Biomarker Correlations of Urinary 2,4-D Levels in Foresters: Genomic Instability and Endocrine Disruption

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Forest pesticide applicators constitute a unique pesticide use group. Aerial, mechanical-ground, and focal weed control by application of herbicides, in particular chlorophenoxy herbicides, yield diverse exposure scenarios. In the present work, we analyzed aberrations in G-banded chromosomes, reproductive hormone levels, and polymerase chain reaction-based V(D)J rearrangement frequencies in applicators whose exposures were mostly limited to chlorophenoxy herbicides. Data from appliers where chlorophenoxy use was less frequent were also examined. The biomarker outcome data were compared to urinary levels of 2,4-dichlorophenoxyacetic acid (2,4-D) obtained at the time of maximum 2,4-D use. Further comparisons of outcome data were made to the total volume of herbicides applied during the entire pesticide-use season.Twenty-four applicators and 15 minimally exposed foresters (control) subjects were studied. Categorized by applicator method, men who used a hand-held, backpack sprayer in their applications showed the highest average level (453.6 ppb) of 2,4-D in urine. Serum luteinizing hormone (LH) values were correlated with urinary 2,4-D levels, but follicle-stimulating hormone and free and total testosterone were not. At the height of the application season; 6/7 backpack sprayers, 3/4 applicators who used multinozzle mechanical (boom) sprayers, 4/8 aerial applicators, and 2/5 skidder-radiarc (closed cab) appliers had two or more V(D)J region rearrangements per microgram of DNA. Only 5 of 15 minimally exposed (control) foresters had two or more rearrangements, and 3 of these 5 subjects demonstrated detectable levels of 2,4-D in the urine. Only 8/24 DNA samples obtained from the exposed group 10 months or more after their last chlorophenoxy use had two rearrangements per microgram of DNA, suggesting that the exposure-related effects observed were reversible and temporary. Although urinary 2,4-D levels were not correlated with chromosome aberration frequency, chromosome aberration frequencies were correlated with the total volume of herbicides applied, including products other than 2,4-D. In summary, herbicide applicators with high urinary levels of 2,4-D (backpack and boom spray applications) exhibited elevated LH levels. They also exhibited altered genomic stability as measured by V(D)J rearrangement frequency, which appears reversible months after peak exposure. Though highly detailed, the limited sample size warrants cautious interpretation of the data. *Key words*: 2,4-D, foresters, reproductive hormones, V(D)J rearrangements. *Environ Health Perspect* 109:495–500 (2001). [Online 9 May 2001]
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Chlorophenoxy herbicides remain one of the most commonly used pesticide products due to their efficacy in weed control, relatively low cost, and low acute toxicity in humans (*1*). Historically, epidemiologic studies conducted in the midwestern United States have suggested an association between chlorophenoxy use and non–Hodgkin lymphoma (*2*,*3*). Chronic, long-term animal studies do not support carcinogenic effects for this herbicide in its pure form (*4*). Early commercial products containing 2,4,5-T (trichlorophenoxyacetic acid) alone or in combination with 2,4-D (dichlorophenoxyacetic acid) were found to have dioxin and dioxin-like contaminants. These findings gave mechanistic support to the proposed connection between chlorophenoxy herbicides and lymphoma due to the immunotoxic effects of dioxins (*5*,*6*). A limited analytical chemical survey of chlorophenoxy herbicides products in current use did not suggest that the level of dioxin contamination in these commercial products poses a major health threat (*7*).

In continuing work from our laboratory, we found that only one out of the seven commercial-grade chlorophenoxy herbicide products induced dose-related increases in micronuclei frequency in cultured human lymphocytes. These data suggest that the majority of the commercial chlorophenoxy products studied were not genotoxic at the chromosomal level (*8*).

