

An advance Cytogenetic approach for the determination of mutagenic potential of chemicals that induce Cell Cycle arrest in G2 phase by application of premature chromosome condensation (PCC) in peripheral blood lymphocytes

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SCE analysis in G2 lymphocyte prematurely condensed chromosomes after exposure to atrazine: The non-dose-depended increase in homologous recombinational events does not support its genotoxic mode of action

Abstract

Several studies have been carried out to evaluate the mutagenic and carcinogenic potential of atrazine, the most prevalent of triazine herbicides classified as a “possible human carcinogen”. The majority of these studies have been negative but positive responses have been also reported including mammary tumors in female Sprague-Dawley rats. Sister chromatid exchanges (SCEs) caused by the presence of DNA lesions at the moment of DNA replication, have been extensively used for genotoxicity testing, but for non-cytotoxic exposures to atrazine controversial results have been reported. Even though exposures to higher concentrations of atrazine could provide clear evidence for its genotoxicity, conventional SCE analysis at metaphase cells cannot be used because affected cells are delayed in G2-phase and do not proceed to mitosis. As a result, the genotoxic potential of atrazine may have been underestimated. Since clear evidence has been recently reported relating SCEs to homologous recombinational events, we are testing here the hypothesis that high concentrations of atrazine will cause a dose-depended increase in homologous recombinational events as quantified by the frequency of SCEs analyzed in G2-phase. Towards this goal, a new cytogenetic approach is applied for the analysis of SCEs directly in G2-phase prematurely condensed chromosomes (PCCs). The methodology enables the visualization of SCEs in G2-blocked cells and is based on drug-induced PCCs in cultured lymphocytes. The results obtained for high concentrations of atrazine, do not demonstrate a dose-depended increase in homologous recombinational events. They do not support, therefore, atrazine’s genotoxic mode of action. However, they suggest that an important part in the variation of SCE frequency reported by different laboratories when conventional SCE analysis is applied after exposure to a certain concentration of atrazine, is due to differences in cell cycle kinetics of cultured lymphocytes rather than to a true biological variation in the cytogenetic end point used.

1- 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. The triazine herbicides, which are used both for pre-emergence and post-emergence control of grasses during cultivation of maize, wheat, sorghum, sugar cane, conifers and others, have made their way into surface and groundwater supplies due to their widespread use in agriculture and low solubility in water (Goldman 1994). Atrazine is the most prevalent triazine found in rural groundwater and through its occurrence in food such as corn, nuts, fruit, and wheat, is a potential hazard to humans. To evaluate its genotoxic and mutagenic potential, several studies have been carried out in different experimental systems ranging from bacterial to mammalian assays. The majority of these studies have been negative but positive responses have been also reported. Atrazine has been found to induce mammary tumors in female Sprague-Dawley rats and it has been classified by the US Environmental Protection Agency (EPA) as a “possible human carcinogen” (Goldman 1994).

Sister chromatid exchanges (SCEs) caused by the presence of DNA lesions at the moment of DNA replication, have been extensively used for genotoxicity testing. Although SCEs are observed in cells treated with radiation or chemical agents which produce various types of DNA lesions (Latt 1981, Natarajan 2002), it has been suggested that DNA interstrand crosslinks may be the major lesions leading to SCE formation in cells irradiated with UV or ionizing radiation (Wojcik et al. 2003). Since it was indicated that atrazine induces DNA damage (Ribas et al. 1995, Clements et al. 1997) a number of in vitro and in

vivo studies have been carried out to investigate the mutagenic potential of atrazine using the analysis of sister chromatid exchanges (SCEs) as a cytogenetic end point. Conflicting findings have been reported, however, and the results are not always conclusive. Particularly, when the frequency of SCEs is slightly increased with respect to the controls, the activity of atrazine has been characterized as minimal. Even though the use of higher doses could confirm a more profound effect of this chemical, they cannot be applied since cells will be arrested in G2-phase and not proceed to mitosis, preventing thus their analysis by scoring of SCEs at metaphase. As a result, using exclusively the conventional methodology for the analysis of SCEs, the genotoxic and mutagenic potential of atrazine may have been underestimated. To overcome this shortcoming, a methodology is needed to enable the analysis of SCEs in interphase and particularly in the G2-phase of cultured peripheral blood lymphocytes. The visualization of interphase chromosomes in peripheral blood lymphocytes and their use for biomonitoring purposes following exposure to genotoxic agents, became possible using a method for cell fusion and premature chromosome condensation (PCC) induction (Pantelias and Maillie 1983, 1984). Thus far, researchers have examined interphase chromosomal damage in lymphocytes using the PCC methodology which has been proved to be a powerful cytogenetic tool for the identification of factors involved in the conversion of DNA damage into chromosomal damage (Terzoudi and Pantelias 1997), affecting thus sensitivity to genotoxic agents (Terzoudi et al. 2000).

