

Micronucleus frequency and proliferation in human lymphocytes after exposure to herbicide 2,4-dichlorophenoxyacetic acid in vitro and in vivo

Nina T. Holland^{a,*}, Paurene Duramad^a, Nathaniel Rothman^b, Larry W. Figgs^c, Aaron Blair^b, Alan Hubbard^d, Martyn T. Smith^a

^a Division of Environmental Health Sciences, School of Public Health, University of California, Berkeley, CA 94720-7360, USA

^b National Cancer Institute, Bethesda, MD 20892, USA

^c St. Louis University, St. Louis, MO 63108-3342, USA

^d Division of Biostatistics, School of Public Health, University of California, Berkeley, CA 94720-7360, USA

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Abstract

Widespread use of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and its association with non-Hodgkin's lymphoma (NHL) and other cancers has raised public concern. Here, micronucleus (MN) formation has been used as a biomarker of genotoxicity, and replicative and mitotic indices (MIs) as biomarkers of cell cycle kinetics in human lymphocytes. Cells were cultured either as whole blood or isolated lymphocytes and treated with pure or commercial forms of 2,4-D at doses between 0.001 and 1 mM for 48 h. Exposure to 2,4-D produced a minimal increase in MN in whole blood and even smaller one in isolated lymphocyte cultures. This induction took place only at levels approaching cytotoxicity and was accompanied by a significant inhibition of replicative index (RI). At a low (0.005 mM) dose of commercial 2,4-D, a small, marginally significant increase in RI (12–15%) was found in two independent sets of experiments ($P = 0.052$). Additionally, we found that lymphocyte RI was more affected by commercial 2,4-D containing 9.4% of the chemically pure 2,4-D, than with an equal concentration of the latter suggesting that other ingredients present in the commercial pesticide may be responsible or may enhance the effect of 2,4-D. Mitotic index, however, did not show any significant change with either commercial or pure 2,4-D. The lymphocytes of 12 male applicators exposed solely to 2,4-D during a 3-month period had a significantly higher RI than the same group prior to exposure and than a control group ($P < 0.01$), in accordance with the in vitro finding of increased RI at low doses. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

2, 4-Dichlorophenoxyacetic acid (2,4-D) is a phenoxy herbicide that functions by maintaining high

levels of the plant hormone auxin, resulting in overstimulation of plant growth and ultimately death. 2,4-D is currently the most widely-used herbicide in the world, with almost 60 million pounds being used annually in the US. [1]. A two- to eight-fold increase in the incidence of non-Hodgkin's lymphoma (NHL) among farmers who frequently used phenoxy herbicides such as 2,4-D has been reported among farmers

* Corresponding author. Tel.: +1-510-642-8781;

fax: +1-510-642-0427.

E-mail address: ninah@uclink4.berkeley.edu (N.T. Holland).

in the central US [2] and in Canada [3]. Other studies, however, are negative or equivocal [4–6]. However, the incidence of NHL has been increasing over the last 20 years and its etiology remains largely unknown [7,8].

Most mechanistic evaluation of 2,4-D has focused on genotoxic effects, yet non-genotoxic alterations could also play a role. Previous *in vivo* and *in vitro* studies of 2,4-D toxicity have produced equivocal results. In a review article, Ibrahim et al. [9] reported that 2,4-D is an unlikely carcinogen since it has not been shown to be mutagenic in most systems. For example, two studies, both of which employed the sister chromatid exchange (SCE) assay, failed to find genotoxic effects [10,11]. However, Turkula's and Jalal study of commercial 2,4-D in cultured human lymphocytes revealed an increased rate of SCE [12]. This result was confirmed by additional findings in bone marrow and testes cells by both the SCE and Comet assays [13]. Pilinskaya also found an increased frequency of aberrant metaphases in human lymphocytes exposed to 2,4-D *in vitro*, as well as in the bone marrow of treated mice [14]. Recently, several negative findings in rat and hamster models using the micronucleus (MN) assay, HGPRT and Ames test were reported [15–17].

The conflicting genotoxicity results may be attributable to different methodologies and treatment protocols [18]. For example, the selection of compositionally different 2,4-D salts and acids or vehicle solvents in each experiment could lead to different absorption and metabolism rates. Furthermore, the 2,4-D used in the commercial products differs from pure 2,4-D, and can be further divided into two categories: (1) salts, which consist of dimethylamine, triisopropanolamine and isopropylamine, and (2) esters, isooctyl ester and butoxyethyl ester. It is therefore essential that the 2,4-D composition and vehicle solvent be denoted.

In addition to genotoxicity, stimulation of cellular proliferation may contribute to the carcinogenicity of chemicals. It has been suggested that 2,4-D or other phenoxy herbicides may cause NHL through lymphocyte or immune alteration [19,20]. Changes in lymphocyte proliferation may be a possible mechanism, since lymphocytes are one of the targets of 2,4-D [21]. Several commonly used measures of proliferation include: mitotic index (MI), replicative index (RI), and cellular proliferation kinetics. MI, or the percentage

of metaphases among harvested, fixed lymphocytes, is easily assessed when CA or SCE assays are performed. Both of these assays require the addition of colchicine or colcemid to arrest the progression of cells from metaphase to anaphase ensuring sufficient number of metaphases for cytogenetic analysis. RI can be measured along with a variation of the MN assay with cytochalasin B [22] or with the SCE assay [23]. Other methods for measuring proliferation less commonly used in conjunction with genotoxicity assessment include radioactive labeling and growth curves [24,25]. Our recent report of increased lymphocyte proliferation among a group of herbicide applicators using 2,4-D further underscores the need to evaluate possible non-genotoxic mechanisms [26].

