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Toxicity and Intracellular Localization of Carbaryl and 1-Naphthol in Cell Cultures Derived from Goldfish

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Carbaryl has been shown to be toxic to five major fish families (MACEK & McALLISTER 1970). At low concentrations, it is a metabolic stressor of freshwater catfish, and causes increased swimming activity and a higher frequency of opercular beats (ARUNACHALAM et al. 1980). Carbaryl and its major hydrolysis product, 1-naphthol, have been found in irrigation and drainage water, as a result of both aerial spraying and sruface runoff (OSMAN & BELAL 1980). Runoff resulting from the application of 1 lb of carbaryl/acre has been shown to depress brain acetylcholinesterase activity in fish (MARANCIK 1976; HULBERT 1978). In aquaria, carbaryl is rapidly converted to 1-naphthol (KARINEN et al. 1967); a recent study, using protozoal cultures as indicators, suggested that 1-naphthol is as toxic as carbaryl (WEBER et al. 1982).

Carbaryl has been shown to localize in the nuclear fraction of human cells *in vitro*, by binding to nuclear proteins (MURAKAMI & FUKAMI 1982).

The purpose of the present study is to assess the toxicity of carbaryl and 1-naphthol to cell cultures derived from goldfish, and to examine the intracellular localization of carbaryl in these cells.

MATERIALS AND METHODS

Cells: CAR cells (ATCC CCL-71), originally derived from goldfish fin, were obtained from the American Type Culture Collection. Cells used were between the 70th and 75th passage from repository stocks.

An uncharacterized cell line derived from goldfish air bladder, ABIII, was initiated in this laboratory. Cells used were between the 50th and 60th passage from primary culture.

CAR and ABIII cells were incubated at 25 C under normal atmosphere.

Medium: Culture medium consisted of Medium 199 supplemented with 10% fetal bovine serum, and 100 units of penicillin and streptomycin, and 0.025 mcg Fungizone/mL.

Carbaryl and 1-naphthol: Technical grade carbaryl (99.07% pure) and technical grade 1-naphthol (99.7% pure) were gifts of

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Union Carbide Corp. Stock solutions were freshly prepared at the start of each experiment in 100% ethanol at 0.1 g/mL and serially diluted with medium to final concentrations of 200, 100, 50, 25, 10, and 5 parts per million (ppm). These dilutions resulted in a maximum of 0.1% ethanol (in the 200 ppm- treated cultures, with further dilutions thereafter); previous studies (SHEA & BERRY 1982) have demonstrated that incorporation of 0.1% or less ethanol into culture medium has no detrimental effect on cells.

Toxicity studies: Toxicity of carbaryl and 1-naphthol was examined according to a previously described method for goldfish-derived cell cultures (SHEA & BERRY 1982). In brief, cells were planted in 24-well trays (Falcon) at a density of $9x10^4$/well. Twenty-four h later, medium was removed, and cultures received medium containing either carbaryl or 1-naphthol at the concentrations listed above, with 4 replicate cultures/concentration. At intervals, cultures were examined for toxicity using phase-contrast microscopy. Toxicity was determined by the extent of granulation, vacuolization, cell rounding, and detachment.

Intracellular localization of carbaryl and 1-naphthol: Duplicate 24 h-old CAR cultures in 75 cm^2 flasks (Corning) received 10 mL medium containing 25 ppm carbaryl without serum, and were incubated for 48 h at 25 C. No cytotoxicity was observed. Duplicate samples of sterile culture medium containing wither carbaryl or 1-naphthol at 25 ppm were also incubated for 48 h at 25 C. After incubation, media samples were stored at 70 C. Culture medium was removed, and cultures were rinsed with Hank's Balanced Salt Solution (HBSS), with medium and rinse combined and frozen. Cells were fractionated into acid-soluble, alcohol-soluble, and acid- and alcohol-insoluble fractions according to the methods of MURAKAMI & FUKAMI (1979) as modified by SHEA & BERRY (1982). Alternatively, the nuclear fraction was isolated by lysis of the cells in HBSS containing 0.1% Triton X-100 (Sigma) followed by centrifugation at 2000 rpm for 10 min, washing of the nuclear pellet in HBSS, recentrifugation, and disruption of nuclei by freezin- (-70 C) and thawing (room temp). All samples were extracted with 1 mL dichloromethane, and thin-layer chromatography was performed according to the method of KARINEN et al. (1967).

