Journal of Toxicology and Environmental Health, Part A, 72: 986–997, 2009
ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287390902929741

Biomonitoring of Genotoxic Risk in Agricultural Workers from Five Colombian Regions: Association to Occupational Exposure to Glyphosate

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In order to assess possible human effects associated with glyphosate formulations used in the Colombian aerial spray program for control of illicit crops, a cytogenetic biomonitoring study was carried out in subjects from five Colombian regions, characterized by different exposure to glyphosate and other pesticides. Women of reproductive age (137 persons 15–49 yr old) and their spouses (137 persons) were interviewed to obtain data on current health status, history, lifestyle, including past and current occupational exposure to pesticides, and factors including those known to be associated with increased frequency of micronuclei (MN). In regions where glyphosate was being sprayed, blood samples were taken prior to spraying (indicative of baseline exposure), 5 d after spraying, and 4 mo after spraying. Lymphocytes were cultured and a cytokinesis-block micronucleus cytome assay was applied to evaluate chromosomal damage and cytotoxicity. Compared with Santa Marta, where organic coffee is grown without pesticides, the baseline frequency of binucleated cells with micronuclei (BNMN) was significantly greater in subjects from the other four regions. The highest frequency of BNMN was in Boyacá, where no aerial eradication spraying of glyphosate was conducted, and in Valle del Cauca, where glyphosate was used for maturation of sugar cane. Region, gender, and older age (≥35 yr) were the only variables associated with the frequency of BNMN measured before spraying. A significant increase in frequency of BNMN between first and second sampling was observed in Nariño, Putumayo, and Valle immediately (<5 d) after spraying. In the post-spray sample, those who reported direct contact with the eradication spray showed a higher quantitative frequency of BNMN compared to those without glyphosate exposure. The increase in frequency of BNMN observed immediately after the glyphosate spraying was not consistent with the rates of application used in the regions and there was no association between self-reported direct contact with eradication sprays and frequency of BNMN. Four months after spraying, a statistically significant decrease in the mean frequency of BNMN compared with the second sampling was observed in Nariño, but not in Putumayo and Valle del Cauca. Overall, data suggest that genotoxic damage associated with glyphosate spraying for control of illicit crops as evidenced by MN test is small and appears to be transient. Evidence indicates that the genotoxic risk potentially associated with exposure to glyphosate in the areas where the herbicide is applied for coca and poppy eradication is low.

Glyphosate (*N*-phosphonomethyl glycine), a nonselective herbicide, is the active ingredient of a number of herbicide formulations and one of the most widely used pesticides on a global basis (Baylis, 2000; Woodburn, 2000; Duke & Powles, 2008). It is a postemergence herbicide, effective for the control of annual, biennial, and perennial species of grasses, sedges, and broadleaf weeds. The relatively high water solubility and the ionic nature of glyphosate retard penetration through plant hydrophobic cuticular waxes. For this reason, glyphosate is commonly formulated with surfactants that decrease the surface tension of the solution and increase penetration into the tissues of plants (World Health Organization International Program on Chemical Safety, 1994; Giesy et al., 2000).

A large number of glyphosate-based formulations are registered in more than 100 countries and are available under different brand names. One of the most commonly applied glyphosate-based products is Roundup, containing glyphosate as the active ingredient (AI) and polyethoxylated tallowamine

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This paper was prepared as part of a Study entitled "Production of Illicit Drugs, the Environment and Human Health," financed with contributions from the Governments of Colombia and the United States of America. The conclusions and opinions expressed herein are those of the authors and not necessarily those of the Organization of American States and its General Secretariat, which as of the date of this copyright, have not formulated any opinion with respect to them.

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(POEA) as a surfactant. Glyphosate and its formulations have been extensively investigated for potential adverse effects in humans (Williams et al., 2000). This pesticide was reported to exert a low acute toxicity to different animal species. Experimental evidence showed that glyphosate did not bioaccumulate in any animal tissues (Williams et al., 2000). Chronic feeding studies in rodents did not find evidence of carcinogenic activity or any other relevant chronic effects (U.S. EPA, 1993; World Health Organization International Program on Chemical Safety, 1994).

With in vitro studies with tissue cultures or aquatic organisms, several of the formulated products are more toxic than glyphosate AI (Giesy et al., 2000; Williams et al., 2000). Differences in the response of test organisms to the AI and the commercial formulation, e.g., Roundup, are likely due to the toxicity of different formulants and surfactants contained in commercial products. There is a general agreement that adjuvants may be more toxic for animals than glyphosate itself (Giesy et al., 2000; Williams et al., 2000; Richard et al., 2005). Cytotoxicity of the commercial formulation Roundup to human peripheral mononuclear cells was 30-fold higher (LC_{50} = 56 mg/L) than for the AI (LC_{50} = 1640 mg/L) (Martinez et al., 2007). Several in vitro and in vivo studies with parallel testing of glyphosate AI and Roundup showed that only the commercial formulation was genotoxic (Rank et al., 1993; Bolognesi et al., 1997b; Gebel et al., 1997; Grisolia 2002). Cytotoxic and genotoxic effects were observed with Roundup and other formulations of glyphosate, but not with glyphosate AI alone in comparative studies involving different experimental systems (Peluso et al., 1998; Richard et al., 2005; Dimitrov et al., 2006). The observed differences were attributed to some ingredients of Roundup, mainly surfactants, and/or to a synergic effect of glyphosate and components of the formulation (Sirisattha et al., 2004; Peixoto 2005).

