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# THE SELF-PROTECTIVE ROLE OF THE MIDGUT GLAND LECTIN (TcLec) IN THE RUSTY MILLIPEDE, *T. CORALLINUS*

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***Abstract:*** **The midgut gland of the rusty millipede, *Trigoniulus corallinus* contains lectin specific for Gal β1-3 linkage and N-acetyl sialic acid. Biological role of the midgut gland lectin (TcLec) of the rusty millipede, *T. corallinus* was studied based on induction and clearance analysis, using erythrocytes of diverse origin as pathogen model. All the erythrocytes when injected into the hemocoel, inspite of their agglutinability and concentration revealed enhanced production of lectin following an initial reduction in HA titre in all the tested tissues except hemolymph. Experiments on clearance of the injected lectin coated and uncoated erythrocytes of diverse agglutinability into the hemocoel showed a positive correlation between the extent of the agglutination and lectin coating on *in vivo* clearance. Hemolysis of the injected erythrocytes *in vitro* revealed that hemocytes recognize and lyse the lectin coated erythrocytes much faster than the uncoated erythrocytes confirming that the lectin act as an 'opsonin' which is a prerequisite for hemocyte mediated lysis and clearance.**

***Keywords:*** **Agglutination, Erythrocytes, Induction, Clearance, Midgut gland, Opsonin, Lectin.**

## 1. INTRODUCTION

Invertebrates adopt a rapid and efficient innate system to recognize and destroy "non-self" material, including pathogens. The innate immune system of invertebrates can respond to the presence of pathogens with cellular and humoral responses (Lee 2001). In general, invertebrate immune system relies on non-self recognition molecules to ensure efficient defense response. Lectins are well known to actively participate in the defense functions of vertebrates and invertebrates where they play an important role in the recognition of foreign particles (Jing et al. 2011). Lectins act as a bridge between the carbohydrates in foreign cells and those on phagocytic cells (Sharon 1984). Lectins present in the hemolymph/tissues of invertebrates are regarded as potential molecules that protect the primitive organisms from invading microbes that threaten their survival (Marques and Barracco 2000).

The lectin in the rusty millipede may play an important role in the innate immune defense of the organism which can be verified by inducing immune response by administering pathogens or model pathogens (erythrocytes). If lectins play a defense role in the biology of the millipede and act as 'opsonin', there will be augmentation in lectin production following the entry of pathogen. To test this hypothesis, the rusty millipede, *T. corallinus* was challenged with lectin coated and uncoated erythrocytes (pathogen model) of diverse agglutinability and the rate of induction, clearance and hemolysis were analysed.

## 2. MATERIALS AND METHODS

### Millipede collection and maintenance

The millipede, *T. corallinus* was collected from swampy areas of the house hold and coconut groves of different localities (Nattalam, Elavuvilai, Arumanai, Panachamoodu, Nagercoil) in Kanniyakumari District, Tamilnadu following monsoon and was identified by the Zoological Survey of India, Chennai. They were kept in large cement tanks containing moist bricks, trunk of plantain tree or dried decaying leaves and fed with raw potatoes, cucumber, cabbage and papaya. Millipedes adapted to the laboratory conditions readily, as evidenced by their molting, copulation and deposition of eggs.

**Purification of lectin**

The midgut gland lectin (TcLec) was purified by affinity chromatography using cyanogen bromide activated sepharose 4B in an econo column (Bio-Rad) previously equilibrated with TBS at 4°C. The elution of lectin (TcLec) was done with elution buffer that contained 10 mM di- sodium EDTA and collected 1 ml fractions on ice in polypropylene tubes containing 10 µl of 100 mM calcium chloride at a rate of 0.3 ml/min. The fractions were vortexed immediately after collection and kept on ice. Fractions containing lectin were pooled on the same day and dialyzed against 1 mM $CaCl_2$ at 4°C and the dialysate was then aliquoted, lyophilized and stored at -20°C.

