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New cell lines from larval fat bodies of *Spodoptera exigua*: Characterization and susceptibility to baculoviruses (Lepidoptera: Noctuidae)

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Abstract

Two new cell lines, designated IOZCAS-Spex-II and IOZCAS-Spex-III, were initiated from the fat bodies of larvae of the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) in TNM-FH medium containing 10% fetal bovine serum. The spherical cells were predominant among the various cell types and measures approximately 15 μm in diameter. The cell lines were mainly composed of tetraploid cells with chromosome numbers ranging from 116 to 131 ($n = 31$). The cell lines were confirmed to have originated from the *S. exigua* by DAF–PCR technique. They were susceptible to the multiple nucleocapsid nuclear polyhedrosis viruses from *S. exigua*.

Keywords: *Spodoptera exigua*; Fat body; Cell line; Karyotype; DAF–PCR; Nuclear polyhedrosis viruses

1. Introduction

Numerous cell lines have been previously established from lepidopteran species since the first insect cell line was established in the early 1960s. Establishment of new insect cell lines often leads to increasing insect cell culture capacity for various needs, such as susceptibility to specific virus infections (Granados et al., 1994; Winstanley and Crook, 1993) and high productivity of viruses and recombinant proteins (Wickham et al., 1992; Wickham and Nemerow, 1993). However, cell lines originating from different insect species tend to differ in their capacity to produce virus or express recombinant proteins (Hink et al., 1991). Therefore, there is a need to develop more lepidopteran cell lines as substrates for recombinant baculoviruses and for baculovirus-expressed proteins (Iwabuchi, 2000).

Cell lines from embryos, neonate larvae, and larval hemocytes of *Spodoptera exigua* have been reported (Gelernter and Federici, 1986; Goodman et al., 2001; Hara et al., 1993; Yasunaga-Aoki et al., 2004), but no published reports are available for cell lines from larval fat bodies of *S. exigua*. This report describes the establishment and characterization of two cell lines originating from the larval fat bodies of the insect.

2. Materials and methods

2.1. Initiation and maintenance of the cell lines

Successful cultures were initiated in August (IOZCAS-Spex-II and IOZCAS-Spex-III, in which IOZCAS indicates Institute of Zoology, Chinese Academy of Sciences), 2004, according to the following procedure. Fat bodies for culture were obtained from final instar larvae of *S. exigua*, which were reared on an artificial diet at the laboratory of Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

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Medium employed to initiate primary cultures was TNM-FH plus 10% fetal bovine serum (FBS) and 10% TNM-FH saturated phenylthiourea (primary medium). The TNM-FH saturated phenylthiourea solution was omitted from the growth medium. The insects were surface-sterilized by immersing them into 75% ethanol for 10 min and blotting them with sterile absorbent paper to remove the alcohol, the fat body tissues were then excised from the larvae. Small pieces of the tissues were thoroughly rinsed with sterile Ringer's solution to remove hemocytes, and then rinsed with the primary medium. The tissues were subsequently transferred with forceps to a T-25 cm^2 culture flask (Corning, NY) containing 1.0 ml of primary medium. After an attachment period (about 12–15 h) and before the tissues had becoming desiccated, an additional 3.0 ml of the growth medium was added without disturbing the attached tissues. The cultures were incubated at 27 °C. Primary cultures were initially fed every 7–10 days (using 2 ml medium replacement). The first subculture of the primary culture was performed after 23 days for IOZCAS-Spex-II and IOZCAS-Spex-III by transferring the contents of the culture flask to a new one containing 2 ml fresh growth medium. Thereafter, 3/4 to 4/5 volume of the medium was replaced at 7–10 day intervals.

2.2. Characterization

Microscopical images of cells from individual cell lines were captured with an Olympus inverted phase contrast microscope with a digital camera. Cell sizes were calculated according to a calibrated magnification factor. Average cell dimensions were determined from measurements of 30 cells.

Karyologic analysis of the two cell lines was carried out both at 7 passages. Modifications of a technique reported by Takahashi et al. (1980) were employed.

The two cell lines at passage 7 were identified as having originated from the *S. exigua* by comparing the profiles of the cell lines and its host with a DNA amplification fingerprinting (DAF) technique employing polymerase chain reaction (PCR) and aldolase as a primer. The primers (aldolase) set 1 (5′CCG GAG CAG AAG AAG GAG CT) and set 2 (5′CAC ATA CTG GCA GCG CTT CA) and PCR conditions used were modified from Liu et al. (2003) and McIntosh et al. (1996). Each PCR consisted of 50 μl mix containing 2 mM $MgCl_2$, 200 μM dNTP, 2 U *Taq* DNA polymerase, 20 pmol of each aldolase primer, and 70 ng cellular DNA templates. The PCR was performed for 35 cycles under the following conditions: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min after 2 min of initial denaturation at 94 °C. Following the last cycle, a final 72 °C extension was carried out for 5 min. The PCR products were analyzed by 2.5% agarose gels.

