APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1981, p. 652-656
0099-2240/81/030652-05$02.00/0
Vol. 41, No. 3

Enhancement of Varicella-Zoster Virus Replication in Cultured Human Embryonic Lung Cells Treated with the Pesticide Carbaryl

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In studies designed to determine the factors responsible for control of herpesvirus replication in an infected cell, we examined the interaction of varicella-zoster (VZ) virus-infected human embryonic lung cells with the pesticide carbaryl. The replication of the cell-associated VZ virus was enhanced 2- to 13-fold as compared to control cultures in Sevin 4 Oil-treated cultures and in cultures treated with the pesticide's active ingredient, carbaryl. The replication of VZ virus in cultures treated with the base oil plus inert ingredients found in the pesticide formulation was not enhanced. Possible differences in cytotoxicity induced by Sevin 4 Oil, pure carbaryl, or the base oil preparation were ruled out since treated and control cultures were shown to have similar numbers of viable cells when measured by trypan blue exclusion tests or by the ability of treated cells to form foci. A dose response study showed a decrease in viral enhancement in cells treated with decreasing carbaryl concentrations.

The interactions between viruses and environmental chemicals such as pesticides are being studied with the growing concern that these interactions may have an impact on human health. Recent epidemiological evidence has suggested a relationship between certain viral diseases, especially influenza B (3, 9), influenza A (7), and varicella, or chickenpox (3), and Reye's syndrome. Reye's syndrome is characterized by encephalopathy with fatty infiltration of the visceral organs in children (8) and is frequently fatal. The biphasic disease pattern of viral infection characteristic of Reye's syndrome seems to have an environmental factor; whereas the preceding viral infections are more prevalent in urban areas, the incidence of Reye's syndrome is higher in rural areas (3, 5, 7). Results of in vivo studies have implicated certain pesticides and emulsifiers as the possible environmental factor in the development of Reye's syndrome, and a model for the development of the disease has been proposed (4, 5). Mice pretreated with insecticide (5) or pesticide dispersal agents and emulsifiers (4) have subsequent greater mortality rates when challenged with nonlethal doses of encephalomyocarditis virus. The disease which develops in the pretreated animals is not entirely characteristic of encephalomyocarditis virus infections but has paralysis with fatty changes in the liver.

Further studies of this phenomenon have used cells growing in culture. These studies have demonstrated that a variety of commercial emulsifiers are capable of enhancing the sensitivity of cultured cells to infection by certain viruses (10, 11); not all emulsifiers could serve as enhancers and not all viruses could be enhanced by the enhancing emulsifiers. These studies suggested that the susceptible viruses were those containing single-stranded ribonucleic acid and that the enhancement of virus replication occurred by two mechanisms: (i) more virus was taken up by treated cells; and (ii) the interferon response of treated cells was reduced.

Since none of these studies used varicella-zoster (VZ) virus, the double-stranded deoxyribonucleic acid virus associated with Reye's syndrome in epidemiological studies, we designed a study to investigate the interaction between VZ virus, a member of the human herpesvirus group, and Sevin 4 Oil, a pesticide widely used for the control of spruce budworm and gypsy moth. Experiments were designed to determine whether Sevin 4 Oil was capable of enhancing the production of VZ in human cells and if so which component or components of the pesticide formulation was responsible for the enhancement.

MATERIALS AND METHODS

Cell cultures. Primary human embryonic lung (HEL, Flow 2000) cells were obtained from Flow Laboratory, McLean, Va. Stationary cultures were grown and maintained in Eagle minimal essential medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 0.08% $NaHCO_3$, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (growth medium).

Virus. Two separate strains of wild-type VZ virus were isolated in HEL cells from independent clinical

specimens provided by Mary Dietrich, Cutler Health Center, University of Maine, Orono. The virus was maintained as cell-associated virus in HEL cells by using infected cells as the inoculum. Stocks were stored at −80°C.

Pesticide and components. Complete Sevin 4 Oil, analytical grade carbaryl (99.9% pure), and base oil plus inert ingredients were provided by Union Carbide. Working dilutions of pesticide and components were made in Eagle minimal essential medium containing 10% fetal bovine serum.

