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Herbicides and adjuvants: an evolving view

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The present report examines the *in vitro* genotoxicity (micronucleus assay) of herbicides and adjuvants and reports on an *in vivo* human study on potential endocrine effects of pesticides, including herbicides. Adjuvants are used in conjunction with 2,4-dichlorophenoxy acetic acid (2,4-D) and other herbicides. Earlier pesticide applier survey results ($n = 709$) show that 59% of the applicators used adjuvants, and the majority of this group used paraffinic oils and/or surfactant mixtures. As a beginning effort to explore the role of adjuvants and herbicides in hormonally based reproductive effects, a prospective, controlled study was performed to analyze blood specimens from three different exposure groups (applicators using herbicides only; applicators using both herbicides and insecticides; and applicators using fumigants in addition to herbicides and insecticides; and a control group composed of other agricultural workers including organic farmers). The applicators and controls were age- and smoking-matched. Study subjects ($n = 78$) were tested before, during, and after completion of pesticide application season for the effects of pesticide products on hormone levels in the bloodstream. Of the applicator exposure groups examined, only the herbicide group showed significant endocrinologic differences from controls. Free testosterone levels were significantly elevated in post-season measurements ($p = 0.032$), and follicle-stimulating hormone (FSH) was significantly decreased at the height of the season ($p = 0.016$) and in the post-season ($p = 0.010$) as compared to controls. These endocrinologic findings are discussed in terms of their possible relationship to potential endocrine effects of herbicides, herbicide contaminants, and adjuvants. *In vitro* genotoxicity examination compared four different commercially available surfactant mixtures with 12 different commercial herbicide products, including six different chlorophenoxy herbicides. Only one herbicide yielded a significant dose–response curve. All four adjuvants showed positive dose–response effects. These preliminary data suggest that adjuvants are not inert but are toxicologically active components added to herbicide mixtures. Whether adjuvant toxicant effects are additive or are independent of herbicide effects is poorly understood.

Keywords: *adjuvants, herbicides, hormone analysis, human study, micronucleus assay.*

Introduction

Historically, epidemiologic studies have linked use of chlorophenoxy herbicides with excess risk of non-Hodgkin's lymphoma (NHL). Geographically, the majority of studies linking 2,4-dichlorophenoxy acetic acid (2,4-D) use with NHL have taken place in the upper Midwestern United States and/or Scandinavian countries (IARC, 1986; Dich et al., 1997). Other efforts from the US and elsewhere failed to show these associations (Dich et al., 1997).

In terms of genotoxicity, the majority of animal and *in vitro* studies do not suggest that 2,4-D by itself is a mutagen or carcinogen (Garry and Griffith, 1996). Clearly, the dichotomy between the epidemiologic findings and animal/*in vitro* studies leads to consideration of other factors that might offer some additional insights into these reported results. Infectious agents, contaminants in commercial-grade preparations, and use of adjuvants are factors of concern. The least studied and, at the same time, the most consistent in terms of pesticide user population exposure is the use of adjuvants. These agents are used for spreading and sticking to improve the solubility and penetration of pesticides including herbicides. Use of adjuvants with chlorophenoxy herbicides is restricted to specific crop needs, *e.g.*, spring wheat, and specialized application needs, *e.g.*, roadside spraying. The chemical composition of these mixtures can include polyoxyethylenes, polyvinyl compounds, and paraffin oils in the same commercial product (Foy, 1992; Harvey, 1992; Schonherr and Bauer, 1992). While chemicals in these classes are known to be biologically active and can contain endocrine-disrupting alkyl phenols, they are considered inert in terms of pesticide activity.

