

Establishment of Two Cell Lines from Embryonic Cells of *Pectinophora gossypiella* (Lepidoptera: Gelichiidae)

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Two cell lines from the most important insect pest of cotton, *Pectinophora gossypiella* S., were obtained from an embryonic tissue culture. The cells were cultured in GRACE's modified medium. The first cell line, designated ORS-PgS (PgS), was obtained by selecting the non-adherent cells during subculturing and consisted of a homogeneous population of rounded cells. The second cell line, designated ORS-PgA (PgA), obtained without any artificial selection, was a heterogeneous population of spherical and spindle-shaped cells with a great capacity to adhere. The two cell lines had a doubling time of 40 and 48 h respectively and were sub-cultured for more than 50 passages. Their polypeptidic profiles appeared identical but were different from those of other lepidopteran cell lines tested. The two cell lines support the multiplication of *Autographa californica* nucleopolyhedrovirus, producing 10^5 plaque forming units/ml. In 1 to 2% PgS cells, polyhedra were synthesized, whereas PgA cells multiplied only virions.

Key words: *Pectinophora gossypiella*, Lepidoptera, cell line, cotton, AcMNPV

INTRODUCTION

Cell cultures are generally considered as useful tools for the study of insect viruses and their permissivity, especially for biotechnological applications such as *in vitro* production of baculoviruses (MARAMOROSCH, 1987).

The cotton pink bollworm *Pectinophora gossypiella* SAUNDERS was susceptible to several viruses isolated from the host. The first one, discovered in 1966 (IGNOFFO and ADAMS, 1966), belongs to the cytoplasmic polyhedrosis viruses. Recently, a picorna-like virus, with a low rate of pathogenicity and considered as a possible associated virus, was identified in this insect (MONSARRAT et al., 1994). Another virus, *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), known for its large spectrum of hosts, is infectious to the pink bollworm larvae (VAIL et al., 1972; BELL and KANAVAL, 1977). As a part of our program on cellular permissivity, we pay particular attention to this virus-insect model.

As no established cell line could support the multiplication of the viruses isolated from *P. gossypiella*, and a *P. gossypiella* cell line has not been reported, we describe in this paper, the development of cell lines derived from this species and their susceptibility to different viruses.

MATERIALS AND METHODS

Primary culture. Larvae collected from the field, were reared in the laboratory until adult emergence. Eggs (0.5 mm in diameter) from several layings were stored between 12



and 14°C to stabilize embryonic development, until enough eggs were collected. Eggs were collected at the brown colour stage, just before hatching. Primary cell cultures were established according to PANT et al. (1977).

Several thousands eggs were surface sterilized by immersion in 5% sodium hypochlorite for 10 min and washed twice in 0.137 M NaCl, 5 mM KCl, 5 mM glucose, 4 mM NaHCO₃, pH 7.2 buffer. The eggs were crushed, homogenized in a teflon homogenizer and then centrifuged for 5 min at 400×g. The pellet was treated for 5 min in a special buffer (2.6 mM KCl, 12 mM NaHCO₃, 137 mM NaCl, 0.4 mM NaH₂PO₄, 5.5 mM glucose and 3.5 mM citric acid, pH 7.2). Fragments and cells were centrifuged for 5 min at 400×g, and the pellet was rinsed twice in PBS. The final pellet was resuspended in GRACE's modified medium (LÉRY and FÉDIÈRE, 1990), containing 20% fetal bovine serum (FBS). Nunc tissue culture flasks (25 cm²) were seeded with the equivalent of 1,500 eggs in 4 ml of medium and incubated at 25°C.

Subculture. During the first 2 months, 1 ml of fresh medium was exchanged every 2 weeks. From the 3rd month, the 6 first subcultures were respectively performed every month, then every 2 or 3 weeks, at a ratio 1:2. In some cases, cells from the supernatant were seeded in new flasks and fresh medium was added to the original flask. In other cases, all cells were detached from the walls with a rubber policeman and seeded in new flasks. From the 7th to the 12th passage, the subcultures were carried out every week using the same selecting technique. In some flasks, the medium was only renewed every week to permit the selection of low multiplication adherent cells. The medium collected from these flasks was centrifuged and stored at 4°C. However, as the technique used for subculturing, some collected medium and fresh medium was mixed to replace the medium withdrawn from the original flasks when the non-adherent cells were removed. During this period, the quantity of serum was decreased to 15%. After the 12th passage, cells were subcultured every 5 to 7 days and seeded with 2 million cells per flask. At this step, the quantity of serum was decreased regularly until 10% and the cells cultivated at 27°C.