2.3. Storage of the lines

For storage, cells from the two lines were frozen at −80 °C in the TNM-FH medium containing 10% dimethyl sulfoxide (DMSO), 20% FBS and successfully regenerated.

2.4. Baculovirus infection

Spodoptera exigua late 3rd instars were offered artificial diet inoculated with *S. exigua* NPV (SeNPV), virus was topically applied to the diet at a concentration of 1×10^7 occlusion bodies (OBs) per ml. Larvae were incubated at 26 °C (Granados et al., 1986). Three days after exposure, the hemolymph of larvae was collected by cutting a proleg and diluting it in TNM-FH medium (1:10) saturated phenylthiourea (to prevent hemolymph melanization). The mixture was centrifuged (200*g*, 5 min) at 4 °C and the supernatant was filtrated through a 0.22-μm filter. The inoculum constituted the first passage of SeNPV-BV (budded virus) and was either used immediately or stored at −20 °C.

To test the susceptibility of each newly established *S. exigua* cell line to SeNPV infection, log phase cells were seeded in 96-well plastic plates with approximately 1.5×10^5 cells/ml and infected with 0.1 ml of the respective virus inoculum after 24 h. The cells were incubated at 28 °C for 3 h, rocked at 15-min intervals and fed fresh medium after discarding the virus suspension. SeNPV-BVs were quantitated by 50% tissue culture infective dose ($TCID_{50}$) in cell line IOZCAS-Spex-II. For SeNPV-OBs, the cultures were collected and the cells were lysed with 1% sodium dodecyl sulfate followed by differential centrifugation. The numbers of OBs from cell line IOZCAS-Spex-II were counted by hemocytometer (Granados et al., 1994).

2.5. Bioassay

Bioassay of the in vitro-grown SeNPV was carried out using the late 2 instar larvae and LC_{50} values were calculated. Six serial dilutions (1×10^4 OBs/ml, 5×10^4 OBs/ml, 1×10^5 OBs/ml, 5×10^5 OBs/ml, 1×10^6 OBs/ml, and 5×10^6 OBs/ml,) of OB samples from IOZCAS-Spex-II were tested in triplicate using 24 larvae/replication by the droplet feeding method (Granados et al., 1994).

3. Results and discussion

3.1. Primary cultures and subcultures

Differentiated cells attached to the flask wall were first noted that make up the cell lines. Cell migration occurred within 48 h after the culture was set up. The migrated cells were either fibroblastic or hemocytes-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. Overgrown cells piled up, or detached from the substrate and floated in the medium. The number of mitotic cells in the cultures was initially low but increased over time.

The first subculture was carried out when the bottom of the culturing flask was covered with masses of cells by transferring the contents of the culture flask to a new one containing 2 ml fresh growth medium. Subcultures of the

two lines were routinely established on a weekly basis using a ratio of cell suspension to fresh medium of 1:4 to 1:5.

3.2. Morphology

Slight differences in morphology and growth were observed in the two cell lines. Cells were usually suspending in the medium although they occasionally adhered to the glass. The cell cultures were heterogeneous in nature, consisting of spherical, spindle-shaped, and macrophage-like cells as depicted in Figs. 1 and 2. As for IOZCAS-Spex-II, the spherical cells (85.7%) were predominant among the various cell types and measured approximately 14.7 μm in diameter. The other cell type was spindle-shaped (12.7%), ranging from 16.0 to 41.9 μm (mean 25.3 μm) in length and 8.4 to 16.0 μm (mean 11.2 μm) in width. Approximately 1.6 percent of the cell was macrophage-like varying from 18.2 to 31.4 μm in diameter. As for IOZCAS-Spex-III, the spherical cells (79.4%) were predominant among the various cell types and measured approximately 16.6 μm in diameter. The other cell type was spindle-shaped (20.4%), ranging from 20.9 to 37.3 μm (mean 28.4 μm) in length and 9.2 to 18.5 μm (mean 13.2 μm) in width. Approximately 0.5 percent of the cell was macrophage-like varying from 21.8 to 40.8 μm in diameter.