To begin to evaluate the possibility that adjuvants might be genotoxic or possibly have endocrinologic effects, we used a threefold approach. First, survey data gathered from an earlier study was used to generate a list of commonly used adjuvants, and their chemical composition was ascertained according to material safety data sheets (MSDS) and other sources. Second, *in vitro* examination of the frequency of

1. Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; EO, ethylene oxide; FSH, follicle-stimulating hormone; HDL, heavy density lipoprotein; IRB, Internal Review Board; LH, luteinizing hormone; MSDS, material safety data sheet; NHL, non-Hodgkin's lymphoma

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micronuclei was conducted in cultured human lymphocytes exposed to different doses of pesticides or adjuvants in order to provide preliminary evidence for chromosome damage and aneuploidy. Third, *in vivo* screening of applicators, who were divided according to three major pesticide use groups (herbicides, herbicides and insecticides, and fumigants), for serum levels of testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) over the pesticide use season, was used to define categorically whether any of the major classes of pesticide might have endocrinologic effects. These data, coupled with pesticide use information, were used to identify specific chemical candidates for detailed examination *in vitro* in the estrogen- sensitive MCF-7 assay. Finally, these first steps and ongoing work in the estrogen-sensitive MCF-7 cell line (Lin and Garry, 1998) are designed to provide background information in support of and to focus on current, large-scale laboratory-based human population studies evaluating fertility, teratogenic potential, and cancer-related endpoints.

Methods: population studies

Pesticide Use Survey

In brief, a current list of licensed pesticide appliers in Minnesota (MN) was obtained from the state Department of Agriculture. Certification for licensure is by examination, accompanied by recertification through continuing education. Specific licensure is granted for use and application of pesticides according to pesticide class (herbicides, insecticides, fungicides, and fumigants). Given that the major agricultural area lies in the southern half of the state, we limited our study to this geographic area. Of the 25,854 state-licensed agricultural pesticide appliers from southern MN, 1000 were randomly selected for survey. Through written questionnaire, each applicator was asked to report the brand name of the pesticide(s) applied and adjuvant(s) used along with the average number of days each pesticide was applied. Where possible, the composition of each brand of adjuvant was determined from MSDS, face labels supplied with the container, direct contact with the manufacturer, and published reference materials. Of the subjects, 71.9% responded and provided data ($n = 719$) for these studies. Further details of these methods and results are reported elsewhere (Garry et al., 1994). Details of adjuvant use and their chemical composition are presented here for the first time.

Experimental Design of Laboratory-Based Human Population Studies

Application of pesticides is a seasonal event in MN. Herbicides are routinely used from mid-April through June. In terms of volume use and acreage treated per day, maximum use occurs in late May (height of season). For insecticide use, the height of the application season occurs from mid-July through mid-August. For fumigant use, the period of maximum application occurs from mid-September to early November. From our initial survey data, we defined pesticide use groups and selected candidates for this study according to the following pesticide use criteria (see Potter et al., 1993 for further details).

(1) Fumigant group: fumigant use more than 5 days per year, with or without some use of other pesticides ($n = 14$).

(2) Insecticide/herbicide group: use of insecticides more than 10 days per year with or without some use of herbicides, but no fumigant or fungicide use ($n = 20$).

(3) Herbicide-only group: use of herbicides more than 10 days per year and no other pesticide use ($n = 26$).

(4) Control group: employed in agriculture but no routine application of pesticides ($n = 18$). Of the 18 selected control subjects, six were organic farmers, four were dairy farmers, two were grain handlers, and six worked in agribusiness.

On-site pesticide use assessment and detailed pesticide application records were obtained. All study subjects denied chronic medication use and stated no chronic disease. Study subject groups, including controls, were age-matched within 5 years and were matched according to cigarette smoking history. All study subjects were male. This project was reviewed and approved by the Human Subjects Committee of the Internal Review Board (IRB) of the University of Minnesota. IRB- Approved Written Informed Consent was obtained prior to performance of these studies.

