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STUDIES ON INDUCTION OF TOLERANCE AGAINST NUCLEAR POLYHEDROSIS IN SILKWORM *BOMBYX MORI* L. AND ITS BIOCHEMICAL ASPECTS

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ABSTRACT

To induce tolerance in silkworm against nuclear polyhedrosis virus, two sets of experiments were carried out. For both the experiments four mulberry silkworm breeds namely NB_4D_2, CSR_2, Pure Mysore, *C. nichi* and a commercial hybrid (PM X CSR_2) were used. In the first set of experiments silkworms were fed with known amount of formalin killed *Bombyx mori* nuclear polyhedral inclusion bodies (PIBs) thrice at various time intervals for multiple immunization. In the second set of experiments such treatment was given only once. At the age of 5th instar second day the silkworms of all the batches were challenged with known amount of live PIBs. Among immunization methods, multiple immunization method was more effective as all the races indicated by high survival rate. Also, variation in quantitative biochemical composition was observed with respect to haemolymph proteins and, enzymes such as succinate dehydrogenase and amylase activity levels when compared with the unimmunized controls. The results pave the way towards the use of immunization method to induce tolerance in silkworm against the dreaded nuclear polyhedrosis virus.

Keywords: Nuclear polyhedrosis, tolerance, haemolymph proteins, amylase, succinate Dehydrogenase

INTRODUCTION

In India, the silkworm cocoon crop loss due to NPV has been reported to an extent of 32.9-55.3 % among the total silkworm diseases **[1]** and most common in summer season **[2]**. Though insects exhibit both humoral and cellular immune response

against various pathogens, no immune system is effective against viral infections **[3]** unlike higher vertebrates. However, some attempts have been made to immunize insects with NPV **[4, 5]**. Aizawa **[6]**, observed some degree of protection when silkworm pupae were injected with a vaccine prepared from infected insect blood. An effort was made to develop an oral vaccine against NPV in silkworms **[7]**. The immunity in *Bombyx mori* against bacteria can be induced by injection of synthetic oligodeoxynucleaotides to silkworm haemocoel **[8]**. Most of the biochemical studies associated with nuclear polyhedrosis in silkworm *Bombyx mori* are limited to haemocyte counts **[9]**; food consumption & utilization **[10]** and effects on some biomolecules **[11]**.

However, studies combining induction of tolerance with biomolecules are rather scarce. Hence the present investigation was undertaken to study the oral immunization and biochemical background of immunization.

MATERIALS AND METHODS

Four silkworm breeds namely, NB_4D_2, CSR_2 (Bivoltines), Pure Mysore, C. nichi (Multivoltines) and a hybrid (PM X CSR_2) silkworms were selected as experimental system to induce tolerance and a hybrid (PM X CSR_2) was selected as experimental system for biochemical studies. The silkworm rearing was conducted in the laboratory following the method described by **Krishnaswami, 1978, 1979, [12, 13]**. Five hundred larvae were kept in triplicate after 3rd moult.

The Nuclear Polyhedral Inclusion Bodies were collected, confirmed and purified **[14]**. Finally, the stock suspension was prepared which contained 7.125 X 10^6 polyhedral inclusion bodies per ml. Enumeration of polyhedral inclusion bodies were done by following Neuber's haemocytometer. For the induction of the resistance, polyhedral bodies were treated in 2% formaldehyde solution for 24h at 30 $\pm$ 1 ºC and used.

In the first sets of experiments, mulberry leaves of M_5 variety were washed with sterile distilled water and surface sterilized with 70 per cent ethyl alcohol using sterile cotton wad. Then the leaves were cut to square shape (10 cm^2) and 0.1 ml inactivated PIBs suspension (from the stock of 0.835 x 10^7/ml PIBs suspension in sterile distilled water) was uniformly smeared and fed to the silkworms. The PIBs smeared leaves were shade dried and chopped to the required size and fed to the silkworms. Suitable untreated batches were also maintained. All experimental batches were maintained with 50 worms in triplicate. Such type of inoculation was carried out thrice *i.e.*, at the age of third instar second day, again at the age of fourth instar second

day and also, at the age of fifth instar first day.