In this report, a new cytogenetic approach for the analysis of sister chromatid exchanges directly in the G2-phase of cultured peripheral blood lymphocytes has been applied. The methodology is based on the induction of premature chromosome condensation (PCC) using Calyculin-A, a potent inhibitor of protein phosphatases type 1 and 2A (Coco-Martin and Begg 1997, Asakawa and Gotoh 1997, Durante et al. 1998, Gotoh et al. 1999) and the visualization of SCEs in G2-phase prematurely condensed chromosomes (G2-PCCs) using a modified fluorescent-plus-Giemsa (FPG) technique (Perry and Wolff 1974, Jan et al. 1982, Terzoudi et al. 2003). This cytogenetic approach enables in a unique way the testing of the hypothesis that high concentrations of atrazine will cause a dose-dependent increase in homologous recombinational events, as quantified by the frequency of SCEs in G2-PCCs. Since clear evidence has been provided relating SCEs to homologous recombinational events (Sonoda et al. 1999), an increase in homologous recombination repair processes is expected to result in an increase in the frequency of SCEs, and this finding would favor atrazine's genotoxic mode of action.

2- Materials and Methods

Culture conditions and premature chromosome condensation 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% Phytohemagglutinin, and incubated at 37°C for 72h in a humidified incubator, in an atmosphere of 5% CO₂ and 95% air. For PCC induction in G2-phase lymphocytes, calyculin-A (Sigma-Aldrich) was used. In order to determine the optimum conditions for PCC induction and scoring, calyculin-A was added to the whole blood cultures at various doses and treatment times. 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.

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 °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 visualization of SCEs, the slides were stained by the Fluorescence-Plus-Giemsa (FPG) technique according to Perry and Wolf (1974) protocol and Jan et al. (1982). A few drops of Hoechst 33258 (5 µg/ml) in Sorensen buffer (pH

6.8) were placed on each slide and covered with coverslips. They were then placed on a slide warmer set at 55°C and exposed to black light fluorescent lamp (Radium SupraBlack HBT 125-281) at a distance of 2cm for 10 minutes. Coverslips were removed by soaking the slides in Sorensen's buffer and stained with 3% Giemsa solution (Gurr R66 in Sorensen's buffer) for 15 minutes. The slides were finally mounted with cover slips and coded for analysis to avoid bias. For SCE scoring, the criteria suggested by Carrano and Natarajan (1988) were applied. Only second division metaphases and G2-phase PCCs, identifiable by their uniform differential staining pattern, containing 46 chromosomes were analysed.

For testing whether the mutagenic potential of atrazine may be underestimated when the conventional SCE analysis is applied, and also for the assessment of exposures that arrest cells at G2-phase, whole blood cultures were treated for the last 24 h of the total 72 h culture period. Atrazine (2-Chloro-4-ethyle-amino-6-isopropylamino-1, 3, 5-triazine), obtained from Sigma-Aldrich, Germany, was used at the concentrations of 5 µgr/ml to 220 µgr/ml and 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. Calyculin-A was dissolved in ethanol absolute.

For each experiment and chemical concentration within an experimental set, a minimum of 3 lymphocyte cultures were run. Routinely, 30-50 cells were scored for SCEs for each culture. Standard deviations of the mean values from three independent experiments were calculated for each experimental point. Data were evaluated statistically by Student's t-test.

3- Results

Drug-induced premature chromosome condensation (PCC) in cultured peripheral blood lymphocytes was used to visualize and quantify the frequency of SCEs in G2-phase. Treatment of cultured cells with 50 nM calyculin-A for 1 hour was chosen as optimum for PCC induction and analysis of SCEs in G2-phase lymphocytes, considering chromosome morphology as well. SCEs as visualized in G2-PCCs after atrazine exposure are shown in figure 1. As a positive control, lymphocyte cultures were treated with 0.1 µgr/ml of mitomycin-C, and the SCEs in G2 lymphocyte PCCs are shown in figure 2. In contrast to the appearance of chromosomes at metaphase (Figure 3), the sister chromatids in drug-induced prematurely condensed chromosomes are aligned in close contact, parallel to each other, and their centromeres are not clearly visible.

In order to test whether high concentrations of atrazine will cause a dose-dependent increase in homologous recombinational events, four sets of experiments were carried out. In the first set of experiments it was examined whether the genotoxic potential of atrazine can be evaluated in G2-phase lymphocytes even at exposures exceeding atrazine's cytotoxic limits that cause accumulation of cells in the G2-phase. The results are shown in Table I. Even though at concentrations of atrazine of 120 µgr/ml and 220 µgr/ml no cells at mitosis were present to be studied by conventional SCE analysis, using premature chromosome condensation the yields of SCEs in G2-phase lymphocytes were easily obtained.

