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In Vitro Release of Lectins From *Phallusia mamillata* Hemocytes After Their Fractionation on a Density Gradient

MATTEO CAMMARATA, NICOLÒ PARRINELLO, AND VINCENZO ARIZZA
Institute of Zoology, University of Palermo, 90123 Palermo, Italy

ABSTRACT Hemocytes were fractionated by centrifugation on a discontinuous Percoll density gradient from the hemolymph of *Phallusia mamillata*. Results obtained from microcultures of the fractionated hemocytes, sugar-inhibition experiments, SDS-PAGE, and immunoblotting indicate that "compartment cells" release cellular-type (CL) lectins that are specific for α-lactose and lactulose. The released lectins have the same properties as the CL lectins that were previously isolated from sonicated unfractionated hemocytes, but they differ in terms of some molecular and immunological properties from the lectins (SL) purified from the serum. SLs were never found in the supernatants from microcultures of the fractionated hemocytes. © 1993 Wiley-Liss, Inc.

Sugar-binding proteins (lectins), humoral and cell-bound, have been found in the hemolymph of many species of invertebrates (for reviews, see Olafsen, '86; Vasta, '91; Parrinello, '91). Hemocytes can contain and can also, presumably, secrete lectins (Amirante and Mazzalai, '78; Amirante and Basso, '1984; Stein and Cooper, '88; Leippe and Renwrantz, '88).

Tunicates have lectins in their hemolymph (Parrinello and Patricolo, '75; Parrinello, '88, '91) and, as demonstrated by immunofluorescence and rosetting studies, lectins can also be found on the surface of the hemocytes of *Ascidia malaca* (Parrinello and Arizza, '88) and *Phallusia mamillata* (Parrinello and Arizza, '89).

The hemolymph from *Phallusia mamillata* contains distinct humoral and cellular α-lactose/lactulose-specific lectins (Parrinello and Canicattì, '83; Parrinello and Arizza, '89) which are capable of agglutinating rabbit erythrocytes. Humoral lectins isolated from the serum (SL) consist of a large subunit (58 KDa) and a small one (15 KDa), which are probably linked by disulfide bonds. The tissue responsible for the release of SL has not been identified. Cellular lectins (CL) isolated from hemocyte lysates are also composed of two subunits (36 KDa and 35 KDa), but they show non-identity reaction when tested in immunodiffusion experiments with SL-specific antibodies (Parrinello and Arizza, '89). Recently, we reported that preparations of total hemocytes from *Phallusia* hemolymph release CL-type lectins during short-term culture in a suitable medium. The lectins are composed of two subunits which are similar to CL subunits in both size and immunological properties, as indicated by immunoblotting with CL-specific antibodies (Arizza et al., '91).

Fractionation of hemocytes by density-gradient centrifugation has frequently been employed (Kustin et al., '76; Schlumpberger et al., '84; Junko and Michibata, '91; Michibata et al., '91) in attempts to understand the functions of the various types of ascidian hemocytes. The hemolymph of *Phallusia mamillata* contains several morphologically distinguishable cell types (Endean, '60; Scippa and Iazzetti, '89). In order to identify the hemocytes responsible for the release of lectins under microculture conditions, we fractionated the hemocyte population of *Phallusia* on a Percoll density gradient. Our results indicate that multivacuolated cells, referred to "compartment cells" (Endean, '60), can release CL-type lectins.

MATERIALS AND METHODS

Animals, collection of hemolymph, and preparation of hemocyte suspension

Specimens of *Phallusia mamillata* were collected from the southern part of the Mediterranean sea (Mazara del Vallo). The hemolymph was withdrawn aseptically from the heart with a sterile syringe, with a sterile calcium-and magnesium-free artifi-

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cial seawater (FSW) that contained 10 mM EDTA (FSW-EDTA; osmolarity adjusted up to 1.18 with NaCl) as anticoagulant (ratio of medium to hemolymph, 1:9, v/v). After centrifugation at 400*g* for 10 min, the supernatant was removed and hemocytes were washed three times in sterile FSW-EDTA. Cells were counted and adjusted to 1.0–8.0 $\times 10^5$/ml in the culture medium, unless otherwise indicated.

Density-gradient centrifugation

Percoll solution (Pharmacia Fine Chemicals, Uppsala, Sweden) was mixed with appropriate volumes of FSW-EDTA to a final concentration of 90% (density, 1.122). This stock solution was mixed with appropriate volumes of FSW-EDTA to make solutions of decreasing densities (1.113, 1.105, 1.098, 1.090, 1.079, 1.071, and 1.060 gm/ml). Densities were measured with a refractometer (RD10 Boehringer Mannheim, Indianapolis, IN). Discontinuous density gradients were prepared by pipetting 1 ml aliquots of Percoll solution with a density of 1.122 gm/ml into 10 ml centrifuge tubes, with sequential overlayering with 1 ml aliquots of each solution of Percoll with decreasing density. Then, 2 ml of suspension of washed hemocytes ($7.7 \pm 1.6 \times 10^6$ per ml) were layered onto each gradient and the tubes were centrifuged in a swing-out rotor at 800g for 30 min at 7°C. Bands of cells were collected by gentle aspiration from the top of each tube and washed three times in sterile FSW-EDTA. Cell viability was estimated by the eosin-y (0.5% in FSW) exclusion test.

Hemocyte cultures, titration of lectins released in vitro, and sugar-inhibition tests

Details of the methodology are described elsewhere (Arizza et al., 1991). The formulation of the medium was based on ionic and osmotic analysis of *Phallusia* hemolymph. The medium was prepared aseptically and sterilized by filtration (0.2 μm filter; Gelman Acrodisc, Ann Arbor, MI) immediately prior to use. In brief, cultures were prepared as follows. Artificial seawater (SW) containing 30% (v/v) of a nutritive medium (M199) with L-glutamine (ICN, Costa Mesa, CA) was used as the culture medium (osmolarity adjusted up to 1.18 with NaCl); suspension of the hemocytes from each band on the density gradient was adjusted to 7.2×10^4 cells per ml and 1 ml aliquots were put into the wells of a sterile 24-well, flat-bottomed cultured plate (Falcon Plastic, Oxnard, CA) and maintained at 15°C. At specified intervals, cultures were removed from the wells, centrifuged, and the cell-free supernatants assayed for hemagglutination activity. The pellets of hemocytes were resuspended in the medium and cell viability was estimated. The microhemagglutination assay was performed in duplicate using rabbit erythrocytes (RE; Sclavo, Siena, Italy) as described by Parrinello and Canicattì ('82), and titers are expressed as the reciprocal of the last dilution of sample that gave clear agglutination. Unless otherwise indicated, the hemagglutination titer is expressed as the mean of results from three separated cultures derived from the same suspension of hemocytes. Experiments to examine inhibition of hemagglutinating activity were carried out using decreasing concentrations (from 200 mM) of monosaccharides and oligosaccharides (Sigma Chem. Co., St. Louis, MO) as inhibitors (Parrinello and Canicattì, '83). The last dilution of sugar that led to inhibition of agglutination was determined in each case.

