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ENCAPSULATION RESPONSE FROM THE HEMOCYTES OF ESTUARINE CLAM *MERETRIX CASTA*

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ABSTRACT

The encapsulation response of hemocytes was specific towards the positively charged DEAE-Sepharose CL-6B beads indicating the predominance of negative charges on the hemocyte surface. The hemocytes were incubated with beads suspended in plasma and HCM for enhanced encapsulation response. The beads suspended in HLS showed reduced encapsulation response when compared to control. This clearly shows the opsonic role present in the hemolymph *Meretrix casta*.

KEYWORDS

Meretrix casta, encapsulation, hemocyte, clam, bivalve, Mollusc.

INTRODUCTION

In invertebrates, encapsulation is the common immune defence reaction towards foreign bodies, including multicellular parasites, which enter the haemocoel and are too large to be phagocytosed [1]. In general, a capsule of haemocytes encloses the foreign body and cytotoxic products, such as degradative enzymes and free radicals, are released by the haemocytes in an attempt to destroy the invader. This immune defence reaction has been most extensively studied in insects and research has shown this process to be highly complex [2], involving a diversity of cellular and molecular processes [3-8]. Physicochemical properties such as surface charge and hydrophobicity also influence parasite–haemocyte interactions [9-12] however, these factors have received less attention than the highly specific biochemical and molecular mechanisms.

Since the 1920s, scientists have shown considerable interest in the biological relevance of surface charge within vertebrate systems [13], and there is much evidence to support a role for surface charge in immunological based reactions. For example, sialic acids, which are present on leucocytes to help prevent non-specific interactions between cells, are removed upon cellular activation during early 'nonself' recognition, resulting in a decreased cell surface negative charge and unmasking of further cell receptors and ligands. This increases cellular interactions and leads to effective immune defence [14-16]. Such a reaction highlights the

synergistic action of non-specific electrostatic forces and highly specific receptor–ligand interactions.

The multicellular parasite system in invertebrates is the trematode blood fluke, *Schistosoma mansoni* and its intermediate host, the gastropod mollusc, *Biomphalaria glabrata* [17-19]. Marine bivalve molluscs are also common hosts to multicellular parasites, including trematodes [20] however, information on host resistance mechanisms to such parasites is very limited, despite the growing aquaculture industry and the high commercial value of molluscan shellfish [21]. Cheng and Rifkin, [22] who recognized the high prevalence of metazoan parasites in marine bivalves, examined the host response to these parasites histologically, and proposed five different types of encapsulation. Research, however, has not continued into understanding the dynamics of capsule formation in bivalve molluscs.

In molluscs, it is also known that there is an elevated level of lysosomal enzymes in the microenvironment of the capsule which may or may not contribute to the parasite death [23]. Therefore, a detailed study of this response under *in vitro* conditions may enable better understanding of not only the functional ability of molluscan hemocytes, but also the mechanism and the involvement of any defense molecules in the host during cell-mediated encapsulation process.

MATERIALS AND METHODS

Experimental animals

Specimens of *Meretrix casta* were collected from the muttukadu estuary, Chennai, India. In the laboratory, all clams were maintained in seawater (30‰; 26 ± 2 ^{o}C) with continuous aeration until use.

Preparation of test sample

Plasma: The hemolymph sample was collected and centrifuged (58 x g, 10 min, 4^{o}C) in Heraeus primoR. The supernatant (plasma) was used for suspending the chromatographic beads.

Hemocyte Lysate Supernatant (HLS): The hemocyte pellet obtained after removal of plasma was resuspended in TBS-I (50 mM tris, 390 mM NaCl, and pH 8.4, 840 mOsm) and sonicated in Labsonic 2000 ultrasonicator (B.Braun, Germany). The cell homogenate was centrifuged (2,576 x g, 10 min, 4^{o}C) and the supernatant (hemocyte lysate supernatant), was used for suspending the chromatographic beads.

Hemocyte conditioned medium (HCM): Hemolymph sample (300 µl) spread on a glass Petri plate to prepare hemocyte monolayer. After allowing the hemocytes to settle and attach on the plate for 10-15 min. The supernatant was carefully removed by aspiration and the hemocyte monolayer was immediately rinsed once with TBS-II (50 mM tris, 453 mM NaCl, 0.25 mM $CaCl_2$, pH 8.4, 840 mOsm) and then with TBS-I. Finally, the monolayer was overlaid with TBS-I and all the cells were completely covered with buffer. The plate was closed with a lid and left undisturbed at 23℃. After 1 h, the plate was tilted, the supernatant was rapidly collected, centrifuged (2,576 x g, 10 min, 4^{o}C) and the supernatant (hemocyte conditioned medium) were used for suspending the chromatographic beads.

