A Continuous Cell Line from Pupal Ovaries of the Common Cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae)

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A continuously growing cell line was obtained from the culture of the pupal ovaries of the common cutworm, *Spodoptera litura*. The primary culture was initiated in MGM-450 medium fortified with 5% fetal bovine serum (FBS) and 3% *Antheraea pernyi* hemolymph heated at 60°C for 30 min. However, the cell line could grow in the MGM-450 medium with 10% FBS or in the MM medium with 3% FBS. The population doubling time was about 43 h in either medium at 25°C. The cells could be stored in the culture medium containing 10% glycerol at –100°C. The karyotype was typical to lepidopteran cell lines. The cell line was susceptible to *S. litura* nuclear polyhedrosis virus. The cell line could be distinguished from other cell lines derived from other insect species by comparison of the isozyme patterns of isocitrate dehydrogenase, malic enzyme, phosphoglucose isomerase and phosphoglucomutase. The cell line was designated as TUAT-SpLi-221.

Key words: insect cell culture, new cell line, continuous cell line, *Spodoptera litura*

INTRODUCTION

The common cutworm *Spodoptera litura* is an important pest of various crops. Biological control of this pest with nuclear polyhedrosis virus of *S. litura* (Sl-NPV) has been exploited (OKADA, 1977). Although a very economical methods for large-scale production of Sl-NPV by the use of host insects have been developed, it nevertheless may be worthwhile to get continuous cell lines of this species for the future development of industrial-scale production. In addition, it may provide a useful tool for the study of the virus as well as of insect cell physiology. Several cell lines have been established from the insects of the same genus; *S. frugiperda* (VAUGHN et al., 1977; LYNN and OBERLANDER, 1983), *S. litolaris* (HILWIG and EIPEL, 1978/79), *S. exigua* (GETERNTER and FEDERICI, 1986) and *S. ornithogalle* (QUHOU et al., 1984). However, these cell lines are said to be rather insensitive to Sl-NPV tested thus far. I therefore attempted to culture cells from *S. litura* and got a continuously growing cell line from pupal ovaries.

MATERIALS AND METHODS

Pupae of *S. litura* were obtained by rearing the larvae on an artificial diet (OKADA, 1977). They were sexed and only female pupae of about four-days-old were used. The pupae were surface-sterilized by submersion in 70% ethyl alcohol for 5 min. The alcohol

attached to the surface of the pupae was wiped off, and the pupae were dissected on a tray under a dissecting microscope. Ovaries taken from five pupae were pooled. They were washed in CARLSON's solution (CARLSON, 1946), and adherant tissues were removed. The cleaned ovaries were transferred into a small amount of culture medium, and cut into small pieces of about 1 mm. The fragments of ovaries were transferred into a culture vessel with the culture medium and the vessel was sealed. The culture medium used was MGM-450 medium (MITSUHASHI and INOUE, 1988) containing 5% fetal bovine serum (FBS), and 3% *Antheraea pernyi* hemolymph (APH) heated at 60°C for 30 min. The heat-treated *A. pernyi* hemolymph was a gift of Dr. T.D.C. GRACE (formerly Division of Entomology, CSIRO, Canberra, Australia). The vessels used were either small glass bottles (MA-12; Flat Co., Nagareyama, Chiba, Japan) or plastic Petri dishes (Falcon-Primaria; Becton Dickinson, Lincoln Park, NJ, U.S.A.). The cultures were maintained at 25°C by renewing half of the medium once or twice a month depending on the conditions of the cultures.

In order to draw growth curves, cells were counted at 0, 4th, and 7th days of the cultures. Since the cells attached to the bottom of the culture vessels, the cells in 10 squares of a grid ocular micrometer were counted at five places selected randomly in a culture vessel. The area of the 10 squares was 0.1 mm². From the number of the cells per unit area, whole area of the bottom of the culture vessel and the volume of the culture medium, the density of the cells and number of cells per ml was calculated. The cultures for the measurement were triplicated.

Karyotype of the obtained cell line was examined as follows. At the logarithmic phase of a culture, colchicin was added to the culture medium so as to give the final concentration of 5×10^{-4} M. The culture was kept for 3 h at 25°C. Then, the cells were detached by pipetting, and the resulting cell suspension was centrifuged at 1,500 r.p.m. for 5 min. The supernatant was discarded, the cells were dispersed in 7.0 ml of 0.58% KCl, and the suspension was kept for 15 min at room temperature. The cells were collected by centrifugation at 1,500 r.p.m. for 5 min. The cell pellet was dispersed in 1.0 ml of 0.58% KCl and an equal part of CARNOY's acetic acid-alcohol fixative (acetic acid + methanol = 1 : 3) was added. After gentle mixing, 5.0 ml of CARNOY's fixative was added. It was left for 15 min at room temperature, and then the liquid was replaced with the CARNOY's fixative by centrifugation (1,500 r.p.m., 5 min). After another 15 min, the cells were collected by centrifugation (1,500 r.p.m., 5 min), and the pellet was resuspended in a small amount (0.5–1.0 ml) of the CARNOY's fixative. A drop of the cell suspension was placed on a cleaned cover glass, and air-dried. The coverglass-attached cells were stained with 1/10 diluted Giemsa staining fluid for 30 min, and differentiated in 95% methanol.

