Antibodies with the Cell-type Specificity to the Morula Cells of the Solitary Ascidiens *Styela Rustica* and *Boltenia Echinata*

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ABSTRACT. The separation of the blood cells of *Styela rustica* (Styelidae, Stolidobranchiae) in discontinuous Percoll gradient showed 4 fractions. The 4th and bottom most fraction contained 90–100% of morula cells. The protein composition of the morula cell fraction revealed on SDS-PAGE showed two major proteins with m.w. 47 and 26 kDa. These proteins were heavily positively charged. Polyclonal antisera against these proteins were raised. Each antiserum reacted with both proteins only in morula cells on the blot after SDS-PAGE and stained the proper protein without crossreaction on the blot after AU-PAGE. The only type of cells stained with antibodies in circulating blood, in the tunic and on the tunic wound surface in paraffin sections of another species *Boltenia echinata* (Puridae, Stolidobranchiae) were morula cells. The morula-type specific antibodies obtained recognized major positively charged proteins which were apparently structural substrates for the phenoloxidase tanning.

The origin of defense and recognition systems in invertebrate and vertebrate animals is the subject of comparative immunology. Particular attention should be paid to ascidians because of their unique phylogenetic position. One of the main defense and/or recognition systems of many groups of invertebrates is the phenoloxidase system (PO system) (30, 31). Ascidians use the PO system not only for defense reactions but for the sclerotization of proteins in tunic (2, 4, 5, 9, 12, 20, 28). This system in ascidians is localized in the morula cells which seems to be a characteristic type for ascidians of all three families of the order *Stolidobranchia* (8, 10, 16, 17, 35). Granules of morula cells contain components of the PO system: PO in inactive form, binding metals (iron or vanadium), polyphenolic compounds, cationic proteins (1, 4, 7, 11, 14, 20, 22, 29, 33). The morula cells are thus one of the key points of the defense system of ascidians.

Phase contrast microscopy, Nomarsky optics and histological analysis of differentially stained sections

identified 12 types of cells in the blood of solitary ascidian *Styela rustica* (10). The main types of blood cells were the following: haemocytoblasts, hyaline amoebocytes, granulocytes, macrophages, and morula cells — vacuolated, young, intermediate and mature. The average number of blood cells in circulation was 10.8 ± 6.1 mln/ml, including 35–45% of morula cells. The signet ring and compartment cells, typical for the other ascidian species, are lacking in *S. rustica*. Vacuolated, young, intermediate and mature morula cells are presumably the consequent stages of the morula cell differentiation pathway (10). The main part of the mature morula cell population can be considered as cells having terminal differentiation and resting defensive cells. They also migrate into the tunic. According to cytochemical studies, mature morula cells contain the main components of the PO system (10, 11).

The goal of the present work was to find out any characteristic components accumulating in this type of cell and to provide antisera against them as tools for further investigation. We have found two proteins of m.w. 47 kDa and 26 kDa with common antigenic determinants in morula cells of two ascidian species.

MATERIALS AND METHODS

Animals

Specimens of *Styela rustica* were collected at the Marine Biological Station of St. Petersburg State University in Chupa in-

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Abbreviations: SDS-PAGE, SDS polyacrylamide gel electrophoresis; AU-PAGE, acid-urea polyacrylamide gel electrophoresis; PBSTw, phosphate-buffered saline with 0.05% Tween 20; AB26, antiserum against 26 kDa protein; AB47, antiserum against 47 kDa protein; PO system, phenoloxidase system; m.w., molecular weight; ASW, artificial sea water; CBB, Coomassie Brilliant Blue; BCIP-NBT, 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium.

let of the Kandalaksha bay of the White Sea, and kept in sea water changed twice a day (25%, 10°C). Before bleeding the tunic was cleaned of epiphytes and washed extensively with sea water, dried by blotting with absorbent wipes. The sampling area was sterilized with 70% ethanol and the body wall was cut to muscular level without injuring the internal organs. The hemolymph exuding from the animal was collected by pipetting into ice-cold tubes rinsed with artificial sea water (ASW) without Ca^{2+} . The composition of ASW was as follows: 0.3 M NaCl, 20 mM KCl, 15 mM EDTA, 10–50 mM Tris-HCl pH 7.6.