Blood specimens were collected prior to the application season, at the height of the application season for each pesticide use group, and within 3 to 6 months after the application season. In each case, the interval between specimen collections was at least 3 months. Control subject specimens were obtained in the same time interval as the age- matched exposed subject. As a general rule, pre-prandial (fasting) morning specimens were obtained for these studies. Serum or heparinized plasma was separated at the time of phlebotomy and transported to the laboratory within 24 h. Aliquots of serum/plasma were cryopreserved at $-80°C$, and later, hormonal analyses were performed on the specimens. All hormonal analyses were conducted in a single batch run. It was not possible to obtain pre-application blood samples from all study participants. There were only about one-third as many pre-application samples available as there were samples from each

of the other two seasons. Thus, comparisons of measurement levels in the pre-application season will have much less statistical power than comparisons in the application and post-application seasons. More details regarding the experimental design for this study are provided in other published work (Potter et al., 1993; Garry et al., 1996).

Hormone Analyses

The LH, FSH, and testosterone concentrations were measured in serum or plasma obtained from blood specimens donated by study subjects. LH and FSH were measured by using two-site immunofluorometric assays from commercially available kits (DELFIA; WallacOy; Turku, Finland; CAT Nos. 1244-031 and 1244-017) modified as previously described (Kesner et al., 1994). Total and protein-unbound (free) testosterone were measured using solid-phase radioimmunoassays (Diagnostic Products Corporation, Los Angeles, CA; CAT Nos. TKTTI and TKTF1). The assays used in hormone measurements are validated for serum or heparinized plasma. Values measured in these blood fluids are not different from each other.

Cholesterol, Triglycerides, and Heavy Density Lipoprotein (HDL) Analyses

All measurements were performed on COBAS FARA automated clinical analysis instrumentation (Hoffmann-LaRoche and Company). In brief, cholesterol was measured by enzymatic determination of total serum cholesterol (Allain et al., 1974). HDL was measured in the supernate after precipitation of LDL (low density lipoprotein) with divalent cations (Allain et al., 1974). Triglycerides were measured by a peroxidase-coupled colorimetric reaction (McGowan et al., 1983).

Statistical Analysis

Comparisons of hormone and cholesterol-related measurements were based on analysis of variance methods after taking a logarithmic transformation of all measurements. In each application season, a one-way analysis of variance was performed, and each applier group was compared to the control group based on *t*-statistics. Two-sided *p*-values are reported.

Methods: *in vitro* genotoxicity of herbicides and adjuvants

General

In earlier studies by our group, some of the paraffin oils contained in cutting fluids were found to be mutagenic in the Ames assay (Garry et al., 1986). Other commercial product classes, such as surfactants and surfactant oil mixtures, have not, to our knowledge, been evaluated for mutagenic activity. Based on this apparent lack of information, we chose to examine the potential genotoxicity of representative members of the surfactant group of adjuvants *in vitro*. Commonly used herbicides, including eight commercial-grade chlorophenoxy products, were used for a comparison group.

Products

Commercial-grade pesticide products, including herbicides and adjuvants, were obtained from pesticide applicators. Routinely, field laboratory staff members removed aliquots of these products from commercial containers and placed them in chemically clean, gas-tight glass vials (Pierce Chemical Company) after use by the applicator. The aliquots were placed in a cooler, taken to the laboratory and stored at 4°C prior to use in these experiments. Each applier supplied a copy of the label and/or package insert for the product to identify vendor source and provide an indication of shelf life.

Commercial-grade products used in these studies are as follows:

Chlorophenoxy herbicides:

1. 2,4-D LV4 (2,4-dichlorophenoxyacetic acid as the butoxyethyl ester);
2. See 2,4-D (isooctyl ester of 2,4-dichlorophenoxyacetic acid);
3. 2,4-D methyl ester (methyl 2,4-dichlorophenoxyacetate) MCPA (4-chloro-2-methylphenoxy acetic acid);
4. 2,4-D LV6 (2,4-dichlorophenoxyacetic acid butoxyethyl ester);
5. 2,4-D amine 4 (dimethylamine salt of 2,4-dichlorophenoxyacetic acid);

Chlorophenoxy herbicide mixtures:

1. Weedone 638 (2,4-dichlorophenoxy acid and 2,4-dichlorophenoxyacetic acid butylethyl ester);
2. Tordon 101 (trichloropicolinic acid and 2,4-dichlorophenoxyacetic acid);

