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Induction of Chromosomal Aberrations by 2,4-Dichloro-6-Aminophenol in Cultured V79 Cells

Zeng-Chang Heng, Joginder Nath, Xiaorong Liu, and Tong-man Ong

Genetics and Developmental Biology Program, College of Agriculture and Forestry, West Virginia University (Z.C.H., J.N., T.O.), and Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health (X.L., T.O.), Morgantown, West Virginia; Department of Environmental Health, West China University of Medical Science, Chengdu, Sichuan, China (Z.C.H.)

2,4-Dichloro-6-aminophenol (DCAP) is a major metabolite of the herbicide 2,4-dichloro-6-nitrophenol ammonium (DCNPA). In our previous studies this metabolite, but not the parent compound, was found to induce sister chromatid exchanges (SCEs) and micronucleus (MN) formation in Chinese hamster lung fibroblasts (V79 cells). Further studies were carried out to determine whether DCAP can also induce structural chromosomal aberrations (CAs) in cultured V79 cells. The assay was performed under three different treatment conditions: 1) 3 h treatment followed by 17 h post-treatment incubation, 2) 12 h treatment, and 3) 20 h treatment with multiple sampling times. Results showed that CAs were induced by DCAP in V79 cells. The aberrations were mainly chromatid types (gaps, breaks, and deletions), triradials, quadriradials, and complex rearrangements. However, induction of CAs appears to be dependent on the treatment condition. The 3 h treatment following a 17 h post-treatment incubation gave rise to 24% aberrant cells (excluding gaps) and a total of 44 aberrations per 100 metaphases, while the 12 h treatment only gave a borderline response and the 20 h treatment did not yield a significant increase of CAs at any concentration tested. It seems that a short-term treatment with higher concentrations is a better treatment protocol for genotoxicity studies of certain cytotoxic chemicals such as DCAP. Results of this study further indicate that the herbicide DCNPA is potentially hazardous to the exposed population.

Key words: herbicide metabolite, 2,4-dichloro-6-aminophenol, chromosomal aberrations, V79 cells, sister chromatid exchanges

Address reprint requests to Dr. Tong-man Ong, Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505.

INTRODUCTION

2,4-Dichloro-6-aminophenol (DCAP) is a major metabolite of the herbicide 2,4-dichloro-6-nitrophenol ammonium (DCNPA), which has been used in agriculture. While little information is available regarding the potential carcinogenic hazards of DCNPA and DCAP to the exposed population, our previous studies on the genotoxicity of these chemicals showed that DCAP, but not DCNPA, induced sister chromatid exchanges (SCEs) and micronucleus (MN) formation in Chinese hamster lung fibroblasts (V79 cells) [1]. Formation of micronuclei is considered to be a consequence of either chromosomal breakages or spindle dysfunction. Clastogens which break chromosomes and spindle poisons which cause spindle damage are classified as different genotoxicants and may possess different toxicological significance.

The chromosomal aberration (CA) assay has been used widely to detect chromosomal damage including chromosomal breaks. Although the assay has been used for a long time, the protocols adopted by various laboratories differ in many ways [2]. Efforts to establish standard testing protocols have been made and different treatment and sampling times for the detection of CAs in mammalian cells in vitro have been recommended. According to the report of the U.S. EPA's Gene-Tox Program [3], continuous exposure with fixation times at 3, 8, and 12 h after the beginning of exposure should be performed. A survey of current practice in genotoxicity testing laboratories [2] suggested that, in the absence of metabolic activation, cultures should be incubated with the test compound for 8–10 h before colcemid treatment and harvest. Longer incubation time is needed, however, if the test chemical causes cell cycle delay. Sofuni [4] recommends 48 h continuous treatment for CA assay in mammalian cell cultures. Bean and coworkers [5,6] emphasized that choosing a suitable sampling time is vitally important to optimize the yield of aberrations, particularly for detection of weakly active chemicals. Armstrong et al. [7] reported that the induction of CAs by 2,4,6-trichlorophenol was only detected using a protocol which included a post-exposure recovery time of 4–12 h. Our studies presented here were performed to determine whether DCAP can induce CAs in cultured V79 cells and whether the response of CA is different under different treatment conditions.

