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ESTABLISHMENT AND CHARACTERIZATION OF AN *OSTRINIA NUBILALIS* CELL LINE, AND ITS RESPONSE TO ECDYSONE AGONISTS

ANDI TRISYONO, CYNTHIA L. GOODMAN, JAMES J. GRASELA, ARTHUR H. MCINTOSH, AND G. MICHAEL CHIPPENDALE[1]

Department of Entomology (A. T., G. M. C.), University of Missouri, Columbia, Missouri 65211, and USDA, Agricultural Research Service (C. L. G., J. J. G., A. H. M.), Biological Control of Insects Research Laboratory (BCIRL), Columbia, Missouri 65203

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Summary

A cell line derived from embryonic tissues of the European corn borer, *Ostrinia nubilalis* (UMC-OnE), was established in EX-CELL 401 medium containing 10% fetal bovine serum. The cells grew in suspension, and were mainly spherical in shape. The cell doubling times at the 17th and 79th passages were 56 and 36 h, respectively. DNA amplification fingerprinting showed that the DNA profile of the OnE cell line was different from that of the southwestern corn borer, *Diatraea grandiosella* (UMC-DgE), and that of the cotton bollworm, *Helicoverpa zea* (BCIRL-HZ-AM1). The OnE cell line was responsive to treatments of 20-hydroxyecdysone and the ecdysone agonists, methoxyfenozide (RH-2485) and tebufenozide (RH-5992). These compounds caused similar effects on the cells, which included cell clumping and decreased cell proliferation. The clumps were observed on the third day of incubation, and became larger after 7 d of incubation. After 168 h of incubation, methoxyfenozide and tebufenozide were 35 and 11 times more effective, respectively, in inhibiting proliferation of the OnE cells than was 20-hydroxyecdysone.

Key words: polymerase chain-reaction; Pyralidae; EC50; 20-hydroxyecdysone; methoxyfenozide; tebufenozide; cell replication.

Introduction

Many cell lines have been obtained from specific insect tissues (e.g., hemocytes and fat body) as well as heterogeneous sources (e.g., embryos and larvae). An insect cell line that responds to the molting hormone, 20-hydroxyecdysone, is useful for screening and identifying the mode of action of compounds with ecdysone-like activity. The cell lines of the fruit fly, *Drosophila melanogaster* Meig., have been used in the discovery and identification of three ecdysone agonists: 1,2-dibenzoyl-1-*tert*-buthylhydrazine (RH-5849) using the Kc cell line (Wing, 1988), 8-*O*-acetylharpagide using the cMK7 and cMK9 cell lines (Elbrecht et al., 1996), and 3,5-di-*tert*-butyl-4-hydroxy-*N*-isobutyl-benzamide (DTBHIB) using the Kc cell line (Mikitani, 1996).

Ecdysone agonists mimic the action of 20-hydroxyecdysone (Wing, 1988; Dhadialla et al., 1998). They represent a new class of insect growth regulators (IGRs) that disrupt growth and development. For example, the ecdysone agonists RH-5849, tebufenozide (RH-5992), and methoxyfenozide (RH-2845) caused larvae of the tobacco hornworm, *Manduca sexta* (L.), the European corn borer, *Ostrinia nubilalis* (Hübner), and the southwestern corn borer, *Diatraea grandiosella* Dyar, to enter premature and lethal molting cycles (Wing et al., 1988; Trisyono and Chippendale, 1997, 1998). In some insect cell lines, these ecdysone agonists elicit effects similar to those induced by 20-hydroxyecdysone, including morphological and physiological changes, and inhibition of cell proliferation (Wing, 1988; Silhacek et al., 1990; Spindler-Barth et al., 1991; Sohi et al., 1995).

Cell lines established from pupal ovaries of *O. nubilalis* have been used to study the microsporidian parasites of corn borers (T. J. Kurtti, pers. comm.). We established a cell line from embryos of *O. nubilalis* (UMC-OnE), characterized the cell line, and demonstrated its response to 20-hydroxyecdysone, methoxyfenozide, and tebufenozide.

