Proc. Indian natn. Sci. Acad., **B 59**, No. 6, pp. 543-548

Late Larval Resistance of *Spodoptera litura* (Fabricius) to Nuclear Polyhedrosis Virus

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(Received 12 November 1992; after revision 20 April 1993; Accepted on 2 July 1993)

Bioassay of the nuclear polyhedrosis virus (NPV) was conducted against *Spodoptera litura* larvae. The LC 50 values recorded a steady increase with larval age. Larvae could not be infected *per os* after 8th day. Regression lines of all larval ages except day 1 were almost parallel to each other, indicating absence of true resistance. Further, intrahaemocoelic injection of non-occluded virus (NOV) and occluded virus (OV) into 6th instar larvae resulted in death at prepual stage due to polyhedrosis. Hence, lack of susceptibility beyond 8th day is due to a gut mediated barrier which comes into operation as the larvae approach pupation. The absence of NOV antigen in the haemolymph of the 6th instar virus fed larvae revealed thorugh DAS-ELISA strengthens the above inference.

Key Words: Late larval resistance, *Spodoptera litura*, NPV

Introduction

Baculoviruses produce fatal disease in larvae of a number of insect species particularly in Lepidoptera and Hymenoptera. The polyhedral inclusion bodies (PIBs) reach the larval gut on ingestion where they are dissolved to liberate virions referred to as occluded virus (OV). These virions fuse with microvilli on the midgut coloumnar or epithelial cells resulting in the release of nucleocapsids into the cytoplasm. These nucleocapsids initiate the primary cycle of replication in the nuclei of midgut cells. The progeny nucleocapsids bud through the plasma membrane into the haemocoel acquiring an envelope in the process. The virions thus released into the haemocoel are referred to as non-occluded virus (NOV) which start the secondary cycle of replication in all the susceptible tissue resulting in the production of polyhedra and ultimately the death of the larva (Granados 1980).

The lethality of baculoviruses prompted many investigators to evaluate their potential as microbial insecticides and it has been found in several Lepidoptera that the susceptibility to baculoviruses decreased as larvae approached pupation. In an earlier work with *Spodoptera litura* an increase in LC_{50} values with larval maturation was recorded (Udom Komolpith & Ramakrishnan 1975). The actual mechanism of such phenomenon has not been worked out in many cases.

We report here the results of an investigation into the effect of larval age on the susceptibility of *Spodoptera litura* to Nuclear Polyhedrosis Virus (NPV). Experiments were also conducted to study the susceptibility of larvae through intrahaemocoelic route of infection and also to detect viral antigens in the larval haemolymph as a sequel to the investigation.

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Materials and Methods

Larvae of *S. litura* were reared on castor leaves at 27 ± 1°C and 75 ± 5% RH. Under these conditions the larval period was 13 days, including six instars. The 5th and 6th instar lasted for 2 and 3 days respectively. The virus isolate was a multiple embedded NPV produced in the laboratory-reared host insects. The virus was purified by filtration and differential centrifugation. The NPV was quantified with an improved Neubauer haemocytometer and the concentration of the virus stock was adjusted to 2×10^{11} PIB/ml.

Bioassay

Serial ten-fold dilutions of the virus stock were used in the bioassay. The dilutions used varied between 2×10^3 and 2×10^9 PIB/ml. Circular discs of castor leaves of 2.54 cm diam. were dipped in the virus suspension, allowed to dry and fed to starved larvae. After 12 hr larvae were given fresh castor leaves. Mortality was recorded every 24 hr till death or pupation and the data were subjected to probit analysis. Five replicates each consisting of 10 larvae were used for each treatment.

