Characterization of a New Continuous Cell Line from Silkworm (*Bombyx mori*) Embryos

Hajime INOUE and Jun MITSUHASHI¹

Sericultural Experiment Station, Tsukuba, Ibaraki 305, Japan

¹ *Forestry and Forest Products Research Institute, Kukizaki, Inashiki, Ibaraki 305, Japan*

(Received March 11, 1987)

A continuous cell line, designated as SES-BoMo-15A, was obtained from embryos of silkworm, *Bombyx mori*, strain "Kuroko". Cells were grown in MGM-448 medium with 10% fetal bovine serum (FBS) as well as in MM medium with 3% FBS. Cells were mostly spherical in shape and contained many granules. They multiplied in a suspended state, and the population doubling time was about 3 days in the MGM-448 medium at 25°C. Most cells had about 100 microchromosomes. The cell line was distinguishable from other lepidopteran cell lines by the isozyme patterns of some enzymes. The line was susceptible to *B. mori* nuclear polyhedrosis virus, *B. mori* cytoplasmic polyhedrosis virus and *Chilo* iridescent virus.

INTRODUCTION

Since TRAGER (1935) obtained growing cells in primary cultures of *Bombyx mori* ovarioles, several investigators have cultured *B. mori* cells (GRACE, 1967; QUIOT, 1982; INOUE and MITSUHASHI, 1984; NINAKI et al., 1985). In a previous paper (INOUE and MITSUHASHI, 1984), we reported the primary cultures of cells originated from the embryos of silkworm "Kuroko" strain. These cells, containing large granules in the cytoplasm showed active cell proliferation and have been subcultured continuously, though their growth up to 30th passage was comparatively slow, i.e. population doubling time was about four days. In this report, we describe some characteristics of the cell line, such as subculture history, morphology, growth, karyotype, amino acid metabolism, susceptibility to viruses, enzyme activities and isozyme pattern.

MATERIALS AND METHODS

Subcultures. Until 100 passages, subcultures were made with the split ratio of 1:2. One ml of the cell suspension, of which cell density was about 4×10^5 /ml, was transferred to a new TD-7 glass flask and was added with two ml of culture medium. MGM-448 medium enriched with 10% fetal bovine serum (FBS) (MITSUHASHI, 1984) and MM medium enriched with 3% FBS (MITSUHASHI, 1982 a) was used to maintain the cell line.

Cell enumeration and measurement. Cell counts were made 0, 3 and 7 days after subcultures. Each time, 0.25 ml of cell suspension was sampled, diluted fifty times with Isotone (an electrolyte solution for a Coulter counter), and applied to Coulter counter Model ZBI. The counting was repeated 5 times with one sample and three

replicates were made. The cell volume was measured with Coulter chanelizer II connected to Coulter counter ZBI. Amplification, aperture current, lower threshold and upper threshold were set as 8, 0.5, 10 and 100, respectively.

Karyotype analysis. The method used was the same as in a previous report (TAKAHASHI et al., 1980). The cells were treated with 5×10^{-6} M colchicin for 5 hr. The harvested cells were spun down at 1,500 rpm. They were then suspended in 0.6% potassium chloride and kept there for 15 min at room temperature. The cells were again spun down, and suspended in 1 ml of 0.6% potassium chloride. To this cell suspension 5 ml of fixative (methyl alcohol:acetic acid=3:1) was added and mixed gently. It was kept for 15 min at room temperature, then the fluid was replaced with pure fixative by centrifugation. Fixation was continued for another 15 min, then the cells were spun down. They were resuspended in an appropriate amount of the fixative and dropped onto a clean slide glass or cover glass. After air-drying, the cells were stained with Giemsa (diluted ten times with phosphate buffer of pH 6.4) for ten min, differentiated with ethyl alcohol and mounted with Bioleit.

Amino acid analyses. Three replicates of culture with the MGM-448 medium were maintained at 25°C for 7 days. Before and after the culture, an aliquot of the medium was sampled from each culture, added with an equal volume of 1% sulfosalicylic acid, and the resulting precipitates were removed by centrifugation. The supernatant was diluted with lithium citrate buffer (pH 2.9), and analysed with an ATTO model MLC 203 automatic amino acid analyser.

Virus inoculation. The virus inocula were prepared as follows: 1) the hemolymph of silkworm larvae infected with a nuclear polyhedrosis virus was passed through a 450 nm millipore filter, 2) purified *Bombyx mori* cytoplasmic polyhedra (10^8 polyhedra/ml) were dissolved with 0.05 M Na_2CO_3 -0.05 M NaCl solution for 15 min at room temperature and then the supernatant was collected after a 3,500 rpm centrifugation for 10 min, 3) the medium used for culturing a cell line of *Mamestra brassicae*, NIAS-MB-32, infected with a *Chilo* iridescent virus was centrifuged for 10 min at 3,500 rpm and the supernatant was collected. Several drops of each inoculum were added by a Pasteur pipette into 4 ml medium in a cell-culturing flask (Falcon #3013), and the culture was incubated at 25°C.