Purification of lectins and preparation of antibodies

The methods have been described in detail elsewhere. Lectin specific for α-lactose were purified by affinity chromatography on HCl-treated Sepharose. SL-type lectins were isolated from cell-free hemolymph (Parrinello and Canicattì, '83); CL-type lectins were purified from sonicated hemocytes (Parrinello and Arizza, '89). CL-specific antibodies were raised by injection of purified CL (300 μg/ml) as antigen into a rabbit; the immunization schedule was the same as that described by Parrinello and Arizza ('89). Rabbit immunoglobulins were fractionated on a column of DEAE-Sephacel (Pharmacia, Uppsala, Sweden) as described by the manufacturer (Pharmacia Fine Chemicals, '80). To monitor the specificity of antibodies, immunoglobulin samples absorbed with heat-inactivated CL were examined in an immunodiffusion test, as described by Parrinello and Arizza ('89). Assays of absorbed samples never resulted in an immunoprecipitation line.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Quantitative analysis of protein was performed by the method of Bradford ('76) with bovine serum albumin (BSA) as the standard. The values given are the averages of three assays ± SE. The samples were concentrated in a Diaflo system equipped with a UM2 membrane (Amicon Corp., Livingston, MA). SDS-PAGE on slab gels was performed as described by Laemmli ('70) and proteins were stained with Coomassie Blue. Gels were calibrated with standard proteins (BIO-RAD, Richmond, CA) and stan-

dard curves were constructed as previously reported (Parrinello and Arizza, '89). The averages from three independent experiments were evaluated.

Immunoblotting (Western blot) analysis of released lectins was performed as described previously (Parrinello and Arizza, '88). The nitrocellulose sheets, with the electrophoretically transferred lectins, were incubated with CL-specific antibodies. Immunostaining was achieved using peroxide-conjugated goat Igs against rabbit antibodies (BIO-RAD), with 4-chloronaphthol as substrate. Samples of purified CL and CL-specific immunoglobulins were aliquots of the same preparations, stored at −80°C, that had previously been utilized in the analysis of the release of lectins by unfractionated hemocytes, as reported by Arizza et al. ('91).

Light microscopy

Living cells, suspended in FSW-EDTA, were observed through an inverted phase-contrast microscope (Fluovert Leitz, Wetzlar, F.R.G.). Total numbers of cells and relative frequencies of eosin-stained cells were determined by use of a hemocytometer. Morphological analysis of the fractionated cells was performed using fixed cells. Drops of the suspension of cells were smeared on glass microscope slides that had been coated with poly-L-lysine (0.1%). Adherent cells were fixed (formaldehyde 4%, NaCl 0.5 M, sodium cacodylate 0.1 M, pH 7.4) and stained with hemalum-eosin (Beccari and Mazzi, '66). Differential hemocyte counts were obtained by examination of at least 200 cells in several visual fields and average values (± SD) were calculated. *Phallusia* hemocytes were classified into cell types using Endean's ('60) nomenclature, as modified by Wright ('81). However, the terms *microgranulocyte* and *hyaline cell* were used instead of *granular* and *hyaline leucocyte,* respectively, while the term *macrogranulocyte* refers to multivacuolated cells obtained by density-gradient centrifugation. This terminology takes into account the recent modification of nomenclature suggested by De Leo ('92).

Statistical analysis

Functional relationships between two variables were expressed in terms of linear regression equation (Sokal and Rohlf, '81). Statistical significance was determined by reference to the t-distribution, using the standard error of the correlation coefficient (Sokal and Rohlf, '81).

RESULTS

Fractionation on a Percoll discontinuous density gradient of Phallusia *hemocytes*

A typical density gradient, after centrifugation, containing separated hemocytes is shown in Figure 1. The total population of cells has been partitioned into eight discrete bands that are clearly distinguishable from one another. The mean number of cells in each band was calculated from five experiments, after fractionation of $15.4 \pm 1.6 \times 10^6$ washed hemocytes; band B4 contains the highest number of cells ($4.9 \pm 2.9 \times 10^6$ per ml) (Fig. 1).

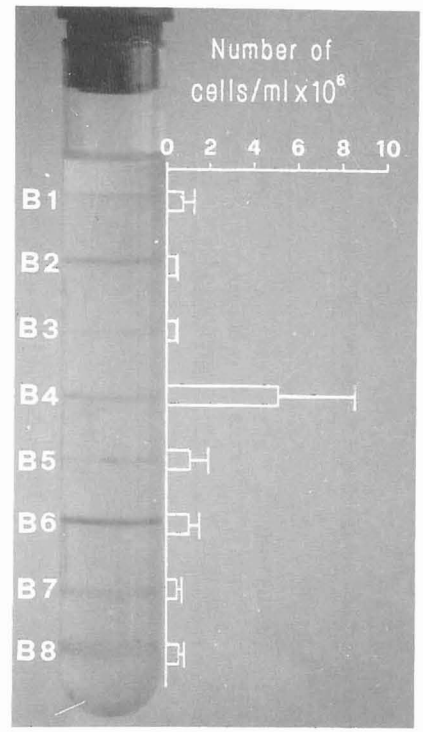

Fig. 1. **Left:** Discontinuous Percoll gradient in which washed hemocytes from *Phallusia mamillata* had been separated. Densities of layers decreased from the lowermost 90% Percoll solution (density = 1.122) upwards. Positions of separated bands of cells after centrifugation are shown on the left. The band number points to the interface between the decreasing densities of layers: B8 (1.122/1.113), B7 (1.113/1.105), B6 (1.105/1.098), B5 (1.098/1.090), B4 (1.090/1.079), B3 (1.079/1.071), B2 (1.071/1.060), B1 (1.060/1.042). **Right:** Hemocytes ($15.4 \pm 1.6 \times 10^6$) from 5 specimens in 2 ml were used in separate experiments, and cell numbers in each band are presented as average values ± SD.

The bands of cells include the following types of cells (Table 1): 1) lymphocyte-like cells (LLC), namely, small, spherical cells (3–5 μm in diameter) with a large nucleus (Fig. 2a); 2) granular leukocytes (6–12 μm in diameter), characterized by the presence of minute, distinct cytoplasmic granules (Fig. 2b); 3) hyaline leukocytes (6–12 μm in diameter), with homogeneous cytoplasm and a round nucleus (Fig. 2c); 4) signet ring cells (8–10 μm in diameter), with a single large vacuole enclosed by a peripheral rim of cytoplasm, and a nucleus in a peripheral position that forms an eccentric crescent-

TABLE 1. Differential distribution of hemocytes from Phallusia mamillata *in bands obtained from the discontinuous Percoll density gradient*

