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Temporal Dynamics of Differential Haemocytes in Multivoltine Crossbreeds of Mulberry Silkworm

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The cellular immune responses of different Indian cross breeds of mulberry silkworm, *Bombyx mori* L. were analysed by estimating differential haemocyte counts. The test silkworm larvae were immunized with Kanamycin resistant *Escherichia coli (E. coli* K_R) (10_{-6} cfu/larva). Different types of haemocytes *viz.,* prohaemocytes, granulocytes, plasmatocytes and spherulocytes were enumerated at specified time intervals of 0h, 6h, 12h, 24h, 30h, 36h and 48h in immunized and control larvae to measure the spatial dynamics of DHC (Differential Haemocyte Count). The DHC profiles of cross breed races *viz.,* APM1 x APS8, APM2 x MVT, APM2 x APS12 and APM3 x APS12 showed that the composition of plasmatocytes, granulocytes and spherulocytes were in the range of 17-35, 69-73 and 2-8 per cent respectively. Upon immunization, there was rapid increase in granulocyte population recording as much as 122 per cent rise. Rapid rise in granulocytes was more evident in cross breeds like APM2 x APS12 and APM3 x APS12 as early as 6h after immunization. However, perceptible decrease in granulocytes and plasmatocytes was noticed in immunized larvae after 24-48h. The study brought out distinct differences in spatial and temporal dynamics of differential haemocyte counts in Indian multivoltine cross breeds of mulberry silkworm.

Key words: Mulberry, Silkworm, *Bombyx mori* L., *E. coli* K_R, Temporal dynamics and Differential haemocytes.

Cellular factors in the haemolymph help in the protection of insect against infectious diseases. Haemocytes of different types *viz*., prohaemocytes, plasmatocytes, granulocytes, spherulocytes, imaginal spherulocytes and oenocytoids are the key candidates of cellular immunity. Raichoudhury and Sengupta (1959) named seven types of haemocytes in *Bombyx. mori.* Nittono (1960) classified the blood cells in *B.mori* L., into six types. The chief defensive cells are the plasmatocytes and the granular cells in *B. mori*, which take part in phagocytosis, encapsulation and nodulation reactions in response to bacterial infection (Krishnan *et al*., 2000).

Haemocyte counts are normally highly variable within the same species as well as among species (Jones, 1977). According to DHC (Differential Haemocyte Count) reported by Wago (1980) in *B. mori*, the granular cells constitute 62 per cent, the prohaemocytes and plasmatocytes 16 per cent, the spherule cells 19 per cent and the oenocytoids 3 per cent. Han *et al.* (1998) investigated that the haemocytic differentiation showed four types of haemocytes: prohaemocytes, plasmatocytes, granulocytes and oenocytes in the larvae of *B. mori.*

Materials and Methods

Cross breeding silkworm rearing

Different Indian cross breed silkworm races, APM1 x APS8, APM2 x MVT, APM2 x APS12 and APM3 x APS12 were procured from the Andhra Pradesh State Sericulture Research and Development Institute, Hindupur.

The silkworm hybrids were reared in rearing house at the Department of Sericulture, Tamil Nadu Agricultural University, Coimbatore, under the standard rearing conditions at temperature 26±1°C with 75 ± 5% relative humidity and photoperiod of 16 L: 8D as per the recommended rearing practice (Raja Ram, 2000).

Immunization

Escherichia coli (Kanamycin Resistant) strain was used in this study. Freshly collected mulberry leaves were dipped in 50 ppm *E.coli* (K_R) broth culture filtrate (10_6 cfu / ml). The leaves were shade dried and given once during first feeding after fourth moult during fifth instar.

Collection of haemolymph

The larvae were wiped with 70 per cent ethyl alcohol. Tip of the first proleg was snipped off, using sterilized blade / scissor. Required amount of haemolymph was collected in cleaned, sterilized and precooled in Ependorf tubes. The haemolymph samples collected from control and immunized larvae were added with lepidopteran saline solution (Na Cl 88 mM, K Cl 8 mM, Ca Cl_2 9.9 mM).

