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Morphological and Molecular Effects of 20-Hydroxyecdysone and Its Agonist Tebufenozide on CF-203, a Midgut-Derived Cell Line From the Spruce Budworm, *Choristoneura fumiferana*

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The morphological and molecular responses of a midgut-derived cell line of the spruce budworm, *Choristoneura fumiferana,* to 20-hydroxyecdysone (20E) and the nonsteroidal ecdysone agonist, tebufenozide (RH-5992), were investigated. The cells responded to these compounds by clumping, generating filamentous extensions, increased mortality and expression of the transcription factor, *Choristoneura* hormone receptor 3 (CHR3). This cell line can be used as a model system to study the mode of action of ecdysone and its agonists. With subsequent passaging in ecdysteroid-containing medium, the degree of clumping increased and the clumping could not be reversed by subculturing in ecdysteroid-free medium. Cell numbers of the adapted cell lines in 20E and RH-5992 containing media were not significantly decreased, compared to the control, but both cell lines accumulated less ^{14}C-labeled RH-5992 and lost the capability of expressing CHR3 in response to these compounds. Taken together, the cell lines appeared to develop a mechanism to adapt to the toxic effects of these compounds. Arch. Insect Biochem. Physiol. 55:68–78, 2004.

KEYWORDS: insecticide resistance; cell culture; ecdysone; cell lines

INTRODUCTION

Similar to 20-hydroxyecdysone (20E), the nonsteroidal ecdysone agonist, tebufenozide (RH-5992), initiates the molting process by binding to the ecdysone receptor (Retnakaran et al., 1997; Dhadialla et al., 1998). This compound induces precocious and incomplete molting that is lethal to larvae (Binnington and Retnakaran, 1991). RH-5992 acts specifically on Lepidoptera and has very little effect on other insects belonging to other orders such as Hymenoptera and Diptera (Sundaram et al., 1998). This difference in sensitivity among different species does not appear to be due to differences in the transport or metabolism of the compound in insects, but is probably due to differences in receptor affinity and exclusion mechanisms (Dhadialla et al., 1998; Sundaram et al., 1998; Hu et al., 2001). RH-5992 induced the expression of glutathione *S*-transferase, which is generally involved in detoxification by forming a conjugate with the toxic compounds (Feng et al., 2001). Insects can also develop tebufenozide resistance after long exposure to the compound (Retnakaran et al., 2001). However, the resistance mechanism is not clear at this time.

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In this study, we examined long-term effects of 20E and RH-5992 on a spruce budworm midgut cell line (FPMI-CF-203) cultured in the presence of the compounds for over 100 passages. Two main findings were observed: first, like *Drosophila* Kc embryonic cell line and *Malacosoma disstria* hemocyte cell line, this midgut cell line showed obvious morphological and molecular responses to these compounds. Therefore, this lepidopteran midgut cell line could be used as a model system for studying the action of ecdysteroid compounds. Second, long-term exposure to these compounds significantly enhanced morphological changes, which could not be reversed even after the compounds were removed, but the cells became less sensitive to the compounds.

MATERIALS AND METHODS

Cell Line and Cell Cultures

The FPMI-CF-203 (CF-203) cell line used in this study was developed from the midgut of the spruce budworm, *Choristoneura fumiferana* (Sohi et al., 1993). All cells were grown in 25 cm^2 tissue culture flasks in Insect X-Press medium (Biowhittaker, Walkersville, MD), supplemented with 2.5% (v/v) heat-inactivated fetal bovine serum (GIBCO BRL Life Technologies Inc., Burlington, ON, Canada) and maintained at 28°C. The starting number of cells was 1.25×10^5/ml in 5 ml of medium. The cells were passaged 350–400 times prior to exposure to 20E and RH-5992.

Ecdysteroidal Compounds and Treatment

The molting hormone, 20-hydroxyecdysone (20E) was purchased from Sigma Chemicals. Tebufenozide (RH-5992, flowable formulation Mimic™-2F, 240 g AI/l) and ^{14}C-labeled RH-5992 (specific activity, 22.2 mCi/g) were gifts from Rohm and Haas Co. (Spring House, PA). Both the 20E and RH-5992 were dissolved in dimethylsulfoxide (DMSO) and serial dilutions were made ensuring that the final concentration of DMSO in the medium remained at 0.1%, at which level the solvent was non-toxic to the cells. Untreated and DMSO controls were included in all experiments. The cells were continuously grown in medium containing 10^{-6}M of 20E and RH-5992 for more than 100 passages. Each passage lasted for about 5 days. The adapted cells were subcultured in the ecdysteroid-free medium containing only DMSO for 24 h prior to adding the compounds at different concentrations. Observations on cell responses started 24 h after treatment and continued to various periods of time.

