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FUNCTIONAL ANALYSIS OF MOULT RELATED HEMAGGLUTININS ISOLATED FROM THE SERUM OF INTERMOULT AND POSTMOULT STAGES OF THE INTERTIDAL MOLE CRAB *Emerita asiatica*

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ABSTRACT

The role of moult-related hemagglutinins, isolated from intermoult (C) and postmoult (A_2) stages of moulting on the immune responses of hemocytes of E. asiatica was examined in the present study. The hemolymph of E. asiatica was found to contain three hemocyte morphotypes, which were identified based on the presence and abundance of cytoplasmic granules. They are the hyaline (12%), semigranular (38%) and granular (50%) hemocytes. Functional analysis performed in vitro unambiguously revealed that the serum and agglutinins isolated from intermoult (C) crabs showed opsonic function by facilitating the phagocytic uptake of human B RBC by hemocytes of mole crab. In this opsonophagocytic process, the serum agglutinin not only serves non-self-recognition function but also acts as a bridge between hemocytes and foreign targets which eventually stimulated phagocytic response of the hemocytes. The phagocytic response of these hemocytes was not significantly altered upon pretreatment of target human B RBC with serum or purified serum agglutinin obtained from postmoult (A_2) crabs revealing that neither the serum nor the purified serum agglutinin obtained from A_2 stage could express opsonic property with hemocytes from crabs at C stage against human B RBC. Thus, the present study unambiguously demonstrates the role of moulting in modulating circulating opsonin, in order to withstand the susceptibility to infectious microbes or the physiological stress imposed during the process of moulting.

Keywords: Mole crab, Moulting, Hemagglutinins, Opsonins.

I INTRODUCTION

The moulting cycle is one of the main intrinsic factors that requires modulation of immune system of crustaceans in order to resist the vulnerability to pathogens. In earlier studies the immune tools commonly used to evaluate the immune ability of crustaceans during moult stages include circulating hemocyte counts, phenoloxidase activity, respiratory burst, phagocytic activity, and clearance efficiency. Earlier studies related to variations in cellular components during moulting includes the hemocyte profile, respiratory burst activity resulting in production of superoxide anions, phagocytic ability of the hemocytes as well as clearance efficiency against impending threat of pathogenic bacteria.

Previous studies have reported a variation in granulocyte production (1), production of hyaline cells (2), and total hemocyte count (THC), (3,4) in isopods & shrimps, at different moulting stages. The authors also studied the susceptibility of these shrimps to vibriosis a common bacterial disease often reported among prawns and shrimps, which was found to be more susceptible during premoult than intermoult. Thus, it was clearly demonstrated that a high THC at premoult could not offer good resistance to the shrimp against vibriosis.

Studies on modulations in cellular immune components were also carried out (5,6 and 7) in shrimps. These studies have clearly indicated the modulation of cellular immune components as well as their functionality upon bacterial challenge at moulting stages.

The status of various humoral immune components including phenoloxidases (4,5,6), antibacterial proteins (8) as well as agglutinins have been highly influenced by the phenomenon of moulting in crustaceans. Thus, the hemolymph soluble as well as hemocytic immune components of decapod crustaceans behave differentially to different moult stages, possibly to meet the differential non-self-challenges to these animals living in different habitats.

Clearance of non-self from circulation and the phagocytic ability of blood cells are the two vital immune responses exhibited by these shell fishes (9,10,11) and there appears to be a clear correlation between variations in cellular immune components during moult stages with the level of functional immune response such as phagocytosis and clearance of non-self in majority of the decapod crustaceans studied.

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Although, many humoral substances have been either demonstrated or implicated to the immune responses of these crustaceans, including lectins, PO, ROS, antibacterial proteins etc. (12,13,14,15,16), many investigators have shown conclusively the opsonic role played by serum agglutinins both in clearance of non-self-such as bacteria as well as phagocytosis of a variety of target cells (11,17,18,19,20,21). However, such studies on the role of hemolymph agglutinins during moulting process, when most of these crustaceans are susceptible to microbial infections, as well as their role in immune functions such as phagocytosis, is limited.