Differential haemocyte count (DHC)

DHC was taken in fifth stadium larvae of silkworm

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after ingestion of *E. coli* (K_R) to identify different types of haemocytes *viz*., granulocytes, plasmatocytes and spherulocytes.

The method generally consisted of drawing haemolymph into a Thoma white blood cell pipette, diluted (1:9) in physiological saline, added with Saline-Versene solution. Haemocytes were counted in a standard Neubaur haemocytometer under electron microscope according to the following formula (Gupta and Sutherland, 1968).

$$\frac{\text{Haemocytes in five 1 mm squares x dilution x Depth of chamber}}{\text{Number of 1 mm square counted}} \times 1000$$

DHC was taken at periodic time interval *viz*., 0, 6, 12, 24, 30, 36 and 48 h after immunization of the larvae with *E. coli* K_R at fifth instar which was identified by following the key presented by Gupta and Sutherland (1967).

Results and Discussion

Differential haemocyte counts of plasmatocytes, granulocytes and spherulocytes were recorded in Indian cross breeds, APM1 x APS8, APM2 x MVT, APM2 x APS12 and APM3 x APS12.

DHC of cross breeds

DHC profiles of five cross breeds chosen in the study are presented in Tables 1 and 2. Perusal of the DHC profile of these cross breeds revealed that plasmatocytes were in the range of 23.0 (APM2 x APS12) to 35.0 per cent (APM2 x APS8); granulocytes occurring at 59.0 (APM2 x APS8) to 68. 0 per cent (APM2 x APS12); Spherulocytes at 2.0 (APM2 x MVT) to 8.0 (APM2 x APS12) per cent.

Table 1. Differential haemocyte count after immunization with *E. coli* K_R and control in APM1 x APS 8 and APM2 x MVT breeds of silkworm, *B. mori* haemolymph (ml^{-1}) at fifth instar in different time interval.

Silkworm race	Time interval (h)	DHC* (10^5/ml)					
		Plasmatocytes		Granulocytes		Spherulocytes	
		Control	Immunized	Control	Immunized	Control	Immunized
APM1 x APS 8	6	2.91	4.45	8.36	9.73	0.45	0.29
	12	3.14	4.64	8.45	10.27	0.73	0.28
	24	3.64	3.82	8.64	7.45	0.82	0.27
	48	5.73	2.00	9.64	2.45	1.09	0.13
r		0.985	-0.975	0.977	-0.98	0.948	-0.953
$1-r_2$		0.030	0.049	0.046	0.040	0.102	0.092
APM2 x MVT	6	3.09	4.73	8.91	9.09	0.32	0.23
	12	3.27	5.36	9.45	9.73	0.35	0.22
	24	3.91	4.45	9.68	8.18	0.36	0.18
	48	4.91	1.27	11.82	4.64	1.18	0.97
r		0.998	-0.935	0.978	-0.961	0.929	0.891
$1-r_2$		0.003	0.126	0.042	0.077	0.137	0.206

* Mean values of three replications with 100 silkworms in each replication r Pearson correlation coefficient 1-r2 Square of pearson correlation coefficient

There was a linear rise in these haemocytes with the growth of the larvae in the entire cross breeds studied. However, DHC profile showed a dynamic change upon immunization in different crossbreeds. Significant rise in plasmatocytes was noticed at 12 h post immunization in APM1 x APS8 and APM2 x MVT. Gradual rise in granulocytes was noticed in all cross breed races at 12 h post inoculation. However, decline in granulocytes were conspicuous during the same time span in the immunized larvae of APM2 x APS12 and APM3 x APS12.

