at 25°C. Hemocytes were then washed repeatedly, fixed, mounted in Acquovitrex, and examined under the light microscope. Yeast and zymosan were omitted in controls. The percentage of phagocytizing cells showing dark-blue formazan precipitate was recorded. 25 μg of superoxide dismutase (SOD; Sigma, ca 6000 U/mg) were added to the incubation medium to check the specificity of the reaction.

Effects of Blood Plasma

To assess the effects of blood plasma (BP) on phagocytosis, yeast cells (both living and autoclaved), SRBC (both fresh and formalinized), latex beads, and *E. coli* cells were preincubated for 30 min in BP (whole or diluted with FSW), thoroughly washed by three centrifugations at 1390 × g, and resuspended in FSW before incubation with *B. schlosseri* hemocytes. FSW was used as a control preincubation medium.

To obtain BP, colonies of about 80 zooids were blotted dry, and marginal vessels were lacerated with fine tungsten needles; blood was collected with a glass micropipette, centrifuged at 780 × g for 15 min, and the supernatant collected. The protein concentration of BP, determined according to Bradford (50) using bovine serum albumin as standard, was adjusted to 1.0 mg/mL by dilution with FSW.

Absorption of Blood Plasma

Suspensions of yeast cells and SRBC in FSW, about 5×10^8 cells/mL, were washed three times by centrifugation in FSW (10 min, 780 × g), resuspended in 1 mL of undiluted BP, and incubated at room temperature with agitation. After 15 min, suspensions were centrifuged and supernatants were further absorbed by repeating the above procedure. Suspensions were finally centrifuged again and supernatants used as yeast- or SRBC-preincubating media.

Scanning Electron Microscopy

Blood was collected as described above, and hemocytes were left to adhere to poly-L-lysine coated coverslips for 30 min at room temperature. They

were then fixed in 1.5% glutaraldehyde plus 0.5% acrolein in 0.2 M cacodylate buffer, pH 7.2, containing 1.7% NaCl, 1% caffeine, and 1% sucrose for 30 min at 4°C (modified after Müller and Greenwood (51)), rinsed in cacodylate buffer, postfixed in 1% OsO_4 in cacodylate buffer for 60 min, and rinsed again three times in cacodylate buffer. Dehydration through a graded ethanol series was followed by critical-point drying in liquid CO_2 with absolute acetone as transitional fluid. Cells were then sputtered with gold and observed with a Leica Stereoscan 110 SEM.

Statistical Analysis

All experiments were repeated three times with three independent cell samples ($n = 3$). Data are expressed as means ± SD. ANOVA was performed with the SAS statistical package (SAS Institute Inc., Cary, NC), and means were compared using the Duncan's test (52).

Results

Optimum Test Particle Concentration, Temperature, and pH

Phagocytosis of yeast cells by *B. schlosseri* hemocytes reaches its maximum at a yeast:hemocyte ratio of 10:1 and a temperature of 25°C (Fig. 1). All subsequent assays were performed at this temperature and yeast concentration. Phagocytic activity is retained over a wide pH range, with an optimum at pH 7.0 (Fig. 2). The phagocytic index increases progressively with time and reaches a plateau of 10–15% after an incubation time of 60 min.

Treatment With Blood Plasma Reduces Phagocytosis of Yeast and SRBC and Enhances Phagocytosis of Latex Particles

BP-treated living yeast and SRBC form large clumps of cells, clearly visible under the light microscope, which do not appear in controls. Preincubation of viable yeast and fresh erythrocytes with BP significantly ($p < 0.001$) reduces phagocytosis, with respect to controls, at all incubation times, and the phagocytic index reaches its maximum value (3–4%) at 30 min. When autoclaved yeast or formalinized SRBC are used, the difference between untreated and treated test particles disappears (Fig. 3). A difference also exists between living and formalinized SRBC, the former being phagocytized at a significantly ($p < 0.05$) higher rate (Fig. 3b).

