Penetration and Replication of *Bombyx mori* Cytoplasmic Polyhedrosis Virus *in vivo**

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Abstract: The process of invasion and replication of *Bombyx mori* cytoplasmic polyhedrosis virus (*Bm* CPV) in the silkworm, *Bombyx mori* was studied. After molting, the third instar larvae of *B. mori* was inoculated with *Bm* CPV, then the hind midgut of the infected larvae were dissected at different intervals, prepared as electron microscope specimens and visualized with the transmission electron microscope (TEM). The results indicated that *Bm* CPV virions were observed associated with the microvilli of the cylindrical cells at 1.5 h post infection (p.i.). The virions entered the microvilli and moved to the cytoplasm 6 h p.i. The virions were observed in the cylindrical cell at 9 h p.i.. The intact virions probably penetrated through the peritrophic membrane, then absorbed and invaded the microvilli for infecting the cylindrical cells. With the virus degradation, the viral cores went into the nucleus and got underway of multiplication. After the eclipse phase, the new formed virogenic stroma (VS) was found in the cytoplasm of cylindrical cells at 24 h p.i.. Furthermore, the progeny virions appeared and their numbers increased gradually. The polyhedrin deposited to embed the virions to form the polyhedron at 48 h p.i..

Key words: *Bombyx mori*; cytoplasmic polyhedrosis virus; penetration; replication

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Bombyx mori cytoplasmic polyhedrosis virus (*Bm* CPV) belongs to the genus *Cypovirus* in the family *Reoviridae*. The free viruses of *Bm* CPV invaded the cylindrical cells of midgut, replicated in the cytoplasm and finally polyhedra embedding virions formed[1-2]. Masahiko conducted a series of experiments to study the entry of the cytoplasmic and the nuclear polyhedrosis viruses into silkworm midgut epithelial cells cultured in vitro, whose finding showed that only the core substance of CPV was injected into the cell while the viral capsid was left on the cell surface[3]. The cultured *Heliothis armigera* external cells were inoculated with *H. armigera* CPV to explore the mechanism of penetration of them. It was found that the capsid projections of viral particle adhered to the surface of microvilli, and the nucleic acid lumped in the cytoplasm, and moved to the cell nucleus. Finally, the virions and empty virions were observed in the phagosome[1]. This experiment was carried out in the midgut of silkworm by negative staining and Transmition Electron Microscopy (TEM).

1 Material

1.1 Propagation and purification of *Bm* CPV

The fourth instar larvae of *B. mori* were fed on mulberry leaves smeared with *Bm* CPV polyhedra (10^9 polyhedra/L). Six hours later, the silkworms were fed on the normal mulberry leaves. Before the maturation of the silkworm, midguts were collected, put in a mortar and grinded then filtered by two pieces of gauze and one piece of absorbent cotton until obtained the solution[4]. The solution was centrifuged with 10 g/L sodium dodecyl sulpate (SDS) and sucrose at the different density gradients (400-500-600 g/L). The sediment was then purified polyhedra of *Bm* CPV, and stored at 4 ℃.

1.2 *B. mori* variety

Silkworm used in this study was the 9Fu × 932 variety from Guangdong Sericulture Co.

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2 Methods

2.1 Preparation and observation of *Bm* CPV negative staining sample

2 mL purified polyhedra of *Bm* CPV was centrifuged and the sediment was mixed with 5 mL of 0.06 mol/L Na_2CO_3 and 0.1 mol/L NaCl (pH 10.8) for 30 min, then the *Bm* CPV was centrifuged with freezing ultracentrifuge and mixed with 20 g/L (pH 7.2) phosphotungstic acid (PTA) for 2 min. After washing and drying, observed by JEM-100CX/II TEM.

2.2 Preparation and observation of TEM sample of silkworm midgut

The third instar larvae after molting were fed on mulberry leaves smeared with a concentration of polyhedra suspension (10^9 polyhedra/L). Six hours later, the silkworms were fed on the normal mulberry leaves. The posterior half of midgut from infected larvae were removed and extracted at the following intervals: 1.5 h, 3 h, 6 h, 9 h, 12 h, 24 h, 36 h, 48 h, 60 h, 72 h and 84 h. The samples were fixed with glutaraldehycle (40 g/L) washed in 0.2 mol/L phosphate buffer four times for 10 min each time, dehydrated in a graded series of ethanol solutions (35%, 50%, 70%, 90%, 100%) for 10 min each time. The specimens were dehydrated with acetone two times for 10 min each time, then permeated with a mixture of one volume of acetone and one volume of epoxy resin, then with one volume of acetone and two volume of epoxy resin, finally with the pure epoxy resin. The specimens were embedded in epoxy resin, placed in dryers with 70 ℃ for 8 hours or more and trimmed the sections and made reservations to cut sheets at the thickness of 500 angstorms (Å), put the sheets on copper net, stained with uranium acetate and lead citrate, observed by JEM-100CX/II TEM.

