Analysis of sister chromatid exchanges directly in G2-phase lymphocyte prematurely condensed chromosomes: A new cytogenetic approach for risk assessment of chemical *in vitro* exposures to cytotoxic levels.

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INTRODUCTION

Sister chromatid exchange (SCE) analysis in human peripheral blood lymphocytes is often applied as a cytogenetic assay for biomonitoring and genotoxicity testing of potentially mutagenic and carcinogenic chemicals. Sister chromatid exchanges (SCEs) represent the interchange of DNA replication products at apparently homologous sites on the two chromatids of a single chromosome, and are indicative of DNA damage corrected by recombinational repair. Such a strand recombination (crossover) is believed to be the basis for the formation of SCEs. It has been found that S-phase dependent agents are very good inducers of SCEs, whereas ionizing radiation is a poor inducer (1). The evidence of SCE formation came from early studies (2) that demonstrated their occurrence during replication of chromosomes and paved the way to detect them easily. A breakthrough in the visualization of SCEs came in 1972-1974. It was then shown that, when a thymidine analogue such as 5-bromodeoxyuridine (BrdU) is incorporated into the DNA for two cell cycles, the sister chromatids, which are different, namely bifilarily and unifilirily incorporated BrdU (BB-TB), can be distinguished using Giemsa staining, fluorescence dyes, or their combination. The fluorescence plus Giemsa (FPG) technique (3) became popular because of the ease with which the technique can be performed and also because the slides can be stored for a long time. At present, however, there are two main practical problems with respect to the use of SCE analysis for genotoxicity testing of possible human carcinogens.

First, the SCE frequencies are obtained scoring only cells that have reached metaphase following exposure to non-cytotoxic chemical doses. Doses exceeding the toxic limits cannot be evaluated since they reduce the number of cells available at metaphase that can be analyzed when using a mitotic inhibitor such as colcemid. Affected cells at those dose ranges can be temporarily arrested in the G2-phase of the cell cycle and, therefore, cannot be analyzed when conventional metaphase SCE analysis is used. Thus, there is a need for a method to score SCEs directly in the G2-phase and to assess the genotoxicity of chemical exposures exceeding the cytotoxic limits.

Second, the base-line of SCEs fluctuates among individuals and between studies and this variation may be sometimes higher than the effect associated with exposure to genotoxic carcinogens. Since only cells proceeded to metaphase can be presently analyzed using SCEs, it could be of interest to examine whether a major part of this background variation is due to differences in cell cycle kinetics rather than due to a true biological variation in the end point used. A method to score SCEs directly in the G2-phase could facilitate as well the elucidation of this issue.

In this report, a simple and easy technique is presented for the analysis of sister chromatid exchanges (SCEs) directly in the G2-phase using premature chromosome condensation (PCC). The visualization of interphase chromosomes in peripheral blood lymphocytes and the analysis of chromosomal damage for biomonitoring purposes become first possible using cell fusion (7, 8). The methodology used in this report, which is within the limits of conventional biomonitoring, is based on the induction of premature chromosome condensation (PCC) in G2-phase peripheral blood lymphocytes using Calyculin-A, a specific inhibitor of protein phosphatases type 1 and 2A (4-6). Subsequently, the analysis of SCEs is carried out using the fluorescent-plus-Giemsa (FPG) technique (3) in G2 prematurely condensed chromosomes (G2-PCCs).

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% Phytohemaglutinin, and incubated at 37^0C in a humidified incubator, in an atmosphere of 5% CO_2 and 95% air for 72h. 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 the doses 10 nM, 50nM, and 100nM during the 0.5, 1, or 3 final hours of incubation period. 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 chemicals used for the implementation of the proposed methodology are: the herbicide Atrazine (2-Chrolo-4-ethye-amino-6isopropylamino-1, 3, 5-triazine), the herbicidal compound Gramoxon, also known as Paraquat dichloride (1,1-dimethyl-4, 4bipyridium), and the X-Ray developer Hydroquinone (1,4 Benzenediol), all obtained from Sigma-Aldrich, Germany.

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), 20 μl of cell suspension were dropped on wet slides and air dried. For SCEs preparation, slides were stained by the Fluorescence-Plus-Giemsa (FPG) technique according to Perry and Wolf protocol (3) with some modifications.

The frequency of cells with prematurely condensed chromosomes expressed as a percentage of all nuclei observed was scored at low magnification (x 200) along lines crossing the centre of the spread drop of chromosome preparations. About 300 cells per experimental point were analyzed. The frequencies of cells with fully condensed chromosomes, partially condensed, or not affected by Calyculin-A, as well as cells at metaphase were scored for each dose and treatment time. For SCE scoring, the criteria suggested by Carrrano and Natarajan (9) were applied. Only second division metaphase, identifiable by their uniform differential staining pattern, containing 46 centromeres were analysed. Every switch of staining between the sister chromatids is scored as an SCE. Switch of label at the centromere were not scored as an SCE either in metaphase or in G2-phase PCCs.

RESULTS AND DISCUSSION

As a consequence of the massive use of chemicals in the environment, residual amounts and their metabolites, some of which have been classified as possible human carcinogens, have been found in drinking water and foods. In agriculture alone, there are at present more than 1,000 chemicals classified as pesticides and many reports have shown that some of them have genotoxic effects, including cancer and several other genetic diseases. These facts have led to an increased concern for the possible threat to human health posed by exposure to such chemical compounds, and different studies have been conducted in human populations applying different indicators of genetic damage. Using cytogenetic end points such as chromosomal damage and SCE analysis a large number of studies have been carried out on the in vivo and in vitro genotoxicity of possible human carcinogens but the results are not always conclusive, and sometimes conflicting cytogenetic findings have been reported (10). In the case for instance of the herbicides atrazine, cyanazine and simazine, which induce mammary tumors in female Sprague-Dawley rats, the frequency of SCEs for exposures in the range of non-cytotoxic doses is usually found to be equal or slightly increased with respect to the controls. The activity of these chemicals has been characterized as minimal and even though higher doses exceeding the toxic limits could give us clearer effects, they cannot be applied since the affected cells will be arrested in G2-phase, at least temporarily, and will not proceed to metaphase preventing, therefore, their analysis using the conventional SCE-methodology.

