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Yield and activity of *Autographa californica* multicapsid nucleopolyhedrovirus and *Phthorimaea operculella* granulosis virus in cloned and uncloned cell lines of *P. operculella*

Xavier Léry, Adel El-Tarras, Said Abol-Ela & Joseph Giannotti
Entomovirology Laboratory ORSTOM, B.O. 26, Giza Code 12211, Cairo, Egypt

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Abstract

Three selected uncloned Pop 2, Pop 3, Pop 4 and two cloned cell lines Pop cl1A and Pop cl2B were derived from the original cell line established from *Phthorimaea operculella* (ORS-Pop-93). Three new non-selected cell lines ORS-Pop-94A, ORS-Pop-94B and ORS-Pop-95 were also established from embryos of the same insect. Differences in morphology, growth rate and polypeptide profile were determined between these cell lines. All the cell lines were susceptible to the *Autographa californica* nucleopolyhedrovirus (AcMNPV). The cloned cell lines produced higher levels of AcMNPV (TCID-50 and PIB) than the parental cells and at the same rate as the Sf9 reference cell line. Substantial amounts of viral DNA were synthesized in the clone Pop cl 2B after infection with the granulosis virus of the potato tuber moth *P. operculella* (PTMGV) and a complete multiplication was obtained in the ORS-Pop-95 cell line. The comparison between Pop cell lines which support limited or complete replication of certain baculoviruses can offer insights into some of the molecular barriers which restrict the host range of these viruses. These cell lines with variable susceptibility to baculoviruses could also be used for *in vitro* recombinations, increasing their virus host range to be used for the control of this pest.

Introduction

One of the main pest of potatoes in sub-tropical and tropical areas, responsible for very large losses in potato production, is the potato tuber moth (PTM) *Phthorimaea operculella* Z. (Lepidoptera: Gelechiidae) (Das *et al.*, 1992). An alternative to chemicals in PTM control, is a granulosis virus (Baculoviridae, Eubaculovirinae) isolated from this species and already used against this pest (Raman *et al.*, 1987).

Insect cell cultures are commonly used to multiply viruses for virological studies, essential to conduct a complete study on the properties of viruses and especially on those which could be employed as biological agents (Oellig *et al.*, 1989). More fundamental studies could also be performed using *in vitro* systems, having biotechnological repercussions (genetic recombinations, molecule production). Unfortunately, Granulosis viruses are very difficult to multiply *in vitro* (Dwyer *et al.*, 1988; Granados *et al.*, 1986).

When we established our first cell line of *P. operculella* (Léry *et al.*, 1995b), ORS-Pop-93, a susceptibility for the *Autographa california* nucleoplyhedrovirus (AcMNPV) was demonstrated but not for the *P. operculella* potato tuber moth granulosis Virus (PTMGV). AcMNPV is generally considered as a privileged baculovirus for many virological studies because of its large host spectrum and its facility to be multiplied in great volume. But the AcMNPV could not be multiplied in *P. operculella* larvae (Taha *et al.*, 1995), except under special conditions (A. Taha, pers. comm.). So it will be of interest to evaluate the capabilities of this virus *in vitro* and *in vivo* as a possible biological agent.

For these reasons, and because the ORS-Pop-93 cell line is a heterogeneous cell line constituted of many different cell types, we have tried to increase the potentialities of the Pop 1 cell line, and if possible, to select cells which can also replicate PTMGV. We have tried to establish other cell lines from the same insect

got by derivation from the original one by selecting cell types and cloning, and by new primocultures. In 1995, we established for the first time a *P. operculella* cell line susceptible to PTMGV named ORS-Pop-95 (Léry *et al.*, 1995b). Therefore, due to the number of cell lines established during this period, it became very important to test the susceptibility of all the established cell lines against the AcMNPV and PTMGV.

In this paper, we describe the derivation and selection of the new established cell lines, as well as the yield and activity of the two viruses. The results were compared with those obtained with the previously published Pop 1 and Pop 95 cell lines.