Hemocyte band	Hemocyte type (% ± SD; n = 5)									
	LLC[1]	Pigment cells	Micro-granulocytes	Compartment cells		Hyaline cells	Cells with acidic vacuole	Morula cells	Macro-granulocytes	Signet ring cells
				Form *A*	Form *B*					
B1	< 1	26.0 ± 17	45.4 ± 16	—	2.8 ± 2	25.8 ± 22	—	—	—	—
B2	—	26.1 ± 9	48.8 ± 20	3.0 ± 1	6.9 ± 3	6.0 ± 3	—	—	—	—
B3	—	8.9 ± 10	—	14.8 ± 1	3.0 ± 2	11.1 ± 8	62.2 ± 2	—	—	—
B4	—	0.4 ± 0.3	1.1 ± 1	67.0 ± 2	31.2 ± 2	0.3 ± 0	—	—	—	—
B4	—	—	—	9.3 ± 9	21.7 ± 1	3.9 ± 4	—	30.6 ± 12	34.5 ± 7	—
B6	—	—	—	1.0 ± 1	7.0 ± 6	—	—	10.4 ± 2	81.6 ± 2	—
B7	—	—	—	—	—	—	—	—	2.7 ± 2	97.3 ± 1
B8	—	—	—	—	—	—	—	—	1.2 ± 1	98.8 ± 1
Unfractionated	< 1	2.0 ± 0.4	5.1 ± 1	37.7 ± 1	19.5 ± 94	4.9 ± 2	3.0 ± 2	19.3 ± 3	4.2 ± 1	4.2 ± 0

[1]LLC = Lymphocyte-like cell.

TABLE 2. Hemagglutination titers of supernatants from cultures of fractionate hemocytes from Phallusia mamillata (7.2 × 10^4/ml)

Specimen	Bands of hemocytes																	
	B1		B2		B3		B4		B5		B6		B7		B8		Not fractionated	
	Hemagglutination titers (reciprocal value), after different incubation times (h), assayed with rabbit erythrocytes																	
	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)
1	< 2	< 2	< 2	< 2	2	2	16	32	2	4	< 2	< 2	< 2	< 2	< 2	< 2	2	8
2	< 2	< 2	< 2	< 2	2	2	32	32	2	4	< 2	< 2	< 2	< 2	< 2	< 2	4	16
3	< 2	< 2	< 2	< 2	2	2	32	64	2	1	< 2	< 2	< 2	< 2	< 2	< 2	2	8
4	< 2	< 2	< 2	< 2	4	2	128	64	8	8	< 2	< 2	< 2	< 2	< 2	< 2	16	64
5	< 2	< 2	< 2	< 2	2	8	16	128	1	1	< 2	< 2	< 2	< 2	< 2	< 2	1	8

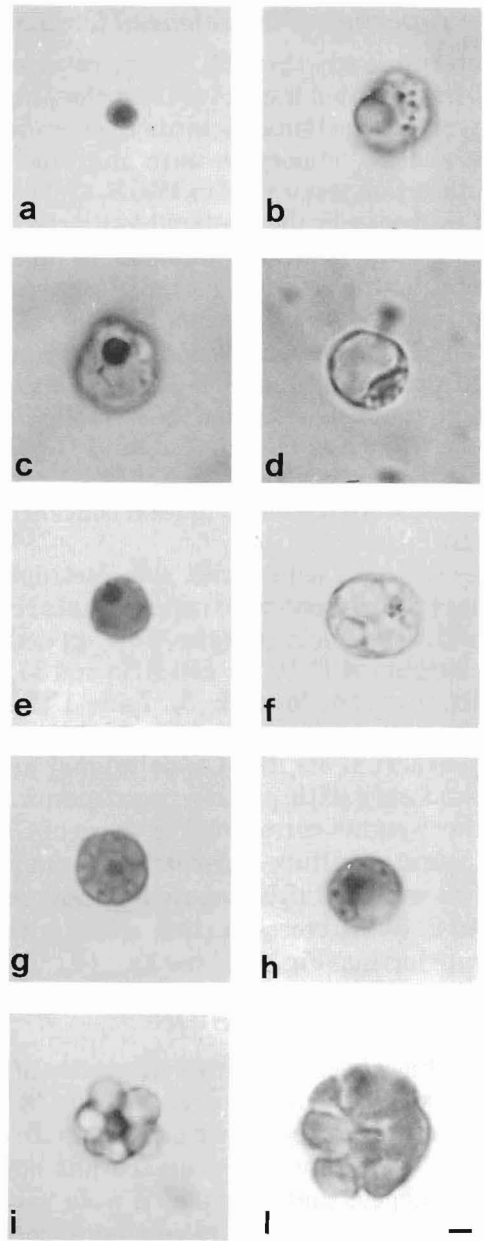

Fig. 2. *Phallusia mamillata* hemocytes from Percoll density gradients, stained by hemalum-eosin. **a:** Lymphocyte-like cell. **b:** Microgranulocyte. **c:** Hyaline cell. **d:** Signet ring cell. **e:** Cell with acidic vacuoles. **f:** Macrogranulocyte. **g:** Compartment cell form *A*. **h:** Compartment cell form *B*. **i:** Morula cell. **l:** Pigment cell. Bar (a–l) = 3 μm.

shaped cap (Fig. 2d); 5) cells with an acidic vacuole (8–9 μm in diameter) having a large vacuole that contains basophilic material, and a round nucleus close to the plasma membrane (Fig. 2e); 6) multivacuolated cells with a variable number of roughly spherical vacuoles that contain optically clear material (Fig. 2f); 7) compartment cells, spherical in shape (8–12 μm), with either two distinct morphological features, namely, angular vacuoles in variable numbers distributed regularly at the periphery of the cytoplasm around a central vacuole (form *A*, Fig. 2g) or a variable number of round or angular vacuoles, as well as a large vacuole on one side of the cell (form *B*, Fig. 2h); the peripheral vacuoles usually contain granules; 8) Morula cells with variable numbers of symmetrically arranged large vacuoles or globules and a berry-like or morula-like appearance (Fig. 2i); and 9) pigment cells, which are yellow or orange, with variously pigmented bodies encased in a thin cytoplasmic envelope (Fig. 2l).

Differential distribution of hemocytes among the fractionated bands of cells (B1–B8)

As indicated in Table 1, B3 consisted mostly of cells with an acidic vacuole; B4 consisted mainly of compartment cells of form *A* (67.0%) and form *B* (31.4%); B6 consisted primarily of macrogranulocytes that contained large granules (81.6%); B7 and B8 consisted of signet ring cells exclusively. The bands B1 and B2 were richer in macrogranulocytes that were characterized by a few small granules, but small numbers of pigment cells, hyaline cells, compartment cells, and cells with acidic vacuoles were also included. Finally, B5 contained compartment cells (21.7%) of form *B*, morula cells, macrogranulocytes, and a few hyaline cells (Table 1). In Table 1, the percentages of each type of hemocyte, as evaluated by an examination of washed hemocytes before density gradient separation, are also listed.