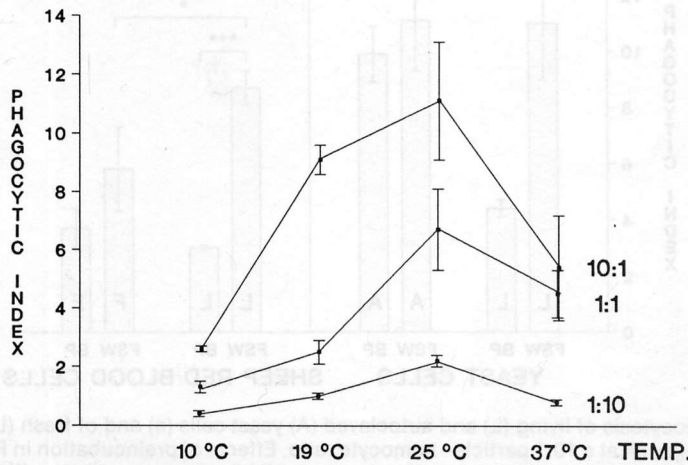

Figure 1. Effect of particle concentration and temperature on phagocytosis of yeast by *B. schlosseri* hemocytes. 1:10, 1:1, 10:1 = particle–hemocyte ratios; incubation time: 60 min. $n = 3$.

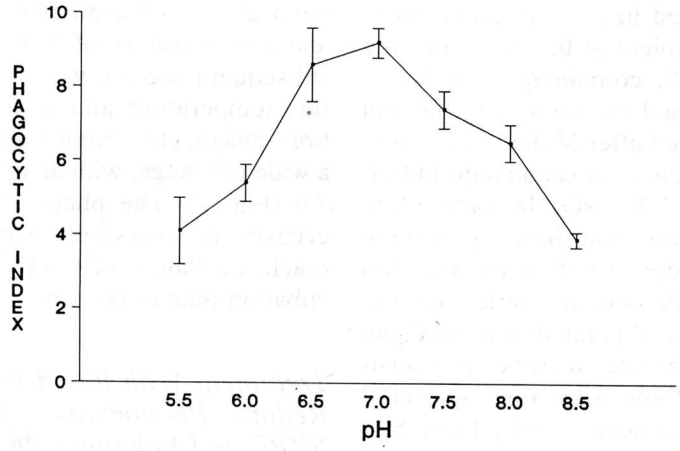

Figure 2. Effect of pH on phagocytosis of yeast by *B. schlosseri* hemocytes. Cells were incubated at 25°C for 60 min in 20 mM Tris, 0.5 M NaCl plus 20 mM $CaCl_2$, adjusted at pH 8.5, 8.0, 7.5, and 7.0, and in 20 mM Bis-Tris plus 0.5 M NaCl and 20 mM $CaCl_2$ adjusted at pH 7.0, 6.5, 6.0, and 5.5. Incubation time: 60 min. $n = 3$.

Preincubation in BP significantly ($p < 0.05$) increases the phagocytic index of latex beads, but has no effects on *E. coli* cells (Fig. 4). However phagocytosis is significantly ($p < 0.05$) reduced when latex beads or bacteria are used as compared with yeast cells or SRBC (compare Fig. 4 with Fig. 3): 1-μm latex particles are engulfed at a significantly ($p < 0.05$) faster than 3-μm ones.

Treatment With Absorbed or Diluted BP Reveals a Humoral Opsonin for Yeast and SRBC

When yeast and sheep erythrocytes are preincubated in yeast- or SRBC-absorbed BP, a significant ($p < 0.001$) increase in the phagocytic index can be observed in 30-min cultures but not in 60-min ones (Table 1).

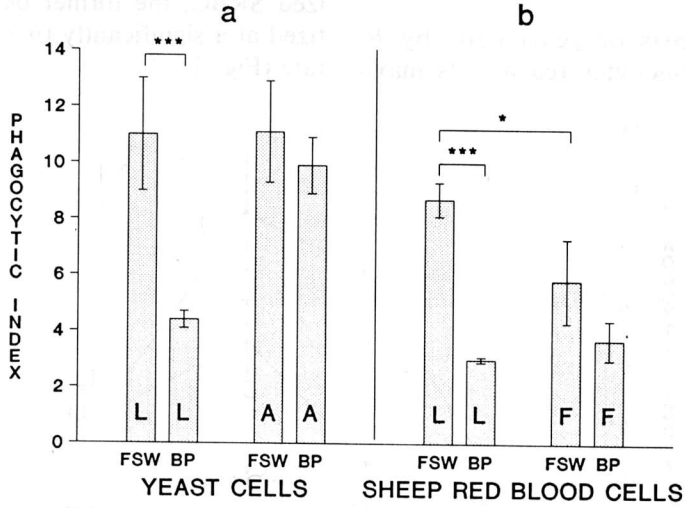

Figure 3. Phagocytosis of living (L) and autoclaved (A) yeast cells (a) and of fresh (L) and formalin-fixed (F) SRBC (b), all at a 10:1 particle–hemocyte ratio. Effects of preincubation in FSW and BP are compared, as well as effects of formalin treatment on SRBC phagocytosis. Significant differences are shown by asterisks. $^{*}p < 0.05$; $^{***}p < 0.001$. Incubation time: 60 min. $n = 3$.

