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Increased lymphocyte replicative index following 2,4-dichlorophenoxyacetic acid herbicide exposure

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Abstract

Objective: Evaluate peripheral blood lymphocyte proliferation (replicative index:RI) and micronuclei frequency (MF) among 2,4-D herbicide applicators.
Methods: Twelve applicators spraying only 2,4-D provided a blood and urine specimen upon enrollment, several urine samples during the spraying season, and a blood specimen at the study's end. Nine controls provided blood and urine specimens upon enrollment and at the study's end. Gas chromatography/tandem mass spectroscopy determined urinary 2,4-D levels and standard *in-vitro* assays determined RI and MF scores. Applicator RI and MF were compared before and after spraying and with controls.
Results: Applicators contributed 45 urine specimens with concentrations ranging from 1.0 to 1700 (μg 2,4-D/g creatinine/L urine) that logarithmically (ln) increased as spraying time increased. Applicator RI increased after spraying ($p = 0.016$), independent of tobacco and alcohol use, and demonstrated a weak dose–response with increasing urinary 2,4-D levels ($p = 0.15$). Among 2,4-D applicators, pre-exposure complete blood counts and lymphocyte immunophenotypes were not significantly different from post-exposure measurements.
Conclusion: Urinary 2,4-D concentration, an exposure biomarker, may be associated with lymphocyte replicative index, a cell proliferation biomarker.

Introduction

2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most widely used postemergence pesticides in the United States [1] that acts by disrupting hormone balance and protein synthesis to cause a variety of plant growth abnormalities. Although, 2,4-D does not appear to cause cancer in rodent bioassays [2], associations with non-Hodgkin's lymphoma have been observed in some [2–6], but not all [7, 8] epidemiologic studies. Animal studies have shown that 2,4-D has a number of biological effects [9–18], but a possible carcinogenic mechanism(s) is not obvious in mammals [19–22]. Humans primarily excrete unmodified herbicide in their urine, suggesting few metabolic intermediates [23]. To provide information on possible inconsistencies between epidemiologic data and biologic effects in humans, we conducted a pilot study of 12 herbicide applicators who exclusively sprayed 2,4-D from April to July 1994 to investigate the relationship between urinary 2,4-D levels and lymphocyte proliferation rates [24] and micronuclei frequencies [25–27].

Materials and methods

Study subjects

Study participants were 13 herbicide applicators from county noxious weed offices in eastern Kansas, aged 17–56, who had no cancer history and no occupational

pesticide exposure 6 months prior to 1 March 1994, and 12 non-applicators. County noxious weed offices are charged with controlling troublesome agricultural weeds (*e.g.* bindweed, wormwood, snakeweed, thistle, knapweed, larkspur, leafy spurge, locoweed, lupine, ironweed, skeleton weed) on public and private land. These pesticide applicators use only herbicides and often spray on a daily basis. Participants completed a questionnaire, maintained a daily activity log, and provided blood and urine samples. Subjects were monitored for 12 weeks or until 2,4-D use was discontinued, whichever came first. Pesticide applicators received $250 and non-applicators received $50 remuneration at the study's end. Shortly after enrolling, one applicator relocated to a nonparticipating county, changed occupations, and withdrew from the study.

Non-applicators were solicited by word-of-mouth and newspaper advertisements. We excluded persons with previous or current cancer medical histories, persons taking prescribed medications, or persons with current disease histories that might interfere with laboratory assays. Of the 150 subjects screened, nine controls were selected and matched to applicators by gender, 5-year age group, alcohol and tobacco use, and geography (preference was given to non-applicator county employees working in or adjacent to an applicator's county of employment). Each control subject received $50 remuneration.

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.

Questionnaires and daily diary

All applicators and matched controls completed a 40-minute, in-person, enrollment questionnaire to collect health and employment history data. Applicators also responded to a 15-minute post-study questionnaire to determine changes in health status, habits, or occupational history. Applicators also kept daily work diaries to record task, task duration, pesticide use, and personal protective equipment use during the 2,4-D spraying season.