All the above-mentioned studies lend support to the fact that immune molecules do undergo modulations with respect to physico-chemical changes occurring in the organism. Besides, some of these studies also revealed the multiplicity in agglutinins during such physiological changes, but not their functional involvement in such activities. Studies on multiplicity of serum agglutinins and their role in immune responses especially during physiological stress situations like moulting among crustaceans are wanting.

II. MATERIALS AND METHODS

2.1 Buffers and other solutions

Four different types of Tris Buffered Saline (TBS) were used. Tris HCl buffer (50 mM, pH 6.8, 7.5), TBS–II (50 mM $CaCl_2$; pH 7.5) TBS-IX (50 mM Tris–HCl, 450 mM NaCl, 50 mM dextrose, 5 mM KCl,2 mM $MgCl_2$ (pH 7.5, 1050 mOsm) TBS-X (50 mM Tris-HCl, 100 mM NaCl; pH 7.5, 290 mOsm) were prepared according to (23). Saline (0.9%), Alsever's solution, Anticoagulant buffer (ACB) (L-Cysteine (ACB, pH 7.5, 1050 mOsm) was also prepared. Phosphate buffered saline (PBS) pH 7.2) was prepared according to (22). Trypan blue dye solution (0.1% in TBS IX), 1% glutaraldehyde solution in PBS, and 2.5% Glutaraldehyde in TBS was prepared prior to use.

2.2 Hemolymph collection and preparation of hemocyte monolayers

Hemolymph sample (50 µl) was collected from the arthroidial membrane by inserting a 23G needle attached to a clean, sterile syringe containing 1 ml of ice-cold cysteine ACB (pH 7.5, 1050 mOsm). The syringe was gently but rapidly rotated to enable thorough mixing of the hemolymph sample, which was immediately transferred to a pre-chilled polypropylene tube. This hemolymph sample containing suspended hemocytes was spread over an area of 2 cm^2 on an alcohol-washed, clean, dry glass slide. The hemocytes were allowed for 30 min at 23°C to attach and extensively spread on the slides in a moist chamber to obtain hemocyte monolayers.

2.3 Viability of hemocytes

The viability of hemocytes in monolayers was determined using trypan-blue dye exclusion technique following (23). Briefly, 6 pairs of hemocyte monolayers were prepared using hemolymph samples from different mole crabs belonging to the intermoult stage, as described earlier. At a given time, point (60, 90, and 120 min), the medium covering a pair of monolayers was drained by gently tilting the slide and the hemocytes were immediately overlaid with 100 µl of trypan-blue solution and left in a moist chamber for 5 min at 23°C. The excess dye was gently flushed away using iso-osmotic TBS. The number of stained (dead) and unstained (live) cells were counted in at least 25 random microscopic fields in each monolayer under bright field optics of a Carl Zeiss Axiolab microscope. The percentage of viable hemocytes in the monolayer was calculated as follows,

$$\text{Percent viable cells} = \frac{\text{Number of live cells counted}}{\text{Total number of cells counted}} \times 100$$

2.4 Preparation of erythrocytes for functional analyses of isolated agglutinins

Human B blood samples were collected in Alsever's solution, and the RBC harvested from this blood sample were fixed in glutaraldehyde following (24). Briefly, RBC from this blood sample were washed thrice in 0.9% saline and once in PBS (pH 7.2) by centrifugation (400 x g, 5 min, RT). The washed RBC pellet (400 µl) was then fixed by suspending in 10 ml of 1% glutaraldehyde solution and let for 1 h at RT. The fixed erythrocytes were extensively washed in PBS, finally resuspended in saline, and stored at 10°C until use.

