In Vitro Cell. Dev. Biol.—Animal 31:836–839, December 1995

1071-2690/95 $05.00+0.00

ESTABLISHMENT OF A CELL LINE DERIVED FROM EMBRYOS OF THE POTATO TUBER MOTH *PHTHORIMAEA OPERCULELLA* (ZELLER)

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(Received 6 October 1994; accepted 3 July 1995)

SUMMARY

A cell line from the main insect pest of potatoes in tropical and subtropical areas, *Phthorimaea operculella* (Zeller), was obtained from embryoculture. These cells were cultured in Grace's modified medium. The cell line, designated ORS-Pop-93, had a heterogeneous population consisting of spherical and spindle cells with great capacity to adhere and a doubling time of 40 h. They were subcultured for more than 60 passages. Their polypeptidic profile was different from profiles of other lepidopteran cell lines. The cell line supports the multiplication of the *Autographa californica* nuclear polyhedrosis virus.

Key words: Phthorimaea operculella; Lepidoptera; Gelechiidae; cell line; potato; embryo; cell culture; Ac NPV; PTM GV.

INTRODUCTION

Research on modern biological control requires some approaches that cannot be explored without the use of cell cultures. *In vitro* studies could easily lead to interesting results on some aspects of physiological or hormonal phenomena (1). They are also necessary for studies on some mechanisms of insecticide resistance and for studies on the properties of viruses, especially those which could be used as biological agents (12).

We found that use of cell cultures was particularly appropriate for studying the potato tuber moth *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), a cosmopolitan dominant pest in subtropical and tropical areas, responsible for very important losses in potato production (3). The use of chemicals was dangerous for humans, and the potatoes became unfit for consumption (18). Among the possible alternatives to chemicals, one of the most likely was the baculoviruses, principally the granulosis virus (Baculoviridae, Eubaculovirinae) isolated on the potato tuber moth (14). By using specific cell cultures and cell lines, an investigator could undertake a comprehensive study on these viruses. The present communication reports the establishment of a cell line from *P. operculella* embryos.

MATERIALS AND METHODS

Primary culture. Larvae collected from the field were reared in the laboratory until adults emerged. The eggs (0.5 mm in diameter) from several layings were stored between 12 and 14° C to retard embryonic development until enough eggs were collected. When eggs became brown just before hatching, they were collected. The method used for establishing primary cell cultures was derived from that of Pant (13).

Thousands of eggs were surface-sterilized by immersion in 5% sodium hypochlorite for 10 min and two washes in phosphate-buffered saline (PBS; 0.137 *M* NaCl, 5 m*M* KCl, 5 m*M* glucose, 4 m*M* $NaHCO_3$, pH 7.2). 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 m*M* KCl, 12 m*M* $NaHCO_3$, 137 m*M* NaCl, 0.4 m*M* NaH_2PO_4, 5.5 m*M* glucose, and 3.5 m*M* citric acid, pH 7.2). The fragments and cells were centrifuged under the same conditions and the pellet was rinsed twice in PBS. The last pellet was resuspended in Grace's modified medium (11) containing 20% of fetal bovine serum (FBS). Nunc tissue culture flasks (25 cm^2) were seeded with the equivalent of 1500 eggs in 4 ml of medium and incubated at 25° C.

Subculture. During the first 3 months, 1 ml of fresh medium was added every 4 weeks. From the fourth month, the three first subcultures were made after 2 months, then every 2 or 3 weeks at a ratio of 1:2. 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 walls with a rubber policeman and seeded in new flasks. From the fourth to the eighth passage, the subcultures were made every 10 to 15 d with the same selection techniques. In some flasks, the medium was renewed only every 2 weeks to permit the selection of slowly multiplying cells. The medium collected from these flasks was centrifuged and stored at 4° C. However, in the technique used for subculturing, some collected medium and fresh medium was mixed to replace the medium removed in original flasks when the nonadherent cells were picked out. After the ninth passage, cells were subcultured every 5 to 7 d and seeded with 2×10^6 cells per flask. At this stage, the quantity of serum was decreased regularly until it reached 10%.