**Preparation of erythrocyte suspension**

Blood samples were collected from different mammals directly in modified Alseivier's medium (pH 6.1). Erythrocytes were suspended and washed thrice by centrifugation at 4000g with ten volumes of physiological saline and with Tris-Buffered Saline (TBS, pH 7.5) and resuspended in TBS as 1.5% suspension (Mercy and Ravindranath, 1993).

**Hemagglutination assay**

Hemagglutination assay was carried out as described by Ravindranath and Paulson (1987). Lectin (TcLec) 25µl was serially diluted with 25µl of TBS (pH 5 to 11) in microtitre wells and mixed with 25µl of 1.5% erythrocyte suspension. The microtitre plates were incubated for 1 hour at different temperature (0 to 100$^{o}$C). The hemagglutination titre is the reciprocal of the highest dilution of the sample that gave agglutination.

**Induction Assay**

**a) Injection of erythrocytes**

Rabbit, pig, rat and human A erythrocytes were chosen for this study. In each experiment 25 µl of 1.5 % / 3 % erythrocyte suspension in 0.9 % sterilized saline was injected into the hemocoel of the millipede.

To study the effect of agglutinability on induction, rabbit erythrocytes with high agglutinability, pig and rat erythrocytes with moderate agglutinability and human A erythrocytes with low agglutinability were used for injection.

To study the effect of lectin coating of the injected erythrocytes on lectin production, the millipedes were injected with lectin coated/native erythrocytes (1.5 % and 3 %).

All injections were made slowly through the soft arthrodial membrane between the column and adjacent segment. The injection site was blotted with cotton before and after the injection of the erythrocytes. Those millipedes that bled at the injection site, due to vigorous movement during injection were discarded. Care was taken to ensure complete injection of the erythrocytes.

**b) Collection of hemolymph and tissues**

To study the effect of erythrocytes injected into the hemocoel of millipedes on humoral agglutinin activity, the hemolymph, foregut, midgut gland and the hindgut were collected at regular intervals (0 to 720 minutes) after injection and HA was analyzed with rabbit erythrocytes that were highly recognized by the midgut gland lectin.

**Clearance Analysis**

**a) Injection of erythrocytes**

To study the role of agglutinability and lectin coating of erythrocytes on the time taken for clearance of pathogens from circulation, rabbit erythrocytes that were highly recognized and human A erythrocytes that were poorly recognized by the lectin were selected. In each experiment 25 µl of 1.5 % erythrocyte suspension in saline was injected into the hemocoel of millipedes. The method of injection was the same as stated for the experiment on induction.

**b) Preparation of lectin coated erythrocytes**

To find out whether the clearance of erythrocytes was enhanced in response to lectin coating of the injected erythrocytes, the erythrocytes were coated with purified lectin diluted to sub agglutination concentration. The sub agglutination differed for different erythrocytes as observed with the HA titre of diverse erythrocytes.

For lectin coating 200 µl of washed and packed erythrocytes were suspended in 20 volumes of lectin (diluted to sub agglutinating concentration) and incubated for 1 hour at 30 $^{o}$C. The lectin coated erythrocytes were washed and suspended in sterilized saline and were injected as stated earlier. The erythrocyte suspensions were examined under the microscope

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to ensure the presence or absence of clumps of erythrocytes. Clumps if present were disrupted by gentle vortexing. The rate of clearance before and after lectin coating was compared.

**c) Collection of hemolymph**

To study the clearance of injected erythrocytes from the circulation, hemolymph (100 µl) was collected at regular time intervals (5 minutes) until the injected erythrocytes completely disappeared from circulation. Hemolymph was added to 700 µl of double distilled water and mixed. After mixing, the total volume was adjusted to 1 ml with double distilled water and the hemoglobin was estimated.

