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Responses of Cell Cultures to Insecticides. II. Chronic Toxicity and Induced Resistance.* (30476)

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Residues of agricultural insecticides are present in the human food supply. Studies in animals have indicated that a regular intake of small amounts of insecticides leads to accumulation of some compounds in animal tissues(1,2,3). DDT and dieldrin have been found in human fat(3). The significance of these findings is not clear but they suggest a potential hazard to human health.

To evaluate the effects of insecticides on human cells, some organophosphorus, organochlorine, and dinitrophenol insecticides have been studied in human cell cultures. In Chang liver and HeLa cells, the insecticides induced cell destruction, and their relative cytotoxicity has been determined(4). As a continuation of the previous studies, this report describes the chronic toxicity of insecticides to human and mouse cell cultures.

Materials and methods. Cell cultures. HeLa cells derived from cervical carcinoma were cultivated in Eagle's basal medium(5) supplemented with 10% calf serum and L-929 mouse cells derived from subcutaneous tissue, in Medium 199(6) supplemented with 10% horse serum. In all tests the media contained 100 units/ml penicillin and 100 μg/ml streptomycin.

For the chronic toxicity tests, cells were suspended in their respective media at 100,000 cells/ml and 5 ml volumes were planted in Falcon 25 cm^2 plastic tissue culture flasks. Insecticides were incorporated in the medium at the time of planting. The insecticide-containing medium was changed every third day or as required. When the cultures showed a full cell monolayer, cells were harvested by the trypsinization method and transplanted to new bottles in the same manner.

For testing the insecticide-treated cultures for alterations of cell susceptibility to the previously exposed insecticide, cells were grown without insecticide for 2 or 3 days, and then reexposed to varying concentrations of the compound, as described in the methods of the acute cytotoxicity test(4).

Insecticidal compounds. The insecticides investigated in this study were purified compounds: O,O,-Dimethyl S-(N-methylcarbamoylemethyl)-phosphorodithioate (Cygon®‡); O,O-Dimethyl (1-hydroxy-2,2,2-trichloroethyl)-phosphonate (Dipterex®§); O,O,-Diethyl S-(2-ethylthio) ethyl phosphorodithioate (DI-Syston®§); O,O,-Dimethyl S-(1,2-dicarbethoxyethyl) phosphorodithioate (malathion‡; 1,2,4,5,6,7,8,8-Octachloro-2,3,3a-4,7,7a-hexahydro-4,7-methanoindene (chlordane‖); 4,4'-Dichloro-α-(trichloromethyl) benzhydrol (Kelthane®¶); 2-(1-Methylheptyl)-4,6-dinitrophenyl crotonate (Karathane®").

The methods of treating the cell cultures are the same as described previously(4).

Test procedure for chronic cytotoxicity.

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TABLE I. Toxicity of Insecticides in HeLa Cells During Long-Term Exposures.

	Chronic toxicity exposures									
	1-29 Days[1]		30-51 Days[2]		52-59 Days[3]		60-70 Days[4]		71-84 Days[5]	
Insecticide	Conc, μg/ml	Toxic	Conc, μg/ml	Toxic	Conc, μg/ml	Toxic	Conc, μg/ml	Toxic	Conc, μg/ml	Toxic
Cygon	2.0	—	20	+	100	—	200	+	300	+++
Dipterex	.5	—	5	—	50	++++	25	++	10	+
Malathion	.1	+	1	—	10	—	20	—	40	+++
DI-Syston	.05	±(S)	.5	—	5	+	10	—	25	+
Chlordane	.1	+	1.0	—	5	—	10	+	20	+
Kelthane	.05	—	.5	—	5	+	10	+	20	++
Karathane	.005	—	.5	—	1	—	5	+	2	—

[1] 5 passages of cultures
[2] 9 " " "
[3] 10 " " "
[4] 11 " " "
[5] 13 " " "

+ – Up to 25% destruction
++ – 25 to 50%
+++ – 50 to 75%
++++ – More than 75%
(S) – Possible stimulation of cell growth

TABLE II. Chronic Toxicity of Insecticides in HeLa Cell Cultures.

