Responses of Cell Cultures to Insecticides. III. Altered Susceptibility to Poliovirus and Diphtheria Toxin.* (30477)

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The previous report of this series described alterations of cultivated human and mouse cells during their chronic exposure to insecticides(1). The observed cell alterations were characterized by morphological changes, increased resistance to insecticides and growth stimulation of the cells by some insecticides in previously treated cell cultures. The results suggested that there were alterations in cell physiology and, accordingly, possible changes in susceptibility of chronically treated cells to other biologically active agents such as mammalian viruses and bacterial toxins.

To investigate this hypothesis, one group of the insecticide-treated HeLa cells was infected with poliovirus and another group was submitted to reaction with diphtheria toxin. HeLa and many other cell strains are known to be highly susceptible to poliovirus and diphtheria toxin. Some "altered" cell strains have been reported, however, to be unaffected by very high levels of diphtheria toxin(2,3). This evidence served as a basis for a study aimed at detecting additional alterations in the insecticide-treated cells using virus and toxin as indicator systems.

Materials and methods. Cell cultures. The characteristics of the insecticide-treated HeLa cultures, and the experimental procedures to determine chronic toxicity have been described in the previous report(1).

The insecticide-containing HeLa cultures were maintained by serial passages over a total period of 108 days. On the 42nd day, at the time of the 8th passage of the cultures, and on the 77th day at the 12th passage, the insecticide-treated cells were subcultured in order to test the cell susceptibility to poliovirus infection. The subcultures for the diphtheria toxin tests were derived on the 84th day and 108th day using the 13th and 16th passage cultures, respectively. For both tests, the subcultures were grown without insecticides for 2 or 3 days. When cell monolayers had formed, the cultures received fresh medium with poliovirus or diphtheria toxin.

Insecticidal compounds. The insecticides investigated were purified compounds: O,O,-Dimethyl S-(N-methylcarbamoylemethyl)-phosphorodithioate (Cygon®‡); O,O-Dimethyl(1-hydroxy-2,2,2-thichloroethyl)-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 procedures of treating the cultures have been described(1,4).

Test procedure for poliovirus infection. Poliovirus titration tests were performed as described by Merchant *et al*(5). Poliovirus, the Mahoney strain, type I was diluted in Eagle's medium(6) containing 5% heat inactivated calf serum. Cultures were incubated for 3 days at 36°C and examined microscopically daily for the appearance of the cytopathic effect. The results were scored according to progressive cytopathic changes and expressed as the infective virus titers—$TCID_{100}$ and $TCID_{50}$. The $TCID_{50}$ was calculated by the method of Reed and Muench(7).

To investigate the capacity of viral biosynthesis in the insecticide-treated cells, virus was harvested 48 hours after infection from those groups of cultures which were infected with virus dilution containing approximately

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TABLE I. Susceptibility of HeLa Cultures to Poliovirus Infection After Long-Term Exposure to Insecticides.

Insecticide	42 Days exposure		77 Days exposure	
	Titer $TCID_{100}$ difference from control (log_{10})	Degree of virus cyto-pathogenicity	Titer $TCID_{100}$ difference from control (log_{10})	Degree of virus cyto-pathogenicity
None (control)	—	++	—	++
Cygon	.0	++	+1.0	++++
Dipterex	.0	+	+1.0	++++
Malathion	.0	++	.0	++
DI-Syston	+1.0	+++	+1.0	++++
Chlordane	+1.0	+++	+1.0	++++
Kelthane	+1.0	++++	.0	+
Karathane	.0	+	+1.0	++++

\+ – Definite morphogenic changes
++ – 50% cell degeneration
+++ – 75% " "
++++ – 100% " "

10 $TCID_{50}$, as determined by previous titration in normal HeLa cells. The harvested poliovirus progeny was titrated in normal HeLa cultures using the tube titration method described above.