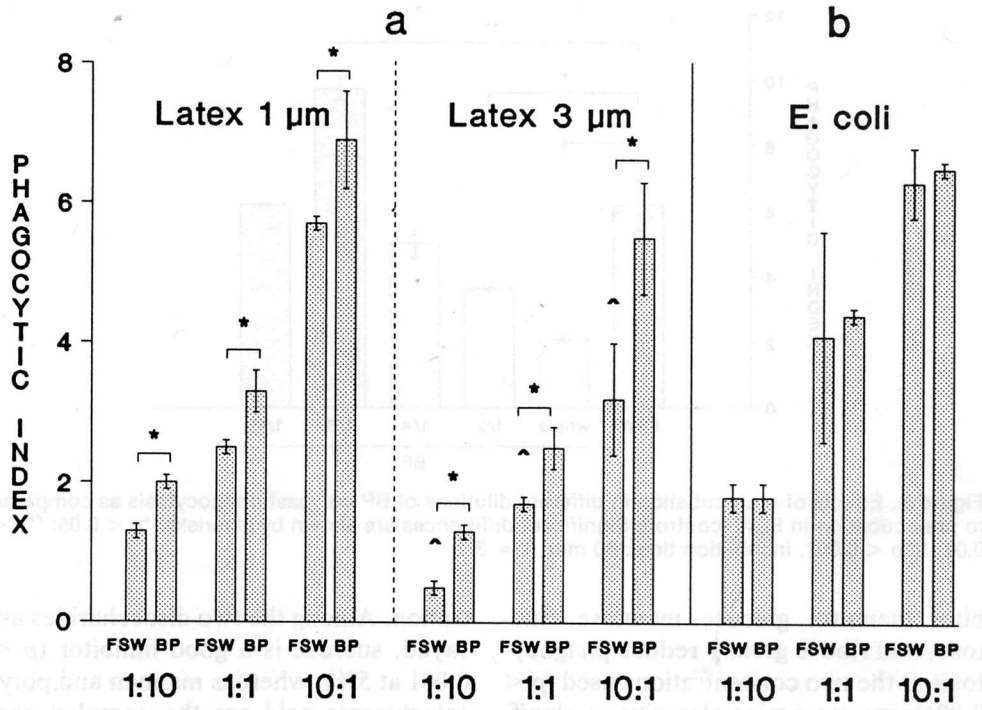

Figure 4. Phagocytosis of 1-µm and 3-µm latex beads (a) and *E. coli* cells (b) at different particle–hemocyte ratios and 60 min incubation. Effects of preincubation in FSW and BP are compared. *: significant differences ($p < 0.05$) between treated and untreated beads; ^: significant differences ($p < 0.05$) between 1-µm and 3-µm untreated beads at the same concentration. Incubation time: 60 min. $n = 3$.

After preincubation of yeast in FSW-diluted BP, a significant ($p < 0.05$) reduction of the phagocytic index in 30-min cultures is still observed after dilution with equal volumes of FSW, whereas a significant ($p < 0.01$) increase in the phagocytic index is registered when BP was diluted 1:8 with FSW (Fig. 5).

Glucose, Mannose, and Their Derivatives Inhibit Phagocytosis of Yeast

The effects of sugars on yeast phagocytosis by hemocytes are shown in Table 2. Some of the monosaccharides or their derivatives show inhibitory effects: sor-

Table 1. Effects of Different Preincubation Media on Phagocytosis of Yeast and SRBC.