2.5 Preparation of hemocytes for in vitro phagocytosis assays

The hemocyte monolayers, for phagocytosis assays, were prepared following the procedure of (25). Briefly, hemolymph sample (50 µl) collected in 1 ml of ice-cold cysteine ACB was immediately transferred to an eppendorf tube held on ice. From this, 50 µl of hemocyte suspension was spread over an area of 2 cm^2 on an alcohol-washed, dry microscopic glass slide and left in a moist chamber for up to 30 min at 23°C to facilitate all hemocytes to settle, attach and spread extensively on the glass slide. After ensuring complete spreading of the hemocytes, the resulting hemocyte monolayer was washed by gently flushing with iso-osmotic TBS, in order to remove the plasma. Each washed monolayer was overlaid with the same buffer and left for 5 to 10 min in the moist chamber. Under this condition, all hemocytes tend to attain a rounded configuration, but remained firmly attached to the glass slide. Subsequently, these hemocyte monolayers were used for in vitro phagocytosis assays.

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2.6 Phagocytosis of serum pre-treated Human B RBC

Subagglutinating concentrations of serum was prepared by appropriately diluting the serum from intermoult (C) or postmoult(A_2) E. asiatica (26) with iso-osmotic TBS. Pre-treatment of RBC with serum was performed by suspending 50 µl of washed and pelleted RBC in 10 ml of the diluted serum and incubated for 30 min at 26°C with frequent gentle shaking. After incubation, the RBC were washed once by centrifugation (400 x g, 5 min, 4°C), in iso-osmotic TBS to remove unbound serum components and finally resuspended in the same buffer as 1.5% v/v suspension. Four hemocyte monolayers were prepared using hemolymph samples obtained from mole crabs in the intermoult stage. First pair of monolayers was overlaid with 200 µl RBC pre-treated with diluted serum. The second pair of monolayers was overlaid with untreated RBC suspended in iso-osmotic buffer, which served as control.

2.7 Phagocytosis of Human B RBC pre-treated with isolated agglutinins

In this experiment, RBC were pre-treated with subagglutinating concentrations of agglutinins isolated from the serum of intermoult (C) and postmoult (A_2) stages of E. asiatica. (27). Pre-treatment of RBC was performed by suspending 50 µl of washed and pelleted RBC in 10 ml of the agglutinin solution at subagglutinating concentrations as done with that of serum. Four hemocyte monolayers were prepared using hemolymph samples obtained from intermoult mole crabs. First pair of monolayers was overlaid with 200 µl of RBC pre-treated with subagglutinating concentrations of agglutinins isolated from C or A_2 stages of moulting. The second pair of monolayers that served as a control was overlaid with 200 µl of untreated RBC suspended in iso-osmotic TBS.

2.8 Phagocytosis of HB RBC pretreated with isolated agglutinin in the presence of carbohydrates

The isolated serum agglutinin from crabs at C stage (4 µg protein/ml) was first mixed with an equal volume of iso-osmotic TBS that did or did not contain mannosamine (200 mM) or mannan (1 mg/ml) and incubated for 30 min at 26°C. RBC were then pretreated with agglutinin from C stage (2 µg protein/ml), agglutinin + 100 mM mannosamine, or agglutinin + 100 mM mannan (1 mg/ml) as described earlier. In controls, RBC (untreated) were suspended in iso-osmotic TBS. All these RBC suspensions were incubated for 30 min at 26°C with frequent gentle shaking, washed and finally resuspended in iso-osmotic TBS as 0.5% (v/v) suspension. Hemolymph sample collected from intermoult shrimps was used to prepare hemocyte monolayers. Six monolayers were overlaid with 200 µl of one of the six agglutinin pretreated HB RBC suspension. A pair of monolayers covered with untreated RBC served as controls.