Materials and Methods

Initiation and maintenance of continuous cell line. A primary cell culture was started in November 1995 from embryonic tissues of *O. nubilalis* using procedures modified from McIntosh et al. (1981), Lynn (1989), and Stiles et al. (1992). Eggs (1–2-d old) were collected from a laboratory culture. The eggs were surface-sterilized by immersion in a solution of 70% ethanol and 1% Triton X-100 for 5 min, in a 0.525% sodium hypochlorite (Clorox®) solution for 5 min, and washed twice with Hanks' balanced salt solution (HBSS). The eggs were crushed and homogenized in HBSS, and centrifuged at 200 × *g* for 10 min. The pellet was resuspended in 2.5 ml culture medium, EX-CELL® 401 (JRH Biosciences, Lenexa, KS) supplemented with 10% heat-inactivated (56° C for 30 min) fetal bovine serum (FBS, Intergen Company, Purchase, NY), 50 U/ml penicillin, and 50 μg/ml streptomycin. The cell suspension was transferred to a T-12.5 cm^2 Falcon disposable flask, and incubated at 28° C. 2.5 μl Ampicillin (10 mg/ml) and 25 μl glutathione (7 mg/ml) were added into the flask only during the initial stage of cell line establishment. The resulting continuous cell culture was designated UMC-OnE.

During the first 2 mo, half of the medium was changed weekly. The first subculture was made 68 d after initiation at a split ratio of 1:2. Because most cells grew in suspension, subculturing was accomplished by transferring 1.25 ml old medium containing OnE cells into a new T-12.5 cm^2 flask and adding 1.25 ml fresh medium into the new flask. Starting at the eighth passage,

[1] To whom correspondence should be addressed at E-mail: chippendaleg@missouri.edu

subcultures were made at a split ratio of 1:5 in a T-25 cm^2 flask. If the cells were not confluent after 4 d of incubation, the cells were supplied with fresh medium. Medium was replenished by withdrawing 2.5 ml old medium after allowing the cells to settle for 5 min and adding 2.5 ml fresh medium. The split ratio for subcultures was increased with increasing passage numbers. After the 105th passage, the cells were subcultured at a split ratio of 1:50 every 6–8 d.

Growth of the OnE cell line. Growth curves were determined at the 4th, 17th, and 79th passages. Cells were seeded at 2×10^5 cells/ml for the fourth passage and 1×10^5 cells/ml for the 17th and 79th passages into 5 ml medium using T-25 cm^2 flasks (two flasks/time point). Viable cell counts were made every 24 h using a hemocytometer (3 counts/flask) for 240 h. The population doubling time was calculated based on the regression equations during the exponential growth phase of the cells (48–144 h) (McIntosh and Ignoffo, 1983).

Characterization of the OnE cell line using DNA amplification fingerprinting. Identification of the OnE cell line was carried out using DNA amplification fingerprinting (DAF) (McIntosh et al., 1996). DNA was extracted from the 4th, 49th, and 110th passages of the OnE cell line (5, 3, and 5×10^6 cells, respectively), the eighth passage of the UMC-DgE cell line (5×10^6 cells, a cell line derived from embryonic tissue of the southwestern corn borer, *D. grandiosella* Dyar, unpublished results), the 98th passage of the BCIRL-HZ-AM1 cell line (6×10^6 cells, a cell line derived from pupal ovaries of the corn earworm, *Helicoverpa zea* [Boddie], [McIntosh and Ignoffo, 1981]), and three newly ecdysed (<1 d) third instar larvae of *O. nubilalis* (15.5 mg). DNA extraction was performed using a Puregene™ DNA isolation kit (Gentra Systems Inc., Research Triangle Park, NC). The samples were hydrated by adding 100 μl DNA hydration solution overnight at room temperature, and stored at 4° C until used.

