Genotoxicity of Atrazine as determined using the analysis of sister chromatid exchanges directly in the G2-phase of peripheral blood lymphocytes.

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Introduction

Human exposure to agricultural chemicals such as pesticides and herbicides has been linked to undesirable health effects including increased cancer incidence and genetic diseases. Atrazine, which is the most prevalent of triazine herbicides, has been found in rural groundwater and through its occurrence in food such as corn, nuts, fruit, and wheat, is a potential hazard to humans. Several studies have been carried out to evaluate the genotoxic activity and mutagenic potential of atrazine in different experimental systems. The majority of these studies have been negative but positive responses have been also reported. In particular, atrazine has been shown to induce mammary tumors in female Sprague-Dawley rats, and EPA's Peer Review Committee has concluded that atrazine should be considered as a Possible Human Carcinogen. To evaluate its modes of action, cytogenetic studies using analysis of sister chromatid exchanges (SCEs) have been carried out, but controversial results have been obtained. In those studies, conventional metaphase chromosome analysis was exclusively used and, therefore, only cells that proceeded to metaphase were analyzed. Damaged cells blocked into the G2-phase could not be analyzed. The genotoxic potential of increasing concentrations of atrazine could not be evaluated as well by using conventional cytogenetic studies and metaphase chromosome analysis only, since at increased chemical concentrations cells can not proceed to mitosis. To overcome these shortcomings of the conventional SCE analysis, we have developed a new method that enables the analysis of SCEs directly in G2-phase, using drug-induced premature chromosome condensation (PCC) in cultured peripheral blood lymphocytes.

Objectives

By means of the new proposed method in this study it is examined:
a) Whether Sister Chromatid Exchange analysis in metaphase chromosomes underestimates the mutagenic potential of atrazine. b) If the genotoxic potential of suspected carcinogens like atrazine could be evaluated in G2-phase, even at exposures exceeding the toxic limits that do not allow cells to proceed to mitosis. c) Whether an important part of the variation in SCE frequency among individuals after *in vitro* lymphocyte exposure to atrazine is due to cell cycle kinetics rather than to a true biological variation of this cytogenetic end point.

Materials & Methods

Culture conditions and PCC induction in G2-phase

Peripheral blood was taken with heparinized syringes from healthy individuals. 0.5 ml of whole blood was added to each culture tube containing 5 ml of McCoy's 5A medium supplemented with 10% fetal calf serum, 1% glutamin, 1% antibiotics (penicillin - streptomycin), 1% Phytohemaglutinin, and incubated at 37^0C for 72h in a humidified incubator, in an atmosphere of 5% CO_2 and 95% air. For PCC induction in G2-phase lymphocytes, 50nM of Calyculin-A (Sigma-Aldrich) dissolved in Ethanol Absolute, was used. Replicate cultures were also made containing 0.05 μg/ml colcemid throughout the last 3-hour culture period, and these were not treated with Calyculin-A. The herbicide Atrazine (2-Chrolo-4-ethye-amino-6isopropylamino-1, 3, 5-triazine), obtained from Sigma-Aldrich, Germany was prepared in dimethyl sulphoxide (DMSO). Mitomycin-C (MMC, Kyowa Hakko Kogyo Co. LTD., Japan) was prepared in RPMI medium and used as a positive control at a final concentration of 0.1 μg/ml.

Sister chromatid exchanges in G2-and M-phase lymphocytes

5-Bromodeoxyuridine (Sigma) was added at a final concentration of 20μM 24 hours after culture initiation. Cultures were incubated at 37 ^{0}C for 72 hours prior to cell harvest. During this culture period, incorporation of BrdU into replicating cells allows for the unequivocal identification of second division metaphase cells. The cultured cells were treated with hypotonic (0.075M) KCl, fixed with methanol-acetic acid (3:1) and 20 μl of cell suspension were dropped on wet slides. Air dried slides were stored in dark. For SCEs preparation, the slides were stained by the Fluorescence-Plus-Giemsa (FPG) technique according to Perry and Wolff protocol.
For SCE scoring, the criteria suggested by Carrano and Natarajan (1988) were applied. Only second division metaphases, identifiable by their uniform differential staining pattern, containing 46 centromeres were analysed. Every switch of staining between the sister chromatids is scored as one SCE. Switch of label at the centromere were not scored as SCE either in metaphase or in G2-phase PCCs.

Results

TABLE I

Concentration of Atrazine in cell culture	SCEs / metaphase cell (Mean ± SD)	SCEs / G2-phase cell (Mean ± SD)
Control (w/o Atrazine)	5.00 ± 0.60	7.10 ± 0.52
100 μgr/ml	No metaphases (cytotoxic level)	10.10 ± 0.71
200 μgr/ml	No metaphases (cytotoxic level)	10.45 ± 0.48

TABLE I: No. of SCEs as analyzed in G2-PCC cells after exposure to atrazine at cytotoxic levels. At these chemical concentrations no metaphases were observed. Standard deviations of the mean values from three independent experiments using the same donor were calculated for each experimental point.

