Changes in Polyamines and DNA Concentrations in the Midgut Epithelium of the Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) Infected with a Densonucleosis Virus¹

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Changes in polyamines and DNA concentrations in the midgut epithelium of the silkworm infected with a densonucleosis virus (*Bombyx* DNV) were investigated. Three kinds of polyamines, spermidine, putrescine and spermine, were detected in the midgut epithelia of both infected and healthy silkworms. However, the ratio of these polyamines was somewhat different from that of *Bombyx* DNV. In the DNV-infected midgut, lower accumulation of spermidine and putrescine, and lower spermidine/spermine ratio were observed as early as 24 hr after DNV inoculation as compared to healthy midgut. DNA concentrations in DNV-infected midguts fluctuated considerably during the course of DNV infection, but the pattern was rather constant as compared to those of healthy ones. These results suggest that infection with *Bombyx* DNV rapidly affects the physiological condition of host cells, and interferes with normal growth and/or the proliferation of cells.

INTRODUCTION

Cell metabolism is affected in various tissues by virus infection. Many investigators have reported the changes in metabolism of nucleic acids and protein in certain target tissues or cultured cells during virus multiplication (KAWASE and HAYASHI, 1965; CARNEGIE and BEAUDREAU, 1969; BRAITHWAITE et al., 1981).

During three decades the biosynthetic pathways of polyamines have been elucidated, and studies indicated that polyamines play an important role in all organisms. Inter-cellular concentrations of polyamines and their biosynthetic enzyme activities are generally high in actively proliferating tissues (HEBY et al., 1975; OKA et al., 1981). Polyamines are also basic compounds of many viruses (BACHRACH et al., 1974; ELLIOTT and KELLY, 1979; COHEN and GREENBERG, 1981). The significance of these polyamines for the function and multiplication of viruses is still poorly understood, though there have been some reports that the metabolism of polyamines in the cultured cells changes markedly during virus multiplication (McCORMICK and NEWTON, 1975; HEBY et al., 1978).

In the silkworm, *Bombyx mori*, putrescine and spermidine were identified as early as 1952 (ACKERMANN, 1952), and the levels of these polyamines were reported to show

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considerable variation during the life cycle of this insect (ACKERMANN, 1955). Recently, changes in polyamine levels in various organs of the silkworm during its life cycle were also reported by HAMANA et al. (1984). However, as far as we know, there is no report on the changes in polyamine level in the midgut epithelium of the silkworm infected with a virus.

Densonucleosis virus (DNV), which belongs to Parvoviridae containing single-stranded DNA, is one of the main pathogens of the silkworm, and it causes economic damage on sericulture in Japan and mainland China (SHIMIZU, 1975; KAWASE and KANG, 1976; IWASHITA and CHUN, 1982; SEKI and IWASHITA, 1983). We reported in a previous paper the polyamine contents of *Bombyx* DNV (BANDO et al., 1983).

The aim of this paper is to investigate the changes in polyamines and DNA concentrations in the midgut epithelium, target tissue of *Bombyx* DNV, in the silkworm during DNV multiplication.

MATERIALS AND METHODS

Silkworm strain and virus inoculation. Several isolates of silkworm DNV have been obtained in recent years (KAWASE, 1985). They differ in some chemical properties and have specific infectivity to silkworm strains (KAWASE, 1985). The DNV used here is Ina isolate, which was isolated from a diseased silkworm reared at Ina City by SHIMIZU (1975). The silkworm strain, N124 × C124, which is susceptible to the DNV (Ina isolate) was used in the present experiment. On the 1st day of the 4th instar, silkworm larvae were separated into two groups; one group was inoculated by feeding on the inoculum of *Bombyx* DNV on mulberry leaves, and the other was supplied distilled water instead of inoculum as control. Both were reared on mulberry leaves at 25°C. The infected and healthy larvae were collected separately from both groups at 24 hr intervals and stored at -80°C.

Extraction and determination of polyamines and DNA. Midgut epithelia from infected and healthy larvae were taken out in ice-cold saline, and weighed after blotting the extra saline with filter paper. The midgut epithelia were homogenized with 5 vol. of ice-cold 10% perchloric acid (PCA), and 1 ml of the homogenate was centrifuged at 600 g for 15 min. The precipitate was resuspended in 0.5 ml of 5% PCA, and centrifuged after standing for 20 min on ice. The extraction was repeated five times, and the extractions were combined in a test tube as the polyamine fraction. The precipitate was then washed with 1 ml of 80% ethanol twice, and with 1.5 ml of ethanol-ether (3:1 v/v). The residue thus obtained was resuspended in 1 ml of 5% PCA, and nucleic acids were extracted by heating at 80°C for 20 min. The extraction with a hot PCA was repeated four times, and these extracts were used for analysis of DNA.