Characterization of cells. The morphology of the cells was described and photographs of the cultures were taken under normal light or through a phase contrast inverted microscope at a magnification of 100 to 500. 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 Petri dishes (10 × 35 mm) seeded with 2.5 to 7.5 × 10⁵ cells. The population doubling time was calculated using an exponential formula. Polypeptide profiles were determined after polyacrylamide gel electrophoresis in 12% using the method of LAEMMLI (1970). Cellular extracts were prepared using a technique of freeze-thawing. After the cells were detached from the flask walls, they were washed 3 times in PBS and centrifuged at 400×g for 5 min. The pellet was resuspended in PBS and treated 3 times by quick freezing in liquid nitrogen (-196°C) and slow thawing at room temperature. Five microliters of cell extract was treated with 15 µl of buffer sample (LAEMMLI, 1970) for 5 min at 100°C. The electrophoresis was performed with a Biorad Miniprotean Slab Cell. The polypeptide profile of established cell lines was compared with PgA and PgS at different passages. The codes for each were as follows: *Spodoptera littoralis* (SL), *Lymantria dispar* (LD) and *Galleria mellonella* (GM), a gift from Dr. J.M. QUIOT (St-Christol-lès-Alès, France), and *Phthorimaea operculella* (PO) (LÉRY et al., 1995).

Virus. The RNA Picorna-like virus (Pgv), isolated from the cotton pink bollworm, *P. gossypiella* (MONSARRAT et al., 1994) and a nucleopolyhedrovirus from *Autographa californica* (AcMNPV), provided by G. CROIZIER (St-Christol-lès-Alès, France), were used to test the