Morphological Observations and Counting of Cell Numbers

All treatments and controls were examined daily under an inverted phase-contrast microscope and the effects were photographically recorded. Cell proliferation, attachment, and morphological changes were estimated quantitatively at the end of each passage. CF-203 cells were harvested prior to passaging by incubating attached cells with 0.05% trypsin solution for 30 s at room temperature. The trypsin was replaced with FBS-supplemented Insect X-Press medium and the monolayer of cells was resuspended. The cell numbers were counted with a Coulter counter (Coulter Electronics, Inc. Hialeah, FL). Cell viability was determined by using the trypan blue assay (Sigma-Aldrich).

Retention Assay of RH-5992 in Cells

The assay for ^{14}C-labeled RH-5992 retention in the cell lines was conducted according to Sundaram et al. (1998). Cell cultures were set up at 2×10^5 cells/ml in RH-5992 free media for 24 h. ^{14}C-labeled RH-5992 was then added to the cells at a level of 300,000 DPM per 5-ml flask. The cells were harvested 24 h later by centrifugation at 3000 rpm. The cell pellets were resuspended and washed three times with phosphate-buffered saline. After the final wash, the cell pellets were suspended in 200 µl of 1.5 M NaOH and incubated at 100°C for 1 h. After cooling and brief centrifugation, 100 µl of supernatant was mixed with 10 ml of scintillation cocktail and the radioactivity was measured in a liquid scintillation counter (Beckman LS 6000SE).

RNA Isolation and Northern Blotting

Total RNA was isolated using the guanidinium isothiocyanate-phenol-chloroform method as described by Chomczyski and Sacchi (1987). Ten micrograms of total RNA from the different treatments were separated on formaldehyde-agarose (1%) gels and transferred to nylon membranes. The Northern blots were hybridized with cDNA probes labelled with α-[^{32}P]dATP. Hybridization and washes were conducted as described by Palli et al. (1998).

RESULTS

Responses of CF-203 Cells to 20E and RH-5992

CF-203 cells that had never been exposed to the ecdysteroid compounds appeared to be morphologically normal during the first day after 10^{-6} M of 20E or RH-5992 was added to the medium (Fig. 1A–D). Some cells started to die and detached themselves from the bottom of the culture flasks by the second day, while some cells continued to divide (Fig. 1E–H). At days 3 and 4 post treatment, numbers of total attached cells in these two treatments were about 30% lower than those in the control (Fig. 1I–P). The cells grown in the presence of 20E and RH-5992 produced filamentous extensions, which became less fusiform, and formed clumps. The trypan blue assay confirmed that about 80% of the attached and clumping cells were viable, whereas only 10–15% of the floating cells were viable. These morphological changes are similar to those seen in a forest tent caterpillar, *Malacosoma disstria*, cell line (MD-66) (Sohi et al., 1995). No significant difference in morphological changes was observed between 20E and RH-5992 treatments, except that the cells appeared to be more sensitive to 20E than to RH-5992 in terms of cell appearance.

Adaptation of CF-203 Cells to 20E and RH-5992

We continued to subculture the cells in the presence of 10^{-6}M 20E for up to 123 passages, generating a 20E cell line, or in 10^{-6}M RH-5992 for up to 107 passages, generating a RH-5992 cell line. Most cells used in this study were between the 69th and 85th passages unless otherwise specified.

Filamentous extensions and clumping were the most significant morphological changes that occurred in the RH-5992 and 20E lines (Fig. 2). The cells of these two cell lines clumped to form aggregates in response to 20E and RH-5992 treatments (Fig. 2B,C, E,F, H,I), as opposed to a monolayer of cells seen in the control, where the cells grew extensively and attached very well to the bottom of the culture flasks (Fig. 2A,D,G). The clumping property developed gradually with continuous subculturing and clumping became more pronounced as the number of passages increased (Fig. 3A,C).

To determine if removal of the compounds from the medium would reverse the clumping, we divided the cells of the 20E line at passage 84 in 10^{-6} M 20E into two groups; one was continuously cultured in the medium with 20E and the other was cultured in the 20E-free medium for an additional 24 passages. The results indicated that the cells continued to aggregate even after 24 passages in the absence of 20E (Fig. 3A,B). Similar results were observed in the RH-5992 line. Cells that were cultured in RH-5992 for 75 passages were subcultured in RH-5992-free medium for another 25 passages and the cells still continued to aggregate (Fig. 3C,D). These results indicate that once the clumping property developed, subculturing in ecdysteroid-free medium did not reverse this feature. No significant difference was found in either the number or size of aggregates between the ones that were cultured continuously in the presence of these compounds and the ones from which the compounds were withdrawn after more than 75 passages in the presence of the compounds (data not shown).