Enzyme activity. About 3×10^7 cells were harvested and these were washed with Carlson's fluid (CARLSON, 1946) three times. The final cell pellet was added with 0.5 ml Carlson's fluid and sonicated. An APIZYM kit (API System S.A., Montalieu, France) was used to test enzyme activity.

Analyses of isozyme patterns. Isozyme patterns of isocitrate dehydrogenase (ICD), malic enzyme (ME), phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM) were examined. For comparison, isozyme patterns of four other lepidopteran cell lines, NIAS-SpSe-1 (originated from *Spilarctia seriatopunctata* fat bodies), NIAS-MaBr-92 (originated from *Mamestra brassicae* hemocytes), NIAS-PX-64 (originated from *Papilio xuthus* ovaries) and FRI-SpLi-221 (originated from *Spodoptera litura* ovaries), were also examined. About 3×10^7 cells were harvested. These were washed in Carlson's fluid three times by centrifugation at 1,500 rpm for 5 min. The final cell pellet had a volume of about 0.1 ml. The supernatant was discarded and 0.1 ml of cell extraction buffer of Authentikit (Corning, E. Walepole, U.S.A.) was added to the pellet. The cells were sonicated and the resultant suspension of cell fragments was centrifuged at 15,000 rpm for 30 min. The supernatant was added with an equal volume of Authentikit enzyme

stabilization buffer and stored at -20°C until use. The frozen material was thawed and $1\ \mu\text{l}$ of it was applied to Authentikit agarose gel. The electrophoresis was run at 160 V for 25 min. After electrophoresis, the agarose gel was stained with respective enzyme substrate reagents at 37°C for 30 min.

RESULTS

History of subculture

A record of passage for 100 times is given in Fig. 1. The passage of cells was possible only with long intervals during the first few passages. The growth of the cells gradually improved and became constant after 20 passages. Then, we considered that the cell line had entered a permanent growth phase, and called it SES-BoMo-15A. The cells had passed in MGM-448 medium, however, this was changed MM medium containing 3% fetal bovine serum (FBS) (MITSUHASHI, 1982 a) after the 50th passage for part of cultures.

Morphology and growth

The cell line consisted mostly of spherical cells containing many granules. They were usually suspending in the medium although they occasionally adhered to the glass. These cells seemed to release the granules into the medium resulting in the continuous existence of many cell debris-like particles in the culture (Fig. 2). The diameter of a cell was about $15\ \mu\text{m}$ and the cell size fluctuated considerably (Fig. 3). Cells grown in MM medium darkened the medium and the cell pellet was also dark in color when spun down, while cells grown in MGM-448 medium did not change the color of the medium and the cell pellet was white.

The cells cultured in MM medium grew somewhat more slowly than those cultured in MGM-448 medium. Population doubling time of the former was more than 7 days, while that of the latter was about 3 days at 25°C (Fig. 4).

Chromosomes

The nucleus of the SES-BoMo-15A cells contained numerous microchromosomes

Fig. 1. A record of passage. Split ratio of 1:2 applied to each passage.

Fig. 2. SES-BoMo-15A cell line showing cells containing large granules and particles (arrows) in the medium. Line indicates $50\ \mu\text{m}$.

Fig. 3. Distribution of cell volume of the SES-BoMo-15A cell line. The analysis was made with a Coulter counter ZBI with a Chanelizer II.

Fig. 4. Growth curves of the SES-BoMo-15A cell line at 100th passage. The cultures were maintained at 25°C.

Fig. 5. A typical metaphase plate of a cell of the SES-BoMo-15A cell line. Line indicates 20 μ m.

Fig. 6. Distribution of chromosome number of the SES-BoMo-15A cell line.

(Fig. 5), making direct counting of chromosomes under a microscope almost impossible. The chromosomes were therefore counted on photographs of metaphase plates taken randomly. The mode of the chromosome number was around 100 (Fig. 6), perhaps showing that the cell line consisted mostly of tetraploid cells. A small peak in the frequency distribution of chromosome numbers was also found around 200, suggesting the presence of a considerable number of octaploid cells.

Amino acid metabolism

Changes of free amino acids in the MGM-448 medium were measured before and after 7 day cultivation of the cells. Most amino acids decreased in quantity to varying extents. Characteristic changes were observed in the marked decrease of glutamic acid and ammonia, and the increase of tyrosine and ornithine.

Susceptibility to virus

The cells were found to be susceptible to *B. mori* nuclear polyhedrosis virus (NPV) and cytoplasmic polyhedrosis virus (CPV). When cells cultured in MGM-448 were

Fig. 7. a: SES-BoMo-15A cells infected with *B. mori* NPV. Polyhedra are visible in the nuclei. Line indicates 10 μ m. b: a SES-BoMo-15A cell infected with *B. mori* CPV. Polyhedra are visible in the cytoplasm. Line indicates 10 μ m.

inoculated with filtered blood of NPV-infected silkworm, polyhedra appeared in the nuclei at 3 days post inoculation (Fig. 7 a). When cells cultured in MGM-448 medium were inoculated with CPV virions liberated from the polyhedra by dissolution in sodium carbonate solution, newly formed polyhedra were recognized 3 days post inoculation (Fig. 7 b). The CPV used in this experiment was a strain to produce tetragonal polyhedra, so that the formed polyhedra were clearly distinguishable from cellular granules. The cells cultured in MM medium were also susceptible to both viruses, however, few cells produced polyhedra. When the cells were inoculated with *Chilo* iridescent virus, some showed iridescence at 10 days post inoculation; however, the overall percentage of cells showing iridescence was very low.

