Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/4037
DOI URL: http://dx.doi.org/10.21474/IJAR01/4037
ISSN NO. 2320-5407

RESEARCH ARTICLE

IDENTIFICATION OF MORPHO-VARIANTS OF PLASMATOCYTE IN HAEMOLYMPH SMEARS OF *ANTHERAEA MYLITTA* AND ITS IMMUNE RESPONSES.

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Manuscript Info

Manuscript History

Received: 16 February 2017
Final Accepted: 19 March 2017
Published: April 2017

Key words:-
Tasar silkworm. plasmatocytes variants. immune responses. hemocytes. *Antheraea mylitta*

Abstract

The most decisive hemocyte type "plasmatocyte" is recognized to carry out a variety of functions viz. phagocytosis, encapsulation, nodule formation, injury repairing and metabolism. In the present study, seven types of PLs variants were identified in haemolymph smear of *Antheraea mylitta.* ThePLs were categorized from other hemocytes by their relatively large size, high nucleo-cytoplasmic ratio, presence of pseudopods/filopods *etc.* and they were visible all through larval-pupal development. The PLs variants were identified also on the basis of their morphology and immunological function towards stress condition i.e. starvation, chilling, heating and disease. Interestingly, PLs variants showed stage and age specific variation in cellular immune responses of tasar silkworm. More PLs variants were recorded under stress condition. The differential count of PLs variants varied considerably before and after moult. Much elongated cytoplasmic prolongations/pseudopods in some PLs variants were observed and its relative percentage showed an inverse rapport. Considerable alteration in temperature (high and low) resulted in appearance of more PLs variants and least number was recorded in pebrine infected insects.PLs variants are seen at different functional states of PLs. Interestingly differential profile of PLs variants fluctuates under stress condition without altering the total count of PLs. It is believed that these variants are morpho-types of PLs and involved in numerous physiological activities during larval-pupal development. Identification of PLs variants and their involvement in immune responses depending on the physiological state of *A. mylitta* possibly will help to perceive the impact of different stresses, disease, drug and disinfectant on health status.

Introduction:-

Almost all animal species interact with environment and microorganisms during their subsistence. Cellular and humoral immunity of insects are the main factors for their huge survival in all territories of the earth (Akai 1969; Beaulaton 1979; Sujatha and Dutta-Gupta 1991; Wago 1991; Neven2000;Figueiredo et al., 2006; Contreras and Bradley2010;Lalouette et al., 2011; Catalan et al., 2012; Baishya et al., 2015; Mishra et al., 2015). Cellular immunity of insect consists of different types of blood cells "hemocytes" (Mishra et al., 2015;Kiuchi et al. 2008;

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Charroux andRoyet (2009) Ratheesh et al., 2015). The plasmatocyte is one of the main hemocyte type which is found in almost all insects with 20-70% of total cell population (Akai 1969; Beaulaton 1979; Sujatha and Dutta-Gupta 1991; Wago 1991; Pampiglione and Gupta 1998; Pandey and Tiwari 2012) These cells (PLs) participate in many functions during post-embryonic development (Charroux and Royet 2009; Clark et al.,2005; Srikanth et al., 2011). PLs are thought to work as a surveillance system detecting cuticle wounds and infections in the hemolymph. The plasmatocytes (PLs) are referred to as immunocytes (Pampiglione and Gupta 1998; Pandey and Tiwari 2012) as they are principally responsible for immunological functions (Charroux and Royet 2009; Clark et al.,2005; Srikanth et al., 2011) against foreign materials/invading organisms. Although, few variants of PLs i.e. vermicytes (VEs) and podocytes (POs) have been observed in the specific stages of insect but their association in cellular immune responses under various stress conditions has not been documented well. Keeping this view in mind the present study was carried out and results are presented in this paper.

Material and Methods:-

Insect Culture:-

The larvae of tropical tasar silkworm, *Antheraea mylitta* (Daba Bi-voltine ecorace) were reared in outdoor conditions in rearing farm and fed on leaves of *Terminalia tomentosa* W&A. As per requirement of various experiments larvae were taken from field and utilized.

Induction of Stress Conditions:-

Twenty four hr old V instar larvae were utilized to study the hemocytic immune responses against low temperature, high temperature, starvation and disease stress (pebrine). For low temperature (4^0C) and high temperature (45^0C) stress, larvae were maintained in environmental chamber for 1 hr. To create starvation stress larvae were starved for 24 hrs. For disease stress, pebrine disease infected larvae were utilized. The respective control lots were also maintained for comparison.