Biological specimen collection, processing, and assay methods

Baseline blood and single-void urine specimens were collected at enrollment from applicators and controls. In addition, overnight urine samples were obtained from applicators every other week following a typical day of 2,4-D application.

To insure that the urine was properly collected and not contaminated, participants were trained to fill containers without touching the urine stream to clothing or hands. Applicators were instructed to urinate at 6:00 p.m. and collect all urine thereafter, including the final urine before reporting for work the next day. To retard bacterial growth, subjects stored overnight urine collections in a single plastic container in a refrigerator at home and transported the urine to the worksite in an ordinary Thermos7® cooler filed with ice packs. Study technicians provided participants with new supplies as needed and prepared the urine specimens for transport to the laboratory. Upon receiving urine specimens, study technicians immediately pipetted 20 ml into each of two 25 ml glass serum vials (Wheaton), capped the vials with Teflon® stoppers, and sealed each with aluminum retainers. Each vial was immediately placed on dry ice and transported frozen to the University of Kansas Medical Center laboratory in Kansas City, Kansas. After determining the total volume, the unfrozen urine was discarded. All frozen samples were stored at −80 °C and were shipped on dry ice to the Centers for Disease Control and Prevention for laboratory analyses at the end of the spraying season. To maintain stability, samples were thawed as needed.

Baseline urinary 2,4-D estimates were determined from pooled urine that contained two enrollment, single-void urine samples selected randomly from applicators and controls, respectively.

Urinary 2,4-D analyses followed procedures described by Hill *et al.* [28]. Frozen urines were thawed, 10 ml urine aliquots were prepared and C^{13} labeled 2,4-D was added as an internal standard. Enzyme hydrolysis, derivatization, clean-up and concentration to 100 μl (microliters) followed. Urine 2,4-D measurements were made using capillary gas chromatography combined with tandem mass spectrometry (GC/MS/MS) employing collision-associated decomposition of parent to daughter ions. Quality assurance methods included retention time evaluation, ion ratios, and controls with known 2,4-D concentrations. Long-term sample storage was similar to conditions described elsewhere [28] for quality control samples measured several times over a 33-month analysis period to determine if 2,4-D concentrations remained within the experimental error of the method (6.2 μg/L (ppb) ± 8.7% (CV)). The detection limit for 10 ml urine samples was 1 μg/L (parts per billion). All 2,4-D concentrations were creatinine adjusted (μg/g creatinine/L urine).

Blood specimen collection and processing

Typically, all blood specimens were collected between 6:00 a.m. and 10:00 a.m. in 10 ml, green top vacutainer

tubes and appropriately packaged for overnight shipment and delivery. For the replicative index/micronucleus assay, lymphocytes were isolated using Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradients and cultured [26]. Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS), layered onto Ficoll-Paque with a ratio of cells + PBS:Ficoll-Paque maintained at 4:3, and centrifuged at 170*g* for 35 minutes at room temperature. The lymphocyte layer was removed, washed twice in PBS at 150–170*g* for 10 minutes each, and then washed with RPMI 1640 media. A hemocytometer was used to achieve an initial culture density of 1×10^6 cells in 2.0 ml of culture medium. Culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/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% CO_2 at 37 °C in 15.0 ml conical polystyrene centrifuge tubes. Cytochalasin B (Sigma, St Louis, MO) (5.0 μg/ml) was added to lymphocyte cultures at 44 hours post-initiation as described by Fenech and Morley [27]. Cytochalasin B prevents complete cytokinesis, which results in multinucleated cells. At 72 hours, lymphocytes were spun directly (48 *g*, 10 minutes) onto glass slides using a cytocentrifuge (Shandon, Sewickley, PA). Slides were air-dried before fixing with methanol at room temperature for 15 minutes. Slides were stored at −20 °C in a sealed box, desiccated, under a N_2 atmosphere. Cell division kinetics were calculated by scoring at least 400 cells per sample (200 cells per duplicate), by counting the percent of cells containing one, two, three or more nuclei per individual. A replicative index (RI) was calculated as follows:

$$\begin{aligned} \mathrm{RI} = \{&1(\%\ \text{mononuclear cells}) \\ &+ 2(\%\ \text{binuclear cells}) \\ &+ 3(\%\ \text{trinuclear cells}) \\ &+ 4(\%\ \text{tetranuclear cells}) + \cdots\}/100. \end{aligned}$$

