Canine Exposure to Herbicide-treated Lawns and Urinary Excretion of 2,4-Dichlorophenoxyacetic Acid

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Abstract

A recent study by Hayes *et al.* (J. Natl. Cancer. Inst., *83:* 1226–1231, 1991) found an increased risk of malignant lymphoma associated with exposure to 2,4-dichlorophenoxyacetic acid (2,4-D) in pet dogs. We conducted a study to determine the extent to which dogs absorb and excrete 2,4-D in urine after contact with treated lawns under natural conditions. Among 44 dogs potentially exposed to 2,4-D-treated lawns an average of 10.9 days after application, 2,4-D concentrations greater than or equal to 10.0 µg/l were found in 33 dogs (75%) and concentrations of ≥ 50 µg/l were found in 17 (39%). Among 15 dogs with no known exposure to a 2,4-D-treated lawn in the previous 42 days, 4 (27%) had evidence of 2,4-D in urine, 1 at a concentration of ≥ 50 µg/l. The odds ratio for the association between exposure to a 2,4-D-treated lawn and the detection of ≥ 50 µg/l 2,4-D in urine was 8.8 (95% confidence interval, 1.4–56.2). Dogs exposed to lawns treated within 7 days before urine collection were more than 50 times as likely to have 2,4-D at concentrations ≥50 µg/l than dogs with exposure to a lawn treated more than 1 week previously (odds ratio = 56.0; 95% confidence interval, 10.0–312.2). The highest mean concentration of 2,4-D in urine (21.3 mg/l) was found in dogs sampled within 2 days after application of the herbicide. These findings demonstrate that dogs living in and around residences with recent 2,4-D lawn treatment absorb measurable amounts of the herbicide for several days after application and thus may constitute a useful animal model for evaluation of the effects of herbicides on the induction of lymphoid cancer.

Introduction

Exposure to pesticides has been suggested as a possible explanation for the widely recognized increased risk for hematopoietic cancers among farmers (1–4). The phenoxyacid herbicide 2,4-D[2] is used extensively in agricul-ture, residential settings, and public places to control the growth of broadleaf weeds. A series of epidemiological studies has linked exposure to 2,4-D and other phenoxy acid herbicides in the agricultural setting to risk of NHL (5–8).

CML may serve as a comparative model for non-Hodgkin's lymphoma in humans due to similarities in biological behavior and pathological appearance (9). CML is a common cancer of dogs with an estimated incidence of 25–30/100,000/year (10). The etiology of CML remains unknown; attempts to identify a retroviral etiology for CML have not been successful. Histologically, most cases of CML are classified as lymphosarcomas, one of the subtypes of NHL in humans (11). Although some dogs have a leukemic phase during their disease, this manifestation is uncommon.

In a recent study, Hayes *et al.* (12) found that exposure to 2,4-D was associated with an increased risk of CML in pet dogs. Owners in households with dogs that developed malignant lymphoma were 30% more likely to have applied 2,4-D or to have employed a commercial lawn care company to treat their yard than owners of control dogs in the hospital-based case-control study. The risk of CML rose to a 2-fold excess with 4 or more owner applications of 2,4-D/ year (12). To date, biomonitoring has not been used in an attempt to validate these findings in household dogs. The present study was undertaken to determine the extent to which dogs absorb and excrete 2,4-D in urine after contact with lawn herbicides containing 2,4-D and to evaluate use of questionnaire responses on herbicide application to ascertain potential for exposure of pet dogs to 2,4-D.

Subjects and Methods

A community-based study was conducted in 1992 to identify dogs living in homes where the lawn had been treated with 2,4-D or other lawn products. Eligible dog owners were identified through local veterinary clinics and a university teaching hospital in the same community. The university hospital was one of three from which subjects were obtained in the case-control study of CML conducted by Hayes *et al.* (12). Participants were identified by their voluntary, written response to a screening question which asked whether they had used a fertilizer, "weed and feed," or other pesticide on their lawn within the past 42 days. The exposure definition included owner application of lawn chemicals as well as commercial pesticide application.