Materials and methods

Reference cell lines

The ORS-Pop-93 cell line, obtained from *P. operculella* (Léry *et al.*, 1995b), and designated Pop 1, was cultivated in Grace's modified medium (Léry and Fédière, 1990), containing 10% fetal bovine serum (FBS) at 27 °C. This cell line was routinely subcultured every 5 to 7 days and seeded at 1 to 2 × 10^6 cells per 25 cm^2 flasks.

The ORS-Pop-95 cell line, also obtained from *P. operculella* (Léry *et al.*, 1997, in press) designated Pop 95, was cultivated in the same medium, at 19 °C. This cell line was routinely subcultured every 2 or 3 weeks.

The *Spodoptera frugiperda* clone 9 (Sf9) cell line, was a gift from Dr. G. Croizier (St Christol-lès-Alès, France) and was cultured under the same conditions as for Pop 1.

Origin and selection of homogeneous cell lines deriving from Pop 1

The selection of the different cell types were performed during the first subcultures. During the first 3 months of the primoculture, 1 ml of fresh medium was added every 4 weeks. From the 4th month, the 3 first subcultures were respectively done after 2 months, then every 2 or 3 weeks at a ratio 1:2. From the 4th to the 8th passage, the selection of cell types occurred. In some cases, cells from the supernatant were seeded in new flasks and fresh medium was added to the original one. In others all the cells were detached from the flask walls with rubber policeman and seeded in new flasks. The subcultures were generally carried out every 10 to 15 days using the same techniques. In some flasks, the medium was only renewed every 2 weeks to permit the selection of low multiplication cells and cubcultured after several months. After the 9th passage, cells were subcultured every 5 to 7 days and seeded with 2 million cells per flask. Over subsequent passages, the quantity of FBS was reduced from 20% to 10%.

Cloned cell lines were established from the Pop 1 cell line, using meythyl cellulose. Different dilutions of cells were dropped on 35 mm Petri dishes (C35) containing 2 ml of 3% methyl cellulose 4,000 cp (Sigma) diluted in Grace's modified medium with 10% FBS, at 27 °C. After 5 to 7 days, colonies arising from single cells, were selected, isolated and removed with a fringed capillary pipette. These colonies, representing clones were seeded in 96 microwells plates, breaking the end of the pipette containing the cells. 200 μl of conditioning medium prepared as follows was added to each well. For the subcultures, conditioned media was prepared by seeding 2 × 10^6 parental cells/25 cm^2 flask. After 4 days, the medium removed was sterilized through 0.2 μm millipore filter and diluted twice in normal Grace's modified medium. The cloned cell lines were initially passaged in C35 containing 2 ml of medium and then routinely subcultured in 25 cm^2 flasks. For each clone obtained, another cloning experiment was performed.

Origin of non selected cell lines

Two new cell lines were established during 1994, in two different experiments. Eggs at two different stages from insects reared for 2 or 3 generations were used for the primary cultures as described previously (Léry *et al.*, 1995b). After 1 to 2 months, the first subculture was performed. The following subcultures were done every 10 to 20 days, depending on the cell culture, without selected pressure. From the 9th passage, cells were subcultured every 7 days in Grace's modified medium, the concentration in FBS of which was regularly decreased from 20% to 10%.

Characterization of cells

The morphology of the cells was described and photographs of the cultures were taken under light or with a phase contrast microscope at X100 to X500. Growth curves were determined from daily hemocytometer counts of cells from 5 replicate cultures stained with trypan blue over a period of 8 days. Cells were cultured in C35 seeded with 4 × 10^5 cells. The population dou-

bling time was calculated at several passages using an exponential formula. Polypeptide profiles were determined after electrophoresis in 12% polyacrylamide gels with a Biorad Miniprotean II dual slab cell (Léry *et al.*, 1996) and the polypeptide profile of all the established cell lines were compared.

Virus

A granulosis virus isolated from the potato tuber moth *P. operculella* (Tunisia isolate), a gift from Dr El-Bedewi (PTMGV) and a nucleopolyhedrovirus from *Autographa californica* (AcMNPV), a gift from Dr. G. Croizier (St Christol-lès-Alès, France), were used to compare the infectivity of the cloned and uncloned cell lines of *P. operculella*. The PTMGV inoculum was prepared as previously described (Léry *et al.*, 1997a, in press) and AcMNPV inoculum was routinely prepared from Sf9 infected cells supernatant, filtered at 0.45 μm.