Effects of RH-5992 and 20E on Cell Numbers

To further investigate if the 20E and RH-5992 lines have developed adapted resistance to the compounds, we studied the effects of these two compounds on cell number. We counted the number of attached cells of these two lines in the presence of 10^{-6} M 20E or RH-5992. The results indicated

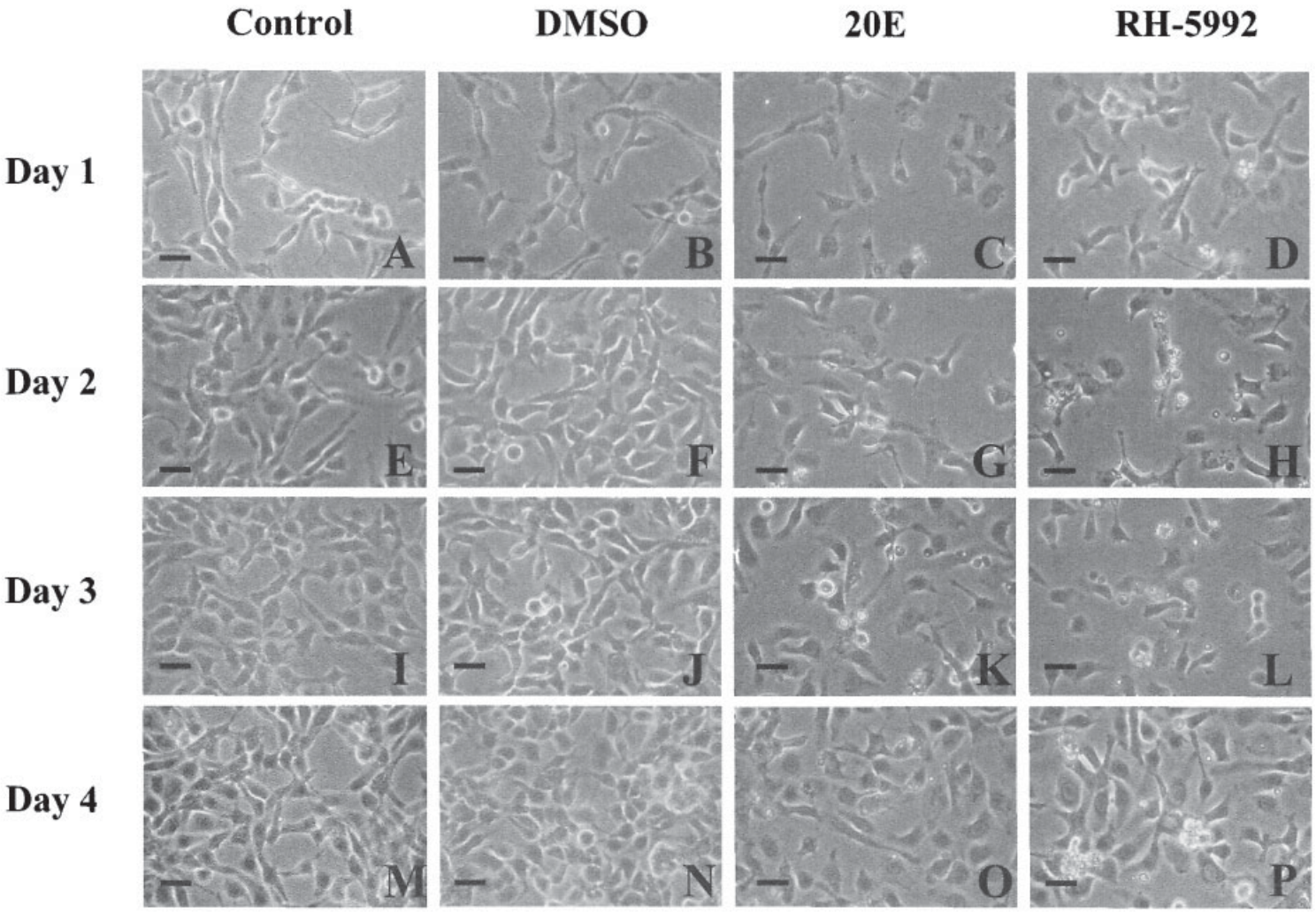

Fig. 1. Phase contrast photomicrographs of CF-203 cells exposed to X-Press medium only (**A, E, I, M**), X-Press medium plus DMSO (**B, F, J, N**), X-Press medium plus 10^{-6} M 20E (**C, G, K, O**) and X-Press medium plus RH-5992 (**D, H, L, P**) for 4 days. All photos have the same magnification. The bars = 60 μm.

