Choristoneura murinana Nuclear Polyhedrosis Virus: Comparative Biochemical and Biological Examination of Replication in vivo and in vitro

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SUMMARY

An in vitro replication system for the Choristoneura murinana nuclear polyhedrosis virus (CmMNPV) was established and used (i) to characterize this baculovirus biochemically; (ii) to study the cytoplasmic spindle-shaped inclusions (CSIs) associated with CmMNPV replication; and (iii) to compare the cytopathic changes during CmMNPV replication in vivo and in vitro as well as the properties of virions, polyhedra and CSIs from both systems. It was shown that the processes occurring during, and the products of, CmMNPV replication in vitro closely resemble those in vivo, i.e. in larval hosts. Genome analysis by restriction endonucleases, as well as infectivity studies with polyhedra from both sources did not reveal major differences between virus produced in vivo and that produced in vitro. The CSIs were found exclusively in the cytoplasm of infected cells and were shown to consist of a single protein of Mr 50000. Although the biological significance of these spindles, which are produced in large quantities, is not known, they do not seem to be of importance for the infectivity of this baculovirus in vivo.

INTRODUCTION

During an outbreak of the European fir budworm, Choristoneura murinana (Lepidoptera: Tortricidae), in the Black Forest (F.R.G.) two baculoviruses, a granulosis virus and, in low incidence, a nuclear polyhedrosis virus (CmMNPV) were detected (Bergold, 1948; Krieg & Langenbuch, 1956). The latter proved to have a remarkably wide host range, being infectious not only for several tortricids but also for larvae of Mamestra brassicae (Lepidoptera: Noctuidae) (J. Huber, unpublished observations). In addition, the virus is of major interest since in all susceptible hosts tested (including M. brassicae), during its replication not only the polyhedral virus occlusion bodies, characteristic for NPVs in general, but also cytoplasmic spindle-shaped inclusions (CSIs) are formed. This feature was first described by Huger & Krieg (1968). Since then, CSIs have been found in association with several other NPV infections. However, CmMNPV has not been well studied and characterized because mass production of C. murinana larvae is difficult and no in vitro replication system was available.

Fortunately, larvae of the codling moth, Cydia pomonella (Cp), were found to be also susceptible to this virus (Huber, 1980). As a rearing stock of coding moth was readily available, many new Cp cell lines were established. CmMNPV replicated in 46 of 81 tested cell lines (Miltenburger et al., 1984). Those cell lines giving the highest polyhedron yield over several passages in vitro were used for a morphological, biochemical and biological characterization of CmMNPV and for a comparison of CmMNPV replication in vivo and in vitro.

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In this paper it will be shown that the cytopathic processes induced by CmMNPV replication in Cp cell lines are very similar to those in CmMNPV-infected host larvae, even with regard to CSIs. Moreover, evidence is presented that no major biochemical or biological differences exist between polyhedra, virions and CSIs from each source.

METHODS

Cell lines. The primary cell lines IZD-Cp 33b and IZD-Cp 14-9 used in these studies had been initiated from embryonic Cp cells. They had a fibroblast-like morphology, a doubling time of 3 and 4 days respectively, and were passaged by scraping with a rubber policeman approximately once a week. The established cell line IZD-Cp 0508 was derived from haemocytes and grown in suspension. All cell lines were maintained in Cp cell culture medium (Miltenburger et al., 1984).

Viral inoculum. Infectious haemolymph was collected from CmMNPV-infected Cp larvae and used as inoculum on IZD-Cp 0508 cells seeded out in Terasaki plates. The virus stock used in the experiments for the infection of cells was tissue culture supernatant virus that had been subcloned three times by the limiting dilution technique in the above cell line and had been subsequently passaged about five times in tissue culture. The TCID₅₀ titre of the CmMNPV inoculum, using IZD-Cp 0508 as the indicator cell line, was 7 × 10⁸/ml.

Production and purification of polyhedra and spindles. Early fifth instar C. pomonella larvae were infected with CmMNPV by contaminating the surface of the rearing substrate with a few drops of a NPV suspension containing 10⁶ polyhedra/ml. Dead larvae were collected into tissue culture medium containing 0.1 mg/ml glutathione and minced in an Ultra-Turrax homogenizer. Larval debris was removed by filtration through three layers of cheesecloth. After the larger debris had settled, the polyhedra and spindles in the supernatant were further purified by washing them three times in 0.1% SDS, layered onto a linear sucrose gradient (30 to 65%, w/w in 0.1% SDS) and centrifuged at room temperature for 60 min at 90000 g. Bands of polyhedra and spindles were removed with a syringe, diluted with distilled water, pelleted and washed once in distilled water (60000 g, 60 min, 4 °C).