**d) Estimation of hemoglobin**

The amount of hemoglobin was estimated using the Cyanmeth-hemoglobin method, a hemoglobin kit manufactured by Sigma Diagnostics (India) Pvt. Ltd., Haroda. Product No. 72431 of Qualigens diagnostics designed for *in vitro* estimation of hemoglobin. The Cyanmeth-hemoglobin technique is the method of choice selected by the International Commission for Standardization of Hematology (ICSH). The method measures all hemoglobin derivatives except sulfhemoglobin.

**Hemolysis**

To study whether the lectin acts as an opsonin, hemolysis experiments were carried out. The hemocytes were separated using the method of Soderhall and Smith (1983). The hemocytes were washed twice to remove the contaminating proteins. The hemocyte suspension was added to the lectin coated or uncoated erythrocytes and incubated for 1 hour at 30 $^{o}$C and then the erythrocyte-hemocyte mixture was centrifuged at 200 x g for 5 minutes. The supernatant was collected for estimation of hemoglobin content which was measured following the Cyanmeth-hemoglobin method. The control included lectin coated or uncoated erythrocytes without hemocytes.

## 3. RESULTS

**Induction of agglutinins**

An initial decline in HA titre was observed following the injection of rabbit, rat, pig and human A erythrocytes in the foregut, midgut gland and hindgut followed by a gradual enhancement in the subsequent periods up to 480 minutes. However, the HA titre of the hemolymph showed a steady increase up to 480 minutes post injection. The HA titre of induced agglutinin varied with the concentration of the erythrocytes administrated.

**HA titre after injection of erythrocytes**

Following injection of 1.5 % and 3 % high agglutinating rabbit erythrocytes into the hemocoel of the rusty millipede, *T. corallinus* an instant alteration in HA titre was observed in the hemolymph and extracts of the midgut gland, foregut and hindgut. The HA titre of the midgut gland showed an initial decline which gradually increased and showed a peak in activity at 480 minutes and a subsequent reduction in the following minutes of post injection (Figure 1, 2, 3, 4).