The six DNA samples (300 ng per sample, except for the 88 ng sample from the larvae), and two negative controls (water and DNA hydration solution) were amplified using polymerase chain reaction (PCR) techniques according to the protocol described by GeneAmp PCR (Perkin–Elmer, Foster City, CA). An oligonucleotide primer pair designed for PCR amplification of mammalian aldolase was used. The PCR samples (10 μl) and the DNA marker (50–100 bp) were electrophoresed in a 2.5% metaphor gel (FMC BioProducts, Rockland, ME) at a constant voltage of 125 V for 1 h in a mixture of 270 ml Tris–borate buffer (0.09 *M* Tris Base, 0.09 *M* boric acid, 0.0025 *M* disodium ethylenediamine-tetraacetic acid, and pH 8.3) and 270 ml H_2O.

Response of the OnE cell line to 20-hydroxyecdysone, methoxyfenozide, and tebufenozide. The morphological response to these compounds was studied as follows: cells were seeded at 1×10^5 cells/ml in T-25 cm^2 flasks containing 5 ml growth medium, and 5 μl of 0.5 μg active ingredient (AI)/ml 20-hydroxyecdysone (within physiological range, 0.01–3 μg/ml [Nijhout, 1994]), methoxyfenozide, or tebufenozide solution prepared in DMSO was added into the flasks. Solvent (5 μl dimethyl sulfoxide [DMSO]) and untreated cells were used as controls. Each treatment was replicated two times. Photographs were taken after 168 h of incubation from 1 replicate per treatment because differences between replicates were not observed.

Cell density response to the above-mentioned compounds was studied as follows: the concentration of 20-hydroxyecdysone, methoxyfenozide, or tebufenozide that inhibited 50% of cell proliferation compared to the control (EC_{50}) was determined by applying a series of concentrations, ranging from 1×10^{-2} to 1×10^4 n*M*, of the compounds prepared in DMSO into the cell suspension. Cells were seeded at 1×10^5 cells/ml using 24-well cell culture plates (Tissue Culture Cluster-24, Cambridge, CA). Each well was filled with 1 ml medium, and 1 μl test compound was added into each well. The number of cells was counted after 168 h of incubation from 2 replicates for each treatment (three readings/replicate). Statistical analysis to determine the EC_{50} values was carried out using a Biomass Program (Missouri Agricultural Experiment Station, 1980).

Results

Initiation and maintenance of continuous cell line. Most cells and tissues of the OnE primary culture began to attach to the bottom of the culture flask 1 d after initiation. The cells remained attached and multiplied slowly until the fourth week. During this period, cells that attached to the flask were fibroblast-like cells, epithelial-like cells, and spherical cells. Some spherical cells were observed in suspension by the fifth week, and these cells became the dominant cells by the time of the first subculture.