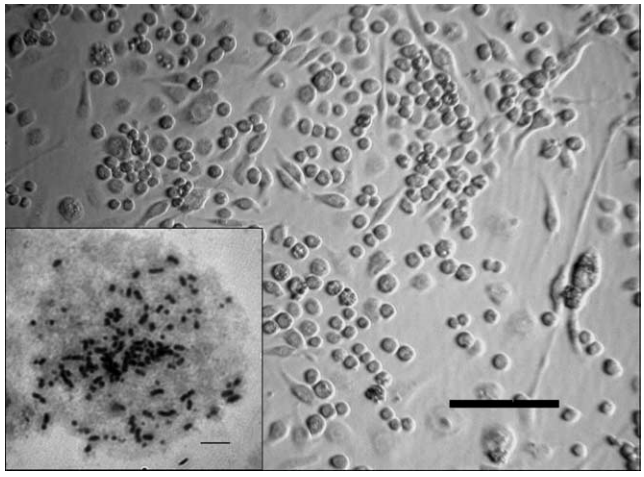

Fig. 1. Photomicrograph of IOZCAS-Spex-II after 10 passages showing predominantly spherical cell morphology but with approximately 12% spindle-shaped cells. Inset shows a typical chromosome spread from this line at passage 7. Marker bar is 10 μm.

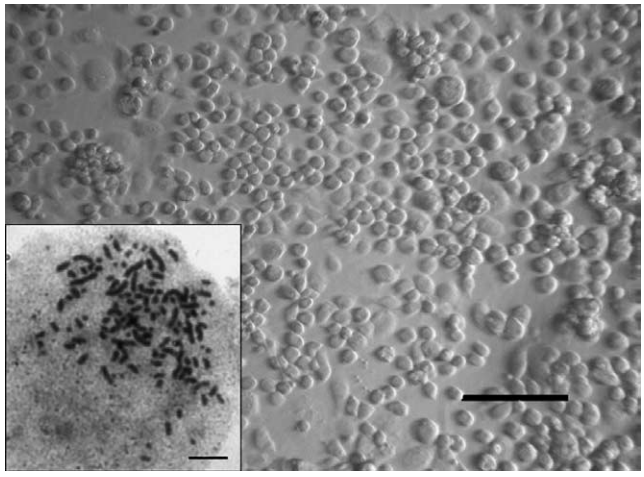

Fig. 2. Photomicrograph of IOZCAS-Spex-III after 10 passages showing predominantly spherical cell morphology. Inset shows a typical chromosome spread from this line at passage 7. Marker bar is 10 μm.

3.3. Characterization

Chromosome counts of the cell lines carried out at passage 7. The cell lines were mainly composed of tetraploid cells and their number ranged from 116 to 131 ($n = 31$) (Hara et al., 1993), as showed in Figs. 1 and 2.

The IOZCAS-Spex-II and IOZCAS-Spex-III cell lines were confirmed as having originated from the *S. exigua* by DAF–PCR analysis as shown in Fig. 3. DNA fragment patterns of cell lines from the cell line IOZCAS-Spex-II and IOZCAS-Spex-III exhibited numerous similarities. Two major bands at approximately 400 and 580 bp of identity were shared between the new *S. exigua* cell lines and their homologous host whereas Schneider's line S2 (S2, a fruit fly embryo cell line, Schneider, 1972) which is the only other cell line from different insect species cultured in our laboratory showed a different profile. Two major bands at approximately 620 and 1000 bp were seen in the S2.

3.4. Susceptibility to SeNPV

The results of the infectivity studies showed that IOZCAS-Spex-II was highly susceptible to the virus originally