Test procedure for diphtheria toxin. The diphtheria toxin (crude) preparation used contained 3000 guinea pig minimal lethal doses (MLD) per ml. For testing, toxin was diluted in Medium 199(8), containing 100 units/ml penicillin and 100 μg/ml streptomycin. The toxin titration tests were performed as described by Gabliks and Solotorovsky(3). Growth medium was removed from cell cultures and replaced with 1.0 ml of Medium 199 containing the toxin at various concentrations. The cultures were incubated at 37°C for 2 days. The degree of toxicity in individual cultures was evaluated according to a scale based on the ratio of destroyed to intact cells. The highest dilution of toxin causing destruction of 50% of cells during an incubation period of 2 days was designated as the toxic dose 50% (TD_{50}). To prove that the lethal effects upon the cells were due to the specific action of diphtheria toxin, toxin neutralization was performed by adding 0.27 unit of diphtheria antitoxin, an amount sufficient to neutralize completely the highest concentration of toxin used. The toxin and antitoxin mixtures were incubated at 25°C for one hour and then added to the cell cultures to determine their cytotoxicity.

Results. 1. Susceptibility to poliovirus infection. All insecticide-treated HeLa cultures were susceptible to the lethal infection of poliovirus, but the virus infectivity titers varied in the treated cultures. The differences in the $TCID_{100}$ titers are listed in Table I. Cells exposed to insecticides for 42 days showed 1.0 log_{10} higher titer in the DI-Syston-, chlordane-, and Kelthane-treated cultures. When retested on the 77th day, more treated cultures showed a higher titer: Cygon, Dipterex, DI-Syston, chlordane, and Karathane. The $TCID_{100}$ titers in the malathion and Kelthane cultures were comparable to that of the control. All treated cultures which showed a higher infectivity titer than the control also degenerated more rapidly.

Titration of viral progeny, harvested 48 hours after infection from cultures infected with 10 $TCID_{50}$ of poliovirus, showed that all insecticide-treated cells synthesized virus. Although the exact titers were not determined, the test indicated that the viral biosynthesis in the insecticide-treated cells was not significantly affected.

2. Susceptibility to diphtheria toxin. The results of diphtheria toxin titraton in HeLa cell cultures after their previous exposure to insecticides for 77 days and 108 days are summarized in Table II. Diphtheria toxin generally induced morphological changes leading to destruction of the cells. In both tests, the Karathane-treated cells were 10 times more resistant to the action of diphtheria toxin than the normal HeLa cells. The cytotoxicity induced by diphtheria toxin was pre-

TABLE II. Susceptibility of HeLa Cultures to Diphtheria Toxin After Long-Term Exposure to Insecticides.

Insecticide	Exposed to insecticides 77 Days	108 Days
	TD_{50}, MLD/ml*	
None (control)	.5	.5
Cygon	.1	.5
Dipterex	.5	.5
Malathion	1.0	.5
DI-Syston	.5	.5
Chlordane	.5	.5
Kelthane	1.0	.5
Karathane	5.0	5.0

* MLD titer in guinea pigs.

vented by addition of diphtheria antitoxin.

Discussion. HeLa cell cultures are susceptible to poliovirus infection. An infection with virus induces viral biosynthesis and leads to degeneration and destruction of the infected cells. Since the reactions of viral biosynthesis depend upon the metabolism of the host cell, an alteration in the metabolism may affect the mechanism of viral synthesis and the cell susceptibility. The results of both virus titration tests indicated that all insecticide-treated cultures were susceptible to the lethal action of poliovirus, but the infectivity titers varied. The $TCID_{100}$ titers in the Cygon-, Dipterex-, DI-Syston-, chlordane-, and Karathane-treated cultures were 1.0 $\log_{10}$ higher than in the control.

The virus yields from the infected cultures were not fully investigated. The titration of viral progeny harvested 48 hours after infection indicated that all insecticide-treated cells synthesized viruses, but the amounts differed from those in untreated cells.

The greater susceptibility of some of the insecticide-treated cultures to poliovirus infection cannot be explained with the data collected in this study. It is not likely that the observed cell alterations with all insecticides are based on impairment of the same mechanism. One analogous situation, although it does not involve insecticides, has been reported with 20-methylcholanthrene (MC) by De Maeyer and De Maeyer-Guignard(9). They showed that vaccinia and Sindbis virus plaques were consistently larger in cultures containing 20-MC than in those without, whereas the yield of virus was not significantly different. This effect was due to the inhibition of interferon production.