Yield and activity of the viruses

AcMNPV. Cell cultures, seeded at 2×10^6 cells into 25 cm^2 tissue culture flasks were infected after 24 h with infectious supernatant at a multiplicity of infection (m.o.i.) of 0.1. The m.o.i. was calculated from TCID-50 values of the AcMNPV multiplied on Sf9. The viral inoculum was allowed to adsorb for 2 h, removed and replaced by fresh medium. Five days after infection, the flasks were scraped with a rubber policeman and the cells and polyhedral inclusion bodies (PIB) recovered by centrifugation 5 min at 1,500 g. The infectious supernatant was removed and titered in Sf9 cells to determine TCID-50 (Summers *et al.*, 1987). Average standard errors of the mean (S.E.M.) were based on triplicate determinations. Sedimented PIB and cells were resuspended in 0.5 ml medium and the number of cells containing PIB were counted to obtain yield/flask. The number of PIB was determined after disruption of the cells using STE buffer (0.15M NaCl; 0.02M Tris; 1 mM EDTA; 0.5% Aprotinine and 0.5% NP40) from hemocytometer counts using light microsccopy.

PTMGV. Cell lines, seeded at 2×10^6 cells into 25 cm^2 tissue culture flasks were infected after 24 h with virions at a concentration of 0.5 OD/ml, prepared as described above. After 2 h contact, the 1.5 ml viral suspension was removed and replaced by 4 ml of fresh medium containing 10% FBS and the cells incubated at 27 °C. After 15 days, the flasks were scraped with a rubber policeman and the cells and media recovered. Cellular extracts were prepared for nucleic acid detection, then granules and virions were purified (Léry *et al.*, 1997a, in press). The digoxygenin-labelled DNA probe was applied according to the protocol recommended by the supplier (Boehringer), and the same protocol was used in the 'dot blot' hybridization technique. A DNA probe prepared from the total extract DNA of the PTMGV with a 25 pg sensitivity was used in the test (Zeddam *et al.*, 1993).

Results

Origin of cell lines

Three different cell lines and two clones, were always multiplied, deriving from the Pop 1 cell line originally established. Pop 2 was derived from adherent cells. The cells with a slow multiplication were kept by removing regularly the supernatant, and waiting 2 to 4 months between each subculture. From the 5th passage, the Pop 2 cell line was subcultured every 1 to 2 weeks, until stabilization ocurred. Pop 3, deriving from a systematically selection of non-adherent cells, was subcultured every month until to 10th passage, when a sufficient quantity of non-adherent cells were floating in the medium, then every 1 to 2 weeks. Pop 4 derived from adherent cells, was cultured during 7 months in the same flask, by removing the non-adherent cells systematically, then subcultured every 2 to 3 weeks. Two clones were finally obtained after 2 successive cloning, Pop cl 1A and Pop cl 2B.

Two new cell lines were established in 1994 using no selection pressure. The first subculture occurred respectively after 1 month and 20 days. As the rate of multiplication was very high in the two experiments, the subcultures were done every 10 to 20 days until the 7th passage, then every week. Only rounded and fibroblast-like cells could be identified. After 7 months, the cells cultured in 10% FBS, were considered to be stabilized and designated as Pop-ORS-94A (Pop A) and Pop-ORS-94B (Pop B).

Characteristics of the cell lines

The new cloned and uncloned cell lines were considered to be established by the 16th passage. The selected cell lines appear to be relatively morphologically homogeneous. Pop 2 (Fig. 1a) and Pop 3 (Fig. 1b)