Preparation of Hemolymph Smear:-

Hemolymph samples were obtained from control and stressed larvae by puncturing their anterior region with the help of sterilized needle. 18µl of hemolymph was drawn on a slide and mixed well with 2µl anti-coagulant. Subsequently a thin uniform film was prepared by pulling the edge of an inclined slide backward. The film was air-dried at room temperature and stained.The methods of smear formation, staining and hemocyte categorization were similar to those applied earlier (Pandey et al., 2010) with slight modification.

Staining of Hemocytes:-

For hemocyte staining, stock solution of Giemsa stain was prepared by employing the method of Yeager (1945) and a portion of it was diluted 10 times with double distilled water. Air dried hemolymph smear was stained with the Giemsa stain for 10-15 minutes and then the slides were rinsed thrice in double distilled water.

Total Hemocyte Count (THC):-

TheNeubauer ruling haemocytometer was used for total hemocyte count (THC) experiment. The hemolymph was drawn from control and stressed larvae into a thoma blood-cell pipette up to its 0.5 mark and diluted up to the 11^{th} mark with Tauber-Yeager's fluid (Tauber and Yeager 1935). The pipette was then shaken for 5-8 minutes and the first three drops were discarded. A double line with improved Neubauer ruling haemocytometer was filled with diluted hemolymph and the circulating hemocytes per cubic millimeter (mm^3) was calculated according to Jones (1962) with slight modification.

Differential Hemocyte Count (DHC):-

Varioustypesof PLs variantswere recognized based on morphology and staining reactions.The differentialcounting of PLs variants and different categories of cells selected from random areas of stained smears of 30 insects were made separately. The percentage of different cell types/PLs variants was calculated as per method applied earlier by Pandey et al. (2010).

Microscopy:-

Light and phase contrast microscopes were used to study the THC, DHC, PLs variants, different cell morphology and cell contour. PLs variants were categorized based on morphology and staining reactions as observed under light and phase contrast microscopes. The Giemsa stained hemolymph smear slides were utilized to study hemocyte morphology.

Data Analysis:-
The data was subjected to the statistical analysis Student's't' test and correlation by using the Microsoft Excel 2003 software.

Results and Discussion:-

Based on light and phase contrast microscopy six types of hemocytes were recognized in hemolymph smears of tasar silkworm *Antheraea mylitta* (Table 1, 2 & 3). PLs are polymorphic cells, fusiform and spindle shaped. Some PLs cells were recognized as relatively smaller with central or eccentric nucleus. They possessed one or two small cytoplasmic prolongations, the pseudopods. Interestingly, these cells exhibited mitotic figures occasionally (Table 1). Seven types of PLs variants (Fig 1 and Table 2 & 3) were identified and changes in their count as well as morphology were observed under different stress conditions viz. low temperature, high temperature, starvation and disease stress (pebrine).ThePLs/variants were distinguished from other hemocyte types by their relatively large size, high nucleo-cytoplasmic ratio, presence of pseudopods/filopods *etc*. and they were visible all through larval-pupal development.

Table 1:-Hemocytes of tasar silkworm, *A. mylitta* and their morphology.

S.N	Hemocyte types	Acronym	Hemocyte morphology
1.	Prohemocyte	PRs	PRs are typically sphere-shaped with a large centrally located nucleus leaving only a small peripheral cytoplasmic area. These cells bear a distinct central nucleolus and are frequently seen in mitotic divisions.
2.	Plasmatocyte	PLs	PLs are polymorphic cells, fusiform, spindle shaped. Seven PLs variants were observed. These cells are with a relatively smaller central or eccentric nucleus. They possessed one or two small cytoplasmic prolongations, the pseudopods. Interestingly, these cells exhibited mitotic figures occasionally.
3.	Granulocyte	GRs	GRs are usually rounded with small central nucleus. Their cytoplasm is characteristically filled with sparsely scattered small sized dense granules.
4.	Spherulocyte	SPs	These cells are known to contain granular, fine textured, filamentous or flocculent materials. SPs are filled with many spherules.
5.	Adipocyte	ADs	ADs are large, roughly rounded cells with a relatively small spherical or slightly elongated nucleus placed eccentrically. A number of fat globules in the form of vacuoles were observed in cytoplasm of these cells.
6.	Oenocytoid	OEs	OEs are spherical or oval relatively large, widely variable sizes possessing small rounded peripheral nucleus. Their cytoplasm is found generally thick and homogeneous.