MATERIALS AND METHODS

Chemicals

DCAP (98% purity) was obtained from the Institute of Sichuan Chemical Industry in Chengdu, China. N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), used for positive controls, was purchased from Aldrich (Milwaukee, WI).

Cell Line and Culture

V79 cells were kindly provided by Dr. C.C. Chang (Michigan State University, East Lansing). The cells were cultured in 75 cm^2 tissue flasks containing 15 ml Eagle's minimal essential medium (MEM), supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and 1% penicillin-streptomycin. Cultures were incubated at 37°C with 5% CO_2 and 95–100% relative humidity. Cells were subcultured every 3–4 days and 5×10^5 cells were plated into each of 100 mm dishes 1 day prior to chemical treatment.

Chemical Treatment

DCAP was dissolved in sterile distilled water at 30 mg/ml as the original solution. All other concentrations of DCAP (0.2, 1, 2.5, 5, 10, and 20 mg/ml) were diluted from the original solution with MEM. Exponentially growing cells were used for chemical treatments. In this study, we used the same treatment protocol, 3 h exposure and 17 h post-treatment incubation, as that used for the previous MN assay [1]. In order to compare the effect of treatment and sampling times on CA frequencies, the following treatment protocols were also used: 1) 12 h exposure and sampling immediately and 2) 20 h exposure with 3 different sampling times: 0, 6, or 20 h after the treatment. Following treatment or treatment and post-incubation, cultures were washed twice with phosphate buffered saline (PBS) and refed with 10 ml complete medium. Solvent and positive (MNNG) controls were run with each trial.

Cytotoxicity Assay

Determinations of cell viability and mitotic index (MI) were made to measure cytotoxicity. The surviving cells of each treatment group at the time of sampling were determined. MI is defined as the number of metaphase figures among 1,000 cells. Selection of the highest dose used in this study was based on its toxicity being greater than 50% of the solvent control.

CA Analysis

Colcemid (0.1 μg/ml) was added to each dish 2 h before harvest. Cells were harvested by trypsinization, washed by centrifugation, resuspended in hypotonic solution (75 mM KCl) for 15 min at room temperature, and fixed 3 times in 3:1 methanol and glacial acetic acid. Two drops of cell suspension were dropped onto a cold slide. After being air-dried, the slides were stained with 10% Giemsa for 10 min. One hundred well-spread metaphases containing 19–23 chromosomes per treatment group were scored for CAs. Aberrations were classified according to the description of Evans and O'Riordan [8] and Scott et al. [9]. A score sheet for CAs included chromatid gaps, chromatid breaks, isochromatid breaks, interstitial deletions (triradials or quadriradials), terminal deletions, dicentrics, centric rings, and minutes. The frequencies of polyploid and endoreduplicated cells were recorded, but were not included in structural aberration totals. The percent of aberrant metaphase cells and the number of CAs with and without gaps were compared to the control using the chi-square test. The frequencies of CAs induced by DCAP in different treatment/sampling protocols were also compared.

RESULTS

Under all three different treatment/sampling conditions, both cell counts and MI decreased with increasing DCAP concentration or treatment time, showing clear cytotoxicity of DCAP to V79 cells. The curves in Figure 1 show the relative number of surviving cells compared to control. The concentrations of DCAP which caused 50% reduction in cell counts were about 250, 50, and 18 μg/ml for 3, 12, and 20 h treatments, respectively. Therefore, the concentration of DCAP used for the 3 h treatment was as high as 5 times that for 12 h and 15 times that for 20 h treatments.