In the second set of experiments, the silkworms of all the races were fed with same quantity of inactivated PIBs at the age of fifth instar first day only.

Again, at the age of fifth instar second day, the silkworms of all the batches *i.e.*, controls as well as treated batches were challenge inoculated with 0.2 ml of PIBs (from the stock of 6 x 10^6 /ml PIBs) of live polyhedral bodies in sterile distilled water. The control worms received the same amount of sterile distilled water only. Later, the worms were allowed to complete larval stage, spinning, pupation and moth emergence. Number of cocoons harvested from each batch was considered for calculation of the response of silkworm races for the induction of resistance.

The silkworms of PM x CSR_2 hybrid were first treated with 0.1 ml of inactivated PIBs (from the stock of 0.835 X 10^7/ml) thrice *i.e.*, once each at the age of third instar second day, fourth instar 3rd day and also at the age of fifth instar first day followed by challenge inoculation with 0.125 ml of live PIBs (from the stock of 1.36 X 10^7/ml) on second day of fifth instar as described earlier. The control batches, immunized batches, immunized followed by challenge inoculated and live virus treated batches are mentioned as C, I, IL and L respectively. The haemolymph was used for all studies.

The larvae from third day of fifth instar were collected daily with a regular interval of 24 h. The haemolymph was collected **[15]** and preserved in a deep freezer at -20°C as stock, and it was used whenever required.

The total protein present in haemolymph was determined by following the method of **Lowry *et al.*, 1951, [16]**. Bovine serum albumin was used as standard protein. The results were expressed as µg of protein/µl of haemolymph.

Quantitative analysis of amylase activity was done in haemolymph following the method of **Noelting and Bernfeld, 1948, [17]**, using 3, 5-dinitrosalicylic acid reagent, as modified by **Ishaaya and Swirski, 1976, [18]**. The reaction mixture contained 2.0 ml of 0.05M sodium phosphate buffer (pH 6.5), 1.0 ml of freshly prepared 1% starch solution and 1.0 ml of appropriately prepared sample solution. Incubation of this mixture was carried out for 30 minutes at 37°C in a water bath. After 30 min, 1 ml of 3,5-dinitrosalicylic acid reagent was added and the mixture was boiled in a water bath for 10 min, then cooled in ice water. The optical density was measured at 540 nm setting the spectrophotometer (Spectronic 2000) to zero with blank solution consisting 1 ml of distilled water in place of sample

solution. The activity of the enzyme was expressed as µg glucose released/µl of haemolymph/min. Succinate dehydrogenase activity levels were estimated by the method of **Nachlas *et al.*, 1960, [19]**, as modified by **Mahesha, 1997, [20]**. The reaction mixture contained one ml of standard sodium phosphate buffer (0.1M, pH 7.4), one ml of 2(4-iodophenyl)-3(4-nitrophenyl) - 5-Phenyltetrazolium chloride (INT, 1mg/ml) and one ml of sodium succinate (15mM). The reaction was initiated by the addition of one ml of sample solution. The tubes were incubated at 37°C for one hour. The reaction was stopped by the addition of 6 ml of glacial acetic acid. The red colour farmazan formed was extracted into 6 ml of toluene by keeping the sample tubes overnight at 4°C. The colour was read at 495 nm in a UV-vis spectrophotometer. The succinate dehydrogenase activity levels were expressed in micromoles of formazan formed per µl haemolymph per hour.

The experimental data were statistically analyzed through SPSS by one way ANOVA **[21]** and Scheffe's post hoc test **[22]** wherever they were applicable.

RESULTS AND DISCUSSION

The results of single immunization and multiple immunization is presented in the **Table 1**.