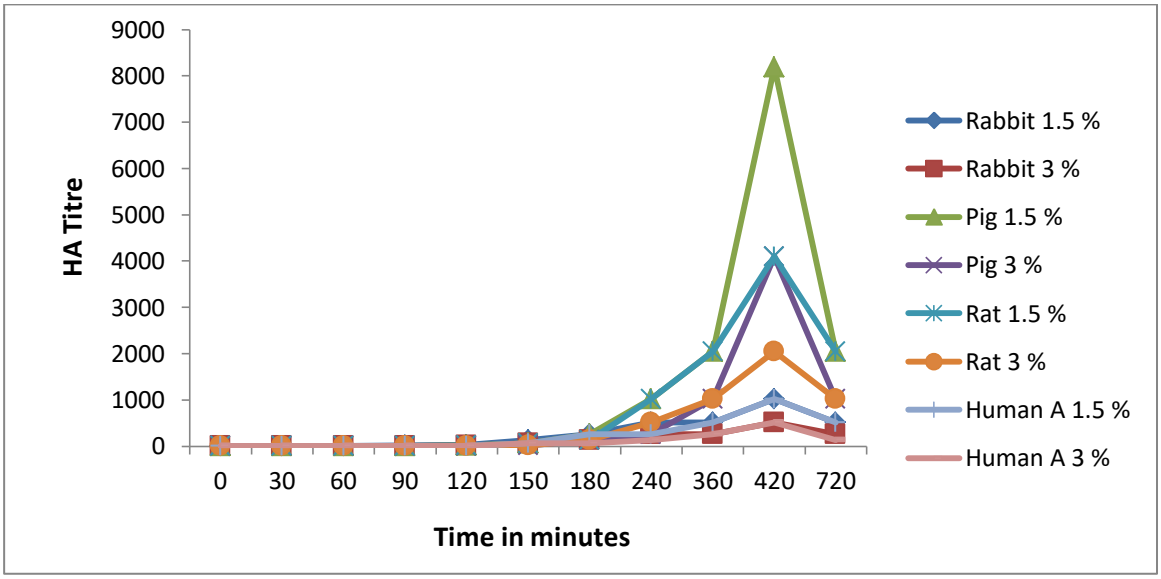


**Figure 1: HA titre of the foregut agglutinin of *T. corallinus* following injection of erythrocytes**

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Injection of 1.5 % and 3 % moderate agglutinating rat and pig erythrocytes into the hemocoel of the rusty millipede, *T. corallinus* resulted in an initial decline in the HA titre of the extracts of the midgut gland, foregut and hindgut. Then the HA titre began to increase and showed maximum HA titre at 480 minutes and a subsequent reduction in the following minutes investigation (Figure 1, 2, 3, 4).

