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A Continuously Growing Cell Line from Larval Hemocytes of *Malacosoma neustria testacea*

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Abstract Larval hemocytes of *Malacosoma neustria testacea* (Lepidoptera, Lasiocampidae) were cultured in MGM-450 medium added with 5% fetal bovine serum (FBS) and 3% *Antheraea pernyi* hemolymph. Out of thirty primary cultures set up, a continuous cell line was obtained, and designated as FRI-MntH-520A. Cells of this cell line could grow in MGM-450 medium fortified with 10% fetal bovine serum alone, and the population doubling time was 6.93 days. Spherical cells were predominant, but sometimes odd-shaped cells appeared. Karyotype of the cell line was typical of lepidopteran cell lines and consisted of numerous small chromosomes. The cell line could be discriminated from other lepidopteran cell lines by comparing the patterns of isozymes of several enzymes. *M. neustria testacea* nuclear polyhedrosis virus (NPV) infected the cell line, whereas *Autographa californica*, *Malacosoma distria*, and *Lambdina fiscellaria somniaria* NPVs did not.

Key words: *Malacosoma neustria testacea*; insect cell line; new cell line; lepidopteran cell line.

Introduction

Malacosoma neustria testacea is a pest of various fruit trees. In order to reduce insecticidal residue on the surface of fruits, the use of insecticides in orchards should be minimized. Then, the importance of exploitation of alternative control methods has increased. Among non-chemical control methods of this pest, the microbial control using *M. neustria testacea* nuclear polyhedrosis virus (Mnt-NPV) seems promissive, because NPV is non-infective to mammals including human, stable in the field on accout of its protective polyhedra, and highly specific (KUNIMI, 1988, 1990). In this context, the present study was undertaken to obtain a cell line permissive for replication of Mnt-NPV. After several larval tissues were cultured preliminarily, hemocytes were chosen to establish cell lines, because of easiness of setting up their primary cultures. As a result, a continuous cell line was obtained. In this report, the process of culturing growing cells, and some characteristics of the established cell line are described.

Materials and Methods

Final instar larvae of *M. neustria testacea* were collected from cherry trees,

Prunus yedoensis, on the campus of the Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan, in May 1987. The larvae were surface-sterilized by submersion in 70% ethyl alcohol for 5 min. The tip of a proleg was punctured with a needle for bleeding, and the blood collected from five larvae were mixed with 10 ml of Carlson's solution (CARLSON, 1946) in a conical centrifuge tube. The hemocytes were centrifuged at 150 g for 3 min and mixed with 10 ml of Carlson's solution. This procedure was repeated three times to wash the hemocytes thoroughly. The final cell pellet was suspended in a culture medium and transferred into a culture bottle. The medium used was MGM-450 (MITSUHASHI & INOUE, 1988) containing 5% fetal bovine serum (FBS) and 3% *Antheraea pernyi* hemolymph heated at 60°C for 30 min. The FBS was purchased from GIBCO (Grand Island, New York, U.S.A.), and the hemolymph of *A. pernyi* was the gift of Dr. T.D.C. Grace, (formerly Division of Entomology, CSIRO, Canberra, Australia), which had been stored at −20°C since 1963. For primary cultures, small glass bottles with a bottom area of 10 cm^2 (MA-12, Flat K.K., Nagareyama, Chiba, Japan) were used. The cultures were maintained at 25°C under darkness, and a half of the medium was renewed every 7 to 30 days depending upon the condition of living cells.

When cells multiplied enough to be subcultured, the cells attached to the bottom of the culture bottles were detached by vigorous flushing of the culture medium from a pasteur pipette. This made most cells detached, and the rest still attached to the vessels were discarded. The suspended cells were divided and dispensed into several vessels. This method did not seem to have selected any particular cell type since the cell population did not alter in morphological characteristics even after many passages. Cell counts were made at 0, 4 and 7 days of the cultivation by means of Thoma's hemocytometers. Population doubling time (PDT) was calculated from these data according to the following Hayflick's equation,

$$PDT = (t_2 - t_1) \log 2 / (\log N_2 - \log N_1)$$

where N_1 and N_2 are the numbers of cells at selected times t_1 and t_2, repectively.

Karyotype and isozyme analyses of the obtained cell line were made by the same method described previously (MITSUHASHI, 1995).