Antikinetochore antibody staining procedures followed those described by Eastmond and Tucker [29]. Methanol-fixed slides were incubated for 5 minutes in PBS containing 0.1% Tween 20. Excess fluid was drained from slides and 40–50 μl of the antikinetochore antibody (Chemicon, Temecula, CA) diluted 1:1 with PBS containing 0.2% Tween 20 was applied. The slide's working surface was covered with a glass coverslip and placed in a humidified chamber at 37 °C for 1 hour. Following two washes in PBS containing 0.1% Tween 20 for 5 minutes each, excess fluid was again drained, slides were covered with a 1:50 dilution of fluorescent goat anti-human IgG (Chemicon, Temecula, CA), and incubated again for 1 hour. Because fluorescent-labeled antibodies fade upon exposure to light, this and all subsequent steps were conducted in yellow light. The slides were rinsed twice in buffer plus 0.1% Tween 20 and counterstained with DNA-dye 4′,6-diamidino-2-phenylindole (DAPI) (2 μg/ml) in an antifade solution [30]. Slides were refrigerated for up to a week prior to microscopic examination.

Randomized and coded slides were scored using a Nikon microscope equipped with epifluorescent illumination and filters for fluorescein (excitation at 470 nm, dichroic at 510 nm, barrier at 520–560 nm) and quinacrine (excitation at 400–440 nm, dichroic at 450 nm, barrier at 470 nm). At least 1000 binucleate lymphocytes (those that have undergone one mitotic division) were scored for the number of micronuclei. When a micronucleus was located using the quinacrine filter, the presence or absence of kinetochore staining was determined by switching to the fluorescent filter. Scoring criteria were as follows: (1) cells appeared round or oval with an intact cytoplasm, (2) nuclei appeared round or oval with an intact nuclear membrane, (3) cells having undergone one nuclear division were scored for the presence of micronuclei, (4) micronuclei had to be 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. Two scorers performed scoring with 10% of slides being rescored. A third scorer additionally assessed all questionable micronuclei.

Other laboratory analyses included lymphocyte phenotypes and complete blood counts (CBC). Lymphocyte phenotypes were determined by flow cytometry using two-color immunofluorescence and a whole-blood stain-and-lyse method [31] under guidelines approved for clinical laboratory analyses [32]. The CBC was performed to determine leukocyte type and number to assess immune function and to characterize erythrocyte size, shape, and number for signs of anemia. Total white blood cells, lymphocytes, monocytes, granulocytes (eosinophils and basophils), and erythrocytes were counted in each blood sample. Hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, and platelet count were estimated. All determinations were made using standard methods with a Coulter counter (Beckman Coulter, Inc., Fullerton, CA 92834, USA).

Statistical Analyses

Our design compared pre-exposure (baseline) measurements with post-exposure measurements of the applica-

tors and post-exposure measurements of applicators with non-applicators controls. Means were presented with standard deviations (mean ± SD) unless otherwise indicated. Urinary 2,4-D concentrations were time adjusted (mean 2,4-D concentration/mean hours spraying 2,4-D) where indicated. Paired *t*-test analyses, stratified by tobacco use history or alcohol use, compared micronuclei and replicative index scores before and after 2,4-D application. An independent sample (unpaired) *t*-test was used to compare applicator micronuclei and replicative index scores with non-applicator controls. A non-parametric test [33] was used to test for a trend of increasing replicative index scores and replicative index difference ($RI_{post\text{-}exposure} - RI_{pre\text{-}exposure}$) across three time-adjusted urinary 2,4-D concentration groups. All statistical data analyses and tests were performed using Stata 5.0 (Stata Corporation, 702 University Drive East, College Station, TX 77840) and SPSS 7.5.1 for Windows (SPSS Inc., 444 N Michigan Avenue, Chicago, IL 60611-3962).

