Induction of Malathion resistance in CCE/CC128 cell line of Mediterranean fruit fly (*Ceratitis capitata* (Wied.)) (Diptera: Tephritidae)

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Abstract

The CCE/CC128 cell line, derived from fertilized eggs of the Mediterranean fruit fly (*Ceratitis capitata*), was used to investigate whether insect cells in culture could develop resistance to Malathion. After 20 cycles of pulse-chase treatment (28 h exposure to 90 µg/ml of Malathion and 48 h recovery in normal medium), a Mal 90 selected population was obtained. DNA content analysis showed that the values were distributed between levels 2C and 4C and no accumulation of cells in a specific phase of the cell cycle was observed. Furthermore, preliminary molecular analysis showed no amplification of the esterase gene in resistant cells. Cross-resistance of Mal 90 cells towards other insecticides was assayed and found to be absent. Our data support the idea that the medfly cell line and, more generally, insect cell cultures, could represent a promising system to investigate insecticide resistance mechanisms.

Introduction

The possibility of using established insect cell cultures in bioassays for screening the activity of insecticides and other xenobiotics has been previously suggested (Baeza-Squiban et al., 1989; Yanangimoto et al., 1990). Bioassays performed with these biological systems offer some advantages, since they are less time consuming and less expensive than those carried out with insect strains. However, the relation between *in vivo* and *in vitro* results still needs to be clarified. In this work we explored the possibility of using an established insect cell line to describe the onset of resistance to Malathion, a well-known organophosphorus insecticide, and test cross-resistance to other insecticides. In addition, cell cycle characteristics were defined and DNA content of both susceptible and resistant cells was determined to investigate the nature of resistance.

The economic and environmental importance as well as the extent of insecticide resistance (Georghiou and Lagunes-Tejeda, 1991) make it a high impact subject in applied entomology. The possibility of preventing or, at least, delaying the occurrence of insecticide

resistance requires better understanding of the biochemical and molecular mechanisms by which it is regulated. We believe that insect cell lines could offer a powerful tool for this kind of investigations.

Materials and methods

Cell line. The CCE/CC128 is an established cell line derived from fertilized eggs of the Mediterranean fruit fly, *Ceratitis capitata* (Cavalloro, 1981). Cells were grown in Schields and Sang medium (Flow Chemicals, Irvine, IR) supplemented with 10% of heat inactivated (30' at 57 °C) fetal calf serum. At 25 °C the cells double in number in about 24 h.

Treatment-cycles. Cells were seeded at a density of 1.5×10^6 cells/25-cm² flask. After 24 h, Malathion (90 µg/ml) was added to the culture medium. 48 h later, the cells were counted, reseeded and grown in normal medium for further 48 h (Mal 90–1 population). The treatment-cycle was then applied to CCE/CC128 cells up to 20 times (Mal 90–20 popu-

lation). Cell samples from different treatment-cycles were stored in liquid nitrogen. The number of cell doubling per treatment-cycle was assumed as a measure of cell proliferation, and calculated according to the formula:

$$CD = \frac{\ln(N_f/N_i)}{\ln 2}$$

where CD = cell doubling number, N_f = final number of cells; N_i = initial number of cells (Rainaldi et al., 1991).

In this work, we will define the unselected cells as the wild type (or WT) population, and the population derived from selection with Malathion as the Mal 90 cell population.

Resistance assays. In order to evaluate the plating efficiency of cells, 2×10^2 Mal 90-5, -6, -9, -18 and -20 cells were seeded in 25-cm² flasks. After 24 hr, the cells were exposed to different doses of Malathion (30, 90 and 180 μ g/ml) for 48 h. They were then allowed to grow in the control medium. After 20 days, the colonies were stained with methylene blue solution 0.5% (w:v) and counted. In each experiment, the control was treated with ethanol.