Hemagglutinating activity of the supernatants from cultures of fractionated hemocytes

Since 7.2×10^4 unfractionated washed hemocytes release an easily detectable amount of lectins into 1 ml of culture medium (Table 2), equivalent numbers of hemocytes from each Percoll-separated band were cultured in microculture wells. Five specimens of *Phallusia* were used and the experiments were carried out in duplicate. Lectins were released into the medium in which B3, B4, and B5 hemocytes had been cultured for 3 h (Table 2). Supernatants from cultures of B4 cells displayed the highest hemagglutination titer (1:128) which was even higher than that of the unfractionated hemocytes (1:16). After a 24-h incubation, hemagglutinating activity of supernatants increased in each of five cultured fractions but only cells from the B4 band gave a titer of 1:128 (Table 2). Throughout the incubations, the cells remained viable, as determined by the eosin-y test. Mortality ranged from 1.0–5.0% in the cultures of Percoll-fractionated hemocytes, whereas unfractionated, washed hemocytes showed the lowest mortality values (0–1.0%).

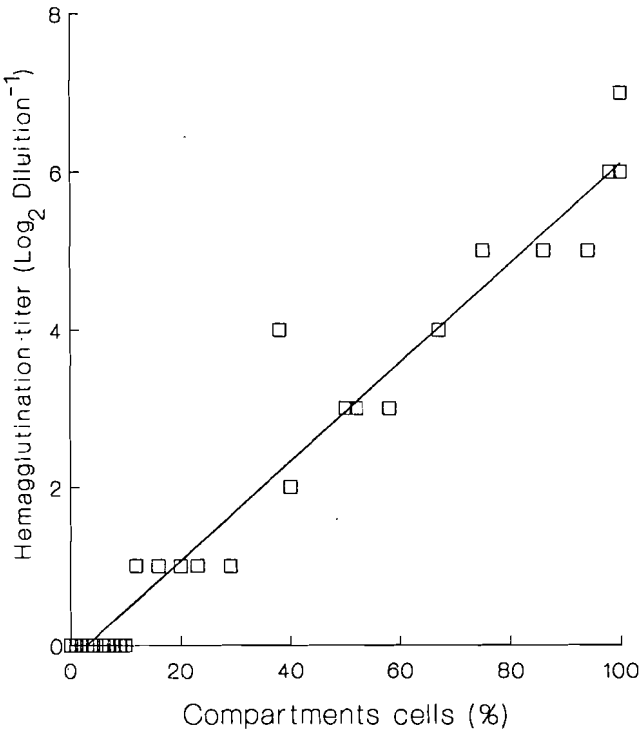

Fig. 3. The relationship, expressed as a linear regression equation, between hemagglutination titer of supernatants from cultures of *Phallusia mamillata* hemocytes (unfractionated and fractionated hemocytes, bands 1–8), and the percentage of compartment cells present in the cultures ($Y = -0.02 + 0.06\times$; $P < 0.001$; $r = 0.98$).

The close relationship (statistically significant at $P < 0.001$; $r = 0.97$) between the percentage of compartment cells in cultures from each band of hemocytes (B3, B4, B5) and the hemagglutination titer of the supernatants is shown in Figure 3. In supernatants from cultures that contained hemocytes from B1, B2, B6, B7, and B8 bands no significant hemagglutinating activity was found.

Properties of the released lectins

To determine whether the lectins released from Percoll-fractionated hemocytes had the properties of CL-type lectins, supernatants from cultures of B3, B4, and B5 hemocytes were analyzed by the sugar-inhibition test and SDS-PAGE, and with CL-specific antibodies by the Western blotting technique.

α-Lactose and lactulose were able to inhibit the hemagglutinating activity (Table 3). Concentrations of sugar required for inhibition (50–200 mM) were proportional to the hemagglutination titers of supernatants from 3-h cultures (1:4–1:64). No significant inhibition was observed when the monosaccharides D-glucose, L-fucose, D-mannose, and D-galactose and the oligosaccharides D-melibiose, D-raffinose, and maltose were tested up to final concentrations of 200 mM.

Under reducing conditions, the electrophoretic patterns of the concentrated supernatants revealed bands of the two major proteins with apparent molecular weights of 37,500 ± 850 KDa and 35,800 ± 800 KDa (Fig. 4A, lanes 2–5). Lane 1 (Fig. 4A) shows the pattern of purified SL. In the immunoblotting experiments, the CL-polyclonal antibodies reacted only with protein components whose molecular weights correspond to those of CL-type lectins, found in culture supernatants from B3, B4, and B5 as well as unfractionated hemocytes (Fig. 4B, lanes 2–5). No cross-reaction occurs with purified serum lectins (Fig. 4B, lane 1).

DISCUSSION

Lectins have been identified in species of virtually all taxa from viruses and bacteria to vertebrates, and they appear to be present in all living organisms. Experimental evidence has been obtained to suggest that they play a wide variety of physiological roles which are related to their car-

TABLE 3. Inhibition by sugars of binding lectins released from fractionated hemocytes from Phallusia mamillata

Specimen	Hemocyte band	Hemagglutination titer[1] before sugar inhibition (reciprocal)	Inhibitory concentration of sugar (μmole/ml)	
			α-Lactose	Lactulose
1	B3	8	50	50
2		4	25	25
1	B4	64	200	100
2		8	50	50
1	B5	4	25	25
2		4	50	25
1	unfractionated	8	100	50
2		16	100	100

[1]Supernatants from cultures of hemocytes assayed with rabbit erythrocytes.

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Percoll solution (Pharmacia Fine Chemicals, Uppsala, Sweden) was mixed with appropriate volumes of FSW-EDTA to a final concentration of 90% (density, 1.122). This stock solution was mixed with appropriate volumes of FSW-EDTA to make solutions of decreasing densities (1.113, 1.105, 1.098, 1.090, 1.079, 1.071, and 1.060 gm/ml). Densities were measured with a refractometer (RD10 Boehringer Mannheim, Indianapolis, IN). Discontinuous density gradients were prepared by pipetting 1 ml aliquots of Percoll solution with a density of 1.122 gm/ml into 10 ml centrifuge tubes, with sequential overlayering with 1 ml aliquots of each solution of Percoll with decreasing density. Then, 2 ml of suspension of washed hemocytes ($7.7 \pm 1.6 \times 10^6$ per ml) were layered onto each gradient and the tubes were centrifuged in a swing-out rotor at 800g for 30 min at 7°C. Bands of cells were collected by gentle aspiration from the top of each tube and washed three times in sterile FSW-EDTA. Cell viability was estimated by the eosin-y (0.5% in FSW) exclusion test.