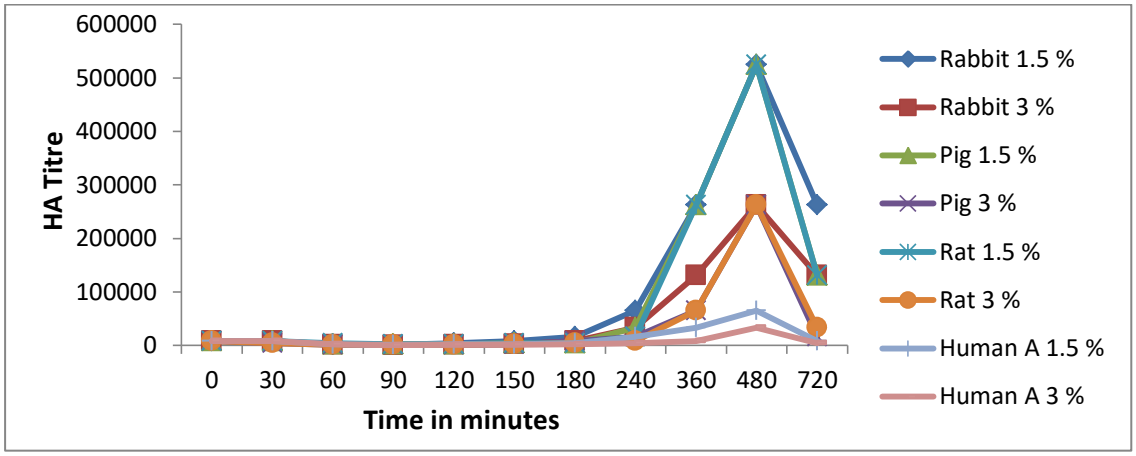


**Figure 2: HA titre of the midgut gland agglutinin of *T. corallinus* following injection of erythrocytes**

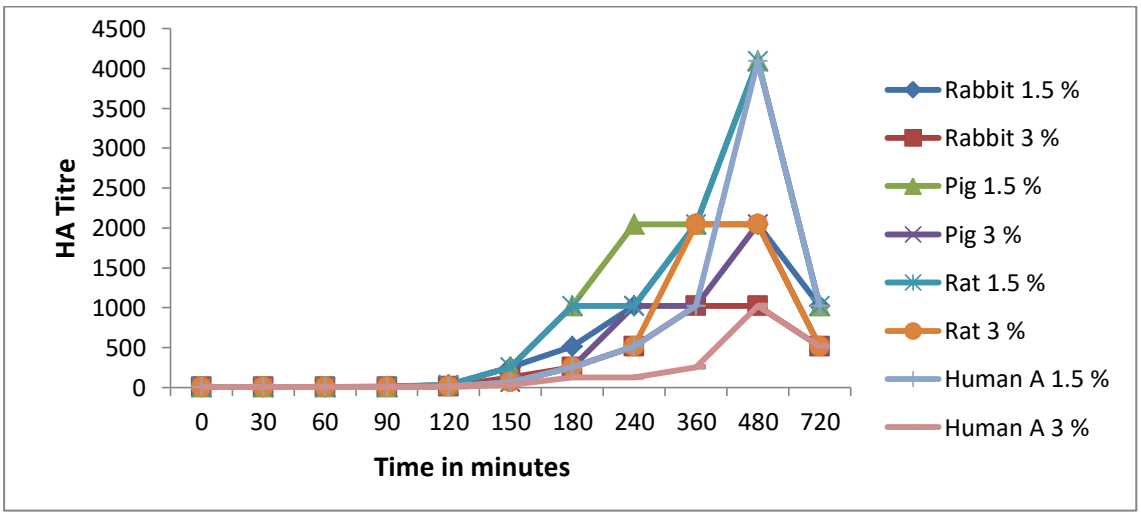


**Figure 3: HA titre of the hindgut agglutinin of *T. corallinus* following injection of erythrocytes**

Similar pattern of change in HA titre was also observed following the injection of the low agglutinating human A erythrocytes (Figure 1, 2, 3, 4).

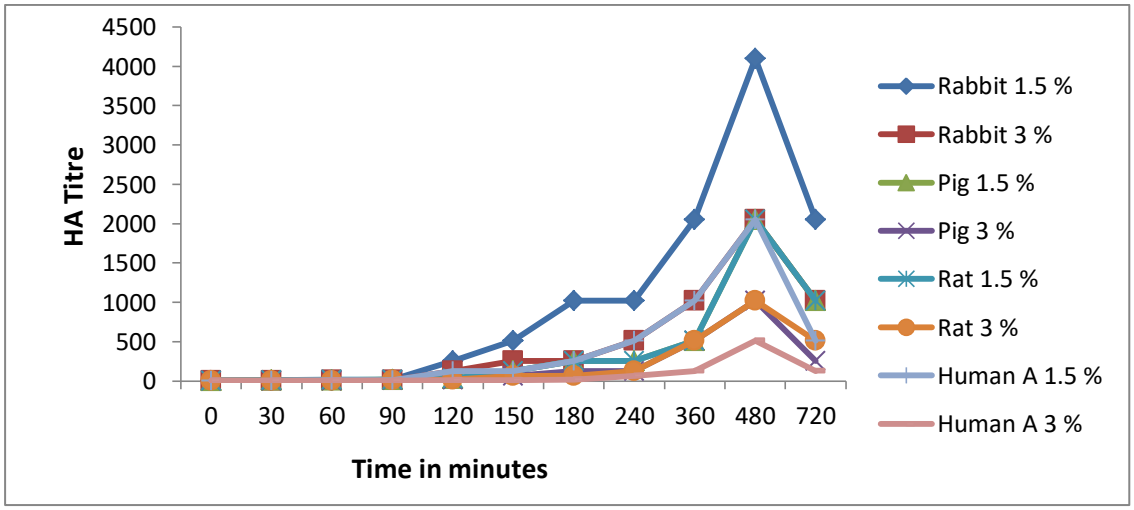


**Figure 4: HA titre of the hemolymph agglutinin of *T. corallinus* following injection of erythrocytes**

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**Effect of injection of lectin coated erythrocytes on HA titre**

Following injection of native erythrocytes, a 4-8 fold reduction in HA titre was observed at 60 minutes which gradually increased and reached a peak at 480 minutes post injection. But subsequent injection of lectin coated erythrocytes following an initial 2 to 4 fold reduction in HA titre, a peak in lectin production was observed at 240 minutes which got reduced at 720 minutes post injection (Figure 5, 6).