A personal interview was scheduled with each of the respondents in their home within 5 days after responding to the screening question. The interview contained questions

regarding the form, timing, and manner of herbicide use, method of herbicide storage, dog's access to the storage area, and watering and rainfall since application. The dog's exposures to the treated lawn, activity patterns, and habits around the time of herbicide application were assessed. At the time of interview, the specific product used was identified by the owner from a listing of the lawn products available at local garden stores and supermarkets. In instances where the lawn product was still available, validation was accomplished by visual inspection of the product container by an investigator. The chemical contents of each product were established by examination of the product container's label at the retailer. At homes where the premises had been treated by a commercial lawn care company, the chemical contents of the application were established by telephone interview of a company representative.

Dogs belonging to owners who had used a product containing 2,4-D were classified as exposed (*n* = 44); those belonging to owners who had not used a 2,4-D-containing product within the past 42 days served as a comparison group (*n* = 15). No additional application of lawn products had been made between the response to the screening question and the time of interview and urine collection except for one dog described below. All dogs were confined to their residences except for walks with owners; none were permitted to roam loose.

A urine sample was collected from each dog by an investigator at the time of interview with the owner. Between 50 and 100 ml of urine were collected from a free flowing urine stream, taking precautions to avoid contamination from the animal's coat. Urine samples were placed on ice and transported to the laboratory where they were stored frozen (–20°C) until analysis. Urine samples were obtained on more than 1 day from two dogs. In instances where multiple samples were available, the first sample taken was used in the analyses presented in the tables.

The concentration of 2,4-D in urine was assessed using an ELISA immunoassay (Envirogard 2,4-D ELISA Plate Kit; Millipore Corp.; Bedford, MA) with a manufacturer's detection limit of 0.1 µg/l 2,4-D in water. After thawing of urine samples at room temperature over a period of approximately 3 hours, duplicate samples were diluted 1:100 with distilled water and placed in antibody-coated wells in a microtiter plate. After the addition of a 2,4-D enzyme conjugate and incubation for 60 min, the plates were washed and substrate and chromogen were added to each well. The colorimetric reaction was read spectrophotometrically with an ELISA plate reader (UVmax Molecular Devices; Menlo Park, CA). Positive (0.5, 10.0, and 100.0 µg/l) and negative controls were run with each plate. The percentage of absorbance was calculated for each sample and the 2,4-D concentration was calculated from the mean of the duplicate readings.

Preliminary quality assurance studies to assess accuracy of the ELISA method were conducted by testing normal human urine spiked with known quantities of 2,4-D, and by comparing ELISA results with GC results for serial urine samples from a farmer exposed to 2,4-D. The ELISA and GC results were comparable.

Selected canine urine samples also were analyzed using GC to obtain additional qualitative confirmation of the presence of 2,4-D (13). Urine was subjected to acid hydrolysis followed by benzene extraction of 2,4-D-free acid. After evaporation of the solvent, the residues were treated with diazoethane. The ethyl ester of 2,4-D was analyzed on two different columns using an electron capture detector. Re-

Table 1 Distribution of 2,4-D in canine urine by exposure status for lawn herbicides in Colorado, 1992

Urine 2,4-D level (µg/l)	Lawn treated with 2,4-D	Last application[a]	Lawn untreated	Total
<10	11	15.9	11	22
10– < 50	16	13.4	3	19
50– > 100	4	7.8	1	5
≥100	13	4.5	0	13
Total	44	10.9	15	59

[a] Mean days since last application of 2,4-D-containing herbicide.

tention times were compared with authentic standards treated identically. Mean recovery of 2,4-D-spiked urine samples analyzed with the GC procedure was 101%. Two urine samples found to have greater than 50 µg/l 2,4-D by ELISA were analyzed by GC. The detection limit of the GC method was 50 µg/l. The presence of 2,4-D in each sample was confirmed by retention time on two different columns in comparison with known standards.

