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Cellular and Biochemical Changes of *Antheraea mylitta* D. on Immunization with Attenuated *Antheraea mylitta* Cytoplasmic Polyhedrosis Virus

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ABSTRACT

The aim of the present study is to analyze the cellular and biochemical changes noticed in tasar silkworm larva (*Antheraea mylitta* D.) immunized with attenuated cytoplasmic polyhedrosis virus (AmCPV). The bioassay was carried out to confirm the no induction of disease in attenuated AmCPV inoculated larvae. Total Haemocytes count and Differential Haemocytes counts were carried out in healthy control, immunized and non immunized silkworm larvae at different time intervals. The hemolymph proteins were estimated in healthy control, immunized and non immunized silkworm larvae. The results confirm that, attenuated AmCPV provides protection against AmCPV infection for a short period (6 to 8 days). The mortality in immunized silkworms was reduced significantly as compared to non immunized inoculated control. The total haemocyte counts increased in haemolymph up to 8th day in immunized silkworms in comparison with non immunized inoculated control indicating the positive haemocyte mediated response in silkworm immunized with attenuated AmCPV. Similarly, differential haemocyte count was different in immunized silkworms from the inoculated control. The prohaemocyte, plasmatocytes and granulocytes were maximum in number whereas oenocytoids were minimum in number. The number of degenerated blood cells was increased in inoculated control up to 8th days of post inoculation. The hemolymph protein in immunized silkworms was significantly higher than non immunized control. The gradual increase 1st day to 8th day was observed in immunized silkworm. In non immunized inoculated control, the total hemolymph proteins have shown increasing trend from 1st to 5th day and decreasing from 6th day onwards.

Key words: *Antheraea mylitta*, cytoplasmic polyhydrosis virus, mortality, haemocytes, proteins

INTRODUCTION

The silkworm *Antheraea mylitta* D. is exploited both as a powerful biological model system and also as a tool to convert leaf protein into silk. The industrial and commercial use of silk, the historical and economic importance of production and its application in all over the world finely contributed to the silkworm promotion as a powerful laboratory model for the basic research in

biology. Silkworm larvae often suffer from various diseases causing heavy losses to the economy of the silk industry. Among diseases in silkworm, cytoplasmic polyhedrosis commonly known as virosis caused by Cytoplasmic Polyhedrosis Virus (CPV), a reovirus is highly contagious and prevails all through the year in tasar culture regions which accounts considerable loss of 25-30% to cocoon production (Sahay *et al*., 2000).

Immune system includes certain types of blood cells. It also includes chemicals and protein in the blood, such as complement proteins. Immunization is a way to trigger the immune response. Small doses on an antigen, such as dead or weakened live viruses are given to activate immune system. Insects exhibit both humoral and cellular immune responses (Boman and Hultmark, 1987; Kimbrell, 1991) in addition to metabolic alterations that are effective against various pathogens like bacteria, fungi, protozoa, etc. Humoral reactions involve slow synthesis of antibacterial and antiviral principles and require several hours for full expression. Cellular responses are direct interactions between circulatory haemocytes and invading non self materials. The interaction is immediate and includes phagocytosis, nodulation and encapsulation (Gupta, 1986). In insects, several types of haemocytes are observed in the haemolymph (Jones, 1979; Butt and Shields, 1996). Nittono (1960) and Saran *et al*. (2002) classified the blood cells in the silkworm, *B. mori* L. and *A. mylitta* in to six types viz., prohaemocytes, plasmatocytes, granulocytes, spherulocytes, imaginal spherulocytes and oenocytes. A variety of functions like mechanization and immobilization of invading organism by encapsulation and/or phagocytosis, wound repair, coagulation have been reported to haemocytes (Pech *et al*., 1995). Few studies have been made on attenuation of viruses and their effect on protection of silkworm, *Bombyx mori* L. from viral disease (Sivaprakasham and Rabindra, 1995; Nataraju *et al*., 2000). Even though some work has been done on the breeding aspects of Tasar silkworm, not much work has been published so far on pathological aspects of tasar silkworm (Reddy *et al*., 2010a, b). To date, reports on cellular and biochemical changes after immunization in silkworm, *A. mylitta* are scanty. Hence, present investigation was conducted to study cellular and humoral response of *Antheraea mylitta* D. on immunization with attenuated AmCPV.

MATERIALS AND METHODS

This study was carried out during the period July, 2009 to October, 2010 in Silk worm Pathology section, Central Tasar Research and Training Institute, Piska Nagri, Ranchi, Jharkhand, India.