Boltenia echinata was collected in the same place and used for the experiments with tunic reparation. The animals' tunic was cut to half of tunic depth and after 5 days it was fixed in Bouin solution (27). Paraffin sections were prepared and processed for AB staining.

Cell Separation

Percoll solution (Pharmacia, Sweden) was mixed with appropriate volumes of ASW to final concentrations of 65%, 50%, 40%, and 30%. Two ml of each solution were overlaid sequentially into glass centrifuge tubes. Two ml of hemolymph were layered onto the gradient and the tube was centrifuged in a swing-out rotor at 800 g for 10 min. Cell layers were collected by gentle aspiration and washed twice in ASW. Bands were numbered from top to bottom of tube. Cell viability and composition of cell fractions were estimated by phase-contrast microscopy. All operations were carried out at 10°C.

Electrophoresis and western-blot

Protein composition of the fractions was determined with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (21). Acid-urea PAGE (AU-PAGE) (24) was performed for the determination of cationic proteins in morula cells. Total histones from rat liver nuclei were used as a marker (a gift from Prof. V.I. Vorobjev, Institute of Cytology, Russian Academy of Sciences). All gels were stained with Coomassie Brilliant Blue (CBB). Electroblothing was done in Laemmli electrode buffer with 20% methanol (34). The blots were washed with phosphate-buffered solution (PBS) with 0.05% Tween 20 (PBSTw). This basic solution was used for all antibody work. The blots were blocked with 3% skimmed milk for periods from 1 hour to overnight (at 4°C in the latter case). After that the blots were incubated with rabbit antiserum (working dilution 1:1,000) for 1 h, washed with PBSTw thrice for 15 min each time, incubated with alkaline phosphatase-conjugated goat anti-rabbit Immunoglobulins (Sigma) and stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (BCIP-NBT) (Sigma) for around 20 min. All operations were performed at room temperature.

Peptide mapping

Peptide mapping was carried out according to the method of Cleveland *et al.* (13) as modified by Bandman *et al.* (6). The proteins in the gel segments — 43 kDa and 47 kDa proteins of

S. rustica morula cells and rabbit actin (a gift from Dr. O. Antropova, Institute of Cytology, Russian Academy of Sciences) — were digested for 30 min with 5 μl of *Staphylococcus aureus* V8 protease (type XVII B, Sigma: P-2922) solution at the following concentrations: 0.2 $\mu\text{g}/\text{ml}$; 1 $\mu\text{g}/\text{ml}$; 5 $\mu\text{g}/\text{ml}$; 10 $\mu\text{g}/\text{ml}$ in reducing buffer for SDS-PAGE. The control contained no protease. Proteolytic fragments were separated on 15% gel and stained with CBB.

Antibody production

Two polyclonal antibodies against two major proteins of morula cells were obtained. In this paper they will be named antiserum against 26 kDa protein (AB26) and antiserum against 47 kDa protein (AB47). The immunization procedure included the following steps. Proteins of the morula cell fraction were separated by SDS-PAGE. The 26 kDa and 47 kDa proteins were cut out from the slab gel, homogenized with PBS and mixed with Freund adjuvant (1:1); this mixture was injected hypodermically into two male rabbits. Boosting with the same mixture was performed intramuscularly in 4 weeks. A week later about 5 ml of blood was taken from the rabbits' ear. The antisera were aliquoted and stored at -20°C . The samples of pre-immune sera were obtained from the same rabbits a week before antigen injection for control staining.