For the production of tissue culture-derived polyhedra and spindles, half-confluent cultures in 25 cm² tissue culture flasks (about 10⁶ cells) of IZD-Cp 0508, IZD-Cp 33b or IZD-Cp 14-9 were infected in the logarithmic phase of growth at a m.o.i. of 0.1. Four to 6 days after infection, cells were harvested and lysed in 0.1% SDS. Further purification was done as described above for larval material.

Electron microscopy. For electron microscopical comparison of morphological changes during CmMNPV replication in vivo and in vitro, thin sections of both virus-infected larval fat body of the homologous host, C. maritima, and samples of virus-infected cultured Cp cells (line IZD-Cp 33b) were prepared. To facilitate handling, the Cp cells were embedded into agarose gel (0.5% in physiological saline). Small cubes of gel containing the cells as well as small pieces of infected larval fat body were fixed for 15 h in 1% osmium tetroxide buffered to pH 7.7 with barbitone sodium (after Palade, 1952), stained for 2 h with 1% phosphotungstic acid and 0.5% uranyl acetate in 70% alcohol, dehydrated in a graded series of ethanol, and embedded in a 7:3 mixture of butyl- and n-methylmethacrylate. Thin sections, cut on a LKB Ultratome, were double stained with uranyl acetate and lead citrate. All electron micrographs were taken with a Zeiss EM 9A electron microscope operating at 60 kV.

Polyacrylamide gel electrophoresis (PAGE). Samples were boiled for 5 min in sample buffer (5% 2-mercaptoethanol, 2% SDS, 0.063 M-Tris-HCl buffer pH 6.8, 10% glycerol, and 0.001 M-bromophenol blue; Maskos & Miltenburger, 1981). Polypeptides were separated by discontinuous SDS-PAGE in a slab gel apparatus (dimensions 10 x 14 x 0.15 cm) using a 3% stacking and an 11% separating gel (Laemmli, 1970).

DNA purification. Gradient-purified CmMNPV polyhedra were dissolved in 0.01 M-NaOH for 5 min on ice. The liberated virions were pelleted by centrifugation at 100000 g for 90 min. The pellet was resuspended at a concentration of 5 mg/ml in buffer containing 0.1 M-Tris-HCl pH 6.8, 0.01 M-EDTA and 0.1 M-KCl and incubated in 1% SDS for 1 h at 60 °C. Proteinase K (Boehringer Mannheim) was then added to a final concentration of 100 µg/ml for a 4 h incubation at 37 °C. The DNA was purified by extraction twice with phenol, once with phenol : chloroform : isooamyl alcohol (25:24:1), and finally once with chloroform : isooamyl alcohol (24:1). After dialysis in 0.1 × SSC (0.15 M-NaCl, 0.015 M-sodium citrate) for 24 h at 4 °C, the DNA was stored in sterile microfuge tubes at 4 °C.

Restriction endonuclease analysis. One microgram of each DNA sample was digested with BamHI, EcoRI, and HindIII restriction enzymes (Bethesda Research Laboratories) for 4 h at 37 °C using the conditions recommended by the supplier. The resulting fragments were resolved by electrophoresis on 0-7% agarose gels, 25 x 12.5 x 0.3 cm (Sigma), using 0.1 M-Tris-HCl pH 8.3, 77 mm-boric acid, 2.5 mM-EDTA buffer for 14 h. The gels were stained in ethidium bromide (0.5 µg/ml) for 1 h, destained in 1 mM-MgSO₄ for 1 h, and photographed under u.v. illumination using a Wratten no. 15 filter. A HindIII digest of λ DNA was used as a mol. wt. standard on each gel. Mol. wt. were estimated graphically, using plots of migration versus log₁₀ mol. wt. of the standard.

Determination of CmMNPV titre and activity. The concentration of the CmMNPV was determined by counting the polyhedra under a light microscope using a Helber counting chamber (0.02 mm depth) and dark field optics at 100000 g for 4 h at 5 °C.
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a magnification of ×400. CSIs were counted in the same way; they could be easily distinguished from the polyhedra by their different shape.

Bioassays were used to measure the activity of virus samples (Huber, 1981). Serial dilutions of the polyhedra suspensions to be tested were mixed into the codling moth diet (Ivaldi-Sender, 1974) at a temperature of 40 °C. One day later, neonate codling moth larvae were placed onto the medium and reared individually at a temperature of 25 °C up to pupation. Mortality was recorded after 14 and 24 days. The values obtained were corrected for natural mortality and subjected to probit analysis.