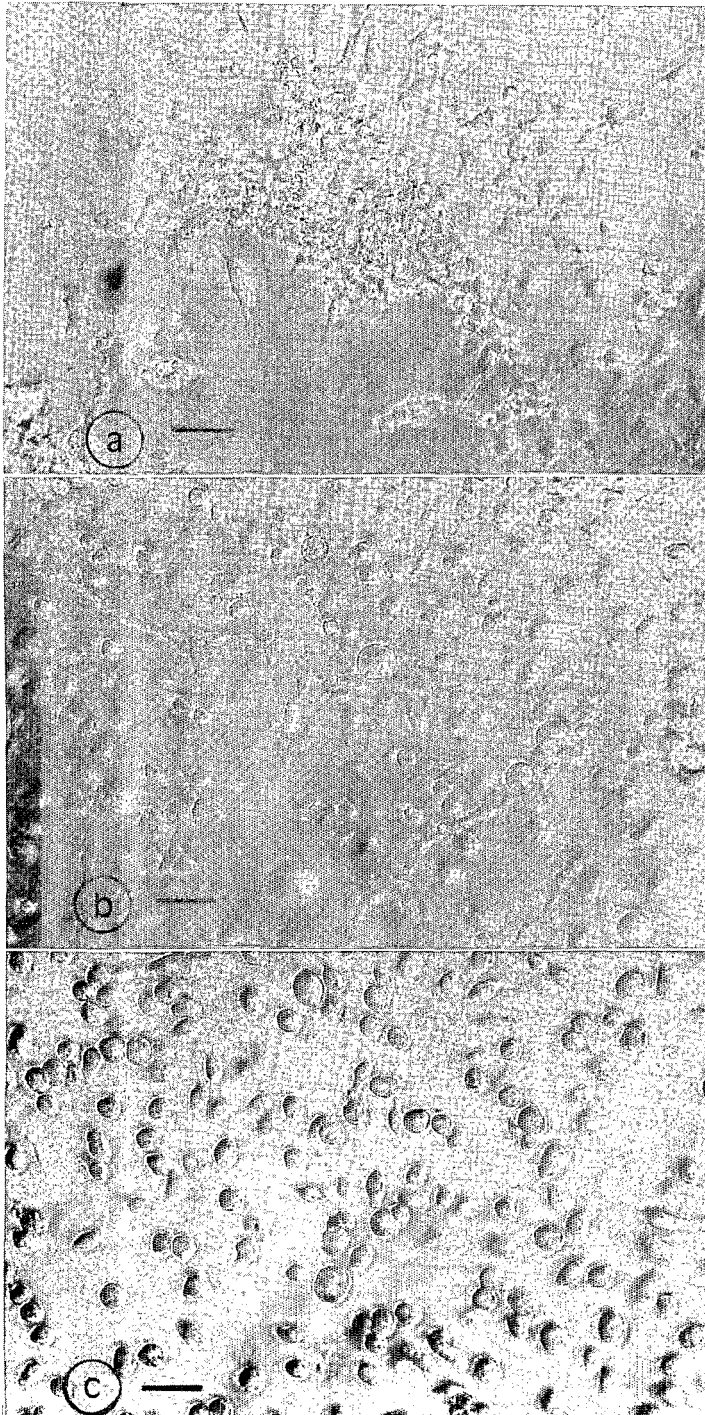


Fig. 1. Cell cultures of *P. gossypiella*. (a) 18 day-old primary culture; (b) 5 day-old culture at the 18th passage of the PgA cell line; (c) 3 day-old culture at the 18th passage of the PgS cell line. Bar represents 40 μ m.

susceptibility of the *P. gossypiella* cell lines. Purified PgV at a concentration of 0.1 OD/ml diluted in GRACE's modified medium and filtered on 0.45 μm millipore filter, was used to infect 24 h cultures of the cell line grown in 25 cm^2 tissue culture flasks. The cells were also infected at a multiplicity of infection (MOI) of 0.1 with AcMNPV. The MOI was calculated from the tissue culture infective dose 50% (TCID₅₀) values of the AcMNPV on *Spodoptera frugiperda* clone 9 cells (Sf9). After 2 h of contact, the 1.5 ml of viral suspensions were removed and replaced by 4 ml of fresh medium containing 10% FBS and the cells were incubated at 27°C. After 6 days post-infection with AcMNPV and 8 days with PgV, the flasks were scraped with a rubber policeman and the cells were centrifuged for 5 min at 400 $\times g$. The supernatants of AcMNPV infections were titrated on Sf9 as described by SUMMERS and SMITH (1987). Pellets diluted in 1 ml Tris-HCl buffer, pH 7.5, were used to evaluate the number of infected cells. For PgV infections, possible cytopathic effects were observed, the supernatants and pellets tested using ELISA and the hypothetic virus purified (MONSARRAT et al., 1994).

ELISA test. For detection of viral proteins, a specific rabbit serum was prepared by injecting purified virus, as previously described (MONSARRAT et al., 1994). The indirect method of ELISA test (enzyme linked immunosorbent assay) using alkaline phosphatase, was conducted according to VOLLER et al. (1976).

RESULTS

Primary culture

Most tissue fragments of embryos began to attach to the culture flask after 1 or 2 days incubation. The explant shrank gradually and cells began to multiply from it. During the first two months cells grew, radiating from the aggregates. Later, smaller distinctive cells of different morphological forms would appear attached to and underneath the fibers. The most commonly observed morphologies were epithelial-like cells, fibroblast-like cells, small and large rounded cells, hemocytes, strongly attached cells (Fig. 1a), and sometimes a few muscle-like cells with contractions and nerve-like cells. At the end of this period, when