Epidemiological studies generally showed no consistent or strong relationships between human exposure to glyphosate or glyphosate-containing products and health outcomes in human populations. No statistically significant association in humans was found with spontaneous abortion, fetal deaths, preterm birth, neural tube defects (Rull et al., 2006), and cancer incidence overall, although a suggested association between cumulative exposure to glyphosate and the risk of multiple myeloma was reported (De Roos et al., 2005).The epidemiologic evidence is insufficient to verify a cause–effect relationship for childhood cancer (Wigle et al., 2008). Four case-control studies suggested an association between reported glyphosate use and the risk of non-Hodgkin's lymphoma (NHL) in age groups from 20 to 70 yr (Hardell & Eriksson, 1999; McDuffie et al., 2001; Hardell et al., 2002; De Roos et al., 2003; Eriksson et al., 2008).

Glyphosate AI and Roundup were extensively tested for genotoxicity in a wide range of in vitro and in vivo systems evaluating different genetic endpoints (gene mutation, chromosome mutation, DNA damage and repair) using bacteria and mammalian somatic cells (Williams et al., 2000). The active ingredient did not induce any relevant genotoxic effects such as gene mutations in a variety of in vitro bacterial assays including the *Salmonella typhimurium* reversion assay, with and without metabolic activation (Wildeman & Nazar 1982; Moriya et al., 1983; Li & Long, 1988) and *Escherichia coli* WP-2 (Moriya et al., 1983; Li & Long, 1988). The active ingredient was also negative in the Chinese hamster ovary cell HGPRT gene mutation assay and in primary hepatocyte DNA repair assay (Li & Long, 1988). The genotoxic potential of the formulation Roundup was investigated in a number of studies evaluating various genetic endpoints in different biological systems and was (1) negative in the *S. typhimurium* reversion assay (Kier et al., 1997), (2) negative in the sex-linked recessive lethal assay with *Drosophila melanogaster* (Gopalan & Njagi, 1981), and (3) negative for in vivo micronucleus (MN) induction in mouse bone marrow (Rank et al., 1993; Kier et al., 1997; Dimitrov et al., 2006). The Roundup formulation was reported in a number of studies to exert weak genotoxic effects in short-term assays.

Differences in the response of test organisms to the active ingredient glyphosate and the commercial formulation Roundup might be due to the toxicity of different co-formulants and surfactants contained in commercial products. Several studies with parallel testing of glyphosate and Roundup showed that only the commercial formulation was genotoxic (Rank et al., 1993; Bolognesi et al., 1997b; Gebel et al., 1997; Grisolia 2002). A recent study on the genotoxic potential of glyphosate formulations found that in some cases the genotoxic effects were obtained under exposure conditions that are not relevant for humans (Heydens et al., 2008).

An in vitro study described a concentration-dependent increase of DNA single-strand breaks (SSB), evaluated by comet assay, in two different human cell lines treated with glyphosate at sublethal concentrations (Monroy et al., 2005). Roundup formulations were shown to affect the cell cycle by inhibiting the G2/M transition and DNA synthesis leading to a genomic instability (Marc et al., 2004a, 2004b). Evidence of DNA damage in peripheral lymphocytes from a small group of subjects potentially exposed to glyphosate was reported in a recent paper (Paz-y-Miño et al., 2007). The number of subjects (21 control and 24 exposed) was small and there were 23 females and only 1 male in the exposed group, making interpretation of the results difficult.

Frequency of MN in human lymphocytes has been widely used for biomonitoring exposure to pesticides (Bolognesi, 2003; Costa et al., 2006; Montero et al., 2006). The MN test, an index of chromosomal damage, is one of the most appropriate biomarkers for monitoring a cumulative exposure to genotoxic agents. Chromosomal damage, as a result of inefficient or incorrect DNA repair, is expressed during the cell division and represents an index of accumulated genotoxic effects. The cytokinesis-block micronucleus (CBMN) methodology (Fenech & Morley, 1985) allows a distinction to be made between a mononucleated cell that did not divide and a binucleated cell that has divided once, expressing any genomic damage associated to recent exposure. The test in its comprehensive application, as was proposed by Fenech (2007) including a set of markers of gene amplification, cellular necrosis, and apoptosis, allows evaluation of genotoxic and cytotoxic effects induced by exposure to a genotoxic agent.

Colombia's anti-drugs strategy includes a number of measures ranging from aerial spraying of a mixture of a commercial formulation of glyphosate (Glyphos) and an adjuvant, Cosmo-Flux (Solomon et al., 2007b), to manual eradication, including alternative development and crop substitution programs (UNODC, 2007). In order to assess the potential genotoxic risk associated with the aerial spraying program with the glyphosate mixture, a cytogenetic biomonitoring study was carried out in subjects from five Colombian regions, characterized by different exposure to glyphosate formulations and other pesticides.

MATERIALS AND METHODS

The study was carried out in five regions of Colombia, with different potential exposure to glyphosate as reported by Sanin et al. (2009). Briefly, the characteristics of the study areas are described here:

Sierra Nevada de Santa Marta—where organic coffee is grown without use of pesticides.

Boyacá—an area of illicit crops, where manual eradication is performed and the use of pesticides and other chemical agents is common.

Putumayo and Nariño—where aerial spraying of glyphosate is performed for coca and poppy eradication. The aerial application rate for eradication of coca is 3.69 kg glyphosate a.e. (acid equivalents)/ha (Solomon et al., 2007b). In order to maximize penetration and effectiveness of the spray formulation, Glyphos is tank-mixed with an adjuvant (Cosmo-Flux® 411F; Cosmoagro, Bogotá).