In order to investigate the role of the CSIs in the infection process, in a preliminary bioassay the spindles were examined for a possible synergistic effect. A NPV suspension with the CSIs removed by sucrose gradient density centrifugation was bioassayed in comparison to the same suspension with the addition of 2 × 10^4 CSIs per ml of diet at all the concentrations (including untreated control).

RESULTS

Cytopathic changes during virus replication in vivo and in vitro

Electron microscopical studies of thin sections revealed that the general sequence of morphogenetic changes during CmMNPV replication was similar in vivo and in vitro, i.e. in larvae of the homologous host C. murinana and in the cell line IZD-Cp 33b derived from the heterologous host C. pomonella, respectively. In both systems the first well defined change displayed by infected cells was the enlargement of the nucleus followed by de novo formation of a large, sponge-like and electron-dense network, the virogenic stroma, in its centre. Virus nucleocapsid assembly was closely associated with this stromal network. Bundles of between two and 14 nucleocapsids became enclosed by an envelope of the unit membrane type which was formed in situ (Fig. 1 a and b). Comparatively low numbers of single nucleocapsids were also enveloped, more so in cultured cells than in larval tissues. As the number of enveloped virions increased, they became progressively occluded into polyhedra in both systems (Fig. 1 and 2). Polyhedra from each source displayed the same paracrystalline lattice of the polyhedrin surrounding the virions. Finally, the nuclei were packed with mature polyhedra which were similar in size but, on average, more numerous in nuclei of larval tissues as compared to those in cultured cells.

A striking cytopathological feature observed in virus-infected cells of both systems was the occurrence of large fibrous masses in the nucleus as well as in the cytoplasm (Fig. 2 a and b). The fine fibrils were often packed in parallel to form straight or curved bundles and, not infrequently, whirls (Fig. 2 b). There was no noticeable morphological difference between the nuclear and cytoplasmic fibrillar material. The fibrous bundles were often found to be closely associated with the surface of growing or mature polyhedra (Fig. 1 b). In many other cells in vivo and in vitro, however, virus occlusion bodies assembled without the participation or presence of discrete fibrous masses, although mostly with obvious involvement of loosely scattered fibrous material.

As described by Huger & Krieg (1968), CmMNPV infections in larvae of the homologous host C. murinana not only induced the formation of the typical polyhedral virus occlusion bodies but also the production of large numbers of spindle-shaped, proteinaceous inclusions in the cytoplasm of all types of infected cells (Fig. 2 b). In host larvae, these varied in length from 1 to 6 μm and in width from 1 to 4 μm. Upon incubation with dilute alkaline or acid solutions, such as 0.1 M-NaOH or 0.1 M-HCl, they spontaneously transformed into extremely elongated fusiform structures up to ten times their original length. Our studies revealed that these CSIs were also produced to the same extent in CmMNPV-infected cells of the heterologous host cell line IZD-Cp 33b (Fig. 2 a). Their formation may have commenced at an early stage of virus-induced cytopathic changes, i.e. with the appearance of the virogenic stroma or at an early stage of nucleocapsid assembly in the nucleus. The CSIs were scattered throughout the cytoplasm and often were aggregated in membrane-bound clusters which appeared to be derived from perinuclear dilated cisternae formed by extension and proliferation of the outer lamella of the nuclear membrane. Thin sections of cultured cells clearly showed that CSIs were also established within cisternae of the nuclear envelope. Many of the growing inclusions were closely associated with ribosome-like particles, some also with fibrous material. In addition, they sometimes developed within degenerating mitochondria.
Fig. 1. Electron micrographs showing portions of CmMNPV-infected nuclei (a) from a cultured cell (IZD-Cp 33b), (b) from a larval fat body cell of *C. murinana*. Both types of cells display occlusion of virions (V) into polyhedra (P). In (b) fibrous material (F) is closely associated with growing polyhedra (P). Bar markers represent 0.5 μm.
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Fig. 2. Electron micrographs of cells in the advanced stage of infection by CmMNPV: (a) cultured cell (IZD-Cp 33b), (b) larval fat body cell of C. murinana. Both types of cells show the formation of polyhedral virus occlusion bodies (P) in the nucleus, the presence of large fibrous masses in the nucleus (FN) as well as in the cytoplasm (FC), and the characteristic spindle-shaped cytoplasmic inclusions (S). NM, nuclear membrane; V, free virus rods; M, mitochondria. Bar markers represent 0.5 μm.
Electron micrographs showed the CSIs to be mostly bipyramidal in shape (Fig. 2a, b). They contained no virions or other structures. High magnification prints from thin sections of appropriately oriented inclusions displayed a crystalline lattice pattern comparable to but narrower than that of polyhedra. The centre-to-centre distance between the opaque lattice lines was calculated to be 6.6 nm for polyhedra and 5.3 nm for the CSIs.