Temporal dynamics of differential haemocyte count in silkworm breeds

Differential haemocyte count profiles of select Indian silkworm breeds and their *in vivo* dynamics in normal as well as bacterial challenged larvae over time have been furnished in Tables 1 and 2.

Perusal of the data of DHC profiles of fifth instar larvae of different silkworm breeds have thrown light on certain aspects of cellular response of *B. mori* that could be summarized as follows.

- ❺y DHC profiles of cross breed races viz., APM1 x APS8, APM2 x MVT, APM2 x APS12 and APM3 x APS12 showed the composition of plasmatocytes, granulocytes and spherulocytes which were to be in the range of 17-35, 69-73 and 2-8 per cent respectively. Upon immunization, there was rapid increase in granulocyte population recording as much as 121 per cent rise.
- ❺y Analysis of composition of plasmatocytes, granulocytes and spherulocytes from five cross breeds revealed wide divergence among breeds. Plasmatocytes were in the range of 23.0 (APM2 x APS12) to 35.0 per cent (APM2 x APS8); granulocytes occuring at 59.0 (APM2 x APS8) to 68.0 per cent (APM2 x APS12); Spherulocytes at 2.0 (APM2 x MVT) to 8.0 (APM2 x APS12) per cent.

Divergence in haemocyte profile of lepidopteran larvae has been widely reported. The plasmatocytes and granulocytes accounted for more than 50 per cent of the haemocytes in circulation in the larval stage of lepidopterans (Lackie, 1988; Ratcliffe, 1993).

Beaulaton (1979) suggested that prohaemocytes in *B. mori* differentiated into plasmatocytes which were in turn differentiated into granulocytes and spherulocytes. DHC profiles of Indian silkworm breeds, NB7, NB18, NB4D2, KA, PM, Nistari, MY1 and C. Nitchi have earlier been documented by Balavenkatasubbiah *et al.* (2001) and Balavenkatasubbiah and Nataraju (2005) and also confirmed the dominance of granulocytes.

The spurt in granulocytes and plasmatocytes was explicit in silkworm breeds upon immunization. It is an established fact that the main cellular defense of insects is mediated by haemocytes. Granulocytes and plasmatocytes are principal haemocyte types capable of adhering to invading pathogens and they are actively involved in phagocytosis mechanism (Lackie, 1988; Strand and Pech, 1995). Anandakumar and Michael (2011) reported that the prohaemocytes undergo division in order to increase the number of granular haemocytes and plasmocytes, which take part in cellular defense mechanism. Thus, in the flacherie inoculated larvae, there was decreased proportion of prohaemocytes but increased proportion of granular haemocytes and plasmocytes.

***In vivo* dynamics of DHC in silkworm breeds**

It is pertinent to measure the unfolding of immune response over time. This temporal dynamics determine the swiftness of immune response. In vivo dynamics of DHC in different silkworm breeds over time upon bacterial challenge (*E. coli* K_R) was compared with that of normal and untreated larvae (Tables 1 and 2).

The swiftness of the reaction was reflected in the spurt in DHC values at 6-24 h in different silkworm breeds. Sudden rise in granulocytes was more evident in cross breeds like APM2 x APS12 and APM3 x APS12. It could be ascertained by faster rate of increase in granulocyte population as early as 6 h after immunization.

Table 2. Differential haemocyte count after immunization with *E. coli* K_R and control in APM2 x APS12 and APM3 x APS12 breeds of silkworm, *B. mori* haemolymph (ml^{-1}) at fifth instar in different time interval.