Test Particle	Preincubation Medium	Phagocytic Index (Incubation Time) (30 min)		(60 min)	
Yeast	FSW	5.6 ± 1.1	***	11.0 ± 2.0	***
	Whole BP	2.2 ± 0.1	***	4.4 ± 0.3	
	Yeast-absorbed BP	10.1 ± 0.5		10.9 ± 3.2	
SRBC	FSW	5.1 ± 0.1	***	11.2 ± 0.9	***
	Whole BP	1.9 ± 0.2	***	4.2 ± 0.4	
	SRBC-absorbed BP	9.6 ± 0.3		10.3 ± 0.5	

*** $p < 0.001$.

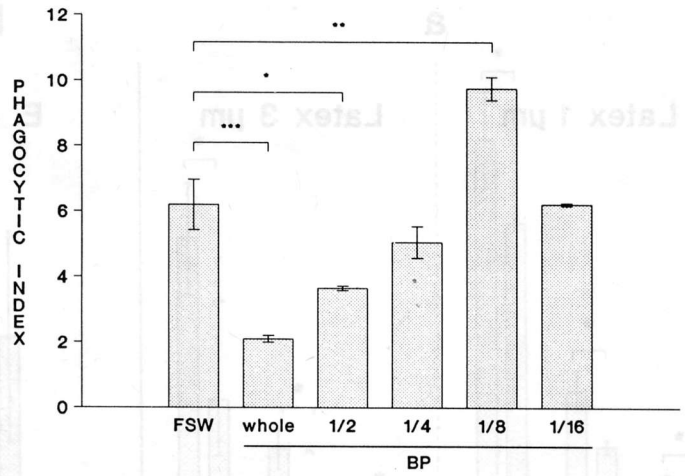

Figure 5. Effects of preincubation in different dilutions of BP on yeast phagocytosis as compared to preincubation in FSW (control). Significant differences are shown by asterisks: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Incubation time: 30 min. $n = 3$.

bitol, mannitol, glucose, mannose, fructose, and ribose greatly reduce phagocytosis at the two concentrations used ($p < 0.001$); mannosamine also gives a significant ($p < 0.01$) inhibition at 1% concentration. Among the two disaccharides assayed, sucrose is a good inhibitor ($p < 0.001$ at 5%), whereas mannan and polygalacturonic acid are the complex carbohydrates that significantly ($p < 0.01$

Table 2. Effects of Carbohydrates and Carbohydrate Derivatives, 0.5 and 1% in FSW, on Yeast Phagocytosis by *Botryllus* Hemocytes. Significant Differences with Respect to Control are Marked by Asterisks.

Carbohydrates	Phagocytic Index	
Control	11.50 ± 0.06	
Monosaccharides	0.5%	1%
D-Sorbitol	2.26 ± 0.07***	1.15 ± 0.01***
D-Mannitol	3.20 ± 0.01***	2.60 ± 0.02***
D-Glucose	2.28 ± 0.12***	1.18 ± 0.07***
D-Glucosamine	7.86 ± 0.12	5.38 ± 0.80**
N-acetyl-D-glucosamine	9.41 ± 0.08	7.01 ± 1.60
Methyl-α-D-glucopyranoside	10.59 ± 0.46	9.13 ± 1.31
D-Mannose	3.58 ± 0.35***	2.57 ± 0.32***
D-Mannosamine	6.29 ± 0.12**	5.92 ± 1.01**
Methyl-α-D-mannopyranoside	10.01 ± 0.12	9.89 ± 0.73
D-Galactose	11.16 ± 0.12	11.25 ± 1.73
D-Galactosamine	11.40 ± 1.25	10.48 ± 0.86
D-Fructose	1.81 ± 0.21***	0***
D-Ribose	2.85 ± 0.12***	2.23 ± 0.14***
Disaccharides		
Saccharose	0.91 ± 0.12***	0.67 ± 0.09***
Lactose	8.05 ± 0.69	6.74 ± 0.88*
Polysaccharides		
Mannan	5.89 ± 0.16**	4.68 ± 0.26***
Polygalacturonic acid	7.26 ± 0.20*	2.63 ± 0.26***
Laminarin	11.49 ± 1.41	11.58 ± 1.11
Chitosan	8.85 ± 0.04	8.73 ± 0.61
Methylcellulose	11.41 ± 0.08	11.45 ± 1.27
Powdered *C. intestinalis* tunic	9.78 ± 0.07	9.21 ± 1.7

$*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

and $p < 0.05$, respectively, at 5% and $p < 0.001$ at 1%) reduce yeast phagocytosis. Laminarin, a β-1,3 glucan, has no effect.