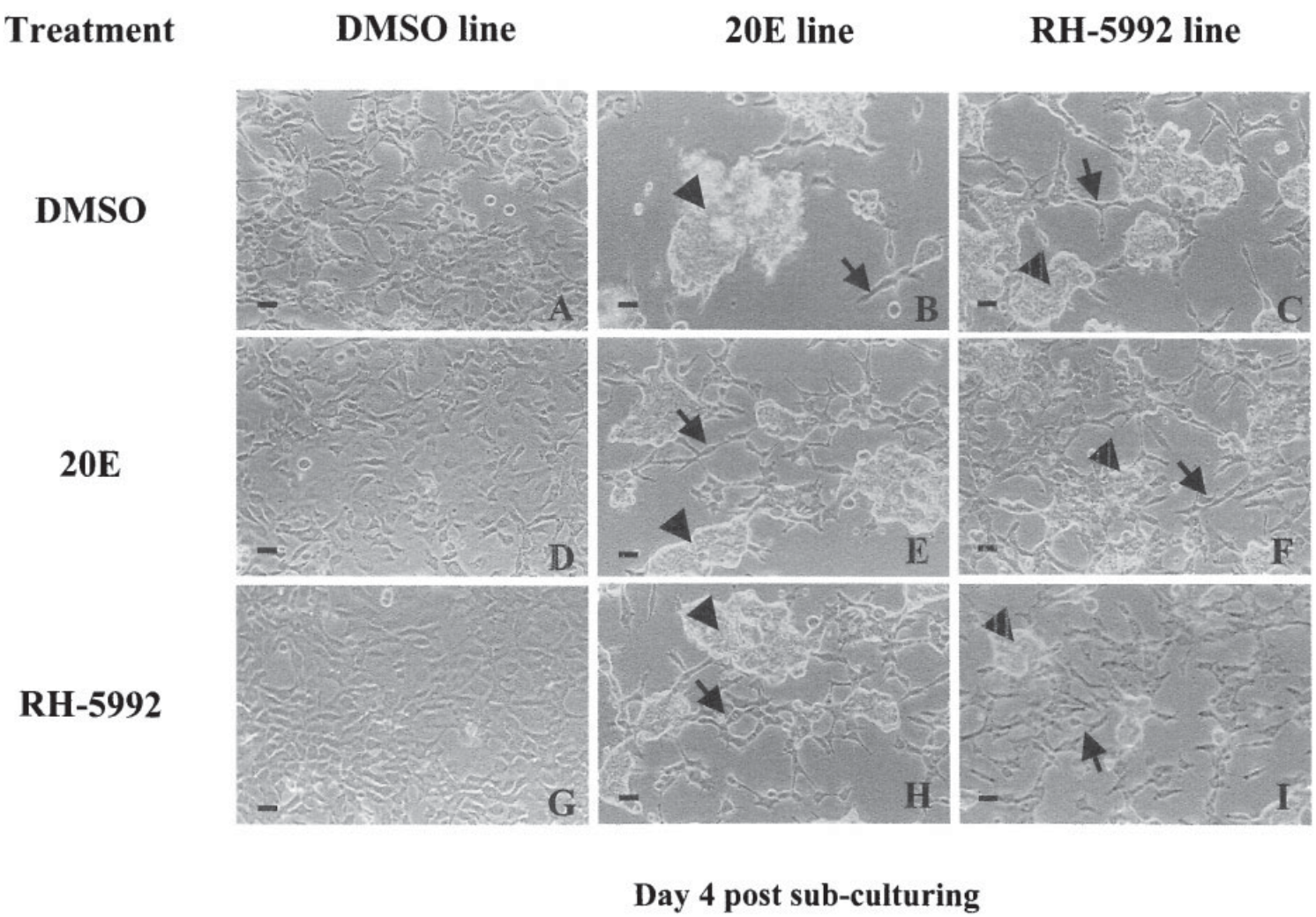

Fig. 2. Phase contrast photomicrographs of CF-203 cells after 60 passages in DMSO (**A, D, G**), 20E (**B, E, H**), and RH-5992 (**C, F, I**), respectively, showing cell responses to DMSO (A–C), 20E (D–F), and RH-5992 (G–I). Large clumps (arrowheads) and filamentous cytoplasmic extensions (arrows) are noted. All photos have the same magnification. The bars = 60 μm.