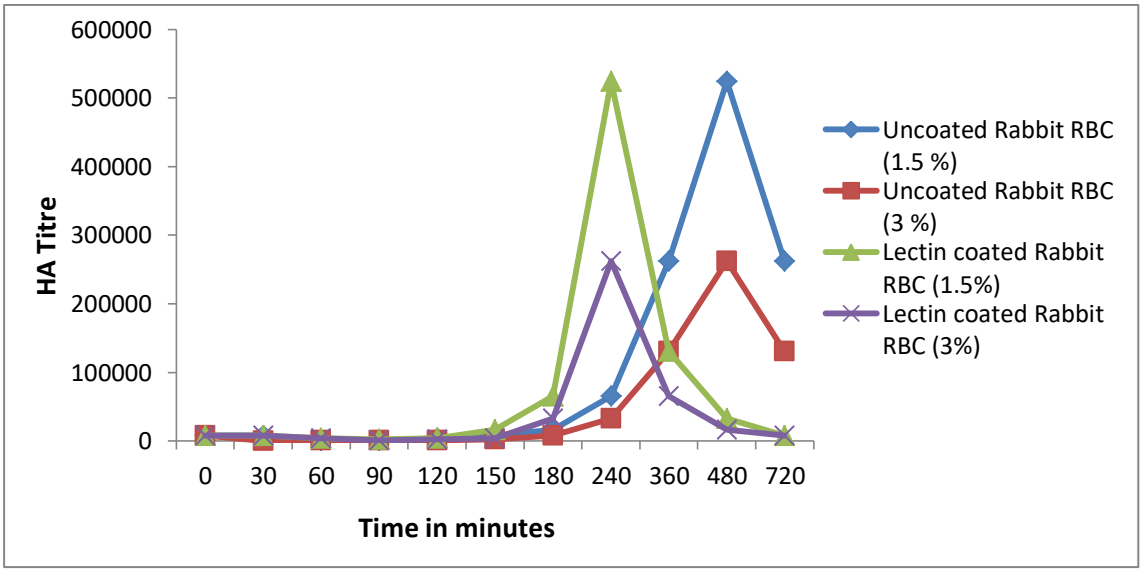


**Figure 5: HA titre of the midgut gland agglutinin of *T. corallinus* following injection of lectin coated and uncoated rabbit erythrocytes**

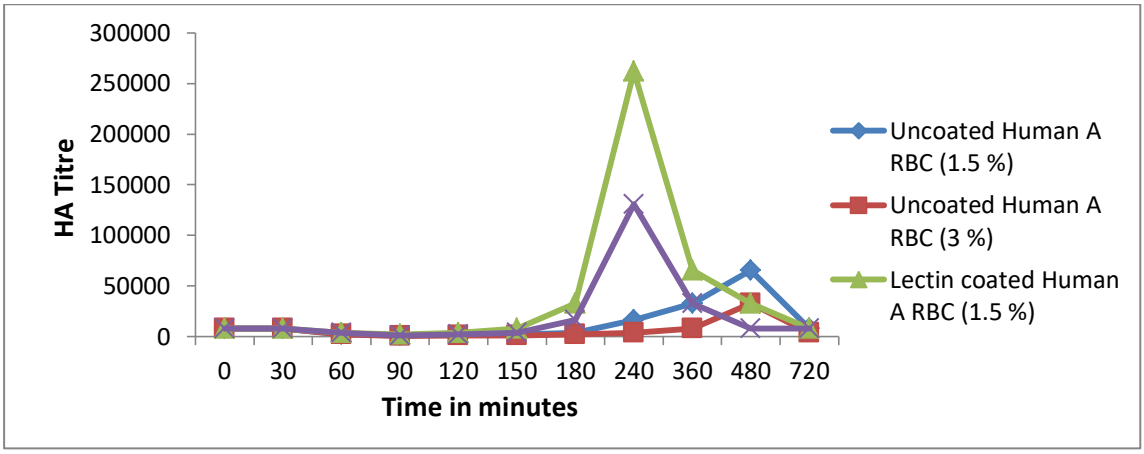


**Figure 6: HA titre of the midgut gland agglutinin of *T. corallinus* following injection of lectin coated and uncoated human A erythrocytes**

**Effect of agglutinability and lectin coating on clearance of erythrocytes**

The high agglutinating rabbit erythrocytes were cleared faster from circulation than the low agglutinating human A erythrocytes. The lectin coated erythrocytes were cleared faster from circulation than the uncoated erythrocytes (Table 1).