A relatively low number of virus-infected cultured cells exhibited aberrant morphogenetic developments, such as 'empty' polyhedra, i.e. polyhedra without occluded virions, sometimes with masses of naked nucleocapsids attached 'end-on' to the surface of the polyhedra; clumped aggregations of virus-containing or 'empty' polyhedra, or nuclei containing naked nucleocapsids and masses of empty or electron-lucent tubular structures possibly representing aberrant forms of viral capsids.

**Biochemical data**

Separating the polyhedral inclusion bodies from the spindles proved to be difficult as both sedimented to rather a high density in sucrose gradients (55 and 57% w/w, respectively) and they quite often adhered to each other. Therefore, spindle preparations were often contaminated with polyhedra and vice versa (Fig. 3, lanes 3 and 4). The polyhedra consisted mainly of a single protein, the polyhedrin (Mr 32000), and a few minor proteins, (Fig. 3, lanes 2 and 4), whereas spindle preparations, if absolutely free of polyhedra (Fig. 3, lane 5), showed only a single protein band of Mr 50000. The polyhedrins from *in vivo* and *in vitro* sources were identical in mol. wt. This was also true for spindle protein from both systems (Fig. 3).

The virions liberated from polyhedra produced in cell culture and the virions liberated from polyhedra produced *in vivo* showed a similar protein composition by SDS–PAGE (data not shown).

Fig. 4 displays the restriction enzyme fragments of *CmMNPV* DNA obtained from polyhedra from larvae (lanes 1, 3 and 5) and from cultured cells (lanes 2, 4 and 6). The patterns of the two DNA preparations were almost identical for each of the three enzymes used. A close comparison of lanes 3 and 4 showed that fragment 4 was slightly larger in lane 4 than in lane 3. Similarly in lanes 5 and 6, fragment 2 was larger in lane 6 than in lane 5. This indicates that the culture-derived virus had a slightly larger genome than virus derived from larvae. The mol. wt. of the genome was obtained by summing the mol. wt. of the fragments of each digestion and computing the average. Our value of 75.9 × 10^6 is close to the published value of 78.9 × 10^6 (Rohrmann *et al.*, 1982), and the restriction endonuclease patterns published there are very similar if not identical to ours.

**Bioassays**

The probit lines from the bioassay data on codling moth larvae using virus produced *in vitro* and *in vivo* were almost parallel, the equations being: \( y = 1.692x - 3.738 \) and \( y = 1.665x - 3.458 \), respectively. The line for the virus produced *in vitro* was well within the 95% confidence range of the *in vivo* line (Fig. 5) and the difference in the LC50 of the two preparations (potency factor: 1.2) was not significant.

The comparison of the *CmMNPV* suspension with the CSIs removed and the same suspension with addition of the spindles showed no significant difference in activity and in the slope of the regression lines (Table 1). The CSIs alone gave a mortality of 12% (the natural mortality in the untreated control was 4%) which was partly due to NPV.

**DISCUSSION**

Replication of *CmMNPV* was obtained *in vitro* in several *Cp* cell lines. Electron microscopical studies showed that the morphological changes associated with *CmMNPV* infection were very similar *in vivo* and *in vitro* (Fig. 1, 2). Viral morphogenesis in the nuclei of infected cells was comparable to that of other NPVs (Tanada & Hess, 1984). However, during the replication *in vitro* of *CmMNPV*, many CSIs, as described by Huger & Krieg (1968), were formed in addition to the polyhedra typical for all NPVs. No such CSIs were observed in uninfected larvae. Associations of CSIs with NPV infections have been reported from two other tortricids: the ugly
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Fig. 3. SDS-PAGE analysis of CmMNPV polyhedra (lanes 2 and 4) and CSIs (lanes 3 and 5) produced in vivo (lanes 2 and 3) or in vitro (lanes 4 and 5). Lanes 1 and 6 contain mol. wt. marker proteins.

