Establishment of Insect Continuous Cell Lines and Its Utilization for Virus Multiplication *in vitro*

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Cell culture of insects is one of the most important techniques for the basic research of life science and biotechnology. Since Grace[2] first established the insect continuous cell line, one fourth century has passed. During this period, many culture media were developed and more than 51 cell lines of Lepidoptera were established[8]; thus the cell culture became a mature technique in the field of establishment of continuous cell lines.

The application of insect cell culture is expected for the virus production *in vitro* aiming at the pest control, and for the production of useful proteins by the Baculovirus-Cell line system[9]. For the economic production of viruses or useful proteins, several problems, such as 1) establishment of highly proliferative cell line, 2) selection of cell highly susceptible to viruses, 3) development of low-cost medium, and 4) large-scale culture of cells, must be solved.

In the present paper, I report the establishment of continuous cell lines of the silkworm, *Bombyx mori*, and its usefulness for the research on the relationship between the multiplication of *Bombyx mori* nuclear polyhedrosis virus and the culture condition of cells. In addition, the cell line of the cabbage armyworm, *Mamestra brassicae*, was also established.

Establishment of insect continuous cell lines

1) Embryo cells of the silkworm[3]

In order to produce a highly susceptible cell line to a *B. mori* nuclear polyhedrosis virus, the eggs, whose diapause was broken by 5°C storage, were incubated at 25°C for 24 hr or 48 hr. The eggs were surface-sterilized in 70% ethyl alcohol for 5 min and were disected in the Carlson's balanced salt solution. The embryos were taken out, freed of adherant yolks, transferred into culture media MGM-443, and cut into small pieces. The fragments were explanted into a culture TD-7 type flask and maintained at 25°C by renewing half of the medium once a week. Cell migration of epithelial-like cells, fibroblast-like cells and hemocyte-like cells from explants was observed and a mitosis of cells was observed a week after the cultures were set up. The number of cells increased to large enough to make subculture at 2 to 4 months after the cultures were set up. The culture medium was switched from MGM-443 medium to MGM-448 medium[7]. The epithelial-like cells which contained granules at the periphery of the nucleus in the cytoplasm proliferated more actively than other cells, and it became a predominant cell type (Plate 1a). The culture became able to be subcultured once a week from about 300 days after the culture was set up. At first, the cells which contain