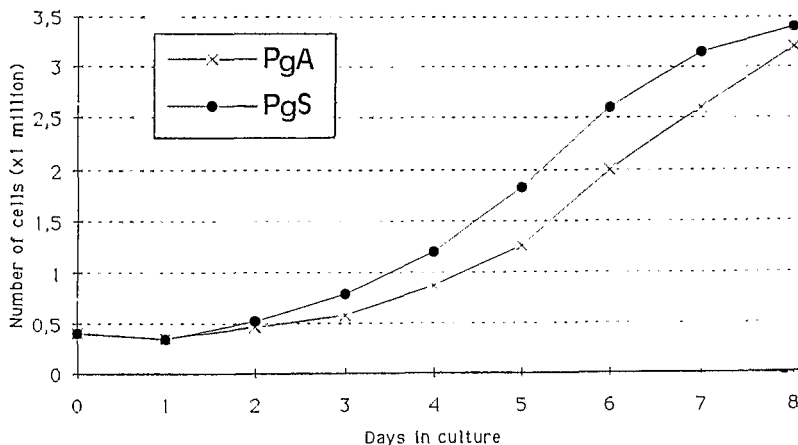


Fig. 2. Growth curves of *P. gossypiella* cell lines at the 18th passage, cultured in modified GRACE's medium, containing 10% fetal bovine serum.

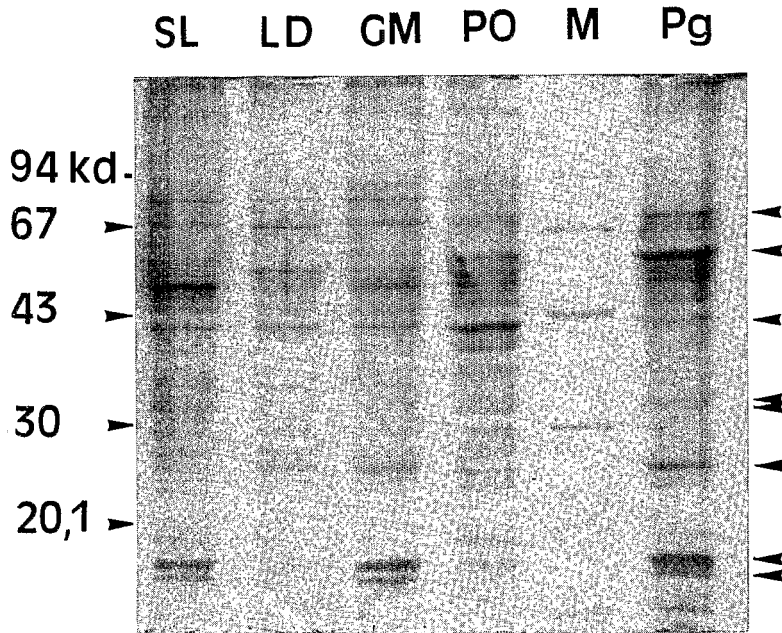


Fig. 3. Electrophoretic profile of different Lepidopteran cell lines on a 12% polyacrylamide gel. SL: *Spodoptera littoralis*; LD: *Lymantria dispar*; GM: *Galleria mellonella*; PO: *Phthorimaea operculella*; Pg: *Pectinophora gossypiella*. ◀ indicates the polypeptides of Pg cell lines significantly different from those of other cell lines tested.

the flask was completely covered with cells, a number of these cells was floating in the medium.

Subculture

During the first subcultures, a number of cell types disappeared, especially the contractile cells. The majority of cells was represented by small rounded cells, slightly adherent, but a small number of strongly adherent cells persisted. Many different cultures were obtained by the selective techniques used. Among these, two different cultures had already been subcultured. One of the two, deriving from the selection of different supernatants mixed together, had a relatively homogeneous population from the 8th passage. The second one, deriving from the total cells, conserved a heterogenous population. During the next passages, cells maintained under the same conditions, did not show modifications. From the 12th passage, the decrease of the quantity of serum to 12.5%, then 10% improved the adherence of cells. At the 15th passage, cells cultivated in 10% FBS were stabilized. The cell morphology and growth did not change for the two selected cell lines.