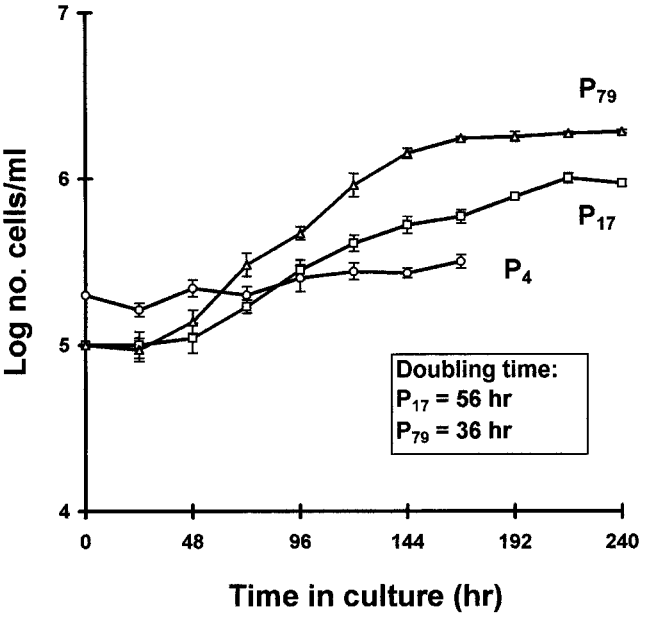

FIG. 1. Growth curves of *O. nubilalis* cells (OnE), at the 4th (P_4), 17th (P_{17}), and 79th (P_{79}) passages. Cells were cultured at 28° C in EX-CELL 401 medium supplemented with 10% FBS and 50 U/ml penicillin plus 50 μg/ml streptomycin. Data points represent the average of 3 counts from each of the 2 replicates (±SD).

The time between two consecutive subcultures varied considerably for the 1st to 15th passages (4–35 d). The OnE cell line was considered to be established by the 16th passage because after this passage the subculture periods were relatively similar (3–9 d). The cells grew in suspension, and were mainly spherical in shape (Fig. 3*A*). The cell morphology did not change even after the cell line had been in culture for 3 yr.

Growth of the OnE cell line. The growth curves of the OnE cell line for three passages were different (Fig. 1). The cell doubling time at the fourth passage was not determined because the cell density did not increase significantly. The cell doubling time decreased from 56 h at the 17th passage to 36 h at the 79th passage showing an increase in the replication rate as the passage number increased. The maximum cell densities over a 10-d period for the 17th and 79th passages were 9.9×10^5 cells/ml (at the ninth d) and 1.9×10^6 cells/ml (at the 10th d), respectively.

Characterization of the OnE cell line using DNA amplification fingerprinting. DAF using a primer pair for aldolase produced different sizes of DNA fragments showing that the DNA profiles of the three cell lines, OnE, DgE, and HZ-AM1, were different (Fig. 2). The number of bands in each sample was determined above the region of 50 bp. The DNA profiles of the OnE cell line at three different passages were identical. The OnE cell line had six major bands with a total of 13 bands ranging from 72 to 731 bp. The *O. nubilalis* larvae had six bands, and the four major bands observed in the larvae were identical to four of the six major bands observed in OnE. The DgE cell line had 17 bands with one band above 1000 bp. The other bands ranged from 54 to 762 bp. Similar to the OnE cell line, the DgE cell line had six major bands, but their sizes were different from those found in the OnE cell line. The HZ-AM1