Characterization of cells. The morphology of the cells was described and photographs of the cultures were taken under normal light or with a phase-contrast inverted microscope at ×100 to 500. Growth curves were determined from daily hemocytometer counts of cells from five replicate cultures stained with trypan blue over a period of 8 d. Cells were cultured in petri dishes (10 × 35 mm) seeded with 2.5 to 7.5 $\times 10^5$ cells. The population doubling time was calculated with an exponential formula. Polypeptidic profiles were determined after electrophoresis in 12% polyacrylamide gel with the method of Laemmli (10). Cellular extracts were first prepared with a technique of freezing-thawing. After the cells were detached from the flask walls, they were washed three times in PBS and centrifuged at 400 ×g for 5 min. The pellet was resuspended in PBS and treated three times by quick freezing in liquid nitrogen (−196° C) and slow thawing at room temperature. Five µl of cell extract was treated with 15 µl of buffer (9) for 5 min at 100° C. Electrophoresis was performed with a Biorad Miniprotean curve. The polypeptide profiles of established cell lines were compared with ORS-Pop-93 at different passages. The codes for each were: *Spodoptera littoralis* (SL), *S. frugiperda* clone 9 (SF9), *S. litura* (Sl), *Lymantria dispar* (LD) and *Galleria mellonella* (GM).

Virus. One granulosis virus isolated from the potato tuber moth (Tunisia isolate PTM GV; gift from Dr El-Bedewi) and three nuclear polyhedrosis

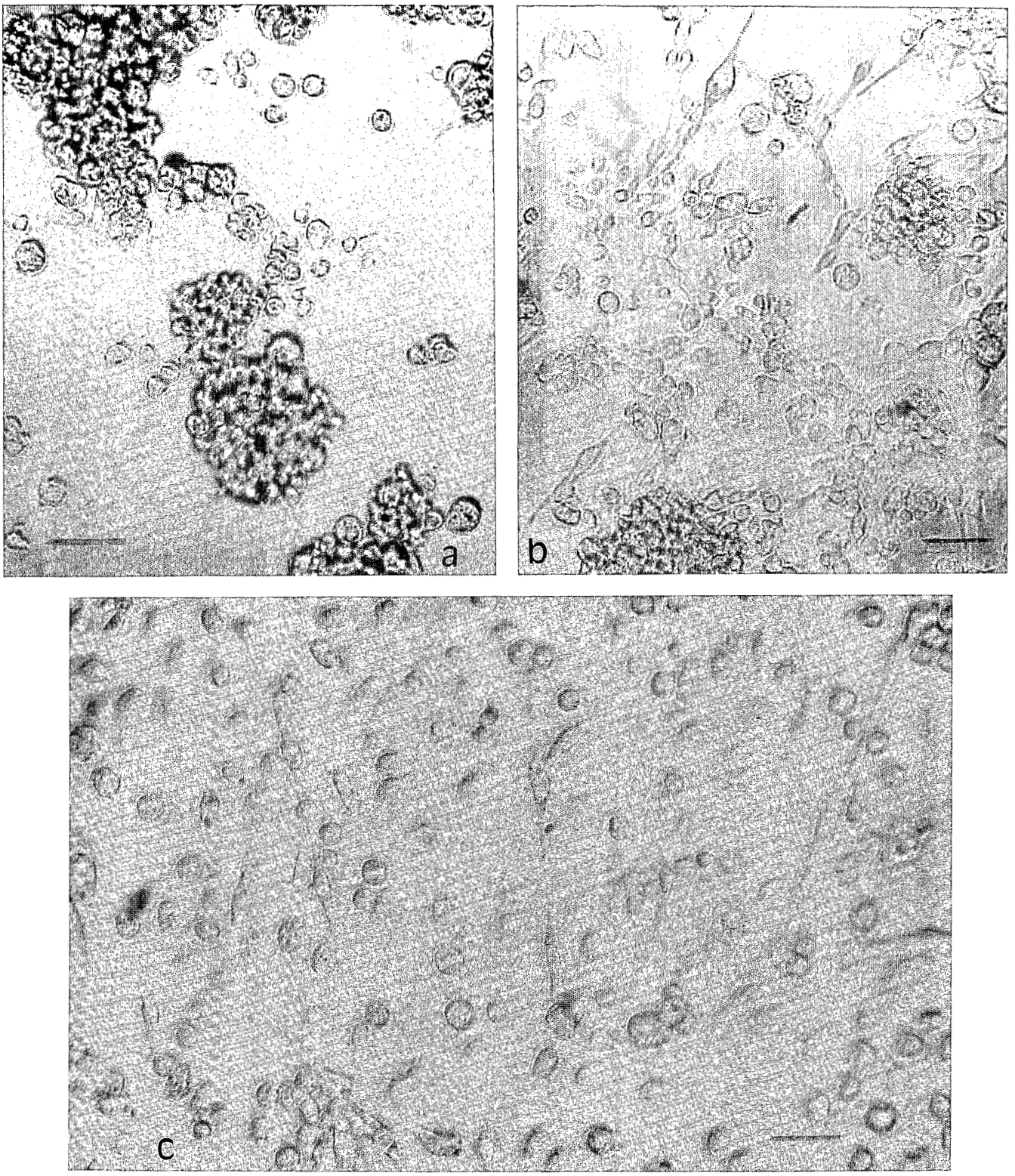

FIG. 1. Cell cultures of *P. operculella*. (a) 9-d-old culture at the seventh passage; (b) 5-d-old culture at the 10th passage; (c) 4-d-old culture at the 47th passage. *Bar* represents 40 μm.