Risks of exposure to 2,4-D associated with contact with a treated lawn and with various canine behaviors were estimated from the adjusted ORs (14) and their 95% CIs (15).

Results

Owners of 44 of the 59 dogs sampled reported having used a lawn product containing 2,4-D within the past 42 days; 39 of the herbicide applications had occurred within the preceding 21 days. Fifteen dogs lived in homes where no 2,4-D-containing product had been used and constituted the comparison group. Among the 44 dogs potentially exposed to 2,4-D on lawns, 2,4-D concentrations greater than or equal to 10.0 µg/l were found in 33 dogs (75%) and concentrations of at least 50 µg/l 2,4-D were found in 17 (39%) (Table 1). Among 15 dogs in the comparison group, 4 (27%) had evidence of 2,4-D in urine at ≥10.0 µg/l. The OR for exposure to 2,4-D defined as finding ≥10.0 µg/l in urine associated with a herbicide-treated lawn was 8.3 (95% CI, 2.2–28.7). For exposure defined as ≥50.0 µg/l of 2,4-D in urine, the OR was 8.8 (95% CI, 1.4–56.2).

When the data were stratified by concentration of 2,4-D in urine (Table 1), concentrations of 2,4-D in urine above 100 µg/l were observed only in dogs from homes with treated yards; 13 of the dogs living on treated yards showed 2,4-D levels above 100 µg/l, while none of the controls had 2,4-D above that value. Six dogs had at least 1.0 mg/l 2,4-D in urine, five dogs had between 1 and 10 mg/l, and one dog had more than 10 mg/l. Four dogs from the comparison group without known exposure to the herbicide had low levels of detectable 2,4-D in urine (mean concentration, 24 µg/l; maximum concentration, 66 µg/l). An inverse relationship between concentration of 2,4-D in urine and the average time elapsed since application of the herbicide was found (Table 1).

The relationship between the date of the last yard application and excretion of 2,4-D in dog urine was examined further (Table 2). Dogs exposed to lawns treated within 7 days before urine collection were more than 50 times as likely to have 2,4-D ≥50.0 µg/l in urine than dogs with exposure to a lawn treated more than 1 week previously (OR = 56.0; 95% CI, 10.0–312.2). Sixteen of 22 dogs (72.7%) with exposure to recently treated lawns had 2,4-D in urine above 50.0 µg/l, while only 1 of 22 dogs (4.5%) from homes

Table 2 Detection of 2,4-D in canine urine by days elapsed since last application in Colorado, 1992

Days since application	No. tested	2,4-D mean μg/l	SD	2,4-D median μg/l	Percentage ≥50.0 μg/l
1–2	4	21,262	(36,133)	5,020	50.0
3–4	6	2,356	(2,089)	2,425	83.3
5–7	12	398	(530)	95	75.0
8–10	7	32	(25)	32	14.3
11–14	7	22	(17)	20	0.0
>14	8	8	(9)	5	0.0
Total	44	2,373	(11,348)	33.5	38.6

Table 3 Detection of 2,4-D in canine urine by hours elapsed between lawn application and initial exposure in Colorado, 1992

Hours between lawn application and initial exposure	No. tested	2,4-D μg/l mean	2,4-D μg/l median	Days since last application mean
≤1	11	83	40	9.5
2–3	9	225	45	12.4
4–11	9	9,994	40	8.1
12–23	4	2,400	2,425	3.5
≥24	11	174	20	16.0

treated over a week before sampling showed 2,4-D in urine at this concentration. The highest mean concentration of 2,4-D in urine (21.3 mg/l) was found in dogs sampled within 2 days after application of the herbicide to the lawn and decreased rapidly in dogs sampled more than 2 days after application. However, one dog had a concentration of 1.7 mg/l 2,4-D in urine when sampled 5 days after application. The eleven dogs for which no 2,4-D was detected in urine (Table 1) had been sampled an average of 16 days after the last application of herbicide, suggesting that any absorbed 2,4-D may already have been eliminated at the time of sampling. Only 1 of these 11 dogs was sampled within the first 6 days after herbicide application.