Intrahaemocoelic injection: Intrahaemocoelic injection into 6th instar larvae was done using NOV and OV. NOV stock was prepared from the haemolymph of infected 4th instar (72 hr post-inoculation). The haemolymph was diluted five times with Hank's insect tissue culture medium and centrifuged at 10,000 g for 15 min to remove haemocytes. It was then filtered through a Whatman membrane filter (0.45 μ) and was used as NOV stock. Occluded virus was prepared from a PIB stock of 10^{10} PIB/ml by mixing it with equal volume of chilled dissolution buffer (0.1 M, Na_2CO_3 + 0.05 M NaCl, *p*H 11.0) and incubating at 4°C for 1 hr. After incubation the material was spun at 4000 g at 4°C for 5 min to remove undissolved polyhedra and other debris. The supernatant was centrifuged at 15,000 g for 1 hr at 4°C to sediment the virus particles. The pellet was suspended in Tris buffer (0.01 M, *p*H 7.5) and filtered through a Whatman membrane filter (0.45 μ) to be used as OV stock.

Serial ten fold dilutions of both NOV and OV stocks were injected into 6th instar larvae at a dose of 2 μl/larva. A total of 30 larvae were used per treatment.

Enzyme-linked immunosorbent assay (ELISA): Double antibody sandwich (DAS-ELISA) method (Clark & Adams 1977) was followed to detect NOV-antigens in the haemolymph of late instar larvae. Nucleocapsids released by sonication from purified virus particles (figure 1) were used to raise antisera in Albino rabbits by multisite intradermal and subcutaneous injections followed by a final intravenous injection (Vaitukaitus et al. 1971). To test the limit of sensitivity of DAS-ELISA and to quantify NOV antigens in larval haemolymph a standard curve was prepared (figure 2) using known quantities of purified nucleocapsid preparation.

Results and Discussion

LC_{50} values of 1, 4, 5, 6, 7 and 8 days old larvae were found to be 0.24, 2.09, 3.80, 14.79, 98.09, and 485.00×10^5 PIB/ml respectively. Thus, 1-day-old larvae were 8, 15, 60, 402 and 1992 times susceptible than 4, 5, 6, 7 and 8 days old larvae respectively. When increase in larval weight was taken into consideration 1-day-old larvae were only twice more susceptible than 8-day-old larvae. Regression lines of all the larval ages except the one for 1-day-old larvae are almost parallel to each other (figure 3). It is intriguing as to why the virulence of a pathogen which can replicate and multiply itself is affected by the dose of the pathogen administered. There is no appreciable shift in the mean response level to the virus and this could be just the appearance of non-susceptibility due to maturation of host larvae. Similar observations were made in *Heliothis punctigera* larvae where the LD_{50} values increased 150-fold during the first week of larval life and the regression lines remained parallel (Teakle et al. 1986). Evans (1981) reported a 34,000-fold difference in