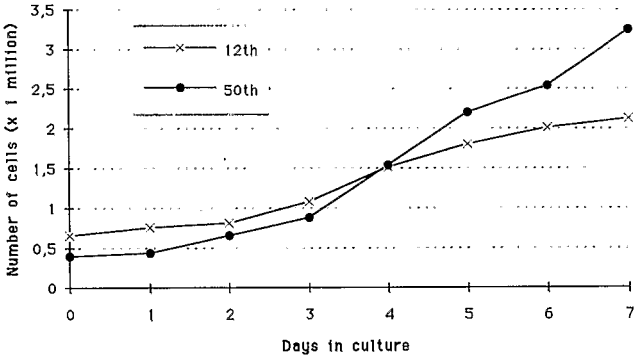

FIG. 2. Growth curves of *P. operculella* cells at 12th and 50th passages, cultured in Grace's modified medium containing 10% fetal bovine serum.

viruses, respectively from *Autographa californica* (Ac NPV), *Mamestra brassicae* (Mb NPV) and *Spodoptera littoralis* (SL NPV) were used to test the infectivity of the ORS-Pop-93 cell line. Purified virions at a concentration of 0.5 optical density/ml, diluted in Grace's modified medium and filtered through a 0.45-μm millipore filter, were used to infect 24-h cultures of the cell line grown in 25-cm² tissue culture flasks. After 2 h contact, the 1.5 ml of viral suspension was removed and replaced by 4 ml of fresh medium containing 10% FBS, and the cells were incubated at 27° C. After 6 d postinfection with NPV and 15 d with GV, the flasks were scraped with a rubber policeman and the cells centrifuged 5 min at 400 ×g. The supernatants of NPV infections were titrated on their specific cell lines (Ac NPV on SF9, Mb NPV on *Mamestra brassicae*, and Sl NPV on *Spodoptera littoralis*) as previously described (15). The pellets diluted in 1 ml Tris buffer at pH 7.5 were used to evaluate the number of infected cells. For GV infections, supernatants and pellets were tested with an enzyme-linked immunosorbent assay (ELISA) test and DNA probe techniques.

ELISA test. For detecting the viral proteins we prepared a specific mouse serum by injecting the total proteins of granules. The indirect ELISA test with alkaline phosphatase, was conducted according to Kelly et al. (9).

DNA probe. The dioxygenin-labeled SIGV DNA probe was applied according to the protocol recommended by the supplier (Boehringer). The same protocol was used in the hybridization "dot blot" technique.

RESULTS

Primary culture. Most tissue fragments of embryos began to attach to the culture flask after incubating 1 or 2 d. The explant shrank gradually and cells began to multiply from it. During the first 3 months, multicellular fibers radiating from the aggregates formed a net-like structure over the entire bottom of the flask. Late in the process, smaller, distinctive cells of many different morphologies appeared attached to and beneath the fibers. The most common were epithelial-like cells, fibroblast-like cells, small and large rounded cells, hemocytes, strongly attached cells, muscle and nerve cells showing contractions, and finally, vesicles. At the end of this period when the flask was completely covered with cells, a number of them were floating in the medium.

Subculture. During the first subcultures, a number of cell types disappeared, especially the contractile cells. Many different cultures were obtained by the selective techniques used, and some were already cultured. One was particularly well formed, deriving from the selection of different supernatants mixed together. At the seventh passage, a homogenous population began to appear. At this stage, small rounded cells were slightly adherent and formed a mass of cells floating in the medium (Fig. 1 *a*). During the next passages, cells maintained under the same conditions did not show modifications. After the 10th passage, decreasing the quantity of serum to 12.5% improved the adherence of cells. Their morphology changed and they looked like fibroblasts. Some rounded cells were budded from them, then detached to float in the medium (Fig. 1 *b*). At the 15th passage, the cells cultivated in 10% FBS were stabilized. The budding effect disappeared and cell morphology and growth did not change.