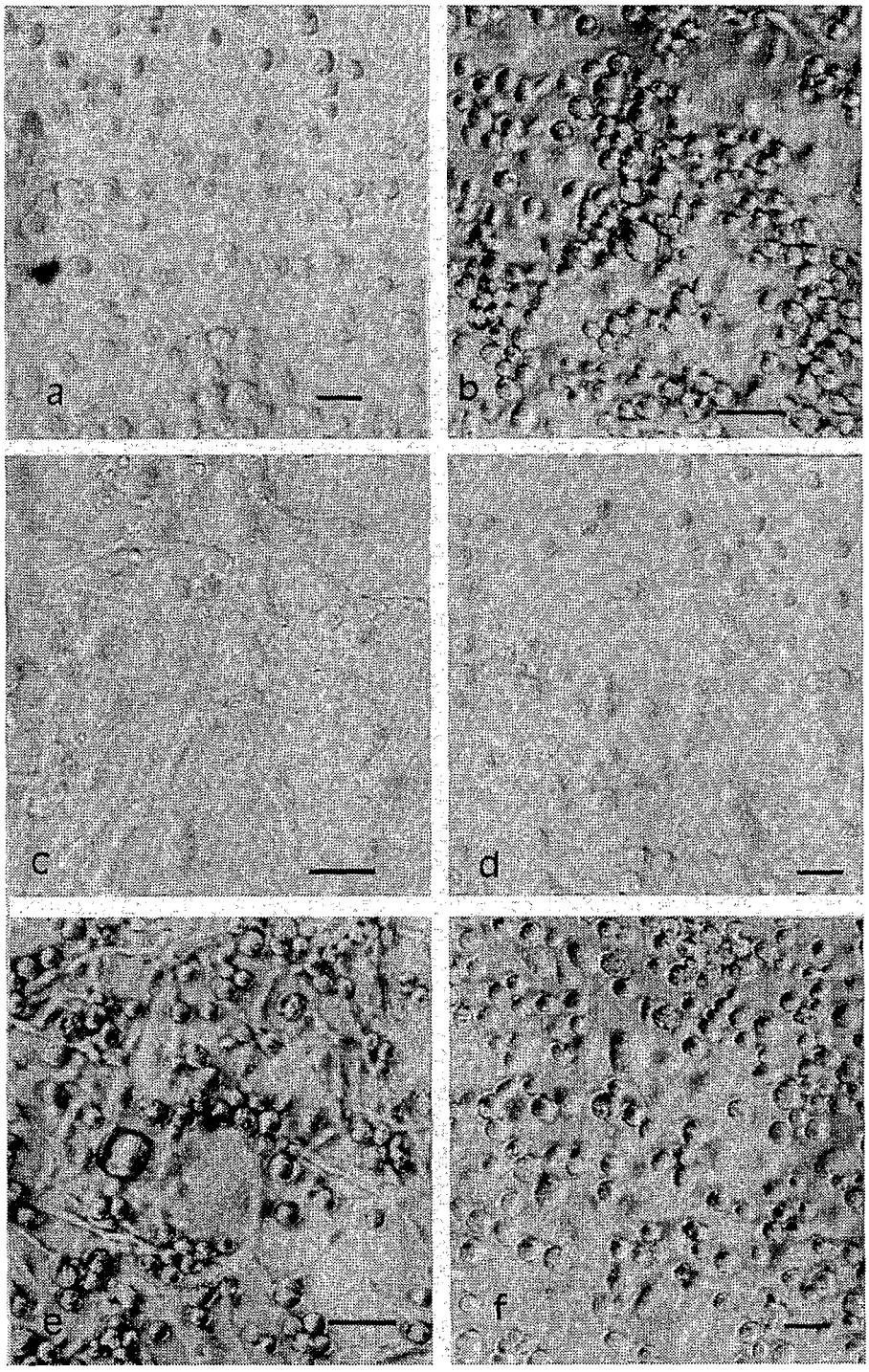

Figure 1. Cell cultures of cloned and uncloned cell lines in log phase, obtained from *P. operculella* and observed by phase contrast. a: Pop2; b: Pop3; c: Pop4; d: Pop cl 2B; e: PopA; f: PopB. Bar represents 30 μm.