One can only speculate about the significance of these observations with respect to the possibility of similar changes taking place in animals during chronic exposure to insecticide residues.

The results of 2 diphtheria toxin titrations in the insecticide-treated cultures showed that the Karathane-treated HeLa cells were 10 times more resistant than the normal ones and suggest the possibility that Karathane induced cell mutations. The possibility of cell mutation is partly supported by the following evidence. Previous investigations indicated that a large variety of primary and established cell cultures derived from susceptible or resistant host species maintain the sensitivity or resistance observed for whole animals(3). Under certain conditions, generally susceptible cultures may become resistant(2,3). Accordingly, Lennox and Kaplan suggested the possible use of diphtheria toxin as a genetic marker of some cultivated cell lines(2).

In surveying these results and those from the chronic toxicity study describing the induced cell resistance to insecticides(1), the DI-Syston-, chlordane-, and Karathane-treated cells showed altered reactivity in 2 or more indicator systems, and, accordingly, indicate some definite changes in the physiology of the cells induced by these insecticides. The Karathane-treated cells were more susceptible to poliovirus and more resistant to diphtheria toxin, but were unaltered in their susceptibility to Karathane. The DI-Syston- and chlordane-treated cells were more susceptible to poliovirus, more resistant to their respective insecticides, and their growth was stimulated in the presence of the corresponding insecticides, but they were unaltered in susceptibility to diphtheria toxin. The Kelthane-treated cells appeared unchanged in these 3 testing systems.

This study has shown that chronic exposure of cell cultures to insecticides induces cell alterations which affect the cell reactivity to 2 biologically active agents—poliovirus and diphtheria toxin.

Summary. HeLa cell cultures were main-

tained in the presence of insecticides for periods up to 108 days. At certain intervals the treated cells were subcultured without insecticides and then infected with poliovirus or submitted to reaction with diphtheria toxin. The Cygon-, Dipterex-, DI-Syston-, chlordane-, and Karathane-treated cultures were more susceptible to poliovirus infection and the infected cells degenerated more rapidly. The malathion-treated cells did not show any change in susceptibility. The Karathane-treated cells were 10 times more resistant to the lethal action of diphtheria toxin than the control. Other treated cultures showed no changes with respect to diphtheria toxin reactivity. The results indicate some alterations in physiology of the insecticide-treated cells which affect the cell response to 2 other biologically active agents—poliovirus and diphtheria toxin.

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Dietary Saturated Medium-Chain Triglycerides and Vitamin E Deficiency in Chicks and Rats.* (30478)

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In the course of a study of the effects of dietary saturated medium-chain triglycerides (C_6-C_{12}; henceforth MCT) on serum cholesterol levels, it was noted that these triglycerides had opposite effects on chickens and rats. The levels in the latter were lower, those in the former higher than in controls(1).

During these studies exudative diathesis was occasionally noted in the chickens fed MCT. This was remarkable because the basic diet contained some added alpha tocopherol, and more than twice the amount of soybean meal, which had been found by Nesheim and Scott(2) to be effective against the occurrence of exudative diathesis; also, the diet was low in polyenoic acid, feeding of which has been shown to promote exudative diathesis by Dam *et al*(3). In view of the fact that exudative diathesis is one of the main symptoms of vit E deficiency in chickens, we decided to study the influence of MCT on the deficiency state in this species and to contrast its effects with those of tocopherol-deficient rats. Such a study seemed interesting not only because of the influence of MCT on cholesterol metabolism but also because MCT has been shown to be capable of counteracting some nutritionally induced disease states in the rat, particularly the consequences of essential fatty acid deficiency(4).

Materials and methods. Chickens: Groups of 15 to 25 male Leghorn or Columbian-cross chicks (♀ × New Hampshire ♂) were placed on the experimental diets at one day of age for periods of 4 weeks. The fat supple-

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