In other *in vitro* studies, we explored the possibility that commercial-grade cholorophenoxy herbicides might show endocrine-disrupting activity. Two commercial-grade products tested showed evidence of weak endocrine-disrupting effects in MCF-7 cells (*9*); a breast cancer cell line that is responsive to estrogen-mediated cell proliferation. With further review, we noted that adjuvants are sometimes used in conjunction with chlorophenoxy herbicides in roadside and other applications. We found that four out of four adjuvants induced significant increases in the frequency of micronuclei (*8*). Two of five adjuvants showed evidence of weak endocrine-disrupting activity in MCF-7 cells (*9*). As a corollary, earlier human studies by our group demonstrated modest alteration of male reproductive hormone levels in herbicide applicators but not in other pesticide use groups (insecticides, fumigants) (*8*) during the application season. The male reproductive hormones measured included follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone. Together, these hormones regulate spermatogenesis and sperm maturation (*10*). Prior molecular and chromosome studies indicated that herbicide application may differ significantly from fumigant application in terms of genotoxicity (*11*). In those studies, G-banded chromosome analysis demonstrated that chromosome damage was least frequent in applicators who only applied herbicides compared to applicators who applied herbicides and insecticides and those who, in addition, applied fumigants.

The present study was designed to focus on exposures limited to herbicides, and if possible, to chlorophenoxy herbicides only, and to determine whether exposure to this herbicide class could contribute to endocrine disruption and to genotoxicity observed in earlier studies. In this effort, we took advantage of earlier studies by others where exposures to backpack sprayers, boom sprayers,

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and aerial applicators demonstrated marked differences in reported urinary levels due to differences in 2,4-D application methods *(12–14)*. These diverse exposure scenarios offered an approach to generate an acute toxicant dose-related biologic response with use of appropriate biomarkers of toxicant effect. Similarly, one might avoid the confounding effects of exposure to more than one herbicide or pesticide class by focusing on acute exposure effects. The work presented below was undertaken to examine these hypotheses.

Materials and Methods

Population study design. Of 270 men who were state-licensed forest/roadside pesticide applicators, 233 took part in a survey of health and pesticide use (86.3%). From this survey group, subjects were selected for clinical laboratory investigative studies. Study inclusion criteria were the following: *a*) no chronic disease, *b*) no chronic medication use, *c*) applied 2,4-D more than 5 days per year (exposed), or *d*) did not apply pesticides this past year (control subjects). Sixty subjects from the initial health and pesticide-use survey met the laboratory study criteria. Six weeks before the beginning of spring herbicide applications, the potential subjects were reinterviewed. Thirty-nine licensed applicators who currently met the laboratory study criteria volunteered for the study. Fourteen of the remaining 21 subjects failed to meet specific health or specific herbicide use criteria in the current time-frame; the other 7 subjects refused. Of the 39 subjects selected, 24 men (mean age 39.1 ± 2.9 years) were defined as exposed on the basis of 2,4-D use, and 15 men (mean age 42.1 ± 2.3 years) who were licensed for forest pesticide application, but did not perform applications themselves, were selected as control subjects. Three of 15 control subjects and 6 of 24 exposed subjects currently used tobacco products.

Specimen collections. In Minnesota, herbicide applications principally occurs in spring and early summer (15 April–15 July). First-voided morning urine and morning blood specimens were obtained from applicators through participating rural clinical laboratories after an 8-hr fast at the end of the peak of the 2,4-D application season. Urine specimens were collected in certified chemically clean, Teflon-lined, glass, screw-cap jars (I-CHEM; Nalge Nunc International, New Castle, DE). Blood specimens were collected in standard clot and heparinized tubes (Becton Dickinson, Franklin Lakes, NJ). The same lot of collection containers supplied to participating clinics was used throughout the study. Specimens were transported and received in the laboratory within 24 hr of collection in U.S. Department of Transportation-approved cold-pack containers. Upon receipt in the laboratory, the coded specimens of blood were processed for cytogenetic analysis. Serum for later hormone analysis was cryopreserved at –80°C in Teflon vials. Urine specimens were transferred to specialized, chemically clean, Teflon-lined cryotubes and cryopreserved at –80°C for later pesticide analysis.

The peak application time frame was determined through telephone review of the pesticide application schedule (application method, days applied, volume to be applied, number of applications, and duration of application). Urine and blood specimens were obtained from control subjects contemporary with those from exposed subjects throughout the application season. Each time a group of exposed subjects' specimens were processed, we included specimens from at least one or more control subjects. A second blood specimen was obtained from exposed subjects only within 6 weeks of the beginning of the following season's application work and compared to the earlier data set. The project was approved by the Institutional Review Board of the University of Minnesota and followed written informed-consent procedures outlined in the approval.