Here, we present our *in vitro* and *in vivo* findings on the cytogenetic effects of the herbicide 2,4-D in humans using biomarkers of chromosomal damage (micronuclei) and cell cycle kinetics (mitotic and RI). A special effort was made to use doses of 2,4-D comparable to environmental and occupational exposures in an attempt to reproduce the stimulatory effect of 2,4-D observed in lymphocytes of applicators [26]. We address several factors which may have contributed to inconsistent findings in previous studies: (1) effect of whole blood (WB) versus isolated lymphocyte cultures, (2) use of pure versus commercial 2,4-D herbicide, (3) the dose range responsible for stimulating and inhibiting lymphocyte proliferation, and (4) the effect of different vehicles on cellular proliferation.

2. Materials and methods

2.1. Study subjects

In the first set of *in vitro* experiments, human blood was collected in heparin-vacutainers from two healthy lab volunteers (31- and 43-year-old non-smoking males) and cultured either as whole blood or isolated lymphocytes. In the second set of *in vitro* experiments, blood samples were collected from five subjects (four new and one repeat from the first set) and cultured as isolated lymphocytes. All study subjects in the second set (three females and two males, ages 26–45 years) were non-smokers.

Samples for *in vivo* studies were collected in eastern Kansas in 1994 from 12 applicators of herbicides and

9 control subjects (non-applicators) over a 6-month period and delivered to the laboratory at UC Berkeley, CA. Applicators were sampled twice, once before initial contact with 2,4-D, and again at the end of the observation period. Subjects were monitored for 12 weeks or until 2,4-D use was discontinued. All applicators and control subjects were males, with an average age of 27.5 ± 12.5 years for applicators and 24.7 ± 4.3 years for controls. Four subjects from each group were current tobacco users. They reported moderate consumption of alcohol (see more details in [26]). A control group consisting of nine individuals was selected and frequency matched to applicators by gender, 5-year age group, and alcohol and tobacco use. All participants signed informed consent documents that were approved by the Human Subjects Review Committees of the National Cancer Institute and the University of Kansas Medical School. Exposure was assessed by interview, job description, and presence of 2,4-D in urine. Urine samples collected from applicators indicated that after spraying the average level of 2,4-D was 240 ± 100 ppb. Average cumulative time of spraying 2,4-D was 204 h and there was a mean of 4.5 ± 1.9 h since exposure per specimen prior to collection. No detectable levels of 2,4-D were noted in applicators before the initial exposure or in the control group. For a more complete description of the study group and of the questionnaire and exposure assessment, see [26].

2.2. Isolated human lymphocyte culture

Human lymphocytes were isolated using Ficoll–Paque (Pharmacia, Piscataway, NJ) density gradients and cultured as described previously [27]. Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS) and layered onto Ficoll–Paque with ratio of blood + PBS:Ficoll maintained at 4:3. The blood was centrifuged at 1340 rpm for 35 min at room temperature. The lymphocyte layer was removed and washed twice in PBS at 1200 rpm for 10 min each, and then washed with RPMI 1640 media. Cell density was counted with a hemocytometer. Typically, each culture consisted of an initial density of 1×10^6 cells in 2 ml of culture medium. Culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco, Grand Island, NY) and 1.5% phytohemagglutinin

(PHA, HA15, Burroughs-Wellcome, Greenville, NC). The lymphocyte cultures were grown in a humidified incubator with 5% CO₂ at 37 °C in 15 ml conical polystyrene centrifuge tubes.

2.3. Whole blood culture

Whole blood cultures were set up by adding 0.9 ml whole blood to 10 ml culture medium consisting of RPMI 1640 supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 1.0% PHA. Culture conditions were the same as described earlier for lymphocytes.

2.4. Treatment conditions

Chemical grade (98% pure by weight) 2,4-D (CAS No. 121-75-5) was purchased from Sigma, (St. Louis, MO). Amine forms of 2,4-D are water soluble, whereas ester forms are soluble in organic solvents. In our preliminary trials, we experienced some difficulties with the solubility of 2,4-D. Similar difficulties have been reported in several studies [18,28]. Therefore, we performed a special comparative test to find the best solvent for 2,4-D. These included dimethyl sulphoxide (DMSO, Sigma, St. Louis, MO), *N,N*-dimethyl formamide (DMF, Fisher Scientific, Hampton, NH), acetone (Fisher Scientific), and 95% ethanol (Fisher Scientific).

Initially, 25 μ g of 2,4-D was dissolved into equal amounts (120 μ l) of each solvent. Each solution was then observed at 4 min, 10 min, and 24 h intervals for formation of a precipitate at the room temperature. Additionally, at 24 h, solvent concentration was increased 10-fold and precipitation ascertained. The best results were obtained with ethanol, while DMSO and DMF formed cloudy suspensions in 2–4 and 5–10 min, respectively. Acetone formed crystals after 24 h of incubation. Therefore, ethanol was selected as a solvent for 2,4-D in all experiments, and its final concentration in the media never exceeded 0.5% by volume. 2,4-D was diluted in ethanol immediately prior to treatment and added to the cultures 24 h after they were initiated, and was present until the end of cultivation at 72 h.

Commercial products do not exceed 11% 2,4-D per EPA regulations. “Spurge & Oxalis Killer” (Lilly Miller Inc.) was chosen because it has the highest

2,4-D content available (9.4% 2,4-D salt, equivalent to 7.8% 2,4-D acid). According to the manufacturer's description, additional components included salt of 2-(2-methyl-4-chlorophenoxy)propionic acid (4.5%), Dicamba (3,6-dichloro-*o*-anisic, 1%), and unidentified inert ingredients (85%). Concentrations of the commercial product were adjusted for comparison with levels of pure 2,4-D. Commercial 2,4-D was also dissolved in either RPMI or ethanol and isolated lymphocyte cultures were treated in concentrations adjusted for the amount of 2,4-D. Colchicine, a well-established aneuploidogen, was used as a positive control to validate our MN results for 2,4-D.