Characteristics of the ORS-PgA and ORS-PgS cell lines

The two new cell lines, ORS-PgA (PgA) and ORS-PgS (PgS) were considered to be established for the 16th passage. PgA comprised a majority of small spherical cells, 10 to 14 μm in diameter, big spherical cells, 20 to 30 μm in diameter and spindle cells, 75 to 100 μm length and 9 to 12 μm width. All cell types could attach to the bottom of the flask, forming

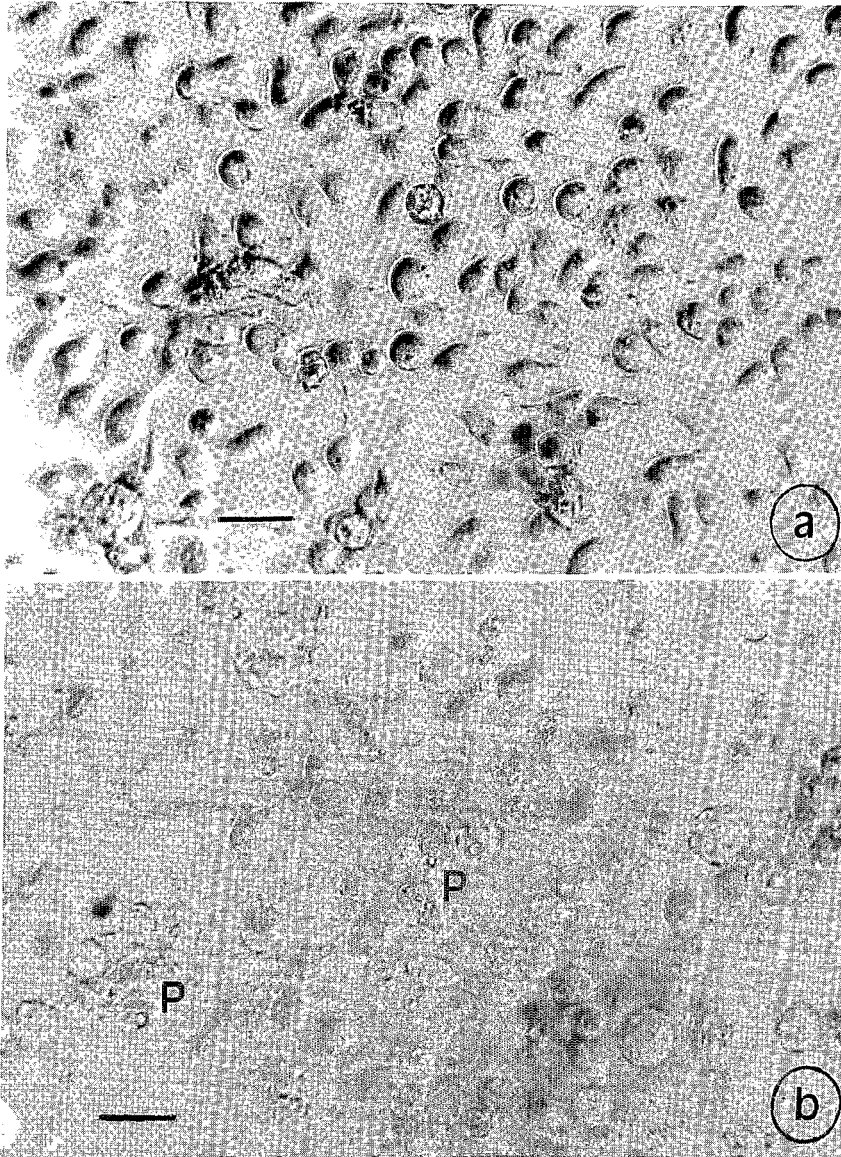


Fig. 4. Cell cultures of *P. gossypiella* at the 18th passage, infected with AcMNPV (a) PgA cell line, 10 days post infection; (b) PgS cell line, 5 days post infection. P indicates polyhedra. Bar represents 25 μm .

a composite monolayer (Fig. 1b). PgS constituted small and medium (10 to 20 μm in diameter) rounded cells (Fig. 1c). The cell population doubling time at 27°C differed between the two cell lines. At the 18th passage, for an optimum seeding of 2×10^5 cells/ml, PgS had a doubling time of 40 h. PgA had a doubling time of 48 h, and its growth curve was divided into two periods, low multiplication during the first 3 days with a doubling time

of 60 h and from the 4th day, a doubling time of 40 h (Fig. 2). The polypeptidic profiles of PgA and PgS were identical for the 3 passages tested (15, 18 and 25th), and this profile was significantly different from those of the other lepidopteran cell lines tested (Fig. 3).