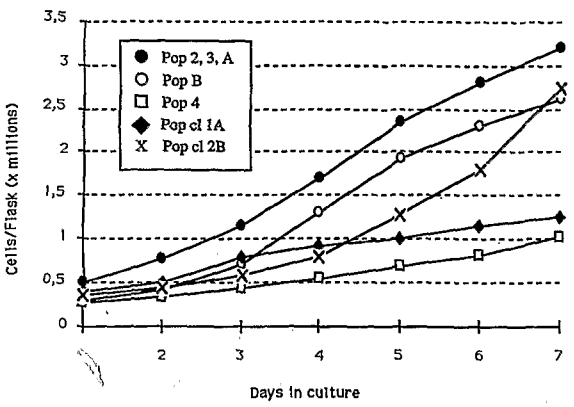

Figure 2. Comparison of the cell growth curves of the different cell lines obtained from *P. operculella.*

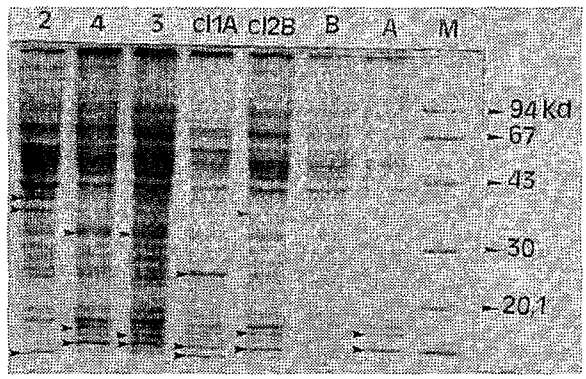

Figure 3. Comparison of the electrophoretic profiles of the different cell lines obtained from *P. operculella* on a 12% polyacrylamide gel. Each cell line is designated as their numeral indicator. M: marker. → significative polypeptide difference.

are constituted in the majority of rounded cells, from respectively 12 μm to 24 μm and 7 μm in diameter. Long fibroblast-like cells, 50 to 100 μm in length, are predominant in Pop 3 (Fig. 1c). The 2 clones, are morphologically identical, with rounded cells, similar to those observed in Pop 2 (Fig. 1d). Pop A appears to be constituted of simular cell populations than the heterogeneous established cell lines, Pop 1 and Pop 95 (Fig. 1e). Pop B is constituted of a similar cell population than Pop 2 (Fig. 1f).

The analysis of the growth curves and cell population doubling times at 27 °C, does not reveal differences among the passages tested (between 15 and 50). At an optimum seeding of 4×10^5 cells/ml, important differences in the growth activity can be found between the different cell lines (Fig. 2). We can identify 3 categories of cell lines. The first one, represented by Pop 2, Pop 3, Pop A and Pop B, has a doubling time of 32 to 36 h and a linear multiplication. The only difference observed, is concerning Pop B, the curve of which is slightly below the others because of a greater mortality in the first 24 h. The second one, is represented by Pop cl 2B, for which the multiplication occurs in 2 phases. During the first 3–4 days, the multiplication is lower than the cell lines of the first category, with a doubling time of 40 h, then it increases to 32 h. Pop 4 and Pop cl 1A, constitue the last category with a rate of multiplication always low and a doubling time near 70 h.

The polypeptide profiles of the different cell lines does not change for the 3 passages tested (15, 20 and 30th), but significant differences can be detected between the cell lines especially in the molecular weights between 14,000 and 50,000 daltons (Fig. 3).

The cell lines have been cultivated for more than 60 passages and can be stored for long periods in liquid nitrogen (–196 °C) by suspension in the culture medium containing 10% dimethyl sulfoxide (DMSO) and 20% FBS and progressive freezing in a NICOOL LM 100 apparatus for 30 min. We have also stored the cells at 4 °C for more than 1 month.

Yield and activity of the PTMGV and AcMNPV

PTMGV. A complete replication of the PTMGV was only obtained in Pop 95 cell line. A spectacular cytopathic effect could be seen and complete granules were obtained after purification of the infected cells as observed previously (Léry *et al.*, 1995a). As no cytopathic effect could be detected in the other infected cell lines maintained more than 15 days at 27 °C, a DNA probe test was used to detect a PTMGV DNA replication in the infected cells. A positive result was only obtained with the Pop cl 2B cell line, at dilutions 1/10. But after purification of the total cells and supernatant, no granule or virions was found.