2.5. Micronucleus assay

Cytochalasin B (Sigma, St. Louis, MO; 5 µg/ml) was added to the cultures at 44 h post-initiation as described by Fenech and coworkers [22,29]. Cytochalasin B prevents the cells from completing cytokinesis resulting in the formation of multinucleated cells. At 72 h, lymphocytes from whole blood cultures were isolated using a Ficoll–Paque gradient and washed twice with PBS. Lymphocytes from whole blood and lymphocyte cultures were spun directly (48 g, 10 min) onto glass slides using a cytocentrifuge (Shandon, Sewickley, PA). Slides were allowed to air dry before methanol fixation at room temperature for 15 min. Slides were stored at –20 °C in a sealed box, desiccated, under an N₂ atmosphere.

2.6. Scoring

Randomized and coded slides stained with DNA-dye 4'6-diamidino-2-phenylindole (DAPI; 1 µg/ml) were scored using a Nikon microscope equipped with epifluorescent illumination and filter for quinacrine (excitation at 400–440 nm, dichroic at 450 nm, barrier at 470 nm). For the micronucleus test, at least 1000 binucleated lymphocytes (those that have undergone one mitotic division) were scored for the number of micronuclei in accordance with recently published validation of the MN assay [29–31]. Scoring criteria were as follows: (1) cells had a round or oval appearance with an intact cytoplasm, (2) nuclei similarly were round or oval with an intact nuclear membrane, (3) only cells having undergone one nuclear division were scored for the presence of micronuclei, (4) mi-

cronuclei were counted only if they were one-third or less the size of the main nuclei, (5) micronuclei were stained similar to the main nuclei, and (6) micronuclei were clearly separated from the main nuclei. Scoring was performed by two scorers, with 10% of the slides being rescored. Variability of repeated scoring of the slides by the same scorer was <20%, the difference between scorers was 24%. This was accounted for as a part of statistical analysis. A third scorer additionally assessed all questionable micronuclei.

2.7. Replicative index

RI, a measure of cell division kinetics, was calculated by scoring at least 400 cells per dose or sample, by counting the percent of cells containing 1, 2, 3 or more nuclei per individual. As a result of addition of cytochalasin B, nuclear division was not affected, but cell division was arrested. Cells that underwent one division would have two nuclei and cells that underwent two divisions would have three or four nuclei. Cells that responded to PHA stimulation but did not yet complete one division had only one nucleus. Cells that did not undergo mitogen stimulation, judging by their size and density of DNA-positive material, were not included in the count. RI was calculated as follows:

$$RI = \frac{1x\% \text{ mononuclear cells} + 2x\% \text{ binuclear cells} + 3x\% \text{ tri} + 4x\% \text{ tetra}}{100}$$

2.8. Mitotic index

Mitotic index is another method of quantifying cellular proliferation. For mitotic index assessment, a slight modification of the cell culture protocol described in Zhang, et al., was used. Briefly, at 72 h after culture initiation, 0.001% colcemid was used to arrest metaphases. Cells were then placed in 0.075 M KCl hypotonic treatment for 30 min at 37 °C and fixed three times in Carnoy's solution of methanol and acetic acid (3:1 dilution, respectively). Fixed cells were then dropped onto pre-labeled glass slides. Slides were stained with Giemsa and coded for scoring. The MI (MI: number of metaphases/total interphases and metaphases) was scored by recording the number of metaphases in 1000 cells. Two slides were scored for each data point.

2.9. Statistical analysis

In vivo data analysis compared baseline measurements for applicators with their post-exposure measurements and post-exposure measurements with non-applicator controls. Paired *t*-test analyses compared micronuclei frequencies and RI scores before and after 2,4-D application. In this case, the null hypothesis of no difference is equivalent to the mean difference being zero. Unpaired *t*-test was used to compare applicators with the control group. A non-parametric test for trend was used to compare changes in RI scores [26].

In vitro data analysis was performed using either linear (for continuous RIs) or Poisson regression (for MN frequencies) models. Because RI is a continuous variable, a linear model was used to investigate the association of RI with dose. Because the measurements of RI are reasonably symmetric and have no outliers, it is expected that the coefficient estimates will be approximately normal. MN is a count variable and so Poisson regression was used to estimate the association of MN with dose. Poisson regression naturally adjusts for differing total cell counts among samples. For comparison of individual data points the *F*-test was employed. Additionally, generalized estimating equation (GEE) analysis was applied, which accounts for the repeated measurements on the same subject (and thus the chance for residual correlation for outcomes measured on the same subject) [32]. Using a GEE augmentation to the standard regression approaches is an attempt to report less biased inferences (standard errors, *P*-values) than can result by standard regression techniques (in our case, linear and Poisson regression) that ignore the potential correlation [33]. *P*-values of 0.05 were used as criteria of statistical significance.

3. Results

3.1. Genotoxic effects of 2,4-D in whole blood and isolated lymphocyte cultures

Whole blood and isolated lymphocyte (IL) cultures were established from venous blood of two donors and treated with a broad range of concentrations of 2,4-D (0.001–1.0 mM). In Fig. 1 the dose–response for MN formation based on three separate experiments is pre-

sented. In whole blood cultures (Fig. 1A), a modest but significant ($P = 0.012$) dose–response was observed in one of the donors (43-year old), but not in the other (31-year old). These two donors differed in their response to pure 2,4-D ($P < 0.01$), however, the actual biological significance is probably quite small because all observed MN values were in a lower range of the normal MN levels for healthy adults as identified by Interlaboratory comparison published by the HUMN International group [31]. In isolated lymphocyte cultures (Fig. 1B), the only noticeable increase in the MN frequency was observed at the highest non-toxic concentration (0.3 mM) of 2,4-D with 13 MN/1000 binucleated cells compared with only 4 MN/1000 in the ethanol vehicle control. Also, there was no difference between donors in response to pure 2,4-D, and the dose–response trend, while slight, was significant by Poisson regression ($P = 0.02$). The GEE approach, which accounts for residual variation among subjects, yielded a marginally significant result ($P = 0.074$).