Valle del Cauca—where glyphosate is applied through aerial spraying for sugar cane maturation. Roundup 747 is the most commonly used product and is applied at a rate of 1 kg a.e./ha, and has no additional adjuvant (personal communication, ASOCAÑA, the Colombian Association for Sugar Growers, December 2008).

Study Population

Two hundred and seventy-four individuals were included in the study. The objective was to sample 30 couples of reproductive age in each area and, where possible, the same couples in the study conducted by Sanin et al. (2009) were sampled. In Putumayo, Nariño, and Valle del Cauca, the population was selected based on the scheduled aerial spraying of glyphosate. This schedule was confidential and provided exclusively for the purpose of the study by the Antinarcotics Police (Putumayo and Nariño) or ASOCAÑA (Valle del Cauca). In Valle del Cauca, a sample size of 30 couples could not be achieved because spraying was not carried out in populated areas of the study region. Most spraying during the study period was carried out on sugar cane crops where no inhabitants were found. All reported areas to be sprayed in Valle del Cauca were visited to search for couples; however, only 14 could be included.

In Sierra Nevada de Santa Marta and Boyacá, the same areas investigated in a previous study (Sanin et al., 2009) were identified, although, due to the instability of the population and high migration, most couples from the previous study were not located. In all regions, the same strategy as described before (Sanin et al., 2009) was followed, visiting household by household until completing 30 couples who fulfilled the inclusion criteria, women of reproductive age (15–49 yr of age) and their spouses, who voluntarily accepted to participate in the study.

Field Data Collection

Field data collection was carried out between October 2006 and December 2007. Epidemiologists and interviewers in the five regions who participated in the Sanin et al. (2009) study were informed about the objectives of the study and trained for data collection. The Ethical Committee of Fundacion Santa Fe de Bogotá approved the study protocol and the informed consent forms used for the study. All the subjects were informed about the aims of the study. All of them gave their informed consent and volunteered to donate blood for sampling. They did not self-report illness at the time of blood sampling and interviews. Every volunteer was interviewed with a standardized questionnaire, designed to obtain relevant details about the current health status, history, and lifestyle. This included information about possible confounding factors for chromosomal damage: smoking, use of medicinal products, severe infections or viral diseases during the last 6 mo, recent vaccinations, presence of known indoor/outdoor pollutants, exposure to diagnostic x-rays, and previous radio- or chemotherapy. A simplified food frequency questionnaire that had already been used in other regions of Colombia was also applied, in order to evaluate dietary folic acid intake. Folic acid intake was characterized because of the role of folic acid deficiency in baseline genetic damage in human lymphocytes (Fenech & Rinaldi, 1994). Specific information about exposure at the time of aerial spraying in Putumayo, Nariño, and Valle del Cauca was addressed in the questionnaire.

Blood Sampling and Cell Culture

Blood samples were collected twice in Boyacá, at the beginning of the study and 1 mo after the first survey, and at 3 different times in Nariño, Putumayo, and Valle del Cauca: immediately before spraying, within 5 d after spraying, and 4 mo later. A sample of 10 ml whole blood was collected from each subject, by venipuncture, using heparinized Vacutainer tubes kept at room temperature and sent within 24 h for the establishment of the lymphocyte cultures. The samples were coded before culturing. The modified cytokinesis-blocked method of Fenech and Morley (1985) was used to determine frequency of MN in lymphocytes. Whole blood cultures were set up for cytogenetic analysis in Bogotá (Colombia) by personnel specifically trained by cytogeneticists from Environmental Carcinogenesis Unit of the National Cancer Research Institute (Genoa, Italy).

Three sterile cultures of lymphocytes were prepared. A 0.4-ml aliquot of whole blood was incubated at 37ºC in duplicate in 4.6 ml RPMI 1640 (Life Technologies, Milano, Italy) supplemented with 10% fetal bovine serum (Gibco BRL, Life Technologies SrL, Milano, Italy), 1.5% phytohemoagglutinin (Murex Biotech, Dartford, UK), 100 units/ml penicillin, and 100 μg/ml streptomycin. After 44 h, cytochalasin B (Sigma, Milano, Italy) was added at a concentration of 6 μg/ml. At the end of incubation at 37°C for 72 h, cells were centrifuged (800 × g, 10 min), then treated with 5 ml of 0.075 m*M* KCl for 3 min at room temperature to lyse erythrocytes. The samples were then treated with pre-fixative (methanol:acetic acid 3:1) and centrifuged . The cellular pellets were resuspended in 1 ml methanol. At this step the samples were sent to the Environmental Carcinogenesis Unit (National Cancer Research Institute, Genoa, Italy). All the samples were centrifuged in methanol. Treatment with fixative (methanol:acetic acid, 5:1) followed by centrifugation was repeated twice for 20 min. Lymphocytes in fresh fixative were dropped onto clean iced slides, air-dried, and stained in 2% Giemsa (Sigma, Milano, Italy). MN analysis was performed blind only on lymphocytes with preserved cytoplasm. On average, 2000 cells were analyzed for each subject. Cells were scored cytologically using the cytome approach to evaluate viability status (necrosis, apoptosis), mitotic status (mononucleated, binucleated, multinucleated) and chromosomal damage or instability status (presence of micronuclei, nucleoplasmic bridges, nucleoplasmic buds) (Fenech 2007). The proliferation index (PI) was calculated as follows:

$$\begin{aligned}\text{PI} = (&\text{number of mononucleated cells} + 2\\ &\times \text{number of binucleated cells} + 3\\ &\times \text{number of polynucleated cells})/\text{ total number of cells.}\end{aligned}$$

Statistical Analysis

Continuous variables were characterized using mean and standard deviation, while categorical variables were expressed as proportions. Dependent variables, micronuclei per binucleated cell (BNMN), and differences in MN between sampling were square-root transformed where required to comply with the required assumptions of normal distribution and equal variances. Comparison of MN between areas was made by one-way analysis of variance (ANOVA). A significance level at 5% was used to assess differences among areas. For multiple comparisons, the Bonferroni test was applied ($\alpha = .05$). Significance of differences in frequency of BNMN between first and second, and second and third sampling were tested by the unpaired *t*-test with equal variances. Difference and 95% confidence interval were used to compare between samplings.