Toxicity studies. Levels of toxicity of tested compounds for HEL cells were determined as follows. HEL cells grown to monolayers in 25-cm^2 tissue culture flasks were flooded with 5 ml of various dilutions of pesticide or component and incubated at 37°C for 18 h using a method previously described by Rozee et al. (11). Cultures were then examined using phase-contrast microscopy for signs of cellular toxicity (vacuolization, granularity, cell detachment).

Viability studies. Treated monolayers were trypsinized, centrifuged at 1,000 rpm for 10 min, and suspended in 1 ml of growth medium. The number of viable cells was determined using the trypan blue dye exclusion test. Samples were then plated in 10-fold dilutions by adding 0.1 ml of each dilution to 60-mm tissue culture dishes containing 5 ml of growth medium. Plates were incubated at 37°C in a humidified atmosphere containing 10% CO_2 until individual foci of cells could be counted (48 h). Plates were then stained with crystal violet and foci were counted.

Viral enhancement assay. HEL cells were grown to monolayer cultures in 25-cm^2 tissue culture flasks and exposed in duplicate to pesticide or components in subtoxic concentrations for 18 h at 37°C. Control cultures were incubated in the presence of growth medium alone. After 18 h, all monolayers were washed three times with sterile phosphate-buffered saline, refed with growth medium, and infected with VZ virus. Cultures were then incubated at 37°C until viral infectious centers were microscopically visible (3 to 5 days). Each duplicate culture was assayed independently.

Virus titration. Infected cultures were trypsinized, centrifuged at low speed for 5 min, and suspended in 1 ml of growth medium. Tenfold dilutions of each sample were made, and 0.1 ml of each dilution was added to new HEL monolayers in 35-mm^2 tissue culture dishes. When foci of infected cells were visible, the plates were washed with phosphate-buffered saline and stained with crystal violet, and the plaques or infectious centers were counted.

RESULTS

Cytotoxicity of Sevin 4 Oil and its components in HEL cells. Toxicity studies were performed to determine the optimal concentration of each compound to be used in viral enhancement assays. A summary of tested compounds is given in Table 1. Carbaryl is the active ingredient in Sevin 4 Oil. The remainder of the pesticide formulation consists of 46.3% petroleum oil and 4.7% inert ingredients provided to us as "base oil plus inert ingredients."

Stock solutions to approximate 100 ppm of each compound were made in growth medium. Since complete Sevin 4 Oil and base oil contain a high concentration of petroleum oil which is probably not soluble in our medium, 100 ppm was the maximum concentration, based on the total amount of each compound added to the stock solutions, assuming that the compound is totally soluble. Actual carbaryl concentrations of undiluted stock solutions were determined by gas chromatography kindly performed by E. Richardson, Maine Public Health Laboratory. These values (given in parentheses in Table 2) demonstrate that carbaryl, the one component that could be assayed directly, was not totally soluble; the actual solubility for other individual components of stock solutions could not be determined.

Monolayers of HEL cells in 25-cm^2 tissue culture flasks were flooded with various concentrations of each compound and incubated at 37°C for 18 h. Cell were then examined using phase-contrast microscopy for vacuolization, granularity, and cell detachment. The results of this

TABLE 1. *Summary of tested compounds*

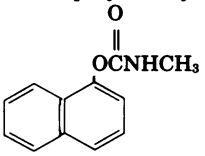
Pesticide or component	Chemical nature
Carbaryl	1 Napthyl methylcarbamate 
Base oil plus inert ingredients	Unknown
Sevin 4 Oil	49.0% Carbaryl 46.3% Petroleum oil 4.7% Inert ingredients

TABLE 2. *Toxicities of Sevin and components in cultures of HEL cells*

Compound	Toxicity[b]					
	Stock solution	1:2[c]	1:4	1:10	1:20	1:100
Sevin 4 oil (8.45)[a]	+	−	−	−	−	−
Base oil plus inert ingredients (0)	+	−	−	−	−	−
Carbaryl (11.78)	+	+	+	−	−	−

[a] Numbers in parentheses indicate actual carbaryl concentration in parts per million of stock solution as determined by gas chromatography.