2.9 In vitro phagocytosis assays

All hemocyte monolayers overlaid with different preparations of RBC suspensions were incubated in moist chambers for 30 min at 23°C. The average number of RBC overlaid on each hemocyte monolayer was same in all experiments. The monolayers were then gently flushed with iso-osmotic TBS to eliminate non-ingested RBC and fixed in 2.5% glutaraldehyde (pH 7.2). After placing a glass cover slip on each monolayer, the hemocytes were examined for phagocytosis of RBC with a Carl Zeiss Axiolab phase contrast microscope at a magnification of 40X. The percentage of hemocytes containing one or more RBC was calculated after a careful evaluation of at least 200 hemocytes in random microscopic fields on each monolayer. The mean value representing rate of phagocytosis recorded from monolayers prepared from three individual crabs for each experimental condition is hereafter reported as percentage phagocytosis.

2.91 Statistical analysis

Each test series in an experimental condition was performed using hemocytes from three mole crabs. The difference between control and experimental values or between two experimental values was tested for statistical significance using paired sample Student's t- test (28).

III RESULTS

3.1 Agglutination of RBC by serum agglutinins

The whole serum from C stage (0.672 mg protein/ml) and A_2 stage (0.280 mg protein/ml) agglutinated buffalo RBC with a HA titer of 32 and 128, respectively. The agglutinins isolated from the serum of crabs at C and A_2 stages also agglutinated buffalo RBC, and gave HA titers of 32 at protein concentrations as low as 4 µg and 0.10 µg, respectively. Thus, the minimal amounts of agglutinins isolated from crabs at C and A_2 stages required for complete agglutination of buffalo RBC were 0.127 µg and 0.031 µg, respectively.

3.2 In vitro observations of hemocytes in monolayers

When a sample of hemolymph from intermoult crabs (C stage), freshly collected in ACB containing 1 mM cysteine (pH 7.5), was placed on a glass slide and observed immediately under phase optics, all hemocytes were free, intact and appeared as round to ovoid cells with fine pseudopodial extensions (Fig. 8). At this stage, they were highly refractile with little cytoplasmic details visible. Subsequently, all the hemocytes rapidly attached on the glass slide within 5 min, tend to spread with protoplasmic extensions and eventually most of the cells firmly attached and fully spread within 30 min. Both nucleus and cytoplasmic contents of hemocytes progressively became more clearly defined during spreading in vitro. The alterations in morphology associated with these responses results in a pleiomorphic population of cells that are very different in appearance from those observed immediately following

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extravasation. When the cells were extensively spread, however, three distinct hemocyte morphotypes at different proportion could be identified based on the number, shape and size of cytoplasmic granules: hyaline cells with none or few granules, semigranular cells with moderate and variable number of granules and granular cells fully packed with granules (Fig. 9). Most granular cells spread only moderately as compared with the other two morphotypes. Relative proportions of these three hemocyte morphotypes in the systemic circulation granular cells accounted for nearly 50%, the semigranular and hyaline cells accounted for only 38 and 12%, respectively.

Figure 8

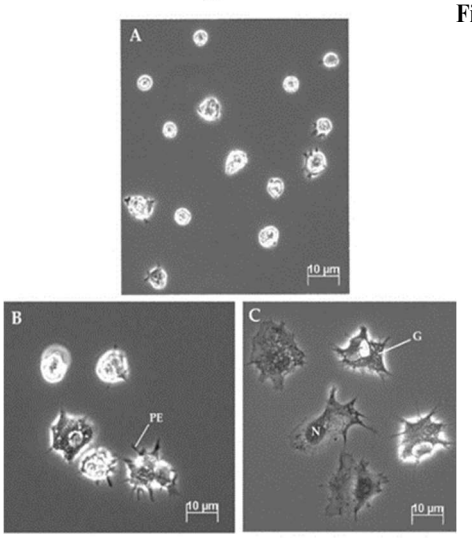

Figure 8. **Phase contrast photomicrographs of live hemocytes of *Emerita asiatica.***

A: Appearance of hemocytes within two min of hemolymph collection in cysteine-anticoagulant buffer. B: The hemocytes showing attachment and successive stages of spreading with protoplasmic extensions (PE) on the glass slide within 10 min *in vitro*. C: Most of the hemocytes fully spread within 30 min forming a hemocytes monolayer. Both nucleus (N) and cytoplasmic granules (G) became more clearly defined in these spread cells.