Other herbicides:

1. Garlon 4 ((3,5,6-trichloro-2-pyridinyloxy)acetic acid);
2. Round-up (*N*-(phosphono-methyl) glycine salt);
3. Pursuit (± 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridine carboxylic acid)
4. Accord (*N*-phosphonomethyl glycine salt);

Adjuvants:

1. Direct (polyvinyl polymers and inert ingredients);
2. X-77 (alkyl polyoxyethylene, free fatty acids, glycol and isopropanol mixture);
3. Nalco-trol (polyvinyl polymer and inert ingredients);
4. Preference (a proprietary surfactant containing less than 0.002% ethylene oxide and less than 0.001% dioxane).

Cell Preparation, Cell Culture, and In Vitro Exposure Conditions

Human lymphocytes were cultured from volunteer blood donors (control subjects) who met the health and exposure criteria set forward in prior publications (Garry et al., 1990). In brief, 1 ml heparinized whole blood containing 1×10^5 to 5×10^5 lymphocytes/ml as a final cell concentration was added to 9 ml complete media [RPMI 1640 (Gibco-BRL), 17% fetal calf serum (Hyclone), supplemented with 2.92 mg L-glutamine (Gibco-BRL), gentamicin 0.08 mg (Gibco-BRL) and 4% phytohemaglutinin (Gibco-BRL)]. Cells were cultured in 10 ml flasks (Corning) at 37°C for 44 h, when 6 μg/ml Cytochalasin B (Sigma) was added to inhibit cytokinesis and allow measurement of toxicant-induced micronuclei. Cells were continued in culture for a total of 72 h. At the termination of culture, cells were lysed in hypotonic KCl and fixed in methanol/acetic acid. Slides were prepared and stained with May Grunwald (Sigma) followed by Gurr buffer and Giemsa stain (BDH laboratories). One thousand binucleate cells were scored for micronuclei according to standard criteria (Countryman and Heddle, 1976; Verhaegen and Vral, 1994). All test chemicals were added at the beginning of culture. Preliminary dose ranging studies were conducted prior to more detailed dose–response studies reported below.

Statistical Analysis for Micronucleus Assay

To test for an increase in the micronucleus frequency with increasing dose level, a χ^2 test for trend in proportions was used. To compare the micronucleus frequency for each dose level to that for control, an exact test was employed. Two sided *p*-values are reported for all tests (Armitage, 1971).

Results: population studies

Survey

In this study group of 719 pesticide applicators, 709 gave detailed information regarding pesticide use and are, for the most part, farmers (approximately 80%). Of the subjects, 59.4% used adjuvants in their applications. Of those appliers who use adjuvants, 57.8% used 10 different commercial products containing paraffin oils, 21.6% used 22 products containing surfactants, 6.7% used vegetable oils, another 6.7% used oil/surfactant mixtures, and 2.6% used eight formulations containing other petroleum oils (Table 1). In total, 52 different adjuvant formulations were used.

Table 1. Adjuvants and product formulations used by applicators

Product class	Frequency of use (%)	Number of formulations
Paraffin oils	57.8	10
Surfactants and wetting agents	21.6	22
Oil/surfactant mixtures	6.7	6
Vegetable oils	6.7	6
Other petroleum oils	2.6	8

Listed in the table is the frequency distribution of the types of adjuvants used by applicators ($n = 709$). The majority (57.8%) contain paraffin oils. Ten different formulations of paraffin oils were used. Twenty-two different formulations of surfactants and surfactant mixtures were used by 21.6% of the applicators who use adjuvants.