1) **Single Immunization:** In Pure Mysore race, the viability rate in the control as well as immunized batches recorded the highest rate of 100%. Whereas the immunized followed by challenge inoculated batches showed 94% and the batch inoculated with live virus showed 56% only. In C. nichi race, the viability rate in the control as well as immunized batches recorded the highest rate of 100%. Whereas immunized followed by challenge inoculated batches showed 76% and the batch inoculated with live virus showed 50% only. In $NB4D_2$ race, the viability rate in the control batch was 96%, followed by immunized (94%), immunized followed by challenge inoculated (66%) and live virus inoculated (38%) batches. In CSR_2 race, the viability rate in the control batch was 96%, followed by immunized (92%), immunized followed by challenge inoculated (66%) and live virus inoculated (38%) batches. In the Hybrid, again the viability rate in the control as well as immunized batches recorded the highest rate of 100%. Whereas immunized

followed by challenge inoculated batches showed 76%, followed by the batch inoculated with live virus showed 44% only.

2) **Multiple Immunization:** In Pure Mysore race, the viability rate in the control as well as immunized batches recorded the highest rate of 100%. Whereas immunized followed by challenge inoculated batches showed 96%, followed by the batch inoculated with live virus 56% only. In C. nichi race, the viability rate in the control as well as immunized batches recorded the highest rate of 100%. Whereas immunized followed by challenge inoculated batches showed 80%, followed by the batch inoculated with live virus showed 50% only. In NB_4D_2 race, the viability rate in the control set was highest (96%) followed by immunized (95%), immunized followed by challenge inoculated (78%) and live virus inoculated (38%) sets. In CSR_2 race, the viability rate in the control and immunized batches recorded the highest rate of 100%. Whereas immunized followed by challenge inoculated batches showed 94%, followed by the batch inoculated with live virus 56% only. In the Hybrid, again the viability rate in the control as well as immunized batches recorded the highest rate of 100%. Whereas immunized followed by challenge inoculated batches showed 84%, followed by the batch inoculated with live virus 44% only.

3) **Biochemical Studies**

Protein: Total protein levels were estimated in all the four batches *viz.,* control, immunized, immunized followed by challenge inoculated and live virus inoculated batches of the hybrid. In control worms, haemolymph proteins showed a significant increase in their levels at every 24 hours till the end of fifth instar. This pattern of haemolymph proteins was observed even in the treated batches **(Table 2)**. The higher concentration of proteins was observed in control set (27.118 µg/µl was the average during 5th instar) followed by the attenuated (30.380 µg/µl), attenuated followed by live virus treated worms (26.074 µg/µl)

and live virus treated set (25.603 µg/µl). Further, the results of one way ANOVA revealed that the variation between the experimental sets is significant at 5% level. **Amylase:** The amylase activity in haemolymph of control set of Pure Mysore X CSR2 silkworms increased with the increase in the age during the fifth instar, and reached maximum on 9th day **(Table 3)**. This pattern of amylase activity was observed even in immunized, immunized followed by live PIBs treated and live PIBs treated batches. However, the rate of activity (Average) was more in case of immunized and immunized followed by live PIBs treated sets. Of the four experimental sets, the average enzyme activity during fifth instar exhibited by immunized set was 22.689 µg/µl/h, followed by immunized followed by live PIBs 22.142 µg/µl/h, control 21.094 µg/µl/h. The results of one way ANOVA revealed that the variation between tested batches is significant at 5% level. **Succinate Dehydrogenase:** The succinate dehydrogenate (SDH) activity levels in the haemolymph of control larvae, showed a higher level on third day and gradual increment from fourth day till 8th day. On the 9th day again 44% lesser activity was recorded. In case of experimental batches, the pattern of enzyme activity levels was almost same except immunized and immunized followed by live PIBs treated sets. In case of immunized followed by live PIBs treated sets the peak activity was noticed on 7th day, whereas live PIBs treated set showed enhanced rate of activity through out the pathogenesis **(Table 4)**. Of all the batches tested, the average activity of the succinate dehydrogenase was more in live PIBs treated set with 1.175 µmoles/µl/hour followed by immunized followed by live PIBs 0.955 µmoles/µl/hour, immunized set with 0.927 µmoles/µl/hour and control with 0.713 µmoles/µml/hour (values are expressed as micromoles of formazan formed/µl/h.). The variation between control and experimental sets and, among

experimental sets is all found to be significant at 5% level.