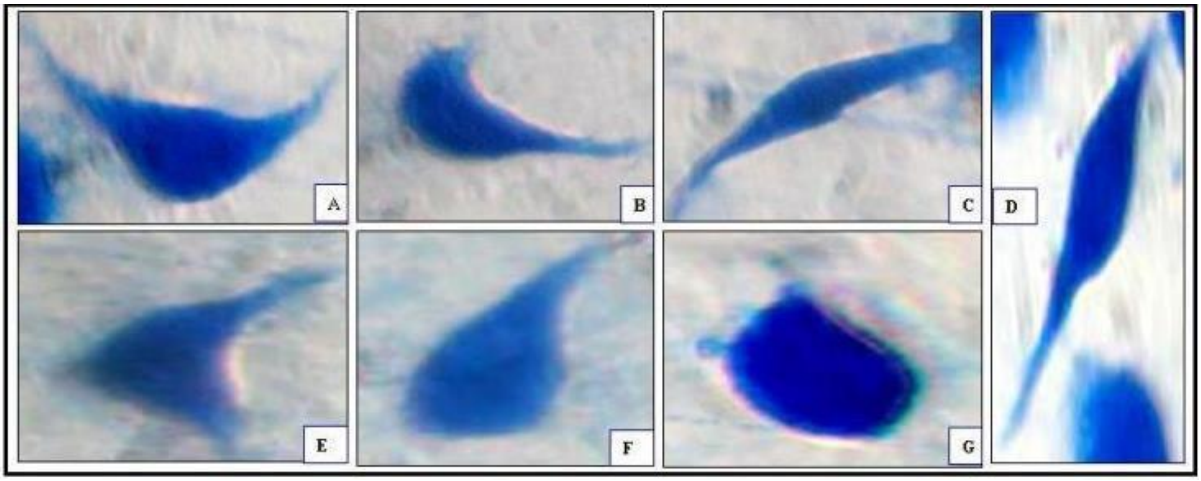

Fig. 1:- A-G Morphology of plasmatocyte variants found in hemolymph smear oftasar silkworm, *A. mylitta* (A) Plasmatocyte variant one (B) Plasmatocyte variant two (C) Plasmatocyte variant three (D) Plasmatocyte variant four (E) Plasmatocyte variant five (F) Plasmatocyte variant six (G) Plasmatocyte variant seven

Table 2:-Differential count of plasmatocyte (PLs) variants found in hemolymph smear of tasar silkworm *A. mylitta*

Plasmatocyte	**Control**	**Low temperature stress**	**High temperature stress**	**Starvation stress**	**Pebrine disease stress**
Total PLs count	33.7±1.8	24.9±1.6**	38.9±2.2*	28.5±2.1*	23.7±1.7**
Plasmatocyte variant one	35.5%	40%	39%	28%	30.5%
Plasmatocyte variant two	24%	21%	14.5%	8.5%	12%
Plasmatocyte variant three	10%	9%	6.5%	11.5%	6.5%
Plasmatocyte variant four	8%	6%	16%	18%	28%
Plasmatocyte variant five	2.5%	3%	1%	2%	4.5%
Plasmatocyte variant six	5%	7%	7%	15%	3.5%
Plasmatocyte variant seven	15%	14%	16%	17%	15%

(Values represent mean ± SD of 10-12 haemolymph determinations, each drawn from 10 larvae)
Note: P values: $^{*}< 0.05$; $^{**}< 0.01$

Table 3:-Morphology of plasmatocyte (PLs) variants found in hemolymph smear oftasar silkworm, *A. mylitta*