The identification and quantitative analysis of polyamines were performed by the technique of thin-layer chromatography as previously described (BANDO et al., 1983). DNA was determined by diphenylamine method (BURTON, 1956) using calf thymus DNA as a standard.

Single radial immunodiffusion. The antigen was prepared as follows: midgut epithelia from infected larvae in several infectious stages were homogenized with a Teflon homogenizer in 5 vol. of 0.7 M phosphate buffer (pH 7.5) containing 0.8% NaCl in final concentration (PBS). These homogenates were centrifuged at 10,000 g for 5 min, and the supernatants were applied to single radial immunodiffusion method. The titer of

anti-DNV serum prepared from rabbit was 256, and an 8-fold diluted serum was used here. One per cent agar in PBS containing 0.5% NaN_3 was melted in a boiling water bath and transferred to 56°C, followed by the addition of antiserum at 56°C into agar at a final concentration of 1.5%. After the agar was layered onto a glass plate 1.5 mm thick, several wells 5 mm in diameter, were cut out by gel punch. The wells were filled with the homogenate. Subsequently, the plate was kept at 37°C for 24 hr and the diameter of each precipitin ring was measured after staining with Coomassie brilliant blue.

RESULTS

Changes in weights of individual body and midgut epithelium during the course of infection.

Figure 1 (A) shows that the individual body weight of healthy larvae increased gradually during 4th instar, and marked increase was observed after 4th moulting. In the case of DNV-infected larvae, a quite similar pattern was observed during 6 days after virus inoculation, but no marked increasing phase after 4th moulting was observed. The body weight of infected larvae was almost constant or slightly less after the 2nd day of 5th instar. The patterns of the changes in the midgut weights from healthy and infected larvae were similar to those of individual body weights (Fig. 1B).

Multiplication of DNV

Virus titration was done by applying the single radial immunodiffusion method,

Fig. 1. Changes in body and midgut weight of DNV-infected larvae. A: body weight, B: midgut weight.

Fig. 2. Multiplication of *Bombyx* DNV. Single radial immunodiffusion technique was employed for the titration for virus, and the comparative amount of virus was determined by the size of the precipitin ring. r: radius of immunoprecipitin ring.

and the relative amount of virus was determined by the size of the immunoprecipitin rings (Fig. 2). When the *Bombyx* DNV was inoculated at the 1st day of 4th instar, virus titer increased rapidly from 24 hr after inoculation, and reached maximum at 96 hr. At the late stage of the experimental period, after 7 days, decreasing of the virus titer was observed.

Changes in the concentrations of polyamines and DNA

A thin-layer chromatogram of dansyl derivatives of the extract from midgut epithelia of infected silkworms is shown in Fig. 3. Three polyamines, spermine, spermidine and putrescine, were identified. Putrescine and spermidine were abundant, but spermine was at lower concentration in this tissue. No minor polyamines were detected by this technique. In both infected and healthy midguts, putrescine and spermidine concentrations changed parallel with that of DNA, which will be described later (Figs. 4 and 5). Compared with healthy midgut, putrescine and spermidine concentrations in infected midgut decreased markedly to a lower level, and the level was maintained for 4 successive days. The profile of spermine in the healthy midgut was almost constant, whereas in the infected midgut, the concentration decreased gradually during the last 4 days.

Figure 6 shows the changes in DNA concentrations in the midgut epithelia from infected and healthy larvae during the course of DNV infection. DNA concentrations

Fig. 3. Thin layer chromatogram of polyamines and related compounds in midgut epithelia. Dansyl derivatives were separated by one dimensional chromatography using a solvent, ethylacetate-cyclohexane (2:3, v/v). S: dansyl polyamines as standard, pu: putrescine, spd: spermidine and sp: spermine.

Fig. 4. Changes in concentration of putrescine in midgut epithelia of DNV infected larvae. These values were calculated by the quantitative analyses of the dansyl polyamines on the thin layer chromatogram plates. The same applies to Fig. 5.

Fig. 5. Changes in concentration of spermine and spermidine in the midgut epithelia of DNV infected larvae. SPD: spermidine, SP: spermine.

Fig. 6. Changes in DNA concentration in midgut epithelia of DNV infected larvae.

in both infected and healthy midguts increased remarkably from the last day of 4th instar to the 1st day of 5th instar, and thereafter decreased suddenly, especially in healthy midgut. Considerable differences between infected and healthy midguts were thus observed in the late stage. On the other hand, no remarkable differences in RNA and protein concentrations between infected and healthy midguts were observed (data not shown).