[b] Symbols: −, visually nontoxic; +, toxic.

[c] Dilution.

experiment are presented in Table 2. The stock solution of Sevin 4 Oil was toxic to HEL cells, but dilutions 1:2 and lower were not. Base oil plus inert ingredients showed a similar pattern of toxicity. Carbaryl was toxic at a dilution of 1:4 but not at lower dilutions.

Since a previous study (11) indicated that maximum viral enhancement was usually seen in cells treated with the highest concentration of chemical that produced no obvious signs of cellular toxicity, the 1:2 dilution of Sevin and base oil and the 1:10 dilution of carbaryl were used in subsequent experiments.

Viability of pesticide- and component-treated cells. To compare the viabilities of untreated versus treated HEL cells which were visually unaffected, cells were grown to monolayers in 25-cm^2 tissue culture flasks, flooded with pesticide, component, or growth medium, and allowed to incubate at 37°C for 18 h. Flasks were then trypsinized, and 10-fold dilutions of the cells contained in each were plated. Trypan blue counts were made of the undiluted samples. Results are presented in Table 3. Percentages of viable cells in each test flask ranged from 72.7% in base oil-treated cells to 87.5% in Sevin-treated cells, with carbaryl-treated cells 78.5% viable and control cells 84.6% viable. The number of plated cells from each flask which divided to form foci ranged from 1.1×10^5 in Sevin-treated cultures to 2.2×10^5 cells in control cultures. Since viabilities were essentially comparable in treated and control cultures, we assumed that toxicity of the compounds would not pose a problem in later studies.

Test for viral enhancement. These experiments were done to determine whether Sevin 4 Oil or any of its components could enhance the growth of VZ virus in HEL cells. Cells were grown to monolayers in flasks, and duplicate cultures were treated with appropriate concentrations of pesticide, components, or growth medium. After 18 h of incubation, monolayers were washed with phosphate-buffered saline and refed with growth medium, and infected cells were added. Flasks incubated at 37°C until infectious centers were microscopically visible (about 4 days) were then trypsinized, and an infectious center assay was performed. Results are shown in Table 4; actual carbaryl concentrations are shown in parentheses where applicable. The enhancement index is calculated as the number of infectious centers in each treated culture divided by the number of infectious centers in the control culture. Each reported value is the average of duplicate plates, and the control value is the average of four plates. Rozee et al. (11) previously defined enhancement as at least a doubling of the amount of virus produced by a treated culture versus a control culture. In this test, Sevin-treated cultures produced about 10 times as much virus as control cultures. Pure carbaryl-treated cultures yielded 10- to 13-fold increases in viral production over controls. The base oil preparation did not enhance the production of virus at all.

TABLE 3. *Viabilities of cultures treated with subtoxic concentrations of Sevin or components*

Compound	Trypan blue counts (% viable cells per culture)	No. of viable foci per flask ($\times 10^5$)
Sevin 4 oil	14/16[a] = 87.5%	1.1
Carbaryl	11/14 = 78.5%	1.9
Base oil plus inert ingredients	8/11 = 72.7%	1.4
Control	11/13 = 84.6%	2.2

[a] Viable cells/total cell number.

Dose response of cells treated with carbaryl. Once it was established that carbaryl was the ingredient in Sevin 4 Oil responsible for viral enhancement, we performed a dose-response experiment to determine the minimum amount of carbaryl required to cause enhancement. HEL cells were again grown to monolayer in 25-cm^2 tissue culture flasks flooded with dilutions of carbaryl ranging from 0.37 to 18 ppm, and incubated at 37°C for 18 h. Cells were then washed with PBS and refed with growth medium. Cultures were then infected, and virus production was determined as described in Materials and Methods. Results are shown in Table 5. Each enhancement value represents the average of duplicate cultures assayed independently. Maximum enhancement was demonstrated in cells treated with 18 ppm carbaryl. Decreasing concentrations gave decreasing indices of enhancement, with 0.37 ppm carbaryl causing no enhancement.