Exposure assessment. Each state-licensed participant (exposed and control) provided their application records for the season's work. These records included product used, application rate, volume of pesticide/herbicide used, use and type of adjuvant used in conjunction with herbicide, and date and method of application. Included in this data set were the number of years of pesticide application work (seniority). Based on change in application practice and change in health status, one study subject (exposed) was excluded from our analyses.

Analytic chemical procedures. Sample preparation. A 10-mL aliquot of urine was enriched with $^{13}C_6$-ring 2,4-D as an isotope dilution internal standard. The urine was acidified, then extracted with dichloromethane:diethyl ether (4:1). The extract was dried over anhydrous sodium sulfate, then concentrated to 100 µL with nitrogen using a TurboVap concentrator (Zymark Corporation, Hopkinton, MA).

Instrumental analysis. The HPLC-tandem mass spectrometric (MS/MS) analysis was performed with an HP1090L HPLC (Hewlett-Packard Co., Palo Alto, CA) connected in-tandem to a TSQ-7000 triple quadrupole mass spectrometer (Finnigan MAT Instruments, San Jose, CA) equipped with an atmospheric pressure ionization (API) interface. Separation was achieved on a 25 cm × 4.6 mm Partisil 5 ODS-3 column (Whatman, Clifton, NJ), which was preceded in-line by a 20-mm guard column with identical sorbent to prolong the column lifetime. The solvent system consisted of acetonitrile:water (60:40) with 0.2% glacial acetic acid at a flow of 1 mL/min.

Negative atmospheric pressure chemical ionization (-APCI) MS/MS was achieved by using nitrogen as a sheath gas and argon as the collision gas. No API auxiliary gas was used. The pressure of nitrogen entering the API unit was kept constant at 40 psi (276 kPa). The argon gas pressure was 2 mT. The API vaporizer and capillary temperatures were 450°C and 250°C, respectively. The discharge of the corona needle was 5 µA. The collision offset was set at 22 V for optimal fragmentation. The electron multiplier voltage ranged from 1,800 to 2,400 V.

During an analysis, four product ions were monitored at a scan time of 0.25 sec/ion in the multiple-reaction monitoring experiment. One quantification and one confirmation ion were monitored for both the native 2,4-D and the $^{13}C_6$-ring 2,4-D. The confirmation ions represent the natural abundance of ^{37}Cl in the 2,4-D molecules.

Data processing/analysis. Data were automatically processed by software supplied with the mass spectrometer. Each ion of interest was automatically selected, retention times calculated, and the area integrated. All data were checked for interference, peak selection, and baseline determination and were corrected if found in error. Because of the specificity of the MS/MS technique, interferences were rare. However, any interferences were easily recognizable because of a dramatic change in the ratio of the quantification ions to the confirmation ions of either the native analyte or the ^{13}C analogue. When necessary, the data were reanalyzed to reflect these corrections.

The data were downloaded into an ASCII file and transferred from the UNIX-based operating system on the TSQ-7000 to a personal computer via an Ethernet connection. The data were imported into an R:BASE (Microrim, Redmond, WA) database specifically designed for this analysis.

Reproductive hormone analysis. We measured luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone concentrations (total and free) in serum or plasma obtained from blood specimens donated by study participants. LH and FSH were measured using two-site immunofluorometric assays from commercially available kits (DELFIA catalog numbers 1244-031 and 1244-017; WallacOy, Turku, Finland) modified as previously described (*15*). Total and free testosterone were measured using solid-phase radioimmunoassays (catalog numbers TKTTI and TKTF1; Diagnostic Products Corporation, Los Angeles, CA). All these hormone assays are validated for use with serum or heparinized plasma. Samples were stored at –80°C until assay. All samples were assayed in

one batch. The intra-assay coefficients of variation for total and free testosterone were 6.76% and 5.19%, respectively, and 1.35% and 1.16% for LH and FSH. For detailed statistical comparisons and analysis of hormonal levels in exposed subjects, only those subjects who provided specimens at the height of the application season and within 6 weeks before the application of herbicides in the following year were included (n = 21 out of 24 exposed subjects).