Hemocyte cultures, titration of lectins released in vitro, and sugar-inhibition tests

Details of the methodology are described elsewhere (Arizza et al., 1991). The formulation of the medium was based on ionic and osmotic analysis of *Phallusia* hemolymph. The medium was prepared aseptically and sterilized by filtration (0.2 μm filter; Gelman Acrodisc, Ann Arbor, MI) immediately prior to use. In brief, cultures were prepared as follows. Artificial seawater (SW) containing 30% (v/v) of a nutritive medium (M199) with L-glutamine (ICN, Costa Mesa, CA) was used as the culture medium (osmolarity adjusted up to 1.18 with NaCl); suspension of the hemocytes from each band on the density gradient was adjusted to 7.2×10^4 cells per ml and 1 ml aliquots were put into the wells of a sterile 24-well, flat-bottomed cultured plate (Falcon Plastic, Oxnard, CA) and maintained at 15°C. At specified intervals, cultures were removed from the wells, centrifuged, and the cell-free supernatants assayed for hemagglutination activity. The pellets of hemocytes were resuspended in the medium and cell viability was estimated. The microhemagglutination assay was performed in duplicate using rabbit erythrocytes (RE; Sclavo, Siena, Italy) as described by Parrinello and Canicattì ('82), and titers are expressed as the reciprocal of the last dilution of sample that gave clear agglutination. Unless otherwise indicated, the hemagglutination titer is expressed as the mean of results from three separated cultures derived from the same suspension of hemocytes. Experiments to examine inhibition of hemagglutinating activity were carried out using decreasing concentrations (from 200 mM) of monosaccharides and oligosaccharides (Sigma Chem. Co., St. Louis, MO) as inhibitors (Parrinello and Canicattì, '83). The last dilution of sugar that led to inhibition of agglutination was determined in each case.

Purification of lectins and preparation of antibodies

The methods have been described in detail elsewhere. Lectin specific for α-lactose were purified by affinity chromatography on HCl-treated Sepharose. SL-type lectins were isolated from cell-free hemolymph (Parrinello and Canicattì, '83); CL-type lectins were purified from sonicated hemocytes (Parrinello and Arizza, '89). CL-specific antibodies were raised by injection of purified CL (300 μg/ml) as antigen into a rabbit; the immunization schedule was the same as that described by Parrinello and Arizza ('89). Rabbit immunoglobulins were fractionated on a column of DEAE-Sephacel (Pharmacia, Uppsala, Sweden) as described by the manufacturer (Pharmacia Fine Chemicals, '80). To monitor the specificity of antibodies, immunoglobulin samples absorbed with heat-inactivated CL were examined in an immunodiffusion test, as described by Parrinello and Arizza ('89). Assays of absorbed samples never resulted in an immunoprecipitation line.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Quantitative analysis of protein was performed by the method of Bradford ('76) with bovine serum albumin (BSA) as the standard. The values given are the averages of three assays ± SE. The samples were concentrated in a Diaflo system equipped with a UM2 membrane (Amicon Corp., Livingston, MA). SDS-PAGE on slab gels was performed as described by Laemmli ('70) and proteins were stained with Coomassie Blue. Gels were calibrated with standard proteins (BIO-RAD, Richmond, CA) and stan-

dard curves were constructed as previously reported (Parrinello and Arizza, '89). The averages from three independent experiments were evaluated.

Immunoblotting (Western blot) analysis of released lectins was performed as described previously (Parrinello and Arizza, '88). The nitrocellulose sheets, with the electrophoretically transferred lectins, were incubated with CL-specific antibodies. Immunostaining was achieved using peroxide-conjugated goat Igs against rabbit antibodies (BIO-RAD), with 4-chloronaphthol as substrate. Samples of purified CL and CL-specific immunoglobulins were aliquots of the same preparations, stored at −80°C, that had previously been utilized in the analysis of the release of lectins by unfractionated hemocytes, as reported by Arizza et al. ('91).

Light microscopy

Living cells, suspended in FSW-EDTA, were observed through an inverted phase-contrast microscope (Fluovert Leitz, Wetzlar, F.R.G.). Total numbers of cells and relative frequencies of eosin-stained cells were determined by use of a hemocytometer. Morphological analysis of the fractionated cells was performed using fixed cells. Drops of the suspension of cells were smeared on glass microscope slides that had been coated with poly-L-lysine (0.1%). Adherent cells were fixed (formaldehyde 4%, NaCl 0.5 M, sodium cacodylate 0.1 M, pH 7.4) and stained with hemalum-eosin (Beccari and Mazzi, '66). Differential hemocyte counts were obtained by examination of at least 200 cells in several visual fields and average values (± SD) were calculated. *Phallusia* hemocytes were classified into cell types using Endean's ('60) nomenclature, as modified by Wright ('81). However, the terms *microgranulocyte* and *hyaline cell* were used instead of *granular* and *hyaline leucocyte,* respectively, while the term *macrogranulocyte* refers to multivacuolated cells obtained by density-gradient centrifugation. This terminology takes into account the recent modification of nomenclature suggested by De Leo ('92).

Statistical analysis

Functional relationships between two variables were expressed in terms of linear regression equation (Sokal and Rohlf, '81). Statistical significance was determined by reference to the t-distribution, using the standard error of the correlation coefficient (Sokal and Rohlf, '81).

RESULTS

Fractionation on a Percoll discontinuous density gradient of Phallusia *hemocytes*

A typical density gradient, after centrifugation, containing separated hemocytes is shown in Figure 1. The total population of cells has been partitioned into eight discrete bands that are clearly distinguishable from one another. The mean number of cells in each band was calculated from five experiments, after fractionation of $15.4 \pm 1.6 \times 10^6$ washed hemocytes; band B4 contains the highest number of cells ($4.9 \pm 2.9 \times 10^6$ per ml) (Fig. 1).

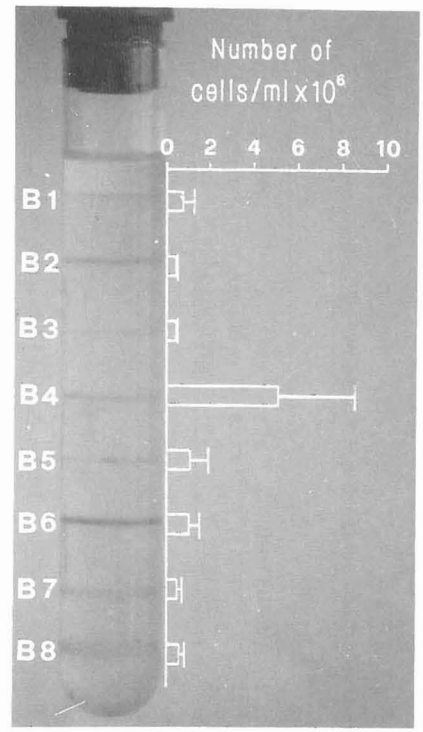

Fig. 1. **Left:** Discontinuous Percoll gradient in which washed hemocytes from *Phallusia mamillata* had been separated. Densities of layers decreased from the lowermost 90% Percoll solution (density = 1.122) upwards. Positions of separated bands of cells after centrifugation are shown on the left. The band number points to the interface between the decreasing densities of layers: B8 (1.122/1.113), B7 (1.113/1.105), B6 (1.105/1.098), B5 (1.098/1.090), B4 (1.090/1.079), B3 (1.079/1.071), B2 (1.071/1.060), B1 (1.060/1.042). **Right:** Hemocytes ($15.4 \pm 1.6 \times 10^6$) from 5 specimens in 2 ml were used in separate experiments, and cell numbers in each band are presented as average values ± SD.