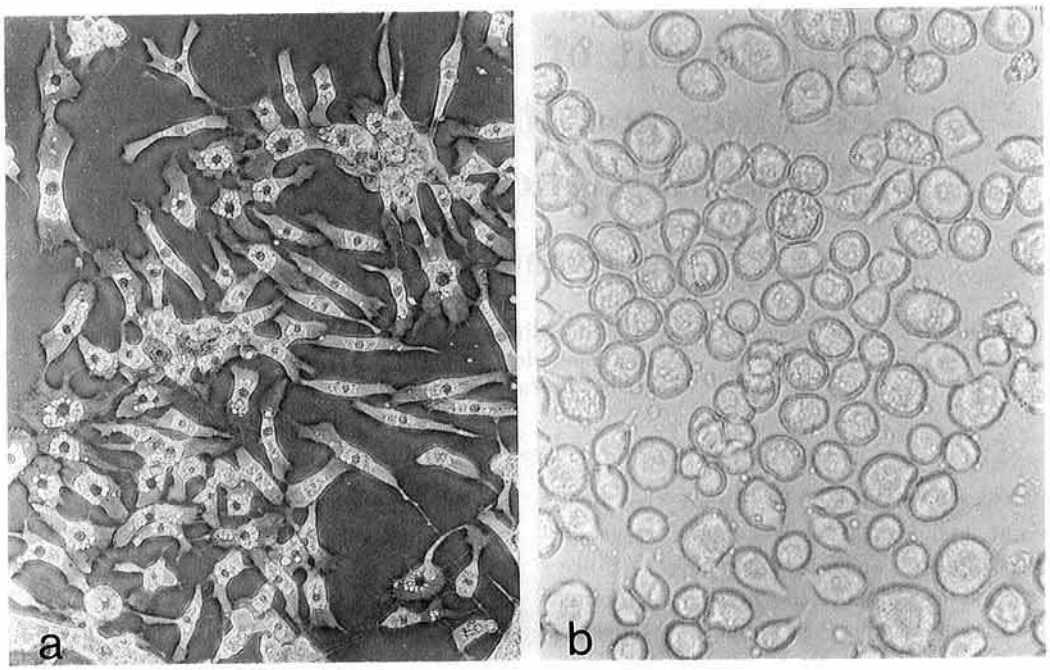

Plate 1. The cell line of *Bombyx mori*
a : The cells containing granules at the periphery of the nucleus proliferated actively.
b : They became floating in the medium.

granules were attached to the basement of the culture vessels, but they changed to be floating-type ones in the medium (Plate 1b).

The subculture reached 100 times in about 700 days after the culture was set up, and the cell line was named SES-BoMo-15A[5]. The characterization of this cell line was as follows: the diameter of cells was about 15 μm; the population doubling time was 3 days at 25°C; the mode of chromosome number was around 100; in amino acid metabolism, most amino acids in the culture medium decreased in quantity and characteristic changes were observed in the marked decrease of glutamic acid and anmonia, and the increase of tyrosine and α-alanine (Table 1); the cell was susceptible to *B. mori* nuclear polyhedrosis virus and cytoplasmic polyhedrosis virus, *Euxoa scandens* cytoplasmic polyhedrosis virus, and *Chilo irridescent* virus.

2) Fat cells of cabbage armyworm, Mamestra brassicae[4]

The full grown larvae which were reared

Table 1. Changes in percentage* of free amino acid content in the medium after 7 days of cultivation

Amino acid	BoMo-15A (%)	MaBr-3 (%)
α-Alanine	256	227
β-Alanine	98	—
Arginin	107	76
Asparagine	88	81
Asparatic acid	5	3
Cystine	59	113
Glutamic acid	15	0
Glutamine	102	410
Glycine	101	77
Histidine	103	91
Isoleucine	59	96
Leucine	51	98
Lysine	99	101
Methionine	45	64
Phenylalanine	58	89
Proline	100	101
Serine	103	91
Threonine	89	97
Tryptophan	67	76
Tyrosine	168	82
Valine	73	99
NH_3	31	6

* The initial content was taken as 100%.