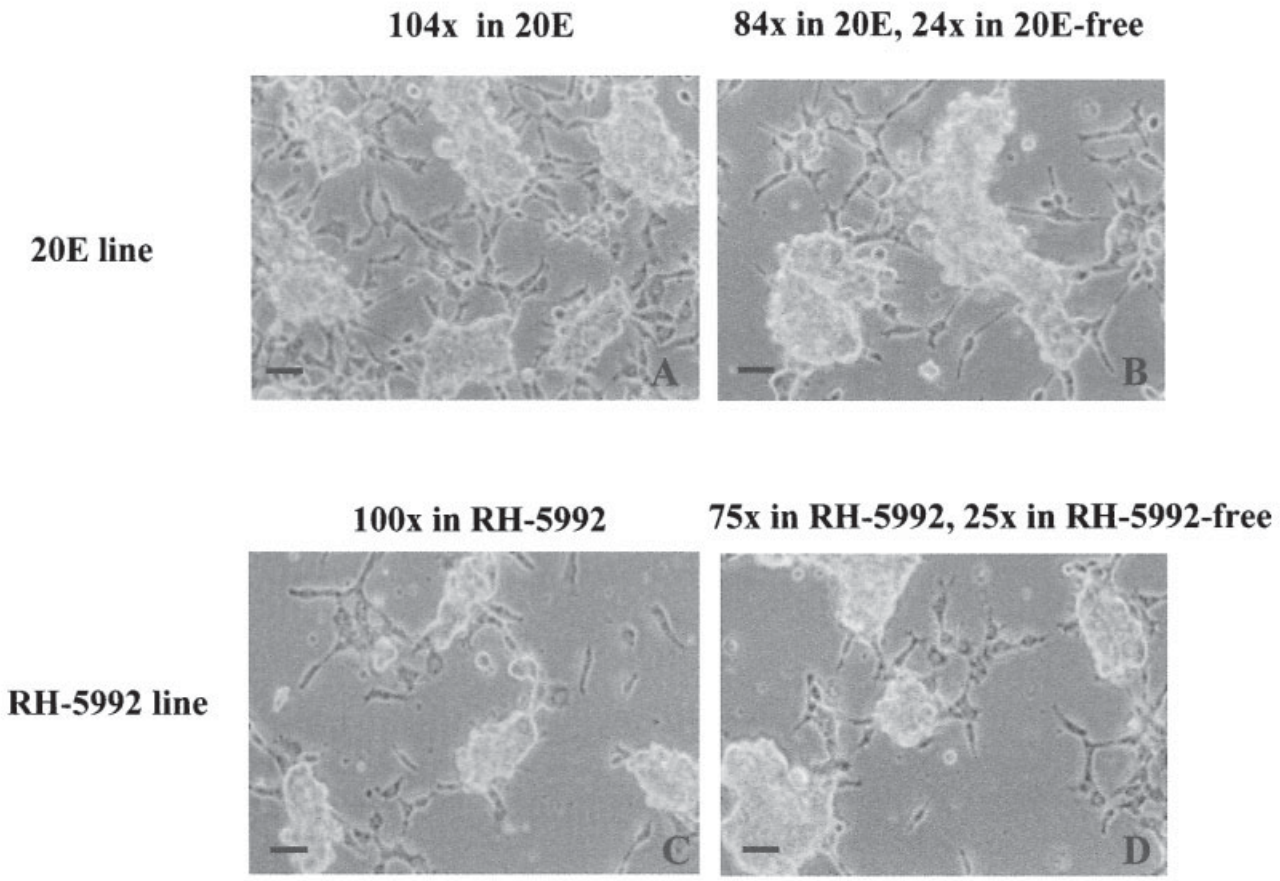

Fig. 3. Cell clumps are shown in the 20E- and RH-5992-free media after high passage numbers in media containing 10^{-6}M of 20E and RH-5992, respectively. **A:** Cells cultured continuously in the 20E medium for 104 passages; **B:** Cells cultured in 20E medium for 84 passages and then in 20E free medium for 24 passages; **C:** Cells cultured continuously in RH-5992 medium for 100 passages; **D:** Cells were cultured in RH-5992 for 75 passages and then an additional 25 passages in RH-5992-free media. All photos have the same magnification. The bars = 60 μm.

that in the DMSO line there were fewer cells in both 20E and RH-5992 treatments compared to the DMSO control on days 3 and 4 post treatment (Fig. 4A). However, RH-5992 and 20E did not significantly decrease the cell number of the RH-5992 and 20E lines at 75 passages compared to DMSO treatment (Fig. 4B,C), although the cells tended to aggregate more. We examined the dose response of RH-5992 and 20E on the cell numbers of these lines (Fig. 5). The 20E and RH-5992 lines were less sensitive to the concentrations of 20E and RH-5992 than the DMSO line, as indicated in a slower decreasing rate of cell number with the increase of the concentrations of the compounds (Fig. 5A,B).

We also determined if these clumping cells were undergoing apoptosis, a possible mechanism by which the cells on the surface of the clumps died, thereby protecting the cells inside the clumps from the toxic effects of the compounds. We monitored DNA degradation, a property of programmed cell death, of the cell clumps attached to the bottom of the culture flasks. There was no obvious evidence of DNA degradation (data not shown).