Total hemocyte count (THC): Hemolymph sample (200 µl) was allowed to fill one of the chambers in an improved Neubauer hemocytometer by capillary action and the hemocytes were allowed to settle for 5 min. As described for counting of WBC [24].

Preparation of sepharose beads: Three types of commercially available chromatography agarose-based beads, namely sepharose CL-6B (neutral), CM sepharose CL-6B (negatively charged) and DEAE sepharose CL-6B (positively charged) were suspended in 0.9% saline and washed extensively in the same medium. The diameter of the wet sepharose beads were 45 to

165 µm and beads from each type were resuspended in saline. After a thorough mixing, the bead suspension was left undisturbed for 15 min to allow the beads with larger size to settle down passively. Subsequently, the bead suspension was aspirated from the column of suspension. The beads thus recovered had a narrow diameter range of 75 to 100 µm and were resuspended in TBS-I. The number of beads in each suspension was counted under Carl Zeiss Axiolab phase contrast microscope and the count was adjusted to 100 to 150 beads and stored at 10^{o}C until use.

Suspension of beads in test samples: A 100 µl aliquot of DEAE Sepharose CL-6B beads suspension was allowed to settle in Eppendorf tubes and then the supernatant was completely removed. The beads were resuspended in plasma, HLS, or HCM.

Isolation of hemocytes: Two aliquots of 300 µl hemolymph sample (TBS-I) was diluted immediately, transferred to prechilled polypropylene tube and centrifuged. The supernatant was discarded and the concentrated hemocytes were resuspended in 300 µl of TBS-I. This hemocyte suspension was used for all *in vitro* encapsulation assays.

Encapsulation assay: Encapsulation assays were performed in V-bottom microtiter plates (Greiner, Nürtingen, Germany). Hemocyte suspension was mixed with sepharose bead suspension in each well and incubated for 45 min, 23^{o}C with gentle mixing at 15 min interval. The entire volume from each suspension was spread on a glass slide and left in a moist chamber for 10 min, 23^{o}C to allow the beads to settle on the slide. After placing a coverslip the samples were examined under a Carl Zeiss Axiolab phase contrast microscope.

Effect of plasma, HLS or HCM: To determine the effect of plasma, HLS or HCM on encapsulation response of washed hemocytes was incubated with beads suspended in undiluted plasma, HLS or HCM and suspended in buffer.

Hemocyte viability: The viability of hemocytes in monolayers was determined using the trypan blue dye exclusion test following [24].

Statiatical analysis: The difference in the encapsulation response of hemocytes in control and each test condition was tested for statistical significance using paired sample Student's t-test [25].

RESULTS

In vitro encapsulation response: The ability of *Meretrix casta* hemocytes to encapsulate chromatography beads (Sepharose CL-6B) with different surface characteristics were tested *in vitro* (Table 1). The washed hemocytes of *Meretrix casta* suspended in iso-osmotic (TBS-I) and incubated with beads suspended in the same buffer, the hemocytes never showed any association with native or CM Sepharose beads. By contrast, the washed hemocytes intensely encapsulated DEAE Sepharose CL-6B beads and this cellular response was over 72% (Table 2).

Effect of plasma: The washed hemocytes of *Meretrix casta*, encapsulated 69% of the total beads suspended in iso-osmotic buffer (TBS-I). On the other hand, when these hemocytes were incubated with beads suspended in plasma, they intensely encapsulated DEAE Sepharose CL-6B beads and the frequency of this response enhanced significantly to (77%) in the presence of plasma ($p<0.001$; Table 3) compared to that of beads suspended in buffer alone.

Effect of HLS: The washed hemocytes incubated with beads suspended in HLS, encapsulation response was reduced in the presence of HLS (57%) compared to that of control (70%). Thus this suppression in cellular encapsulation response was statistically

significant as compared to control ($p<0.001$; Table 4).

Effect of hemocyte conditioned medium: The washed hemocytes encapsulated 70% of the total beads counted. When the beads were suspended in conditioned medium harvested from the hemocytes and incubated with washed hemocytes, the encapsulation response was 75% (Table 5). The difference observed in the increased encapsulation response of hemocytes between beads suspended in iso-osmotic TBS-I and the hemocyte conditioned medium was statistically significant ($p<0.001$).

Table 1.
Physicochemical properties of synthetic encapsulation targets*

Target	Size (diameter;µm	Matrix	Functional group	Charge
DEAE Sepharose CL 6B	45-165	Agarose	Diethylaminoethyl	Positive
CM Sepharose CL 6B	45-165	Agarose	Carboxymethyl	Negative
Sepharose CL 6B	45-165	Agarose	None	Neutral

* As stated by Sigma-Aldrich Company Ltd, USA.

Table 2.
In vitro encapsulation response of Meretrix casta hemocytes against chromatographic Sepharose CL-6B beads.