Ca^{2+} and Mg^{2+} Dependence

Uptake of yeast requires calcium or magnesium ions. As compared to values obtained with FSW, phagocytic index is significantly ($p < 0.001$) reduced when incubation is carried out in ISO containing 0.1, 0.5, and 1 mM EGTA; it increases with increasing concentrations of Ca^{2+} and Mg^{2+} in ISO and approximates FSW values at 20 mM Ca^{2+} or Mg^{2+}. Magnesium ions are significantly ($p < 0.05$) more effective than calcium ions in promoting phagocytosis (Fig. 6).

Production of Superoxide Anion

Superoxide anion is produced by *Botryllus* blood cells during phagocytosis, as revealed by the significant ($p < 0.001$) increase in the number of formazan spots found in phagosome-containing cells in association with engulfed zymosan or yeast particles (Table 3; Fig. 7i, j), suggesting the occurrence of a respiratory burst. The percentage of NBT-positive cells is significantly ($p < 0.01$) reduced when incubation takes place in the presence of SOD (Table 3).

Phagocytic Hemocytes: Hyaline Amoebocytes, Macrophage-like Cells and Signet-ring Cells

Three types of hemocytes appear to engulf test particles: hyaline amoebocytes, macrophage-like cells, and signet-ring cells (Fig. 7, a–h). Hyaline amoebocytes are variable in size and have a homogeneous cytoplasm filled with small granules hardly detectable under the light microscope. They frequently bear long and thin pseudopodia which confer on them a fusiform shape; macrophage-like cells are granular hemocytes, 10–15 μm in diameter, containing giant vacuoles often filled with cellular debris; signet-ring cells, about 10 μm in size, have a unique vacuole displacing the cytoplasm and the nucleus at the periphery (44).

The number of particles inside phagocytizing hemocytes increases with longer

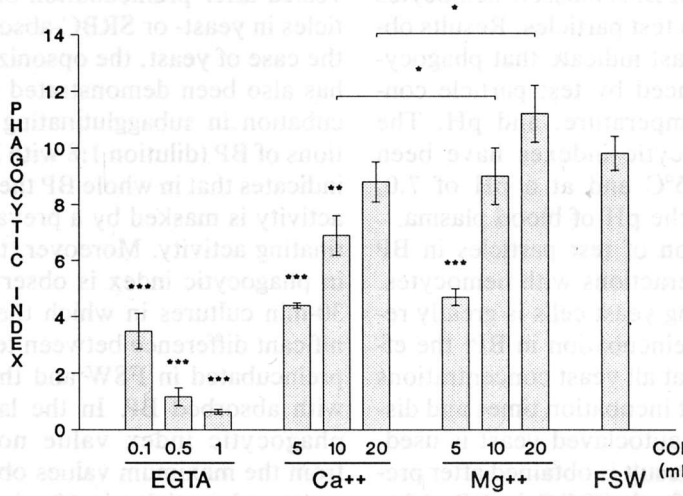

Figure 6. Effects of EGTA and divalent ions Ca^{++} and Mg^{++}, in ISO, on phagocytosis of yeast by hemocytes. Significant differences with respect to FSW (control) are shown by asterisks. Significant differences between the effects of Ca^{2+} and Mg^{2+} ions are also shown. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Incubation time: 60 min. $n = 3$.

Table 3. Effects of Various Experimental Conditions on Superoxide Anion Production by *Botryllus* Hemocytes, as Revealed by NBT Reduction.

Treatment	Percentage of Formazan-Containing Cells	
No yeast (control)	2.97 ± 0.31	***
Yeast	6.74 ± 0.19	
Yeast, 25 μg SOD (6000 U/mg)	3.42 ± 0.69	**

** $p < 0.01$; *** $p < 0.001$.

incubation times: up to 8 yeast cells were counted inside a single macrophage after 60 min of incubation.

When yeast is supplied for the first 5 min of incubation and monolayers are then washed and incubated again in FSW for different times before being fixed and observed under the light microscope, yeast cells are initially found almost exclusively inside hyaline amoebocytes; later, the number of positive macrophage-like and signet-ring cells gradually increases, while the number of particle-containing amoebocytes decreases as incubation reaches 60 min.