Cytoplasmic polyhedrosis virus (AmCPV) inoculum: Fresh cytoplasmic polyhedrosis virus inoculum's was prepared from diseased silkworm. Completely whitened mid-gut obtained from cytoplasmic polyhedrosised silkworm at an advanced stage of infection were homogenized in sterile distilled water. The polyhedral suspension was filtered through a cheese-cloth and the filtrate was centrifuged at 3000 rpm for 15 min and the polyhedra were purified following Aizawa (1971) by repeated and differential centrifugation. The resultant pellet suspended in distilled water was examined by light microscope for purity. The polyhedral suspension in sterile distilled water was prepared to contain 1×10^5 polyhedra mL^{-1}.

Attenuation of cytoplasmic polyhedrosis virus (AmCPV): The AmCPV polyhedra was treated by 0.114 M Na_2Co_3 (pH 10.35) at 27°C for 24 h, then by 0.02% formaline at 27°C for 36 h. This attenuated AmCPV was used to immunize the tasar silkworm.

Immunization and bioassay: The attenuated AmCPV was tested by bioassay to confirm their no-infectivity to silkworm. Then the silkworm was immunized by oral feeding of attenuated AmCPV. The attenuated AmCPV was smeared on to the *Terminalia tomentosa* (assan) leaves and fed to 4th instars of Daba bivoltine silkworm breed after 24 h of moult. The efficacy of immunization was determined by challenging the silkworm larvae by active AmCPV 24 h after attenuated AmCPV inoculation and mortality due to disease. The treated and controlled larvae reared in indoor rearing condition and the cumulative mortality due to AmCPV was recorded, every day/and microscopic examination. Each treatment had three replications of 50 larvae each.

Estimation of haemocytes count: Every day Total Haemocyte Counts (THC) estimation in the haemolymph of all treated and control batches was determined following the method described by Tauber and Yeager (1935) using haemocytometer. The THC per mm^3 of haemolymph was estimated according to the formula suggested by Jones (1962). Every day from 0 to 8th day 6 larvae were collected from each replication, the haemolymph from all the 6 larvae was collected in to three eppendoff tubes (2 larvae haemolymph/tube) on ice and stored at 4°C. A total of 6 tubes represented 3 replication collections. The Differential Haemocyte Count (DHC) was estimated by counting different haemocytes from a haemocyte population of 200. Different haemocytes were identified based on the morphological features as described by Nittono (1960). The observations were made on THC and DHC counts.

Estimation of total proteins: The total protein content in hemolymph was estimated by the method of Lowry *et al.* (1951). To 100 μL of hemolymph, 100 μL of 20% trichloro acetic acid was added and kept for 30 min the contents were centrifuged at 3,000 rpm for 5 min and the pellet was washed twice with 10% trichloroacetic acid. Finally the palate was dissolved in 0.1 N sodium hydroxide. To the 10 μL of pellet sample 5 mL of alkaline copper sulphate reagent (reagent A: 2% sodium carbonate in 0.1 N sodium hydroxide; reagent B: copper sulphate in 0.1% potassium sodium tartrate in 1:1 ratio) was added. To make alkaline copper sulphate reagent, reagents A and B were mixed well in the ratio of 50:1. After 10 min, 0.5 mL of folin-phenol reagent (the commercial solution was diluted once with distilled water) was added and shaken well. After 30 min, the colour intensity was read at 660 nm in a Jasco V-530 Spectrophotometer. The blank sample contained 10 μL of distilled water, 5 mL of alkaline copper sulphate reagent and 0.5 mL of folin-phenol reagent. The protein content was recorded from the standard curve prepared for bovine serum albumin (10-100 μg). The protein content in the samples was expressed as mg mL^{-1} of hemolymph.

Data analysis: For data analysis the statistical computer application package SPSS 10.0 was employed. The data generated were average of three independent experiments. Datawere subjected to Analysis of Variance (ANOVA) and the means were compared for significance using Duncan's Multiple Range Test (DMRT; $p = 0.05$) (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of attenuated AmCPV on mortality in silkworm: The results of mortality in tasar silkworm immunized with Attenuated AmCPV and challenged with active AmCPV are presented in Table 1. It was observed that the attenuated AmCPV provides protection against AmCPV infection for a short period (6 to 8 days). The immunization 24 h prior to AmCPV inoculation was more effective to delay and reduce the mortality due to AmCPV infection. The cumulative mortality