Immunohistochemistry

Adult individuals of *Styela rustica* and *Boltenia echinata* were fixed in Bouin solution for 24 hours and then rinsed in 70° ethanol. Fixed samples were embedded in paraffin and sectioned at 5–7 μm . The traces of picric acid were washed out with 70° ethanol after removal of paraffin, and then these sections were washed with PBSTw. Immunohistochemical staining basically followed the procedure described in chapter "Electrophoresis and western blot". All operations were performed in a moist chamber at room temperature. The stained sections were mounted in 80% glycerol. For control some sections were incubated with pre-immune rabbit serum with all the sequential steps. Preparations were observed under a light microscope.

RESULTS

Resolution of the gradient

The cells washed out of the Percoll gradient solution were alive and motile. The bottom (4th) fraction had the most stable composition; no less than 90% and often up to 100% of the cells were mature morula cells. SDS-PAGE of all fractions and plasma proteins is presented in the Fig. 1. There are about 10 proteins in cell-free plasma. The protein of 23 kDa is the main one. A protein of very high mol.mass is also visible among plasma proteins. All cell fractions contained 4 zones in the range of 16–19 kDa which probably correspond to the histones as judged by their apparent mobility in

Fig. 1. SDS-PAGE of proteins separated by Percoll discontinuous gradient. 26 kDa and 47 kDa proteins are the major ones in the 4th fraction (mature morula cells). 12% gel. Lane 1: fraction 1; lane 2: fraction 2; lane 3: fraction 3; lane 4: fraction 4; lane 5: plasma.

SDS-PAGE (21, 24). These zones are practically absent from the plasma fraction and *vice versa* — plasma proteins are absent from the cell fractions. This is evidence that there is no cross-contamination of plasma and cell fractions and no cell lysis occurs during separation.

The protein compositions of the two top fractions revealed large amounts of protein with mol.mass 43 kDa. The 4th fraction which consisted of mature morula cells displayed 2 major proteins of mol.mass 47 and 26 kDa in similar amounts (Fig. 1, 4). The previous fraction with younger morula cells also contained these proteins as major ones, but in this case the amount of the 47 kDa protein is higher than that of the 26 kDa one and a 43 kDa zone is visible (Fig. 1, 3).

Peptide mapping

A high content of filamentous actin is a characteristic feature of hyaline amebocytes — cells with a high motility (10). The 47 kDa protein seems to be typical for the morula cells; the mol.mass similarity suggests that this protein could be an ascidian isoform of actin with unusual electrophoretic mobility. In order to examine this possibility we did comparative peptide mapping of the rabbit stress muscle actin, the 43 kDa protein from the cell fractions from the top of the gradient and the 47

Fig. 2. Peptide mapping of 43 kDa (I), 47 kDa (II) proteins of *Styela rustica* hemocytes and rabbit actin (III). Proteins were digested during 30 min by *Staphylococcus aureus* protease V8 in concentrations: Lanes 1, 5, 9 — 10.0 $\mu\text{g/ml}$; lanes 2, 6, 10 — 5.0 $\mu\text{g/ml}$; lanes 3, 7, 11 — 1.0 $\mu\text{g/ml}$; lanes 4, 8, 12 — 0.2 $\mu\text{g/ml}$. 15% SDS-PAGE.

kDa protein from the morula cell fraction. The result is shown in Fig. 2. The pattern of rabbit actin peptides and 43 kDa one of *S. rustica* definitely have features in common so it can be concluded that this is the same protein — actin. The peptide pattern of the 47 kDa protein is rather different from the actins from either species.

Antibodies

In order to determine whether the 47 kDa and 26 kDa proteins are really characteristic of morula cells we raised polyclonal antibodies against corresponding zones cut out from SDS-PAGE. Western-blot after SDS-PAGE with the ABs obtained showed that both ABs recognize both proteins among morula cells proteins and do not react with proteins of the 1st gradient fraction in 1:1,000 dilution of antisera. The AB26 affinity is slightly lower than the AB47 one (Fig. 3).