The two cell lines which have now been cultivated for more than 50 passages, may be stored long term in liquid nitrogen (-196°C) by suspending with culture medium containing 10% DMSO and 20% FBS and progressive freezing in NICOOL LM 10 (CFPA, France) for 30 min. They can also be stored at 4°C for more than one month.

Viral infections

Infections of either cell line with PgV could not be demonstrated as tested by ELISA. After purification of the infected cells and supernatants, no viral particles were observed under electron microscope or detected by spectrophotometer analysis. The AcMNPV was multiplied at a detectable rate in the two cell lines. Three days post-infection, polyhedra began to appear in PgS cells and on the 7th day, all susceptible cells were infected. The formation of polyhedra was limited to 1 to 2% of cells at this step (Fig. 4a). In PgA cells, no polyhedra could be detected even at 8 to 10 days post-infection (Fig. 4b). After titration of the infectious supernatants on Sf9, the same result was found with PgA and PgS. The virus produced had a TCID-50 of $2.36 \times 10^5/\text{ml}$, compared to the 10^7 to 10^8 TCID-50 obtained when AcMNPV was multiplied directly in Sf9 cells.

DISCUSSION

The two cell lines, PgA and PgS, obtained, represent the first report of establishment of cultures of the cotton pink bollworm *P. gossypiella*. These cell lines were derived from embryos and had a population composed of various cell types. Similar observations have been reported for different species (Dwyer et al., 1988; Miltenburger et al., 1985; Tsang et al., 1981). Nevertheless, this heterogeneity in the cells did not affect the stability of the cell lines among passages since neither the growth curve nor the polypeptidic profile was modified. Conserving the total population of cells during all the passages led to the establishment of a heterogeneous cell line (PgA), whereas the selection of supernatant cells led to the establishment of a relatively homogeneous cell line (PgS), as previously reported for other species (Léry et al., 1994).

The susceptibility of the cell lines was tested against two important viruses. PgV (isolated on *P. gossypiella*) did not multiply in the cell lines. This result must be compared to those obtained *in vivo* by Monsarrat et al. (1994). They indicate that the virus multiplied at a low rate in larvae, existing within adult populations and probably representing an "associated virus."

In contrast, the AcMNPV, known for its large spectrum of hosts and its high infectivity (Hoffmann et al., 1990), could multiply in the two cell lines. The susceptibility of the cell lines was not correlated with the insect species from which the cell lines were derived, the susceptibility of the cell line to normal AcMNPV replication, or the tissue origin of the cells (Guzo et al., 1991). Only the selected cell line appeared to be truly permissive, supporting complete DNA and protein synthesis which led to the production of infectious progeny. The non-selected cell line, could be considered as semipermissive because the cells did not produce the complete occluded viruses. The quantity of virions produced by the two cell lines was low compared to the standard Sf9 system yielding TCID₅₀ values 2-3 orders of magnitude higher. However, it was comparable to those generally observed in heterologous

cell lines (HARA et al., 1994; RICE et al., 1989). The number of polyhedra synthesized in the PgS cell line remained at a low rate.

This is not the first report of such a phenomenon. CARPENTER and BILIMORIA (1983) reported a semipermissive infection of SfMNPV in the TN-368 cell line with a cycle aborting prior to the virion assembly stage, when the same virus was permissive for its homologous cell line Sf21. In 1986, McCLINTOCK et al. described a semipermissivity in a gypsy moth cell line Ld 652Y with AcMNPV, restricted to the early phase of AcMNPV replication (McCLINTOCK et al., 1986).

More recently, we have obtained a new cell culture of *P. gossypiella* which did not support any AcMNPV multiplication and can be considered as a non-permissive cell line (unpublished data). It will be interesting to clone the PgS cell line to increase the susceptibility to AcMNPV and the number of polyhedra produced (LÉRY et al., in press). Thus, we now have a complete system of permissivity of insect cell lines from the same host origin. This will facilitate the study of factors regulating the permissive, semipermissive or non permissive replication cycle of AcMNPV in heterologous cell lines and improve our understanding of the factors involved in regulating control mechanisms during NPV replication. The availability of these cell lines, obtained for the first time, from *P. gossypiella*, will also permit us to answer some questions on the insect's biology and cell metabolism.

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