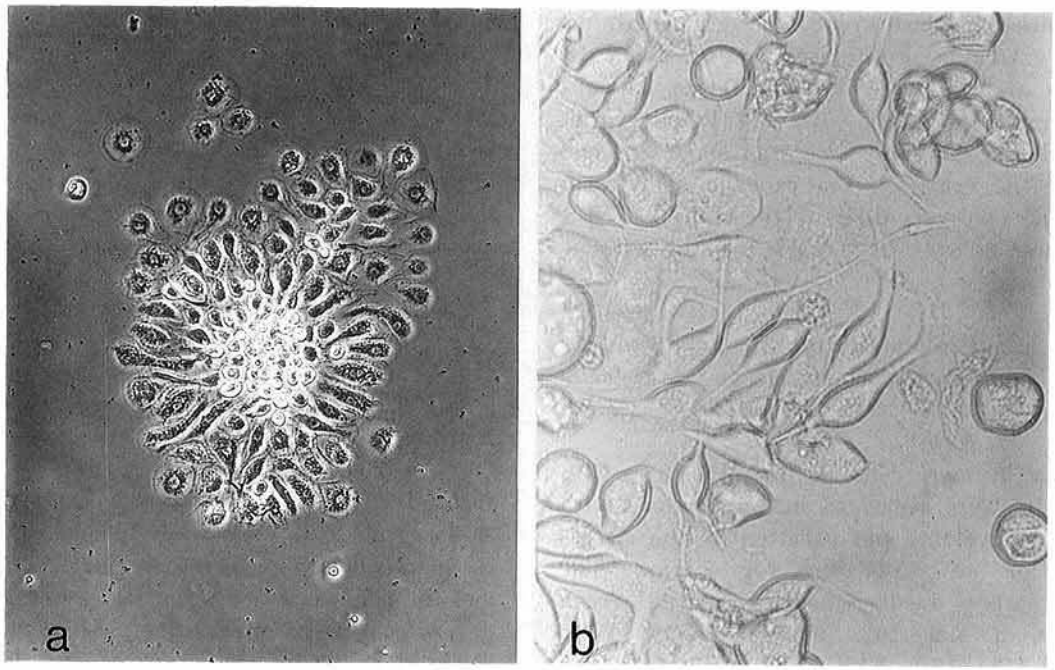

Plate 2. The cell line of *Mamestra brassicae*
a : A rosette-like cell sheet was observed in the primay culture.
b : The elongated form of the cells when the population density was high.

with artificial diet for silkworm were used for primary culture. The fat body tissues were collected from the surface-sterilized larvae and explanted into TD-7 culture vessels. The migrated cells from fat body tissues which were floating in the medium were attached to the basement of culture flasks. At around 1 month after the cultures were set up, cell mitosis was activated and rosette-like cell sheets were frequently observed in several sites of culture vessels (Plate 2a). The cell proliferation of 20 culture lots was markedly active, and 5 of 20 lots were selected and they were able to be subcultured weekly with a 1:5 split ratio at around 2 months after the culture was set up.

They reached 100 times of subculture during 220–320 days after the cultures were set up, and they were named SES-MaBr-1, 2, 3, 4, and 5. All of the cells were spherical and floating in the medium, but they became an elongated form when the population density was high (Plate 2b). The population doubling time of five cell lines was about 30 hr in a Mitsuhashi and Maramorosch (MM) medium with 3% fetal bovine serum (FBS) at 25°C. The amino acid requirement of cells was a little different from that of silkworm cell as shown in Table 1.

Cell proliferation and virus multiplication in continuous cell lines

1) Adaptation of cells to a low-cost medium

For the production of useful proteins or insect viruses, the large-scale culture of cells using a low-cost medium is expected. The cost of the MGM-443 medium containing 10% FBS and MM medium containing 3% FBS was about 1/2 and 1/6 that of the MGM-448 medium containing 10% FBS, respectively. Thus, the cells of silkworm and cabbage armyworm cultured on MGM-448 medium were tried to adapt to the MM medium containing 3% FBS.

When the silkworm cells were subcultured with the MM+3% FBS medium with the split ratio of 1:1, the proliferation of cells became very slow and many cells died at around 5 to

7 times of subculture. However, remaining cells gradually proliferated and at about 18 times of subculture it was able to be subcultured every week with a 1:1 split ratio. Population doubling time was about 8 days.

On the contrary, the cabbage armyworm cells adapted very easily to the MM+3% FBS medium, followed by the MM medium without FBS.

2) *Multiplication of B. mori nuclear polyhedrosis virus in the cells cultured with the MM+3% FBS medium*[11]

The *B. mori* cells in the MM+3% FBS medium were separated into 4 portions with an equal volume and pelletted by the centrifugation of 1,000 rpm× for 1 min. The cells were resuspended with the fresh media of MM+10% FBS, MM+3% FBS, MGM-443 (10% FBS) and MGM-443 (3% FBS), respectively, and 2 days later 80 μl of the sterilized virus solution was inoculated into 4 m*l* of the culture medium. Around 1,000 cells were observed with time and the number of cells which formed polyhedra was counted (Table 2).

Table 2. The number of cells which formed polyhedra in various culture-media

Media	Days post virus inoculation		
	5	14	21
MM+3 %FBS	0.0%	0.0%	0.0%
MM+10%FBS	0.0	0.0	1.9
MGM-443 (3 %FBS)	5.0	23.4	
MGM-443 (10%FBS)	11.2	38.0	

Around 1,000 cells were examined.