The charge of the major morula cell proteins

It has been shown that morula cell granules contain cationic proteins enriched by ϵ -Lysine' aminogroups (11). To check if the major morula cell proteins are charged AU-PAGE sensitive to the positively charged proteins was applied to the cell lysate. AU-PAGE analysis showed several proteins (Fig. 4A). There are two major bands, the higher of which is double, which are localized in the upper part of the gel. The other group of proteins seems to correspond to histones. Histones have been used as markers in AU-PAGE and this group of *S. rustica* proteins has the same mobility.

Immunoblotting has been done with the proteins after AU-PAGE separation (Fig. 4B) and the two ma-

Fig. 3. Western blot of the proteins from morula cell fraction and cells from the 1st gradient fraction with amidoblack 10B (1), antisera AB26 (2) and AB47 (3) in staining dilution 1:1,000. Lane a: proteins of the 1st fraction blood cells; lane b: morula cell proteins of the cells from the 3rd and 4th fractions sampled together. 12% SDS-PAGE.

Fig. 4. Determination of cationic properties of morula cell proteins. A: Two main proteins in the morula cells lysate loaded on AU-PAGE marked with * and ♦ (A, 2). Total rat histone preparation used as a marker (A, 1). 15% gel. B: Western blot with the morula cell lysate after AU-PAGE with: lane 1, AB26; lane 3, AB47; lanes 2, 4, pre-immune serums. C: SDS-PAGE of the proteins * (lane 1, 26 kDa) and ♦ (lane 2, 47 kDa) cut out from AU-PAGE. 12% gel.

jor bands from AU-PAGE were also cut out from the gel, poured with Laemmli reducing buffer and SDS-PAGE was performed (Fig. 4C). The second dimension shows that the upper double band corresponds to the 47 kDa protein and the lower band to the 26 kDa one. In blotting analysis after AU-PAGE both bands

Fig. 5. Immunohistochemical staining of sections of body cavity (A, B) and tunic (C) with AB47 (A, C) and AB26 (B). (A, B) Morula cells are only stained in body cavity (arrows), (C) morula cell in tunic is stained, but the tunic itself is not stained. Magnification: $\times 1,250$.

got stained. AB47 stained the double band which corresponds to the 47 kDa protein, AB26 stained the second band which corresponds to the 26 kDa protein. AB47 affinity is significantly higher. There is no crossreactivity between ABs in this case.

Immunohistochemistry

We stained paraffin sections of two ascidian species which belong to the same order but different families that were at our disposal — *Styela rustica* and *Boltenia echinata*.

Styela rustica paraffin sections did not get stained with the ABs. In paraffin sections of *B. echinata* both ABs stained cells in the body cavity and in the tunic. The morphological characteristics of the stained cells (cellular size and shape, and granular size) testify to the fact that it is the morula cells that really get stained (Fig. 5).

To clarify cell-type specificity in staining the following experiment was done. A wounded *B. echinata* in the process of healing was fixed and the sections were stained with ABs. The staining with AB26 (Fig. 6) and with AB47 (data is not shown) were basically the same. The adjacent sections were treated with immune (Fig. 6A) and pre-immune (Fig. 6B) serums. Pre-immune serum did not give any staining at all. The immune anti-serum with AB26 reacted with cells in the body cavity, tunic and on the wound surface (Fig. 6). The localiza-

Fig. 6. Immunohistochemical staining of sections of *Boltenia echinata* body cavity and wounded tunic at 5 days after wounding. Morula cells in body cavity, in tunic and on the wound surface are stained with AB26 (A) but not stained with pre-immune serum (B). (T) Tunic area; (C) body cavity area. Magnification: $\times 250$.

tion of the stained cells corresponded to the expected localization of the morula cells.