Table 2. Comparison of testosterone, FSH and LH among pesticide applier groups

	Pre-application	Application	Post-application
Free testosterone			
Control	26.9 (3.0)	26.7 (1.5)	24.4 (1.6)
Fumigator	28.5 (3.7)	26.5 (2.7)	26.5 (2.6)
Herbicide	27.6 (2.3)	29.6 (2.2)	29.4 (1.6) $p = 0.032$
Insecticide	25.8 (3.6)	27.4 (1.7)	27.4 (1.7)
Total testosterone			
Control	5.28 (0.47)	5.16 (0.39)	4.92 (0.37)
Fumigator	5.08 (0.75)	4.55 (0.49)	4.64 (0.57)
Herbicide	5.36 (0.10)	4.89 (0.40)	4.89 (0.30)
Insecticide	4.58 (1.27)	5.29 (0.43)	5.46 (0.38)
FSH			
Control	4.34 (0.82)	4.47 (0.62)	4.92 (0.71)
Fumigator	4.54 (1.14)	4.22 (0.59)	4.29 (0.58)
Herbicide	2.74 (0.62)	2.82 (0.34) $p = 0.016$	2.88 (0.39) $p = 0.010$
Insecticide	4.58 (1.27)	3.68 (0.57)	3.95 (0.58)
LH			
Control	3.30 (0.63)	3.38 (0.24)	3.24 (0.34)
Fumigator	3.61 (0.55)	4.09 (0.51)	3.84 (0.52)
Herbicide	2.71 (0.53)	3.47 (0.27)	3.48 (0.31)
Insecticide	4.20 (0.91)	4.00 (0.38)	4.30 (0.49)

Listed in the table are the geometric mean values from measurements of total and free testosterone, FSH, and LH in serum obtained from pesticide applicators and controls. In these comparisons, pre-season, height of application season, and post-season data were compared within each exposure group (fumigant applicators, herbicide applicators, and insecticide applicators) and controls were obtained during the same time frame. All *p*-values are from *t*-tests and are two-sided. For herbicide applicators, free testosterone values rise significantly after completion of their application of herbicides, while FSH values rise during and after completion of the application season but are significantly lower than controls for these time points. Mean FSH values in the herbicide group over the entire study period are significantly lower than controls ($p = 0.017$). FSH and LH values are recorded in standardized international units per liter; total and free testosterone in nanogram per deciliter. The standard errors for all test values are presented in parentheses. The data suggest clinically minor but significant change in the endocrinologic status of herbicide appliers over the application season.

Hormone Analysis, Cholesterol, and Cholesterol-Related Analyses

There were few significant differences in hormone levels (Table 2). Free testosterone levels increased in the herbi-

Table 3. Cholesterol, triglycerides and HDL in pesticide appliers

	Pre-application	Application	Post-application
Cholesterol			
Control	197.2 (8.7)	193.8 (11.1)	199.1 (11.1)
Fumigator	218 (10.1)	187.4 (13.3)	211.2 (14.1)
Herbicide	212.5 (7.4)	211.2 (7.2)	213.2 (7.7)
Insecticide	204.6 (7.3)	208.1 (9.1)	204.8 (8.3)
Triglycerides			
Control	97.0 (12.4)	82.3 (11.0)	74.0 (8.5)
Fumigator	142.3 (23.2)	104.8 (14.0)	137.8 (10.5)
			$p = 0.002$
Herbicide	110.1 (9.7)	118.7 (9.7)	110.4 (0.30)
		$p = 0.022$	$p = 0.010$
Insecticide	99.5 (10.2)	88.7 (8.9)	88.7 (8.9)
HDL			
Control	48.6 (2.2)	50.2 (2.8)	49.5 (2.3)
Fumigator	39.6 (3.5)	43.1 (1.4)	42.3 (2.0)
	$p = 0.048$	$p = 0.022$	$p = 0.023$
Herbicide	48.0 (2.2)	49.8 (1.8)	46.0 (1.9)
Insecticide	48.7 (3.5)	51.1 (3.2)	48.5 (3.4)