Treatment with commercial 2,4-D yielded similar results. Over the broad dose range of concentrations, minimal genotoxicity was observed (data not shown as a figure or table). A slight increase in the MN frequency of 6.7 MN/1000 (2 MN in control) was found in one donor only at the highest 2,4-D dose (0.3 mM). This was an older donor (43-year old). It is well known that generally MN level is age dependent [31]. However, our design with repeated sampling of the few individuals close in age, did not allow for inter-individual difference to be attributed to the age of participants. Thus, observed 2,4-D-induced MN levels did not exceed the normal range of baseline MN variability in healthy untreated individuals (3–12 MN/1000) for either whole blood or isolated lymphocyte cultures [31]. As expected, the positive control colchicine induced high levels of MN, up to 50 MN/1000 for Donor 1 and 40 MN/1000 for Donor 2 (Fig. 1C). In summary, the overall genotoxic effect of 2,4-D as measured by the MN assay was minimal.

3.2. Proliferative effects of pure versus commercial 2,4-D

The baseline RI in whole blood (2.0–2.2) was consistently higher than in isolated lymphocyte cultures (1.2–1.4) for both donors. A dose-dependent inhibition of RI was observed with higher doses of 2,4-D

in both WB and IL cultures ($P < 0.001$). At the low dose (0.005 mM) of commercial 2,4-D, however, RI was slightly increased in two independent experiments. While the background RI level was 1.20–1.27, it increased to 1.31–1.36 after 0.005 mM 2,4-D treatment ($P < 0.05$).

The increased proliferation observed at the low commercial 2,4-D dose in our initial in vitro results was similar to that reported for applicators (who had an 11–14% RI increase) [24]. This prompted us to conduct the next series of experiments with more subjects (five, including one previously involved in the first set of experiments) and using two independent

methods of proliferation assessment (MI and RI). Two 2,4-D doses were selected for treatment (0.005 and 0.3 mM), with 0.3 mM serving as the positive control for inhibition of proliferation. Additionally, we made provisions in the protocol to study the possible interactions of two vehicles, ethanol and RPMI, on the level of replication after 2,4-D.

3.3. Effect of 2,4-D on lymphocyte replicative index in vitro

The RI results for the five subjects are presented in Table 1. As expected, based on the first set of

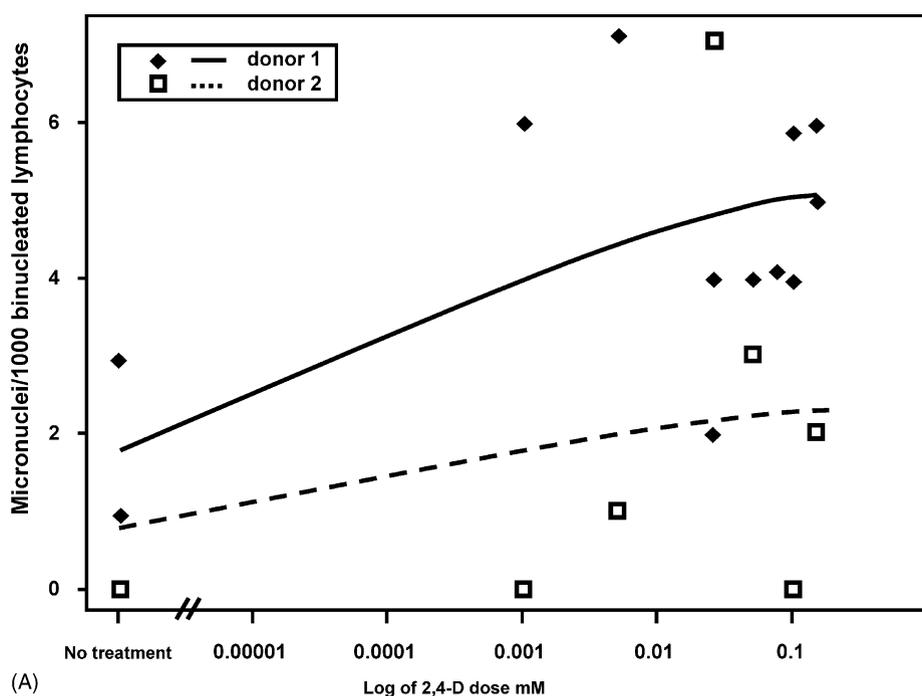
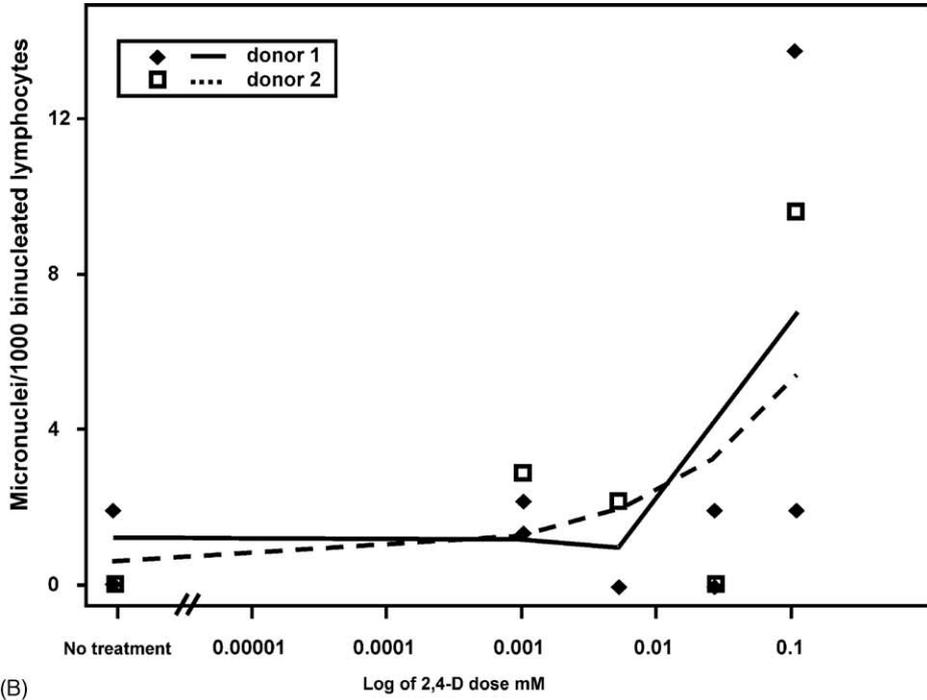
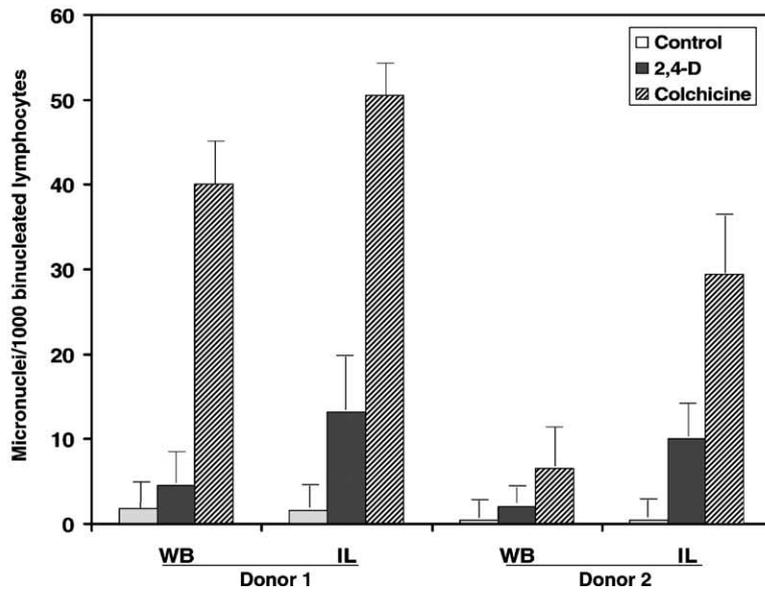


