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Herbicide 2,4-dichlorophenoxyacetic acid (2,4-D)-induced cytogenetic damage in human lymphocytes *in vitro* in presence of erythrocytes

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Abstract

The genotoxic effects of 2,4-D and its commercial derivative 2,4-D DMA were studied by measuring sister chromatid exchange (SCE), cell-cycle progression and mitotic index in human whole blood (WBC) and plasma leukocyte cultures (PLC). Concentrations of 10, 25, 50 and 100 μg herbicide/ml were used during 72 h. In WBC, a significant increase in SCE frequency was observed within the 10–50 μg 2,4-D/ml and 25–100 μg 2,4-D DMA/ml dose range. Contrarily, in PLC, none of the concentrations employed affected the SCEs frequency. A significant delay in cell proliferation was observed in WBC after treatments with 25 and 50 μg 2,4-D/ml and 50 and 100 μg 2,4-D DMA/ml. In PLC, only 100.0 μg 2,4-D/ml altered cell-cycle progression. For both chemicals, a progressive dose-related inhibition of mitotic activity was observed. The results demonstrated that the presence of erythrocytes in the culture system modulated the DNA and cellular damage inflicted by 2,4-D and 2,4-D DMA into human lymphocytes *in vitro* as well as both 2,4-D and 2,4-D DMA were more potent genotoxic agents in the presence of human red cells.

Keywords: 2,4-D; 2,4-D DMA; Human lymphocytes; Sister chromatid exchanges; Cell-cycle progression; Mitotic activity

1. Introduction

Living species are inevitably exposed to pesticides because their use in agriculture has been increasing steadily all over the world. In epidemiological as well as in experimental clastogenesis studies there is increasing interest in biomonitoring markers, which can provide or at least give a clue for the biologically active/passive exposure to genotoxic pollutants. Several epidemiological studies demonstrated that occupational exposure to some pesticides may be related to several cancers types and other diseases (IARC, 1977, 1983, 1986, 1987, 1991). To evaluate genetic damage various *in vivo* and *in vitro* test systems have been assessed in mammalian as well as in prokaryotic cells (Evans and O'Riordan, 1975; Evans, 1986). One of most employed test systems are cultured peripheral lymphocytes, in which the analysis of both sister chromatid exchange (SCE) frequency and cell-cycle proliferation have been widely used as bioassay for clastogenicity (Latt et al., 1980; Wilmer et al., 1981; Palitti et al., 1982; James et al., 1997).

Among the group of chlorinated aromatic hydrocarbon acid pesticides, 2,4-dichlorophenoxyacetic acid (2,4-D) has been in extensive used uninterruptedly since 1944 in agriculture for broad-leaved weeds, control of woody plants, and reforestation programs (IARC, 1977). Despite its decades of usage, there are still data gaps concerning 2,4-D's effects on human health and environmental risk (IARC, 1991). In plants, this chemical mimics the action of auxins, hormones that stimulate growth, but in mammals and other species no mimic hormonal activity was observed (Osterloh et al., 1983). It is known that 2,4-D is taken up by the cells, passes rapidly through the cell membrane, and is not metabolized (Bergesse and Balegno, 1995).

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So far, the exact mechanism/s by which 2,4-D is incorporated into either plant or animal cells are not totally understood, although peroxisome proliferation has been considered as one plausible mediators of 2,4-D genotoxicity (Linnainmaa, 1984; Lundgren et al., 1987; Vainio et al., 1982). It has been suggested that chemicals possessing this type of activity induce peroxisomal β-oxidation increasing the intracellular production of H_2O_2 and other reactive oxygen species (Reddy et al., 1980, 1982). Then, this type of actions may explain an indirect way of action by which the genetic material could be affected by the chemical. It is well documented that when mammalian cells are exposed to reactive oxygen species, lesions appear in the DNA (Meneghini and Hoffmann, 1980; Birboin, 1982; Larramendy et al., 1987). So far, induction of chromosomal aberrations (Emerit et al., 1982a,b; Estervig and Wang, 1984; Phillips et al., 1984; Nicotera et al., 1985), SCE (Emerit et al., 1982a,b; Speit and Vogel, 1982; Estervig and Wang, 1984; Nicotera et al., 1985; Larramendy et al., 1987,1989a,b; James et al., 1997) and delay in cell-cycle progression (Larramendy et al., 1987, 1989a,b) have been determined as a consequence of the damage inflicted in the DNA by reactive oxygen species.