	Chronic toxicity			Acute toxicity(4) ID_{50}
Insecticide	Non-toxic conc, μg/ml	Minimal toxic dose, μg/ml	Tolerated toxic conc, μg/ml	Chronic toxicity MTD
Cygon	2.0	20.0	100	11
Dipterex	5.0	10.0	10	2.2
Malathion	<.1	.1	20	130
DI-Syston	.5	5.0	10	1.8
Chlordane	<.1	.1	10	220
Kelthane	.5	5.0	10	4.6
Karathane	1.0	5.0	5	.8

The effects were evaluated in 2 ways. First, microscopic examination was made to determine the cytopathogenic effect. The lowest concentration of an insecticide which induced destruction or morphological alteration of 25 to 50% of the cells was expressed as the minimal toxic dose (MTD). With some insecticides, cells became gradually resistant and tolerated concentrations above the MTD levels. The highest concentration of an insecticide which was tolerated for at least 2 weeks or for 2 passages of the cultures was expressed as the tolerated toxic concentration (TTC).

The alternative method measured chronic toxicity by determination of growth inhibition. The growth of cultures was measured by determination of total cell protein per culture. The method for cell protein determination and its application for measurements of acute cytotoxicity has been described(4). Concentrations of a compound which inhibited the growth by 10% and 50% during an incubation period of 48 hours were designated as inhibitory doses, ID_{10} and ID_{50}.

Results. 1. Cytotoxicity of insecticides during chronic exposure. Cygon, Dipterex, malathion, DI-Syston, chlordane, Kelthane, and Karathane, in amounts below their acute cytotoxicity levels, developed toxic manifestations which, in general, were characterized by inhibition of growth and progressive morphological changes. The appearance and degree of cytotoxicity was dependent on the concentration and the length of the exposure periods as indicated in Table I.

The results of the chronic cytotoxicity test during the indicated exposures for a total period of 84 days are summarized in Table II. The first column indicates concentrations which did not induce any detectable signs of toxicity. The minimum toxic dose (MTD) in μg/ml at which each insecticide induced cytopathic effects and growth inhibition is: Cygon, 20; Dipterex, 10; DI-Syston, 5; Kelthane, 5; Karathane, 5; malathion, 0.1; and chlordane, 0.1. At the TTC levels, the Dip-

TABLE III. Resistance of HeLa Cell Cultures to Insecticides After Previous Long-Term Exposure.

	20-Day exposure	84-Day exposure	
	Resistance	Resistance	
Insecticide	ID_{50} treated / ID_{50} control	ID_{50} treated / ID_{50} control	ID_{10} treated / ID_{10} control
Dipterex	1.1	.8	.7
Malathion	1.5	1.1	1.7
DI-Syston	1.0	2.2	3.0
Chlordane	.7	1.3	2.5
Kelthane	1.9	.7	.7
Karathane	1.5		

terex, malathion, and chlordane cultures always showed an increased number of large, rounded, abnormal cells which contained fine cytoplasmic granules. In the DI-Syston and Kelthane cultures most cells showed large cytoplasmic granules around the nuclei. Multinucleated cells and giant cells were also present.

2. *Induced cell resistance to insecticides.* The results of altered cell susceptibility to insecticides were expressed as the ratios of the ID_{50} or ID_{10} levels of previously treated cells to the corresponding ID levels of previously untreated controls. Results are summarized in Table III. After an exposure of 20 days, according to ID_{50} values, the Kelthane culture was 1.9 times more resistant than the control; malathion, 1.5 times; and Karathane, 1.5 times. The susceptibility of the Dipterex and DI-Syston cultures was comparable to the control, and the chlordane culture was slightly more susceptible. In similar tests, after exposure for 84 days, the DI-Syston culture was 2.2; chlordane, 1.3; and malathion, 1.1 times more resistant than the controls. However, when the same cultures were compared at the ID_{10} values, the corresponding ratios increased to 3.0, 2.5, and 1.7. In contrast to the increased resistance of DI-Syston-, chlordane- and malathion-treated cells, the Dipterex and Kelthane cultures were slightly more susceptible.

