Double Infection of a Cytoplasmic-Polyhedrosis Virus and an Infectious-Flacherie Virus in the Silkworm, *Bombyx mori* (Lepidoptera : Bombycidae)

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The interaction between a cytoplasmic-polyhedrosis virus (CPV) and an infectious flacherie virus (IFV) of the silkworm, *Bombyx mori*, was investigated. Following the simultaneous infection of the larva with both CPV and IFV, the two viruses multiplied independently in the midgut. CPV multiplied in the columnar cells and IFV in the goblet cells, suggesting that there was no apparent interference between the two viruses at the host tissue level, but that a condition of independent coexistence had been attained. However, the period of lethal infection was shorter in larvae subjected to double virus infection than in those infected with each virus separately.

INTRODUCTION

It has been reported that the cytoplasmic-polyhedrosis virus (CPV) of the silkworm, *Bombyx mori*, multiplies mainly in the cytoplasm of the columnar cells in the middle to posterior portion of the midgut (ARUGA et al., 1963). On the contrary, the main target of the infectious-flacherie virus (IFV) of the silkworm is the cytoplasm of goblet cells located in the anterior to middle portion of the midgut. In view of these observations, it is of interest to examine the interaction of the two virus multiplications in the same host larva.

In the present study the multiplication of the two viruses in the same host and the effect of the double infection on the lethal time of the host larva were investigated.

MATERIALS AND METHODS

Semi-purified cytoplasmic polyhedra (CP) were prepared by repeated washing and centrifugation (at 3,500 rpm for 10 min) of the homogenate of CPV infected midguts.

IFV infected larvae of 10 g were homogenated with 90 ml of distilled water and centrifuged at 12,000 rpm for 15 min. The supernatant was then passed through a Seitz filter (Toyo asbestos sterilising films No. 85SB, Toyo Roshi Co., Tokyo), and the filtrate was used as an IFV suspension. This original IFV suspension is described as IFV 10^{-1} in the present paper.

In the preliminary test, IFV 10^{-1} showed about the same lethal infectivity with CP at a concentration of 2×10^7 /ml. Therefore, the original CP suspension was diluted with distilled water and the concentration was adjusted to CP 2×10^7 /ml. The solution of CP 2×10^7 /ml in IFV 10^{-1} was also prepared. Each virus suspension of

CP 2×10^7 /ml, CP 2×10^7 /ml in IFV 10^{-1} , and IFV 10^{-1} was smeared on mulberry leaves with a small piece of absorbent cotton. After the leaf was dry, newly hatched larvae of a F_1 hybrid between two silkworm races, J-124 and C-124, were fed with the treated leaves for 6 hr, and subsequently the infected larvae were reared on normal leaves at 27°C.

As demonstrated in the previous papers (INOUE, 1972; INOUE et al., 1972), when the frozen tissue section of the midgut was observed by a fluorescent antibody technique, IFV infected cells were recognized as "fluorescent cells" dotted in the midgut, indicating that the main target of IFV was goblet cell. This enabled us to demonstrate IFV multiplication by counting the "fluorescent cell" and by observation of the intensity of fluorescence. Accordingly, at several days after the infection, some larvae from each virus infection group were anesthetized with ether, and embedded in small blocks of pig liver. The blocks were frozen for 8–10 min in *n*-hexane cooled in a dry ice-acetone bath, and then sectioned vertically at 7 μ m using a cryostat microtome maintained at about -22°C. The sections were stained, as described in the previous paper (INOUE and AYUZAWA, 1971), with fluorescein isothiocyanate conjugated with anti-IFV γ -globulin of rabbit. The average number of fluorescent cells of three sections per larva was counted.

Inasmuch as the counting of polyhedra on frozen section under the fluorescent microscope was difficult because of the thickness of section, numbers of polyhedra were counted with paraffin sections; some larvae of the same groups were fixed with CARNOY's fluid, embedded in paraffin, sectioned at 5 μ m and stained with DELAFIELD's hematoxylin-eosin after treatment with 1N-HCl at 60°C for 3 min. The average polyhedron number in three vertical sections per larva was counted.

RESULTS

Simultaneous inoculation of CPV and IFV

The inocula containing either CP 2×10^7 /ml, IFV 10^{-1} , or CP 2×10^7 /ml in IFV 10^{-1} were perorally administered to newly hatched larvae, and on days 3 and 5 after the inoculation numbers of fluorescent cells and polyhedra in frozen or paraffin sections were counted, respectively.