The time elapsed between lawn treatment and the initial exposure of the dog to the lawn was examined (Table 3). The mean levels of 2,4-D in urine were highest for dogs exposed between 4 and 23 h after application of the herbicide. However, the median values for 2,4-D in urine were relatively similar across the groups. On average, dogs were sampled 10.9 days after herbicide application. As shown in Table 2, the time elapsed between initial exposure and the collection of a urine sample was the major determinant of the urinary concentration of 2,4-D, rather than the hours elapsed before the dog first contacted the treated lawn.

Urine samples were collected from two dogs on more than one occasion after exposure to a 2,4-D-treated lawn. One dog initially sampled 7 days after herbicide application had 90 μg/l 2,4-D in urine. This value decreased to 75 and then to 53 ug/l, 11 and 13 days after application, respectively. A second dog sampled 14 days after the first application of herbicide had 15 μg/l 2,4-D in urine; when sampled 4 days after a second lawn application 4 weeks later, the concentration of 2,4-D was 100 μg/l.

Potential risk factors were evaluated in stratified analyses for an association with 2,4-D exposure, controlling for the time elapsed since the last application. A cutoff value of 50.0 μg/l 2,4-D in urine was used to search for associations with potential risk factors for exposure to the herbicide. Elevated but imprecise ORs of approximately 5 were found for dogs who licked their paws or coats frequently or dug in the treated yard. These estimates were based on small numbers of dogs with the exposure. Thirteen homes received commercial application of a lawn herbicide. The mean concentration of 2,4-D in dogs living in homes treated by a commercial applicator (6666 μg/l) was higher than that for owner application (570 μg/l); however, a higher proportion of the former group was sampled within the first week after application.

Discussion

Phenoxyacid herbicides were implicated first in the etiology of NHL in a Swedish case-control study which found a 5-fold increase in risk for persons exposed to phenoxy acids, including 2,4-D (5). Concern about the widespread use of 2,4-D increased after a 1986 report (6) that showed a doubling in risk of NHL among Kansas farmers who sprayed or mixed the herbicide. Farmers who used 2,4-D for more than 20 days a year had approximately a 6-fold increase in risk (6). Subsequently, a 50% increase in risk of NHL was found for men in eastern Nebraska who sprayed or applied the herbicide in agriculture; the risk increased with increasing frequency of use (7). Recently, a significant dose-response between risk of death from NHL and acres sprayed with herbicides was found in a Canadian mortality study of a period when 2,4-D represented 75–90% of the herbicide applications in the province (8).

The role of environmental exposures in the induction of CML is virtually unexplored. Before the report of an association between exposure to 2,4-D and risk for canine lymphoma (12), only one other study evaluating environmental exposures had been reported (16). Exposure to low level radiation from uranium mill tailings was examined in a study of cancer in Mesa County, Colorado. No relationship with CML was demonstrated; however, the study was limited by the small number of cancers of any specific type (16).

In the current study, evidence was found that absorption and excretion of 2,4-D occurs readily among dogs after application of the herbicide to lawns, particularly during the first 2 days after application. The pattern of urinary excretion of 2,4-D was consistent with absorption during the period shortly after application. Dogs sampled within the first 7 days after the last application were found to excrete 2,4-D in urine up to a mean of 21.3 mg/l within the first 2 days after exposure, while dogs sampled after 7 days had declining concentrations. However, in one dog sampled, 7, 11, and 13 days after application 2,4-D values above 50 μg/l were still found on the last day of testing, suggesting prolonged exposure to the herbicide, relatively slow excretion, or both. The half-life in dogs of a structurally similar phenoxy herbicide, 2,4,5-trichlorophenoxyacetic acid, was found to be 77 h (3.2 days) after a single oral dose (17), while that for 2,4-D in humans is 11–12 h (18).