AcMNPV. All the cell lines tested in our experiments could multiply the AcMNPV. The 2 clones, Pop cl 2B and at a lower degree, Pop cl 1A, produced significantly higher virus yields (TCID-50), respectively 8.14 and 6.94, than the other cell lines, corresponding to the 7.64 TCID-50 obtained with the Sf9 reference cell line (Table 1). The maximum titer in the cell lines was not achieved 5 days post-infection. Two days later an increase of an approximatly factor 1 occurred. At 5 days post-infection, 0.05% to 75% of cells contained

Table 1. Average TCID-50 and PIB/flask produced in cloned and uncloned cell lines of *Phthorimaea operculella* 5 days post-infection

Cell Line	TCID-50/ml[a] (average + SEM)	PIB/flask[b] (average + SEM)
Pop 2	4.20 ± 0.05	2.30 ± 0.50
Pop 3	5.72 ± 0.05	36.80 ± 8.00
Pop 4	3.66 ± 0.02	0.14 ± 0.05
Pop cl 1A	6.94 ± 0.05	108.00 ± 15.00
Pop cl 2B	8.14 ± 0.06	474.00 ± 56.00
Pop A	3.94 ± 0.03	18.40 ± 7.00
Pop B	4.04 ± 0.05	9.20 ± 1.00
Sf9	7.64 ± 0.05	3712.00 ± 126.00
Pop 1	5.67 ± 0.05	40.20 ± 8.40
Pop 95	5.02 ± 0.06	36.40 ± 5.20

[a] Log_{10}
[b] Number × 10^5/ml

PIB, when 90% of Sf9 cells were infected. 75% of 2.45 × 10^6 Pop cl 2B cells contained PIB. The number of cells in each flask and the percentage of cells containing PIB for the other cell lines, Pop cl 1A, Pop B, Pop 3, Pop A , Pop 2 and Pop 4 were respectively: 2.3 × 10^6 cells (20.0% containing PIB), 3.4 × 10^6 cells (10.0% containing PIB); 4.5 × 10^6 (4.0% containing PIB); 4.9 × 10^6 cells (2.0% containing PIB); 5.3 × 10^6 cells (0.5% containing PIB) and 2.6 × 10^6 cells (0.05% containing PIB). When expressed as number of PIB per seeded cell, the average number of PIB/cell was: Pop cl 2B, 23.7; Pop cl 1A, 5.4; Pop B, 0.46; Pop 3, 1.84; Pop A, 0.92; Pop 2, 0.115 and Pop 4, 0.007, corresponding to 0.14 (pop 4) to 3712.00 (Sf9) PIB/flask (Table 1). In each case, Pop 95 produced similar results than the Pop 1 cell line.

Discussion and conclusion

As the first cell line of *P. operculella* was considered to be heterogeneous, the cell lines deriving from it could be considered as relatively homogeneous. The methods used to select specific cells, resulted in the establishment of cell lines with different biological activities. All the established cell lines had specific characteristics, regarding their morphology, their growth rate and their polypeptide profile. But the general polypetide profile of the *P. operculella* established cell lines was the same, indicating the same species origin of the cells (léry *et al.*, 1995b). As we found no difference between the different passages for a specific cell line even after 60 passages, this indicated that the cells constituting the population of a cell line, stayed at a same level when the culture conditions did not change.

The morphology of the cells present in a cell line did not reflect the biological activity. The 2 clones obtained, similar for the cell aspect, had distinct polypeptide profiles. Their growth curves and doubling time were also different. If we analyse the growth curves of the 3 different categories of cell lines obtained, we can conclude that, the strongly fibroblast-like adherent cells (Pop 4) grew slower than the rounded cells and could not detach from the flask walls when confluency occured. The number of cells obtained per flask, therefore remained low.