In the double infection group, as summarized in Fig. 1, 19 to 38 fluorescent cells were detected on day 3 and 111 to 140 on day 5. This increasing trend was quite similar to that in the IFV single infection group. The polyhedron numbers in the double infection group were 1.7×10^3 to 2.7×10^3 and 1.7×10^3 to 3.1×10^3 on days 3 and 5, respectively. There was no definite difference in number of polyhedra between double infection group and the CPV single infection group, although the larvae from both groups showed a large variation in polyhedron number. In the larva from the double infection group, fluorescent cells appeared in the anterior to middle portion of the midgut and many polyhedra were formed in the middle to posterior portion. As presented in Fig. 2, polyhedra were recognizable alongside the brush border of columnar cells, showing the same phase as reported by IWASHITA (1971).

On day 4 after inoculation, midguts of larvae from groups of the CPV+IFV or the CPV infection were smeared on glass slides and the size of 100 polyhedra in each group was measured. The majority of polyhedra in both groups ranged from 3.1 to 4.5 μ m in size, but the average sizes of polyhedra were 3.8 μ m and 3.7 μ m in

Fig. 1. Multiplication of an infectious-flacherie virus (IFV) and a cytoplasmic-polyhedrosis virus (CPV) in a double or single virus infection.
 a: Number of "fluorescent cells", which appeared in frozen sections stained with fluorescein-isothiocyanate conjugated with anti-IFV γ -globulin, was described as No. of fluorescent cell/ $7 \mu\text{m}$ thick section/larva. b: Number of polyhedra, observed in paraffin section, was described as No. of polyhedra/ $5 \mu\text{m}$ thick section/larva.

Fig. 3 Cumulative mortality curve of larvae in a simultaneous CPV and IFV infection.

groups of the CPV+IFV and the CPV infection, respectively.

In order to know the influence of double virus infection on the lethal time of larvae, groups of 15 larvae infected with CP $2 \times 10^7/\text{ml}$, IFV 10^{-1} , or CP $2 \times 10^7/\text{ml}$ in IFV 10^{-1}

were reared at 27°C. As presented in Fig. 3, larvae of the CPV+IFV infection group began to die on day 5, and this lethal time was 2 days earlier than those in the CPV or the IFV infection groups.

These results indicated that CPV and IFV multiplied independently at their respective target cells in the same midgut of larva infected simultaneously with the two viruses, resulting in the curtailment of the lethal time of larva.

CPV inoculation prior to IFV

The interaction of CPV and IFV was investigated when the two viruses were inoculated into larva at 2 or 4 days interval. The newly hatched larvae were perorally inoculated with CP 2×10^5 /ml and then followed with IFV 10^{-1} inoculation at an interval of 4 days. These larvae were reared at 27°C and the dead larvae were removed daily. As described in Fig. 4, the cumulative mortality curves for the three infection groups indicated that the average lethal time of CPV+IFV group was 4 days earlier than those of the CPV or IFV infection groups.

Fig. 4 Cumulative mortality curve of larvae infected with CPV followed by IFV infection at an interval of 4 days.

These results seemed to show good multiplication of the two viruses in the same larvae. In order to discern the multiplication of the two viruses, newly hatched larvae were perorally inoculated with CP 2×10^7 /ml and followed with inoculation of IFV 10^{-1} at an interval of 2 days. On day 5 after CPV inoculation, polyhedron numbers were compared in both CPV+IFV and CPV infection groups. As shown in Fig. 5a, polyhedron numbers ranged from 1.9×10^3 to 6.3×10^3 in the CPV infection group, while 1.5×10^3 to 5.8×10^3 in the CPV+IFV infection group, although both larvae gave a large variation in polyhedron numbers. The average polyhedron number from 4 larvae of the CPV+IFV infection group corresponded to about 75 percent of that of the CPV infection group. On day 4 after IFV inoculation, fluorescent cell numbers were counted in both the CPV+IFV and the IFV infection groups. As summarized in Fig. 5b, 54 to 113 cells were recognized in the CPV+IFV infection

Fig. 2. Frozen section of the midgut of the larva stained with fluorescein isothiocyanate conjugated with anti-IFV γ -globulin on day 5 after simultaneous cytoplasmic-polyhedrosis virus (CPV) and infectious-flacherie virus (IFV) infection. Under the UV observation of fluorescent microscope, polyhedra gave blue or gray-blue autofluorescence, brush border gave red, and IFV-infected cell gave greenish-yellow specific fluorescence. Polyhedra appeared in the cytoplasm of the columnar cell alongside the brush border and IFV multiplied in the cytoplasm of the goblet cell.