The bands of cells include the following types of cells (Table 1): 1) lymphocyte-like cells (LLC), namely, small, spherical cells (3–5 μm in diameter) with a large nucleus (Fig. 2a); 2) granular leukocytes (6–12 μm in diameter), characterized by the presence of minute, distinct cytoplasmic granules (Fig. 2b); 3) hyaline leukocytes (6–12 μm in diameter), with homogeneous cytoplasm and a round nucleus (Fig. 2c); 4) signet ring cells (8–10 μm in diameter), with a single large vacuole enclosed by a peripheral rim of cytoplasm, and a nucleus in a peripheral position that forms an eccentric crescent-

TABLE 1. Differential distribution of hemocytes from Phallusia mamillata *in bands obtained from the discontinuous Percoll density gradient*

Hemocyte band	Hemocyte type (% ± SD; n = 5)									
	LLC[1]	Pigment cells	Micro-granulocytes	Compartment cells		Hyaline cells	Cells with acidic vacuole	Morula cells	Macro-granulocytes	Signet ring cells
				Form *A*	Form *B*					
B1	< 1	26.0 ± 17	45.4 ± 16	—	2.8 ± 2	25.8 ± 22	—	—	—	—
B2	—	26.1 ± 9	48.8 ± 20	3.0 ± 1	6.9 ± 3	6.0 ± 3	—	—	—	—
B3	—	8.9 ± 10	—	14.8 ± 1	3.0 ± 2	11.1 ± 8	62.2 ± 2	—	—	—
B4	—	0.4 ± 0.3	1.1 ± 1	67.0 ± 2	31.2 ± 2	0.3 ± 0	—	—	—	—
B4	—	—	—	9.3 ± 9	21.7 ± 1	3.9 ± 4	—	30.6 ± 12	34.5 ± 7	—
B6	—	—	—	1.0 ± 1	7.0 ± 6	—	—	10.4 ± 2	81.6 ± 2	—
B7	—	—	—	—	—	—	—	—	2.7 ± 2	97.3 ± 1
B8	—	—	—	—	—	—	—	—	1.2 ± 1	98.8 ± 1
Unfractionated	< 1	2.0 ± 0.4	5.1 ± 1	37.7 ± 1	19.5 ± 94	4.9 ± 2	3.0 ± 2	19.3 ± 3	4.2 ± 1	4.2 ± 0

[1]LLC = Lymphocyte-like cell.

TABLE 2. Hemagglutination titers of supernatants from cultures of fractionate hemocytes from Phallusia mamillata (7.2 × 10^4/ml)

Specimen	Bands of hemocytes																	
	B1		B2		B3		B4		B5		B6		B7		B8		Not fractionated	
	Hemagglutination titers (reciprocal value), after different incubation times (h), assayed with rabbit erythrocytes																	
	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)	(3)	(24)
1	< 2	< 2	< 2	< 2	2	2	16	32	2	4	< 2	< 2	< 2	< 2	< 2	< 2	2	8
2	< 2	< 2	< 2	< 2	2	2	32	32	2	4	< 2	< 2	< 2	< 2	< 2	< 2	4	16
3	< 2	< 2	< 2	< 2	2	2	32	64	2	1	< 2	< 2	< 2	< 2	< 2	< 2	2	8
4	< 2	< 2	< 2	< 2	4	2	128	64	8	8	< 2	< 2	< 2	< 2	< 2	< 2	16	64
5	< 2	< 2	< 2	< 2	2	8	16	128	1	1	< 2	< 2	< 2	< 2	< 2	< 2	1	8

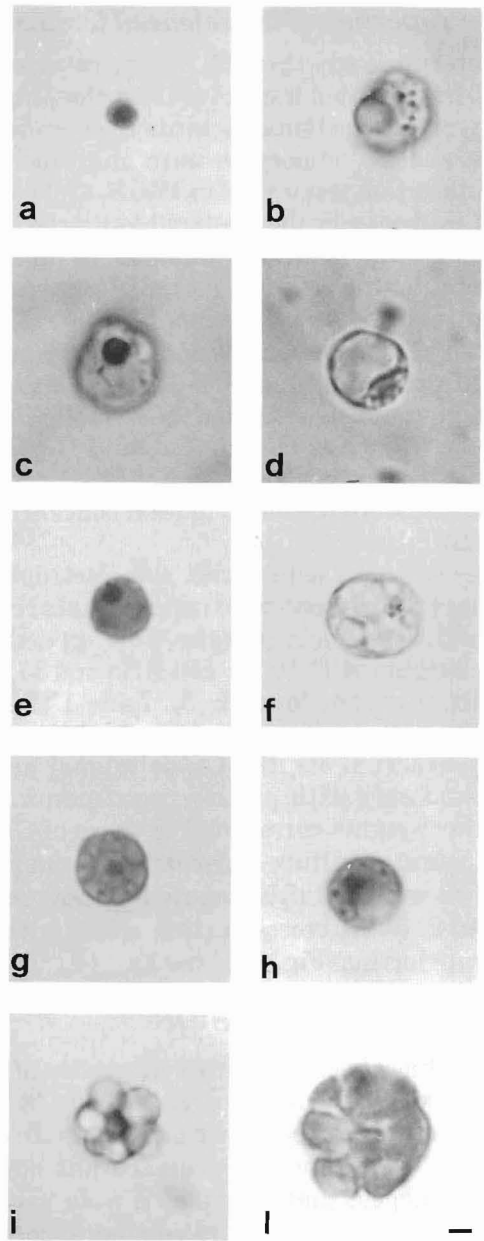

Fig. 2. *Phallusia mamillata* hemocytes from Percoll density gradients, stained by hemalum-eosin. **a:** Lymphocyte-like cell. **b:** Microgranulocyte. **c:** Hyaline cell. **d:** Signet ring cell. **e:** Cell with acidic vacuoles. **f:** Macrogranulocyte. **g:** Compartment cell form *A*. **h:** Compartment cell form *B*. **i:** Morula cell. **l:** Pigment cell. Bar (a–l) = 3 μm.

shaped cap (Fig. 2d); 5) cells with an acidic vacuole (8–9 μm in diameter) having a large vacuole that contains basophilic material, and a round nucleus close to the plasma membrane (Fig. 2e); 6) multivacuolated cells with a variable number of roughly spherical vacuoles that contain optically clear material (Fig. 2f); 7) compartment cells, spherical in shape (8–12 μm), with either two distinct morphological features, namely, angular vacuoles in variable numbers distributed regularly at the periphery of the cytoplasm around a central vacuole (form *A*, Fig. 2g) or a variable number of round or angular vacuoles, as well as a large vacuole on one side of the cell (form *B*, Fig. 2h); the peripheral vacuoles usually contain granules; 8) Morula cells with variable numbers of symmetrically arranged large vacuoles or globules and a berry-like or morula-like appearance (Fig. 2i); and 9) pigment cells, which are yellow or orange, with variously pigmented bodies encased in a thin cytoplasmic envelope (Fig. 2l).