Bivariate analysis between dependent variables and putative risk factors was performed by one-way ANOVA, comparing exposed and nonexposed subjects. In cases where risk factor was continuous, such as age, folic acid intake, alcohol consumption, and coffee consumption, the correlation coefficient was used.

A multiple linear regression was conducted to assess association with BNMN at the first sampling with different variables: region, age (as continuous variable as well as categorical age), ethnicity as a dichotomous variable, exposure to genotoxic products as defined earlier, gender (female vs. male), and intake of folic acid (categorized in quartiles). Regression analysis was conducted with transformed variables, with square root transformation of BNMN and natural logarithm of age, to obtain a normal distribution.

RESULTS

Demographic characteristics and habits of the study groups are described in Table 1. The study population comprised 274 subjects (137 female and 137 male; average age 30.4 ± 7.8 yr). The mean age of the subjects was similar in the different regions. A large part of the studied population was mestizo, with the exception of the Nariño area consisting of individuals of African origin. In the total population, 38% of interviewees had not completed primary education. Putumayo had the largest proportion with education and Valle del Cauca the lowest as shown in Table 1. Only 10% of all subjects were smokers, (20% in Putumayo); a large majority of subjects were drinkers of beer or liquor with a consistent consumption of guarapo (traditional alcoholic beverage prepared by fermentation of maize) in Santa Marta and Boyacá. No statistically significant differences of folic acid intake were observed between different regions (the mean values ranged from 750 and 1189 μg/wk).

One hundred and nine (39.8%) of 274 participants reported current use of pesticides in their occupation or other activities. Nariño (76.6%) and Putumayo (61.7%) were the two regions where prevalence of use of genotoxic pesticides was higher; Boyacá (24.2%) and Valle del Cauca (28.6%) reported lower use. None of the study subjects in Santa Marta reported use of pesticides. No data regarding quantity of pesticide used were available. Fifty (18.3%) out of 273 who gave information

TABLE 1
Demographic Characteristics and Possible Confounding Exposures in the Study Populations

Area	Santa Marta	Boyacá	Putumayo	Nariño	Valle del Cauca
Number of subjects	60	62	60	64	28
Age (mean (SD))	27.0 (5.6)	29.1 (8.8)	31.4 (7.2)	32.5 (7.4)	33.4 (8.7)
Ethnicity (%)					
Mestizo	100	100	88.3	3.1	60.7
African			6.7	96.9	39.3
Indian			5.0		
Education (%)					
None		4.8	1.7		
Primary incomplete	26.7	38.7	53.3	42.2	21.4
Primary complete	21.7	29.0	20.0	23.4	32.1
High school incomplete	25.0	8.1	20.0	25.0	28.6
High school complete	26.7	19.4	3.3	9.4	17.9
Technical			1.7		
Occupation (%)					
Agriculture	10.0	41.9	60.0	62.5	7.1
Housewife	40.0	50.0	38.3	34.4	50.0
Other	50.0	8.1	1.7	3.1	42.9
Health insurance (%)					
Uninsured	50.0	9.7	36.7	71.9	7.1
Subsidized	38.3	83.9	60.0	18.7	50.0
Insured	11.7	6.4	3.3	9.4	42.9
Coffee consumption (cups/day)					
Mean (SD)	1.8 (2.3)	1.7 (0.8)	2.3 (4.1)	1.3 (0.4)	1.7 (1.2)
Percent of population	80.0	67.7	88.3	76.6	82.1
Smoking (%)					
Nonsmokers	91.7	95.2	80.0	87.5	92.9
Alcohol (%)					
Liquor	28.3	25.8	53.3	78.1	78.6
Beer	51.6	67.7	63.1	82.8	64.3
Guarapo	6.7	59.7	1.7	3.2	10.7
Users of illicit drugs (%)	6.7	0	5.0	7.8	0
Diet					
Folic acid intake (μg/wk)	1189	873	750	1160	812

about x-ray examination reported to having been exposed at some time; however, only 21 out of 46 who gave information on dates of x-ray reported exposure in the last 6 mo before the interview and first blood sample. Sixty-one percent of population reported viral infections, the highest prevalence in Nariño (89.5%) and the lowest in Putumayo (49.2%). However, 89.3% of viral infections were the common cold and 6.1% dengue fever. Hepatitis was reported by six interviewees without any specification of the type of the infection.