Figure 9

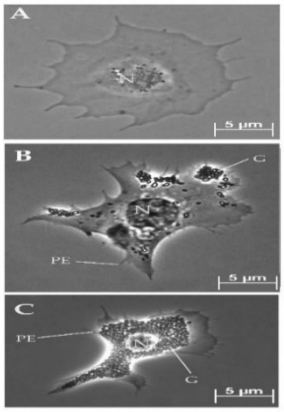

Figure 9. **Phase contrast photomicrographs of extensively spread live hemocytes of *Emerita asiatica.* Based on the number of cytoplasmic granules (G), three distinct hemocyte morphotypes could be identified. A: Hyaline cell with none or few cytoplasmic granules. B: Semigranular cell with moderate and variable number of granules of various shapes and sizes. C: Granular cell whose cytoplasm is packed with granules. N: Nucleus, PE: Protoplasmic extensions.**

3.3 Viability of hemocytes

The monolayers with extensively spread hemocytes from intermoult crabs were maintained in vitro in the ACB for 2 h at 23°C. Under these conditions over 83% of hemocytes were viable for up to 2 h as revealed by trypan blue dye exclusion test (Table 18).

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Table 18. Viability of *Emerita asiatica* hemocytes *in vitro.*

Animal No.	Per cent viability of hemocytes*		
	30 min	60 min	120 min
1.	93.4	88.6	83.7
2.	95.4	90.8	83.7
3.	94.5	89.5	81.5
Mean ± SD	94.7 ± 1.0	89.63 ± 1.1	83.06 ±1.0

* Monolayers of hemocytes were held in anticoagulant buffer at 23^0C

3.4 Preparation of hemocytes for in vitro phagocytosis assays

When the hemocyte monolayers comprising fully spread cells were rinsed and overlaid with iso-osmotic TBS, all hemocytes tend to attain rapidly a rounded configuration within 5 - 10 min. These rounded hemocytes when overlaid and incubated for 15 min with RBCs suspended in buffer, exhibited no or poor attachment of HB RBC to hemocyte surfaces. By contrast, this 'attachment', often as rosettes, was seen remarkably with many rounded hemocytes incubated for 10 min with RBC suspended in serum of E. asiatica (Fig. 10A). Upon extension of the incubation time to 30 min with RBC suspended in serum, it was vividly notable that several hemocytes contained 1 - 5 RBC intracellularly (Fig. 10B), thereby demonstrating phagocytic ability of E. asiatica hemocytes in vitro. In this test system, the ingested RBC appeared darker and markedly lost their surrounding bright rings as compared with free or extracellularly attached RBC. Thus, these apparent differences enabled easy and unambiguous determination of the ingested RBC.

Figure 10

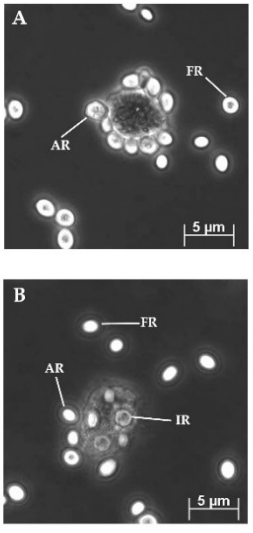

Figure 10. Phase contrast photomicrographs of live hemocytes of *Emerita asiatica.*

A: Hemocyte incubated for 10 min with human B RBC suspended in buffer showing a typical rosette formation. B: Hemocyte (from intermoult crab) in a monolayer incubated for 30 min with HB RBC suspended in iso-osmotic TBS showing phagocytosis. Note that the ingested RBC (IR) appeared darker and markedly lost their surrounding bright rings as compared with free (FR) or attached (AR) RBC.