The data from this study were compared with those obtained from exposure to 2,4-D under experimental conditions (19). In the current study, urine concentrations were higher and persisted longer than previously reported (19). Dogs placed on grass plots with application rates one to four times recommended doses absorbed and excreted 2,4-D in urine with maximal concentrations up to 4.3 mg/l 1 day after exposure (19). The mean urine concentration of 21.3 mg/l observed within 48 h after application under natural conditions approached the maximum level of 26.4 mg/l reported

for a dog placed on a grass plot treated at four times the recommended application rate and sampled at 1 day. Thus, the levels of 2,4-D in urine found in some dogs presumably exposed at the recommended application rate under natural conditions exceeded those reported for three dogs exposed at four times the recommended rate. In the experimental study, the herbicide was not detected in urine at the end of 1 week in two dogs on grass plots treated at the recommended application level; urine concentrations were not reported for the interval between 1 and 7 days (19). Among 22 dogs tested within 1 week of household lawn herbicide application, 6 had values of at least 1 mg/l 2,4-D in urine. There was considerable evidence of exposure in measurements made after 24 h; the mean concentration of 2,4-D in dogs sampled 72–96 h after application was 2.4 mg/l.

However, several differences between the experimental (19) and observational studies limit the comparability of the data. First, the two studies used a GC and an ELISA procedure, respectively, to measure urinary 2,4-D concentrations. We validated ELISA values by comparison with GC findings in human and canine samples; however, a systematic study to examine differences attributable to the analytic methods at varying concentrations was not conducted. Second, neither study adjusted urine concentrations of 2,4-D to urine creatinine values; therefore, differences in 2,4-D concentrations among and between dogs in the two studies may have reflected the urine osmolality. First morning urine samples were generally but not consistently obtained in the study reported here. First morning urine is likely to be relatively concentrated and may have increased the values of urinary 2,4-D. Third, a heavy rain 24 h after application may have limited exposure in the experimental study. In the natural setting, rain and lawn watering occurred inconsistently and at irregular times after application. Behavioral or physiological differences in the dogs may have contributed to differences in exposure and elimination; all subjects in the experimental study were English pointers. Finally, the application rates for the herbicide by home owners may have exceeded recommendations severalfold; no means of assessing this variable exist.

Four dogs with no history of exposure to a lawn herbicide had evidence of 2,4-D in urine. Not all of the 2,4-D found in the urine of these dogs may have been attributable to herbicides applied around the home. Some dogs may have been exposed to 2,4-D when taken to areas such as parks with an unknown history of herbicide treatment. In addition, drift of herbicides from neighboring yards may have occurred. Alternatively, low levels of 2,4-D in control dogs may represent a nonspecific ELISA reaction to the urine substrate. The accuracy of the ELISA procedure for 2,4-D in urine was evaluated by gas chromatographic confirmation only with samples containing ≥50 µg/l of the analyte.

This study provides evidence that dogs living in and around residences with recent 2,4-D treatments absorb measurable amounts of the herbicide through normal activities and behaviors. Further, it supports the use of owner questionnaire responses on herbicide application as a surrogate for potential exposure of pet dogs to 2,4-D. This method of assessing potential exposure was used in the case-control study of Hayes *et al.* (12), which found an association between reported exposure to the herbicide and increased risk of CML.

Current evidence is inadequate to classify 2,4-D as carcinogenic for animals or humans (20). However, two studies have demonstrated some increase in the incidence

of lymphoreticular neoplasms including lymphosarcoma in rodents (21, 22) and increased rates of sister chromatid exchanges and other chromosomal aberrations have been demonstrated *in vitro* in human lymphocyte cultures (23, 24) and *in vivo* in exposed humans (25, 26). Although some evidence for genotoxicity of 2,4-D exists (27), a mechanism for cancer induction remains unclear. Carcinogenicity may be due to excessive production of hydrogen peroxide and peroxisome proliferation (28). Additional laboratory-based and epidemiological research is needed in this potentially important model of environmentally induced lymphoid neoplasia.

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