Retention of RH-5992 in the 20E and RH-5992 Lines

To investigate whether an exclusion mechanism is present in the 20E and RH-5992 lines, we examined the ability of these two adapted cell lines to retain ^{14}C-labeled RH-5992. We found that the

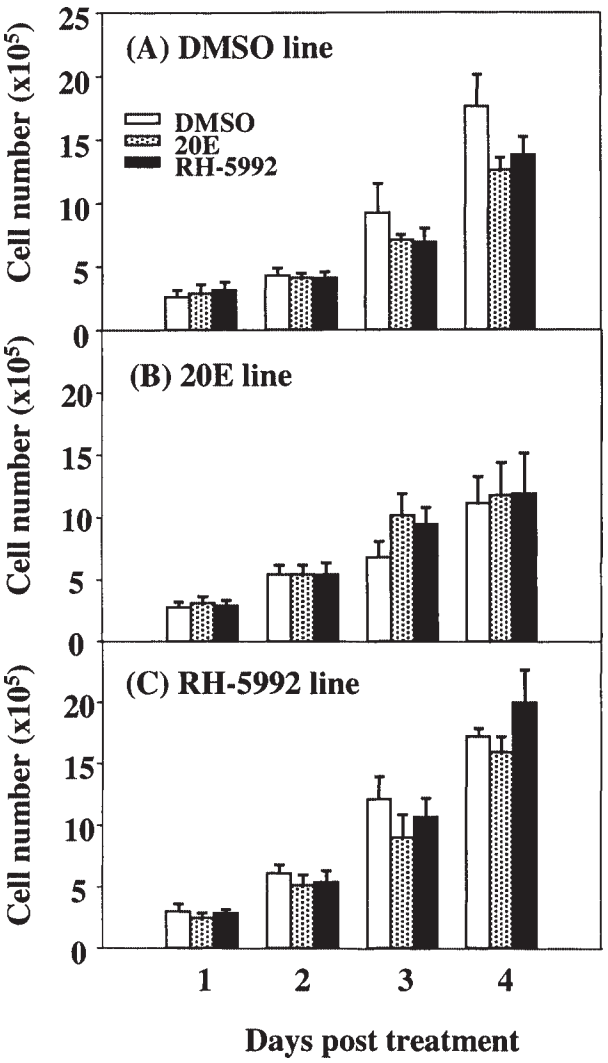

Fig. 4. Viable cell numbers of different lines in response to 10^{-6}M of 20E and RH-5992 treatments. The passage number for the 20E line, RH-5992 and DMSO line were 75, 69, and 75, respectively, and cells were counted at the indicated time post passaging. The original cell concentration in all treatments was 1.25×10^5 cells/ml in 5 ml of culture medium.

adapted cell lines retained 63–74% of the ^{14}C-labeled RH-5992 radioactivity compared to the DMSO line at the 35th passage in the 20E or RH-5992 media, respectively (Fig. 6).

Expression of CHR3 in the 20E and RH-5992 Lines

Northern blotting analyses revealed a strong CHR3 signal detected in the DMSO cell line 6 h post treatment with RH-5992, whereas no CHR3 was detected in the adapted cell lines at the 38th passage in the presence of 20E and RH-5992, suggesting that they lost the ability to express CHR3 in response to RH-5992 treatment, while the control cells showed responsiveness to RH-5992 in CHR3 expression (Fig. 7).

DISCUSSION

The present study demonstrated that the spruce budworm CF-203 midgut cell line was responsive to 20E and RH-5992 and can be used as an in vitro lepidopteran model system to study the mode of action of nonsteroidal ecdysone agonists and that morphological transformation such as aggregation became more severe after prolonged exposure to 20E and RH-5992 and this transformation was irreversible even after the ecdysteroids were removed from the medium, while the cells became less sensitive to the compounds.

The nonsteroidal ecdysone agonists, such as RH-5992, RH-0345, RH-2485, and RH-5849, have been used as insect control agents because they induce precocious, incomplete molting that leads to larval death (Binnington and Retnakaran, 1991). In vitro cell responses to ecdysteroids have been studied using a *Drosophila* Kc embryonic cell line (Courgeon, 1972; Cherbas and Cherbas, 1981). Ecdysteroid treatment of *Drosophila* Kc cell line enhances synthesis of ecdysone-inducible polypeptides, causes morphological transformation in cells, inhibits cell proliferation, and increases acetylcholinesterase activity (Courgeon, 1972; Cherbas and Cherbas, 1981; Wing, 1988). A search for an ecdysteroid responsive forest insect cell line that can serve as an in vitro model system for studying ecdysone actions was conducted using a tent caterpillar (*M. disstria*) cell line (IPRI-MD-66) and two spruce budworm cell lines (FPMI-CF-70 and IPRI-CF-1) (Sohi et al., 1995). MD-66 cell line was found to be ecdysone responsive whereas CF-70 and CF-1 lines showed little or no morphological response to the nonsteroidal ecdysone agonist and were, therefore, deemed unsuitable (Sohi et al., 1995). The results of this study indicated that the CF-203 midgut cell line responded to 20E and RH-