Chromosome studies. Specimen collection and cell culture. The standardized specimen collection and lymphocyte culture methods we used are detailed in earlier publications (*11*).

Chromosome analysis. As a general rule, we examined 100 complete consecutive G-banded metaphase cells per subject. For 96% of subjects, exactly 100 metaphase cells were examined, with 83–96 cells examined in the remaining subjects. Less than 10% of the metaphases examined contained < 46 chromosomes. All metaphases with rearrangements were photographed and karyotyped for breakpoint verification. These analyses were performed at the 400-band stage or greater. In this study, metaphase chromosomes with demonstrable discontinuity between chromosome segments but without loss of chromosome material are referred to as breaks, regardless of chromosome alignment. Otherwise, the International System for Human Cytogenetic Nomenclature (*16*) was followed for banded chromosome studies. For purposes of graphic presentation, the 400-band stage nomenclature was used. Chromosome readers were blind to exposure status.

PCR-based V(D)J trans–rearrangement assay. Previously, members of our investigative group developed (*17*) a polymerase chain reaction (PCR)-based assay to define the frequency of occurrence of variable (V), diversity (D), joining (J) recombinase-mediated trans-rearrangements between a V segment from a T-cell receptor gamma (7p14-15) locus and a J segment from the T-cell receptor beta (7q35) locus. This rearrangement results in a chromosome 7 inversion. This abnormality occurs at low frequency in all individuals. The PCR-based assay described below provides a measure of genomic instability (*18*).

In brief, genomic DNA was isolated by modified method of the Buffone procedure (*19*) and resuspended in deionized water at a concentration of 100 ng/µg. DNA concentration was measured spectrophotometrically and rechecked by agarose gel electrophoresis. We routinely extracted 1 µg DNA/1.5–2.0 × 10^5 peripheral blood mononuclear cells. In the first step to assay for recombination, DNA (125, 250, 500, and 1,000 ng) was suspended in a 50-µl solution containing 200 µM deoxynucleotides, 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 M MgCl, 0.01% gelatin, and the *a* set of primers (Vγa and Jβ1a) at a concentration of 1.4 ng/µL, 10% DMSO, and 2.5 U of Taq polymerase. Negative and positive controls were run with each experiment. The reaction was carried out at 95°C × 4 min for denaturation, followed by 25 cycles of amplification consisting of 95°C × 15 sec, 57.5°C × 15 sec, and 72°C × 30 sec plus 6 sec increase per cycle. After 25 cycles, 10 min at 72°C was allowed for chain elongation. Ten percent of the first step reaction was nested using the same conditions with a *b* set of primers (Vγb and Jβ1b) at a concentration of 6 ng/µL. PCR products were run in 1.5% agarose electrophoresis gel, and a picture was taken of the ethidium bromide-stained gel before transfer to nylon paper. A *c* set of primers was used to verify Vγb/Jβ hybrids. A product was called positive for Vγ/Jβ rearrangements if it hybridized to both probes (Vγc and Jβ1c). Values were expressed as the reciprocal of the dilution titer per microgram of DNA. As before, blood samples obtained at the height of chlorophenoxy use were compared to samples obtained 8 months or more later for the exposed groups. Control samples for comparison were obtained throughout the pesticide application season.

Statistical methods. Analyses of hormone levels were based on the differences between the logarithm of the hormone level at the peak of the application season and the logarithm of the hormone level several months after the application season. We used analysis of variance methods to test for changes in hormone level according to 2,4-D application method. Pearson correlation coefficients were used to quantify the relationship between urinary 2,4-D level and changes in hormone level across all application methods. To determine if chromosome aberration frequencies varied according to level of herbicide exposure, exact permutation significance levels based on the Wilcoxon rank-sum test were computed. We used Poisson regression analyses to examine the relationship between aberration frequencies and urinary 2,4-D levels. The frequencies of V(D)J rearrangements were compared among exposure groups using the exact Wilcoxon test and also by comparing the proportion of men with two or more V(D)J rearrangements using an exact trend test. Pearson correlation coefficients were used to quantify the relationship between urinary 2,4-D level and V(D)J rearrangement frequencies. All reported p-values are two-sided.

