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COMPARATIVE AND ONTOGENIC BIOCHEMISTRY AND IMMUNOLOGY

Carbohydrate Mimicry of the Parasite in the *Himasthla elongata* (Trematoda: Echinostomatidae)–*Littorina littorea* (Mollusca: Prosobranchia) System

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Abstract—Using labeled lectins, a comparative study of the surface of tegument of *Himasthla elongata* at different stages of development cycle (daughter rediae, cercariae, and metacercariae) and glycocalyx of plasma membranes of hemocytes of molluscs *Littorina littorea* and *Mytilus edulis* that are the first and second intermediate hosts of this trematode species, respectively, has been carried out. It is found that in the course of the development cycle of the parasite there occurs a change of the set of terminal sacharides of the glycocalyx of the *H. elongata* tegument surface as well as differences are revealed in the pattern of binding of three out of five tested lectins with hemocytes of blue mussel and periwinkle. At the same time, the presence of similar carbohydrate determinants on the surface of hemocytes of *L. littorea* and daughter rediae of *H. elongata* is shown. The established similarity in composition of glycocalyx of hemocytes of the mollusc and the trematode parthenitae is, most likely, a result of coevolution of the parasite and the host and is of the adaptive nature. Use of the mechanism of the carbohydrate mimicry by parthenitae of this species allows them to avoid attacking by effector cells of the internal defense system of the mollusc-host.

INTRODUCTION

By the present time, extensive information has been accumulated on interaction between parasite and host in the mollusc–trematode parthenita systems. It is known that trematodes use at least two mechanisms to avoid recognition in the host organism and to suppress activity of its defense system. One of them is inhibitory action on hemocytes, the main effector cells of the host defense system, by several factors (secretory-excretory products) released by the parasite. The second is use of the so-called "passive" strategies, such as molecular mimicry, i.e., structural similarity of surface molecules of the parasite and of the host tissues, and molecular masking, i.e., binding of host glycoproteins on the parasite surface by membrane receptors that are, as a rule, of the lectin nature (see reviews [1, 2]).

Alongside with significant achievements for the last years in studies on composition of secretory-excretory products of trematodes of several species and mechanisms of their interaction with hemocytes of mollusc hosts, many problems of the existence of such phenomena as molecular mimicry and molecular masking as well as the extent of their occurrence in various combinations of mollusc–parthenitae of trematodes have remained unsolved. All current evidences in favor of the presence of such "passive" strategy of interaction with immune system of the vertebrate host are obtained in studying maritae of three species of trematodes—*Schistosoma mansoni* [3], *Fasciola gigantica*, and *F. hepatica* [4].

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The only reliably documented example of the structural similarity of trematode proteins with proteins of intermediate hosts remains homology of tropomyosins in mother sporocysts of *S. mansoni* and mollusc *Biomphalaria glabrata* [5]. However, the intracellular localization of this protein and its rather high conservatism cast doubt that the revealed homology is a consequence of the adaptive coevolution of the parasite and the host and reflects the true molecular mimicry. As to similarity of superficial proteins of the trematode parthenitae and of mollusc cells, the presence of similar determinants on the surface of the trematode parthenitae and the mollusc tissues has been shown for the same *S. mansoni–B. glabrata* system by using antisera obtained after immunization of experimental animals by mollusc hemocytes or worm proteins [6–8]. However, the molecular mimicry revealed by this method can be a result of the presence on the trematodes surface and in *B. glabrata* tissues of widely spread common epitopes, whose main components most likely are carbohydrates [9]. Apparently, to make final conclusion about the presence of the mechanism of molecular mimicry in this parasite–host system, it is necessary to elucidate the precise biochemical structure of determinants bound by cross-reacting antisera.

It is to be noted that in all above-mentioned cases, protein structures were analyzed first of all, whereas the key determinants during the "self–not-self" recognition in all so far studied representatives of the Mollusca phylum are carbohydrate components of glycoproteins of the cellular surface, which are bound by lectins—the main factors of the humoral part of the defense system of these animals [10]. This peculiarity of the mollusc defense system allows suggesting that similarity of the carbohydrate component of glycocalyx of the parthenita tegument and the host cells can play the key role in prevention of recognition and destruction of the parasite by hemocytes. In this connection, the goal of the present work was a comparative analysis of carbohydrates exposed on the surface of rediae, cercariae, and metacercariae of *Himasthla elongata* as well as of hemocytes of molluscs *Littorina littorea* (Gastropoda, Prosobranchia) and *Mytilus edulis* (Bivalvia), the first and second intermediate hosts of trematodes of this species, respectively.