DISCUSSION

Styela rustica blood does not coagulate, unlike insect and crustacean haemolymph (10, 26, 32). The haemocytes easily agglutinate in sea water but the addition of EDTA to ASW solution prevents cells from aggregating. Similar conditions for separation of ascidian blood cells were used by other investigators (23, 25, 30).

The Percoll density gradient used in this work had high resolution. Fraction 4 (65% Percoll, density 1.089 g/ml) contained up to 100% of morula cells. Such a degree of purity makes it possible to define the main cell-specific proteins. Thus we succeeded in finding two major proteins of *S. rustica* morula cells. These proteins are most likely the components of the PO system which morula cells bear.

One of the components of the PO system is an o-diphenol substrate for PO. Several low m.w. polypeptides (3–10 kDa) are described as o-diphenol compounds among ascidians: tunichromes, ferreascidin, halocyamin and others, all of which produce quinones upon enzyme oxidation (4, 14, 20, 33). They are unlikely to have anything in common with the proteins observed because of the striking difference in their mol. masses.

Unfortunately we could not determine the protein nature in the main bands after SDS-PAGE electrophoresis according to the usual functional tests for enzyme activity. The reported mol. mass of the proPO and PO subunits of species from different phylum according to SDS-PAGE lay in the range of 40–200 kDa (18, 19, 31), hence the 47 kDa protein could be PO or proPO itself. The large positive charge which is shown in the present work makes the 47 kDa and 26 kDa proteins candidates for cationic proteins, the third component of the PO system. According to the cytochemical data the morula cell proteins of *S. rustica* contain a high amount of free lysine ϵ -aminogroups; this feature gives them a high positive charge and makes them a suitable substrate for the PO system (11).

Our data suggest that despite the crossreactivity of the AB47 and AB26 on the blots after SDS-PAGE electrophoresis, the 26 kDa and 47 kDa proteins are in more complicated connection than a mere dimerization. Protein composition of the cells from gradient fraction 3 — young morula cells — displays higher amounts of the 47 kDa than the 26 kDa protein. In fraction 4 (mature morula cells) both proteins are represented nearly equally (Fig. 1, 4; Fig. 4A). Both have common antigenic determinants after SDS-PAGE (Fig. 3). These data allow us to suggest that the 26 kDa could be a derivative of the 47 kDa protein. On the other hand, different antigenic determinants exist in the proteins and are recognized by the correspondent ABs when exposed. This happens after AU-PAGE when crossreactivity of the ABs is not revealed and the specificity of the ABs to each protein increases. Different antigenic determinants are thus exposed after SDS and acid-urea processing. Probably the fixation with urea will also help to increase the specificity of staining on paraffin sections.

The failure of staining of *S. rustica* sections (unlike *B. echinata*) by conventional procedure may be due to the type of packing of these proteins in morula cell granules which masks the antigenic determinants. There is also no staining in *S. rustica* and *B. echinata* extracellular components of the tunic evidently because of the strong tanning of the proteins in it.

Morula cells in tunic release the content of their granules during tunic formation and regeneration. O-diphenol compounds, oxidized by the PO system, take part in crosslinking of protein substrate and thus tan the upper layer of tunic (9, 15, 28). They also participate in self/nonself recognition reactions; in all biological reactions involving morula cells the latter form an extracellular matrix consisting of proteins stabilized by the PO system (2, 5, 9, 12). The main proteins of the morula cells observed in this work may be precursors of the proteins tanned by phenols during PO reaction. Protein sequencing of the proteins could give the exact

answer. Nevertheless, biochemical and immunohistochemical data give evidence that the 47 kDa and 26 kDa proteins are markers of the morula cells since ABs against these proteins stain only this type of cell. We already know that ABs work in cell-type specific manner in two ascidian species. The possible limits of applying ABs to the other species remains to be defined. ABs obtained present a useful tool for the investigation of the PO system in defense reactions, in self/nonself recognition reactions, and in the organization of extracellular matrix proteins in tunic formation of such an evolutionary significant species as ascidians.

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