For the examination of susceptibility to nuclear polyhedrosis viruses (NPV), Mnt-NPV was obtained from Dr. M. Shimazu (Laboratory of Insect Pathology, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, Japan). *Autographa californica* NPV (Ac-NPV) was obtained from Dr. F. Hink (The Ohio State University, Columbus, Ohio, U.S.A.). *Malacosoma distria* NPV (Md-NPV) and *Lambdina fiscellaria somniaria* NPV (Lfs-NPV) were obtained from Dr. S. S. Sohi, (Forest Pest Management Institute, Sault Ste. Marie, Ontario, Canada). The Mnt-NPV and Lfs-NPV inocula were prepared by

dissolving partially purified polyhedra in 0.1% sodium carbonate, and the solution was sterilized through a membrane filter of 0.2 μm pore size (Sartorius GmbH, Göttingen, Germany). The inocula of Ac-NPV, and Md-NPV were supernatant of infected cell cultures containing free viruses. Inoculation to the cells was conducted by adding one drop of the virus suspension into the healthy cell cultures.

Results and Discussion

1. Process of cell growth

Primary cultures: When hemocytes were span down before dispersion into culture medium, some hemocytes formed aggregates, which were brought into culture vessels as cell masses of various sizes. Most hemocytes and the cell masses attached themselves to the bottom of the culture vessels. However, spherule cells, oenocytoids and some prohemocytes did not adhere to the vessels, and remained freely suspended. These suspending cells were eliminated from the cultures when the culture medium was renewed. Plasmatocytes and granular hemocytes spread thin cytoplasmic processes and became very flat (Fig. 1). With the advance of culturing, cells migrated from the cell masses to their surroundings. Cell multiplication occurred mostly in the cells that had migrated from the cell masses. In some parts, the multiplied cells formed cell sheets. As the culture became old, cells changed their shapes and identification of their types became very difficult.

Twelve out of thirty cultures showed promissive cell growth. In the latter,

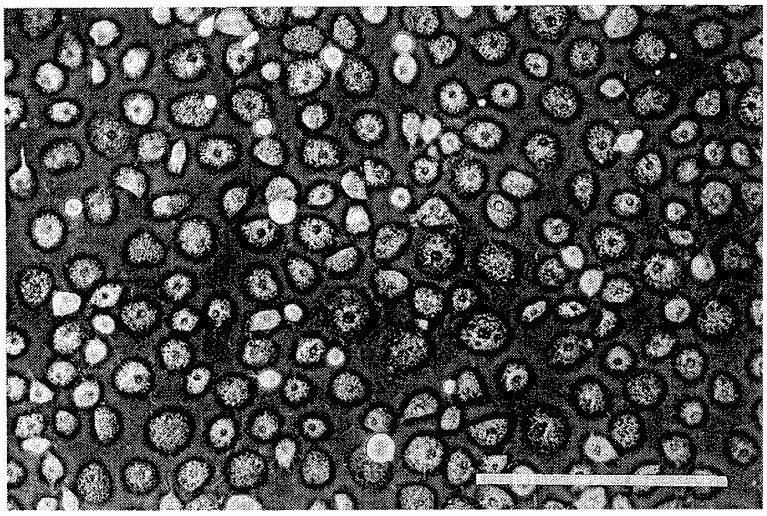

Fig. 1. Primary culture of larval hemocytes of *Malacosoma neustria testacea.* Flat cells are plasmatocytes and granular hemocytes. White refrective cells are prohemocytes. Line indicates 100 μm.

various types of cells were observed, although their original hemocyte type could not be identified, because they often changed their morphology during culturing. In the growing cultures, many patches of cells were formed on the bottom of the culture bottle. They gradually became larger, and some fused to each other, covering a wide area on the bottom of the culture bottle.

Subcultures: Out of the twelve primary cultures showing promissive cell growth, four cultures could be subcultured. Most cells were detached from the substrate by vigorous flushing of culture medium. The resulting cell suspension was dispensed to larger vessels, and an appropriate amount of fresh medium was added to each vessel. The dispersed cells again attached themselves to the substrate and continued to multiply in the same manner as in the primary cultures. After the 2nd passage, subcultures were made with 1 : 2 split ratio. Cell growth was extremely slow during the early passages but it became gradually faster. Also in the early phase of subculturing, cell growth was not stable, resulting irregular intervals of subcultures. Cell growth, became more stable with the advance of the culture, and subculture became possible at regular intervals. These cells grew well in the MGM-450 medium fortified with 10% FBS alone without addition of *A. pernyi* hemolymph. Only one culture maintained its active proliferation during subculturing. Consequently a cell line was obtained from that culture, and designated as FRI-MntH-520A (Fig. 2). The other cultures were terminated either by unappropriate maintenance or by accidental microbial contamination.