Structural CAs induced by DCAP in V79 cells using 3 h treatment followed by

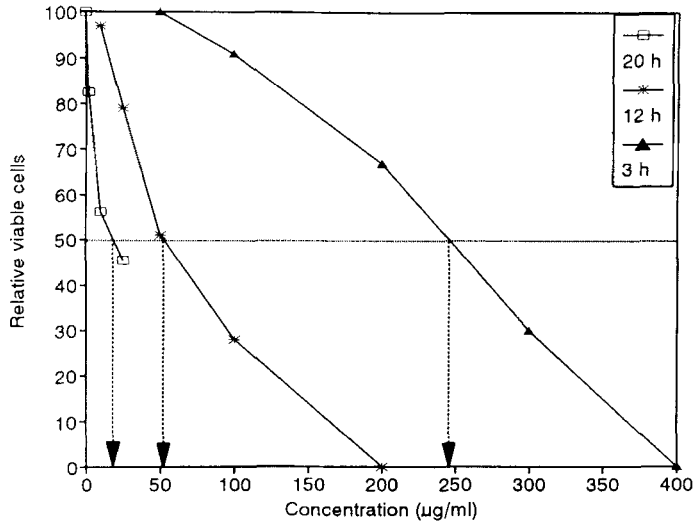

Fig. 1. Relative survivors of V79 cells treated with DCAP. Arrows show the concentrations of DCAP which caused 50% reduction in surviving cells.

17 h post-treatment incubation are shown in Table I. The percent of aberrant cells without gaps and the total aberrations per 100 metaphases in the exposed groups at 200 and 300 μg/ml significantly increased over the background in a dose-related manner. The observed aberrations were mainly chromatid types (gaps, breaks, and deletions), triradials, quadriradials, and complex rearrangements (Fig. 2).

Frequencies of CAs in V79 cells after 12 h treatment (no post-treatment incubation time) with DCAP are shown in Table II. At 50 μg/ml, DCAP caused a detectable increase ($P < 0.05$) in the percent of aberrant cells with gaps and in total aberrations per 100 metaphases. At 100 μg/ml, the yield of aberrations was not significantly different from that in the solvent control. DCAP at 100 μg/ml, however, was highly toxic to V79 cells after 12 h treatment.

The results of 20 h treatment with DCAP are given in Table III. No significant increase in aberrant cells per 100 metaphases was seen over the concentration range of 2–25 μg/ml. In order to confirm whether the negative response was based on an unsuitable sampling time due to cell cycle delay, we compared the effect of sampling

TABLE I. Frequencies of CAs in V79 Cells Treated With DCAP for 3 H

		Aberrant cells (%)		Aberrations per 100 metaphase cells[a]						
				Chromatid type			Chromosome type			
Compound	Concentration (μg/ml)	With gaps	Without gaps	g	b	e	g	b	e	Total aberrations
DCAP	0	2	0	1	0	0	1	0	0	2
	100	4	0	4	0	0	0	0	0	4
	200	7	5*	4	6	2	0	0	0	12**
	300	29**	24**	10	12	18	0	0	4	44**
MNNG	1	39**	30**	12	13	28	1	1	4	59**

[a]g = gap; b = break; e = exchange.
*$P < 0.05$ and **$P < 0.01$, compared with solvent control using chi-square test.

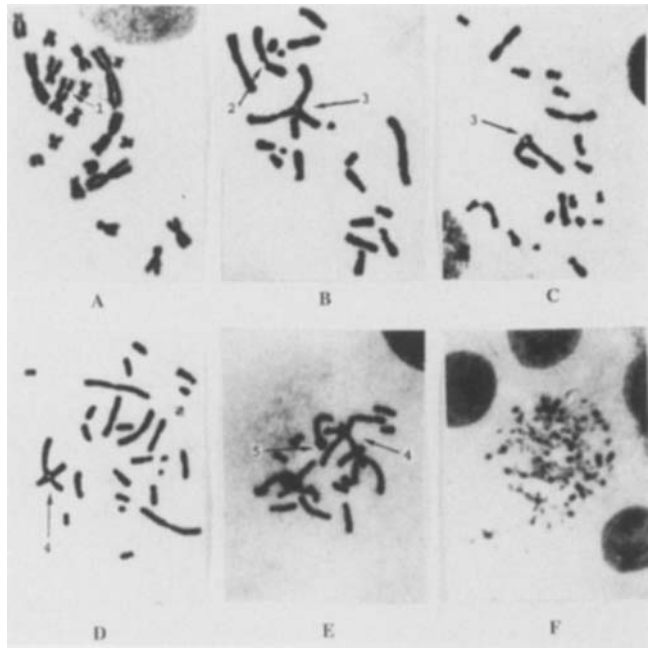

Fig. 2. CAs in V79 cells treated with DCAP for 3 h. **A–E:** Chromatid gaps (1), chromatid break (2), triradials (3), quadriradials (4), and sister union (5). **F:** Pulverized cell.

time on CA induction (Table IV). No difference was observed in the numbers of aberrant cells (with and without gaps) among different sampling (post-treatment incubation) time groups treated with the same concentration of 10 μg DCAP/ml which only gave a 56% relative survival. However, MI appeared to decrease with the increasing post-treatment incubation time.