Genome DNA content and cell cycle analysis. Flow cytometer analysis of genome DNA content of cells was performed with an Epics Elite flow cytometer (Coulter Electronics, Hialeah, FL, USA). Three million cells each of wild type and Mal 90-20 were fixed in ethanol, stained with propidium iodide in combination with ribonuclease digestion (using the Coulter DNA-Prep reagents) and the DNA histogram was recorded.

Slot-blot analysis. High molecular weight DNA from wild type and Mal 90-20 cells was prepared by using a salting out procedure (Miller et al., 1988). Dilutions of each DNA sample were loaded on nylon filter in the minifold II slot-blot apparatus. Then the filter was probed with the heterologous E4 esterase cDNA of *Myzus persicae* (Field et al., 1988). The probe was labeled by a non-isotope method involving random priming and subsequent detection by chemiluminescence using the conditions recommended by the supplier (Boehringer Mannheim, Germany).

Cross-resistance bioassays. These bioassays were performed in parallel on wild type and Mal 90-20 cells. The method was the same as described in resistance bioassays for Malathion, but in this case the

Figure 1. Cell doubling after 48 h of the CCE/CC128 cell line during the selection with Malathion. Data were collected from Mal 90-6 to Mal 90-20 cell populations and expressed as moving averages of the next four values.

insecticides tested were Dieldrin, Propoxur and Allethrin. The control run for each experiment was treated only with the insecticide solvent.

Chemicals. Technical grade Malathion, Dieldrin, Propoxur and Allethrin were provided by SCAM Limited (Modena, Italy). Immediately before use, technical grade Malathion and Propoxur were diluted in absolute ethanol (1:10) while Dieldrin and Allethrin were diluted in acetone.

Results

Selection with Malathion. Exponentially growing CCE/CC128 cells undergo two cell doublings during 38 hr of growth. From preliminary experiments we observed that the presence of Malathion in culture medium dose-dependently reduced cell proliferation ability. At the dose of 90 μ g/ml, less than 20% of cells divided. This dose was used throughout the experiment (data not shown). Results of selection are shown in Figure 1. In early treatment-cycles, 0.30 cell doublings were observed instead of the expected 2. Subsequently, in late treatment-cycles, a progressive increase in cell doublings was observed, peaking at 1.9 doublings in the Mal 90-18 population.

Malathion resistance assays. Cell susceptibility to Malathion was measured as ability to form colonies in medium containing increasing doses of Malathion (30, 90 and 180 μ g/ml). Since Mal 90-5, -6 and -9 cells had the same sensitivity to the chemical ($p < 0.05$ by

Figure 2. Cell survival $\pm$ SE at different doses of Malathion and different treatment-cycles.

Table 1. Percentage distribution ($\pm$ S.E.) of Wild Type and Mal 90–20 cells in different phases of cell cycle

Cell population	G1	S	G2
Wild Type	40.3 $\pm$ 0.4	53.9 $\pm$ 3.0	5.8 $\pm$ 3.4
Mal 90–20	51.2 $\pm$ 0.8	39.0 $\pm$ 9.6	9.8 $\pm$ 8.8

Student's t-test), values were pooled and the resulting curve was compared to that obtained for the wild type (Figure 2). These two curves overlapped almost completely. In late treatment-cycles survival data obtained with Mal 90–18 and Mal 90–20 cells were pooled since they were similar ($p < 0.05$ by Student's t-test). These cells were able to form colonies in the medium containing 90 μ g/ml of Malathion (Figure 2). These results indicated that after 20 treatment-cycles Mal 90 cell population was capable of proliferating and forming colonies in medium containing 90 μ g/ml of Malathion to exactly the same extent as the unselected population in normal medium.

Genome DNA content. Mal 90–20 cells were analysed for their nuclear DNA content. Genomic DNA content was distributed between values of 2C and 4C, with percentage of cells in different cell phases being similar to those of wild type cells (Table 1). The data are averages of three replicated assays. A two-way ANOVA applied to the values revealed no significant difference between wild type and Mal 90–20 cells.