MATERIALS AND METHODS

Snails *L. littorea* were collected at low tide at littoral of the Bolshoi Gorelyi Island (the Chupa Bay, the Kandalaksha Gulf, the White Sea). For the entire time before experiment the animals were kept in cages and supplied with sufficient food (*Fucus vesiculosus* and attached algae). Mussels (*M. edulis*) were collected in plantations of then Nikolskaya Bay and were kept in cages before experiments. All studies were carried out in August, 2001.

Rediae of *H. elongata* were removed by dissection of *L. littorea*, destroying carefully the hepatopancreas tissues with a preparation needle. The dissection was performed under a binocular microscope at ×16 magnification. To rule out possible artifacts due to an injury of parthenita glycocalyx by digestive enzymes released from damaged tissues of the mollusc hepatopancreas as well as to absorption of host's glycoproteins on the trematode tegument surface, the rediae were cultivated *in vitro* for 2–3 days before experiment according to the procedure described earlier [11]. Only parthenitae moving actively, uninjured, and free of mollusc tissue fragments were chosen for cultivation. The chosen rediae were repeatedly washed off with large volumes of sterile sea water and then transferred into the wells of the 24-well trays for cell cultures ("Sarstedt," USA) in the cultivation medium L-15. Throughout the cultivation the trays with parthenitae were at temperature 13–14°C.

To obtain cercariae, the infected molluscs were placed in small vessels with the sea water; the released cercariae were collected in plastic test tubes and concentrated by cooling on ice. Using the same method of concentration, the cercariae were washed off several times with filtrated sea water.

Metacercariae of *H. elongata* were obtained by the procedure of encystment *in vitro*; for this purpose the cercariae leaving littorinae were collected and concentrated as mentioned above, then larvae were placed in plasma (cell-free hemolymph) of mussels. Action of plasma of this mollusc species produces encystment of most cercariae for 2 h. The obtained metacercariae were washed off from mussel plasma proteins with filtrated sea water. Rediae, cercariae, and metacercariae of *H. elongata* were fixed for 30 min in 5% paraformaldehyde containing 4 mM sodium carbonate.

Table 1. Lectins used in the experiments

Lectin	Abbreviation	Source	Specificity
Concanavalin A	ConA	*Canavalia ensiformis*	α-D-mannose
			α-D-glucose
Bandeiraea simplicifolia lectin	BSL	*Bandeiraea simplicifolia*	α-D-galactose
			N-acetyl-D-galactosamine
Roman snail lectin	HPA	*Helix pomatia*	N-acetyl-D-galactosamine
Wheat germ lectin	WGA	*Triticum vulgaris*	N-acetyl-D-glucosamine
Wisteria floribunda lectin	WFL	*Wisteria floribunda*	N-acetyl-D-galactosamine

Hemolymph of *L. littorea* was collected from the buccal sinus of the previously anaesthetized molluscs, using sterile needles for disposable syringes. Hemolymph of mussels was collected from sinus of posterior adductor of the animals, using sterile disposable syringes of 1 ml volume. The hemolymph was placed directly into the fixing solution of 4% formaldehyde (Sigma, St. Louis, USA) at a 1:2 ratio; the cells were fixed for 30–45 min.

Study of glycocalyx of *H. elongata* and hemocytes of *M. edulis* and *L. littorea* was carried out using labeled lectins. The following conjugates of lectins with fluorochrome (FITC) (Sigma, St. Louis, USA) were used in experiments (Table 1).

After washing off from fixing solution with TBS buffer (50 mM Tris-HCl, pH 8.0) of containing (mM): 4 $CaCl_2$, 2 $MgCl_2$, 36 NaCl, and 2 KCl, the rediae, cercariae, and metacercariae were placed in solution of labeled lectin (25 μg/ml in TBS). After the 30-min incubation the objects were washed off three times with TBS to remove non-bound conjugate and embedded in the medium preventing fading of fluorescent label (the anti-fade medium, DAKO, Denmark). To check specificity of the binding, control preparations were treated with working lectin solution with addition of corresponding monosaccharide at 0.1 M concentration.

The fixed hemocytes of *L. littorea* and *M. edulis* were washed out twice (10 min, 1000 rpm) from the fixing solution with TBS and suspended in lectin solution. After the 30-min incubation in the lectin solution the hemocytes were washed off twice with TBS, suspended in a small volume of the anti-fade medium and placed on object glasses. For control preparations, the corresponding monosaccharide at the 0.1 M final concentration was added in the lectin solution 30 min before addition of the hemocyte suspension.