The means and standard deviations of frequency of MN and related parameters according to regions are shown in Table 2 and presented graphically in Figure 1. Compared with Santa Marta, where people grow organic coffee without the use of pesticides and which is considered as a reference area, the baseline frequency of BNMN was significantly greater in subjects from the other four regions. The highest frequency of BNMN was in Boyacá, where no aerial eradication spraying of glyphosate was carried out, and Valle del Cauca, where aerial spraying was for maturation of sugar cane. There was no significant difference between mean frequency of BNMN in Boyacá and Valle del Cauca. There was no significant difference in frequency of BNMN between Putumayo and Nariño,

TABLE 2

Mean (SD) Frequency of Binucleated Cells with Micronuclei (BNMN), Total Micronuclei (MNL) per 1000 Binucleated Peripheral Lymphocytes, Frequency of Mononucleated Cells per 1000 Lymphocytes (MNMO), and Proliferation Index (PI) by Region before the Exposure (Phase 1), 5 d after Spraying (Phase 2) and 4 mo Later (Phase 3)

Region	Santa Marta	Boyacá	Putumayo	Nariño	Valle del Cauca
Phase 1					
Number of subjects	60	62	58	63	28
BNMN	1.83 (0.97)	5.64 (1.72)	3.61 (1.51)	4.12 (1.65)	5.75 (2.48)
MNL	1.97 (1.05)	6.16 (1.91)	3.90 (1.66)	4.36 (1.85)	6.02 (2.50)
MNMO	0.41 (0.44)	0.99 (0.64)	0.47 (0.51)	0.51 (0.39)	1.12 (0.88)
PI	1.54 (0.14)	1.45 (0.14)	1.68 (0.15)	1.47 (0.12)	1.51 (0.15)
Phase 2					
Number of subjects	ND	55	53	55	27
BNMN		4.96 (2.00)	4.64 (2.45)	5.98 (2.03)	8.64 (2.81)
MNL		5.41 (2.25)	5.02 (2.95)	6.35 (2.18)	8.98 (2.93)
MNMO		0.87 (0.65)	0.44 (0.46)	0.70 (0.45)	1.65 (0.62)
PI		1.72 (0.14)	1.66 (0.20)	1.40 (0.18)	1.51 (0.14)
Phase 3					
Number of subjects	ND	ND	50	56	26
BNMN			5.61(3.08)	3.91 (1.99)	7.38 (2.41)
MNL			5.96 (3.23)	4.13 (2.20)	8.17 (2.72)
MNMO			0.82 (0.54)	0.55 (0.42)	0.98 (0.60)
PI			1.43 (0.17)	1.41 (0.14)	1.45 (0.20)

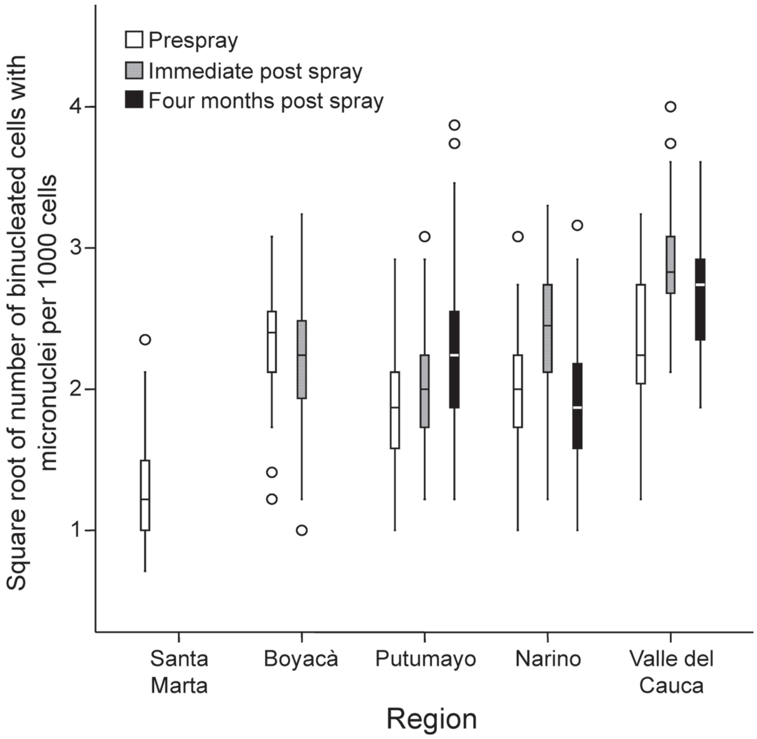

FIG. 1. Box plot of frequency of BNMN in the five study regions with samples taken prespray, 4–5 d post-spray, and 4 mo post-spray. Box plots: The center horizontal line marks the median of the sample. The length of each box shows the range within which the central 50% of the values fall, with the top and bottom of the box at the first and third quartiles. The vertical T-lines represent intervals in which 90% of the values fall. The ❍ symbols show outliers. See text for description of statistically significant differences.

although Boyacá and Valle del Cauca showed a significantly higher frequency than Nariño and Putumayo. A higher frequency of BNMN in Boyacá was also observed in a second sampling 1 mo later.

There were differences in frequency of BNMN between sampling periods. A statistically significant difference in frequency of BNMN between first and second sampling was observed in Valle, Putumayo, and Nariño immediately (<5 d) after spraying. Four months after spraying in Nariño, there was a statistically significant decrease in the mean frequency of BNMN compared with the second sampling, but in Valle del Cauca the decrease was not significant nor was the increase observed in Putumayo significant (Figure 1 and Table 2).