3 Results

3.1 Morphobgy of *Bm* CPV

After decomposition of the purified polyhedra of *Bm* CPV, extracted the virions, negative stained and prepared as ultrathin sections and then observed by JEM-100CX/II TEM for shape and size (Fig. 1).

Fig. 1 showed the general appearance of *Bm* CPV. The virions show a circular outline, the diameter is about 50-65 nm, the submit of shell is pentangular or hexagonal, from where stretching a tube-shaped projection. Some are empty virions with no nucleic acid.

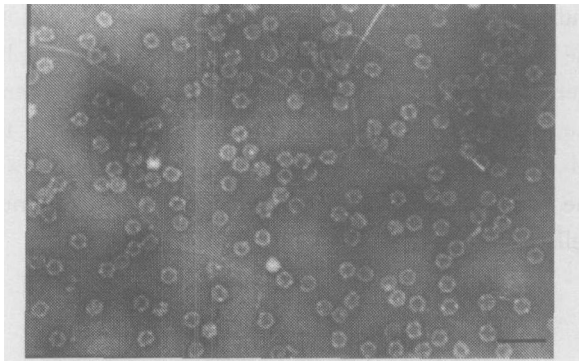

Fig. 1 *Bm* CPV by negative staining technique (bar= 200 nm)

3.2 The process of entry of *Bm* CPV into the cylindrical cells of midgut

The third instar larvae of *B. mori* after molting were fed on mulberry leaves smeared with a concentration of polyhedra suspension (10^9 polyhedra/L). The midguts were extracted from the infected silkworm at intervals, made into the ultrathin section and observed by TEM (Fig. 2, Fig. 3 and Fig. 4).

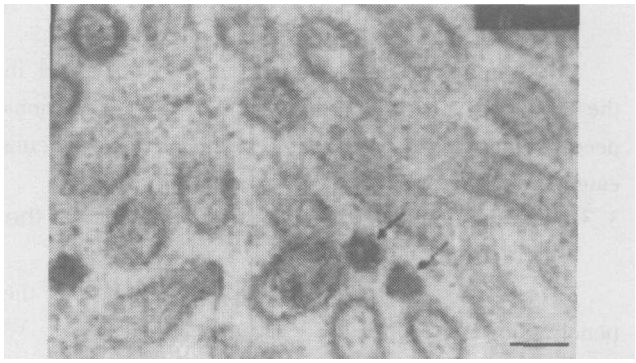

Fig. 2 Penetration of *Bm* CPV at 1.5 h p. i. "↑" stands for the entry of virions in microvilli (bar= 100 nm)

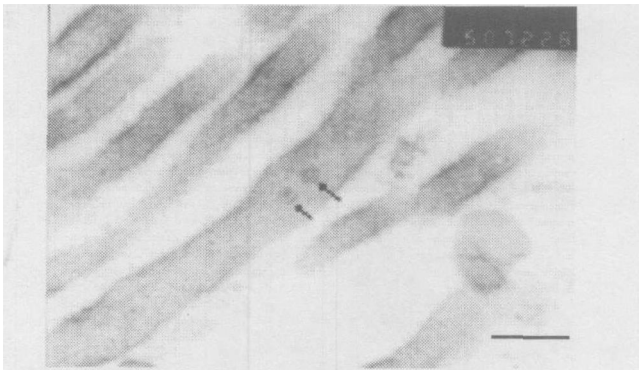

Fig. 3 Penetration of *Bm* CPV at 6 h p. i. "↑" stands for the entry of virions in microvilli cylindrical cells (bar= 200 nm)

The third instar larvae after molting ate the mulberry leaves which were smeared with *Bm* CPV, the polyhedra dissolved on the effect of basic intestinal fluid

and released the free virions. At 1.5 h after infection, the free virion penetrated through the peritrophic membrane. Some virions of the intact virions entered the microvilli of the cylindrical cells, however, the others entered the microvilli (Fig. 2). Fig. 3 showed the virions occurred in the microvill of the cylindrical cells.