Results

Urinary 2,4-D concentration and exposure status. Table 1 compares application method, urinary 2,4-D levels, and total volume of herbicides used for exposed and control subjects. Urine specimens obtained within 24 hr of the peak application show an exposure gradient according to application method. The relative rankings for urine 2,4-D levels by application method are back pack sprayer > boom sprayer > aerial application> skidder> control subjects. These data are consistent with the expected differences in acute exposure for manual ground application (backpack) versus mechanical (boom sprayer), closed cabin (skidder-radiarc), or aerial application (helicopter or fixed wing) (*14*). There is a 10-fold difference in the mean urinary concentration levels (380.1 ppb) for all backpack and boom spray applications versus the pooled values of all aerial and skidder closed-cab applications (33.2 ppb).

Reproductive hormone analysis. The largest changes in hormone levels during the application season (Table 2) were increases in LH levels for backpack applicators (p = 0.053) and boom sprayer applicators (p = 0.089). The increase for both application methods combined was significant (p = 0.015). Using serum from 21 of 24 applicators, LH levels are directly correlated (r = 0.56; two-sided p = 0.006) with urinary 2,4-D levels at the time of maximum application. FSH and total and free testosterone were not correlated with the level of 2,4-D in urine at the time of maximum use of chlorophenoxy herbicides. However, after the application season, the levels of total testosterone were directly correlated (r = 0.37; two-sided p = 0.03) with the level of 2,4-D in the urine at the time of peak season use.

Chromosome analysis. In these analyses of the chromosome data, we considered correlations among urinary 2,4-D levels and chromosome damage, applicator group and chromosome damage, and pesticide use volume and chromosome damage. Table 3 expresses the relationship between the total volume of herbicides applied during the application season and chromosome damage as measured in G-banded metaphases from human lymphocytes. Chromosomal translocations, inversions, deletions (TIDs), breaks, and gaps occur more frequently among applicators who apply more than 1,000 gallons of herbicide during the application season. As noted in Table 1, most of these men are aerial applicators who apply a broad spectrum of herbicides including 2,4-D. With regard to the possible relationship between urinary concentrations of 2,4-D and chromosome aberrations, regression analyses indicated nonsignificant, negative regression coefficients. Adjustment for tobacco use and cigarette smoking status had little impact on the analysis of the association between 2,4-D levels and chromosome aberration frequencies. Thus, acute, high-level exposure to 2,4-D as measured by urinary concentration, with or

without adjuvant use, is not associated with detectable chromosome damage in G-banded lymphocytes.

V(D)J rearrangement frequency. Analysis of the data from exposed study subjects by application method showed that the frequency of two or more rearrangements is directly related to mean level of 2,4-D in urine (backpack sprayers > boom sprayers > aerial > skidder > control; $p = 0.018$) during the application season (Table 4). We compared the mean frequency of rearrangements per microgram DNA for backpack and boom spray application methods (3.36 ± 0.79), closed cab (aerial and skidder) methods (1.85 ± 0.45), and forestry controls (1.47 ± 0.31). The frequency of rearrangements in applicators performing hand-held applications was significantly greater than in control ($p = 0.023$) subjects. The rearrangement frequency for mechanized application was not significantly different from that for hand-held pesticide application ($p = 0.14$). Specimens collected and examined from the exposed groups 6 weeks before the beginning of applications in the following year show some differences. For backpack sprayers, none of the six had any detectable rearrangements. Consistent with our earlier work *(17,18)*, these data show that rearrangement frequency varies with exposure status and is, in general, a transient event in healthy, exposed workers. Workers with more seniority and exposure to higher volume of different herbicides retain V(D)J region rearrangements over time (aerial applicators and skidder appliers). Minimally exposed foresters (controls) have a somewhat higher V(D)J rearrangement frequency (i.e., 1.47) than unexposed control subject values reported in our previous studies (i.e., < 1).