The frequency of mononucleated cells with micronuclei (MOMN) was used as an index of background level of chromosomal damage accumulated in vivo (Table 2). The lowest frequency of MOMN for the first sampling was observed in Santa Marta; however, there was no marked difference in frequency of MOMN in Santa Marta, Putumayo, and Nariño and no statistically significant difference between Valle and Boyacá. However, Valle and Boyacá had a significantly higher frequency of MOMN than Putumayo, Nariño, and Santa Marta at first sampling. Immediately after spraying, Valle showed a significantly higher frequency of MOMN compared to Putumayo and Nariño, and Nariño was also higher than Putumayo. Between first and second sampling, the increase in frequency of MOMN in Nariño and Valle was statistically significant, but there was no difference in Putumayo nor in Boyacá 4 mo after the first sampling. Data suggest greater exposure to genotoxic agents in these populations is independent of the exposure to glyphosate products.

The proliferation index (PI) in all the studied groups was in the range of normal values described in the literature. No significant reduction of PI was observed in association with environmental exposures in groups of subjects from the different regions. A statistically significant correlation coefficient (0.288) between PI values from the first and the second samplings was observed, confirming the association with individual characteristics and not with any toxicity related to the exposure or to the culture techniques. Due to the low frequency observed, data with respect to other nuclear alterations, including in cytome analysis (Fenech, 2007), are not described in Table 2: the mean frequency of nucleoplasmic bridges (NPB) for all subjects was 0.010 per 1000 cells, that of nuclear buds was 0.022 per 1000 cells, and only rare necrotic and apoptotic cells were found in some samples.

Gender was the most important demographic variable affecting the BNMN index. Frequencies of BNMN in females were greater than those in males (mean 4.43 ± 2.36 vs. 3.61 ± 1.82, respectively, in total population) (Table 3). The groups of subjects were evenly matched for gender by including only couples in the study. No association was found between frequency of MN and age as a categorical variable, nor was there an association with smoking, but prevalence of smoking was low (~10% in the total population). A higher baseline frequency of MN was observed in subjects of African origin, suggesting greater susceptibility. Other lifestyle factors such as alcohol, coffee consumption, or illicit drug intake were not associated with initial measures of BNMN and MOMN.

One hundred and thirty-four of the 152 subjects in Nariño, Putumayo, and Valle reported information on contact with Glyphos and Cosmo-Flux after eradication spraying. The other 18 did not provide information in the second survey or blood samples were inadequate for testing micronuclei. Sixty-six (49.2.0%) reported no contact with the spray and 68 (50.8%) reported coming into contact with the spray because they entered sprayed fields or reported contact with the spray droplets. The mean BNMN in Nariño and Putumayo was greater in respondents who self-reported exposure, but differences were not statistically significant (Table 4). In Valle, only one respondent reported contact with glyphosate.

Region, gender, and older age (≥35 yr) were the only variables associated with the frequency of BNMN before spraying (Table 5). In fact, using Santa Martha, where no use of pesticides was reported, as reference, Boyacá, Valle del Cauca, Putumayo, and Nariño showed a statistically significant higher mean frequency of BNMN. There were also significant differences between Boyacá and Valle and Putumayo and Nariño. Females had a statistically higher mean frequency of BNMN than males after adjusting for all other variables. Greater age was also associated with greater frequency of BNMN. Neither exposure to genotoxic products, nor ethnicity, nor intake of folic acid was associated with frequency of BMMN at the first sampling. The multiple linear regression analysis of difference between second and first sampling only demonstrated statistically significant association with region after adjusting for all other variables, indicating that Putumayo, Nariño, and Valle had significantly greater differences between second and first sampling than Boyacá.

DISCUSSION

The main objective of this study was to test whether there was an association between aerial spraying of glyphosate and cytogenetic alterations, evaluated as frequency of MN in peripheral leukocytes. Biomonitoring was carried out in three regions of Colombia in populations exposed to aerial spraying of glyphosate: Putumayo and Nariño, where the application was performed for eradication of coca and poppy, and Valle del Cauca where the herbicide was used for maturation of sugar cane. Two control populations not exposed to aerial spraying of glyphosate were also selected: the first one from Sierra Nevada de Santa Marta, where organic coffee is grown without the use of any pesticides, and the other from Boyacá, with a region of illicit crops, where manual eradication is performed and subjects were potentially exposed to several pesticides but not glyphosate for aerial eradication. The ex vivo analysis of leukocytes in the presence of cytochalasin B, added 44 h after the

TABLE 3
Association of Mean (SD) Frequency of Binucleated Cells (First Sampling) with Micronuclei (BNMN/1000 Binucleated Lymphocytes) and Demographic Variables