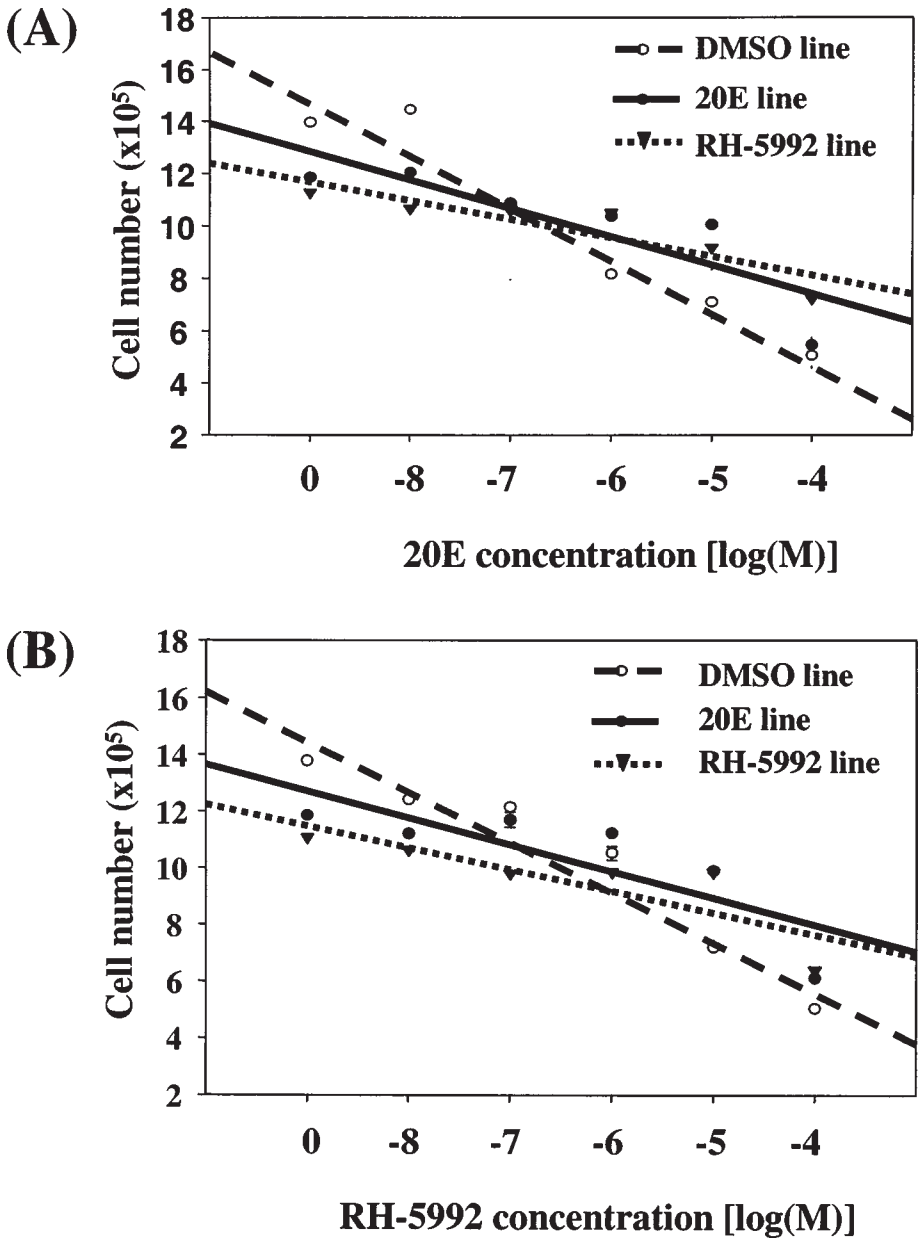

Fig. 5. Viable cells of 20E and RH-5992 lines in response to different concentrations of the compounds at 83 passages in 20E and 69 passages in RH-5992. The original cell concentration in all treatments was 1.25×10^5 cells/ml in 5 ml of culture medium. The viable cells were counted at day 4 post exposure to the compounds.

5992 by developing filamentous extensions and aggregating into clumps. Previous experiments (Palli et al., 1996; Sundaram et al., 1998) as well as this study demonstrated that this cell line is responsive to 20E and RH-5992 by expressing the transcription factor, CHR3, which can serve as a reliable assay for the early effects of both 20E and RH-5992 (Jindra et al., 1994; Palli et al., 1996, 1997). These phenotypic and molecular observations are similar to those found for the MD-66 and *Drosophila* Kc cell lines. These results confirmed that CF-203 midgut cell line could indeed be used as an in vitro model system to study the mode of action of ecdysteroid agonists. *Drosophila* Kc cell line is an embryonic cell line, while MD-66 is derived from hemocytes of *M. disstria* and CF-203 is derived from the midgut tissue of the spruce budworm but they all were responsive to 20E and nonsteroidal ecdysone agonists. This study, along with others, shows that diverse cell lines developed from different tissues and species contain the basic requirements for ecdysone responsiveness.