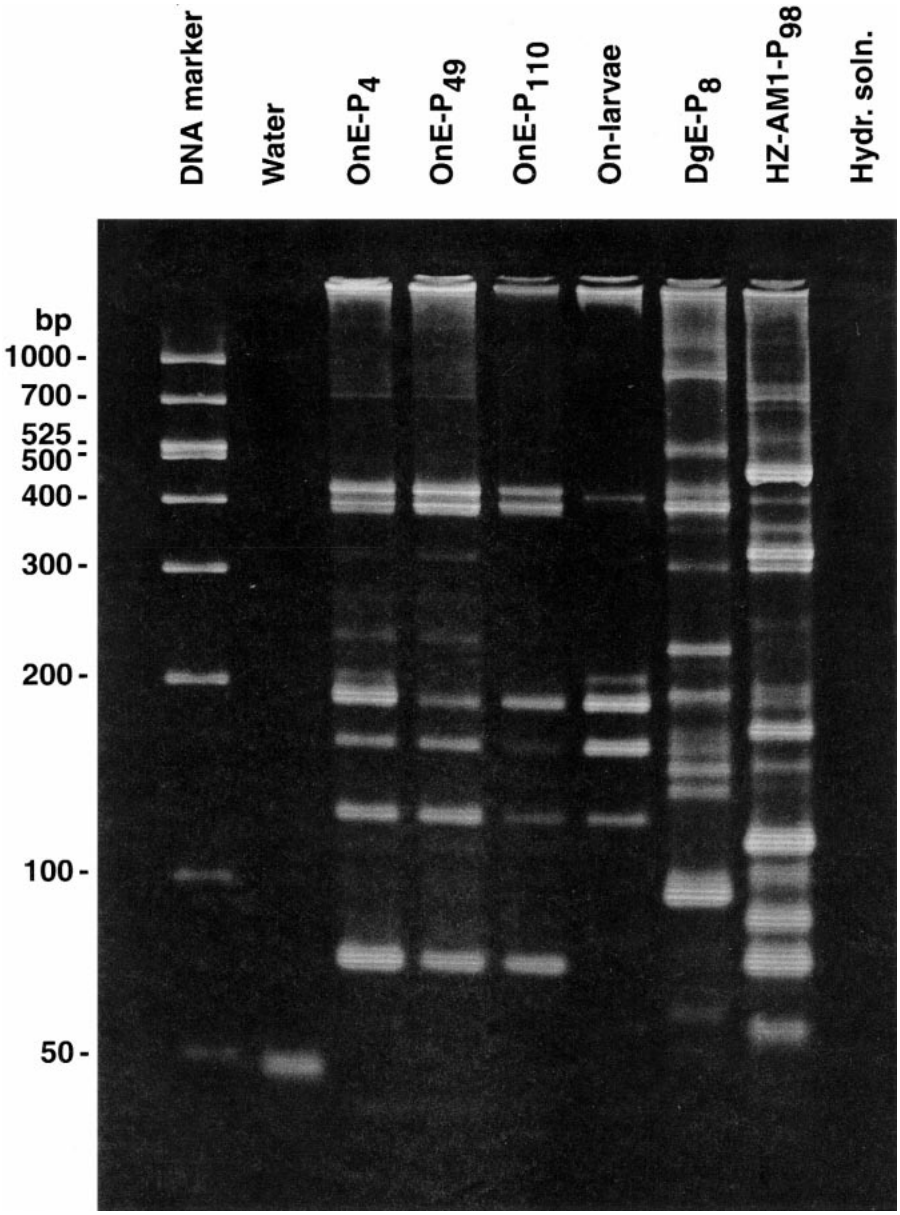

FIG. 2. DAF profiles of *O. nubilalis* cells at the fourth (OnE-P_4), 49th (OnE-P_{49}), and 110th passage (OnE-P_{110}), *O. nubilalis* larvae (On-larvae), *D. grandiosella* cells at the eighth passage (DgE-P_8), and *Helicoverpa zea* at the 98th passage (HZ-AM1-P_{98}). Water and hydration solution (Hydr. soln.), used to dilute the DNA samples, represent negative controls run in place of DNA.

cell line had more bands than the OnE and the DgE cell lines, with a total of 20 bands, ranging from 52 to >1000 bp. The size of the major DNA fragments found in the HZ-AM1 cell line was different from those found in the OnE or the DgE cell line.

Response of the OnE cell line to 20-hydroxyecdysone, methoxyfenozide and tebufenozide. The morphology of the OnE control cells after 168 h of incubation (Fig. 3*A*) was identical to those at 0 d (data not shown). The morphology of cells treated with DMSO was identical to those of the control, suggesting that DMSO had no effect on the morphology of the cells (data not shown). The OnE cells treated with 20-hydroxyecdysone (Fig. 3*B*), methoxyfenozide (data not shown), or tebufenozide (data not shown) exhibited similar responses of cell clumping. Cell clumping due to the application of these compounds was observed on the third day of incubation. The clumps, which were loosely attached to the bottom of the flask, became larger with increasing incubation time.

With regards to cell density, DMSO did not have any significant effect on the proliferation of the OnE cells (data not shown). Application of 20-hydroxyecdysone, methoxyfenozide, or tebufenozide to the OnE cell line inhibited cell proliferation in a dose-dependent fashion (Fig. 4). After 168 h of incubation, the EC_{50} values of 20-hydroxyecdysone, methoxyfenozide, and tebufenozide were 22.50, 0.63, and 1.99 n*M*, respectively. These results showed that methoxyfenozide and tebufenozide were 35 and 11 times more effective, respectively, in inhibiting the proliferation of OnE cells than was 20-hydroxyecdysone; and methoxyfenozide was three times more effective than tebufenozide.