Isozyme patterns were examined by the use of AuthentiKit (Corning, Walpole, MA, U.S.A.). For preparation of enzyme solution, about 10^7 cells were harvested. The cells were washed once in CARLSON's solution by means of centrifugation (1,500 r.p.m., 5 min). The cell pellet was added to 0.1 ml cell extraction buffer of the AuthentiKit, and left for 15 min in ice. Then the cells were disintegrated by pipetting. After centrifugation (3,000 r.p.m., 15 min), the supernatant had 0.1 ml enzyme stabilization buffer of the AuthentiKit added. The enzyme solution was kept at –20°C until use. When the enzyme activity was found too weak, the solution was concentrated by putting a few pieces of Lyphogel (Gelman Science, Ann Arbor, MI, U.S.A.) into the solution. Electrophoresis was run at 165 V for 25 min according to the instruction of the AuthentiKit. After electrophoresis, the agar film was wetted with the respective enzyme substrate solution, and incubated at 37°C for 30 min. Then the film was washed in distilled water and air-dried.

for recording the stained bands and for preservation. For comparison, the isozyme patterns of the following lepidopteran cell lines currently cultured in my laboratory were also examined: NIAS-MaBr-92 (MITSUHASHI and SHOZAWA, 1985) derived from the larval hemocytes of the cabbage armyworm; *Mamestra brassicae*; NIAS-PX-58 (MITSUHASHI, 1973) derived from the pupal ovaries of the swallow tail, *Papilio xuthus*; NIAS-MaBr-85 (MITSUHASHI, 1981) derived from the larval fat bodies of *M. brassicae*; NIAS-SpSe-1 (MITSUHASHI, 1984) derived from the larval fat bodies of an arctiid moth, *Spilarctia seriatopunctata*; and FRI-SpIm-1229 (MITSUHASHI and INOUE, 1988) derived from the larval fat bodies of the mulberry tiger moth, *Spilosoma imparilis*.

The inoculum of SI-NPV was prepared from partially purified polyhedra. Diseased larvae were homogenized with CARLSON's solution, and polyhedra were isolated by the three cycles of low (1,000 r.p.m., 5 min) and high (4,000 r.p.m., 15 min) speed centrifugations. The polyhedra were dissolved in 0.5% aqueous sodium carbonate, and the solution was sterilized by passing it through a membrane filter (0.2 μm pore size). In order to inoculate a culture, a drop of the sterilized virus solution was added to the culture medium.

RESULTS AND DISCUSSION

1. Primary cultures

Cell migration occurred within 24 h after the culture was set up. The migrated cells were either fibroblastic or hemocyte-like. They distributed themselves densely around explants at early stages, but gradually moved to the surrounding area and finally distributed over almost the entire area of the vessel (Fig. 1). Epithelial cell-like cells were seldom seen. The rate of mitoses in the migrated cells was initially very low, but gradually increased. In the fastest case, subculture became possible a month after the initiation of the culture.

Fig. 1. The cells in the primary culture. The bar indicates 200 μm .

The growth of the cells in the primary cultures resembled that of the pupal ovaries of *M. brassicae* (MITSUHASHI, 1976).

2. Subcultures

Among 30 primary cultures prepared, 18 became possible to subculture, however, only one culture remained as a continuous subculture. The others terminated either by microbial contamination or deterioration. The first subculture of the continuous line was made on the 223rd day of the cultures. At the confluent state of this culture, most cells attached to the substrate, however, the cells could be detached from the substrate by pipetting. The resulting cell suspension was divided into two vessels and an equal amount of fresh medium was added. Thereafter subculturing was made in the same way (1 : 2 split ratio) until the 100th passage. The growth of cells at early passages was slow, and the intervals between successive subculturing were quite long. This cell growth became consistent after the 20th passage. This cell line took 34 months to pass 100 times. The continuously growing cell line that was obtained was designated as TUAT-SpLi-221.

3. Characteristics of TUAT-SpLi-221 cell line

Morphology. The cell line was a mixed population, and therefore consisted of cells with various shapes and sizes (Fig. 2). Majority of the cells, however, were spherical. The volume of the cells also fluctuated considerably.