Beads used	Encapsulation response (%)@
Sepharose CL-6B	-
CM Sepharose CL-6B	-
DEAE Sepharose CL-6B	72 ± 4

@ Data represent mean (±SD) of ten determinations from ten clam.
- No association

Table 3.
Effect of plasma on in vitro encapsulation response of Meretrix casta hemocytes against DEAE Sepharose CL-6B beads.

Suspension of beads (%) @	Encapsulation response
Buffer (Control)	70 ± 4
Plasma	77 ± 5*

@ Data represent mean (±SD) of ten determinations from ten clam.
** The difference between control and test beads suspended in plasma was statistically significant ($p<0.001$)*

Table 4.
Effect of HLS on in vitro encapsulation response of Meretrix casta hemocytes against DEAE Sepharose CL-6B beads.

Suspension of beads (%) @	Encapsulation response
Buffer (Control)	70 ± 4
HLS	57 ± 5*

@ Data represent mean (±SD) of ten determinations from ten clam.
** The difference between control and test beads suspended in HLS was statistically significant (p<0.001)*

Table 5.
Effect of hemocyte conditioned medium (HCM) on in vitro encapsulation response of Meretrix casta hemocytes against DEAE Sepharose CL-6B beads.

Suspension of beads (%) @	Encapsulation response
Buffer (Control)	70 ± 4
HCM	75 ± 4*

@ Data represent mean (±SD) of ten determinations from ten clam.
** The difference between control and test beads suspended in HCM was statistically significant (p<0.001)*

DISCUSSION

The hemolymph of the marine estuarine clam *Meretrix casta* does not coagulate *in vitro*, only hemocyte aggregation occurs presumably to maintain hemostasis, as reported for other molluscan species [26]. Such cellular aggregation could be easily prevented by diluting the hemolymph sample with iso-osmotic buffer during hemolymph collection [27] which enabled to harvest hemocytes in native form. In this study, an attempt has been made to assess the hemocyte-mediated cellular immune responses i.e. encapsulation a most important host defense that operates against foreign objects which are too large to be phagocytosed by single blood cells [28]. Several investigators have subsequently demonstrated, both *in vivo* and *in vitro*, this as an easily observable defense response in a few groups of invertebrates, and most frequently in insects and crustaceans [29].

Many earlier *in vitro* studies have successfully utilized chromatographic gel matrices such as Sephadex or Sepharose beads with varying surface charge as foreign targets to elucidate the underlying mechanism of cellular encapsulation response by insects and crustaceans. In the event of this response, the hemocytes interacted preferentially or more intensely with the surface of the positively-charged beads [30], negatively-charged beads [31], neutral and positively-charged beads [32], positively- and negatively-charged beads or with beads regardless of the types of surface charge [10]. Furthermore, several studies have provided direct or circumstantial evidence for the potential role of hemocyte-derived soluble factors in promoting the encapsulation response of hemocytes [33]. Occasionally, such factors with encapsulation-promoting or regulating activity have been detected in the plasma of a few insects [34-36].

The hemocytes of *M.casta* selectively encapsulated only the positively-charged (DEAE

Sepharose CL-6B) beads as reported in insects [30,37] suggesting that the hemocytes possess net negative charges on their surfaces which apparently facilitate selective and direct attachment of hemocytes to the positively-charged groups on DEAE Sepharose beads resulting in initiation of encapsulation process.

The plasma prominently promoted and often intensified the encapsulation response of the hemocytes, implicating the presence of opsonic factor with encapsulation-promoting activity in the plasma of *M.casta* as reported in few mollusc [38-41] and insect [34-36]. The identity of this plasma factor is unknown at present and this observation indicate the capability of *M.casta* hemocytes to encapsulate specific targets. On the other hand, the presence of HLS abrogated the encapsulation response of *M.casta* hemocytes suggesting for the presence of encapsulation-inhibiting factor in the hemocytes and it could be envisaged that this unique factor plays a key role in regulation of cellular encapsulation process in *M.casta*.

The hemocytes showed an enhanced level of encapsulation response against the beads suspended in conditioned medium harvested from the hemocytes implicating the *in vitro* release of encapsulation promoting factor by the hemocytes, probably stimulated by the adherence and spreading activity of this hemocyte on the surface of the Petri plates used in this study to harvest the hemocyte conditioned medium. It could be speculated that hemocytes of *M.casta* may possess both encapsulation-promoting and inhibiting molecules and only the encapsulation-promoting molecules are released into the surrounding medium by the hemocytes. The overall outcome of this *in vitro* study on cellular encapsulation response in *M.casta*. The plasma contains mainly encapsulation-promoting factor or it may also contain encapsulation-inhibiting factor. The mechanical destruction of the hemocytes possess only encapsulation-inhibiting factor leading to suppression of this response and the hemocytes are able to release molecules that can always enhance encapsulation response which is an important event required for the destruction of the invading organism.

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