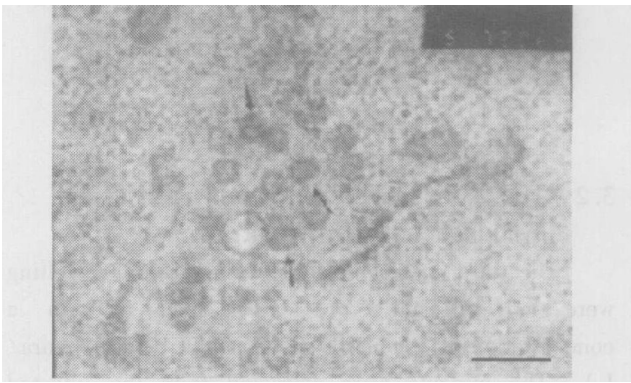

Fig. 4 Penetration of *Bm* CPV at 9 h p. i.
"↑" stands for the virions entering the cylindrical cells (bar = 200 nm)

At 9 h p. i., the intact virions were observed in the cytoplasm of cylindrical cells (Fig. 4). The virions need about nine hours to complete the process of being eaten by silkworm and entering the target cell.

3.3 Replication of *Bm* CPV and generation of the cytoplasm

There is a short latent period between the penetration and replication (at 9 h to 24 h p. i.). Furthermore, the virions formed in silkworm between 24 h p. i. and 48 h p. i., and the observation of sections at intervals was shown in Fig. 5, Fig. 6, Fig. 7, Fig. 8 and Fig. 9.

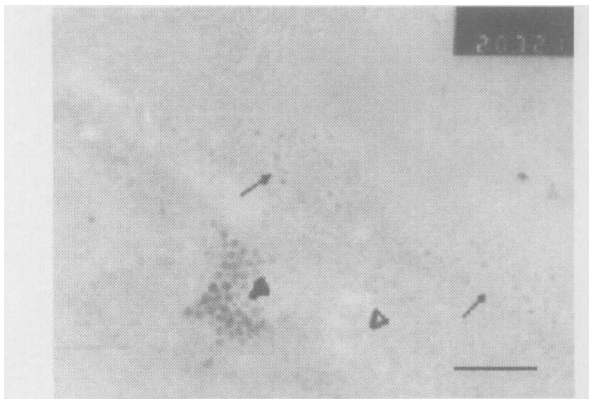

Fig. 5 Replication of *Bm* CPV at 24 h p. i.
"△" stands for virogenic stroma (VS) formed in the cytoplasm of the cylindrical cells. "↑" stands for the new formed virions (bar = 500 nm)

At 24 p. i., the light stained VS were observed in the cytoplasm of the cylindrical cells and the irregular shaped and big or small virogenic stroma (Fig. 5). There are no cell organelles in the stromas; the offspring virions formed at the edge of stroma and distributed. Moreover, 36 h after infection, the virions gradually extended to the center of stroma, and the VS gradually shrunk, the formerly formed stroma distributed with virions evenly in VS (Fig. 6).

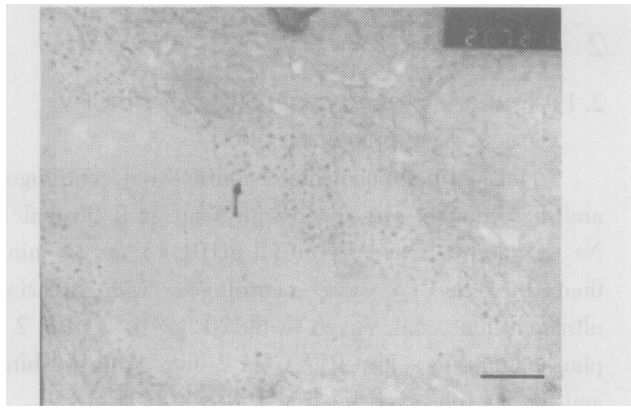

Fig. 6 Replication of *Bm* CPV at 36 h p. i.
"↑" stands for the VS shrinking and distributed with virions (bar = 500 nm)

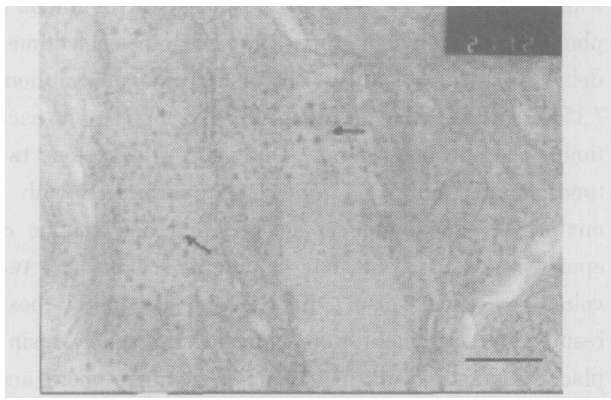

Fig. 7 Replication of *Bm* CPV at 48 h p. i.
"↑" stands for the dark stained polyhedrin embedding virions (bar = 500 nm)