Table 1. Comparison of urinary 2,4-D concentrations by application method.

Application method and number of subjects	Concentration (ppb)	Total 2,4-D applied (gal)	Total herbicides applied (gal)
Backpack ($n = 7$)	28	30	30
	63	78	78
	64	26	26
	160	50	186
	270	81	81
	890	153	165
	1,700	110	682
Mean	453.6	75.4	178.3
Boom spray ($n = 4$)	86	83	83
	140	64	98[a]
	490	93	123
	290	60	88[a]
Mean	251.5	75	98
Aerial ($n = 8$)	<0.57	96	440
	19	1,331	2,548
	25	10	563[a]
	51	484	3,307
	19	7,500	7,500
	40	274	1,158[b]
	92	1,356	4,612[b]
	97	850	1,629
Mean	42.9	1487.6	2719.6
Skidder ($n = 5$)	4.8	352	952
	58	980	2,289[a]
	4.1	1,027	2,120[b]
	0.85	4	1,004
	20	430	430
Mean	17.6	558.6	1359.0
Control ($n = 15$)	< 0.57	0	0
	< 0.57	0	0
	< 0.57	1	2
	< 0.57	0	0
	< 0.57	0	16
	< 0.57	0	0
	< 0.57	0	0
	< 0.57	0	0
	< 0.57	0	0
	< 0.57	0	0
	< 0.57	0	0
	1.2	0	0
	1.8	0	0
	0.86	0	0
	< 0.57	0	0
Mean	0.5	0.1	1.1

Backpack and boom sprayers who apply pesticides manually have higher urinary levels of 2,4-D than do aerial and skidder applicators ($p < 0.001$). A value of 0.3 was used in calculating means for individuals with 2,4-D levels below the limit of detection.
[a]More than 100 pounds of granular herbicide was also applied. [b]More than 500 pounds of granular herbicide was also applied.

Discussion

Previous studies by Knopp (*20*) have demonstrated that urinary 2,4-D levels can exceed 1,000 ppb in workers employed in chlorophenoxy herbicide manufacture. By contrast, earlier reports dealing with forest pesticide applications suggest that urinary concentrations of 2,4-D arising from exposure occur within a range of 45–326 ppb (*21*). In the present work we examined first-voided urine specimens from workers at the time of maximum use of 2,4-D (*22*). This strategy, commonly used in the occupational setting (*23*), takes advantage of the reported half-life of 2,4-D in humans (12–33 hr) (*24*), repeated subject exposure, and urinary excretion rate to optimize exposure assessment. In Minnesota, the maximum number of herbicide applications occur within a 6-week period in late spring (May, June) and

Table 2. Comparison of reproductive hormone levels in urine from men seasonally applying 2,4-D.

Herbicide application method	No. of subjects	FSH (mIU/mL)		LH (mIU/mL)		Total testosterone (ng/mL)		Free testosterone (pg/mL)	
		Spring	Winter	Spring	Winter	Spring	Winter	Spring	Winter
Backpack	6	4.4 (1.3)	3.9 (1.1)	5.8 (1.2)	3.5 (0.7)	5.7 (0.9)	5.8 (0.7)	26.4 (7.5)	28.8 (12.6)
Boomspray	4	4.9 (1.6)	4.4 (1.3)	4.5 (1.4)	2.9 (0.9)	7.4 (1.1)	7.2 (0.9)	26.0 (9.2)	27.4 (15.5)
Aerial	7	5.0 (1.2)	5.1 (1.0)	4.0 (1.1)	4.4 (0.7)	5.1 (0.9)	4.6 (0.7)	31.5 (6.8)	36.6 (11.6)
Skidder	4	4.9 (1.6)	5.0 (1.4)	4.8 (1.5)	4.3 (0.9)	5.4 (1.1)	5.7 (0.9)	22.9 (9.0)	24.5 (15.4)
Controls	15	5.0 (0.9)		4.7 (0.6)		5.4 (0.3)		24.8 (1.9)	