Listed in the table are the geometric mean values and standard errors of the mean in parentheses for total cholesterol, triglycerides, and heavy density lipoprotein (HDL) for specimens obtained from the same three time points shown in Table 2 from each applicator group and controls. All data in the table are recorded in milligram per deciliter. Comparison of values for cholesterol, triglycerides, and HDL within each exposure group shows no significant differences ($p > 0.05$), indicating that seasonal variation due to pesticide use was not a factor in these results. Compared to control subjects, fumigators show significantly increased triglycerides and lower HDL ($p < 0.05$) values; herbicide applicators demonstrate significantly increased triglycerides and cholesterol ($p < 0.05$). The findings suggest that long-term exposure to certain classes of pesticides could be a factor affecting lipid metabolism. All *p*-values are from two-sided *t*-tests.

cide appliers in the application and post-application seasons as compared to their pre-application values and were significantly lower than controls in the post-application season. This significance was due, at least in part, to a decrease in the control mean in the post-application season. Herbicide appliers had significantly lower FSH values than controls in the application and post-application season. FSH values in the herbicide appliers were lower than normal in the pre-application season. However, the difference was not significant because of the smaller number of pre-application samples. With regard to cholesterol-related analyses, there were few significant differences in cholesterol-related measurements (Table 3). These could not be attributed unequivocally to pesticide application. Herbicide appliers had significantly higher triglyceride measurements than controls in the application and the post-application season, but there was little change in the triglyceride mean values for herbicide appliers from season to season. The control mean was quite low in the post-application season, resulting in a significantly higher mean value for the fumigator group as well as the herbicide group. Fumigators had significantly lower mean HDL levels than controls in all three seasons, but there was little variation among HDL levels from season to season among fumigators.

Results: *in vitro* studies

Micronucleus Assay

Listed in Table 4 are the summary results from the *in vitro* assay of human lymphocytes treated with different doses of commercial-grade adjuvants and some commonly used herbicides. Figure 1 depicts results from all dose–response studies considered positive in the micronucleus assay. As noted in Table 4, the different commercial-grade chlorophenoxy herbicide products are representative of those used by applicators in southern and northwestern MN. Of eight different brands and/or combinations of chlorophenoxy herbicides tested, only one showed a significant dose–response (2,4-D LV4) and one showed a weakly

Table 4. Comparison of *in vitro* screening of selected herbicides and adjuvants

Herbicides and adjuvants	Results
Micronucleus frequency	
Chlorophenoxy herbicides	
2,4-D LV4	+
2,4-D methyl ester	−
MCPA	−
See 2,4-D	−
2,4-D LV6	−
2,4-D amine	−
Chlorophenoxy herbicide mixtures	
Weedone 638	+/−
Tordon 101	−
Other herbicides	
Garlon 4	−
Pursuit	−
Roundup	−
Accord	−
Adjuvants	
Direct	+
X-77	+
Nalco-Trol	+
Preference	+

Summary results from all commercial-grade products tested are presented. All products were initially examined in four dose point studies ranging from 1 to 1000 μg/ml. In this table, product data showing a significant increase ($p = 0.05$ or less) at some dose level (+/−), products that showed a significant dose–response with a doubling of micronucleus frequency (+), and negative data (−) are summarized. More detailed review of positive (+) dose–response data are graphically presented in Figure 1.