The preparations were examined using a Lyumam-IZ fluorescence microscope (LOMO, St. Petersburg) with a set of the color filters providing excitation of fluorescence in the spectral range of 450–500 nm.

RESULTS

Results of the performed experiments are presented in Table 2. The main terminal sacharides of glycocalyx of *L. littorea* hemocytes are N-acetyl-galactosamine and D-mannose/D-glucose, all littorina hemocytes being equally able to bind lectins ConA and HPA. Hemocytes of *M. edulis* differ considerably from hemocytes of *L. littorea* by types of monosaccharides in composition of the glycocalyx. It is quite possible that most terminal carbohydrates of the mussel hemocyte glycocalyx are N-acetylated sacharides specifically recognized by HPA and WGA. However, it is essential that the mussel hemocyte population is heterogeneous with respect to their interaction with lectins. Only a part of the mussel hemocyte population has receptors for HPA binding on their surface, whereas all littorina hemocytes are equally able to interact with this lectin. On the mussel hemocyte membrane, sites for WGA binding are also revealed; however, in this case, lectin reacts equally with all hemocytes. In

Table 2. Interaction of lectins with surface of *L. littorea* and *M. edulis* hemocytes as well as with the tegument surface of *H. elongata* at different stages of the parasite life cycle

Lectin	Specificity	Hemocytes of		Rediae of *H. elongata*	Cercariae of *H. elongata*	Metacercariae of *H. elongata*
		M. edulis	*L. littorea*			
WGA	GlcNAc	+	–	–	+ (rec.)	–
HPA	GalNAc	+	+	+	+ (rec.)	–
ConA	D-Man	–	+	+	–	–
	D-Glc					
BSL	D-Gal	–	–	–	+	–
	GalNAc					
WFL	GalNAc	–	+	+	–	–

Note: Signs "*plus*" and "*minus*"—the presence or the absence of lectin binding with the surface of hemocytes or trematode tegument; *rec.*—lectin binding only in certain receptor zones near abdominal and oral suckers of cercariae.

the case of the lectin preincubation with corresponding monosaccharides, an inhibition of the lectin binding with the cells surface was observed.

Study of the surface of *H. elongata* tegument at different stages of life cycle with use of labeled lectins has shown that the main terminal saccharide residues of glycocalyx are as follows: in rediae—N-acetylgalactosamine, N-acetylglucosamine, D-mannose/D-glucose, while in cercariae—D-galactose and N-acetylated saccharides. It is to be noted that all lectins interacting with the redia tegument are bound uniformly throughout the entire tegument area, whereas such uniform binding is not typical of cercariae. Only one lectin, BSL, interacts with the entire surface of the larva tail and body, whereas two others, HPA and WGA, are bound mainly in certain regions around the oral sucker. None of the tested lectins interact with the surface of metacercariae. In all cases the lectins binding was inhibited by addition of free monosaccharides to the medium.

Thus, there are appreciable changes of carbohydrate pattern of the tegument surface during the development cycle of *H. elongata*. Both the number of lectins able to bind with the tegument surface and the character of their binding with the parasite surface changes at transition from rediae to cercariae.

By the types of monosaccharides exposed on the glycocalyx surface, rediae of *H. elongata* turned out to be extremely similar with littorina hemocytes. Three lectins, WFL, HPA, and ConA, interact both with the redia surface and with the membrane of *L. littorea* hemocytes, whereas hemocyte membranes of the two mollusc species have common binding sites only for HPA.

DISCUSSION

Only three out of five tested lectins—ConA, WFL, and HPA—have been found to interact with the surface of *L. littorea* hemocytes, the population of cells of the *Littorina* hemolymph being homogeneous with respect to ability to bind lectins. The presence of sites for ConA binding has been shown in studying hemocytes of pulmonate mollusc *Lymnaea stagnalis* [12–13], *Bulinus africanus* [14], and *B. glabrata* [15]. The absence of ConA interaction with hemocytes of *B. glabrata*, which was described by Zelck and Becker [16], most likely is caused by that the authors have used calcium-free buffer in their experiments, although it is known that ConA requires the presence of divalent ions in the medium for sacharide binding [17]. Lectin of the roman snail also is able to interact with glycoproteins of the plasma membrane of hemo-

cytes of *B. glabrata* [16] and *L. stagnalis* [12], but in the latter case only 10–20% of the whole population of hemocytes of the pond snail bound HPA. This can indicate that despite morphological similarity, hemocytes of *L. stagnalis* represent a population that is more differentiated functionally in comparison with cells of the littorina hemolymph.