Variable	Santa Marta	Boyacá	Putumayo	Nariño	Valle del Cauca	Total
Sex						
Females	1.98 (1.03)	6.22 (1.79)	3.91 (1.71)	4.57(1.77)	6.45 (2.82)	4.43 (2.36)
Males	1.68 (0.90)	5.06 (1.46)	3.31 (1.25)	3.66 (1.39)	5.05 (1.94)	3.61 (1.82)
p	.236	.007	.131	.028	.138	.002
Age						
18–24 yr	2.00 (1.14)	5.50 (1.96)	3.32 (1.25)	3.64 (1.72)	6.19 (2.15)	3.67 (2.16)
25–34 yr	1.66 (0.87)	5.70 (1.66)	3.53 (1.17)	4.20 (1.77)	4.20 (0.76)	3.97 (2.08)
35 yr and older	1.93 (0.67)	5.62 (1.73)	3.84 (1.86)	4.25 (1.52)	6.04 (2.84)	4.41 (2.19)
p	.438	.929	.574	.564	.313	.093
Ethnicity						
Mestizo	1.83 (0.97)	5.64 (1.72)	3.72 (1.52)	4.75 (1.06)	5.82 (2.44)	3.94(2.24)
Africa and Indian	0	0	2.86 (1.31)	4.10 (1.66)	5.64 (2.65)	4.20(1.90)
p			.162	.588	.850	.368
Smoking						
Yes	2.00 (1.06)	5.33 (0.76)	3.31 (1.00)	4.77 (1.51)	4.50 (1.41)	3.83 (1.60)
No	1.82 (0.97)	5.65 (1.76)	3.80 (1.56)	4.03 (1.66)	5.90 (2.57)	4.07 (2.20)
p	.693	.756	.395	.233	.459	.592
Folic acid intake (quartiles)						
1	1.92 (0.99)	6.11 (1.95)	3.23 (1.12)	4.50 (1.75)	5.86 (2.34)	3.89 (2.23)
2	1.64 (0.66)	5.70 (1.75)	3.47 (1.49)	3.80 (1.47)	5.86 (2.74)	3.97 (2.21)
3	1.69 (0.92)	5.69 (1.82)	4.00 (1.37)	3.85 (2.04)	6.58 (2.84)	4.47 (2.22)
4	1.94 (1.20)	4.94 (1.13)	3.69 (2.429)	4.28 (1.51)	4.63 (2.05)	3.75 (1.89)
p	.779	.399	.515	.645	.612	.220

TABLE 4
Mean Frequency of Binucleated Cells with Micronuclei (BNMN) at the Second Sampling per 1000 Binucleated Lymphocytes and Self-Reported Exposures to the Glyphosate Spray in Three Areas Where Aerial Application Had Occurred

	Nariño (*n* = 55)		Putumayo (*n* = 53)		Valle del Cauca (*n* = 26)	
Route of exposure	*n*	Mean BNMN (SD)	*n*	Mean BNMN (SD)	*n*	Mean BNMN (SD)
No exposure	28	5.81 (1.85)	13	3.84 (1.30)	25	8.56 (2.90)
Spray in air	5	7.30 (0.57)	1	5.50 (0)		
Spray on skin	8	5.62 (1.60)	15	4.90 (1.87)	1	9.50 (0)
Entered sprayed field	14	6.06 (2.77)	24	4.87 (3.18)		
p Value (ANOVA)		0.472		0.612		0.760
Any exposure	27	6.16 (2.22)	40	4.90 (2.69)	1	9.50 (0)
p Value (no exposure vs. any exposure)		0.525		0.181		0.760

Note. The data comprise respondents in the second survey from which blood samples were obtained.

TABLE 5
Multiple Linear Regression Analysis Adjusted for Region, Age, Gender, Ethnicity, and Folic Acid Intake

Variable	Coefficient	*p*	95% CI
Region			
Boyacá	3.75	≤.0001	3.19, 4.31
Putumayo	1.58	≤.0001	1.00, 2.16
Nariño	2.06	≤.0001	1.49, 2.64
Valle del Cauca	3.65	≤.0001	2.92, 4.39
Age (yr)			
25–34	0.28	.250	–0.20, 0.76
35 and older	0.75	.008	0.20, 1.31
Gender			
Females	1.00	≤.0001	0.60, 1.40

start of cultivation, made it possible to distinguish between nondividing mononucleated cells—as an index of accumulated chromosomal damage—and binucleated cells, which had completed one nuclear division during in vitro culture and expressed MN associated with recent exposure to genotoxic agents.

The baseline level of chromosomal damage, evaluated as frequency of BNMN, was associated with the different regions considered in our study. The frequency of BNMN before spraying was also associated with region, gender, and age. Gender difference in the background incidence of MN in peripheral leukocytes, with the frequency being consistently higher in females, and a strong correlation between MN frequency and increasing age are well documented (Bonassi et al., 1995, 2001; Bolognesi et al., 1997a).

Data demonstrated no significant effect of smoking, confirming findings from the literature (Bonassi et al., 2003) although prevalence of smoking in our study population was small (7–20%, Table 1). No association with alcohol consumption was observed. A higher susceptibility of people of African origin compared to the mestizo group was suggested by a greater baseline frequency of BNMN and increased frequency at the second sampling period.

There was some indication of an association between BNMN and exposure to pesticides in general. The lowest frequency of BNMN was observed in Sierra Nevada de Santa Marta, where people self-reported that they did not use pesticides. The mean frequency of BNMN in this group of subjects (1.83 ± 0.97) was similar to that observed in healthy unexposed subjects for the same range of age (Bolognesi et al., personal communication). The higher mean frequency of BNMN observed in Boyacá and Valle del Cauca (5.64 ± 1.72 and 5.75 ± 2.48, respectively) and that in Nariño and Putumayo (4.12 ± 1.65 and 3.65 ± 1.51, respectively), compared to Santa Marta, are in agreement with similar biomonitoring studies carried out in subjects exposed to pesticides using the MN test or other genetic endpoints (Bolognesi, 2003; Bull et al., 2006).

There was no clear relationship between BNMN and the reported use of pesticides classified as genotoxic. Participants in Boyacá and Valle del Cauca showed higher frequency of BNMN than those in Putumayo and Nariño. However, a greater proportion of participants in the latter regions self-reported the use genotoxic pesticides (76.6% in Nariño and 61.7% in Putumayo). There is no information available on other relevant factors such as frequency of use, rate applied, time of exposure, and protective measures used, and we could therefore not characterize exposures to explain the differences. There were further inconsistencies; for example, in Boyacá, where more frequent use of pesticides was expected, only 24.2% of participants self-reported use, compared with the greater values in Nariño and Putumayo. However, it is possible that in areas such as Boyacá, individuals might be potentially exposed to persistent pesticides applied in the past and still present in the environment.