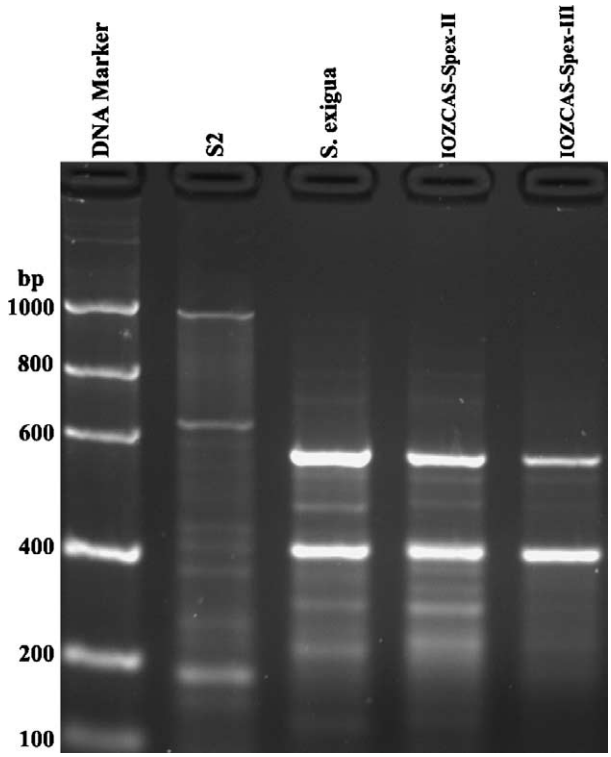

Fig. 3. DAF–PCR profiles of *S. exigua* cell lines and their insect host. Cell lines S2 served as comparisons.

from *S. exigua* while IOZCAS-Spex-III being only slightly susceptible to SeNPV. Typical cytopathogenic effects were observed, such as enlarged nuclei with numerous OBs formed in the nuclei at late stage of infection (Figs. 4 and 5). The cell line IOZCAS-Spex-II yielded 2.35×10^7–2.67×10^7 OBs/ml. The resulting nonoccluded virus was still infection to IOZCAS-Spex-II cells. The number of OBs produced by cultured cell was 211 ± 11 per cell. The $TCID_{50}$ of SeNPV extracellular virus was 8.6×10^5 $TCID_{50}$/ml (For IOZCAS-Spex-III, data was not shown).

Probit analysis revealed LC_{50} values of 6.01×10^5 OB/ml (95% CI = 7.16×10^5–5.04×10^5 OB/ml) for larval-derived OBs, and 2.30×10^5 OB/ml (95% CI = 2.94×10^5–1.79×10^5 OB/ml) for cell-derived OBs. Virulence of the cell-derived OBs was a little higher than that of the larval-derived OBs.

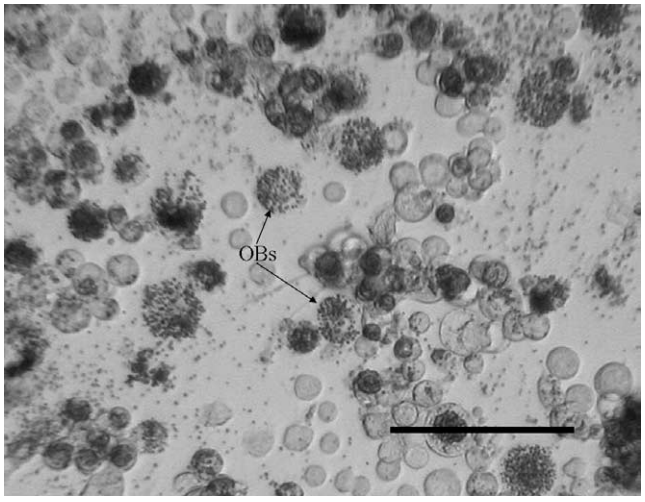

Fig. 4. Cytopathology of IOZCAS-Spex-II cell lines infected with SeNPV showing presence of OBs. Bar, 50 μm.

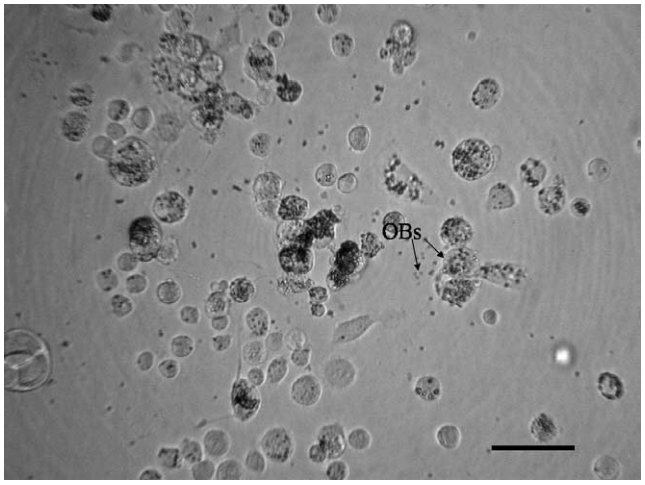

Fig. 5. Cytopathology of IOZCAS-Spex-III cell lines infected with SeNPV showing presence of OBs. Bar, 50 μm.

We also tested their respective susceptibilities to *Helicoverpa armigera* NPV (HaNPV), *Spodoptera litura* NPV (SpltNPV), and *Apocheima cinerarius* NPV infection, but the new cell lines were not susceptible to them (data is not shown).

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