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3.5 Phagocytosis of serum pre-treated HB RBC

As shown in Figure 11, the phagocytic activity of hemocytes against HB RBC pretreated with subagglutinating concentration of serum from crabs at C stage increased the rate of phagocytosis from a control value of 23 to 35%. The rate of enhanced phagocytic activity with serum - pretreated HB RBC was statistically significant over the control values. When the hemocytes were incubated with HB RBC pretreated with subagglutinating concentration of serum from crabs at A_2 stage (Fig. 12), the phagocytic rate was 24% which was not statistically significant over the control value (20%).

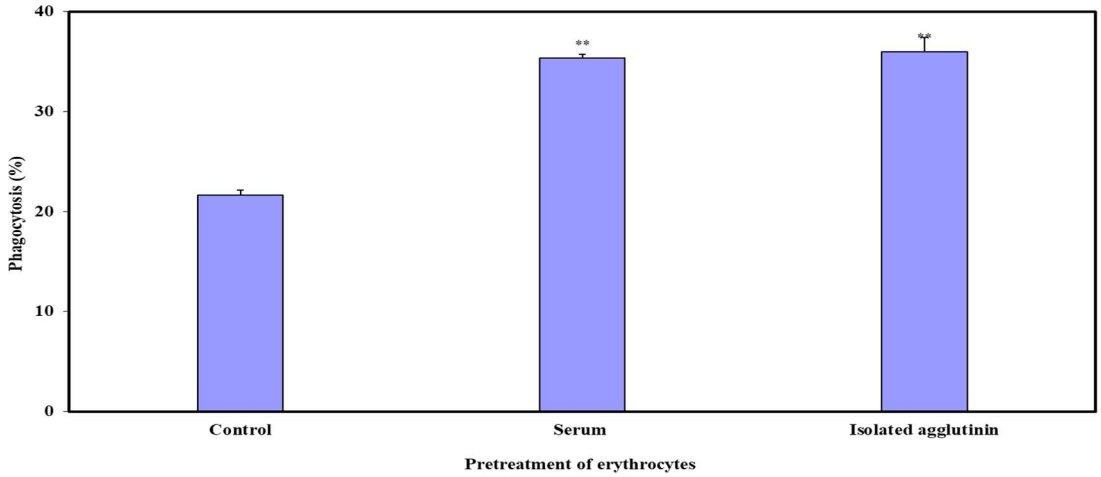

Figure 11. *In vitro* phagocytosis of HB RBC by hemocytes of *Emerita asiatica*. RBC were pretreated with serum from crabs at C stage or agglutinins isolated from the serum of crabs at C stage. Untreated RBC served as controls. Vertical bars represent means (±SD) of phagocytosis rates of duplicate samples from three mole crabs. The difference in phagocytosis rates between control and test hemocyte monolayers incubated with RBC pretreated with serum or isolated serum agglutinin was statistically significant (**p<0.002).

3.6 Phagocytosis of HB RBC pretreated with isolated serum agglutinins from crabs at C and A_2 stages

As shown in Figure 11, when the hemocytes collected from intermoult mole crabs of E. asiatica were exposed to HB RBC pretreated with subagglutinating concentration of agglutinin isolated from stage C crabs, the rate of phagocytosis enhanced from 23% (control) to 36% even at a protein concentration as low as 0.06 µg. This enhancement in the phagocytic rate was statistically significant over control values at p>0.002 level (Fig. 11). When the hemocyte monolayers were incubated with HB RBC pretreated with subagglutinating concentration of agglutinin isolated from stage A_2 (Fig. 12), the rate of phagocytosis enhanced to 27% which was statistically not significant over the control values even at p>0.01 level (20 %).

