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Biological role of the hemolymph lectin of the freshwater crab *Travancoriana charu*

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***Abstract*: The role of the O-acetyl N-glycolyl neuraminic acid (O-acetyl NeuGc) specific lectin isolated and purified from the hemolymph of the freshwater crab *Travancoriana charu* in the innate immunity of the crab was documented using mammalian erythrocytes as antigenic stimulants. The erythrocytes when injected into the hemocoel regardless of their agglutinability and concentration, manifested increase in HA titer at varied time intervals. Augmentation in hemagglutinating activity, post administration of erythrocytes indicates an increase in the circulating lectin. Faster clearance of erythrocytes coated with lectin than the uncoated ones, indicates the role of lectin in the process of elimination of foreign pathogens. Lectins played an eminent role in enhancing hemocyte mediated hemolysis, functioning as an opsonin.**

Keywords:* Hemagglutinin, lectin, induction, clearance, *Travancoriana charu.

I. INTRODUCTION

Lectins are glycan-binding proteins or glycoproteins that recognize the cell surface glycoconjugates of the microorganisms and then induce various immune responses, such as immobilization, phagocytosis, clearance, and encapsulation [1]. Lectins play a crucial role in innate immune response by acting as opsonins, recognizing foreign substances by binding to their carbohydrate components and triggering phagocytosis of pathogens by scavenger cells [2]. Lectins participate in the tagging and exclusion of foreign organisms covered with different carbohydrate receptors [3, 4, 5]. This ability of lectins to decode the stereochemical information carried by carbohydrates enables cells to perform a wide variety of recognition and regulatory processes [6, 7]. Most lectins play a crucial role in diverse biological processes, particularly in host defense mechanisms, inflammation, metastasis [8], apoptosis [9], antiproliferative, antitumor, immunomodulatory [10, 11], antifungal and antiviral [12] activities. Because of their specific recognition of sugar determinants in the wall or the capsule of bacteria, lectins have been suggested to participate in the innate immune response as opsonins, enhancing the rate of phagocytosis of microorganisms exerted by hemocytes. In crustaceans, the circulating hemocytes are crucial in protecting the animal against invading microorganisms by participating in recognition, phagocytosis, melanisation and cytotoxicity [13].

II. MATERIALS AND METHODS

Erythrocyte collection

Blood from various mammalian species (dog, buffalo, goat) were collected directly in sterile modified Alsevier's medium pH 6.1 (30 mM sodium citrate, 77 mM sodium chloride, 114 mM glucose, 100 mg neomycin sulphate and 330 mg chloramphenicol). Before use the erythrocytes were washed thrice by centrifugation at 1500 x g for 5 minutes and resuspended in Tris Buffered Saline (TBS) pH 7.5 as 1.5% erythrocyte suspension.

Injection of erythrocytes

To examine the role of invading pathogens in inducing agglutinin production, different concentrations of mammalian erythrocytes were injected into the hemocoel of crabs. The erythrocytes selected for this study include high hemagglutinating (HA titer=256-512) dog erythrocytes, moderately agglutinating (HA titer=16) buffalo erythrocytes and

low agglutinating (HA titer=2) goat erythrocytes. As a preliminary study, different concentrations (1.5–10%) of the selected erythrocyte suspension in 0.9% sterilized saline was injected into the hemocoel of the crabs and the concentration tolerated by the crab was analyzed by observing the rate of mortality. As the animals could tolerate up to 5% concentration, erythrocyte suspension of 1.5%, 2.5% and 5% in 0.9% sterilized saline was selected for induction experiments. 100 µl of each of the erythrocyte suspension in different concentrations was injected slowly into the soft arthodial membrane between the coxa of the fourth pleopod on the dorsal surface of the carapace. The injection site was blotted with cotton, both before and after the injection of erythrocytes. The crabs that bled at the site of injection were discarded. Care was taken to ensure complete injection of erythrocytes.

Pretreatment of erythrocytes with lectin

The erythrocytes were coated with lectin diluted to subagglutination concentration. For lectin coating 200 µl of washed and packed erythrocytes were resuspended in 20 volumes of lectin (diluted to subagglutination concentration HA=2) and incubated for 1 hour at room temperature. The lectin coated erythrocytes were washed and resuspended in sterilized saline. The erythrocyte suspensions were examined under microscope to ensure the presence or absence of clumps of erythrocytes. Clumps if present were disrupted by gentle vortexing.

Collection of hemolymph samples

To study the effect of injection of erythrocytes on hemagglutinating activity, hemolymph samples were collected at regular intervals following erythrocyte injection and HA assay was carried out with dog, buffalo and goat erythrocytes.