Activities of enzyme

Analyses with the APIZYM kit revealed the strong activities of alkaline phosphatase, acid phosphatase, phosphoamidase, leucine arylamidase, β -galactosidase, and N-acetyl- β -glucosaminidase in the SES-BoMo-15A cells, while the very weak activities of esterase lipase, cystine arylamidase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase. No activity was detected in lipase, trypsin, chymotrypsin, α -galactosidase and β -glucuronidase.

Isozyme patterns

The isozyme patterns of ICD and ME of SES-BoMo-15A were clearly distinguishable from those of other cell lines, although the pattern of PGI was similar in all lines tested and the mobility of PGM of SES-BoMo-15A, NIAS-SpSe-1 and NIAS-MaBr-92 was similar (Fig. 8).

DISCUSSION

In addition to the several hitherto reported continuous cell lines of *B. mori* (GRACE, 1967; QUIOT, 1982; INOUE and MITSUHASHI, 1984; NINAKI et al., 1985), a cell line called BM-N has been used for studies on isozymes and *B. mori* NPV (TABACHNICK and KNUDSON, 1980; MAEDA, 1984). The BM-N cell is suspected to be an unpublished *Bombyx* cell line established by T.D.C. Grace (Dr. H. WATANABE, personal communication). On the cell line of S.P.C.-Bm36, NINAKI et al. (1985) obtained data showing absence of

Fig. 8. Isozyme patterns. Diagrammatic representation of the electrophoretic patterns of 4 enzymes obtained from 5 cell lines of lepidopteran insects. ICD: isocitrate dehydrogenase; ME: malic enzyme; PGI: phosphoglucose isomerase; PGM: phosphoglucomutase. 0: origin. BoMo: SES-BoMo-15A cell line from *Bombyx mori*; SpSe: NIAS-SpSe-1 cell line from *Spilarctia seriatopunctata*; MaBr: NIAS-MaBr-92 cell line from *Mamestra brassicae*; Px64: NIAS-PX-64 cell line from *Papilio xuthus*; SpLi: FRI-SpLi-221 cell line from *Spodoptera litura*.

the DNA fragments homologous to fibroin and multi-copy (BMC 1) genes and difference of the restriction map of rDNA from those of *B. mori* and *B. mandarina*.

The discrimination of insect cell lines has been done primarily with isozyme analyses using a cellulose acetate electrophoresis (BROWN and KNUDSON, 1980) or by isoelectrofocusing (McINTOSH and IGNOFFO, 1983). TABACHNICK and KNUDSON (1980) examined isozymes of 18 enzymes on 16 insect cell lines, and suggested the suitability of using isozyme patterns of ICD, ME, PGI and PGM for identification of these cell lines. In two of these four enzyme systems, ICD and ME, the SES-BoMo-15A cell line was clearly distinguishable from other lepidopteran cell lines we are culturing in the same laboratory. SATO (1985) examined enzyme activities of his eight insect cell lines using an APIZYM kit, and obtained a different reaction of β -glucuronidase and N-acetyl- β -glucosaminidase from different cell lines. The cell line FTRS-HmL45 derived from *Homona magnanima* showed strong activity of β -glucuronidase in contrast with the other lines. Four cell lines derived from *Adoxophyes* sp., *Pandemis hepaxana*, *Archippus breviplicanus* and *Hoshinoa longicellana* showed weaker activity of N-acetyl- β -glucosaminidase than the other cell lines. Of these two enzymes, the SES-BoMo-15A cell line showed weak activity of N-acetyl- β -glucosaminidase and no β -glucuronidase activity.

For amino acid metabolism, glutamine in the medium did not change very much during 7-day cultivation of the SES-BoMo-15A. This was a characteristic of this cell line because most insect cell lines usually consume a larger amount of glutamine (MITSUHASHI, 1982 b).

The diploid number of *B. mori* chromosomes has been known to be 56, however, most cells of this line established by GRACE (1967) contained many more than 100 chromosomes when examined at 15 and 18 months after the cell line was established. The SES-BoMo-15A cells also had about 100 chromosomes.

The SES-BoMo-15A cells were susceptible to BmNPV, BmCPV and CIV. The *B. mori* cell line by GRACE (1967) and the S.P.C. Bm36 cell line (QUIOT, 1982) were also susceptible to BmNPV, but the latter seemed to have greater ability to support the growth of many insect viruses, such as NPV of *Spodoptera littoralis*, *Lymantria dispar*, *Antheraea pernyi*, *Mamestra brassicae*, *Dipteropsis watersi*, *Galleria mellonella* and *Autographa californica*, CPV of *Euxoa scandens*, *Europtera punctillata*, *Cryptophlebia leucotreta* and *Earias biplaga* (RAGHOW and GRACE, 1974; QUIOT, 1982).

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