Results

Twelve white males ranging in ages from 17 to 56 years (mean: 27.5 ± 12.5 years) were enrolled as applicators (Table 1). Two were past users and four were current users of tobacco. All subjects drank beer and consumption ranged from 1 to 30 cans per week. Three applicators consumed hard liquor. Nine controls, eight white and one white-Hispanic, ranged in age from 19 to 32 with a mean of 24.7 ± 4.3 years. Four non-applicators used tobacco and four consumed beer.

Forty-five urine specimens were collected from applicators following 204 spraying hours of 2,4-D and a mean of 4.5 ± 1.9 hours per specimen prior to collection. The herbicide was typically sprayed from a long, flexible wand attached to a truck bed reservoir. Urinary 2,4-D concentration increased as hours spent spraying herbicide increased (Figure 1). The 2,4-D concentration grand mean among all applicators after spraying 2,4-D was 240 ± 100 (±SE) ppb. The means among serial urine samples from applicators taken after spraying ranged from 12 ± 5.2 pbb (n = 4) to 1285 ± 336 ppb (n = 4).

Mean micronuclei scores and replicative index scores among applicators before and after spraying 2,4-D and scores between applicators and non-applicator controls are shown in Table 2. No significant differences in micronuclei scores were observed, but post-exposure scores among applicators were lower (21.5%) than pre-exposure scores and lower among applicators (17.2%)

Table 1. Comparison of pesticide applicators and controls by age, race, tobacco use, and alcohol use

Participants[a]	Age	Tobacco use		Alcohol use	
		Cigarettes	Other	Beer (cans)	Liquor (shots)
1. Applicator	56	40/day	Never	24/week	No
2. Applicator	25	Never	Never	6/week	No
3. Applicator	30	Never	Never	< 1/week	No
4. Applicator	24	Never	Never	6/week	No
5. Applicator	20	Never	1.0 oz/day (chew)	12/week	No
6. Applicator	21	Never	Never	8/week	No
7. Applicator	24	7/day[b]	1.0 oz/week (snuff)[b]	12/week	No
8. Applicator	26	Never	1.2 oz/day (chew)	12/week	3/week
9. Applicator	50	Never	1.5 oz/day (chew)[b]	30/week	No
10. Applicator	17	Never	Never	4/week	No
11. Applicator	18	5/day	1.0 oz/day (snuff)[b]	6/week	3/week
12. Applicator	20	Never	Never	6/week	3/week
1. Control	31	Never	1.5 oz/day (chew)	12/week	No
2. Control	25	Never	Never	No	No
3. Control	32	Never	Never	10/week	No
4. Control	26	Never	Never	No	No
5. Control	22	20/day	1.0 oz/day (snuff)	2/week	No
6. Control	21	Never	Never	4/week	No
7. Control	25	20/day[b]	1.0 oz/day (snuff)	No	No
8. Control	23	Never	1.0 oz/day (chew)[b]	No	No
9. Control	19	Never	Never	No	No

[a] All subjects were male.
[b] Former user.

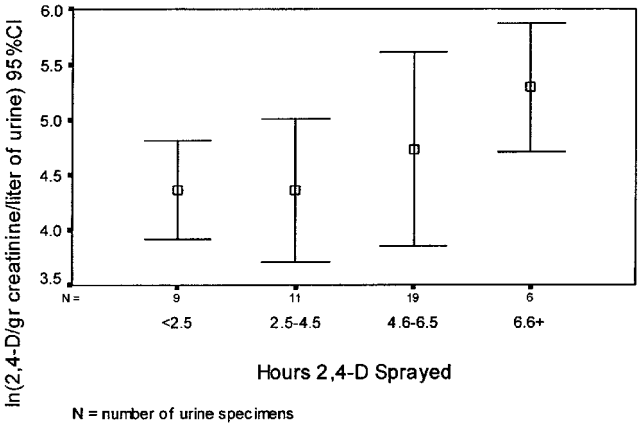

Fig. 1. 2,4-D Levels among urine samples grouped by spraying duration category.