Clearance

Injection of erythrocytes

Dog, buffalo and goat erythrocytes were selected for the clearance study. 100 µl of 1.5% suspension of both lectin coated and uncoated erythrocytes were injected into the crabs. Time taken for clearance was measured by collecting the hemolymph from the crab at regular intervals (every ten minutes) until the erythrocyte was not detected in the circulation. To estimate hemoglobin, 100 µl of the collected hemolymph was added to 700 µl of distilled water and the total volume was adjusted to 1 ml with distilled water.

Estimation of hemoglobin

Hemoglobin content was estimated by Cyamethemoglobin method, using hemoglobin kit (Sigma Diagnostic, India). The Cyamethemoglobin technique is the method of choice selected by the International Commission for Standardization in Hematology (ICSH). To 5 ml of Drabkins solution, 20 µl of the sample or standard was added, mixed well and kept at room temperature (30±2ºC) for 5 minutes. The absorbance was measured against reagent blank at 546 nm in a spectrophotometer. The rate of clearance was noted with both lectin coated and uncoated samples. Hemoglobin content is calculated as follows.

$$\frac{\text{OD of the sample}}{\text{OD of the standard}} x \frac{\text{Concentration of the standard}}{\text{Volume of the sample}} = \mu\text{gHb/ml}$$

Hemolysis

To study the interaction of crab hemocytes on native and lectin treated erythrocytes, the hemocytes were separated using the method of Soderhall and Smith [14]. The dactylus of the crab was cut and the hemolymph was collected in 1.35 ml of ice cold (4ºC) anticoagulant buffer, Citrate EDTA: (trisodium citrate 30 mM, citric acid 26 mM , NaCl 71 mM, glucose 100 mM and disodium EDTA 10 mM). The mixture of hemolymph and buffer was shaken gently to assist rapid mixing and centrifuged at 200 x g for 2 minutes at 4ºC. The hemocyte pellet was then resuspended in 1.5 ml of iso–osmotic buffer (Tris HCl 50 mM, NaCl 156 mM and $CaCl_2$ 1 mM, pH 7.5) used for HA assay. The hemocytes were washed twice to remove the contaminating proteins and then used for hemolysis experiments. The hemocyte suspension was added to lectin coated or uncoated erythrocytes and incubated for 1 hour at room temperature (30±2ºC). The hemocyte mixture was centrifuged at 200 x g for 5 minutes. The supernatant was collected and hemoglobin content was estimated following Cyamethemoglobin method. The control included lectin coated or uncoated erythrocytes with hemocytes.

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III. RESULTS

Hemagglutination profile after injection of dog erythrocytes

Injection of high agglutinating dog erythrocytes enhanced the agglutination activity of the hemolymph. The HA titer varied with time and concentration of the injected erythrocyte. Enhanced HA activity with erythrocytes (dog, buffalo, goat) was noticed between 2-8 hours post injection of various concentration (1.5% / 2.5% / 5%) of high agglutinating dog erythrocytes. Two fold increase in HA titer following injection of 1.5% and 2.5% dog erythrocytes and four fold increase in HA titer following injection of 5% dog erythrocytes was observed. Two to four fold augmentation in HA activity was noticed against buffalo and goat erythrocytes respectively.

TABLE 1: HA titer of the hemolymph of the crab, *Travancoriana charu* following injection of diverse concentrations of erythrocytes

Time (Hr)	0	1	2	4	6	8	16	24	48	72
Erythrocytes injected	**HA titer with dog erythrocytes**									
1.5% Dog	256	256	128	512	256	256	256	256	256	256
2.5% Dog	256	256	128	512	512	256	256	256	256	256
5.0 % Dog	256	256	128	1024	1024	512	256	256	256	256
1.5% Buffalo	256	128	64	512	256	256	256	256	256	256
2.5% Buffalo	256	128	128	512	256	256	256	256	256	256
5.0% Buffalo	256	128	512	512	256	256	256	256	256	256
1.5% Goat	256	256	256	128	512	256	256	256	256	256
2.5% Goat	256	256	128	128	512	512	256	256	256	256
5.0% Goat	256	256	128	512	512	256	256	256	256	256
	HA titer with buffalo erythrocytes									
1.5% Dog	16	16	32	16	16	16	16	16	16	16
2.5% Dog	16	16	16	32	16	16	16	16	16	16
5.0 % Dog	16	16	16	32	64	32	16	16	16	16
1.5% Buffalo	16	32	16	16	16	16	16	16	16	16
2.5% Buffalo	16	32	32	16	16	16	16	16	16	16
5.0% Buffalo	16	64	128	32	32	16	16	16	16	16
1.5% Goat	16	16	16	8	32	16	16	16	16	16
2.5% Goat	16	16	16	32	32	16	16	16	16	16
5.0% Goat	16	16	32	64	32	32	16	16	16	16
	HA titer with goat erythrocytes									
1.5% Dog	2	2	4	2	2	2	2	2	2	2
2.5% Dog	2	2	2	4	2	2	2	2	2	2
5.0 % Dog	2	2	4	8	4	2	2	2	2	2
1.5% Buffalo	2	2	2	2	4	2	2	2	2	2
2.5% Buffalo	2	2	2	2	4	4	2	2	2	2
5.0% Buffalo	2	2	2	2	8	4	2	2	2	2
1.5% Goat	2	2	2	2	4	2	2	2	2	2
2.5% Goat	2	2	2	4	2	2	2	2	2	2
5.0% Goat	2	2	2	4	4	2	2	2	2	2