DISCUSSION

DCAP induces MN formation in V79 cells [1], therefore, it may be a clastogen and/or spindle poison [1]. In this study, DCAP induced a significant increase of CAs

TABLE II. Frequencies of CAs in V79 Cells Treated With DCAP for 12 H

				Aberrant cells (%)		
Compound	Concentration (μg/ml)	MI	Cells scored	With gaps	Without gaps	Total aberrations
DCAP	0	21	100	2	0	2
	10	22	100	3	0	3
	25	21	100	6	2	7
	50	10	100	10*	5*	11*
	100	1	49[a]	8	4	4
MNNG	1	15	50	19**	11*	21**

[a]No more well-spread metaphase cells for scoring aberrations.
*$P < 0.05$ and **$P < 0.01$, compared with solvent control using chi-square test.

TABLE III. Frequencies of CAs in V79 Cells Treated With DCAP for 20 H

DCAP (μg/ml)	Relative survival (%)	MI	Aberrant cells (%)	
			With gaps	Without gaps
0	100.0	28	2	0
2	82.5	26	2	0
10	56.0	22	3	1
25	45.3	12	7	0

in V79 cells. This seems to indicate that the micronuclei observed in our previous study could be due to chromosomal breakages. It is known that CAs in somatic cells may involve the etiology of neoplasia and in germ cells may lead to spontaneous abortion, perinatal mortality, or congenital malformations in the offspring [10]. Since DCAP is a major metabolite of DCNPA, this herbicide may pose potential teratogenic and carcinogenic hazards to the exposed population.

It has been reported that CAs can be induced in cells exposed to a low pH medium [11,12]. DCAP is a halogenated phenol and is acidic in aqueous solution (approximate pH 3.2). The pH of culture medium containing different concentrations of DCAP was approximately 7.4, which is not different from the MEM alone; therefore, the CAs induced by DCAP were not due to a decrease in pH of the medium. Reduction of the nitro group in vivo in DCNPA, the parent compound, is likely to be responsible for the cytotoxicity and clastogenicity of DCAP. It is interesting to know that DCAP induced CAs in short-term (3 h) but not in long-term (20 h) treatment. This is most likely due to the concentrations of DCAP used. Because of the cytotoxicity, lower concentrations must be used for longer exposure times. In contrast, a short-term treatment allowed us to use higher concentrations (Fig. 1). At lower concentrations, a longer treatment time may cause cell death, however, may not induce or increase frequencies of CAs because only M2 cells were scored. Thus, it seems that a short-term treatment with higher concentrations is a better treatment protocol for genotoxicity studies of certain cytotoxic chemicals such as DCAP. Whether this is a better protocol for all other cytotoxic halogenated phenols is not known.

It has been emphasized that the time at which metaphase cells are sampled after treatment is critical for detection of CAs [5,6,13]. Because cell cycle delay often results from chemical treatment, a prolonged sampling time is needed to allow delayed cells to progress to mitosis. In this study, different sampling times following 20 h treatment with DCAP were used, but no increase in the frequency of CAs was observed in any of the sampling times used. These results further indicated that the

TABLE IV. Frequencies of CAs in V79 Cells Treated With DCAP for 20 H With Different Sampling Times After Treatment

DCAP (μg/ml)	Sampling time (h)	Relative survival (%)	MI	Aberrant cells (%)	
				With gaps	Without gaps
0	0	100.0	28	2	0
10	0	56.0	22	3	1
	6	47.7	16	7	1
	24	27.4	12	3	2

non-clastogenic responses did not result from unsuitable sampling time but from excessively low concentrations used. The positive response from 3 h treatment followed by 17 h post-treatment incubation seems to support the recommended protocol of Galloway et al. [14], that the treatment length should be 3–6 h, followed by sampling at the time equivalent to 1.5 normal cell cycle lengths from the beginning of treatment.

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