**Table 1: Hemoglobin level (µg/ml) in the hemolymph of the millipede at different time intervals after the injection of lectin coated and uncoated erythrocytes**

| Time (minutes) (n = 10) | Hemoglobin content (µg/ml) | | | |
|---|---|---|---|---|
| | Rabbit RBCs | | Human A RBCs | |
| | Uncoated | Lectin Coated | Uncoated | Lectin Coated |
| 5 | 31.1±0.2 | 41.96±0.11 | 58.03±1.7 | 64.2±0.05 |
| 10 | 28.3±0.11 | 34.6±0.057 | 43.86±0.05 | 54.6±0.26 |

| | | | | |
|---|---|---|---|---|
| 15 | 17.9±0.11 | 19.1±0.15 | 37.73±0.06 | 31.66±0.40 |
| 20 | 6.1±0.05 | 0 | 21.3±0.1 | 13.4±0.3 |
| 25 | 0 | 0 | 16.4±0.36 | 9.5±0.4 |
| 30 | 0 | 0 | 11.53±0.55 | 3.1±0.26 |
| 35 | 0 | 0 | 7.6±0.4 | 0 |
| 40 | 0 | 0 | 1.9±0.26 | 0 |
| 45 | 0 | 0 | 0 | 0 |
| 50 | 0 | 0 | 0 | 0 |

**Role of hemocytes on hemolysis**

Recognition and rapid hemolysis of the lectin coated erythrocytes by hemocytes than the uncoated erythrocytes reveal that the lectin act as an opsonin in clearing the pathogen that enter into the circulation. The opsonic nature of the lectin is confirmed by the ability of the hemocyte to recognize and lyse the lectin coated erythrocytes than the uncoated erythrocytes (Table 2).

**Table 2: Effect of lectin coating on hemolysis of erythrocytes by hemocytes**

| **Treatment (n = 10)** | **Hemoglobin content (µg/ml)** | |
|---|---|---|
| | **Rabbit** | **Human A** |
| **Control** | 37.8±0.05 | 19.5±0.05 |
| **Lectin coated RBCs** | 2.26±0.05 | 1.9±0.1 |
| **Uncoated RBCs + hemocytes** | 2.13±0.1 | 1.42±0.11 |
| **Lectin coated RBCs + hemocytes** | 29.06±0.06 | 15.7±0.1 |

## 4. DISCUSSION

A sialic acid specific lectin with affinity for the sialyl residues of the sialoglyco-protein lactoferrin was isolated and purified from the midgut gland of the rusty millipede, *T. corallinus* by affinity chromatography. In this investigation on the biological role of the sialic acid specific midgut gland lectin of the rusty millipede, *T. corallinus* reveals that there is enhanced lectin production following injection of erythrocytes as pathogen model into the hemocoel. The ability to distinguish self from non-self is the fundamental aspect of immunity. Although Myriapods lack antibody-based humoral immune systems, they are believed to have defense molecules that function similar to antibodies (Basil-Rose 1999; Delphine 2006; Xue-Lan et al. 2006; Vinoliya 2006; Arul-Gandhi 2013). Lectin-carbohydrate recognition represents a ligand receptor interaction that is universal in organisms (Feizi 2000; Cambi and Figdor 2003).

The results of the present investigation revealed the presence of antibody like substances capable of binding and clearing the injected red blood cells into the hemocoel of the millipede, *T. corallinus*. The HA titre reflects the level of dilution of lectin at which erythrocytes can be agglutinated. It serves as an index to measure the quantity of lectin found in the tested samples and its affinity to the erythrocytes. Sub agglutination concentration of lectin refers to the dilution of lectin at which the lectin binds to the surface but cannot agglutinate the erythrocytes. While high HA titre refers to the highest dilution at which agglutination can be achieved, they also indicate that the expression of lectin receptor on the cell surface. If the lectin receptors are dense, it can be agglutinated even with very highly diluted lectin. Entry of pathogens as experimented by injecting high, moderate and low agglutinating erythrocytes reveals a peak of activity in lectin production at 480 minutes after the injection of rabbit, rat, pig and human A erythrocytes. Diverse agglutinating activity has been reported in insects (Jayalakshmi 2005), crabs (Mercy and Ravindranath 1994; Denis 2001; Priyatharshini 2007; Devi 2007; Shoba 2011; Punitha 2012; Mettilda 2012), snail (Thanalakshmi 2006), freshwater mussel (Shyamala 2016), centipede (Vinoliya 2006) and millipedes (Basil-Rose 1999; Delphine 2006; Arul-Gandhi 2013) after administrating erythrocytes.