The values represent the titer observed in five animals at a particular time when analyzed with dog/buffalo/goat erythrocytes. The standard deviation values are not pointed because all values in a particular group are strikingly identical.

Hemagglutination profile after injection of buffalo erythrocytes

Increase in hemagglutination activity with dog erythrocytes was observed between 2-4 hours post injection of various concentrations (1.5% / 2.5% / 5%) of buffalo erythrocytes. Augmentation of HA titer with buffalo erythrocytes was noticed between 1-6 hours post injection and, 4-8 hours post injection when assayed with goat erythrocytes.

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Hemagglutination profile after injection of goat erythrocytes

Administration of low agglutinating goat erythrocytes (1.5% / 2.5% / 5%) resulted in two fold increase in HA activity with dog erythrocytes, 2-4 fold augmentation of HA activity with buffalo erythrocytes and 2 fold increase in HA titer with goat erythrocytes .

Effect of lectin coating on induction

Increase in HA titer was observed 4 hours post injection of uncoated erythrocytes and one hour post injection of lectin coated erythrocytes respectively. Four fold increase in HA titer was noticed after injection of lectin coated erythrocytes compared to the uncoated ones.

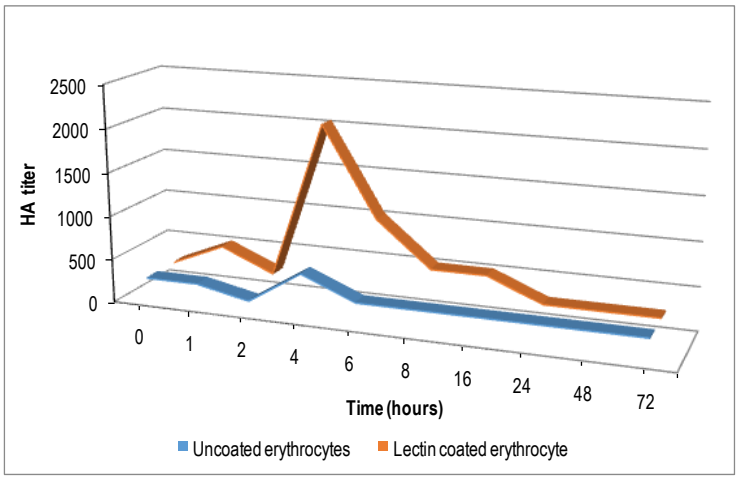

Fig 1: Variation in lectin production following injection of lectin coated and uncoated dog erythrocytes on the HA titer of *Travancoriana charu*

Clearance of erythrocytes

There was a positive relation between agglutinability of different erythrocytes and the time taken for complete clearance of erythrocytes. High agglutinating dog erythrocytes were cleared rapidly (60 minutes) than buffalo erythrocytes (80 minutes) and low agglutinating goat erythrocytes (100 minutes). Lectin coated erythrocytes were cleared much faster than the uncoated ones.