Fig. 1. MN frequency in peripheral blood lymphocytes exposed to pure 2,4-D or colchicine: dose–response of MN induced by 2,4-D in whole blood (WB) (A), isolated lymphocytes (IL) (B) or by colchicine in both WB and IL (C). The results for two donors (diamonds and squares) are based on the scoring of 1000 binucleated lymphocytes for each data point. Curves are the fit of dose–response based on multiple regression modeling. For whole blood (A) no significant change for Donor 1 was observed ($P = 0.63$), however a slight dose-dependent increase was observed in Donor 2 ($P = 0.012$) compared to the baseline MN frequency of 4 MN/1000 BN. For isolated lymphocytes (B) no difference was observed in the responses of two donors and an increased induction of MN was found only at 2,4-D levels approaching cytotoxicity when compared to the baseline MN frequency of 4 MN/1000 BN. (C) The maximum increase in MN frequency in lymphocytes of two male donors treated with 2,4-D (0.05–0.3 mM) was compared to that induced by colchicine, a positive control. Colchicine was added 24 h after the beginning of cultivation and remained in culture for 24 h. The level of MN induction by colchicine (5×10^{-6} mM) was always several times higher than by 2,4-D, which did not exceed normal background levels. Both donors had a more noticeable increase in MN frequency in isolated lymphocytes than in whole blood cultures ((A) micronuclei (MN) frequency in whole blood (WB) lymphocytes exposed to pure 2,4-D (B) micronuclei (MN) frequency in isolated blood lymphocytes (IL) exposed to pure 2,4-D (C) micronuclei (MN) frequency in lymphocytes exposed to pure 2,4-D and colchicine).



(B)



(C)

Fig. 1. (Continued).

Table 1
Replicative index after in vitro treatment of isolated lymphocytes from five donors

2,4-D dose (mM)	Vehicle	2,4-D chemical form	Subject					Mean (RI)
			1	2	3	4	5	
0.000	RPMI	NA	1.56	1.43	1.60	1.61	1.89	1.62
0.000	Ethanol	NA	1.62	1.38	1.68	1.55	1.88	1.62
0.005	RPMI	Commercial 2,4-D	1.69	1.51	1.67	1.66	1.53	1.61
0.005	Ethanol	Pure 2,4-D	1.58	1.50	1.52	1.64	1.70	1.59
0.005	Ethanol	Commercial 2,4-D	1.64	1.52	1.57	1.66	2.06	1.69
0.300	RPMI	Commercial 2,4-D	1.34	1.13	1.42	1.27	1.35	1.30
0.300	Ethanol	Pure 2,4-D	1.53	1.27	1.39	1.28	1.50	1.39
0.300	Ethanol	Commercial 2,4-D	1.31	1.22	1.16	1.20	1.29	1.24

experiments, there was a significant inhibition of lymphocyte proliferation for all five individuals at the highest dose level (0.3 mM) independent of the vehicle used for both pure and commercial 2,4-D ($P < 0.001$).