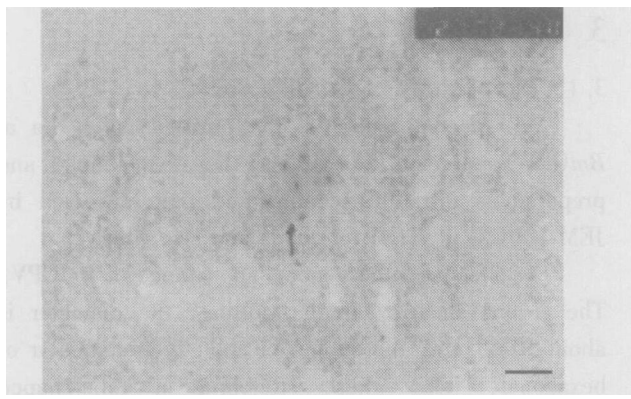

Fig. 8 Replication of *Bm* CPV at 60 h p. i.
"↑" stands for the zoom of the irregular shaped heavy electron density (the immature polyhedra) embedded virions (bar = 500 nm)

As shown in Fig. 7 (48 h p. i.), the viral polyhedra protein began to deposit in parts of VS showed heavy staining and gradually embedded the virions. Also at 60 h p. i., the deposition of viral polyhedra protein increased and enlarged, the arris became clear, and the appearance of electron density became heavier. These were immature polyhedra (Fig. 8).

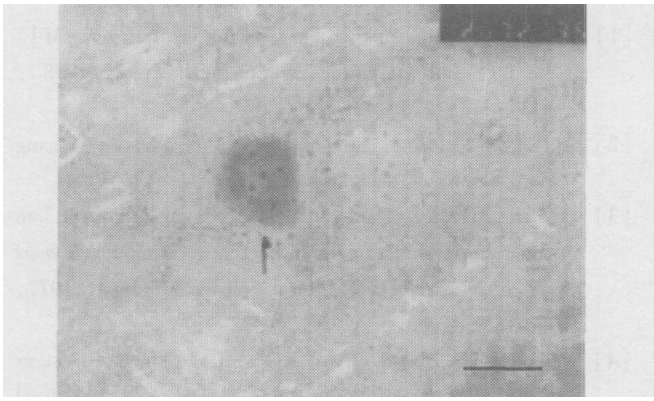

Fig. 9　Replication of *Bm* CPV at 72 h p. i.
"↑" stands for the crystal lattice of polyhedra (bar= 500 nm)

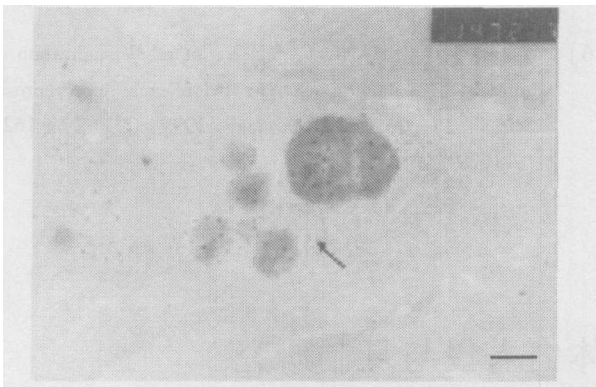

Fig. 10　Replication of *Bm* CPV at 84 h p. i.
"↑" stands for the mature and big or small polyhedra (bar= 500 nm)

From fig. 9 at 72 h p. i., the structure of the netted crystal lattice of polyhedra can be clearly observed by TEM. Finally, at 84 h of treatment, several mature polyhedra were formed in the cytoplasm of the infected cells (Fig. 10). The main shape of polyhedra is hexagonal, there are also virions showing an oval or irregular shape. The polyhedra were not observed in cell nucleus.

4　Discussion

4.1　The invasion of *Bm* CPV

The process of invasion in the cylindrical cells of the midgut was discussed by Masahiko who suggested that the virions only discharged the nucleic acid into the cells[3]. In our experiment, we found that the virions firstly adhered to the microvilli, penetrated the microvilli membrane with the whole particle, entered the microvilli and moved to the cytoplasm. Finally, the virions penetrated the double nucleus, removed the shells and started to initiate the replication. Zhang H *et al* argued that the RNA transcriptase orientated at the bottom of the capsid projections[5-6]. This explanation is in agreement with our findings in the present study since the virions entered the cells with the whole particle. The RNA transcriptase is indispensable for the replication of CPV, and this enzyme is in the capsid[6]. Many predecessors had indicated that the nucleic acid of *Bm* CPV is not infectious. We consider the way that the virions entered the cells with the whole particle and lead to infection is more acceptable.