TABLE 2: Time taken for clearance of native and lectin coated erythrocytes

Time in minutes	Hemoglobin content (µg/ml)					
	Dog		Buffalo		Goat	
	Uncoated erythrocytes	Lectin coated erythrocytes	Uncoated erythrocytes	Lectin coated erythrocytes	Uncoated erythrocytes	Lectin coated erythrocytes
10	45.16 ± 0.14	37.5 ± 0.08	45.24 ± 0.05	48.75 ± 0.05	43.75 ± 0.05	46.87 ± 0.10
20	33.69 ± 0.16	23.75 ± 0.26	34.57 ± 0.04	37.56 ± 0.07	40.12 ± 0.05	39.37 ± 0.04
30	26.25 ± 0.08	11.25 ± 0.28	22.51 ± 0.11	21.24 ± 0.05	34.87 ± 0.09	26.62 ± 0.03
40	11.25 ± 0.22	7.5 ± 0.12	18.58 ± 0.11	18.75 ± 0.18	34.12 ± 0.02	15.75 ± 0.10
50	7.8 ± 0.20	0	15.36 ± 0.07	7.43 ± 0.07	28.87 ± 0.11	8.25 ± 0.04
60	3.75 ± 0.12		11.25 ± 0.10	0	22.87 ± 0.02	7.87 ± 0.14
70	0		7.57 ± 0.10		15.75 ± 0.13	6.72 ± 0.10
80			3.69 ± 0.14		13.5 ± 0.08	0
90			0		11.62 ± 0.11	
100					9.37 ± 0.06	
110					0	

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Hemocyte mediated hemolysis

Lectin coated erythrocytes (dog, buffalo, goat) were eminently recognized and lysed by the hemocytes than the uncoated erythrocytes.

Table 3: Effect of hemocytes on hemolysis of lectin uncoated and coated erythrocytes

Treatment	Hemoglobin content (µg/ml)		
	Dog erythrocytes	Buffalo erythrocytes	Goat erythrocytes
Untreated erythrocyte suspension (control)	45 ± 0.03	36.5 ± 0.02	31.62 ± 0.11
Lectin coated erythrocytes without hemocytes	1.3 ± 0.04	1.1 ± 0.14	0.37 ± 0.04
Lectin coated erythrocytes with hemocytes	31.87 ± 0.08	22.4 ± 0.11	18.74 ± 0.13
Uncoated erythrocytes with hemocytes	5.1 ± 0.11	4.5 ± 0.10	3.78 ± 0.02

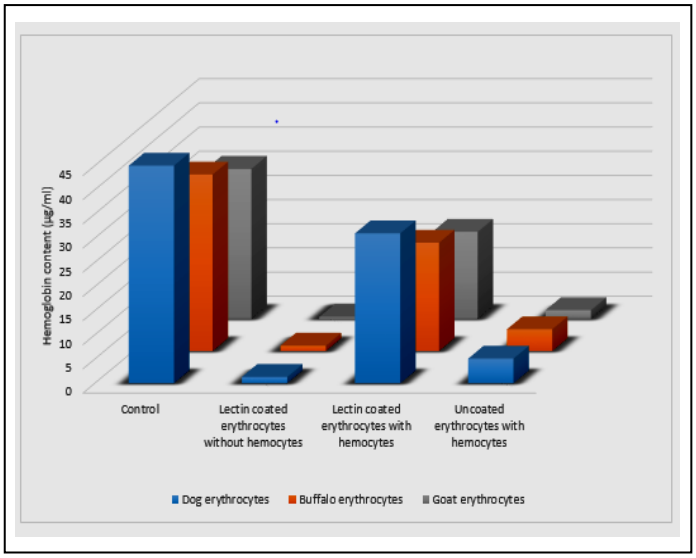

Fig 2: Hemocyte mediated hemolysis of lectin coated and uncoated erythrocytes

IV. DISCUSSION

Invertebrates lack an adaptive immune system but they have developed various defense mechanisms by which they recognize surface determinants on potential pathogens [15, 16]. Recognition of pathogenic microorganisms by lectins is assumed to be due to lectin carbohydrate interaction. Numerous reviews reporting agglutinin involvement in arthropod defense and recognition mechanisms have been reported [17, 18, 19, 20]. The report on the role of lectins in enhancement of cellular immune responses also elaborates the function of lectin not only as recognition molecules but also as opsonins [21].

It has been observed that lectins activate phagocytosis by binding to foreign cells or by increase in lectin production upon injection of foreign substances [22]. This has been demonstrated by studying the regulation of synthesis of lectins by inducing invertebrates to injury or by introducing microorganisms to them [23]. In this study the biological role of hemolymph lectin of the crab *Travancoriana charu* was elucidated using erythrocytes showing differential agglutinability with the lectin as pathogen models. High HA titer with dog erythrocytes indicates the predominance of the lectin specific sugar moieties on dog erythrocytes. Following the injection of 1.5%, 2.5% and 5% dog erythrocytes, the hemolymph lectin exhibited a slight decrease in HA titer from the initial titer of 256 to 128 at 2 hours and a subsequent 4-8 fold increase in HA titer between 4-8 hours post injection. Decline in HA titer followed by the augmentation of HA activity was also observed following the injection of buffalo and goat erythrocytes. The decrease in HA titer following erythrocyte injection may be due to the utilization of the agglutinins and the succeeding increase in HA activity may be due to the secretion / release of agglutinins from other tissues when the hemolymph agglutinin in the circulation is used up for elimination of pathogens. In some crustacean species, lectins are stored in the cytoplasmic granules of hemocyte and released to the extracellular space upon challenge with pathogens [24, 25]. Lectin production and induction in arthropods