At the low dose (0.005 mM) of commercial 2,4-D, four out of five study subjects exhibited an increase

in RI, for both RPMI and ethanol vehicles (Fig. 2). Pure 2,4-D results were inconclusive with three individuals responding with increased proliferation and four actually declining. In Fig. 2, these changes in RI are presented along with the RI results from the first set of experiments, showing the range of variability of

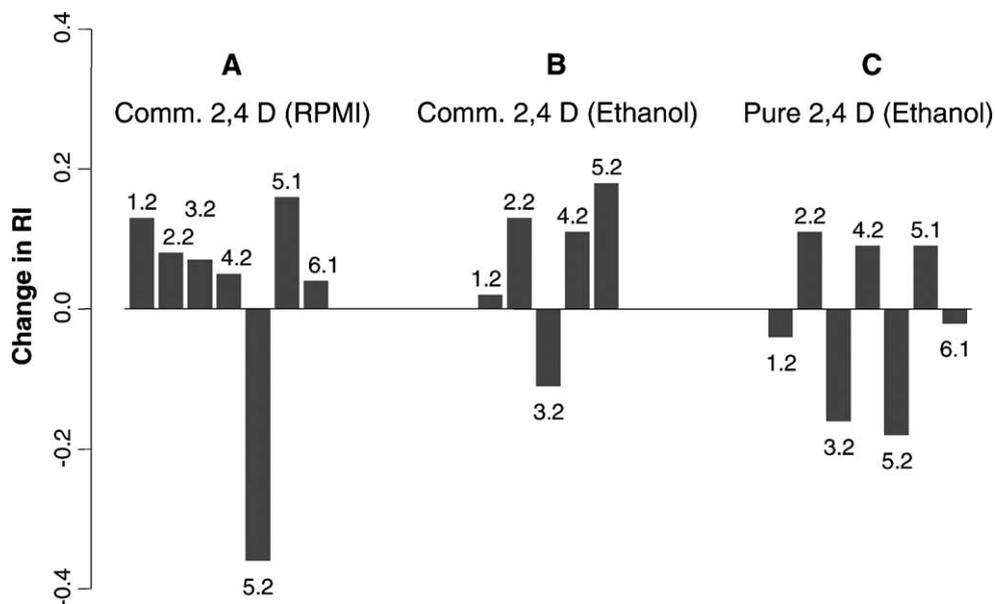


Fig. 2. Change in replicative index after low 2,4-D (0.005 mM) treatment in comparison with three herbicide/solvent combinations. For each category, the baseline (vehicle) RI score was subtracted from its corresponding low dose (2,4-D in vehicle) RI score. Each column reflects the relative increase or decrease in RI for each subject in the first and second series of experiments. For convenience, the first digit identifies the subject number and the second digit the experiment series. Concentrations of administered commercial 2,4-D were adjusted to equal the active amounts in pure 2,4-D. (A) Commercial 2,4-D dissolved in RPMI induced an increase in lymphocyte proliferation for all subjects when compared to their respective control (RPMI) levels. One of the subjects (#5.2) had also a decrease in RI in the independent experiment. (B) Commercial 2,4-D dissolved in ethanol increased the RI in four out of five individuals. (C) Pure 2,4-D dissolved in ethanol did not show a consistent increase (change in replicative index after low 2,4-D (0.005 mM) treatment in comparison with control for three herbicide/vehicle solvent combinations).

Table 2
Mitotic index after in vitro commercial and pure 2,4-D treatment of lymphocytes from five donors

2,4-D dose (mM)	Vehicle	2,4-D chemical form	Subject					Mean (RI)
			1	2	3	4	5	
0.000	RPMI	NA	5.7 ± 2.1 ^a	6.5 ± 0.7	8.3 ± 0.4	9.2 ± 0.6	10.0 ± 1.4	7.9 ± 1.9
0.000	Ethanol	NA	6.4 ± 1.2	6.6 ± 1.3	7.6 ± 3.7	9.7 ± 1.7	10.3 ± 1.5	8.1 ± 2.3
0.005	RPMI	Commercial 2,4-D	6.5 ± 5.9	7.3 ± 1.8	3.8 ± 0.6	8.2 ± 0.2	7.5 ± 0.9	6.6 ± 2.6
0.005	Ethanol	Pure 2,4-D	5.4 ± 0.1	6.6 ± 0.9	8.8 ± 0.2	6.8 ± 0.1	10.2 ± 0.7	7.6 ± 1.0
0.005	Ethanol	Commercial 2,4-D	8.2 ± 0.8	6.2 ± 1.1	6.9 ± 1.6	6.6 ± 0.6	13.5 ± 2.1	8.3 ± 3.0
0.300	RPMI	Commercial 2,4-D	1.6 ± 0.7	2.4 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	0.8 ± 0.3	1.0 ± 1.0 ^b
0.300	Ethanol	Pure 2,4-D	2.0 ± 0.6	1.3 ± 0.6	1.5 ± 0.8	2.0 ± 0.4	2.2 ± 1.3	1.8 ± 1.9 ^b
0.300	Ethanol	Commercial 2,4-D	2.5 ± 0.0	0.8 ± 1.2	0.2 ± 0.4	0.2 ± 0.8	0.4 ± 1.1	0.8 ± 0.7 ^b

^a All numbers show a mean percent of metaphases in the two sets of 1000 cells scored for each data point.

^b Inhibition was significant in comparison with respective control and 0.005 mM 2,4-D treatment ($P < 0.001$).

individual RI responses to the low 2,4-D dose. Of the five subjects, numbers 2 and 4 consistently showed increased RI for all combinations. Subject 5 was highly variable. One possible explanation is that this individual has an unusually high background RI, which may make it difficult to observe further increases in RI.

Since in both the first and second series of experiments, a similar trend toward increased RI was

observed after low (0.005 mM) dose 2,4-D treatment and the same protocol was used, the data from both sets were pooled together and analyzed using multiple logistic regression. This way variation between experiments and donors was accounted for in our statistical analyses. With the data combined, a marginally significant 12–15% increase in RI was found at 0.005 mM level of commercial 2,4-D ($P = 0.052$).