The adaptation of cells to growing in the presence of an insecticide was also evident in the mouse L-929 strain. The L strain cells were originally susceptible to 10 μg/ml of Dipterex. During an exposure to 10 μg for 7 days and to 20 μg/ml for the subsequent 13 days, the cells were completely resistant to Dipterex at 60 μg/ml and some were resistant to 100 μg/ml on the 21st day. Accordingly, after 21 days the Dipterex-treated cells were 6 times more resistant to Dipterex than the control.

3. *Growth stimulatory effect of insecticides to previously treated HeLa cells.* On the 84th day the DI-Syston- and chlordane-treated cultures were subcultured and maintained without insecticides for 2 days. When the subcultures were exposed to various concentrations of the insecticides, to which they were originally exposed, growth was greater than in the control culture that had never been treated. The results showed that in the presence of 10 μg/ml of DI-Syston the growth of the normal culture was reduced by 44%, whereas in the chronically treated culture it was increased by 88%. Similarly, 10 μg/ml of chlordane inhibited the growth of the normal culture by 8%, whereas the growth in the chronically treated culture was increased by 16%.

Discussion. The chronic toxicity of insecticides to HeLa cells is characterized by growth inhibition and cell destruction. When the magnitudes of the chronic and acute toxicities are compared (Table II), it is evident that most insecticides are significantly more toxic during the chronic exposures.

During the long-term exposure to insecticides, cells gradually adapt to growing in their presence. After exposure for 84 days, the tolerated toxic concentrations are 2 to 200 times above the MTD levels. The TTC of malathion and chlordane are, respectively, 200 and 100 times the MTD. Some degree of adaptation to Cygon, DI-Syston and Kelthane is also evident, but not to Dipterex and Karathane. The ability of HeLa cells to grow in the presence of an insecticide was associated with the development of increased resistance to that insecticide. When insecticide-treated HeLa cells were subcultured without insecticides for several days and then exposed to the same insecticides at higher concentrations, the malathion, DI-Syston and chlordane cultures were 1.7 to 3.0 times more resistant than the normal cells. In a similar type of experiment with Dipterex, the mouse

L cells became 6 times more resistant during an exposure for 20 days.

The resistance of the DI-Syston- and chlordane-treated cells was associated with a growth stimulatory effect of both insecticides upon the treated cells. The effect was particularly significant in the DI-Syston-treated culture, in which growth increased by 88%, and in the chlordane culture where it increased by 16%.

The induced resistance of mammalian cell cultures to insecticides may be associated with several factors: selection of more resistant cell types present in the original culture; induced mutations in the cells followed by selection; and induction of enzyme systems which inactivate the insecticides. The fact that cell resistance was evident in cultures after the removal of DI-Syston and chlordane from the medium for 3 days allows one to postulate that the induced cell resistance is of a more than temporary nature.

The possibility of enzyme induction to explain the cell resistance is a plausible one, since such enzymes are known to be associated with the drug resistance of some microorganisms. The growth stimulatory effect of DI-Syston and chlordane upon previously treated cells also suggests a certain dependence of previously treated cells on these insecticides—a phenomenon which is similar to the well-known dependence of certain resistant bacteria to antibiotics. The theory of enzyme induction is also supported partly by reports that chlordane has stimulated drug metabolizing enzymes in the rat(7).

This study has shown that chronic exposure of cell cultures to insecticides is associated with development of resistance.

Summary. HeLa cell cultures were exposed to subtoxic concentrations of insecticides for 84 days. All insecticides tested induced cytotoxicity which was characterized by growth inhibition and cell destruction. The minimal toxic doses (MTD) in μg/ml were: Cygon 20, Dipterex 10, DI-Syston 5, Kelthane 5, Karathane 5, malathion 0.1, and chlordane 0.1. All insecticides, except Karathane, were significantly more toxic in the chronic than in the acute cytotoxicity tests. On continued exposure, the cells gradually adapted and grew in the presence of the insecticides at concentrations 2 to 200 times above the MTD. After cessation of exposure the malathion-, chlordane-, and DI-Syston-treated cultures were 1.7 to 3.0 times more resistant to the same insecticide than the untreated control cells. The induced resistance to DI-Syston and chlordane was associated with a growth stimulatory effect of each insecticide on the respective chronically treated culture. It is postulated that the cell resistance to insecticides may be associated with cell mutations or induction of enzyme systems capable of inactivating or metabolizing the insecticides.

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