The patterns of cell growth in the primary cultures and subcultures were similar to those reported for other lepidopteran cell lines, especially those for hemocytes (MITSUHASHI, 1967; MITSUHASHI & SHOZAWA, 1985). However,

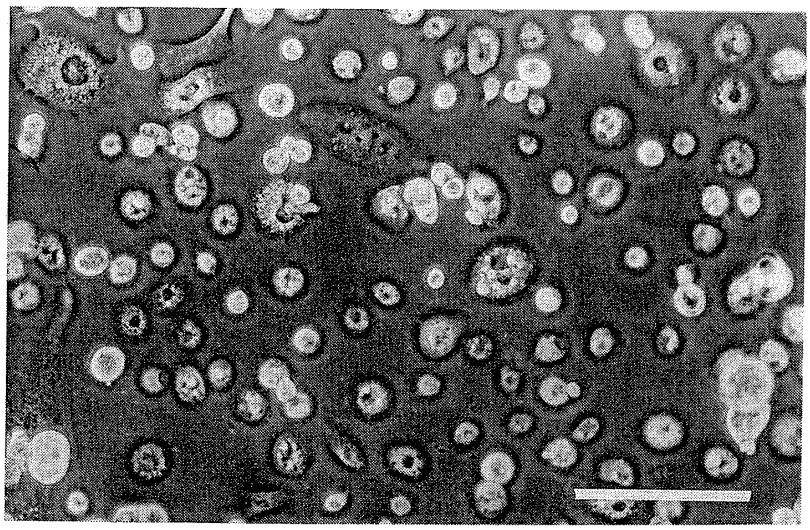

Fig. 2. Cells of the FRI-MntH-520A cell line. Line indicates 100 μm.

cell morphology was more diverse in the present cell line of *M. neustria testacea.*

2. Characterization of the cell line

Growth rate: The pattern of growth in MGM-450 medium with 10% FBS is shown in Fig. 3. Population doubling time was 6.93 days. The growth rate was extremely low compared to those of *Chilo suppressalis* and *Mamestra brassicae* hemocyte cell lines (MITSUHASHI, 1967; MITSUHASHI & SHOZAWA, 1985).

Morphology: Most cells attached themselves to the bottom of the culture vessels but some floating cells often appeared when the cell density became high. Spherical cells were predominant with occasional appearance of odd-shaped cells. The latter consisted of elongated large oenocytoid-like cells (Fig. 4A), ciliate-like cells (Fig. 4B), cells having spine-like cytoplasmic projections (Fig. 4C), and cells resembling sclerocytes of Porifera (Fig. 4D). Such odd-shaped cells often appeared when the culturing conditions were good and cells were multiplying vigorously, but the factor(s) inducing such transconfiguration is unknown. This type of morphological changes of cells has not been reported in any other insect cell line.

Karyotype: The karyotype of FRI-MntH-520A cell line was similar to those of other lepidopteran cell lines. Chromosomes were numerous and small in size (Fig. 5). It was, therefore, difficult to count the number of chromosomes accurately, but most cells appeared to be tetraploid. The chromosomes seemed to have difuse centromere, since no constriction was observed in any chromo-

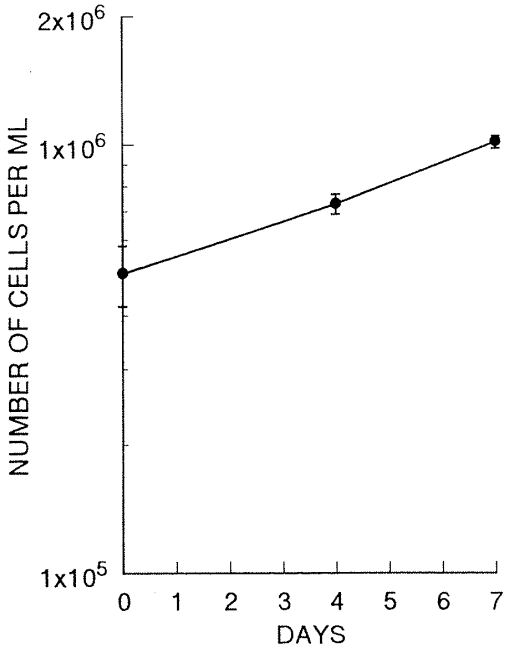

Fig. 3. Growth of the FRI-MntH-520A cell line in MGM-450 medium with 10% FBS at 25°C.