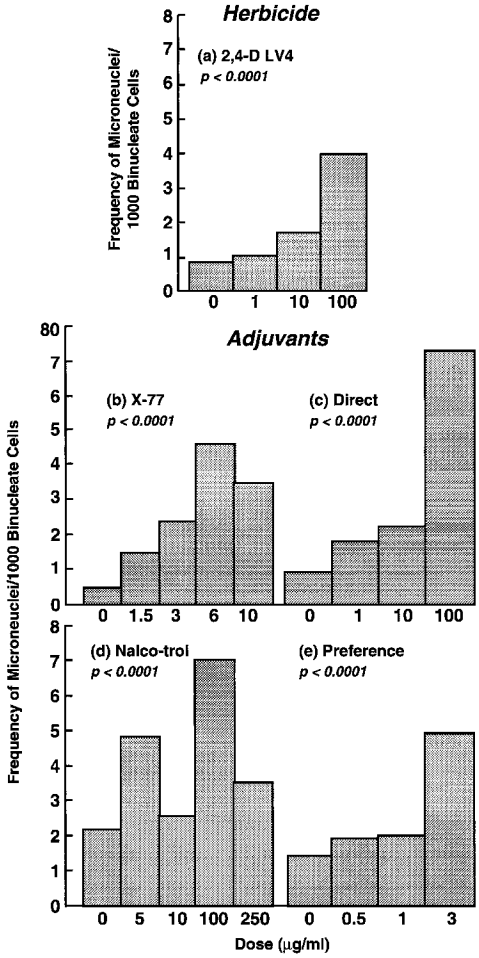

Figure 1. Shown in the figure are typical dose-related increases in the frequency of micronuclei in cultured human lymphocytes treated with herbicides or adjuvants. Separate cultures of treated and untreated (control) lymphocytes from the same donor were used in each study shown. Repeated studies with different donor lymphocytes for experiments (b) and (c) have been performed with similar results. The data recorded for (d) and (e) are atypical and probably due to death of cells expressing micronuclei (Tates et al., 1991). The *p*-values given for each experiment express the level of significance for dose–response. At least four different dose levels, including an untreated control, are shown for each separate experiment.

positive result (Weedone 638). As a point of comparison, reagent-grade 2,4-D as the methyl ester showed a negative response. None of the four other commercial herbicides studied (Garlon 4, Roundup, Pursuit, and Accord) were considered positive in this assay system. In limited assessment of adjuvants selected at random from the surfactant class, all four of the adjuvants studied showed dose-related, statistically significant increases in micronucleus frequencies (Table 4; Figure 1). Of all the commercial herbicide products examined, the most consistently negative results were obtained for products containing *N*-phosphonomethyl glycine, commonly known as glyphosate (Roundup and Accord).

Discussion

In Vitro Studies

In the present effort, commercial-grade chlorophenoxy herbicides and other herbicides, including glyphosate, were examined in the micronucleus assay. In terms of genotoxicity, these data suggest an apparent dichotomy between these commercial herbicide preparations and commercially available adjuvants that may be used in conjunction with these products. The present data show that some chlorophenoxy herbicide products in current use are marginally genotoxic. These predominately negative data are consistent with earlier animal studies and an *in vitro*

study measuring the mutagenic or carcinogenic potential of these herbicides in the chemically pure form (Innes et al., 1969; Mullison, 1986; Mustonen et al., 1986; Schop et al., 1990). The ingredient information for commercial-grade products that gave marginally positive data (2,4-D LV 4 and Weedone 638) lists more than 40% inert ingredients on the product label. However, all of the commercial-grade herbicides studied list at least 20% inert ingredients in the product label or MSDS. The overwhelming majority of these commercial-grade herbicides are negative in the micronucleus assay even though they contain inert ingredients. More intriguing and more speculative are the adjuvant data. Two products (Direct and Nalco-trol) contain polyacrylamides. In whole animal studies, monomeric acrylamide induces dose-related increases in micronucleus frequencies (Kligerman et al., 1991; Lahdetie et al., 1994). One product contains glycols, isopropanol, and alkylarylpolyoxyethylenes (X-77). X-77 has been used historically as a reference standard for surfactant/adjuvant comparison studies (Matsui et al., 1992). Review of the biologic activities of the components of X-77 is of interest. Studies of impurity-free isopropanol and studies of polyoxyethylene derivatives do not suggest mutagenic effects at the chromosomal level (Meyer et al., 1988; Kapp et al., 1992; Hirai et al., 1994). Polyoxyethylenes are derivatives of ethylene oxide (EO) and at least one polyoxyethylene adjuvant lists EO as a contaminant at the 1% level. EO is a known human mutagen (Garry et al., 1979; Landrigan, 1992) and can induce significantly increased frequency of micronuclei in humans and animals (Applegren et al., 1978; Tates et al., 1991). Some glycol derivatives can induce micronucleus formation and other mutagenic effects (Moore, 1989; Gollapudi et al., 1993; Ma et al., 1993; Chiewchanwit and Au, 1995). Whether any of these adjuvants contain biologically significant concentrations of the contaminants mentioned or other mutagenic materials is uncertain. In a somewhat different vein (manuscript in preparation), surfactants such as X-77 contain alkylphenol ethoxylates and can be considered weak pseudoestrogens (Nimrod and Benson, 1996). Finally, these findings suggest that nonionic surfactants have toxicant properties that may lead to genotoxicity and pseudoestrogenic effects. In support of this hypothesis, nonionic surfactants such as alkylphenol ethoxylates are known to interact with and alter biologic macromolecules such as phospholipid components of cell membranes and intracellular proteins including enzymes (Cserhati, 1995).