Table 2. Comparison of the micronuclei and replicative index mean scores for 2,4-D applicators and controls

Assay	n	Mean (±SD)[a]	p[d]
Micronuclei			
Applicators			
Before 2,4-D applied	12	11.6 (±2.8)	0.289
After 2,4-D applied	12	9.1 (±6.2)	
Applicator *vs.* controls			
Applicators	12	9.1 (±6.2)	0.454
Controls	9	11.0 (±4.8)	
Replicative index			
Applicators			
Before 2,4-D applied	12	1.33 (±0.2)	**0.016**[b]
After 2,4-D applied	12	1.47 (±0.2)	
Applicators *vs.* controls			
Applicators	12	1.47 (±0.2)	**0.046**[c]
Controls	9	1.29 (±0.2)	

[a] Replicative index score = $1 \times \%M_1 + 2 \times \%M_2 + 3 \times M_3 + 4 \times M_4$, where M_x is the number of a cell division cycle per 1000 cells.
[b] Paired t-test.
[c] Independent sample t-test.
[d] p = Probability of observing applicator score differences before and after spraying 2,4-D.

than among non-applicators. In contrast, replicative index scores among applicators were significantly higher following 2,4-D exposure ($p = 0.016$) and higher than non-applicators' scores ($p = 0.046$). A comparison of pre- and post-exposure lymphocyte immunophenotypes among applicators and between applicators and controls did not show significant differences or trends (not shown). Categorical analyses of mean urinary 2,4-D concentration and RI scores (Table 3) showed an increase in RI scores as time-adjusted urinary 2,4-D levels increased, but no statistically significant trend ($p = 0.15$) was observed.

The replicative index increased among applicators after spraying regardless of tobacco and alcohol use (Table 4). Both before and after spraying, replicative index was higher among applicators who did not use tobacco and among those who drank six or fewer beers than among tobacco users or more frequent beer drinkers. Alcohol and/or tobacco use significantly decreased replicative index scores, but because of the considerable overlap in usage, independent effects of their use could not be determined.

Among applicators, lymphocyte immunologic phenotypes and complete blood counts (CBC) before spraying 2,4-D were not statistically different after spraying 2,4-D, nor were there significant differences between 2,4-D applicators and controls after applicators had sprayed the herbicide (not shown).

Discussion

This study was conducted to evaluate several biologic outcomes in relation to urinary levels of 2,4-D in humans. Measurable levels of 2,4-D occurred in applicators' urine but not among non-applicators. Applicators' urinary 2,4-D levels increased with the number of spraying hours, but overall were intermediate among other occupational 2,4-D users similarly exposed [14, 15, 18, 34] and generally above population-based references [35]. Urinary 2,4-D concentration was associated with

Table 3. Dose–response relationship between urinary 2,4-D concentration categories, mean replicative index scores, and replicative index difference[a] among herbicide applicators

Concentration category (mean ppb/mean hours spraying 2,4-D)	n	Mean RI after spraying (±SD)	Trend test across categories	Mean RI difference (±SD)	Trend test across categories
<20	4	1.32 (0.21)	$Z = 1.44$	0.05 (0.15)	$Z = 0.34$
20–40	3	1.53 (0.17)	p[b] = 0.15	0.20 (0.14)	$p = 0.40$
>40	5	1.57 (0.22)		0.16 (0.18)	

[a] Replicative index difference = $RI_{baseline} - RI_{end\ of\ study}$. Trend test across categories is a nonparametric test for trend across ordered groups developed by Cuzick [33] is an extension of the Wilcoxon rank-sum test; [b]p is equal to the probability of observing a value greater than $|Z|$.