DISCUSSION

EX-CELL 401 supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin proved to be a suitable medium to ini-

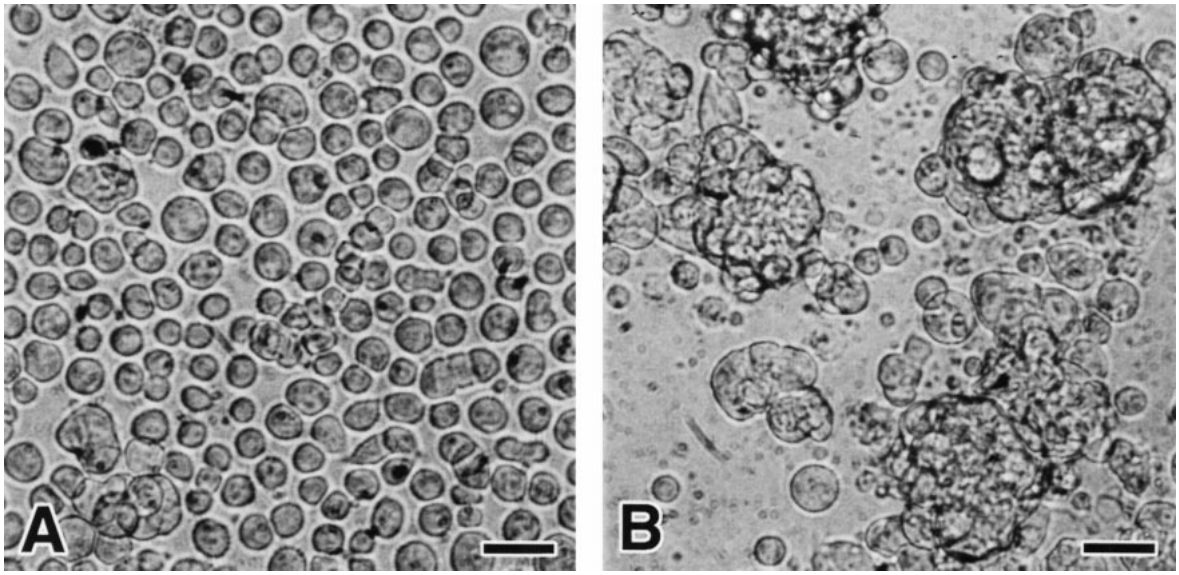

FIG. 3. Effect of 20-hydroxyecdysone on the *O. nubilalis* cell line (OnE). (*A*) Untreated cells at 168 h and (*B*) 20-hydroxyecdysone (0.5 μg AI/ml) at 168 h. Methoxyfenozide (0.5 μg AI/ml) and tebufenozide (0.5 μg AI/ml) exhibited an effect similar to that of 20-hydroxyecdysone. The cells were cultured at 28° C in EX-CELL 401 medium supplemented with 10% FBS and 50 U/ml penicillin plus 50 μg/ml streptomycin. *Bar*, 30 μm.