nest caterpillar, Archips cerasivorana (Smirnoff, 1970), and the light brown apple moth, Epiphyas postvittana (Longworth & Singh, 1980); from three subspecies of a geometrid: the eastern hemlock looper, Lambdina fiscellaria fiscellaria, the western oak looper, L. f. somnaria, and the western hemlock looper, L. f. lugubrosa (Cunningham, 1970, 1971); from a phycitid: the almond moth, Cadra cautella (Adams & Wilcox, 1968); and from a cossid: Cossus cossus (Payne & Deseö, 1983). Our studies revealed that CSIs were also formed abundantly during CmMNPV replication in the heterologous cell line IZD-Cp 33b. As in host harvae, the CSIs were produced exclusively in the cytoplasm of CmMNPV-infected cells. Similarly, Sohi & Cunningham (1972) observed formation of CSIs in two cell lines of Malacosoma disstria haemocytes in the course of infection by L. f. somnaria NPV. The obligate association of CSIs with reproduction of the cited NPVs, either in vitro and in vivo, and their absence in controls strongly suggests that the biosynthesis of their protein is directed by these NPVs. This is supported by the fact that no difference in the mol. wt. of the CSI protein produced in vivo and that produced in vitro could be found.

The formation of CSIs is not unique to certain NPVs, it also occurs during the replication of a number of entomopoxviruses (EPV) (Granados, 1981). With these viruses, CSIs are also proteinaceous in nature, devoid of virus particles and appear to be chemically different from the
Fig. 4. Restriction enzyme fragments of CmMNPV DNA from larva-derived (lanes 1, 3 and 5) and tissue culture-derived polyhedra (lanes 2, 4 and 6). Digestions were with BamHI (lanes 1 and 2), EcoRI (lanes 3 and 4) and HindIII (lanes 5 and 6). The λ HindIII mol. wt. markers (×10^6) are given at the left.

Fig. 5. Dose-response curves (probit lines) of neonate codling moth larvae to CmMNPV produced in vivo in C. murinana larvae (●) and in vitro in the cell line IZD-Cp 33b. (○). The shaded area indicates the 95% confidence range for the line of the virus produced in vivo. (Mortality recorded after 14 days. Dose in number of polyhedra/ml of diet.)

EPV occlusion body proteins. In EPV infections of Melolontha melolontha, spindle development may also take place between the inner and outer membranes of the nuclear envelope, similar to CmMNPV CSIs.

As CSIs are regularly associated with CmMNPV infections and are formed in large quantities, it seems reasonable to speculate on their possible function in virus replication. An easy explanation could be that they enhance the infectivity of the CmMNPV. However, preliminary bioassays using purified polyhedra and polyhedra mixed with spindles revealed no significant difference in infectivity of the two preparations. With the codling moth, no
synergistic effect of the CSIs could be found, although spindles were produced in this alternative host. Possibly they are just deposits of a redundant protein. The low mortality found with the CSIs alone may be attributed to the contamination of the spindle preparation with NPVs.

CmMNPV DNA was shown by restriction enzyme analysis to be slightly altered after five passages in the Cp cell line. Serial passage of a number of other NPVs has resulted in spontaneous mutants, termed FP for few polyhedra, that produce fewer occlusion bodies than the wild-type virus. These mutants are typically detected by plaque assay within three or four passages in cultured cells. After plaque purification they have been shown in some cases to have acquired insertions of host cell DNA in their genomes (Miller & Miller, 1982; Fraser et al., 1983). Burand & Summers (1982) reported that continuously passaged virus isolates of Autographa californica NPV which did not exhibit the FP phenotype could also have altered genomes containing insertions of repeated viral DNA. In contrast, the CmMNPV viral genome has remained rather stable over five passages, although this stability may not be maintained at higher passage numbers.

Although we detected minor DNA changes after five passages in vitro, the CmMNPV polyhedra obtained by propagation of this virus in Cp cell lines were as infectious as those of larval origin, indicating that any genetic changes had not affected virus infectivity.

Baculoviruses have been considered as candidates for cloning vectors in the biotechnology industry because of their ability to package larger segments of foreign DNA in a vector that is expressed in eukaryotic cells. All constructions to date have used the polyhedrin promoter and have achieved high concentrations of secreted protein in both tissue culture supernatants (Pennock et al., 1984; Smith et al., 1984) and in the haemolymph of living insects (Maeda et al., 1985). During NPV infections, the production of CSI protein is nearly as high as that of polyhedrin, suggesting a similarly strong promoter for the former. Now that CmMNPV can be replicated in cell culture, the identity and potential usefulness of this novel promoter can be explored.

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