Cellular adhesiveness. The cells showed a tendency to attach to the bottom of the culture vessels. However, the attachment was not very firm, and the cells were easily detached from the substrate by flushing the culture medium onto them, from a pipette. In the course of establishing continuous cell lines from the pupal ovaries of *M. brassicae* (MITSUHASHI, 1977) and *P. xuthus* (MITSUHASHI, 1973), the cells attached to substrate initially

Fig. 2. Mixed cell population of TUAT-SpLi-221 cell line showing various shaped cells. The bar indicates 200 μ m.

Fig. 3. Growth curves of TUAT-SpLi-221 cell line at 25°C. Circles: MGM-450 medium with 10% FBS, triangles: MM medium with 3% FBS. Vertical lines are standard deviation.

Fig. 4. A typical metaphase plate of TUAT-SpLi-221 cell line. The bar indicates 10 μ m.

became floating cells after repeating subcultures many times. The TUAT-SpLi-221 cells, however, did not change their property to attach substrate. When these cells reached confluent state, the contact inhibition that is commonly observed in non-cancer mammalian cell cultures did not occur. Overgrown cells piled up, or detached from the substrate and floated in the medium.

Growth media and growth rate. Although the primary culture was initiated with MGM-450 medium containing 5% FBS and 3% APH, later it was found that the cells could grow in the MGM-450 medium containing only 10% FBS. After the growth rate of the cells became consistent, the cells could be adapted to MM medium (MITSUHASHI and MARAMOROSCH, 1964) with 3% FBS. Attempt to culture the cells in serum-free media has

Table 1. Electrophoretic mobility of isozymes^a

Cell lines ^b	Enzymes ^c			
	ICD	ME	PGI	PGM
TUAT-SpLi-221	10-14 16-20	13-15	12-14	16-18
NIAS-MaBr-85	6-12 14-17	8-10	8-11	20-23
NIAS-MaBr-92	6-12 14-17	8-10	8-11	20-23
FRI-SpIm-1229	7- 8 13-15	14-16	3- 5	10-12
NIAS-SpSe-1	4- 8	9-10	11-13	19-20
NIAS-PX-58	6- 9	23-24	7-10	16-18

^a Isozyme mobility was expressed as distance (mm) from the origin.

^b See Materials and Methods for description of cell lines.

^c ICD: isocitrate dehydrogenase, ME: malic enzyme, PGI: phosphoglucose isomerase, PGM: phosphoglucomutase.

Fig. 5. *S. litura* NPV polyhedra formed in a nucleus of the infected TUAT-SpLi-221 cells. The bar indicates 10 μ m.

not been successful so far.

Growth curves in the MM medium with 3% FBS and in the MGM-450 medium with 10% FBS at 25°C are shown in Fig. 3. The population doubling time in both media was estimated to be 43 h.

Karyotype. There was great diversity in the number of chromosomes. Majority of

cells showed metaphase plate like Fig. 4. Each chromosome was very small and seemed to have a diffuse centromere. It was a typical metaphase plate for lepidopteran cell lines currently known (MITSUHASHI, 1981, 1983, 1984; MITSUHASHI and SHOZAWA, 1985; MITSUHASHI and INOUE, 1988; INOUE and MITSUHASHI, 1988). The average chromosome number was estimated to be around 130, and the cell population was considered to consist of a near-tetraploid cell population.

Isozyme analysis. By the use of AuthentiKit, isozyme patterns of malic enzyme (ME), isocitrate dehydrogenase (ICD), phosphoglucumutase (PGM), and phosphoglucose isomerase (PGI) were compared with those of some other lepidopteran cell lines that were culturing in my laboratory. The mobilities of the isozymes are summarized in Table 1. The pattern of ME of TUAT-SpLi-221 somewhat resembled that of FRI-SpIm-1229, that of PGI to that of NIAS-SpSe-1 and that of PGM to that of NIAS-PX-58. The pattern of ICD of the new cell line was different from those of other cell lines. It was possible to distinguish the TUAT-SpLi-221 cell line from other cell lines by examining isozyme patterns of these four enzymes.

Storage. TUAT-SpLi-221 cells could be stored at -100°C by adding glycerol to the cell suspension so as to make a final concentration of 10%.

Susceptibility to viruses. TUAT-SpLi-221 cells were susceptible to the SI-NPV (Fig. 5). The infected cells died without bursting. Consequently, polyhedra were not liberated into the culture medium, and remained in the nuclei of the dead cells. The ratio of polyhedra-forming cells was about 30% at the early stage of subculture, but it decreased with the advance of passage. Some sublines lost their susceptibility to the virus at about the 50th passage. However, cells frozen during early passages retained their susceptibility.

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