DISCUSSION

Until the 6th day after inoculation, we could not distinguish the DNV-infected larvae from healthy ones by their outer appearance, body weight or midgut weight. However, by single radial immunodiffusion test we could detect the DNV infection only 24 hr after inoculation. The virus multiplied actively from 24 hr after DNV inoculation, and reached maximum at 96 hr (Fig. 2). These results indicate that the virus multiplication may be almost finished by 6 days after inoculation.

WATANABE et al. (1976) reported histochemical observations showed that many midgut epithelial cells infected with DNV (Ina isolate) fall off into the midgut lumen late in the infectious stage. Therefore, it seems highly probable that the decrease of titer of DNV antigen at the late stage reflects the falling off of these midgut cells containing DNV.

Although no remarkable difference was observed in RNA and protein concentrations between infected and healthy midguts, we detected rather high DNA and low polyamine concentrations in the infected midgut. In the last stage of the DNV infection, many columnar cells including viruses in the infected epithelium fell off into the gut lumen,

Table 1. Spermidine/spermine and putrescine/spermine ratios in the midgut epithelia infected with DNV

Days after inoculation	Spermidine/spermine		Putrescine/spermine	
	Infected	Healthy	Infected	Healthy
0	5.4	5.4	3.4	3.4
1	5.5	7.5	2.6	3.4
2	5.2	6.1	1.9	2.0
3	3.4	4.6	1.1	1.8
4	3.8	4.9	1.0	1.5
5	4.3	5.0	1.3	2.1
6	4.5	7.0	2.0	3.9
7	6.0	5.9	2.7	2.9
8	7.3	4.5	2.1	2.1
9	8.4	4.4	5.0	1.8
10	9.7	3.4	6.2	1.0
<i>Bombyx</i> DNV ^a	3.5		1.8	

^a Calculated from the polyamine content in DNV particles (BANDO et al., 1983).

and the midgut epithelium consisted of many goblet and regenerating cells. Therefore, the high DNA concentration in the infected midgut at the late stage as compared to the healthy midgut may suggest the high numbers of cell nuclei in regenerated cells by expression of mg/g midgut, because DNV antigen titer decreased at the late stage.

There have been reports that polyamine syntheses were stimulated markedly by the infection of tumor viruses (HEBY et al., 1978), but in the case of *Plodia interpunctella* infected with granulosus virus, no significant difference was recognized between infected and healthy larvae (WILSON and CONSIGLI, 1984). In *Bombyx* DNV infection, however, it appears that the virus infection affects the metabolism of polyamines in the target tissue, and reduces their concentrations during the virus multiplication.

It is known that the rate of polyamine synthesis and cellular polyamine levels show a high correlation with the rate of cell proliferation (HEBY et al., 1975); but little is known about the function of polyamines in insects. However, it has been suggested that the polyamines have a stimulatory effect on macromolecular syntheses (WYATT et al., 1973; DION and HERBST, 1970; BYUS and HERBST, 1976). We observed that the accumulation of polyamine and DNA reached maximum level during the moulting stage (5–6 days after inoculation) in the healthy midgut. Thus, the low level of polyamine concentrations in the infected midgut may indicate that DNV infection interferes with growth and/or the proliferation of host cells. On the other hand, high concentrations of DNA, putrescine and spermidine at the late stage in the infected midgut are due to the stopping of cell growth in the infected cells.

Recently, HAMANA et al. (1984) reported that, using ion-exchange chromatography, several minor polyamines besides the three common polyamines could be detected in various organs of the silkworm, *Bombyx mori*. We were unable to detect such minor polyamines by thin layer chromatography; this is probably due to the difference of sensitivity in the two methods.

According to JANNE et al. (1964), high-growing cell has a high spermidine/spermine ratio, whereas a low-growing cell has a low one. It is clear that the spermi-

dine/spermine ratios in DNV-infected midgut are already lower than those of a healthy one 24 hr after DNV inoculation (Table 1). The low ratios of these amines in the infected midgut also suggest that the normal growth of midgut cells is inhibited by the DNV infection. In contrast, rather high ratios of these amines were observed during the last stage. This would be mainly caused by the cessation of cell growth and polyamine metabolism in the infected cells at this stage.

In the polyamines in the *Bombyx* DNV, BANDO et al. (1983) reported that the ratio of spermine, spermidine and putrescine was clearly different from those of *Galleria* and *Junonia* DNVs. The ratios of these polyamines in both healthy and infected midguts were also somewhat different from that of *Bombyx* DNV.

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