RESULTS AND DISCUSSION

Toxicity: Similar levels of toxicity of carbaryl were observed for both CAR and ABIII cells (Table 1). At day 1 after addition of carbaryl, cultures treated with 200 ppm were slightly granulated compared to controls; on day 2 this amount of carbaryl was toxic. Toicity was not observed at levels below 200 ppm until day 7, when 50 ppm was toxic, and 25 ppm induced slight granulation. This pattern of toxicity was unaltered until day 10, when the study was terminated.

200 ppm 1-naphthol resulted in complete destruction of both CAR and ABIII cltures by day 1, with mild granulation induced by 100 ppm in ABIII cells at this time (Table 2). On day 2, mild granula-

tion was also observed in CAR cells treated with 100 ppm. On day 5, 100 ppm was toxic to both cell lines, with mild granulation observed for both at 50 ppm. By day 7, 25 ppm induced mild granulation in both CAR and ABIII cultures, with 10 ppm also inducing granulation in CAR cells; 50 ppm was toxic at this time. On day 10, 25 ppm was toxic, and 10 ppm induced granulation, for both cell lines.

In comparison to carbaryl, 1-naphthol was initially far more toxic to both cell lines, and after 10 days incubation, exhibited an endpoint toxicity level 2-fold over that of carbaryl (25 ppm for 1-naphthol versus 50 ppm for carbaryl).

Table 1: Toxicity*of carbaryl to cell lines derived from goldfish.

Day	cell line	Toxicity 200**	100	50	25	10	5	0
1	CAR	+/-	-	-	-	-	-	-
	ABIII	+/-	-	-	-	-	-	-
2	CAR	+	-	-	-	-	-	-
	ABIII	+	-	-	-	-	-	-
5	CAR	+	-	-	-	-	-	-
	ABIII	+	-	-	-	-	-	-
7	CAR	+	+	+	+/-	-	-	-
	ABIII	+	+	+	+/-	-	-	-
10	CAR	+	+	+	+/-	-	-	-
	ABIII	+	+	+	+/-	-	-	-

*Toxicity determined by extent of granulation, vacuolization, cell rounding, and detachment. Symbols: -, visually nontoxic; +, toxic; +/-, mild granulation; TD, totally detached.

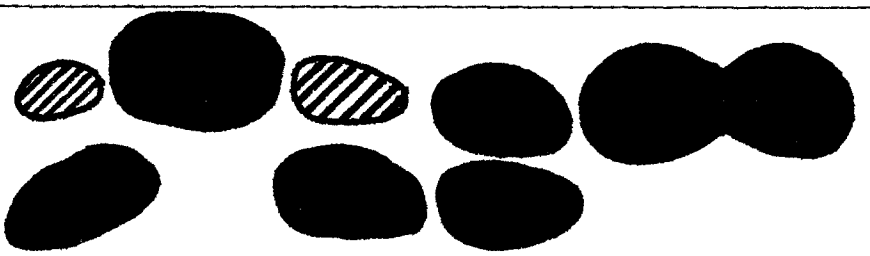
**ppm carbaryl incorporated into culture medium.

Table 2: Toxicity*of 1-naphthol to cell lines derived from goldfish

day	cell line	Toxicity 200	100	50	25	10	5	0
1	CAR	TD	-	-	-	-	-	-
	ABIII	TD	+/-	-	-	-	-	-
2	CAR	TD	+/-	-	-	-	-	-
	ABIII	TD	+/-	-	-	-	-	-
5	CAR	TD	+	+/-	-	-	-	-
	ABIII	TD	+	+/-	-	-	-	-
7	CAR	TD	+	+	+/-	+/-	-	-
	ABIII	TD	+	+	+/-	-	-	-
10	CAR	TD	+	+	+	+/-	-	-
	ABIII	TD	+	+	+	+/-	-	-

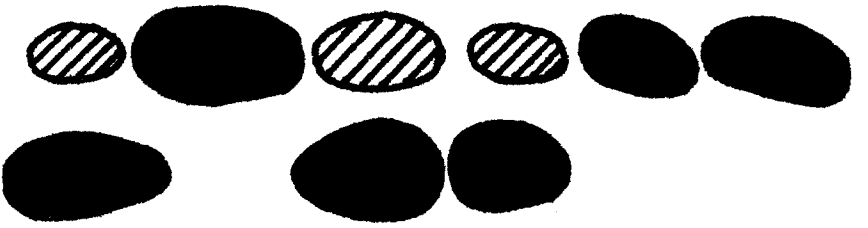
*See legend for Table 1.