Table 1: Effect of attenuated AmCPV (50 μL mL^{-1}) on mortality in AmCPV inoculated (1×10^9 POB mL^{-1}) tasar silkworm, *A. mylitta* D

	Cumulative mortality (%) due to AmCPV infection							
	Days after inoculation of AmCPV							
Treatment	6	8	10	12	14	16	18	20
Attenuated AmCPV	0	0	0	0	0	0	0	0
Attenuated AmCPV +Active AmCPV	0	0	0	0	1.33±0.35^c	2.67±0.37^c	6.33±0.54^c	10.67±0.52^c
Active AmCPV +Attenuated AmCPV	3.33±0.45^b	6.33±0.47^a	13.00±0.54^b	20.33±0.45^b	28.67±0.27^b	33.67±0.52^b	37.33±0.35^b	42.33±0.27^c
Inoculated control	15.33±0.35^a	21.33±0.24^b	25.67±0.27^a	35.67±0.25^a	41.33±0.25^a	45.33±0.54^a	52.67±0.25^a	58.33±0.25^c
Normal control	0	0	0	0	0	0	0	0

Every Value represents the mean of three replicates±SE followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at p = 0.05

Table 2: Total haemocyte count after immunization and AmCPV infection in silkworm, *A. mylitta*

	Days post inoculation							
Treatment	1	2	3	4	5	6	7	8
Attenuated AmCPV	13650±0.25^a	15237±0.37^a	15950±0.33^a	16534±0.54^a	16408±0.51^a	16432±0.25^a	15503±0.51^a	16625±0.25^a
Inoculated control	12700±0.35^b	14480±0.57^b	14350±0.35^b	12671±0.45^c	9851±0.54^c	6203±0.35^c	5009±0.57^c	3812±0.35^c
Normal control	12352±0.75^c	12530±0.29^c	12982±0.55^c	13455±0.35^b	13670±0.51^b	13702±0.45^b	12380±0.35^b	13801±0.47^b

Every Value represents the mean of three replicates±SE followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at p = 0.05

was 0.00% on 6th day which increased to 10.67% on 20th day. The mortality in immunized silkworms was reduced significantly as compared to non immunized inoculated control (15.33% on 6th day which increased to 58.33% on 20th day). The immunization after inoculation was comparatively ineffective as the mortality was higher (3.33% on 6th day which increased to 42.33% on 20th day).

Few workers in past have studied on immunization of *Bombyx mori* against the infection of nuclear polyhedrosis virus. More or less similar observation have been reported by Aizawa (1954) who reported that the immunization of silkworm pupae with attenuated virus was effective in preventing some of the pupae dying due to viral infection. Liu and Zhong (1989) have also reported reduction of loss in *Bombyx mori* due to cytoplasmic polyhedrosis by 30-60 and 40-60% by oral feeding of attenuated BmCPV at laboratory and farmers level respectively. Nataraju *et al.* (2000) tested the potential of attenuated BmNPV as oral vaccine in silkworm, *B. mori* against BmNPV which resulted reduction of infection by 85%.

Cellular changes in immunized silkworm, *A. mylitta*

Total Haemocyte Counts (THC): The results on total haemocyte count in immunized and AmCPV infected silkworms are presented in Table 2. The total haemocyte counts increased from 1st day to 6th days and decreased on 7th day after the next moult and again increased on 8th day in the silkworm immunized with attenuated AmCPV and normal control. In the immunized silkworms, the total haemocyte counts was 13650 mm^{-3} of haemolymph and increased to 16432 mm^{-3} by 6th day. On 7th day total haemocyte counts were 15503 mm^{-3}. On 8th day the count was

increased up to 16625 mm^{-3}. The similar trend was observed in non immunized normal control. While in inoculated control the counts were increased after inoculation with AmCPV up to 2nd day of infection. Then there was a decrease for a period ranging from 3-8 days. In non immunized normal control, the total haemocyte count was significantly low as compared to the immunized silkworms because the increase may represent the defense response of silkworm, *A. mylitta* against the invading pathogen.