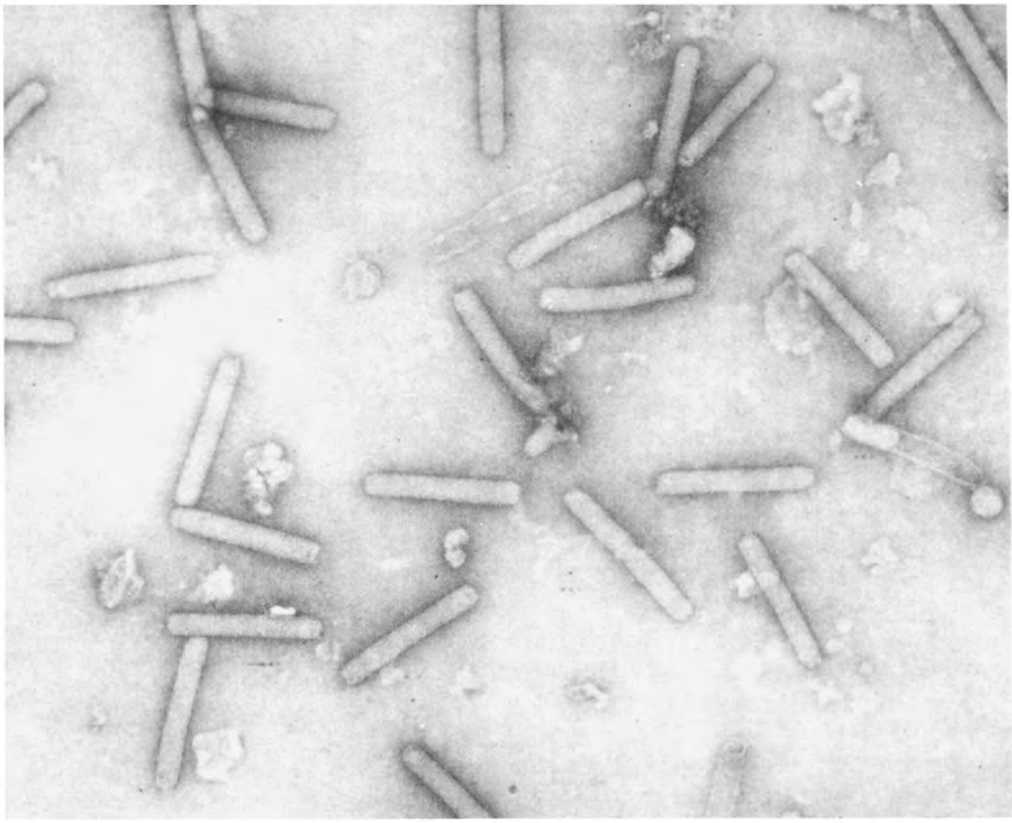

Figure 1 Nucleocapsids of S1 NPV released from virus particles by sonication × 60,000

the LD_{50} values from 1st to 5th instar larvae of *Mamestra brassicae* while 5th and 6th instar larvae were virtually resistant to virus infection. It may therefore be concluded that increase in LD_{50} or LC_{50} values with the increase in age of larvae is not due to true resistance mechanism but due to the dilution effect of viral inoculum related to increase in larval weight.

In the present study no mortality was recorded by *per os* inoculation of 5th instar day two and 6th instar larvae. The larvae pupated and normal healthy adults emerged. This could be due to the fact that larvae infected with a lethal dose of virus pupated before the incubation period of the virus. Thus changes associated with pupation seem to affect the susceptibility of the larvae to the virus.

Susceptibility through Intrahaemocoelic Route

We found 100% mortality of 6th instar larvae even at 1000 times dilution of NOV stock when injected into haemolymph. But with OV only when the original stock was injected 100% mortality was recorded which decreased drastically on dilution (table 1). In both the cases the larvae which died after injection failed to pupate and remained in prepupal stage. When such prepupae were dissected and observed under phase contrast microscope the fat body was heavily infected and polyhedra were seen in large numbers. In the case of *Heliothis armigera* also there was no change in susceptibility of last instar when the virus was administered into haemolymph (Teakle et al. 1986).

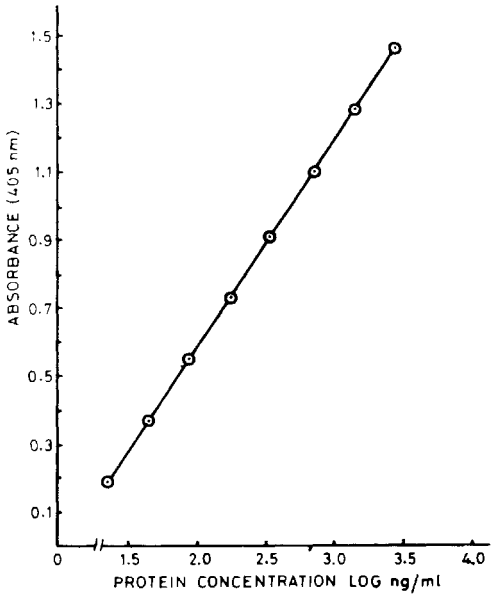

Figure 2 Relationship between nucleocapsid concentration (ng/ml) and absorbance in DAS-ELISA