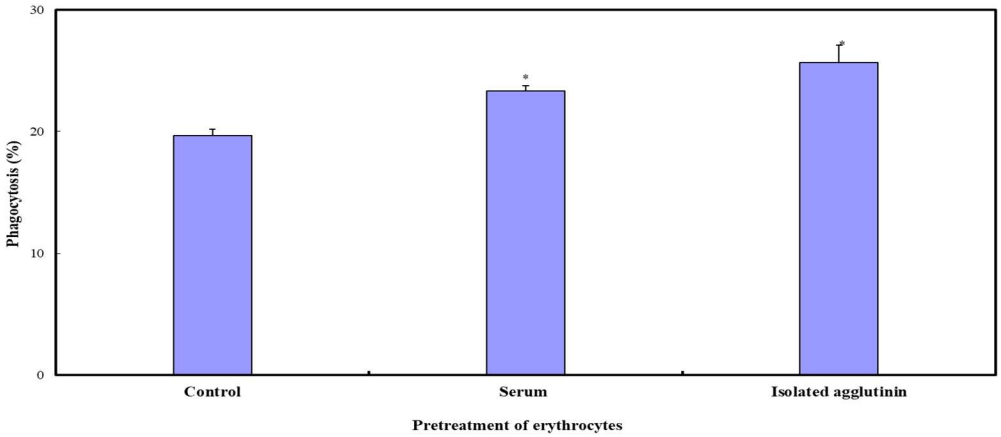

Figure 12. *In vitro* phagocytosis of RBC by hemocytes of *Emerita asiatica*. RBC were pretreated with serum from crabs at A_2 stage or agglutinins isolated from the serum of crabs at A_2 stage. Untreated RBC served as controls. Vertical bars represent means (±SD) of phagocytosis rates of duplicate samples from three mole crabs. The difference in phagocytosis rates between control and test hemocyte monolayers incubated with RBC pretreated with serum or isolated serum agglutinin was statistically significant (*p<0.01).

3.7 Phagocytosis of HB RBC pretreated with isolated agglutinins from C and A_2 stages in the presence of carbohydrates

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The hemocytes in control monolayers incubated with untreated HB RBC expressed the phagocytic response of 20%. The rate of phagocytic uptake of RBC by hemocytes was significantly enhanced ($p<0.001$) to 34% upon exposure of hemocytes to RBC pretreated with agglutinin isolated from the serum of crabs at C stage (Fig. 13). The presence of 100 mM ManN during pretreatment of RBC did not influence the phagocytic activity of hemocytes. On the other hand, mannan concurrently tested at a concentration of 1 mg/ml significantly abrogated the phagocytosis stimulating effects of isolated agglutinin.