The observed data agreed with the earlier workers as they investigated that once entomophagus fungi have penetrated in the host integument and gained access nutrient-rich haemocoel, they are confronted with host humoral and or cellular defenses (Butt *et al*., 1988; Butt and Humber, 1989; Vey and Gotz, 1986). As humoral response, the phenoloxidase system will be activated to induce the phagocytic process and melanization which works as toxin to invading microorganism (Tanada and Kaya, 1993). The cellular responses to infection have been worked out in many insect by earlier workers (Chain and Anderson, 1982; Dunn and Drake, 1983; Horohov and Dunn, 1983). Similar observations have also been made Mallikarjuna *et al*. (2002) in his study on the effect of systematic fungicide on the total haemocyte count in *Beauveria bassiana* infected silkworm, *Bombyx mori*. Salt (1970) reported that Haemocytes are exteremely efficient in removing pathogens by accomplishing a series of reactions designated as phagocytosis, nodule formation or encapsulation. The present observations are in agreement with the earlier investigation that the number of haemocytes may increase (Balavenkatasubbaiah *et al*., 2001) and decrease (Gilliam and Shimanuki, 1967) to counter foreign body when infected. Recently stress has been induced to tasar silkworm to study its impact on Haemocytes count (Pandey *et al*., 2010). Similar studies carried out in other insects also confirms our results (Al-Attar, 2010). The results revealed that, on the basis of the above findings of the earlier workers it is evident that CPV induce the defense response through multiplication of haemocytes as is indicated by the increase in total haemocyte counts of the haemolymph of the worms.

Differential Haemocyte Counts (DHC): The observations with regard to DHC in silkworm, *Antheraea mylitta* D. immunized with attenuated AmCPV and challenged with active AmCPV are depicted in Table 3. In immunized silkworm and non immunized normal control all the recorded haemocytes showed a gradual increasing trend during developmental period. The number of prohemocyte, Plasmatocytes, granulocytes and spherulocytes viz., 28-38, 33-39, 46-52 and 22-31, respectively in immunized silkworm which were comparatively higher than non immunized control. While during progressive infection, the gradual decrease in number of prohemocyte, spherulocytes and oenocytoids viz., 28-12, 24-9 and 13-3, respectively was noticed in inoculated control. The plasmatocytes and granulocytes decreased up to 3rd day and increasing trend was observed from 5th day onwards. The number of oenocytoid was less in all the treatment including inoculated and normal control and ranged from 3 to 15. Few vermiform cells, synonyms of plasmatocyte (Lea and Gilbert, 1966) were recorded in both the treatments and control. The number of degenerated cells was comparatively less in immunized and non immunized silkworms than the inoculated control.

The number of prohemocyte decreased due to the conversion of prohemocyte to other types of haemocyte during course of infection and number of plasmatocytes and granulocytes increased as both are involved in defense mechanism against entry of pathogens. The palsmatocyte and granulocytes function in defense against largest foreign body in *Bombyx mori* L. was worked out by Sato *et al*. (1976) and Akai and Sato (1976).

Table 3: Differential haemocyte counts after immunization and AmCPV infection in silkworm, *A. mylitta*

		Days post inoculation							
Treatment	Haemocyte	1	2	3	4	5	6	7	8
Attenuated AmCPV	PR	28±0.25	30±0.33	31±0.23	33±0.25	35±0.38	35±0.66	36±0.28	38±0.32
	PL	33±0.57	35±0.34	37±0.29	38±0.23	38±0.39	38±0.35	37±0.56	39±0.25
	GR	47±0.33	49±0.37	50±0.35	52±0.45	52±0.37	51±0.38	51±0.54	52±0.26
	SP	22±0.45	24±0.25	25±0.25	26±0.65	28±0.29	30±0.27	28±0.27	31±0.28
	OE	4±0.29	5±0.23	5±0.36	5±0.24	6±0.37	9±0.54	8±0.56	12±0.35
	VER	0	2±0.21	0	2±0.28	0	0	0	0
	DEG	8±0.21	8±0.37	6±0.25	5±0.28	8±036	7±0.65	6±0.28	6±0.35
Inoculated control	PR	28±0.33	25±0.34	24±0.23	23±0.38	18±0.75	17±0.65	15±0.25	12±0.37
	PL	33±0.27	39±0.29	44±0.89	42±0.54	38±0.78	35±0.59	32±0.23	25±0.39
	GR	35±0.57	45±0.47	48±0.75	45±0.35	44±0.85	41±0.25	32±0.45	27±0.35
	SP	24±0.67	20±0.28	18±0.56	17±0.28	15±0.65	14±0.54	12±0.75	9±0.31
	OE	13±0.63	11±0.35	10±0.28	8±0.49	8±0.35	5±0.27	4±0.54	3±0.27
	VER	1±0.57	0	0	0	0	0	0	0
	DEG	22±0.54	24±0.65	25±0.56	26±0.35	30±0.38	37±0.95	39±0.56	42±0.28
Normal control	PR	22±0.26	25±056	27±0.65	29±0.37	30±0.39	33±0.68	34±0.28	35±0.26
	PL	32±0.26	33±0.32	35±0.23	37±0.36	39±0.37	41±0.38	37±0.29	38±0.25
	GR	35±0.37	36±0.28	38±0.25	39±0.25	42±0.28	40±0.25	38±0.35	38±0.27
	SP	17±0.38	18±0.27	20±0.24	22±0.29	25±0.39	26±0.25	27±0.36	28±0.29
	OE	5±0.45	6±0.59	8±0.89	10±0.29	10±0.37	12±0.58	13±0.35	15±0.38
	VER	0	0	0	0	4±0.38	1±0.38	0	0
	DEG	10±0.57	12±0.23	13±0.13	14±0.37	16±0.28	9±0.28	7±0.25	8±0.23