The infection percentage of cells was MGM-443 (10% FBS) > MGM-443 (3% FBS) > MM+10% FBS > MM+3% FBS, and the additive effect of FBS in the medium on the virus multiplication was observed. The formation of polyhedra in the cells of MM media was found to be at a very low level.

3) *Virus susceptibility of cells which was returned to the MGM-443 medium from the MM medium*

The *B. mori* cells cultured in the MM+3% FBS medium were retransferred to the MM+10% FBS and MGM-443 (10% FBS) media, respectively. After the several passages in those media, the cells were inoculated with *B. mori* nuclear polyhedrosis virus (NPV) and the polyhedra formation was observed on day 5 post virus inoculation. As shown in Table 3, polyhedra was observed only in the cells with the MGM-443 (10% FBS) medium and it was of interest that with increasing the passage times the ratio of polyhedra-formed cells was increased. This result suggests the possibility of the activation of cell metabolism by the retransfer to the nutrition-rich medium from the low-cost medium.

4) *Effect of the addition of insect hemolymph into the low-cost medium*[6]

In order to improve the low level formation of polyhedra in the cells with the MM+3% FBS medium, the silkworm hemolymph was added in that medium.

The hemolymph was collected from the 5th instar larvae of the silkworm at 5°C and then

Table 3. Virus susceptibility of cells returned to the MGM-443 medium from the MM medium

Media	Percentage of cells which formed polyhedra	
	11th passage	22nd passage
MM+3 %FBS→MM+3 %FBS	0.0%	0.0%
MM+3 %FBS→MM+10%FBS	0.0	
MM+3 %FBS→MGM-443 (10%FBS)	6.6	44.7
MGM-443 (10%FBS)→MGM-443 (10%FBS)		12.1

More than 1,200 cells were examined.
Observation at 5 days post virus inoculation.

Table 4. Effects of silkworm hemolymph on the formation of polyhedra

Media	No. of cells examined	No. of cells formed polyhedra	Percentage
MM+ 3%FBS	1690	0	0.0%
MM+ 3%FBS+10% hemolymph	1593	514	32.3

Observation at 7 days post virus inoculation.

heated at 60°C for 1 hr in order to prevent the melanization of hemolymph. The supernatant of the centrifugation at 3,500 rpm for 30 min of the hemolymph was collected and added to the MM+3% FBS medium up to 10% and filtered through a 450 pore size filter.

The cells of the MM+3% FBS medium were separated into 2 portions with the same volume and precipitated by the centrifugation of 1,000 rpm for 1 min, and resuspended with the media of MM+3% FBS and MM+3% FBS+10% hemolymph, respectively. Then, *B. mori* NPV was inoculated to the cultures and the cultures were incuvated at 25°C. On day 4 post virus inoculation, the cells formed polyhedra were counted. As shown in Table 4, the formation of polyhedra was markedly promoted by the addition of silkworm hemolymph. These results suggest that the MM medium needs to be supplemented with nutrients for normal formation of polyhedra of *B. mori* NPV.

Conclusion

Many insect cell lines have been developed in this one fourth century, however, a few cell lines showed a high susceptibility to the insect viruses. The newly established *B. mori* cell line was highly susceptible to *B. mori* NPV, but the cell line of *M. brassicae* was less susceptible to the *M. brassicae* NPV[4].

The infection of cells with insect viruses *in vitro* was influenced by the culture media. The supplementation of FBS, glutamin, *d*-tocopherol, cholesterol, aluminum chrolide and zinc sulfate to the culture media increased the formation of polyhedra of *B. mori* NPV[10], *Lymantria dispar* NPV[1] and *Autographa californica* NPV[12]. The supplementation of insect hemolymph to the low-cost medium, MM medium, improved the polyhedra formation. These results show that the research on the relationship between nutrition and virus multiplication is of great importance.

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