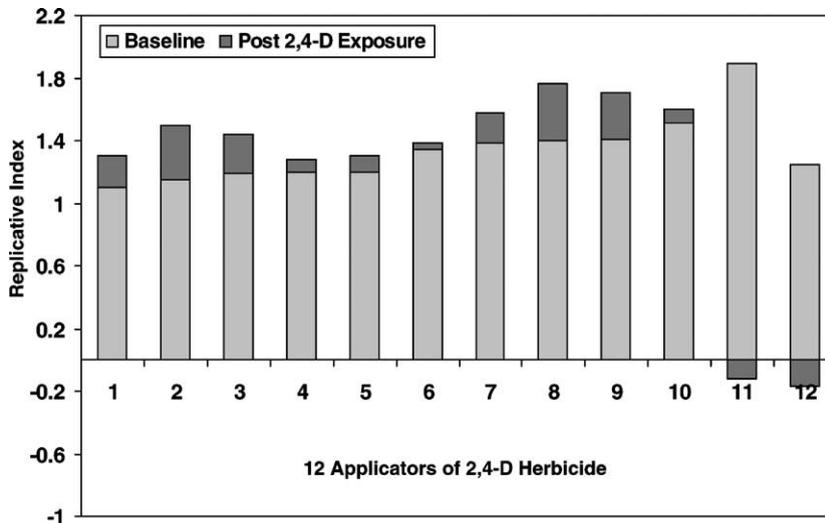


Fig. 3. Change in replicative index of 2,4-D applicators: pre- and post-spraying of the herbicide. The distribution of proliferation index in applicators and the control group indicate that statistically significant stimulation of proliferation occurred after applicators were exposed to 2,4-D for up to 3 months. The average RI in lymphocytes of applicators after exposure was 1.47 ± 0.2 compared to 1.33 ± 0.2 and 1.29 ± 0.2 before exposure for applicators ($P < 0.001$). Out of 12 applicators, 10 showed an increase in RI (change in replicative index of 2,4-D applicators: pre- and post-spraying of herbicide).

3.4. Mitotic index in vitro

The effect of 2,4-D treatment on MI in lymphocytes of five donors is summarized in Table 2. A significant inhibition of proliferation was observed after 0.3 mM 2,4-D treatment in both commercial and pure 2,4-D cultures, independent of the vehicle used ($P < 0.001$). However, unlike the RI findings, there was no significant difference in MI between control and low 2,4-D treatment.

3.5. Effect of 2,4-D herbicide in vivo

Previously, we reported an association between urinary 2,4-D concentration and increased peripheral blood lymphocyte RI in 2,4-D-exposed applicators [24]. Average RI in lymphocytes of applicators after exposure was 1.47 ± 0.2 compared to 1.33 ± 0.2 and 1.29 ± 0.2 before exposure for applicators and control group, respectively ($P < 0.001$). Ten out of 12 applicators had a higher level of RI after 3 months of 2,4-D exposure (Fig. 3). No statistically significant increase in MN frequency was observed after exposure. In fact, the frequency was 21% lower after exposure (11.6 ± 2.8 and 9.1 ± 6.2 MN/1000 binucleated cells, respectively); however, this difference was not statistically significant. The same MN frequency was observed in the control group matched by age, smoking and alcohol consumption (11.0 ± 4.8 MN/1000 binucleated cells).

4. Discussion

Several epidemiological studies previously reported an association between occupational 2,4-D exposure

and NHL and other cancers [2,3,9,34]. There is a current lack of clear experimental evidence that 2,4-D causes a biologic effect that might be related to cancer. To further explore the nature of the association of 2,4-D with cancer, we studied two main factors of carcinogenicity, proliferation and genotoxicity, as a result of 2,4-D exposure of human lymphocytes both in vivo and in vitro. Our study of the cytogenetic effects of herbicide 2,4-D in cell cultures showed an MN increase above normal baseline only at high 2,4-D doses, i.e. those approaching cytotoxic levels (Table 3). This is in agreement with a recent finding that only one out of seven commercial-grade chlorophenoxy herbicide products induced a dose-related increase in micronuclei [35]. Doses of this magnitude would seldom occur among applicators and probably explains the absence of any increase in the frequency of micronucleated cells observed in our cohort of 2,4-D applicators [26]. Negative findings were also reported in occupationally exposed tractor sprayers who had a relatively low 2,4-D concentration in their breathing zone that did not exceed the Finnish hygienic limit for 2,4-D [28]. In studies observing the results of exclusive 2,4-D exposure, Crossen and coworkers report negative findings by the SCE assay [10,11]. The only positive finding of occupational exposure to 2,4-D might be explained by the subjects' exposure to a mixture of pesticides [36]. A number of papers reported significant increases in CA, SCE and MN associated with exposure to pesticides [37–41]. However, these findings may depend on the composition of the pesticide mixture and level of exposure [42]. Lower level exposure to less potent pesticides apparently does not produce any cytogenetic changes in exposed workers [43,44]. Results of this study add 2,4-D to the list of chemicals with no observed genotoxicity in vivo by the MN assay.