So far, the ability of 2,4-D to induce DNA damage measured by the SCE assay is not fully documented and controversial. Galloway et al. (1987) found an increase SCE in Chinese hamster ovary (CHO) cells after treatment in presence of S9 fraction. However, in a recent study reported by us, a significant increase of SCE was observed in CHO cells treated with 2.0–4.0 μg/ml 2,4-D and 2,4-D DMA dose-range even in the absence the S9 fraction (González et al., 2005). Turkula and Jalal (1985) observed a highly significant increase in SCEs in human lymphocytes *in vitro* treated with 50.0 μg/ml of pure 2,4-D but not when dosages of 100.0 and 250.0 μg/ml were employed. Similarly, Madrigal-Bujaidar et al. (2001) reported a significant increase in SCE frequencies in bone marrow and germ cells of mice after oral administration of 100 and 200 mg/kg of 2,4-D. On the other hand, no SCE induction was observed after a 1 h pulse-treatment of CHO cells with 2,4-D pure and a commercial 2,4-D formulation (2,4-D salt as the active ingredient) with and without S9 activation (2.0–221.0 μg/ml dose-range) (Linnainmaa, 1983). Mustonen et al. (1989) reported that pure 2,4-D was unable to increase the frequency of chromosomal aberrations in human lymphocytes *in vitro* (27.0–75.6 μg/ml dose-range), whereas commercial 2,4-D formulation significantly enhanced it only in the absence of S9 fraction (27.0–27,630.0 μg/ml dose-range). Recently, SCE induction has been reported for chick embryo B-lymphocytes after long-term exposure to either pure 2,4-D and commercial formulation containing 37% of 2,4-D (500.0–4000.0 μg/embryo dose-range) (Arias, 1995).

In agriculture, the 2,4-D is used as the active component of several technical formulations. Accordingly, workers and environment are exposed to the simultaneous action of the active ingredient and a variety of other chemicals contained in its commercial derivatives. Therefore, the present study was carried out to compare the levels of the possible genotoxicity of 2,4-D as a pure active ingredient and 2,4-D DMA, as one of its most widely used technical formulation in Argentina. The DNA-damaging potential of these compounds was monitored on human lymphocytes *in vitro* in presence or absence of red human cells, using the analysis of the SCE frequency and the cell-cycle progression as cytogenetic endpoints.

2. Materials and methods

2.1. Chemicals

2,4-Dichlorophenoxyacetic (2,4-D; CAS No. 94-75) was obtained from Riedel-de Haën (Pestanal®, Hannover, Germany). Dimethylamine 2,4-D salt (2,4-D DMA) was kindly provided by Delente Laboratories SRL (Buenos Aires, Argentina). Acetone was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Blood samples

Blood samples were obtained from six healthy men under 30 years of age (non-smokers, non-alcohol drinkers, and under no medication or food supplements intake) selected according to previously described recommendations reported elsewhere (Bianchi et al., 1979). Twenty ml of blood were drawn by venipuncture from each donor immediately prior culturing at the Buenos Aires Province Blood Bank (Argentina) under approval granted by the ethical committee for studies involving human subjects. The donors were thoroughly questioned regarding their lifestyle, working environment and recent exposures to potential mutagens to exclude possible confounding factors affecting the endpoints employed in the analysis, e.g. drugs intake, recent viral infections, vaccinations, or X-ray exposure.

2.3. Whole blood cultures (WBC) and plasma leukocyte cultures (PLC). Pesticide treatment for SCE assay

Human WBC were set up by inoculating 1.0 ml of whole blood in 9.0 ml of culture medium [90% Ham's F10 medium (Gibco, Grand Island, NY, USA), 10% fetal calf serum (Gibco), 100 U penicillin/ml (Gibco), 10 μg streptomycin/ml (Gibco) and 10 μg BrdUrd/ml (Sigma Chemical Co.)]. Human PLC was carried out according to Moorhead et al. (1960) and Larramendy and Reigosa (1986). Briefly, after gravity sedimentation of whole blood (approximately 20 ml sample) for 1–2 h at room temperature, aliquots of 1 ml of plasma-leukocyte suspension were added to 9 ml of complete culture medium. The final concentration of leukocytes was approximately 1.2×10^6 cells/ml. Immediately after seeding, 2,4-D and 2,4-D DMA were dissolved in acetone prior to use and then were diluted in culture medium so that the addition of 100 μl to cultures allowed to reach the required concentration specified in results section. 2,4-D and 2,4-D DMA were used at the final concentration of 10, 25, 50 and 100 μg/ml. WBC and PLC from donors 1–3 and from donors 4–6 were treated with pure 2,4-D and 2,4-D DMA, respectively. The final solvent concentration was <1% for all the treatments. Negative controls [untreated cell and solvent-vehicle-treated cells (50 μl acetone/10 ml)] were processed concurrently with herbicide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Cultures were duplicated for each experimental point, during at least three independent experiments. Immediately after herbicide treatment, 0.3 ml of phytohaemagglutinin M (Gibco) was added to each culture (0 h). After treatment, cells were incubated at 37 °C in a 5% CO_2 for 72 h. During the last 3 h of culture, the cells were treated with colchicine (0.1 μg/ml, Sigma Chemical Co.), collected by centrifugation, treated with hypotonic solution (0.075 M KCl, 37 °C, 15 min) and fixed in methanol-acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique. The same batches of culture medium, sera and reagents were used throughout the study.