Slot-analysis. Because the esterase gene was found to be amplified in several organophosphorous resistance cases (Mouchès et al., 1986; Field et al., 1988), the gene copy number was measured in Mal 90–20 and

Figure 3. Slot-blot of cellular DNA of wild type and Mal 90–20 populations. Hybridization signals were obtained with three different quantities of DNA. The probe used was the heterologous E4 esterase cDNA of *Myzus persicae*.

wild type cells (Figure 3). The hybridization signals were proportional to the amount of DNA and similar in both cell populations, suggesting that the gene copy number of esterase gene were not affected by the treatment.

Cross-resistance bioassays. The experiment performed to test the hypothesis that Mal 90–20 cells could be cross-resistant to Dieldrin, Propoxur and Allethrin gave the results shown in Figures 4A, B and C. Statistical comparison between results for each treatment dose did not show a univocal trend: thus in assays with Dieldrin, the wild type cells were significantly more resistant than Mal 90–20 cells at the doses of 8 and 10 μ g/ml. However, at 4 μ g/ml no differences between the wild type and the Mal 90–20 cells were found ($p < 0.05$ by Student's t-test). In treatments with Propoxur cells were less susceptible than the wild type cells ($p < 0.05$ by Student's t-test) only at 20 μ g/ml. In bioassays with Allethrin significant differences were found at the doses of 2 and 5 μ g/ml, with wild type cells less susceptible than Mal 90–20 cells ($p < 0.05$ by Student's t-test).

Discussion

In field conditions the Mediterranean fruit fly is commonly controlled by insecticides. Despite the frequent chemical treatments applied to prevent damage caused by this insect, no cases of resistance have been detected in the field. However, the medfly developed resistance to some insecticides when artificially selected in the lab (Orphanidis et al., 1980; Koren et al., 1984). These findings suggest that the medfly has genetic potential for developing resistance to insecticides. In this

Figure 4. Results of bioassays performed with three different insecticides on WT and Mal 90 cells. (A) Bioassays with Dieldrin; (B) Bioassays with Propoxur; (C) Bioassays with Allethrin.

study we demonstrated the development of Malathion resistance in an established cell line of *Ceratitis capitata*. The CCE/CC128 cell line was challenged with 20 pulse-chase Malathion treatment-cycles. The evidence that Mal 90–20 cells had acquired resistance to Malathion was obtained by plating efficiency assays. At the

end of selection, colony formation by the Mal 90–20 population in Malathion-containing medium was as efficient as in normal medium. Quantitative analysis of genome DNA showed that Malathion did not select cells with abnormal DNA content or altered cell cycle. Gene-dosage effects were also investigated. It is well-known that amplification of an esterase gene has been found responsible for organophosphate resistance in several insects (Mouchès et al., 1986; Field et al., 1988). However, the quantitative analysis of hybridization signals revealed that no amplification occurred in the esterase gene of Mal 90–20 cell population.

Cross-resistance experiments did not give a univocal result. Dieldrin weakly affected cell doubling of wild type cells, while it heavily affected doubling in the Mal 90–20 population at the highest doses. On the other hand, Propoxur was toxic for both the control and Mal 90–20 cells except at the highest dose, where Mal 90–20 cells were more resistant than WT cells. Allethrin was more toxic for the Mal 90–20 cell population at 2 and 5 $\mu\text{g}/\text{ml}$ than to WT cells. These results suggest that the Malathion resistance mechanism did not confer the ability of surviving the tested compounds.

Conclusions

Experimental evidence collected so far showed that induction of Malathion resistance was possible in an established cell line of *Ceratitis capitata*. Further work needs to be done to clarify the molecular and biochemical mechanisms of insecticide resistance in CCE/CC128 cells. In spite of these considerations, it seems reasonable to suggest that insect cell cultures may represent an interesting new tool in studies on insecticide resistance. The easy handling and rapidity of response of the cell culture system could allow their use as fast predictive method to forecast the development of insecticide resistance, thereby offering exciting opportunities in future studies on resistance. The next step in this direction could be the validation of results obtained in the cell line with the data collected on insect strains.

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