In this study we analyze SCEs in G2-PCCs in order to investigate directly in the G2-phase of cultured peripheral blood lymphocytes the clastogenic and mutagenic potential of various chemicals considered as possible human carcinogens. The objectives were first to standardize this new cytogenetic approach and then to verify whether SCE analysis in metaphase chromosomes underestimates the mutagenic potential of various chemicals. Morever, it is tested whether risk assessment for possible carcinogens could be carried out even at chemical exposures exceeding the toxic limits that cause the arrest of lymphocytes at G2-phase.

The first set of experiments was carried out to standardize the appropriate conditions for PCC induction and the visualization of SCEs in G2-PCCs of peripheral blood lymphocytes. As shown in Table 1, treatments with 50 nM calyculin-A for 2 hours as well as with 100 nM for 1hour, resulted in the highest percentage of cells with prematurely condensed chromosomes. However, the chromosomes

in the majority of the cells became fuzzy and shortened when the 72 h blood cultures were subsequently treated with calyculin-A for more than 1 h. For this reason, considering chromosome morphology as well, the 1 h treatment with 50 nM calyculin-A was chosen as optimum for PCC induction and SCE analysis in G2-phase lymphocytes.

TABLE 1

			PCCs in the different phases of the cell cycle					
Calyculin-A / duration of the treatment (nM) / Hours	**Intact Nuclei (%)**	**Partly condensed nuclei (%)**	**G1 (%)**	**S (%)**	**G2 (%)**	**M (%)**	**Total PCCS (%)**	**Cells analysed**
10 nM / 1 h	55,9	5,2	1,4	33,5	1,1	2,6	38,8	268
10 nM / 2 h	60,5	0,4	0	28,3	2	8,5	38,8	247
10 nM / 3 h	52,8	6,3	0	36,9	3,5	0,3	40,8	284
50 nM / 0.5 h	47	29	4	8	8,4	2,5	23	319
50 nM / 1 h	44	8,8	7	29,4	10	0	49,9	339
50 nM / 2 h	65	2,6	4,8	41,4	8,2	0	54,5	229
50 nM / 3 h	47,6	2,8	4,1	41,2	4,1	0	49,5	315
100 nM / 0.5 h	52,6	15,7	2,1	24,5	2,4	2,4	31,5	285
100 nM / 1 h	36,4	7,8	2	43,7	3,4	6,4	55,6	343
100 nM / 3 h	66	4,4	0	27,3	2,2	0	29,5	227

Table 1: Analysis of SCEs in G2-PCCs after different Calyculin-A doses and different treatment times

In order to test whether genotoxic risk assessment for possible carcinogens could be carried out even at exposures exceeding the toxic limits that arrest lymphocytes in G2-phase, a second set of experiments was carried out and the results are shown in Table 2. When the chemicals atrazine and

hydroquinone were used in blood cultures at very high concentrations (toxic), SCEs could not be scored since no cells at mitosis were present at this experimental point. However, as it is shown in Table 2, by using premature chromosome condensation, the genotoxic assessment and the yields of SCEs per cell at G2-phase even at doses exceeding toxic limits, were easily obtained.

TABLE 2

Chemical	**Concentration in cell culture**	**SCEs / metaphase cell**	**SCEs / G2-phase cell**
Control (w/o chemical)		5.00	6.20
Atrazine	100 μgr/ml	No metaphases (cytotoxic level)	10.10
Atrazine	200 μgr/ml	No metaphases (cytotoxic level)	10.45
Hydroquinone	200 μM	No metaphases (cytotoxic level)	13.12

Table 2: No. of SCEs as analyzed in G2-PCC cells, in comparison to those analyzed in cells at metaphase, after exposure to various chemicals at cytotoxic levels.

In a third set of experiments the new methodology was applied to test whether SCE analysis in metaphase chromosomes is the most sensitive approach to estimate the genotoxic potential of various suspected carcinogens. The results are presented in Table 3. A much higher SCE yield per cell was scored in G2-PCCs than in cells at metaphase. These results suggest that using conventional SCE analysis at metaphase cells, the mutagenic potential of chemicals that temporarily arrest cells at the G2-phase of the cell cycle could be underestimated.

TABLE 3

Chemical	**Concentration in cell culture**	**SCEs / metaphase cell**	**SCEs / G2-phase cell**
Control (w/o chemical)	-	5.00	6.20
Hydroquinone	50 μM	8.70	14.50
Paraquat	500 μM	6.10	8.41

Table 3: No. of SCEs as analyzed in G2-PCC cells, in comparison to those analyzed in cells at metaphase, after exposure to various chemicals.

In conclusion, in this work a new cytogenetic approach is presented that overcomes some of the disadvantages of the conventional SCE analysis at metaphase cells. This new cytogenetic approach that enables the analysis of SCEs in cells arrested in the G2-phasse of the cell cycle, can be easily applied and requires only standard cytogenetic laboratory equipment. Therefore, it can be performed in most biomonitoring laboratories in order to assess the genotoxic effect of chemical exposures even at doses that exceed the toxic limits. In particular, it may be a unique method for screening possible human carcinogens with respect to their possible genotoxic activity.

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