DISCUSSION

Results presented here and in other papers show that a variety of pesticides (11), emulsifiers (4, 10, 11), and other chemical toxins (2) enhance the growth of certain viruses in cultured cells. Earlier studies (4, 10, 11) had indicated that single-stranded ribonucleic acid viruses were enhanced by emulsifiers, whereas double-stranded deoxyribonucleic acid viruses, such as herpes simplex virus type 1 and vaccinia virus, were not. The particular deoxyribonucleic acid viruses selected are not cell associated and replicate efficiently in culture. VZ virus, on the other hand, is known to replicate only as a cell-associated virus in cell cultures, and depends more

TABLE 4. *Effect of Sevin and components on the growth of VZ in HEL cells*

Compound	Infectious centers per culture		Enhancement index[a]
	Treated	Control	
Sevin (4 ppm)[b]	3.4×10^6		9.18
Sevin (4 ppm)	3.8×10^6		10.2
Carbaryl (5 ppm)	5.0×10^6		13.5
Carbaryl (5 ppm)	3.9×10^6		10.6
Base oil	3.3×10^5		0.89
Base oil	3.4×10^5		0.91
Control		3.7×10^5	1

[a] Enhancement index, Number of infectious centers in treated/number of infectious centers in control.
[b] Numbers in parentheses indicate amount of carbaryl in parts per million.

TABLE 5. *Effect of decreasing concentrations of carbaryl on viral enhancement*

Carbaryl (ppm)	Infectious centers per culture		Enhancement index[a]
	Treated	Control	
18	9.7×10^5		2.0
3.7	8.0×10^5		1.7
0.7	7.8×10^5		1.6
0.37	4.7×10^5		1.0
0		4.7×10^5	1.0

[a] Enhancement index, see Table 4.

on the physiological state of the infected cell. Rozee et al. (11) determined that cells had to be exposed to enhancing emulsifiers for 4 to 6 h at 37°C before viral enhancement could be detected, and that these cells returned to the control state after 4 to 6 h. This suggests that an alteration in cellular metabolism in treated cells may affect viral enhancement. This could explain why enhancement was seen with VZ virus but not with herpes simplex or vaccinia virus.

We were unable to obtain Sevin and the inert ingredients without their fuel oil dispersal agent. Because of high concentrations of this oil in the complete pesticide and base oil preparations which led to low solubilities in our aqueous medium, we were unable to determine exactly how much of these compounds was actually dissolved in the growth medium. Our reported values of parts per million are based on the theoretical concentrations which would have been obtained had all the pesticide or base oil gone into solution. Since optimum levels of pesticide or components were found to be just less than cytotoxic (11), we were able to dilute our test solutions accordingly and look for enhancing ability without knowing the precise oil concentrations. Carbaryl concentrations, however, could be accurately determined using gas chromatography and are reported as such.

Although our reported data are representative, both higher and lower enhancement indices were obtained in other similar experiments, ranging from a low of 2-fold to a high of 50-fold. Several factors could account for this variation. Due to the cell-associated nature of VZ virus, input multiplicities can vary from one experiment to the next. Another factor affecting the magnitude of enhancement could be cell age; as HEL cells approach senescence, the enhancement in any given experiment appears to be higher. This could be a membrane phenomenon, or could be a function of cellular metabolism. Experiments are in progress to further define optimum conditions for enhancement.

A previous study (1) has shown that certain organophosphorus insecticides increase the permeability of liposome membranes. This, and the fact that carbaryl is somewhat lipid soluble (6), suggests a possible association between the enhancing ability of carbaryl and cellular lipids. We are presently attempting to study lipid changes in cells that are virus infected, cells that are pesticide treated, and infected-treated cells. It may be possible to correlate any detected changes with clinical data reporting an increase in fatty acid methyl esters in the organs of children suffering from Reye's syndrome (12).

ACKNOWLEDGMENTS

This work was supported in part by the Maine State Agricultural Experiment Station and by the State of Maine, Department of Conservation Appropriation Account Number 1505-4017-4099.

We thank Rodney Bushway for helpful discussions.

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