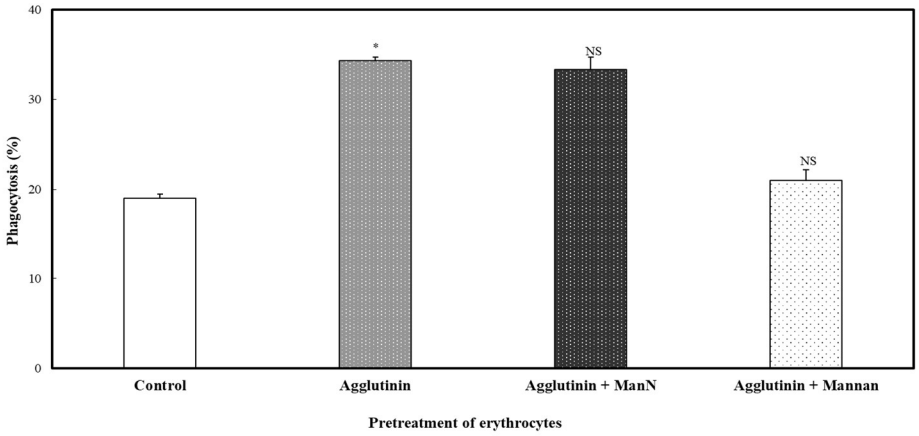

Figure 13. *In vitro* phagocytosis of RBC by hemocytes of *Emerita asiatica*. RBC were pretreated with agglutinin isolated from crabs at C stage in the absence or presence of 200 mM mannosamine (ManN) or 1 mg. ml^{-1} mannan. Untreated RBC served as controls. Vertical bars represent means (±SD) of phagocytosis rates of duplicate samples from three mole crabs. The difference in phagocytosis rates between control and agglutinin alone or agglutinin and agglutinin + mannan was statistically significant (*$p<0.001$). The difference between agglutinin and agglutinin + ManN was not statistically significant (NS: $p>0.05$)

IV DISCUSSIONS

In the present study, the role of moult-related hemagglutinins, isolated from intermoult (C) and postmoult (A_2) stages of moulting on the immune responses of hemocytes of E. asiatica was examined. Since an in vitro test system permits desirable experimental manipulation and conclusive assessment of the hemocytic functions, an advantage lacking in in vivo systems besides having inherent complexity, the response of hemocytes were examined in vitro. The hemolymph of E. asiatica undergoes rapid coagulation upon extravasation and this process essentially involves degranulation and lysis of hemocytes. Therefore, 1 mM cysteine was used as an anticoagulant as a pre-requisite to obtain hemocytes in native form that would eventually enable the in vitro analysis of phagocytic response. The hemocytes harvested using this anticoagulant were free, intact and highly refractile and were able to form monolayers on glass surfaces, and this hemocytic behaviour was considered suitable for analysis of their phagocytic activity in vitro. Over 80% of the hemocytes obtained using cysteine anticoagulant remained viable, a feature conducive for their functional analyses.

The naturally occurring agglutinins in the sera of many invertebrates and prochordates are known to serve as opsonins, and by facilitating phagocytic uptake of target cells precoated with either serum or purified serum agglutinins obtained from intermoult animals. Such functions of agglutinins have been successfully demonstrated in disparate groups of invertebrates including the insects (29), the crustaceans (18,19,21,30,31), the mussels (32,33) and the colonial and solitary ascidians (34,35). A few investigators have also demonstrated that such an opsonic interaction, mediated by recognition and binding to non-self and subsequent phagocytosis, involves certain specific sugar residues of target and the opsonic molecule present in the serum or purified serum agglutinin (12,36). During the course of the present study to investigate the ability of purified serum agglutinin (PSA) isolated from the intermoult crabs (C stage), the hemocytes collected from intermoult crabs were found to avidly phagocytose human B RBC (a response higher than those recorded for buffalo or rat RBC) pretreated with serum or PSA obtained from intermoult crabs compared to untreated RBC. Such an enhancement in phagocytic rates of hemocytes against human B RBC were nearly quenched by the presence of mannan, whereas a non-inhibiting sugar mannosamine failed to inhibit the phagocytosis of either the serum- or PSA- treated human B RBC. Thus, these observations clearly demonstrate that the serum agglutinins of intermoult mole crab E. asiatica serve as opsonic molecules which appear to accomplish opsonic function by bridging hemocytes and the human B RBC leading to enhanced phagocytic response by hemocytes. When human B RBC pretreated with serum or PSA obtained from A_2 stage were presented to hemocyte monolayers prepared from C stage, the phagocytic response of these hemocytes was not significantly altered. These observations clearly show that neither the serum nor the PSA obtained from A_2 stage could express opsonic property with hemocytes from crabs at C stage. This observation together with opsonic property demonstrated by serum or PSA obtained from intermoult mole crabs demonstrate that the opsonic molecules present in the serum of the mole crabs are distinctly related to stages of their moult cycle. It

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would be of scientific interest to inquire into the opsonic role for lectin isolated from A_2 crabs against the hemocytes of the crabs from the same stage.

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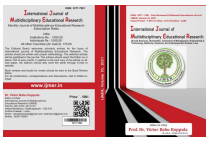
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