Table 3

Summary of micronucleus (MN) frequency and replicative index (RI) in human lymphocytes exposed to pure and commercial 2,4-D in vitro and in vivo

Exposure to 2,4-D	Lymphocytes cultured as	Pure 2,4-D	Commercial 2,4-D
In vitro	Whole blood	Minimal increase in MN frequency at cytotoxic level, decreased RI with high dose	Minimal increase in MN frequency at cytotoxic level, increased RI at low dose
	Isolated lymphocytes	Minimal increase in MN frequency at cytotoxic level, decreased RI with high dose	Minimal increase in MN frequency at cytotoxic level, increased RI at low dose
In vivo	Isolated lymphocytes	N/A	No effect on MN, increased RI

Research on possible biologic mechanisms for 2,4-D has been primarily focused on the genotoxicity, while cytotoxicity and proliferation were assessed in only a few papers. Moreover, most in vitro studies examining the cytotoxic effect of 2,4-D on human lymphocytes have been conducted using serial concentrations of 2,4-D ranging from 0.1 to 5 mM. In this dose range, cell death through apoptosis via a direct effect on mitochondria is likely [45]. Therefore, we specifically incorporated in our analyses 2,4-D doses that were non-cytotoxic and 100-fold lower than were previously used and thus more relevant to occupational settings. While we observed an expected inhibition of both MI and RI at the high 2,4-D concentrations, at a lower (0.005 mM) dose of commercial 2,4-D, a small, marginally significant increase in RI (12–15%) was found in two independent sets of experiments ($P = 0.052$). Additionally, we found that lymphocyte RI was more affected by commercial 2,4-D containing 11% of the chemically pure 2,4-D, than with an equal concentration of the latter suggesting that ingredients other than 2,4-D present in the commercial pesticide may be responsible for, or may enhance the effect of 2,4-D. Mitotic index, however, did not show any significant change with either commercial or pure 2,4-D.

The most typical response observed both in vitro and in vivo after exposure to chemicals, radiation, or other genotoxic agents is inhibition of proliferation. This event is commonly observed with medical treatments [46,47], pesticide exposure [41,48], arsenic in drinking water [24], and other toxicant exposures [49]. No changes in proliferation, however, were reported after exposure to pulsed magnetic fields [50] or pesticides [44]. More unusual are findings of increased proliferation, for example in individuals treated with metronidazole [51], malnourished children [52], and children with Down's syndrome [25,53].

Our current observation of increase in RI in lymphocytes of 2,4-D applicators, supported by an increase in RI in vitro induced by a low dose of commercial 2,4-D may be important in light of its possible involvement in the mechanism of cancer. The fact that MI did not show any induction after low-level 2,4-D treatment may be explained by the difference between these two methods of monitoring of cell kinetics in culture. While RI is more comprehensive because it covers all stages of cell cycles during 28 h (presence of

cytochalasin B), MI is more focused on catching cells entering the metaphase stage during approximately 4 h of colcemid presence.

Lymphocyte proliferation is an extremely complex system, influenced by many factors. There is increasing evidence that indicates pesticides are capable of immunomodulatory effects ranging from suppression of humoral immunity to induction of neutrophilic oxidative stress [54–56]. Recently Lee, et al. reported an increased relative count of B cells in CD-1 mice exposed to commercial 2,4-D during gestation [57].

Another possible explanation for increased indices of proliferation may be a heightened response to PHA, resulting in a shorter lag time between exposure to PHA and transition from the G0 stage of the cell cycle to the G1 stage of the cell cycle and/or a shorter cell cycle subsequent to stimulation [52]. PHA preferentially stimulates T-cells, which are the target cells for leukemia including T-ALL, whereas B lineage cells are the predominant target cells of NHL. In a study of children with asthma and allergic rhinitis, increased frequencies of MN in B-lymphocytes prompted authors to hypothesize a potential link to elevated risk of lymphoma among individuals with allergic diseases [58]. While our data on 2,4-D-enhanced lymphocyte proliferation are obtained for T-cells (due to culture conditions using PHA, a mitogen that induces T-cell proliferation), it may reflect a more general effect on other cell types including B cells. This possibility could be addressed in the future studies by using the B-specific mitogen pokeweed [59] or separation of T- and B-cells with magnetic beads or flow cytometry. It will also be interesting to explore whether long-term cultures (more than 7 days) can provide additional insights into the mechanisms of 2,4-D interactions with cell proliferation and immune response.

Recently, adjuvants have been suggested as inducers of cellular proliferation [60,61]. Adjuvants are chemicals commonly added to pesticides to enhance the active ingredients and improve solubility. These chemicals include polyvinyl compounds, polyoxyethylenes and paraffin oils and may not be listed specifically, but included under the category of “inert ingredients” [60]. The commercial 2,4-D we selected contained 85% inert ingredients. In a recent survey of 2,4-D applicators, 59% were found to add adjuvants prior to

spraying [61]. Such mixtures can enhance the absorption and toxicity of pesticides by increasing dermal penetration. The combination of 2,4-D and adjuvants together may be responsible for inducing increased proliferation. This has been reported using MCF-7 cell lines treated with 2,4-D and adjuvants [60]. We also observed increased proliferation in human lymphocytes after commercial and pure 2,4-D treatment, although the effect of pure 2,4-D was less pronounced. This suggests that other chemicals present in the commercial product may contribute to the proliferative effect of 2,4-D in human lymphocytes. Whether these adjuvants cause proliferative effects independently or are additive to those caused by 2,4-D is not currently established [61]. The possibility of pure 2,4-D inducing a stimulatory effect cannot be disregarded since there was evidence of proliferation in some of our subjects.

Variation in individual responses to 2,4-D treatment was evident in both our *in vivo* and *in vitro* datasets. The lymphocytes of subjects treated with low doses were similar to our *in vivo* observations of applicators, i.e. higher proliferation rates post-sprays or after 2,4-D treatment *in vitro*. There is a suggestion that some individuals may be more sensitive to 2,4-D exposure than others. Additional factors, including status of the immune system, genetic predisposition, metabolic or DNA-repair differences, and simultaneous exposure to other environmental toxicants may contribute to this variation in RI scores and deserve attention.

In summary, we can conclude that genotoxicity of 2,4-D as measured by the MN assay at environmentally relevant concentrations is negligible. However, our finding of increased proliferation after low 2,4-D exposure may be of importance since proliferation is an important contributor to malignant transformation. Additionally, there are indications that in the presence of 2,4-D, biological responses to other chemicals may be changed or enhanced resulting in an increased level of mutational events. This warrants more work on the possible mechanism of association between 2,4-D and cancer.

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