Discussion

We have analyzed the in vitro phagocytic activity of *B. schlosseri* hemocytes toward various test particles. Results obtained with yeast indicate that phagocytosis is influenced by test particle concentration, temperature, and pH. The highest phagocytic indexes have been obtained at 25°C and at a pH of 7.0, which is near the pH of blood plasma.

Preincubation of test particles in BP may affect interactions with hemocytes. Uptake of living yeast cells is greatly reduced after preincubation in BP: the effect is evident at all yeast concentrations and at different incubation times and disappears when autoclaved yeast is used. An analogous result is obtained after preincubation of fresh SRBC in BP. After BP treatment, both living yeast cells and SRBC form aggregates that are not formed by untreated cells. This leads to the suggestion that agglutinins might exist in *B. schlosseri* BP, able to coat yeast cells and sheep erythrocytes, thus limiting the availability of these particles for the phagocytic process. The absence of effects of BP on phagocytosis of autoclaved yeast cells may be explained by modified surface properties of the target particles so that they are not recognized by the agglutinins. The agglutinating activity of *Botryllus* BP towards yeasts, SRBC, and rabbit erythrocytes was actually observed in preliminary agglutination assays (data not shown). An agglutinating activity affecting the phagocytosis of yeast and bacteria has recently been described in the hemolymph of *Galleria mellonella* larvae (46).

A humoral opsonin enhancing phagocytosis of yeast and SRBC has been revealed after preincubation of these particles in yeast- or SRBC-absorbed BP; in the case of yeast, the opsonizing activity has also been demonstrated after preincubation in subagglutinating concentrations of BP (dilution 1:8 with FSW). This indicates that in whole BP the opsonizing activity is masked by a prevailing agglutinating activity. Moreover, the increase in phagocytic index is observed only in 30-min cultures in which there is a significant difference between test particles preincubated in FSW and those treated with absorbed BP. In the latter case a phagocytic index value not different from the maximum values obtained with untreated particles in 60-min cultures is reached. The above difference is not observed in 60-min cultures, suggesting a role of the opsonin in increasing the