The third instar *B. mori* larvae were inoculated with *Bm* CPV polyhedra, observed the process of invasion using TEM. The polyhedra dissolved by the influence of the basic intestinal fluid and discharged the virions, pinocytosis on the surface of the microvillus membrane and cell membrane was not observed. There is no phagosome in the cytoplasm of cylindrical cells. The structure of some capsids is vague. This may be the defense reaction of cell to destroy the virions. Meanwhile, the virions were observed among the microvilli of the cylindrical cells at 1.5 h p. i. The virions entered the microvilli and moved to the cytoplasm after 6 h p. i. The process carried on with the whole particle. This indicated that the penetration with the whole particle is one way of penetration of *Bm* CPV. On the process of invasion of intact virions, we had not observed the split of the microvillus membrane. We inferred that there is an extreme small gap, but big enough to let the virions enter. When the virions enter the cells, it probably happens the phenomenon with dissolving the membrane that is brief and tiny, but the cells can repair themselves and maintain the intact membrane after the virions penetration. The process may involve some chemical reactions or effect of some enzymes. It is very useful for curing the disease to study and unfold the mechanism of invasion of *Bm* CPV.

4.2　The replication of *Bm* CPV

After entering the cytoplasm, the nucleic acid (dsRNA) moved to the cell nucleus where dsRNA replicated and proliferated. After the eclipse phase, the netted VS formed in the cytoplasm. It is the membrane that has independent structure, irregular shapes, and no boundary with the cytoplasm. The bigger VS formed when several VS mixed. It can occupy an extensive area of the cell. The heavy electron of the surface of

the VS concentrated and many virions formed. The virions were wrapped by membrane. Then the mature virions moved to the cytoplasm, embedded by the polyhedra protein. Finally, the intact polyhedra formed, and one of them can contain thousands of virions at random[4].

The process of replication and development is between the form of new virions (24 h p.i.) and shrinking of the virogenic stroma (VS) (48 h p.i.), both the proliferation and form of new virions also happened in this period. The polyhedra deposited to embed the virions after 60 h p.i., and the new polyhedra formed finally.

5 Conclusion

The study aimed to observe the process of invasion, proliferation, and replication of *Bm* CPV in the infected larva in vivo, the results are as follows.

The virion got into the microvilli by penetration of the peritrophic membrane with the whole particle, and then invaded the cytoplasm of the cylindrical cells. This is different from the way that penetration of insect primitive cultured cells. The virions absorbed on the surface of microvilli, then discharge the nucleic acid. This may be another way of invasion of *Bm* CPV in tissue cells.

After entering the cytoplasm of *B. mori*'s cylindrical cells, the virions can be seen in the nucleus and initiated the replication after the eclipse phase.

The offspring virions formed in VS, and the polyhedra protein embeded the intact virions, and then the new virus particle formed.

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质型多角体病毒在家蚕体内入侵与复制研究

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摘 要：采用在家蚕活体内研究家蚕质型多角体病毒（*Bm* CPV）的入侵增殖复制过程，探讨 *Bm* CPV的入侵过程和增殖复制机制。实验用健康的三龄起蚕经口接种 *Bm* CPV后不同时间取中肠后部固定，制作电镜样品，在透射电子显微镜下观察，结果发现 *Bm* CPV病毒被食下 1.5 h后，在中肠上皮组织圆筒形细胞微绒毛之间有病毒粒子存在，6 h后病毒进入微绒毛内，并向细胞质移动，9 h后观察到圆筒形细胞质中有病毒粒子。作者认为病毒入侵是以整个病毒粒子穿越中肠围食膜，吸附并进入微绒毛，感染圆筒形细胞，与前人病毒入侵只是髓核进入细胞，而病毒衣壳仍留在细胞外的推测不同。随后病毒核心物质进入细胞核，启动复制循环。经过隐潜期后病毒复制，在感染 24 h后，细胞质中形成病毒发生基质，子代病毒开始形成，逐渐增多。感染 48 h后，圆筒形细胞质中的多角体蛋白逐渐沉积并将病毒粒子包埋，最终形成新的多角体。

关键词：家蚕；质型多角体病毒；入侵；复制

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