Mussel hemocytes also are able to bind HPA to a various extent, whereas lectin from wheat germs interacts with all cells. These data agree completely with the results obtained at studying *M. edulis* hemocytes with use of lectins labeled with colloid gold [18]. However, no clear relationships between the morphological type of the cells and their ability to bind HPA have been revealed. Probably, the HPA-positive cells represent a subpopulation of granulocytes carrying granules of a small diameter [18].

During realization of the life cycle of *H. elongata* there occurs a change of carbohydrate determinants of the glycocalyx surface. This phenomena is characteristic of all species of trematodes studied in this aspect—*S. mansoni* [19–23], *S. margrebowiei* [24], *Trichobilharzia ocellata* [25], and *T. szidati* [26]. The change of the tegument glycoprotein composition seems to reflect change of the strategies used by trematodes to prevent attacks of the internal defense system of the host at different stages of the life cycle. Thus, in the case of *H. elongata*, of especial attention is to be paid to similarity in pattern of terminal saccharides of the tegument glycocalyx of daughter rediae and of glycocalyx of hemocytes of the mollusc-host *L. littorea*. At this stage of the life cycle the carbohydrate mimicry is likely to be one of the main mechanisms preventing adhesion of the littorina hemocytes to the parthenita tegument. This suggestion can be confirmed by that the *L. littorea* hemocytes are not able to adhere *in vitro* to the tegument of the *H. elongata* rediae fixed with formaldehyde. Apparently, the carbohydrate mimicry plays a certain role in prevention of attack of effector cells of the vertebrate and human immune system against maritae of trematodes *S. mansoni* [3, 27], *F. gigantica*, and *F. hepatica* [4] that carry on their surface oligosaccharides analogous to carbohydrate chains of some glycoproteins of the definitive host. Unfortunately, at present there are no clear evidences for the use of this mechanism by trematodes at interaction with defense system of the intermediate host. Only for the *B. glabrata—S. mansoni* system, use of histochemical methods with labeled lectins allowed demonstrating a significant similarity of daughter sporocysts and tissues of the host hepatopancreas, but at the same time there also were revealed determinants characteristic only of the parasite surface [28]. Probably, when trematodes use the carbohydrate mimicry, the complete coincidence of the terminal saccharide pattern of the worm tegument and the host tissues is not obligatory, while the key factor could be the presence of specific saccharides representing some "self"-marker for the molluscan defense system acting similarly to molecules of the main histocompatibility complex of the vertebrates immune system [29].

The tegument surface of *H. elongata* cercariae differs essentially from the mollusc-host hemocytes by the set of sacharides; moreover, distribution of some sacharides on the larva body surface is not uniform. Thus, sites of WGA and HPA binding are located mainly near the oral sucker. The non-uniform distribution of receptors of SBA (soy bean lectin) and WGA has also been shown in cercariae of *T. szidati* [26]; in a similar way, lectin from *Anguilla anguilla*, that binds specifically fucose residues interacts only with the surface of the *S. mansoni* cercariae body, whereas the tail of the larva does not have receptors of this lectin [30]. Cercariae seem to use other mechanisms of interaction with the host defense system; adhesion of hemocytes to the tegument at this stage of the life cycle can be hampered by motility of the larva and, probably, by molecular masking. The latter strategy of defense is also characteristic of cercariae of *T. ocellata* [31]. Further, at transition to metacercariae, the parasite surface loses completely the ability to interact with lectins. If to take into consideration the relative inactivity of the cyst surface and the low metabolic activity of the parasite at this stage, it can be suggested that the successful survival of the larva is provided only by its localization in the blood vessel-poor muscle tissues of the second intermediate host. An argument in favor of this suggestion can be the fast encapsulation of the parasite by the mollusc hemocytes *in vitro* on transfer of metacercariae directly into the mussel hemolymph.

Thus, in the considered *L. littorea–H. elongata* system, the carbohydrate mimicry seems to play a

certain role in interaction with the host only at the stage of parthenogenetic generations. At transition to cercariae and further to metacercariae there occurs a change of the main mechanisms enabling the parasite to avoid activation of the host defense system and attack of hemocytes.

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