There was no evidence of an association between BNMN and folic acid deficiency. An assessment of folic acid intake from the semiquantitative food frequency questionnaire showed that, according to accepted recommendations (Herbert, 1987), the diet of the study populations was not deficient in folic acid and there were only small differences between regions. Consistent with these data, no association was found between MN and folic acid intake, either as a continuous variable or by quartiles.

The frequency of BNMN increased after spraying with glyphosate but not consistently. The results obtained with a second sampling, carried out immediately after the glyphosate spraying, showed a statistically significant increase in frequency of BNMN in the three regions where glyphosate was sprayed. However, this was not consistent with the rates of application use in the regions. The increase in frequency of BNMN in Valle (application rate = 1 kg a.e. glyphosate/ha) was greater than that in Nariño and Putumayo (3.69 kg a.e. glyphosate/ha).

There was no significant association between self-reported direct contact with eradication sprays and frequency of BNMN. The frequency of BNMN in participants who self-reported that they were exposed to glyphosate because they entered the field immediately after spraying (to pick the coca leaves), felt spray drops in their skin, or they thought they were exposed because they had contact with the chemical in the air, was not significantly greater than in subjects living in the same areas but who were not present during spraying. Decreases in frequency of BNMN in the recovery period after glyphosate spraying were not consistent. The third sampling, 4 mo after spraying, demonstrated a statistically significant decrease in frequency of BNMN only in Nariño.

Overall, these results suggest that genotoxic damage associated with glyphosate spraying, as evidenced by the MN test, is small and appears to be transient. The frequencies of BNMN in Nariño and Putumayo during the second and the third sampling fell within the range of values observed in Boyacá, an area where people were exposed to a complex mixture of different pesticides (including glyphosate). A greater increase in frequency of BNMN was observed in Valle del Cauca, but it cannot be attributed only to the glyphosate exposure, because the application rate of the herbicide in this area was one-third compared with that in Nariño and Putumayo. This conclusion is further supported by the frequency of MN in mononucleated cells (MOMN), which provides an indication of the background level of chromosome/genome mutations accumulated in vivo (Manteuca et al., 2006). A statistically significant increase of MOMN was observed in Boyacá and Valle del Cauca before and after the aerial spraying, suggesting exposure to other genotoxic compounds in these populations was independent of the exposure to glyphosate. Evidence indicates that the genotoxic risk potentially associated with exposure to glyphosate in the areas where the herbicide is applied for eradication of coca and poppy is of low biological relevance. One of the strengths of our study was the detection of a transient chromosomal damage, evaluated as MN frequency in peripheral blood of the exposed subjects, since it was possible to compare the baseline before spraying with the effects detected immediately after spraying. Glyphosate persists in the environment for only a short time (half-life for biological availability in soil and sediments is hours, and 1-3 d in water; Giesy et al., 2000), is rapidly excreted by mammals and other vertebrates (Williams et al., 2000; Acquavella et al., 2004) and chronic effects, if any, would not be expected.

One of the major drawbacks of environmental epidemiology studies is the characterization of exposures to the agents being investigated. In this study two approaches were used to characterize exposures to glyphosate: ecological and self-reported. In the ecological study design, frequency of BNMN in participants was compared from regions with different patterns of pesticide use. As previously discussed (Sanin et al., 2009), this ecological design may result in misclassification of exposures (Arbuckle et al., 2004), but as an exploratory assessment of exposure it is useful (Ritter et al., 2006).

Others have attempted to improve assessment of exposure to pesticides in epidemiological studies. One study used a self-administered questionnaire for the assessment of exposure to glyphosate, which was defined as (a) ever personally mixed or applied products containing glyphosate; (b) cumulative lifetime days of use, or "cumulative exposure days" (years of use times days/year); and (c) intensity-weighted cumulative exposure days (years of use times days/year times estimated intensity level) (De Roos et al., 2005). A pesticide exposure score based on self-reported work practices was recently developed to estimate annual exposure level (Firth et al., 2007). Based on an algorithm to estimate lifetime exposure to glyphosate from questionnaire information, a moderate correlation was found with concentrations of glyphosate in urine and no significant correlation with self-reported exposure (Acquavella et al., 2004).

In our study, questions related to whether there was direct contact with the spray were used but this did not consider area of skin exposed, region of skin exposed, differences in rates of penetration, or personal hygiene.

Given the situation, the best approach possible, a prospective cohort, was used but the need to use better procedures to estimate the exposure is acknowledged. Based on the applicable Bradford–Hill guidelines (Hill, 1965), it is not possible to assign causality to the increases in frequency of BNMN observed in our study. There was a smaller frequency of BNMN and MOMN in the region of no pesticide use compared with the regions where pesticides (including glyphosate) were used, which is consistent with other reports in the literature. Although temporality was satisfied in the increase in frequency of BNMN after spraying, this response did not show strength as it was not consistently correlated with the rate of application. Recovery was also inconsistent with decreases in frequency of BNMN in the areas of eradication spraying but not in the area where lower rates were applied on sugar cane.

Further studies are needed to better characterize the potential genotoxic risk associated with the application of glyphosate for sugar cane maturation. The smaller number of subjects recruited in this study and small amount of information about the exposure precluded any conclusions. Many pesticides are used in conventional agriculture in Colombia and many pesticides are used in the production of coca (Solomon et al., 2007a, 2007b); however, there is not sufficient information to correlate the frequency of MN to the pesticide exposure.

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