was reported in the flesh fly *S. peregrina* where lectin could be induced not only by foreign particles or injury, but also by the changes in the developmental stages of the fly. Enhanced lectin production upon induction with pathogens has also been reported in the silk moth *B. mori* [26], the mantis shrimp *S. mantis* [27], crab *Scylla serrata* [28].

A distinct positive correlation between the time taken for clearance of foreign cells (erythrocytes) and HA titer with erythrocytes points out the interaction between lectin and its receptors on the surface of the pathogens. Expeditious clearance of erythrocytes preferentially agglutinated by the lectin specifies the interaction between the lectin in the hemolymph and the lectin receptors on the surface of the erythrocytes and, it may be the initial step involved in clearance. Faster clearance of erythrocytes coated with lectin than the uncoated ones clearly signifies the role of lectin in the process of elimination of foreign pathogens.

In the freshwater crab *Travancoriana charu*, the escalated rate and time taken for clearance and the effective hemolysis of lectin coated erythrocytes compared to the uncoated ones, points out the role of the lectin as a recognition mediator. Lectins, by binding to surface glycoconjugates on foreign substance, may serve as a discriminatory link between non-self material and the hemocytes involved in phagocytosis or encapsulation reactions [29]. Therefore lectins may assume the role of 'opsonins' in the hemocyte mediated hemolysis of erythrocytes. Correlation between erythrocyte clearance and hemolysis with the extent of erythrocyte agglutination by the lectin supports this contention. Hemolymph lectins from *Homarus americans* [30], *Mytilus edules* [31], *Aplysia californica* [32], *Penaeus monodon*, PmLec [33] and *Procambarus clarkii*, Pc-Lec [34] act as opsonins which enhance phagocytosis against foreign particles. To be true opsonic factors, arthropod lectins, must be able not only to specifically bind to the surface of non-self-particle but also adhere to virtually specific receptors on the phagocyte (hemocytes) surface, thus facilitating phagocytosis in an analogous manner to the Fc portion of mammalian immunoglobulins or the C3b fragments of the vertebrate complement system [35]. The combact of infections relies on efficient recognition mechanisms, and the lectins of crustacean hemolymph could play a crucial role in this context. The endogenous lectin in the hemolymph of *Travancoriana charu* is capable of acting as non-self recognition molecule for a wide range of organisms encountered and thus fulfills the function of vertebrate antibodies in this decapod crustacean.

V. CONCLUSION

Lectins act as humoral factors similar to immunoglobulins in vertebrates; they activate phagocytosis by binding to foreign bacterial cells, leading to the engulfment and death of the cells. One of the most probable roles of *Travancoriana charu* lectin is to act as humoral factor in the defense mechanism. In the present study, erythrocytes showing differential hemagglutinability with the lectin were used as pathogen models for experiments on induction, clearance and hemolysis. Challenging the animal (*Travancoriana charu*) with high agglutinating dog erythrocytes resulted in a reduction in the hemagglutnating ability of the animal, followed by an augmentation in HA titer. The depletion of circulating lectin might have resulted in the reduction of HA titer, which was short lived. Subsequently an increase in the HA titer was observed, which could be due to the stimulation of the innate immune mechanism of the crab by the invading pathogens that would have influenced the lectin production or release, resulting in increased HA titer. Rapid clearance of erythrocytes, preferentially agglutinated by the lectin (dog erythrocytes) than the low agglutinating goat erythrocytes reveals the extent of avidity between the lectin in the hemolymph and the lectin receptors on the erythrocyte surface. Faster clearance of lectin coated erythrocytes from the circulation than the uncoated ones clearly indicates the role of lectins in the elimination of exogenous pathogens. The above assumption is further supported by the positive correlation observed between the surge in the rate and time taken for the clearance of native and lectin coated erythrocytes. Lectins have been suggested to participate in innate immune response by enhancing the rates of phagocytosis of microorganisms by hemocytes. Lectin coating not only facilitates clearance but also enhances hemocyte mediated hemolysis. This highlights the role of lectin as an 'opsonin' facilitating the process of phagocytosis. Thus it can be postulated that the lectin may coat the pathogen and facilitate clearance.

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