TABLE I

Concentration of Chemical in cell culture	SCEs / metaphase cell (Mean ± SD)	SCEs / G2-phase cell (Mean ± SD)
Control	6.10 ± 0.60	6.60 ± 0.52
Atrazine 60 µgr/ml	8.50 ± 1.10	9.90 ± 0.71
Atrazine 120 µgr/ml	No metaphases	10.30 ± 0.90
Atrazine 220 µgr/ml	No metaphases	10.25 ± 0.80
MMC 0.1 µgr/ml	15.30 ± 0.91	27.42 ± 1.23

TABLE I: Yield of SCEs as scored in G2-PCC cells after exposure to atrazine at high concentrations. 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.

The second set of experiments was designed to test whether in vitro exposure of peripheral blood to atrazine from 5 µgr/ml to 200 µgr/ml will cause a dose-dependent increase in homologous recombinational events, as quantified by the frequency of SCEs obtained at each experimental point. As it is shown in Table II, the results obtained in particular at high atrazine's exposures, do not demonstrate a dose-dependent increase in the frequencies of SCEs.

In the third set of experiments it was examined whether SCE analysis in metaphase chromosomes is a more sensitive method to estimate the genotoxic potential of atrazine. The results are presented in Table III. A higher SCE yield per cell was scored in G2-PCCs than in cells at metaphase. In the fourth set of experiments, the involvement of cell cycle kinetics in the variation of SCEs among individuals after exposure to 20µgr/ml of atrazine was examined. The results are also shown in Table III. A lesser SCE variability (CV=8.5%) was observed when the analysis was carried out in G2 phase prematurely condensed chromosomes than in metaphase cells (CV=20%). The range for SCEs per cell among healthy individuals after atrazine exposure was 5.5-9.9 when analyzed in cells at metaphase, whereas the range was 8.4-10.4 when SCEs were scored in G2-phase PCCs.

TABLE II

Concentration of Atrazine in cell culture	SCEs / metaphase cell Range* (Mean ± SD)	Coefficient of variation (%)	SCEs / G2-phase cell Range* (Mean ± SD)	Coefficient of variation (%)
Control (w/o atrazine)	4.4 – 8.9 (6.0 ± 1.6)	26.7	6.0 – 7.2 (6.4 ± 0.5)	7.8
5 µgr/ml	5.2 – 7.8 (6.5 ± 1.2)	18.5	7.2 – 8.3 (7.7 ± 0.6)	7.8
10 µgr/ml	6.0 – 9.2 (8.0 ± 1.4)	17.5	7.9 – 10.0 (8.9 ± 0.9)	10.1
20 µgr/ml	5.5 – 9.9 (7.5 ± 1.5)	20.0	8.0 – 10.4 (9.3 ± 0.8)	8.5
50 µgr/ml	7.1 – 10.5 (8.1 ± 1.4)	17.3	9.4 – 10.0 (9.6 ± 0.7)	7.3
100 µgr/ml	No metaphases	-	9.9 – 12.5 (10.5 ± 0.9)	8.6
150 µgr/ml	No metaphases	-	9.9 – 11.9 (10.1 ± 0.9)	8.9
200 µgr/ml	No metaphases	-	11.6 – 12.0 (10.4 ± 0.8)	7.7

* Range of SCEs / cell from 4-6 donors.

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

TABLE III

Donor No.	SCEs / metaphase cell (Mean* ± SD)	SCEs / G2-phase cell (Mean* ± SD)	Difference in sample means**
1.	6.7 ± 0.5	9.2 ± 1.0	2.5
2.	6.9 ± 0.7	8.4 ± 0.6	1.5
3.	9.9 ± 1.2	10.4 ± 0.9	0.5
4.	6.6 ± 0.9	8.4 ± 0.5	1.8
5.	5.5 ± 0.8	9.0 ± 0.8	3.5
6.	8.2 ± 0.9	9.3 ± 0.9	1.1
7.	8.7 ± 1.0	10.2 ± 1.2	1.5
Overall mean±SD	7.5 ± 1.5	9.3 ± 0.8	1.77 ± 0.98
	Coefficient of variation CV = 20 %	Coefficient of variation CV = 8.5 %	

* mean from 3 independent experiments

** Significance of the difference in sample means, t-test, $\alpha=0.05$
($t=4.78$, $0.01 < p < 0.001$)

TABLE III: Variation of SCE frequencies among normal individuals as scored in metaphase cells and in G2-phase cells using premature chromosome condensation after exposure to 20 μ gr/ml of Atrazine.