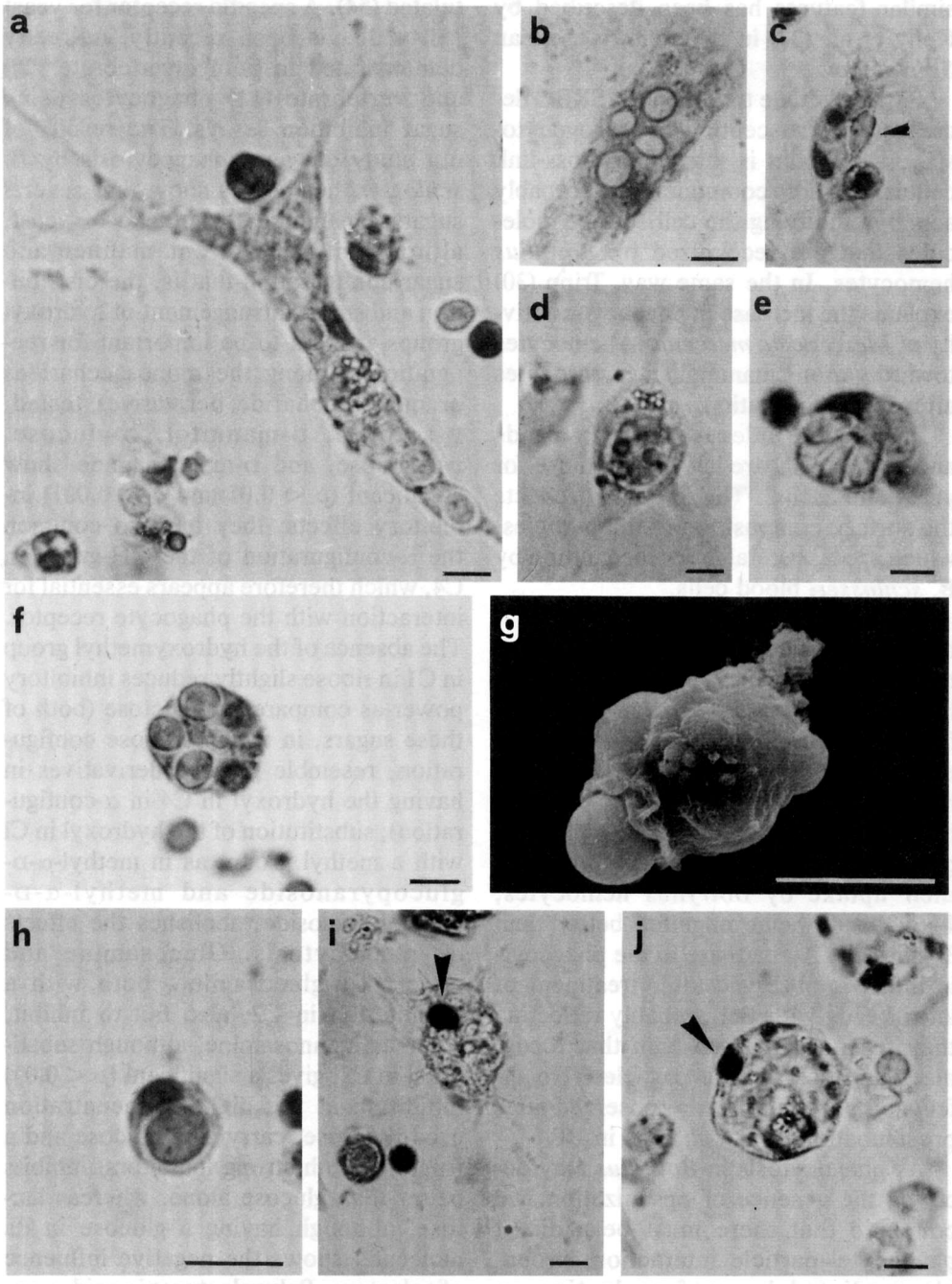

Figure 7. Hemocytes involved in phagocytosis. a–d: hyaline amoebocytes containing yeast cells (a,b), SRBC (c, arrowhead), and latex beads (d); e–f: macrophage-like cells filled with SRBC (e) and yeast cells (f); g: scanning electron micrograph of a hemocyte engaged in ingesting a yeast cell: lumps on the blood cell surface are already ingested yeast cells; h: signet-ring cell with a yeast particle inside its vacuole; i, j: yeast-containing hemocytes, showing dark formazan deposits after NBT treatment (arrowheads). Bar length: 5 μm.

phagocytic rate. A humoral opsonin with similar features has been described by Kelly et al. (27) in the solitary ascidian *Styela clava*.

Formaldehyde treatment of SRBC decreases their susceptibility to phagocytosis. As formalin is known to cross-link cellular peptidic components, it probably acts by modifying the cell surface molecules that are recognized by *Botryllus* hemocytes. In the same way, Tripp (30) explains the increase in phagocytic activity of *Mercenaria mercenaria* hemocytes toward various mammalian erythrocytes after formalin fixation.

Phagocytic indexes for latex beads and *E. coli* cells are lower than those for yeast and SRBC. This probably reflects the surface composition of the particles, which are less suitable for recognition by *B. schlosseri* blood cells.

Wiesner (53) recently reported that the humoral defense response evoked after injection of inert particles into the hemocoel of *G. mellonella* larvae was influenced by their physicochemical properties. In agreement with these data, our results on the phagocytosis of latex beads show that a change in a physical parameter such as size can influence their uptake by *Botryllus* hemocytes, 1-μm beads being engulfed better than 3-μm ones. An increase in the phagocytic index is observed after treatment of latex beads with BP, probably reflecting the presence of an opsonin that recognizes and coats these particles. No effects on phagocytosis are observed after preincubation of *E. coli* cells in BP.

