Letter to the Editor

PRIMARY CULTURES OF MIDGUT CELLS FROM HELIOTHIS VIRESCENS CAN BE FROZEN AND STORED

Dear Editor:

Midgut cells from Heliothis virescens have been successfully cultured in vitro. Stem cells multiply in vitro, and selected daughter cells differentiate to columnar and goblet cells that are morphologically similar to those in the midgut in vivo. Columnar and goblet cells have finite lifetimes, die, and are replaced by differentiating stem cells. Since only the stem cells are able to divide, they must be nourished by growth factors, as are vertebrate stem cells maintained in vitro. However, few insect growth factors are known, and we find it difficult to keep these primary cultures alive more than 3 or 4 mo. Here we describe a method for freezing and later thawing cultures of H. virescens midgut cells, allowing continuous use of standardized cultures and reducing the need for constant preparation of new cultures.

Viruses such as Autographa californica (Engelhard et al., 1990) and toxins from Bacillus thuringiensis (Carroll and Ellar, 1997) enter insects as they feed and attach to receptors on the midgut cells; virus will multiply in midgut cells prior to moving to other tissues, while B. thuringiensis toxin targets the gut cells for destruction. It is useful to have a means of culturing and preserving uniform cultures of insect midguts in order to accurately study these interactions and those of other agents in vitro. Furthermore, little is known of the developmental physiology of this tissue; studies of the mechanisms controlling gut cellular organization and tissue repair have just begun (Baldwin et al., 1996; Loeb and Hakim, 1999).

In vivo, the Lepidopteran midgut is composed of a simple epithelium. Between larval molts, the epithelium consists of brush bordered columnar cells with goblet cells dispersed among them. Neuroendocrine and sensory nerve cells, though present, are rare, and a few stem cells lie among the bases of the epithelial cells. However, in the initial stages of the molt, stem cells proliferate to become the most numerous cell type present and quickly intercalate between existing cells, where they differentiate to mature goblet and columnar cells. A midgut several times larger than the previous one results, with goblet and columnar cells organized in the same pattern as before (Hakim et al., 1988; Baldwin et al., 1993).

We have been able to culture midgut cells from Manduca sexta (Sadrud-Din et al., 1994, 1996) as well as from Heliotothis virescens (Loeb et al., 1999) in vitro by taking advantage of the stem cell proliferative stage and have shown that the midgut in vitro is a stem cell system (Loeb and Hakim, 1996). Only midgut stem cells undergo mitosis and give rise to differentiated midgut cell types (Loeb and Hakim, 1996). Mature columnar and goblet cells tend to die from time to time, to be replaced by differentiating daughter stem cells. Reproducing stem cells and their progeny can be maintained in vitro for several mo as primary cultures. It has not yet been possible to establish a cell line that retains these in vivo characteristics. Under present conditions, viable stem cells gradually decrease in number until the cultures become extinct. New cultures must be prepared regularly to replace those that no longer produce active stem cells. As a partial remedy, we now report that vigorous cultures can be frozen and, when thawed, retain the characteristics of the original culture at time of freezing.

Heliothis virescens eggs were obtained from the Cotton Insects Laboratory, Phoenix, Arizona, and larvae were raised on artificial media at 30°C and with a 16:8-h LD (light:dark) cycle, as described in Loeb (1994). A few h prior to the obvious molt, the old larval head slips forward, exposing part of the newly molted head as a white band between the existing head and the thorax (slipped head stage). Midguts were dissected from surface-sterilized fourth-instar larvae at this stage, and cultures were prepared as described in Loeb and Hakim (1999). The cells grow well in Grace’s medium (Life Technologies, Grand Island, NY), diluted 1:1 with Ringer to bring the osmotic pressure closer to that of the hemolymph of H. virescens (285 m osmolar) (Loeb and Binbaum, 1981). It was supplemented with a mixture of B vitamins (riboflavin, pyridoxine, thiamin, folic acid, niacin, calcium pantothenate, biotin, and vitamin B12, all from Sigma Chemical Co., St. Louis, MO), fetal calf or bovine serum (7%) (Life Technologies), antibiotic-antimycotic (2 × 10−5%) (Sigma), gentamicin (0.2%) (Calbiochem, La Jolla, CA), and 20-hydroxyecdysone (1 ng/ml) (Calbiochem). The growth factors that continue stem cell proliferation are contained in aqueous extracts of M. sexta pupal fat body (FBX) (20 μg/ml) (Loeb and Hakim 1996). Mixed cultures of H. virescens midgut containing stem, differentiating, and mature differentiated cells were frozen in three separate trials extending over a 6-mo period. Cells were centrifuged at 400 × g for 5 min; the supernatant was discarded, and the cell pellet was resuspended in 1 ml of medium without FBX. Cell counts made of 5 μl aliquots indicated approximately 107 cells/ml. The slurry of cells was further diluted 5 to 10 times with medium minus FBX. Hybri Sigma Max™ DMSO (Sigma) was added dropwise to ultimately yield a 10% solution. Approximately 1 ml of the slurry was pipetted into each of 5 to 10 sterile glass ampoules, and the ampoules were sealed with an Ampoulomatic ampoule sealer (Biosience Inc., Bethlehem, PA). One ampoule was withheld (nonfrozen control) after DMSO addition in the first and second attempt at freezing down the cells; the DMSO was diluted out, and the cells transferred to culture dishes (Figs. 2 and 3, never frozen). The remaining sealed ampoules were placed into freezing canes and inserted into the well of a Cryomed model 1010 (Forma Scientific, Marietta, OH) programmable cell freezing system that controlled