Characteristics of the ORS-Pop-93 cell line. The new cell line considered to be established at the 16th passage, has a heterogeneous cell population of spherical (small and big) and spindle cells. All the cell types can attach to the bottom of the flask, forming a monolayer (Fig. 1 *c*). The cell population doubling time at 28° C was at the 12th passage, 54 h after 3 × 10^5 cells/ml were seeded and at the 50th passage, 40 h after 2 × 10^5 cells/ml were seeded (Fig. 2). The polypeptidic profile of the ORS-Pop-93 cell line was the same for the three passages tested (10, 50 and 60th). It was significantly different from those of the other lepidopteran cell lines tested (Fig. 3).

The cell line which has now been cultivated for more than 60 passages, may be stored for a long time in liquid nitrogen (−196° C) by suspension with the culture medium containing 10% DMSO and 20% FBS and progressive freezing in NICOOL LM 100 for 30 min. It can also be stored at 4° C for more than 1 month.

Viral infections. Negative results were obtained with the PTM GV. All the infections tested by ELISA and probe indicated that the virus did not multiply in the ORS-Pop-93 cell line. Among the 3 NPV tested, only the Ac NPV multiplied at a detectable rate in the cell line. Three days postinfection, polyhedra began to appear in the cells and on the seventh day, all susceptible cells were infected. Four to 5% of cells were infected at this stage. After titration, the virus produced had a TCID50 of 5 × 10^5/ml corresponding to 3.5 × 10^5 plaque-forming units per milliliter.

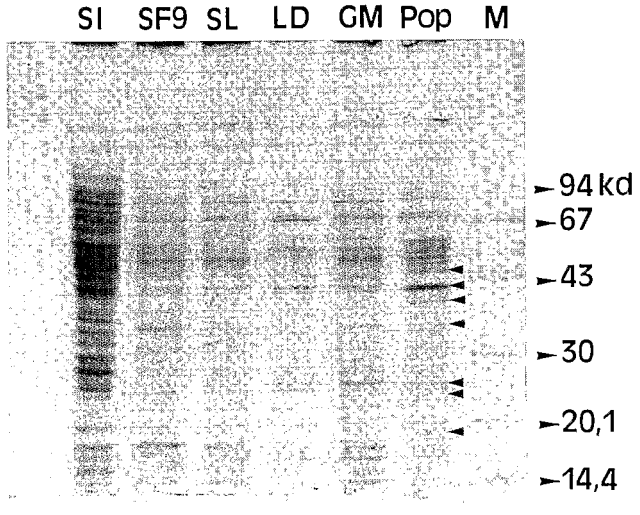

FIG. 3. Electrophoresis of different lepidopteran cell lines in a 12% polyacrylamide gel. Sl: *Spodoptera litura;* SF9: *Spodoptera frugiperda* clone 9; SL: *Spodoptera littoralis;* LD: *Lymantria dispar;* GM: *Galleria mellonella;* Pop: ORS-Pop-93. *Arrows* indicate polypeptides of the Pop cell line that are significantly different from those of other cell lines tested.

Discussion and Conclusion

Two cell cultures from *P. operculella* were previously reported (6,13), but their multiplication was stopped and neither of them is available. The new cell line established in our laboratory, ORS-Pop-93, represents the only *in vitro* model of *P. operculella*. This cell line is derived from the embryos and is composed of various cell types. Other cell lines have been reported for different species (5,16). Nevertheless, the heterogeneity of cells did not affect the stability of the cell line among the passages, because neither the growth curve nor the polypeptidic profile was modified. From cultures begun previously and as a result of successive selections, other cell lines with different predominant cell types are being developed. As the potato tuber moth is representative of a group of potato crop pests, all of these cell cultures could be of general interest.

The susceptibility of the cell line to infection was tested against several viruses. The PTM GV did not multiply, confirming the difficulty of multiplying GV *in vitro* (4,7). On the other hand, the nuclear polyhedrosis viruses (NPV) are known for their great ability to multiply *in vitro* and their numerous hosts (8). *Pectinophora gossypiella* (17), a cotton pest in Egypt, is susceptible to the more commonly used virus, Ac NPV, but *P. operculella* larvae were not. We found that Ac NPV could multiply in ORS-Pop-93 cell line, producing quantities of polyhedra. The number of cells which could be infected was relatively low (5%), indicating that only a few populations of the cell line were susceptible to the virus. These populations could be cloned to increase the susceptibility of the cells. The multiplication of the Ac NPV obtained *in vitro* represents a new focus of interest, because of the possibility of adapting it to *P. operculella* larvae (2).

Establishing these cultures will facilitate studies on insect biology, cell metabolism, and means of action against the PTM.

Acknowledgments

We thank H. El Bolbol for his technical assistance and the researchers of the International Potato Center (IPC).

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