Table 4. Comparison of tobacco and alcohol use and replicative index scores among applicators and non-applicators

User group	n	Pre-exposure replicative index[a]	Post-exposure replicative index	Summary *p* value
2,4-D Applicator				
Ever use tobacco				
No	6	1.44 ± 0.24	1.61 ± 0.17	
Yes	6	1.24 ± 0.14	1.34 ± 0.18	
p-Value[b]		0.11	**0.03**	**0.006**
Alcohol use >6 cans/week				
No	6	1.49 ± 0.08	1.64 ± 0.06	
Yes	6	1.18 ± 0.02	1.32 ± 0.06	
p-Value		**0.005**	**0.004**	**0.0001**
Non-applicator				
Ever use tobacco				
No	4	1.28 ± 0.15	n.a.	
Yes	5	1.29 ± 0.19	n.a.	
p-Value		0.89		
Alcohol use >6 cans/week				
No	6	1.31 ± 0.12	n.a.	
Yes	3	1.24 ± 0.26	n.a.	
p-Value		0.56		

[a] Replicative index score $= 1 \times \%M_1 + 2 \times \%M_2 + 3 \times M_3 + 4 \times M_4$, where M_x is the number of nuclei per cell of a cell division cycle per 1000 cells.

[b] Probability of observed *t* (test that mean differences are equal to zero using two sample *t*-test and assuming equal variances). Summary *p*-value is the probability of obtaining the observed distribution of means.

increased peripheral blood lymphocyte replicative index scores, but not with micronucleus formation. The replicative index for lymphocytes was higher among applicators than non-applicators and it was higher among applicators after spraying than before spraying. Age, tobacco, or alcohol use could not explain these differences. Other factors that might explain replicative index differences among the exposed and unexposed are medical and pharmacological history, previous occupational or heterogeneous occupational pesticide exposures, or chance. However, we excluded unhealthy subjects initially and upon completion of the study (pre-existing or concurrent morbidity) and included among controls only individuals with no previous occupational pesticide exposure.

Changes in sister chromatid exchange and micronucleus formation have been associated with other pesticides [1] and replicative index changes have been associated with aging [36–38]. Our findings, while preliminary, are the first to show a relationship between urinary 2,4-D concentration and increased replicative index in humans. Increased replicative index scores may be important because they suggest stimulated cell growth that could contribute to carcinogenesis. Our finding of no relationship between the frequency of micronuclei and urinary 2,4-D level does not support a human chromosome-damage outcome at mean urinary 2,4-D levels ranging from 12 to 1285 ppb.

The link between 2,4-D function and lymphocyte replicative index is unclear. However, as in animal cells, plant cell growth regulatory receptor proteins may act on plasma membrane ion channels, inositol phospholipid signaling pathways, or inside the nucleus to regulate gene expression [39–44]. Whatever the mechanism, our results and other recent investigations [19, 20] do not support a genotoxic pathway.

This pilot investigation was designed to appraise genetic and epigenetic mechanisms and orient our interdisciplinary research regarding epidemiologic associations between NHL and 2,4-D exposure [4] and potential relationships between urinary 2,4-D levels, lymphocyte proliferation rates [24], and micronucleus frequencies [25–27]. The replicative index was used as a measure of cell proliferation and the micronucleus assay as a measure of 2,4-D genotoxicity. These assays were selected because they were simple, inexpensive, and had been effectively used in numerous occupational studies [39] including a malathion study by co-investigators Titenko-Holland and Smith [40].

The relationship between urinary 2,4-D levels and lymphocyte proliferation observed in this pilot suggests that a larger investigation is needed to further evaluate the association. One challenge will be finding a sufficiently large herbicide applicator population exposed only to 2,4-D because weeds grow sympatrically and heterogeneous herbicide applications are typical. The

second challenge will be developing a biologically plausible mechanistic model that describes 2,4-D's association with diseases that involve lymphocyte proliferation.

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