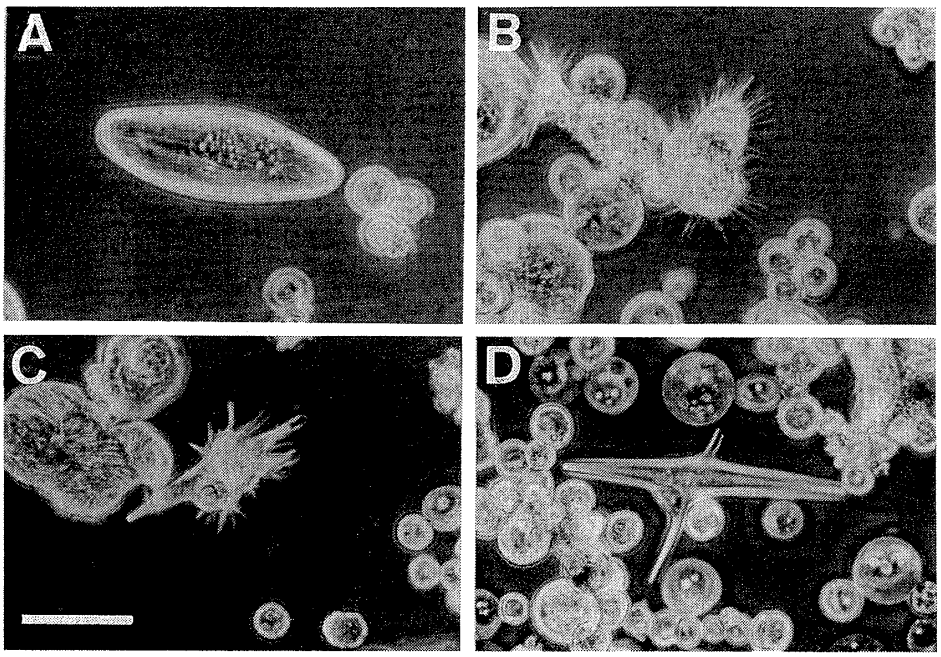

Fig. 4. Morphology of odd-shaped cells. A: an oenocytoid-like cell; B: ciliate-like cells; C: a cell having spine-like cytoplasmic projections; D: a cell resembling sclerocites of Porifera. Line indicates 20 μm.

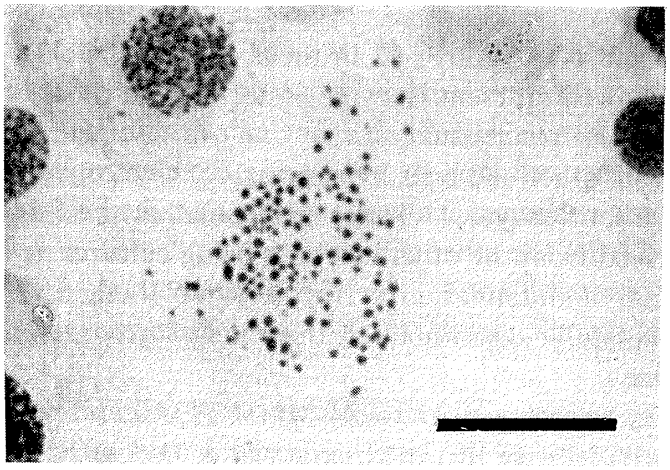

Fig. 5. A typical metaphase plate of a FRI-MntH-520A cell. Line indicates 20 μm.

some.

Isozyme analysis: Isozymes of isocitric dehydrogenase (ICD), malic enzyme (ME), phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM), which are known to be useful for discrimination of different insect cell lines were examined by means of the AuthentiKit (Corning, E. Walepole, U.S.A.). The FRI-MntH-520A was similar to NIAS-PX-58 in isozyme patterns of ME, to NIAS-PX-58 and NIAS-SpSe-1 in those of ICD, and to NIAS-PX-58,

Table 1. Electrophoretic mobility of isozymes.[a]

Cell lines[b]	Enzymes[c]			
	ICD	ME	PGM	PGI
FRI-MntH-520A	5– 8	23–25	9–12 20–23	7–10
FRI-SpIm-1229	7– 8 13–15	14–16	10–12	3– 5
NIAS-SpSe-1	4– 8	9–10	19–20	11–13
NIAS-MaBr-85	6–12 14–17	8–10	20–23	8–11
NIAS-MaBr-92	6–12 14–17	8–10	20–23	8–11
TUAT-SpLi-221	10–14 16–20	13–15	16–18	12–14
NIAS-PX-58	6– 9	23–24	16–18	7–10

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

[b] FRI-MntH-520A, *Malacosoma neustria testacea* cell line; FRI-SpIm-1229, *Spilosoma imparilis* cell line; NIAS-SpSe-1, *Spilosoma seriatopunctata* cell line; NIAS-MaBr-85, *Mamestra brassicae* fat body cell line; NIAS-MaBr-92, *M. brassicae* hemocyte cell line; TUAT-SpLi-221, *Spodoptera litura* cell line; NIAS-PX-58, *Papilio xuthus* cell line.