Every value represents the mean of three replicates±SE. PR: Prohaemocyte, PL: Plasmatocyte, GR: Granulocyte, SP: Spherulocyte, OE: Oenocytoid, VER: Vermiform cell, DEG: Degenerated cell

Table 4: Total protein contents (mg mL^{-1}) in immunized silkworm, *A. Mylitta*

	Days post inoculation							
Treatment	1	2	3	4	5	6	7	8
Attenuated AmCPV	32.18±0.33^{a}	36.85±0.47^{a}	41.52±0.34^{a}	45.42±0.21^{a}	49.26±0.57^{a}	51.25±0.33^{a}	54.48±0.37^{a}	57.13±0.27^{a}
Inoculated control	33.26±0.37^{a}	36.28±0.63^{a}	40.73±0.37^{b}	43.36±0.27^{b}	45.10±0.43^{b}	48.49±0.37^{b}	43.93±0.42^{b}	40.27±0.33^{c}
Normal control	22.75±0.29^{b}	28.56±0.37^{b}	33.31±0.42^{c}	35.27±0.32^{c}	36.95±0.27^{c}	38.24±0.29^{c}	40.38±0.67^{c}	43.58±0.57^{b}

Every Value represents the mean of three replicates±SE followed by the same letter in a column do not differ significantly according to Duncan's multiple range test at p = 0.05

Biochemical changes in immunized silkworm, *A. mylitta*

Total protein contents: The data on the effect of immunization on hemolymph total protein contents in AmCPV infected silkworm are presented in Table 4. The hemolymth protein in immunized silkworms increased gradually from 32.18 mg mL^{-1} on 1st day to 57.13 mg mL^{-1} on day 8th. Similar trend was observed in non immunized silkworms but the total hemolymph proteins were significantly low. In inoculated control, the total hemolymph proteins have shown increasing trend from 1st to 5th day and decreasing trend from 6th day onwards.

The results indicated that changes occur in the hemolymph protein, during the course of AmCPV infection. The difference in hemolymph protein between immunized, non-immunized

silkworm and inoculated control becomes more pronounced as the diseases progresses. This would probably indicate that during infection the synthesis and release of proteins from fat bodies are greatly increased. There are reports of production of antimicrobial substances such as lectin, defensin and attacin with the entry of foreign bodies (Wago, 1995). Pombo *et al.* (1998) reported that several viral induced proteins were also produced during infection of baculovirus and a sharp decrease in overal protein synthesis was observed. Sinha *et al.* (1996) studied the changes in protein content in haemolymph of tasar silkworm, *Antheraea mylitta* and reported that protein content in it increases enormously during larval development. A great reduction in all the protein fractions was reported in the haemolymph of heavily diseased larvae. Quantitative and qualitiative changes in protein profile of various tissues of tropical tasar silkworm, Antherea mylitta D was recently studied (Kumar *et al.*, 2011). The effect of infection by cytoplasmic polyhedrosis virus on the midgut protein metabolism in silkworms was studied by Watanabe (1971) and reported that the active synthesis of midgut proteins, as well as polyhedron proteins, is induced by infection and continues until later stages.

CONCLUSION

In this study, Cellular and biochemical changes in tropical tasar silkworm *Antheraea mylitta* D. on immunization with attenuated *Antheraea mylitta* Cytoplasmic Polyhedrosis Virus (AmCPV) was carried out. Attenuated virus induced protection against severe strain of virus. The survability of the larvae was due to increase of haemocytes and protein level in attenuated larvae of tasar silkworm.

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