Differential distribution of hemocytes among the fractionated bands of cells (B1–B8)

As indicated in Table 1, B3 consisted mostly of cells with an acidic vacuole; B4 consisted mainly of compartment cells of form *A* (67.0%) and form *B* (31.4%); B6 consisted primarily of macrogranulocytes that contained large granules (81.6%); B7 and B8 consisted of signet ring cells exclusively. The bands B1 and B2 were richer in macrogranulocytes that were characterized by a few small granules, but small numbers of pigment cells, hyaline cells, compartment cells, and cells with acidic vacuoles were also included. Finally, B5 contained compartment cells (21.7%) of form *B*, morula cells, macrogranulocytes, and a few hyaline cells (Table 1). In Table 1, the percentages of each type of hemocyte, as evaluated by an examination of washed hemocytes before density gradient separation, are also listed.

Hemagglutinating activity of the supernatants from cultures of fractionated hemocytes

Since 7.2×10^4 unfractionated washed hemocytes release an easily detectable amount of lectins into 1 ml of culture medium (Table 2), equivalent numbers of hemocytes from each Percoll-separated band were cultured in microculture wells. Five specimens of *Phallusia* were used and the experiments were carried out in duplicate. Lectins were released into the medium in which B3, B4, and B5 hemocytes had been cultured for 3 h (Table 2). Supernatants from cultures of B4 cells displayed the highest hemagglutination titer (1:128) which was even higher than that of the unfractionated hemocytes (1:16). After a 24-h incubation, hemagglutinating activity of supernatants increased in each of five cultured fractions but only cells from the B4 band gave a titer of 1:128 (Table 2). Throughout the incubations, the cells remained viable, as determined by the eosin-y test. Mortality ranged from 1.0–5.0% in the cultures of Percoll-fractionated hemocytes, whereas unfractionated, washed hemocytes showed the lowest mortality values (0–1.0%).

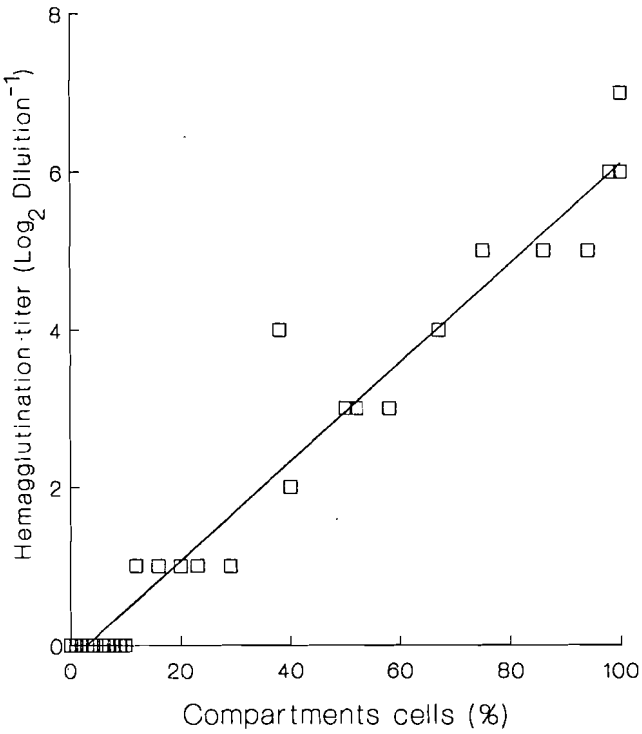

Fig. 3. The relationship, expressed as a linear regression equation, between hemagglutination titer of supernatants from cultures of *Phallusia mamillata* hemocytes (unfractionated and fractionated hemocytes, bands 1–8), and the percentage of compartment cells present in the cultures ($Y = -0.02 + 0.06\times$; $P < 0.001$; $r = 0.98$).

The close relationship (statistically significant at $P < 0.001$; $r = 0.97$) between the percentage of compartment cells in cultures from each band of hemocytes (B3, B4, B5) and the hemagglutination titer of the supernatants is shown in Figure 3. In supernatants from cultures that contained hemocytes from B1, B2, B6, B7, and B8 bands no significant hemagglutinating activity was found.

Properties of the released lectins

To determine whether the lectins released from Percoll-fractionated hemocytes had the properties of CL-type lectins, supernatants from cultures of B3, B4, and B5 hemocytes were analyzed by the sugar-inhibition test and SDS-PAGE, and with CL-specific antibodies by the Western blotting technique.

α-Lactose and lactulose were able to inhibit the hemagglutinating activity (Table 3). Concentrations of sugar required for inhibition (50–200 mM) were proportional to the hemagglutination titers of supernatants from 3-h cultures (1:4–1:64). No significant inhibition was observed when the monosaccharides D-glucose, L-fucose, D-mannose, and D-galactose and the oligosaccharides D-melibiose, D-raffinose, and maltose were tested up to final concentrations of 200 mM.

Under reducing conditions, the electrophoretic patterns of the concentrated supernatants revealed bands of the two major proteins with apparent molecular weights of 37,500 ± 850 KDa and 35,800 ± 800 KDa (Fig. 4A, lanes 2–5). Lane 1 (Fig. 4A) shows the pattern of purified SL. In the immunoblotting experiments, the CL-polyclonal antibodies reacted only with protein components whose molecular weights correspond to those of CL-type lectins, found in culture supernatants from B3, B4, and B5 as well as unfractionated hemocytes (Fig. 4B, lanes 2–5). No cross-reaction occurs with purified serum lectins (Fig. 4B, lane 1).

DISCUSSION

Lectins have been identified in species of virtually all taxa from viruses and bacteria to vertebrates, and they appear to be present in all living organisms. Experimental evidence has been obtained to suggest that they play a wide variety of physiological roles which are related to their car-

TABLE 3. Inhibition by sugars of binding lectins released from fractionated hemocytes from Phallusia mamillata

Specimen	Hemocyte band	Hemagglutination titer[1] before sugar inhibition (reciprocal)	Inhibitory concentration of sugar (μmole/ml)	
			α-Lactose	Lactulose
1	B3	8	50	50
2		4	25	25
1	B4	64	200	100
2		8	50	50
1	B5	4	25	25
2		4	50	25
1	unfractionated	8	100	50
2		16	100	100

[1]Supernatants from cultures of hemocytes assayed with rabbit erythrocytes.