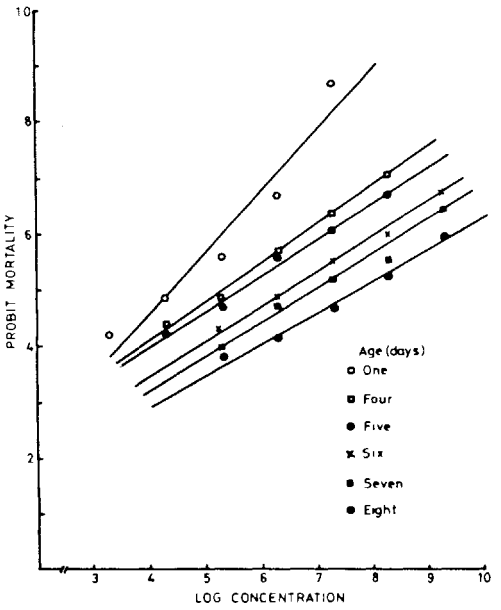

Figure 3 Regression lines of NPV against different larval age groups of *S. litura*

Further the possibility of changes associated with pupation is ruled out as these larvae could be infected by intrahaemocoelic route. Therefore a probe into the receptor sites on the midgut cells and also the probable changes in them with larval age might provide an answer to the midgut barriers.

In the present study the NOV was found to be highly infectious compared to OV as shown by the response of the larvae to different dilutions of these preparations. In earlier studies also, it was shown that OV is highly infectious by *per os* route and its haemocoelic infectivity is considerably low (Stairs 1980). Thus the two forms reflect different functional requirements both in survival and cell attachment in the alkaline environment in the gut and the slightly acid conditions in the haemolymph.

NOV Antigens in the Haemolymph

ELISA technique has been extensively used in the case of baculoviruses because of high degree of its sensitivity. McCarthy and Henchal (1983) used DAS-ELISA to detect NOV of *Autographa californica* (*in vitro* and *in vivo*), using antibody raised against purified nucleocapsid preparation. In this experiment 72 hr was the earliest time at which infectious virus could be detected in the haemolymph. In the present study also DAS-ELISA which was standardized to have a sensitivity of 20 µg/ml was used to detect NOV antigens in the larval haemolymph. When haemolymph from 4th instar day two larvae which bled at 12 hr interval after inoculation was used as test sample in DAS-ELISA, 72 hr was the earliest time for NOV antigens to be detected in the haemolymph (table 2). NOV antigens could be detected 84 hr after inoculation in the case of 5th instar day two larvae. All the inoculated larvae belonging to 6th instar pupated normally and at no points of time were NOV antigens detected in

Table 1 *Susceptibility of sixth instar larvae to intrahaemocoelic injection*

Larval stage	Mortality (percentage)						Control
	Dilution of NOV stock			Dilution of OV stock			
	10^{-1}	10^{-2}	10^{-3}	0	10^{-1}	10^{-2}	
6th instar day one	100.00	100.00	100.00	100.00	56.66 (4.71)	16.66 (8.16)	0.00
6th instar day two	100.00	100.00	100.00	100.00	46.66	0.00 (4.71)	0.00
6th instar day three	100.00	100.00	73.33 (9.428)	100.00	23.33 (9.42)	0.00	0.00

(Values in parentheses indicate SD)

Table 2 *Detection of NOV in the haemolymph by DAS-ELISA*

Hours after *per os* inoculation	4th instar Day 2	5th instar Day 2	6th instar			Control
			Day 1	Day 2	Day 3	
60	—	—	—	—	—	—
72	+(0.55)	—	—	—	—	—
84	+(1.10)	+(0.58)	—	—	—	—
96		+(1.35)	—	—	—	—
108			—	—	—	—
120			—	—	—	—

+ test positive for the antigen
− test negative for the antigen
(Values in parentheses indicate A405 values)

the haemolymph. These findings show that in late larvae the secondary cycle of replication that is essential for the mortality of the host does not take place in the haemolymph and supports further the earlier view that gut is acting as a barrier for the viral entry.

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