[c] ICD, isocitrate dehydrogenase; ME, malic enzyme; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase.

NIAS-MaBr-85 and NIAS-MaBr-92 in those of PGI (Table 1). However, the other cell lines showed different isozyme patterns from those of the FRI-MntH-520A cell line. These results suggest that one can discriminate the FRI-MntH-520A cell line from other cell lines by comparing the electrophoretic patterns of isozymes of these four enzymes. This also means that the FRI-MntH-520A cell line was not derived from the other cell lines being cultured in my laboratory by accidental cellular contamination. By the same method, LYNN and HUNG (1991) has successfully discriminated their new *Tricogramma* cell lines from their host cell lines.

Susceptibility to viruses: The FRI-MntH-520A cell line was susceptible to Mnt-NPV. The polyhedra of the virus appeared 3 days after inoculation, and increased in number (Fig. 6). The percentage of polyhedra producing cells was about 30%. However, it may be increased by cloning the cell line , since HARA *et al.* (1995) reported that a cell population became highly susceptible to *Spodoptera exigua* NPV after cloning. The Ac-NPV, Md-NPV and Lfs-NPV failed to infect cells of the FRI-MntH-520A cell line.

In conclusion, a new continuously growing cell line was obtained from the hemocytes of *M. neustria testacea.* The cell line established was similar to other lepidopteran cell lines in many respects. A prominent characteristic of this cell line was occasional appearance of odd-shaped cells. This new cell line was

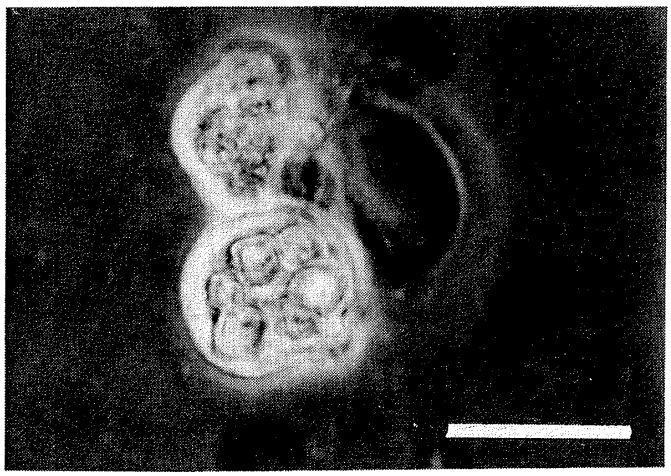

Fig. 6. Polyhera formed in nucli of FRI-MntH-520A cells after infection with Mnt-NPV. Line indicates 20 μm.

susceptible to Mnt-NPV, and it may be therefore useful for large scale production of Mnt-NPV in the future.

References

CARLSON, J. G. 1946. Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitoses. *Biol. Bull.*, **90**: 109–121.

HARA, K., M. FUNAKOSHI & T. KAWARABATA, 1995. A cloned cell line of *Spodoptera exigua* has a highly increased susceptibility to the *Spodoptera exigua* nuclear polyhedrosis virus. *Can. J. Microbiol.*, **41**: 1111–1116.

KUNIMI, Y., 1988. Safety of microbial insecticides. Bio Industry **5**: 77–86. (In Japanese.)

———— 1990. Current status and prospects of microbial insecticides. Plant Protection **4**:, 377–382. (In Japanese.)

LYNN, D. & A. C. F. HUNG, 1991. Development of continuous cell lines from the egg parasitoids *Trichogramma confusum* and *T. exiguum*. *Arch. Insect Biochem. Physiol.*, **18**: 99–104.

MITSUHASHI, J., 1967. Establishment of an insect cell strain persistently infected with an insect virus. *Nature*, **215**: 863–864.

———— 1995. A continuous cell line from pupal ovaries of the common cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). *Appl. Entomol. Zool.*, **30**: 75–82.

———— & H. INOUE, 1988. Obtainment of a continuous cell line from the larval fat bodies of the mulberry tiger moth, *Spilosoma imparilis* (Lepidoptera: Arctiidae). *Appl. Entomol. Zool.*, **23**: 488–490.

———— & A. SHOZAWA, 1985. Continuous cell lines from larval hemocytes of the cabbage armyworm, *Mamestra brassicae*. *Develop., Growth & Differ.*, **27**: 599–606.

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