Values shown are mean (SE). Samples were collected from applicators during the highest level of 2,4-D use (spring) and about 6 weeks before the next spring season herbicide applications (winter). LH values during the spring increase with increasing urinary 2,4-D concentrations ($r = 0.56$; two-sided $p = 0.006$). Winter total testosterone levels were directly correlated ($r = 0.37$; two-sided $p = 0.03$) with peak season urinary 2,4-D levels.

early summer. The volume of 2,4-D use varies with application method (Table 1). The measured urinary 2,4-D values strongly suggest that ground application by backpack or boom sprayer yields significant, acute exposure for the workers. Interestingly, six of seven backpack sprayers stated they used rubber gloves and wore rubber boots as protective gear. Five of seven backpack sprayers wore a protective suit. These data and other data from other application groups (not shown) suggests that the method of herbicide application is the most significant factor in personal exposure.

The lack of correlation between urinary 2,4-D levels and chromosomal aberrations is not unexpected, as the overwhelming majority of animal and *in vitro* studies do not show evidence of genotoxicity for 2,4-D (*4*). In an earlier *in vitro* study by our group, some adjuvants used in conjunction with 2,4-D applications were found to be genotoxic (*8*). As a point of reference, adjuvants are chemical mixtures that commonly contain surfactants and oils used to increase the potency of herbicides. In the present study, it was possible to divide the ground application group who applied less than 100 gallons of herbicide (n = 7) into appliers who used only 2,4-D products and adjuvants (n = 3) and those who did not (n = 4) use adjuvants with 2,4-D. No significant difference in the frequency of chromosome damage between these two small groups was noted. In the remaining exposed subjects grouped by volume of total herbicide applied (i.e., 100–1,000 and >1,000 gallons), it was not possible to separate adjuvant and nonadjuvant use for applications of 2,4-D and other herbicides. All but two of the appliers in these exposure groups used adjuvants. However, the increased frequency of chromosome aberrations noted is related to the total volume of different herbicides used including 2,4-D. To what extent the cumulative chromosome effect observed can be related to adjuvant use is uncertain. Moreover, the diversity of adjuvant product formulations used in conjunction with 2,4-D, viewed in contrast to the direct correlation between 2,4-D levels in the urine with LH and V(D)J levels in blood, weighs against direct participation of specific adjuvants in these acute effects. Indirect participation of adjuvants through increased skin penetration of the herbicide remains a concern.

The increase in V(D)J rearrangements observed herein connotes a transient increase in genomic instability with forester exposure to chlorophenoxy herbicides and or adjuvants. These findings are similar to those we reported in agricultural pesticide applicators and in patients undergoing chemotherapy in response to short-term, relatively high level exposure to known or suspected genotoxic agents (*17,18*). The parallel between these earlier findings and the current study is most clearly demonstrated in backpack sprayers who underwent short-term, high-level exposure to chlorophenoxy herbicides. In some aerial applicators and skidder applicators, V(D)J region rearrangements were retained over time. The mean age (40.5 years) of aerial and skidder applicator groups is not significantly different from the mean age (37.4 years) of backpack sprayers, but the mean seniority (years licensed) of backpack sprayers is significantly less (7.7 years) than that of aerial applicators (18.5 years). Seniority, differences in the volume of pesticide used by the different exposure groups, use of herbicides other than 2,4-D, or exposures unique to aviation may explain the persistence of rearrangements noted in aerial applicators. Considered at a mechanistic level, it is possible that repeated exposures to pesticides over time led to development of long-lived "memory" T cells. These cells could account for persistence of the V(D)J region rearrangements (*25*). Foresters chosen as control subjects for this study held supervisory positions and were not actively engaged in herbicide applications. V(D)J rearrangements in excess of 1 per microgram of DNA in the minimally exposed control group may reflect exposure events unaccounted for in available records. This suggestion is supported in part by detectable 2,4-D in the urine of some of these foresters.