Such a metabolic change might help in improving the defense mechanism of the host. Disappearance of protein bands during different periods of larval development indicates either the non production or utilization of blood proteins. The hydrolysis of proteins might have occurred during the larval period to form amino acids, which in turn, might be utilized to form silk proteins. The hydrolysis of blood proteins to form amino acids are in agreement with the results of **Beadle and Shaw**, 1950, **[23]**, who reported the hydrolysis of proteins during the larval life of the *Bombyx mori* for the maintenance of amino acid concentration in the blood. **Florkin**, 1937, **[24]** has reported that the free amino acids in the blood of *Bombyx mori* are the efficient precursors for the production of silk.

The induction of resistance/immunization was more effective in multiple immunization technique when compared to single immunization method. Because, in defense mechanism, unlike vertebrate which has red blood corpuscles and white blood corpuscles in a closed circulatory system, insects with open body cavity lack lymphocytes, the major source of vertebrate immunity to virus infection. But insects have only free blood cells called haemocytes, however, they lack memory.

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Table 1: Effect of Single and Multiple Immunization on Viability

Race ↓	Treatment ↓	Single Immunization: No. of cocoons harvested	Single Immunization: No. of cocoons with live pupae	Single Immunization: No. of moths emerged	Single Immunization: Viability %	Multiple Immunization: No. of cocoons harvested	Multiple Immunization: No. of cocoons with live pupae	Multiple Immunization: No. of moths emerged	Multiple Immunization: Viability %
Pure Mysore	C	50	50	50	100	50	50	50	100
	I	50	50	50	100	50	50	50	100
	IL	50	48	47	94	50	48	48	96
	L	36	32	28	56	35	33	28	56
C. nichi	C	50	50	50	100	50	50	50	100
	I	50	50	50	100	50	50	50	100
	IL	44	41	38	76	44	41	40	80
	L	30	35	25	50	28	26	25	50
NB_4D_2	C	50	49	48	96	50	50	48	96
	I	50	49	47	94	50	50	47	94
	IL	43	40	33	66	43	41	39	78
	L	26	23	19	38	26	22	19	38
CSR_2	C	48	48	48	96	49	48	48	96
	I	46	46	46	92	49	47	46	92
	IL	37	34	33	66	45	41	39	78
	L	25	23	19	38	26	22	19	38
PM x CSR_2	C	50	50	50	100	50	50	50	100
	I	50	50	50	100	50	50	50	100
	IL	42	31	38	76	44	41	42	84
	L	24	23	22	44	25	23	22	44

NOTE: The variation between control and experimental and among experimental sets are all significant at 5% level; Fifty worms in triplicate for each dose were used for inoculation

Table 2: Concentration of Total Proteins (µg/µl) in Haemolymph of Pure Mysore X CSR_2 Larvae

Dose of PIBs	Fifth Instar Development							Average concentration
	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	
Control	9.330	12.200 (+30.76)	19.966 (+63.65)	24.600 (+23.20)	32.569 (+32.39)	42.330 (+29.97)	48.833 (+15.36)	27.118
Induced	9.069 (-2.79)	14.300 (+57.68) (+17.21)	24.800 (+73.42) (+24.21)	33.200 (+33.87) (+34.95)	38.530 (+16.05) (+18.30)	46.165 (+19.81) (+9.05)	46.600 (+0.94) (-4.57)	30.380
Induced followed by Live	8.565 (-8.19)	12.430 (+45.12) (+1.88)	19.233 (+54.73) (-3.67)	22.165 (+26.29) (-9.89)	32.690 (+47.48) (+0.37)	40.200 (+22.97) (-5.03)	48.566 (+20.81) (-0.54)	26.074
Live	9.030 (+16.41)	14.465 (+60.18) (+18.56)	16.066 (+1.06) (-19.53)	23.530 (+46.45) (-4.06)	35.500 (+50.87) (+8.99)	40.300 (+13.52) (-4.79)	42.330 (+5036) (-13..31)	25.603