All the cloned and uncloned cell lines obtained multiply the AcMNPV. The two cloned cell lines produced higher levels of AcMNPV (TCID-50, PIB) than their parental cells and at a same rate as the Sf9 cell lline, when the uncloned cell lines produced low or similar levels. These results were comparable to those previously obtained on other models, indicating an increase in the multiplication of NPV in cloned cell lines as compared to the parental cells (Lenz *et al.*, 1991; Rice *et al.*, 1989). As observed by Corsaro and Fraser (1987), our study indicated that differences in TCID-50 and PIB concentrations between different cell lines were not necessarily correlated. Cell lines that produced high levels of PIB did not necessarily generate correspondingly high titers of TCID-50 and *vice versa*. For example, comparing Pop A, Pop B and Pop 2, the lower level of PIB/seeded cells, produced the higher titer of TCID-50 although, Pop 3 produced a high titer of TCID-50 but a lower PIB comparing with Pop cl 1A. On the other hand, Pop cl 2B produced consistently high levels of both TCID-50 and PIB while, Pop 4 generated very low levels. The TCID-50 was not correlated to the number of PIB produced. For example, Pop cl B2 which had the highest titer (8.14) produced few amounts of PIB (474.00) compared with Sf9 (3712.00). The differences observed between the cell lines may be due to one or a combination of several factors, inability of cells to replicate, differences in rate of mitosis, differences in cell susceptibility due to viral attachment, penetration and uncoating (Gettig *et al.*, 1987). Except for the Pop 4 cell line, no direct relationship could be made between cell growth rate and the level of production, since neither the cell lines with highest nor those with lowest doubling times produced comparable levels of PIB. The differences were most probably due to viral susceptibility of the cells (Guzo

et al., 1992) or the production of inhibition factors (Guzo *et al.*, 1991) to explain the differences observed in the number of susceptible cells in each cell line. Not only the production of infectious virions, but also the number of occluded viruses were highly variable in cell cultures (Wood *et al.*, 1982). This indicated probably an incomplete cycle of the virus replication, with a late abortive phase. In 1983, Carpenter and Bilimoria reported a semipermissive infection of Sf MNPV in the TN-368 cell line with a cycle aborting prior to the viron assembly stage, when the same virus was permissive for its homologous cell line Sf21. In 1986, Mc Clintock *et al.*, with AcMNPV, described a semipermissivity in a gypsy moth cell line LD 652y, restricted to the early phase of AcMNPV replication.

The results obtained with PTMGV, confirmed the difficulty to multiply granulosis viruses *in vitro* (Dwyer *et al.*, 1988; Granados *et al.*, 1986). Nevertheless, using in cloned cell line, Pop cl 2B, we have obtained incomplete multiplication. Recent results obtained by Winstanley (1994), demonstrated the establishment of permissive cell lines susceptible to GV, using low temperatures (19–21 °C), indicating that the cells cultivated at 27 °C had lost their susceptibility. Our cell line established in those conditions, Pop 95 confirmed this hypothesis, because a complete replication of the PTMGV was obtained with the formation of granules (Léry *et al.*, 1995a; Léry *et al.*, 1997a, in press). More, recent results, indicated that another granulosis virus could be multiplied on the Pop 95 cell line, the *Spodoptera littoralis* GV (Léry *et al.*, 1997b, in press).

We have demonstrated that TCID-50 and PIB production of AcMNPV could be increased by selecting out high producing clones. At the same time, we have revealed that one of the clones, Pop cl 2B was semipermissive to the PTMGV. Under specific culture conditions, we have also obtained a cell line which supports both AcMNPV and PTMGV multiplication. The comparison between insect cell lines which support limited or complete replication of certain baculoviruses can offer insights into some of the molecular barriers which restrict the host range of these viruses (Guzo *et al.*, 1992). More, AcMNPV, could multiply at a high rate in cells of *P. operculella*, while it slightly infects the insect *in vivo* (A. Taha, pers. comm.). Finally, using our different PTM cell models as a filter, *in vitro* recombinations could be attempted, using AcMNPV and several GVs to expand AcMNPV host trange. By the means as realized before on other NPV models (Maeda *et al.*, 1993), it is reasonable to admit that the new recombinant viruses obtained could be used as a virus alternative for the control of *P. operculella*.

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Address for correspondence: Xavier Léry, Entomovirology laboratory ORSTOM, B.O. 26, Giza code 12211, Cairo, Egypt.