As phagocytosis in *Botryllus* may occur in the absence of opsonization, we conclude that there must be a direct phagocyte–particle interaction, probably mediated by surface lectins on phagocyte plasma membrane. Hemocyte-associated lectins, related to soluble agglutinins, have been demonstrated in different invertebrate phyla (54) including tunicates (55). For most of them a role in the recognition of non-self particles during phagocytosis has been postulated (54). A specific receptor for yeast cell wall has been recently indirectly demonstrated in both invertebrate (20) and vertebrate (11) phagocytes using sugar inhibition assays. The results of our study on yeast phagocytosis by *B. schlosseri* hemocytes show that several sugars can inhibit the uptake of yeast, although in a different manner and sugar configuration-that is, the distribution and spatial arrangement of hydroxyl groups-appears to be important for recognition. Among the monosaccharides or monosaccharide derivatives tested, D-sorbitol, D-mannitol, D-glucose, D-mannose, and D-mannosamine show significant ($p < 0.01$ and $p < 0.001$) inhibitory effects: they have in common the α-configuration of the OH-group in C4, which therefore appears essential for interaction with the phagocyte receptor. The absence of the hydroxymethyl group in C1 in ribose slightly reduces inhibitory power as compared to fructose (both of these sugars, in their pyranose configuration, resemble glucose derivatives in having the hydroxyl in C4 in α-configuration); substitution of the hydroxyl in Cl with a methyl group, as in methyl-α-D-glucopyranoside and methyl-α-D-mannopyranoside, abolishes the effects on phagocytosis. Glucosamine and *N*-acetyl-D-glucosamine, both with a substitution in C2, also fail to inhibit, whereas mannosamine, although substituted in C2, gives a significant ($p < 0.01$) inhibition at the higher concentration used. Sucrose, carrying a glucose and a fructose, both strong inhibitors, inhibits better than glucose alone, whereas lactose, although having a glucose in its molecule, shows the negative influence of galactose. Polygalacturonic acid, a galactose derivative, appears to reduce phagocytosis significantly ($p < 0.05$ at 0.5%), probably because of substitution in C6; mannan is also a good inhibitor ($p < 0.01$ at 0.5%), probably being the polysaccharide actually recognized on

the yeast cell wall as it has mannans and β-1,3 glucans as their main components (56). The absence of effects on phagocytosis by laminarin indicates that, unlike what happens in *Biomphalaria glabrata* (20), a β-1,3 glucan receptor is not involved in yeast phagocytosis by *Botryllus* blood cells.

When incubation takes place in isotonic Tris or Bis-Tris buffer, calcium or magnesium ions are required to restore phagocytic activity to levels comparable to those obtained with FSW; furthermore, phagocytosis is strongly inhibited by low quantities of EGTA in the incubation medium. This is consistent with the hypothesis of cell surface lectins involved in particle recognition whose carbohydrate binding activity require divalent ions such as Ca^{2+} and Mg^{2+}.

The phagocytic activity of *Botryllus* hemocytes is associated with respiratory bursts, as suggested by increased superoxide anion production during phagocytosis, revealed by NBT reduction to formazan in cells with phagosomes. As respiratory burst in both vertebrates and invertebrates occurs at the very beginning of the phagocytic process upon perturbation of the phagocyte plasma membrane by foreign particles (12,32), only cells containing just-phagocytized particles are expected to respond positively to the NBT test; cells containing formerly ingested particles, in which the metabolic pathway leading to superoxide anion production is dormant, should not contain any formazan spot. This assumption is supported by our data showing a difference between the percentage of cells containing yeast cells and NBT precipitates and the percentage of cells able to phagocytize yeast, the former being far lower than the latter. The specificity of the reaction is demonstrated by the significant reduction of formazan spots upon addition of SOD to the culture medium.

Among the great variety of blood cell types present in ascidians, hyaline amoebocytes, macrophage-like cells, and signet-ring cells are the cell types involved in phagocytosis in *B. schlosseri*. This agrees with the conclusion of a previous study which, on the basis of a histoenzymatic analysis, assigned these hemocyte types to the same differentiation pathway (44) and with the results of Smith (42) and Smith and Peddie (43) on two species of solitary ascidians. As their relationship is concerned, in our opinion macrophage-like cells and signet-ring cells represent cell types which are engaged in processing engulfed materials and which have their precursors in hyaline amoebocytes, i.e., actively phagocytizing cells which, after particle ingestion, withdraw their cytoplasmic projections and change their shape from flat and fusiform to spherical. This view is indirectly supported by in vivo studies showing that carmine particles injected into a marginal colonial blood vessel are found almost exclusively inside hyaline amoebocytes when blood is collected 10 min after injection, whereas 30 min later most of them are found inside macrophage-like cells and signet-ring cells (unpublished data). In addition, the first cell types to ingest yeast cells after a 5 min pulse of monolayers with yeast are hyaline amoebocytes. Their number then progressively falls with longer incubation times in FSW without yeast, while the number of particle-containing macrophage-like cells and signet-ring cells increases.

Experiments are now under way to characterize the agglutinins and the opsonins revealed in the course of the present work and to better clarify the mutual relationships among the three hemocyte types involved in phagocytosis.

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