With regard to hormone analysis, prior studies by our group (*8*) and by Straube et al. in 1999 (*26*) show some notable similarities and differences in reported changes in reproductive hormone levels during and after acute exposure to pesticides. Both those studies report significant increases in testosterone levels after the pesticide application season was completed. Increase in the reported testosterone levels in the current study after the application season were consistent with these earlier findings. In our earlier study, FSH levels were decreased at the height of the application season and LH values were increased in serum from herbicide applicators. In the present study and in the Straube et al. study (*26*), significant increases in LH were obtained at the height of the application season. Neither of the two earlier studies reported urinary levels of pesticides or herbicides. Direct correlation of urinary levels of 2,4-D with serum levels of LH at the time of highest exposure suggest a direct effect on hormonal levels by chlorophenoxy herbicides. Chronically increased secretion of LH by the pituitary in response to exposure to these products leading to significant increases in testosterone levels is consistent with our present understanding of testosterone cycling in response to LH stimulation of testosterone synthesis by the Leydig cells of the testes (*10*). Curiously, sustained high LH levels have been seen in association with exposure to dioxins (*27*).

Table 3. Comparison of chromosome aberration frequencies (%) in G-banded metaphase lymphocytes among applicators by volume of herbicides applied.

G-Banded metaphase chromosome aberrations	Total volume of herbicides applied			
	None	Low (1–100 gal)	Mid-range (100–1,000 gal)	Heavy (> 1,000 gal)
TID	0.65 ± 0.30	1.20 ± 0.43	1.00 ± 0.46	2.22 ± 0.38
Breaks	0.93 ± 0.49	1.17 ± 0.69	1.33 ± 0.74	2.89 ± 0.61
Gaps	1.29 ± 0.36	1.03 ± 0.51	1.33 ± 0.55	3.00 ± 0.45
Other	0.29 ± 0.22	0.29 ± 0.31	0.50 ± 0.34	0.33 ± 0.28
No. of subjects	14	7	6	9

The pooled frequency of TID (translocations/inversions/deletions) and independently examined frequencies of breaks and gaps are increased in chromosomes from applicators using more than 1,000 gallons herbicides (TID, p = 0.003; breaks, p = 0.017; gaps, p = 0.006).

Table 4. Comparison of T lymphocyte-derived V(D)J trans-rearrangement frequency among herbicide applicators and control subjects.

Application method	Proportion of subjects with ≥ 2 V(D)J rearrangements/µg DNA		Urinary 2,4-D, mean (SE)
	Spring	Winter	Spring
Backpack	6/7 (86%)	0/6 (0%)[a]	453.6 (236.5)
Boom spray	3/4 (75%)	2/4 (50%)	251.5 (90.5)
Aerial	4/8 (50%)	2/8 (25%)	42.9 (12.5)
Skidder	2/5 (40%)	2/5 (40%)	17.6 (10.6)
Controls	5/15 (33%)	NS	0.5 (0.2)

NS, no specimen. The proportion of subjects with two or more V(D)J region trans-rearrangements per microgram DNA were categorized by application method. These data were compared to urinary 2,4-D concentration at the time of maximum use (spring). During the peak application season rearrangement frequencies rank as follows: forester controls < skidder < aerial < boom spray < back pack (p = 0.018 using exact trend test). V(D)J region rearrangement frequencies are positively correlated (r = 0.54) with urinary 2,4-D levels (p = 0.003).
[a]No winter specimen was obtained from one of seven backpack sprayers.

The median and normal range for the clinical LH assay used in this study is 3.3 and 1.0–8.4 mIU/mL (n = 89 men). These data and our reported findings suggest that although the reproductive hormone data may be significant for the population; they are not of immediate clinical concern for the individual. It is not clear what impact these minor but statistically significant and repeatedly observed *(8,26)* reproductive hormone disruptions might have on male reproductive potential. From a different perspective and potentially of greater concern may be the effects of a minor increase in LH secretion on the menstrual cycle and ovulation. Whether small fluctuations of the level of LH can affect women's fertility is uncertain.

Increased V(D)J rearrangement frequencies and LH levels positively correlated with the level of 2,4-D in the urine but did not correlate with total herbicide use or seniority. Together, these data further suggest that increased LH and V(D)J were due to acute, short-term exposure to 2,4-D used in conjunction with adjuvants. Finally, the apparent coincident effect of 2,4-D exposure on LH levels and the increased V(D)J region rearrangements once again lead to intriguing speculation regarding the relationship of reproductive hormonal status and the function of the immune system (*28*).

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