Silkworm race	Time interval (h)	DHC* (10^5/ml)					
		Plasmatocytes		Granulocytes		Spherulocytes	
		Control	Immunized	Control	Immunized	Control	Immunized
APM2 x APS12	6	2.82	4.64	9.54	11.54	0.77	0.55
	12	2.91	6.36	10.73	12.55	0.86	0.52
	24	3.18	4.73	11.55	9.55	0.91	0.41
	48	4.273	1.909	12.273	3.859	1.364	0.327
r		0.983	-0.852	0.921	-0.968	0.976	-0.976
$1-r_2$		0.034	0.275	0.151	0.063	0.048	0.048
APM3 x APS12	6	2.45	4.82	10.27	10.09	0.55	0.43
	12	2.64	5.36	10.28	11.36	0.36	0.42
	24	3.73	5.13	10.37	9.55	0.45	0.36
	48	4.99	1.55	11.18	4.18	0.99	0.18
r		0.992	-0.888	0.950	-0.934	0.839	-0.987
$1-r_2$		0.015	0.211	0.096	0.128	0.295	0.025

* Mean values of three replications with 100 silkworms in each replication r Pearson correlation coefficient $1-r_2$ Square of pearson correlation coefficient

Shapiro (1968) reported increase in granulocytes number upon NPV infection in *Galleria mellonella* whereas plasmatocytes were decreasing with the progression of disease. However, some reports suggested that there indeed was a decrease in THC (Total Haemocyte Count) and DHC upon infection by bacterial pathogen in some insects. The efficiency of blood cells in defending against septicaemia depends on the type and dosage of pathogen. Light doses of *Bacilli* (1.7 to 2.8 x 10_4/ larva) were phagocytosed within half an hour after injection in *Psedoletia unipuncta* while, higher doses resulted in decrease of THC (Wittig, 1962).

Decrease in number of granulocytes and plasmatocytes was noticed in immunized insects after 24-48 h. This may be due to the involvement of these haemocytes in phagocytosis. Granulocytes and plasmatocytes were the only haemocytes involved in phagocytosis reaction in Lepidoptera (Elrod- Erickson *et al.,* 2000). They are also involved in nodulation *i.e*. multiple haemocytes binding to aggregations of bacteria (Schmidt *et al.,* 2001). There are some patterns – recognition receptors (PRRs) in the plasma that enhance phagocytosis or nodulation. PRRs include LPS binding protein, gram-negative bacteria, recognition protein, peptidoglycan recognition protein, lectin, haemolin, *etc*. Koizumi *et al.* (1999) reported isolation of LPS binding protein from *B. mori.* A number of molecular elicitors of cellular defense have been isolated and purified from *B. mori* (Lavine and Strand, 2002).

Comparatively high number of plasmatocytes and granulocytes were observed in APM1 x APS8, APM2 x APS12 and APM3 x APS12 in the present study. The result is in conformity with the findings of Balavenkatasubbiah and Nataraju (2005) who have reported that higher number of granulocytes and plasmatocytes in PM, NB4D2 and Nistari during progressive *Bm*NPV infection.

Jaydeb *et al.* (2000) attributed the survival of the silkworm breed P5 against diseases to their cellular

defense. Patil and Jamuna (2000) categorized several multi and bivoltine breeds based on THC. Balavenkatasubbiah *et al.* (2001) reported 50 per cent increase in plasmatocytes and nearly 100 per cent rise in granulocytes in tolerant silkworm breeds like PM and Nistari when challenged with NPV. Sivaprasad *et al.* (2003) developed a haemocyte-based index to distinguish the resistant and susceptible silkworm breeds. Thus, the findings of the present study are in conformity with that of earlier workers regarding dynamics of haemocytes in Indian silkworm breeds upon infection.

Drop in granulocytes population was conspicuous in several silkworm breeds upon immunizations in the present study. This may be due to the loss of haemocytes through degranulation in the course of immune reaction (Rowley and Ratcliffe, 1976). Moreover, the drop in haemocytes at 24-48 h in immunized larvae might have coincided with the onset of humoral immune response guided by *de novo* synthesis of antibacterial proteins.

Temporal dynamics of haemocytes in terms of THC and DHC throws light on the capability of some silkworm breeds over others in clearing off the pathogens. Further studies are needed to work out the short-term immune dynamics as well which would reflect the instant cellular defense.

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