NOTE: Values within parenthesis (1st row) represent percent change over previous day; Values within parenthesis (2nd row) represent percent change over control; The variation between control and experimental sets and among different treatment are all significant at 5% level

Table 3: Amylase Activity Levels in Haemolymph of Pure Mysore x CSR_2 Larvae (Values expressed as µg of glucose released /µl of haemolymph/h)

Dose of PIBs	Fifth Instar Development							Average concentration
	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	
Control	3.333	4.999 (+49.98)	12.333 (+86.69)	24.498 (+194.63)	25.000 (-9.08)	25.000 (0.00)	52.497 (+109.98)	21.094
Induced	4.166 (+24.99)	7.166 (+120.01) (+83.35)	13.499 (+36.36) (+33.92)	25.999 (+100) (-9.08)	26.832 (+7.33) (+120.01)	30.832 (+14.90) (+120.01)	51.333 (+66.49) (+120.01)	22.689
Induced followed by Live	4.166 (+24.99)	4.999 (-15.55) (-6.83)	13.332 (+85.66) (-7.75)	22.499 (+65.02) (-18.17)	26.665 (+38.84) (-6.99)	30.665 (-12.17) (-16.26)	51.666 (-4.35) (-13.94)	22.142
Live	6.666 (+100)	7.499 (+12.49) (-8.51)	12.332 (+64.44) (-21.01)	16.000 (+29.74) (-36.48)	22.832 (+42.7) (-11.09)	24.498 (+7.29) (-23.63)	43.464 (+77.41) (-28.09)	19.041

NOTE: Values within parenthesis (1st row) represent percent change over previous day; Values within parenthesis (2nd row) represent percent change over control; The variation between control and experimental sets and among different treatments are all significant at 5% level

Table 4: Succinate Dehydrogenase Activity Levels in Haemolymph of Pure Mysore x CSR_2 Larvae (Values expressed as µ moles of farmazan formed/µl of haemolymph/h)

Dose of PIBs	Fifth Instar Development							Average concentration
	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	
Control	0.928	0.440 (-52.58)	0.488 (+10.90)	0.560 (+14.75)	0.688 (+22.85)	1.216 (+76.74)	0.672 (-44.73)	0.713
Induced	1.440 (+55.17)	0.448 (+68.88) (+1.81)	0.348 (+22.32) (+28.68)	0.928 (+166.66) (+65.71)	1.056 (+13.79) (+53.48)	1.536 (+45.45) (+26.31)	0.736 (-52.08) (+9.52)	0.927
Induced followed by Live	1.632 (+75.86)	0.576 (-64.70) (+30.9)	0.576 (0.00) (+18.03)	0.768 (+33.33) (+37.14)	1.216 (+58.33) (+76.74)	1.120 (+7.89) (-7.89)	0.800 (-28.57) (+19.04)	0.955
Live	1.568 (+68.96)	1.536 (-2.04) (+249.1)	1.088 (+29.16) (+122.95)	1.312 (+20.58) (+134.28)	0.992 (-24.39) (+44.18)	0.672 (-32.25) (-44.73)	1.056 (+57.44) (+57.14)	1.175

NOTE: Values within parenthesis (1st row) represent percent change over previous day; Values within parenthesis (2nd row) represent percent change over control; The variation between control and experimental sets and among different treatments is all significant at 5% level