Localization of carbaryl and 1-napthol in CAR cells: Initial experiments involving fractionation of CAR cells into acid-soluble, alcohol-soluble, and acid- and alcohol-insoluble fractions revealed exclusive localization of all compound removed from the medium in the acid- and alcohol-insoluble cytoskeletal fraction (data not shown). We therefore explored this phenomenon further by isolating nuclei from CAR cultures exposed to medium containing carbaryl. The hydrolysis of carbaryl to 1-naphthol in sterile culture medium during 48 h incubation is shown in Fig. 1. Approximately 10% of the



A B C D E F

Fig. 1: Hydrolysis of carbaryl to 1-naphthol in sterile medium at 25 C. A, carbaryl standard; B, 1-naphthol standard; C, carbaryl in sterile medium at 0 h; D, carbaryl in sterile medium at 48 h; E, 1-naphthol in sterile medium at 0 h; F, 1-naphthol in sterile medium at 48 h.



A B C D E F

Fig. 2: Localization of 1-naphthol in CAR cell nuclei. A, carbaryl standard; B, 1-naphthol standard; C&D, duplicate samples of carbaryl in culture medium after 48 h incubation with cells; E&F, duplicate samples of the nuclear fraction of CAR cells after 48 h incubation with medium containing carbaryl.

carbaryl introduced into the culture medium at 0 h had hydrolyzed to 1-napthol during the course of the chromatographic assay. By 48 h after addition, approximately 50% of the carbaryl had hydrolyzed. No further breakdown of 1-naphthol was noted, either subsequent to the hydrolysis of carbaryl, or when 1-naphthol was initially introduced into the medium.

In the presence of cells, under the same conditions of incubation, 1-naphthol did not accumulate in the culture medium over 48 h. However, 1-naphthol was observed in the nuclear fraction at 48 h, at levels equivalent to those found in sterile medium after 48 h incubation. Carbaryl was not detected in the nuclear fraction (Fig. 2). When cells were fractionated into acid-soluble, alcohol-soluble, and acid- and alcohol-insoluble fractions, 1-napthol was exclusively located in the acid- and alcohol-insoluble fraction, and carbaryl did not localize in any cellular fraction (Data not shown).

Apparently, carbaryl is not taken up by CAR cells under these conditions. Rather, its major hydrolysis product, 1-naphthol, is removed from the medium as it is formed. This phenomenon would explain the dramatic difference in toxicity observed for both cell lines on day 1 after addition of 200 ppm 1-naphthol as compared to 200 ppm carbaryl (Tables 1 and 2), as well as the 2-fold difference in toxicity noted for 1-naphthol versus carbaryl after 10 days incubation.

MURAKAMI & FUKAMI (1980, 1982) have reported the uptake and localization of carbaryl in the nuclear fraction of human cells in vitro; their method of assay utilized carbaryl radioactively labelled with ^{14}C at the carbon 7 and 10 positions. It should be noted, however, that these positions are within the ring structure of the compound, and therefore do not allow differentiation between carbaryl and 1-naphthol. Thin-layer chromatography, utilized in the present study, readily permits this differentiation, as the Rf values for carbaryl and 1-naphthol are not equivalent. It would be of interest to examine whether carbaryl itself localizes in human cells in culture, or whether hydrolysis is a prerequisite for uptake. ^{14}C labelling of the methylamine moiety, or the carbonyl moiety, or the use of thin-layer chromatography, could accomplish this. It is entirely possible that our piscine cultures are simply passively adsorbing 1-naphthol, while human cells are capable of actively accumulating carbaryl.

WEBER et al. (1982) have shown that 1-naphthol is as toxic as its parent compound, using protozoal cultures as an indicator system. Considering the relatively rapid rate of hydrolysis of carbaryl in aquaria (KARINEN et al. 1967), our data suggest that its major hydrolysis product, 1-napthol, contributes to its toxicity is other aquatic systems as well.

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