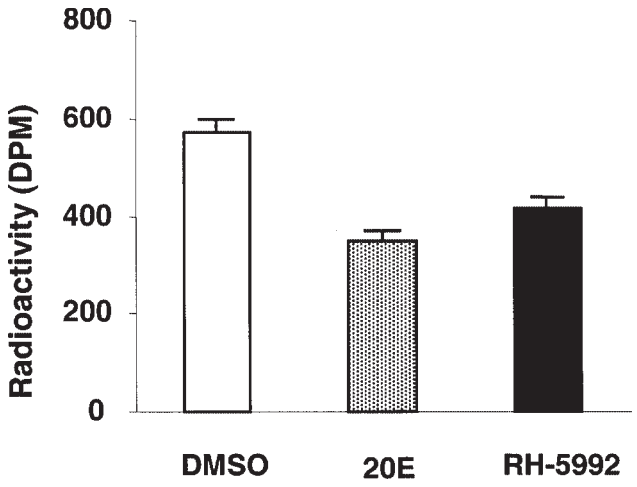

Fig. 6. Retention of ^{14}C-labeled RH-5992 in the different lines at 35 passages in the compounds. For the RH-5992 retention assay, the cells were harvested 24 h post addition of ^{14}C-labeled RH-5992. The cells were washed, lysed, and the amount of ^{14}C retained was determined as described in Materials and Methods.

Unlike 20E, RH-5992 is selectively toxic to lepidopteran larvae but is non-toxic to other insect species belonging to the Orders Diptera, Coleoptera, Homoptera, Orthoptera, and Hemiptera (Slama, 1995). Insect resistance to RH-5992 seems to develop progressively with insect development (Retnakaran et al., 2001). While this selective toxicity of RH-5992 was not due to the differential metabolic fate of the compound in susceptible lepidopteran and resistant non-lepidopteran larvae, differences in receptor affinity (Dhadialla et al., 1998; Sundraram et al., 1998) and active exclusion (Sundraram et al., 1998; Hu et al., 2001) may contribute to the selective toxicity in lepidopteran and dipteran cells. This study examined the possible resistance development by continuously culturing the cells in the presence of these compounds for a prolonged period of time. Cottam and Milner (1997) found that long-term exposure of *Drosophila* C18+ cell line to 20E caused cells to multiply faster, de-attach from the substrate and lose the tendency to aggregate at higher passages. The results of this study indicated that the morphological transformation such as aggregation became more severe after prolonged exposure to 20E and RH-5992 and this transformation was irreversible even after the ecdysteroids were removed from the medium. Similar irreversible clumping was also observed in *Drosophila* Kc cell line (Courgeon, 1972). On the other hand, the cell line became less sensitive to these compounds than the original cell line in terms of lower cell mortality and a lack of ability to express CHR3. To date, we have not determined how the cells lost their sensitivity to the ecdysteroids. Aggregation into clumps might be one of the reasons for these changes. The aggregated cells might take up less toxic compounds or actively pump out the toxic compounds as in the case of resistant DM-2 cell line (Sundaram et

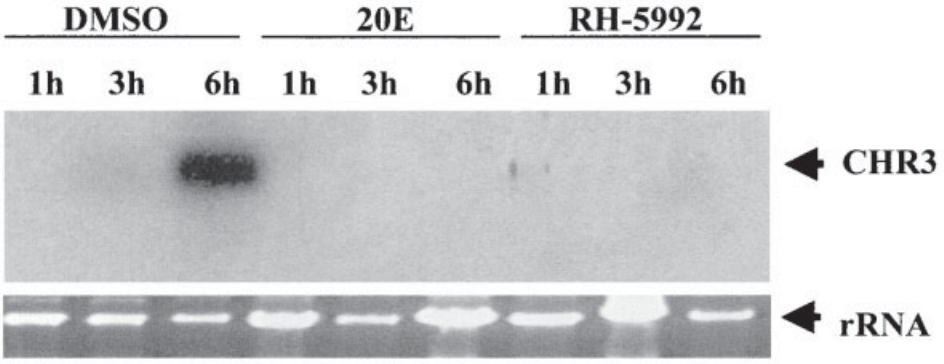

Fig. 7. Expression of CHR3 in the different cell lines at 38 passages in the compounds. The cells were sub-cultured in 20E or RH-5992-free media for 24 h and then treated with 10^{-6} M of RH-5992. Total RNA was extracted from the cells at 1, 3, and 6 h post treatments. Ten micrograms per lane of total RNA were separated on a 1% formaldehyde-agarose gel and probed using a CHR3 probe.

al., 1998), since retention of RH-5992 in the clumped cells of the adapted lines was somewhat lower than the control. Although these adapted cell lines showed some resistance to the ecdysteroid agonist, they did not grow in monolayers, but instead aggregated into large clumps, which easily floated into the culture medium making it unsatisfactory for use in resistance studies. Single and well-attached cells should be selected from the cultures at high passages for development of a suitable resistant cell line.

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