Fig. 5 Numbers of fluorescent cells and polyhedra in the larvae infected with CPV followed by IFV infection at an interval of 2 days. Polyhedra were counted on day 5 after CPV inoculation and fluorescent cells were on day 4 after IFV inoculation.

group, while 76 to 126 cells were observed in the IFV infection group. The average number of the cell in the CPV+IFV infection group was 74 and corresponded to 72 percent of that of the IFV infection group.

These results showed that CPV and IFV multiplied well in the same host larva when infected with CPV followed by IFV, although the average numbers of polyhedra and fluorescent cells were slightly smaller compared with the case of single infection with only one of the pathogens.

IFV inoculation prior to CPV

The interaction of the two viruses was investigated in the larva infected with IFV followed by CPV. A group of 15 larvae was perorally inoculated with IFV 10^{-4} and followed with the inoculation of the CP $2 \times 10^7/\text{ml}$ at an interval of 4 days. In this trial, the lethal time of the group was shortened by the double virus infection, indicating that the dead larva were first observed on the 8th day after the 1st virus inoculation, and all larvae died by the 12th day. Thus the lethal time, compared with the initial time of larval death, was 3 days earlier than that of the single IFV infection, and 6 days earlier than that of the single CPV infection.

In the next trial, newly hatched larvae were perorally inoculated with IFV 10^{-1} and 2 days later were further inoculated with CP $2 \times 10^7/\text{ml}$. On the 5th day after IFV 10^{-1} inoculation, numbers of fluorescent cell were 149 to 204 in the IFV+CPV infection group, and 179 to 227 in the IFV infection group. The average number of the cell obtained in the double infection group was 82 percent of that of the IFV single infection group. On the 4th day after the inoculation of CP $2 \times 10^7/\text{ml}$, polyhedron numbers in the IFV+CPV infection group varied from 10.3×10^3 to 17.5×10^3 . Although the variation was similar to that of the CPV infection group, the average polyhedron number was estimated as 83 percent of that of the CPV infection group.

From these results obtained in the case of IFV infection followed by CPV infection, it is suggested that both viruses were able to multiply in the same host larva, although the IFV+CPV infection group showed a slightly smaller average number of fluorescent cells and polyhedra compared with that of the larvae infected with IFV or CPV alone.

DISCUSSION

The virus interactions that have been described thus far in insects are (1) interference, (2) independent coexistence and (3) complementation (synergism) (TANADA, 1971).

According to ARUGA et al. (1965), IFV, when fed to silkworm larvae prior to CPV, interfered with the infection of the latter virus, but there was no apparent interference when IFV was fed to the silkworm larvae after CPV. This was the case where interference of the two virus infection was investigated at the organismal level. However, interference of viruses at the organismal level does not necessarily mean existence of the interference at the tissue or cellular level.

For instance, LOWE and PASCHKE (1968a) studied in detail the interaction of a nuclear-polyhedrosis virus and a granulosis virus in larvae of the cabbage looper, *Trichoplusia ni*. Their results indicated that although there was an increased percentage of mortality of the test insects when the two viruses were fed together to the larvae, the increase was largely not statistically different from that of the treatment with each virus separately, suggesting neither interference nor synergism between the two virus infections. However, they also reported that the two viruses were sometimes found in adjacent cells but not in the same cell (LOWE and PASCHKE, 1968b). Such cases where two different viruses did not infect the same cell were considered as interference at the cellular level.

In the present study, interaction of IFV and CPV multiplications in the midgut of the same host silkworm larva was studied. As a whole, the two viruses multiplied independently and showed a coexistence because of their different target cells; IFV mainly multiplied in the goblet cell, while CPV multiplied in the columnar cell of the midgut. Furthermore, there was no indication that the two viruses infected the same cell, as far as the multiplication IFV was determined by a fluorescent antibody technique and that of CPV by development of polyhedra. However, the period of lethal infection was shorter in the larvae of the double virus infection than in the larvae infected with each virus separately. The present study also indicated that, when CPV and IFV were inoculated at a 2 day interval into the silkworm, larva infected with both viruses gave slightly small numbers of polyhedra and fluorescent cells compared with those infected with each virus alone. Therefore, CPV and IFV multiplications in the midgut of doubly infected larva were not completely independent, but the first virus infection caused slight depression of the multiplication of the secondary infected virus in the different cell through a pathophysiological change of the host tissue.

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