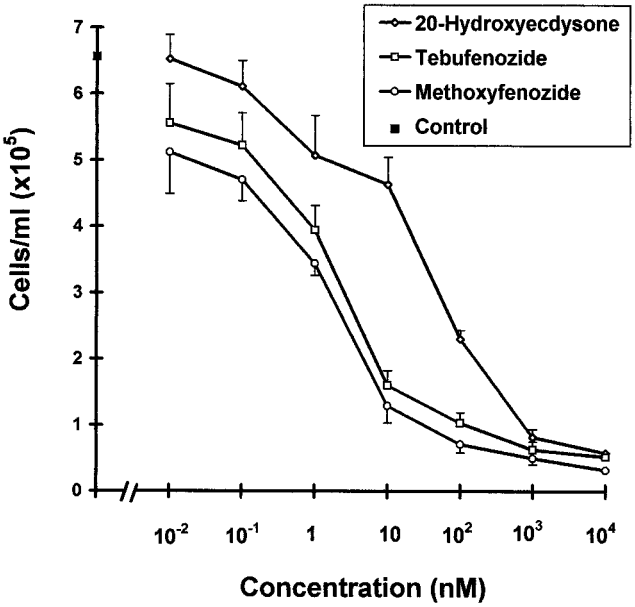

FIG. 4. Cytotoxicity of 20-hydroxyecdysone, methoxyfenozide, and tebufenozide on *O. nubilalis* cells. Cells were seeded at 1×10^5 cells/ml and cultured at 28° C in EX-CELL 401 medium supplemented with 10% FBS and 50 U/ml penicillin plus 50 μg/ml streptomycin. Cells were counted after 168 h of incubation. *Bar* represents SD.

tiate and culture *O. nubilalis* embryonic cells. The doubling time of the resulting continuous cell line, OnE (36 h at its 79th passage) is comparable to that of some cell lines established from *Heliothis virescens* and *H. zea* (the doubling time ranged from 27 to 40 h) (McIntosh and Ignoffo, 1983). The differences in the doubling time may be due to several factors, including differences in the tissue source of the lines and the medium in which they were maintained.

The present study extends previous reports (Kariuki, 1996; McIntosh et al., 1996) demonstrating that DAF is a useful technique for identifying cell lines. The DNA profiles of the OnE cell line from three different passages (the fourth, 49th, and 110th passages) were identical. These results indicate that the OnE cell line has maintained its identity, and the fingerprint generated was a unique and reproducible characteristic. The DNA profile of *O. nubilalis* larvae had four major bands identical to that of the OnE cell line, as noted previously for other cell lines and their associated species (McIntosh et al., 1996). Even though a few bands in the DgE and the OnE cell lines were similar in size, the DNA profile of the DgE cell line was distinguishable from that of the OnE cell line. The DNA profile of the HZ-AM1 cell line was different from that of the OnE and the DgE cell lines, but it was similar to the results obtained by McIntosh et al. (1996) on the same cell line.

The OnE cell line responded to exposure to 20-hydroxyecdysone, methoxyfenozide, or tebufenozide, resulting in cell clumping and inhibition of cell proliferation. These results are typical effects of ecdysteroids and the ecdysone agonists at the cellular level; similar observations have been made in other insect cell lines (Courgeon, 1972; Cherbas et al., 1980; Wing, 1988; Dinan et al., 1990; Silhacek et al., 1990; Spindler-Barth et al., 1991; Sohi et al., 1995). However, other morphological changes, such as cell elongation, were not observed in the OnE cell line at the dose used in this study (0.5 μg/ml).

Methoxyfenozide and tebufenozide were more effective in inhibiting proliferation of the OnE cells than was 20-hydroxyecdysone. These results support previous reports showing that tebufenozide was more potent in inhibiting cell proliferation than was 20-hydroxyecdysone (Sohi et al., 1995), and methoxyfenozide was a more potent ecdysone agonist than was tebufenozide in vivo (Brunner et al., 1995; Ishaaya et al., 1995; Trisyono and Chippendale, 1997, 1998).

Several insect cell lines have been used to study the action of

ecdysteroids (Dinan et al., 1990), and a bioassay using *D. melanogaster* B_{II} cell line has been developed for screening ecdysteroid agonists and antagonists (Clément and Dinan, 1991; Clément et al., 1993). The responsiveness of OnE cells to 20-hydroxyecdysone, methoxyfenozide, and tebufenozide offers the potential use of this cell line for screening and studying compounds with ecdysone-like activity.

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