3- Discussion

Even though the evidence for the mutagenic and carcinogenic potential of triazines is still equivocal, the extensive human exposure to these herbicides is likely to continue and possibly increase in the near future. For USA alone there are estimates ranging from 90 to 121 million pounds of active ingredient used annually (Goldman 1994, Kligerman et al. 2000). Most of the studies reported in the literature tend to support the concept that triazines are either not genotoxic or have minimal genotoxic activity (Kligerman et al. 2000, Tennant et al. 2001). Two studies have indicated that atrazine can cause DNA damage as measured by alkaline single cell gel (SCG) assay. Clements et al. (1997) reported that atrazine caused significant DNA damage in bullfrog tadpole erythrocytes. Atrazine was also shown to cause DNA damage with and without S9 activation in human lymphocytes treated in vitro (Ribas et al. 1995). EPA has proposed to upgrade the classification of atrazine to a "likely human carcinogen", and reevaluate of the mutagenic and carcinogenic potential of atrazine (Ribas et al. 1995).

In an attempt to study the genotoxicity of atrazine, in the present report a new cytogenetic approach is used for testing the hypothesis that high concentrations of atrazine will cause a dose-dependent increase in homologous recombinational events, as quantified by the frequency of SCEs in G2-PCCs. For this purpose it was examined whether the genotoxic potential of atrazine can be evaluated in G2-phase lymphocytes even at exposures exceeding atrazine's cytotoxic limits that cause cell accumulation in the G2-phase. The results shown in Table I demonstrate that at high concentrations of atrazine, SCEs could not be scored using conventional analysis since no cells were present at metaphase. However, using premature chromosome condensation the yields of SCEs in G2-phase lymphocytes were easily obtained. With respect to whether in vitro exposure of peripheral blood to atrazine from 5 μ gr/ml to 200 μ gr/ml would cause a dose-dependent increase in homologous recombinational events, as quantified by the frequency of SCEs obtained at each experimental point, the results presented in Table II do not support this hypothesis. Particularly, the fact that an increase of chemical concentration from 50 to 200 μ gr/ml did not increase the frequency of SCEs, as scored in G2 lymphocyte PCCs, does not support atrazine's genotoxic mode of action.

The results presented in Table III show that a higher SCE yield per cell was scored in G2-PCCs than in cells at metaphase. They suggest, therefore, that SCE analysis in G2-PCCs is a more sensitive method to estimate the genotoxic potential of atrazine since it includes in the analysis the G2-blocked cells as well. On the average the SCE frequency obtained in the G2-phase is significantly higher ($0.01 < p < 0.001$) than that obtained in metaphase. In Table III results are also shown from experiments designed to test whether the variability in the kinetics of cultured peripheral blood lymphocytes among individuals may affect the frequency of SCEs when conventional SCE analysis in metaphase cells is exclusively applied. A lesser SCE variability was observed when the analysis was carried out in G2-PCCs than in metaphase cells. The range for SCEs per cell among healthy individuals after atrazine *in vitro* exposure was 5.5-9.9, with a coefficient of variation (CV) value of 20% when analyzed in cells at metaphase, whereas the range was 8.4-10.4, with a CV value of 8.5% when SCEs were scored in G2-phase PCCs. Similar CV values were also obtained for the other atrazine concentrations used as shown in Table II. These results suggest that an important part in the variation of SCE frequency reported by different laboratories when conventional SCE analysis is applied after exposure to a certain concentration of atrazine, is due to differences in cell cycle kinetics of cultured lymphocytes rather than to a true biological variation in the cytogenetic end point used.

In conclusion, the use of a simple protocol for SCE analysis in G2-phase lymphocyte PCCs enables the evaluation of the genotoxic potential of atrazine even at high concentrations of this pesticide. Even though a minimal genotoxic activity was observed at the low dose range, the fact that an increase of chemical concentration from 50µgr/ml to 200µgr/ml did not increase the frequency of SCEs, as scored in G2 lymphocyte PCCs, does not support atrazine's genotoxic mode of action. Furthermore, since a lesser SCE variability is observed when the analysis is carried out in G2-phase, it is possible that an important part in the variation of the SCE frequencies and the discrepancies between different laboratories reporting on the genotoxic effect of a certain dose of atrazine, is due to differences in cell cycle kinetics of cultured lymphocytes rather than to a true variation in the induction of SCEs.

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Figure 1: SCEs as visualized in G2-PCCs of peripheral blood lymphocytes exposed to 20 µgr/ml of atrazine. The sister chromatids in drug-induced PCCs are parallel to each other and their centromeres are not clearly visible.

Figure 2: SCEs as visualized in G2-PCCs of peripheral blood lymphocytes treated with 0.1 µgr/ml of Mitomycin-C.

Figure 3: SCEs as visualized in peripheral blood lymphocytes at metaphase. The clear appearance of the centromeres in metaphase chromosomes differentiates them from the drug-induced G2-PCCs.