S. No.	**Plasmatocyte variants**	**Detection of plasmatocyte variants**
1.	Plasmatocyte variant one (Fig 1A)	PLs variant (one) is most abundant PLs. Low temperature stress elicited its clumping
2.	Plasmatocyte variant two (Fig 1B)	Loss of psedopods has been observed in PLs variant (two) under high temperature stress and its disintegration was also observed
3.	Plasmatocyte variant three (Fig 1C)	PLs variant (three) showed little peripheral cytoplasmic area which is frequently involved in spreading behavior
4.	Plasmatocyte variant four (Fig 1D)	PLs variant (four) with more cytoplasmic prolongations/ extremely elongated cells. Loss of pseudopods was observed under pebrine disease stress
5.	Plasmatocyte variant	PLs variant (five) was rarely detected with tri-ramous shape

	five (Fig 1E)	
6.	Plasmatocyte variant six (Fig 1F)	More number of PLs variant (six) were detected when starvation stress was given
7.	Plasmatocyte variant seven (Fig 1G).	PLs variant (seven) was approximately rounded in shape detected with few in number. It is known for its stability

Interestingly, PLs variants showed stage and age specific variation in cellular immune responses of tasar silkworm. More PLs variants were recorded under stress condition (Table 2 & 3). The differential count of PLs variants varied considerably both before and after moult. Much elongated cytoplasmic prolongations/pseudopods in some PLs variants were observed and the relative percentage of different PLs showed an inverse relationship (Table 2). Considerable alteration in temperature (high and low) resulted in appearance of more PLs variants and least number were recorded in pebrine infected insects. These variants are seen at different functional states of PLs. Interestingly, differential profile of PLs variants fluctuates under stress condition without altering the total count of PLs.

Insects represent almost all territories of the earth and account around 75% of total animal population. It is still indistinct that being a poikilothermic organism, without acquired immune responses, how do they survive in all geographical regions of the world? Probably, it indicates its very strong innate immune responses i.e. cellular and humoral. Insect blood cells (hemocytes) which play very crucial role in cellular immune responses are very vast and interesting subject for scientific community (Akai 1969; Beaulaton 1979; Sujatha and Dutta-Gupta 1991; Wago 1991; Neven2000;Figueiredoa et al., 2006; Contreras and Bradley2010;Lalouette et al., 2011; Catalan et al., 2012; Baishya et al., 2015; Mishra et al., 2015). It was the year 1758 when Schwammerdam for the first time described insect blood cells (hemocytes) as transport globules. Although, in last 250 years, lot of research work has been conducted on hemocytes but many of the aspects like categorization of plasmatocyte variants are explored very little. It is reported that cellular immunity of insect involves different types of hemocytes (Mishra et al., 2015; Kiuchi et al., 2008; Charroux andRoyet 2009; Ratheesh et al., 2015) and PLs is one of the main hemocyte type which is found in almost all insects with 20-70% of total cell population [Baishya et al., 2015; Pampiglione andGupta 1998; Pandey and Tiwari 2012). These cells (PLs) participate in many functions during post-embryonic development (Kiuchi et al., 2008; Clark et al., 2005; Srikanth et al., 2011). PLs probably work as surveillance system also detecting cuticle wounds and infections in the hemolymph. Further, despite the fact that the plasmatocytes (PLs) work as immunocytes, they carry out very crucial role in cellular immune responses of insects and are responsible for production of antimicrobial peptides, phagocytosis and aggregation of pathogens i.e. PLs form aggregates at sites of tissue injuries that work as a physical barrier preventing microorganism infections. Although, few variants of PLs i.e. vermicytes (VEs) and podocytes (POs) have been observed in the specific stage of some insect species but seven PLs variants recognition in economically very important sericigenous insect *A. mylitta* and their association in cellular immune responses under various stress conditions is perhaps the first report. As their differential profile changes under various stress conditions, therefore, PLs variants may be considered as an indicator of insect's various physiological states which possibly will help to distinguish the impact of different stress, disease, drug and disinfectant on health status of *A. mylitta*. Proper understanding of PLs variants will lead to help the betterment of applied sciences of insects and related industry. Literature says that, our awareness of PLs variants and their functions in general is quite little. It is expected that, any marked change in differential count of PLs variants may affect the insect directly or indirectly. It is expected that variation in PLs consequent to stresses/adversities can be used as an indicator for health and it can be potentially utilized in applied and biomedical sciences. Hence, these areas should be explored and utilized for human welfare.

Conclusions:-

Seven types of PLs variants were identified and change in their count as well as morphology was observed under low temperature, high temperature, starvation and disease stress (pebrine). As the tasar silkworm *A. mylitta* is economically important sericigenous insect, therefore, identification of PLs variants and their involvement in immune responses depending on the physiological state of *A. mylitta* possibly will help to distinguish the impact of different stresses, disease, drug and disinfectant on health status.

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