The initial decrease in HA titre observed following injection of erythrocytes could be due to the utilization of agglutinin to remove the injected pathogen. The subsequent rise in HA titre must be due to the synthesis or release of the lectin into the hemolymph from other tissues. Presence of hemagglutinin seems to be very low in the hemolymph, hindgut, foregut in the native condition i.e. when the animals were not challenged with erythrocytes. When they were exposed to the challenge, an initial reduction in HA titre is observed in the midgut gland, foregut and hindgut within 60 minutes of exposure and an increase of the same in the subsequent analysis. But no initial reduction was observed in HA titre of the hemolymph. Hemolymph, being the circulatory fluid, encounters the pathogenic challenge first, prior to any other tissue, because

hemolymph bathes all the tissues and the erythrocytes were injected into the hemocoel. So it can be assumed that the agglutinin from all the tissues may be allowed to flow into the hemolymph to fight with the intruder. Till the animal could succeed in the process of clearing the pathogen, the tissues may continue to do this function. Both in the native state and after pathogenic challenge, the agglutinin content was found to be very high in the midgut gland compared to other tissues. So, it can be the site of synthesis of the lectin. In the natural environment, the millipedes have increased exposure to pathogens during rainy season and under such conditions they may release the lectin for the immediate use. Synthesis and release of the lectin depends on the intensity of the challenge as reported in the millipede, *T. descriptus* (Basil-Rose 1999). Hemagglutinating activity in the midgut gland of millipede, *T. descriptus* (Basil-Rose 1999), *A. disticta* (Delphine 2006; Arul-Gandhi 2013), hepatopancreas of the crab, *S. serrata* (Mullainadhan 1982), hemolymph of the crab, *P. jacquimontii* (Denis 2001), *E. emeritus* (Jayasuriya 2002), *B. cunicularis* (Priyatharshini 2007), *E. tetragonum* (Devi et al., 2013) and *L. lamellidens* (Mettilda 2012) support such a possibility. Further, the presence of sialic acid specific lectins in the hemolymph and midgut gland of the millipedes and the presence of sialyl residues on the outer surface of the bacteria found in the vicinity of these animals suggest that the lectin performs a defensive role.

Clearance of pathogens in millipedes may involve several specific processes, which may be opsonization, lysis and its clearance through gut as reported in the millipede, *T. descriptus* (Basil-Rose 1999). Recognition by hemolymph and lysis of foreign cells, clearance of the soluble products of the lysis, such as hemoglobin by gills and hepatopancreas is reported in crabs (Mullainadhan et al. 1984; Mullainadhan and Ravindranath 1984; Mercy and Ravindranath 1994). In addition, entry of foreign cells may result in synthesis or release of more lectin into circulation so as to facilitate the instant removal of the foreign cells.

## 5. CONCLUSION

From the above observations it can be interpreted that the millipede, *Trigoniulus corallinus* is physiologically adapted to confront foreign cells by enhancing the production of lectins in their body. These lectins may perform as “opsonins” by coating the foreign cells and makes them to be easily documented by hemocytes, followed by hemolysis and rapid clearance from the animal. Our investigations on induction, clearance and hemolysis, strappingly hold up the role of lectins as ‘recognition molecule’ in the defense strategy of the millipede, *Trigoniulus corallinus*.

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