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(1) Products mentioned in this article are not endorsed by the U.S. Department of Agriculture.
from Heliothis virescens primary midgut cultures that were never frozen, thawed after 24 h, thawed after 7 d, and thawed after 35 d in liquid N₂. Aliquots were counted at regular intervals for approximately 1 mo. The standard method for diluting DMSO was followed in each case. Each point represents the mean of aliquot counts from two separate wells.

冷却至1°C/min至冷冻。这些含冷冻物的安瓿被放置于液氮中冷冻，并迅速在温暖的水中解冻。在前两次尝试中，解冻过程的稀释物是在使用标准体积的1, 1, 2 ml的介质中，每次添加，形成滴在一块带有温和打旋的物质中，加到解冻细胞中。然而，结果表明每5 μl的解冻细胞中，大约10倍的细胞数量低于预期，而最少数细胞被破坏（图1和2，‘never frozen’和‘thawed after 7 d’）。当程序被修改为在解冻时，细胞的体积被添加，逐步，250 μl的增量与在添加溶液的每一线性增加中，这些健康细胞存活率在10倍以上。最后， goblet cells were destroyed (Figs. 1 and 2, ‘never frozen’ and ‘thawed after 7 d’). When the procedure was modified to one where the same volume of medium was added more gradually, in 250 μl increments with gentle swirling at each addition, recovery of living cells was 10 times greater, and goblet cells appeared normal (Fig. 2, ‘thawed after 42 d’; Fig. 3).

After dilution of the DMSO-containing slurry, the mixture was centrifuged at 400 × g to pellet the cells. The supernatant was discarded, and the cells were resuspended in fresh medium; the pelleting procedure was repeated, and the cells were resuspended in 1 ml medium-FBX. These cells were equally distributed in each of four wells of a six-well plate (approximately 10⁶ cells/well); medium-FBX was added to a volume of 3 ml/well. Five-microliter aliquots of two of the wells were withdrawn at regular intervals for cell counting, and general observations of all the cultures were made for approximately 1 mo after thawing. Three separate cell suspensions have been frozen using this procedure.

Cells were fed once per wk by replacing one-third of the medium in each well with fresh medium-FBX. Surviving thawed and cultured cells appeared normal; stem cells divided, and differentiating cells were observed among the mature goblet and columnar cells in each well (Fig. 4). Although a monolayer of dead and dying cells appeared above the healthy cells after 24 h in the first two trials (Figs. 1 and 2, ‘never frozen’ and ‘thawed after 7 d’), little cell loss was observed in the 7-wk thawed cells in the second trial and in all of the third trial, where more gradual dilution of DMSO preservant was made after thawing (Fig. 2, ‘thawed after 42 d’; Fig. 3). Although there appeared to be a precipitous loss of cells in the first trial (Fig. 1), the remaining cells looked healthy, and the cell number in the resulting cultures appeared relatively constant. After 1 wk, goblet cells were again observed in the culture, eventually reaching the normal ratio for H. virescens of one goblet per 10 columnar cells. A constant number of cells was also seen in cultures represented in Fig. 3. However, populations of healthy cells may also fluctuate in number (Sadrud-Din et al., 1994) as in the cultures represented in Fig. 3. However,