Human Studies: Hormones and Cholesterol

As noted in the Results section, preliminary data regarding the hormonal status of applicators show that herbicide applicators demonstrate seasonal decrements in testosterone and FSH, while other applicator groups and controls do not. The consistent low levels of FSH in the herbicide applicator group do not appear to be a technical artifact, since the assay for FSH was performed in a single run on archived, coded, and blinded specimens. However, population-based biologic artifact is not ruled out. To establish the biologic significance of the FSH data will require additional, more detailed *in vivo* and *in vitro* study. Along these lines, two studies of different pesticide applier populations are nearing completion. We will ascertain, in more detail, hormonal relationships across the pesticide use season. *In vitro* screening studies employing the MCF-7 cell line are also underway to establish possible endocrine-disrupting effects of adjuvants.

The consistently elevated cholesterol and triglyceride values in the herbicide appliers across each time point measured compared to controls (Table 3) is of speculative interest. Several studies point to dioxins and dioxin-containing chlorophenoxy herbicides as a possible etiologic factor for elevation of cholesterol and triglycerides and decreased HDL that were found in populations exposed to these agents some years earlier (May, 1982; Martin, 1984). Reports concerning current exposure to dioxins (Egeland et al., 1994) show that serum dioxin levels are correlated with fluctuation of the levels of LH, FSH, and total testosterone, depending on dose level measurements stratified by exposure quartile. Currently, we have no evidence that commercial chlorophenoxy herbicides or other pesticides in current use contain dioxins or that herbicides used some years earlier by our study subjects might contain dioxins. The mechanism of action of several herbicide classes (carbamothioates and polycyclic alkanoic acids) include effects on lipid synthesis (Duke and Kenyon, 1988; Wilkinson, 1988; Duke, 1990), raising the steroid hormonal question independently of dioxin effects. In this connection, direct effects on cholesterol synthesis, lipid peroxidation and/or conversion of cholesterol to testosterone are considerations.

Aside from the herbicide or herbicide contaminant issues raised above, the possible mutagenic activity of adjuvants considered here and possible endocrine-disrupting activity of these products add further complexity to interpretation of pesticide use data from human studies. It is to be noted that fumigant applicators are, for the most part, commercially licensed and perform applications for hire. This group uses herbicides in the spring, insecticides in the summer, and fumigants in the fall. Herbicide applicators are, for the most part, privately licensed farmers and only apply herbicides to their own lands. While apparent herbicide-induced alteration of hormonal and cholesterol-related measures did occur in these two exposed groups, the levels of change do not suggest an association with known clinical disease entities. Moreover, subtle variation in population biology, including differences in physical activity

and lifestyle among the applicator groups, could account for some of the results obtained.

Summary

Altogether, the *in vivo* and *in vitro* data warrant careful attention in planning human pesticide exposure/effect studies. From our current data and other studies cited, it is clear that adjuvants are not biologically inert. The toxicant role of adjuvants in relation to herbicide use needs to be considered. Given the number of adjuvant formulations studied ($n = 4$) and the number encountered in our survey ($n = 52$), study of formulated adjuvant products alone or together with herbicides for mutagenic or other health endpoints will be challenging. Nonetheless, this avenue may lead to a clearer understanding of the human teratogenic and carcinogenic potential of herbicides in common use.

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