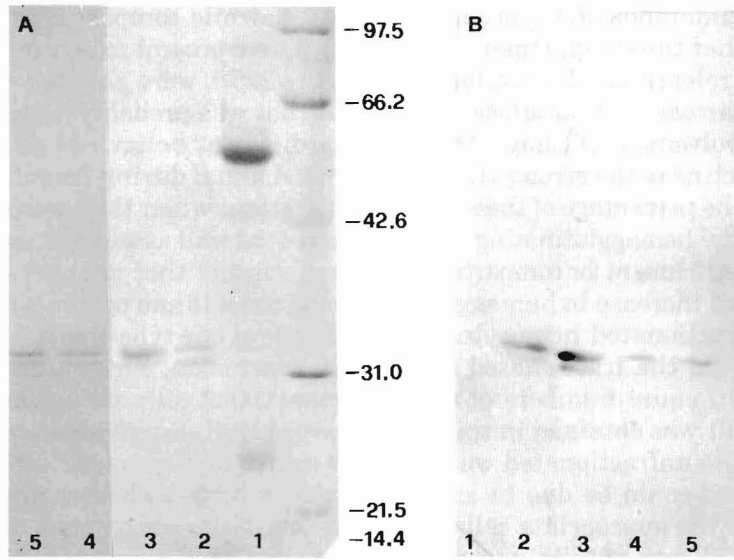

Fig. 4. Results of SDS-polyacrylamide gel electrophoresis **(A)** and immunoblotting **(B).** Serum lectins isolated from *Phallusia mamillata* (SL) and lectins released into the supernatants of cultures (24 h at 16°C in SW-M199) of unfractionated and fractionated hemocytes (bands B3, B4, B5) were analyzed. **Lane 1:** Serum lectins. **Lane 2:** Supernatant from a culture of unfractionated hemocytes. **Lane 3:** Supernatant from a culture of hemocytes partitioned in band 4. **Lane 4:** Supernatant from a culture of hemocytes partitioned in band 3. **Lane 5:** Supernatant from a culture of hemocytes partitioned in band 5. The pattern of standard proteins (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme) and their molecular weights (KDa) are presented. Immunoblotting was performed with CL-specific antibodies raised against cellular lectins (CL) that had been purified from sonicated hemocytes. Protein contents of concentrated samples from the supernatant of a culture of B4 cells and supernatant from the unfractionated hemocytes: 40.6 ± 1.2 and 18.7 ± 4.2 μg/ml, respectively.

bohydrate-binding properties and correlated with the increasing complexity of the organisms. Several roles have been proposed for invertebrate lectins, including participation in cell aggregation, fertilization, embryonic development, metamorphosis, wound repair, and transport of simple or complex carbohydrates. In addition, although supporting evidence has been reported for only a few species, it is often assumed that hemolymphatic sugar-binding proteins are involved in cell-mediated defence reactions (Amirante, '92; Kobayashi and Yamamoto, '93; reviews: Olafsen, '86; Parrinello, '91; Parrinello and Arizza, '92; Vasta, '91). Lectins have been proposed as the most likely participants in cellular and humoral immune recognition mechanisms in invertebrates. In tunicates, the biological function of lectins in the hemolymph, as well as in several types of hemocytes, is still far from being understood. For reviews, see Olafsen ('86), Parrinello ('88, '91), and Vasta ('91).

One criterion employed in attempts to deduce the possible biological roles of invertebrate lectins is their tissue distribution, together with the identification of the sources of lectins in different organs, tissues, and fluids. Cell-separation techniques and microculture of specific populations of hemocytes provide a new approach to the study of hemocyte function as it relates to the distribution, biosynthesis, and release of lectins. In the present study we have shown, for the first time in tunicates, that the hemocytes known as compartment cells can contain and release lectins. In *Phallusia mamillata,* the lectins that these cells release appear to be of CL-type different in some molecular and immunochemical properties from the SL-type humoral lectins.

Discontinuous Percoll gradients, as used for the fractionation of hemocyte populations from the hemolymph of *Phallusia mamillata,* cannot be considered optimal for purification of all types of cell. However, we obtained enriched populations of microgranulocytes (~ 45–48%, bands 1 and 2), cells with an acidic vacuole (~ 60%, band 3), and isolated cell populations of compartment cells (~ 100%, band 4) and macrogranulocytes (80%, band 6). Signet ring cells were distributed into two closely adjacent bands, each of which contained such cells almost exclusively. These two populations probably differ in the density of the materials packaged in the single large vacuole and, therefore, they were separated by Percoll gradient.

The results obtained from the microcultures of the fractionated hemocytes, sugar-inhibition exper-

iments, SDS-PAGE, and immunoblotting of culture supernatants indicate that the compartment cells are responsible for the release of CL-type lectins that are specific for α-lactose and lactulose. Further evidence for the involvement of compartment cells in the release of lectins is the strong statistical correlation between the percentage of these cells present in cultures and the hemagglutinating activity of the supernatants. Enrichment for compartment cells is associated with an increase in hemagglutinin titers, and the unfractionated hemocytes released less lectin than did the fractionated ones (fraction B4), even when equal numbers of cells were cultured. This result was obtained in spite of the higher viability of the unfractionated washed hemocytes in culture and could be due to an increase in the number of the appropriate cell type present in a specific population of cells, as well as to the stimulatory effect of Percoll which may increase the rate of lectin release by fractionated cells. Lectins of the SL-type, which differ from CL in terms of mobility during SDS-PAGE and immunological determinants, were never found.

According to the previous report on the release of lectins from total hemocytes (Arizza et al., '91), and the recent results of immunocytochemical studies (Arizza et al., '93), it seems likely that lectins of the CL-type are stored in the vacuoles of compartment cells and are released when cells are stimulated by the conditions used for in vitro culture. The mechanism of their release is unclear, and the functional role of compartment cells and lectins of this ascidian remain have to be demonstrated.

Azema examined hemocytes of *Phallusia* by light microscopy ('37). He described an hemocyte type that he named "cellule plurivacuolaire." His drawings show two cells that appear to be morphologically identical to our compartment cells of form *A* and form *B*, on the basis of the apparent eccentric location of a large vacuole. Scippa and Iazzetti ('89) discriminated between compartment cells of I and II type on the basis of the electron density of the vacuolar materials. Type I cells have peripheral vacuoles and a large eccentric vacuole that contains electron-dense material. This cell appears to correspond to our form *B*, whereas no clear relationship exists between form *A* and the compartment cell of type II. The two forms, *A* and *B*, cannot be considered as two distinct types of hemocytes. No difference, in terms of lectin release, was observed between cultured compartment cells of form *A* and *B*, as demonstrated by the linear correlation between differential cell counts and hemagglutinating titers. Both types of hemocyte were present in band 4, while compartment cells of form *A* or *B*, which were present mostly in bands 3 ($A \sim 15\%$) and 4 ($B \sim 22\%$), were able to release lectins to an extent that was probably dependent on cell number. The different behaviour of compartment cells of forms *A* and *B* during Percoll density gradient centrifugation, when they were partitioned in bands 3 and 4, as well as some of their morphological features suggest that changes occur in the vacuolar compartments and contents at various stages of the life cycle of one type of cell.

In conclusion, our results demonstrate that the compartment cells of *Phallusia mamillata* contain CL-type lectins and release them in vitro. To date, the source of serum lectin (SL) remains unknown. With respect to both intra-and extracellular lectins, the possibility exists that, although they may be found in a particular tissue or body fluid, their biosynthesis and secretion may be associated with specific cells or tissue. After their secretion they are distributed to the body fluids and adsorbed or pinocytosed by those cells in which they are finally detected.

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