TABLE II

Concentration of Atrazine in cell culture	SCEs / metaphase cell (Mean ± SD)	SCEs / G2-phase cell (Mean ± SD)
Control (w/o atrazine)	4.4 – 8.9 (6.0 ± 1,6)	6.0 – 7.2 (6.4 ± 0.5)
5 μgr/ml	5.2 – 7.8 (6.5 ± 1.2)	7.2 – 8.3 (7.7 ± 0.6)
10 μgr/ml	6.0 – 9.2 (8.0 ± 1.4)	7.9 – 10.0 (8.9 ± 0.9)
20 μgr/ml	6.0 – 9.9 (8.0 ± 1.6)	8.0 – 10.4 (9.0 ± 0.5)
50 μgr/ml	7.1 – 10.5 (8.1 ± 1.4)	9.4 – 10.0 (9.6 ± 0.2)
60 μgr/ml	7.3 – 11.9 (9.9 ± 2.0)	9.9 – 11.9 (11.4 ± 0.8)
100 μgr/ml	No metaphases (cytotoxic level)	9,9 – 12.5 (11.5 ± 0.9)
200 μgr/ml	No metaphases (cytotoxic level)	11.6 – 12.0 (11.6 ± 0.8)

Table II: No. of SCEs as analyzed in G2-PCC cells, in comparison to those analyzed in cells at metaphase, after exposure to atrazine. Standard deviations of the mean values from six different donors were calculated for each experimental point

Figure 1: Sister Chromatid Exchanges (SCEs) as visualized in peripheral blood lymphocytes at metaphase

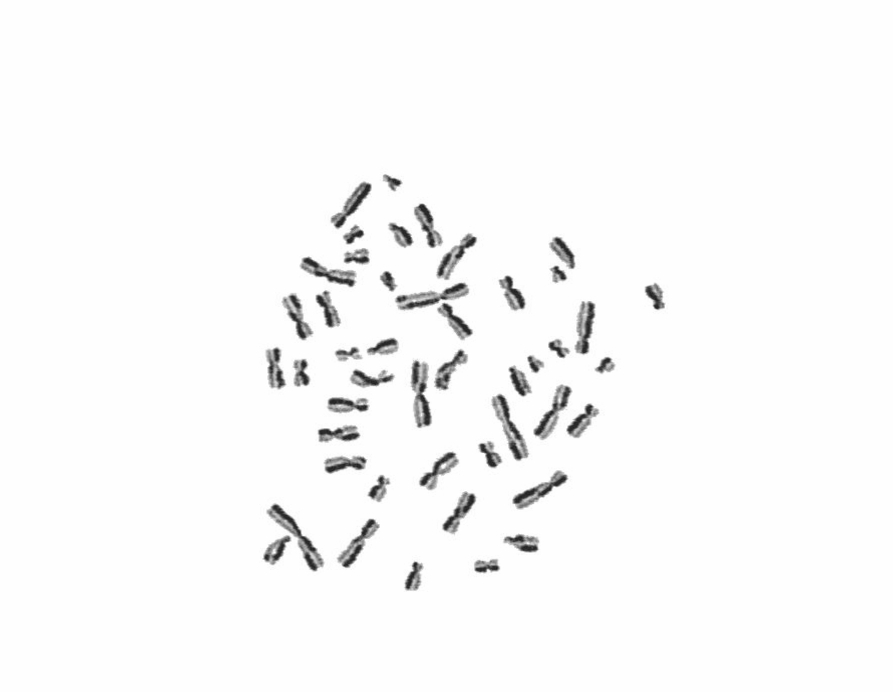

Figure 2: SCEs as visualized in G2-PCCs of an unexposed peripheral blood lymphocytes treated with Calyculin-A for 1h, demonstrating a characteristic appearance of attached sister chromatids

Figure 3: SCEs in G2-PCCs of a lymphocyte exposed to 50 μgr/ml of Atrazine

Conclusions

- Using the new proposed cytogenetic method for the genotoxic assessment of atrazine, the yields of SCEs per cell at the G2-phase of the cell cycle were easily obtained even at doses exceeding the toxic limits.
- A higher SCE yield per cell was scored in G2 - PCCs than in cells at metaphase after exposure to atrazine. Thus, using conventional analysis at metaphase cells, the mutagenic potential of chemicals that temporarily arrest cells at the G2-phase of the cell cycle may be underestimated.
- A lesser variability in the SCEs frequency among different individuals was observed when the analysis was carried out in G2 - PCCs than in metaphase cells.
- An important part of this variation may be due to differences in cell cycle kinetics rather than to a true biological variation in the end point used.
- The results obtained from the analysis of SCEs directly in G2-phase lymphocytes, suggest that atrazine exposure at high concentrations has a minimal genotoxic activity.