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Genotoxicity in workers exposed to methyl bromide

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Abstract

To address the genotoxicity of in vivo methyl bromide (CAS 74-83-9) exposure in humans, we collected blood and oropharyngeal cells as part of a cross-sectional morbidity study of methyl bromide-exposed fumigation workers and their referents. Micronuclei were measured in lymphocytes and oropharyngeal cells, and hypoxanthine–guanine phosphoribosyl transferase gene (*hprt*) mutations were measured in lymphocytes. A total of 32 workers and 28 referents provided specimens. Among current non-smokers, mean *hprt* variant frequencies (Vfs) were found to be elevated among workers compared to referents (geometric mean: workers = 4.49×10^{-6}, referents = 2.96×10^{-6}; two-sided $p = 0.22$); this difference was more pronounced among workers with 4 h or more of recent methyl bromide exposure compared to referents (geometric mean: workers = 6.56×10^{-6}, referents = 2.96×10^{-6}; two-sided $p = 0.06$). Mean oropharyngeal cell micronuclei were higher among workers compared to referents (mean: workers = 2.00, referents = 1.31; two-sided $p = 0.08$); the results were similar when workers with 4 h or more of recent methyl bromide exposure were compared to referents (mean: workers = 2.07, referents = 1.31; two-sided $p = 0.13$). No consistent differences between workers and referents were observed for frequencies of kinetochore-negative lymphocyte micronuclei, or kinetochore-positive lymphocyte micronuclei. The study was limited by a sample size sufficient only for detecting relatively large differences, absence of a reliable method to measure the intensity of workplace methyl bromide exposures, and relatively infrequent methyl bromide exposure (e.g., the median length of exposure to methyl bromide during the 2 weeks preceding the survey was 4 h). In conclusion, our findings provide some evidence that methyl bromide exposure may be associated with genotoxic effects in lymphocytes and oropharyngeal cells. Further study on the genotoxicity of methyl bromide exposure in humans is warranted.

Keywords: Methylbromide; Micronucleus; Biological marker; Fumigation; Smoking; Mutagenicity test; *hprt* mutation

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1. Introduction

Methyl bromide (CAS 74-83-9) is a commonly used fumigant and pesticide. It is used in the fumigation of soils for control of nematodes and to fumigate buildings for termite control. In the US, its heaviest use is in Florida and California [1]. Types of workers exposed to methyl bromide include structural fumigators, pest control applicators and farm workers. Unfortunately, reliable estimates of the number of workers exposed to methyl bromide do not exist (many of these workers are employed in agriculture and by small businesses which are sectors not included in National Institute for Occupational Safety and Health (NIOSH) exposure surveys [2]).

In vivo short term genetic toxicology tests suggest that methyl bromide may be a point mutagen in fruitflies [3], an alkylating agent in mice [4], and a clastogen (inducing micronuclei in bone marrow cells) in rats and mice [5]. Another animal study did not find evidence of genotoxicity from methyl bromide using in vivo test systems [6]. The ability of methyl bromide to alkylate DNA and break chromosomes suggests that it might be carcinogenic, as these are mechanisms of oncogene activation [7,8]. However, the animal carcinogenicity data are ambiguous. Animal studies involving short-term gavage exposure to methyl bromide suggest the potential for forestomach neoplasia [9,10]. In contrast, chronic inhalation studies with methyl bromide have been negative for carcinogenicity [3,11,12]. The toxicity of methyl bromide in inhalation studies may limit extrapolation of these results to humans; chronic exposures approximately one order of magnitude greater than permitted in occupational settings could not be tolerated by the experimental animals. Furthermore, because methyl bromide is highly reactive and lipophilic, the amount of the methyl bromide dose that reaches the nucleus may be too low to produce measurable genotoxicity. While there is conflicting evidence for the carcinogenicity of methyl bromide, there is clear evidence of carcinogenicity for several structural analogues of methyl bromide (e.g., methyl chloride [13], ethyl bromide [14], and ethyl chloride [15]).

Although in vitro exposure of human peripheral lymphocytes to methyl bromide produced sister chromatid exchanges and chromosomal aberrations [16], the in vivo genotoxicity of methyl bromide exposure among humans has not been determined. To address the genotoxicity of in vivo methyl bromide exposure in humans, blood and oropharyngeal cells were collected as part of a cross-sectional morbidity study of fumigation workers exposed to methyl bromide and sulfuryl fluoride. Hypoxanthine–guanine phosphoribosyl transferase gene (*hprt*) mutations and micronuclei were measured in T-lymphocytes obtained from a subset of these workers and an unexposed referent group. In addition, the number of micronuclei present in oropharyngeal cells was measured. These test systems were chosen because they measure the types of genetic damage observed in the previous studies described above.

2. Materials and methods

2.1. Study population

This study, conducted in 1992–1993, compared living individuals (workers) employed as a shooter (the individual who is responsible for introducing the fumigant into the structure) or tent crew worker (the individual responsible for raising and dismantling the tarps used to cover the structure) in the structural fumigation industry, with an unexposed comparison group. The workers were employed at one of 40 fumigation companies located in southern Florida. A short interview was administered to each worker to determine study eligibility. In order to qualify for participation in the study, workers had to report that they were employed in the fumigation industry for at least 6 months, were engaged in non-fumigation pest control activities less than 20% of their worktime, and that at least 50% of the fumigation jobs they ever participated in involved the use of methyl bromide or at least 80% of the fumigation jobs involved the use of sulfuryl fluoride. The referent (comparison) group consisted of neighbors and friends recruited by the exposed workers. All members of the referent group denied ever having employment as a pesticide applicator or employment in the fumigation industry, and denied ever being poisoned by pesticides. A total of 123 workers and 121 referents were examined. A total of 84% (123/147) of eligible

workers participated in the study. The neurologic effects observed in this cohort have been previously reported [17]. The participants on whom biological markers were measured were selected based on the extent of their methyl bromide exposure and on their cigarette smoking status. Workers who reported that a high proportion of their lifetime fumigation jobs (generally 50% or more) involved the use of methyl bromide were preferentially selected. As for referents, we initially chose the referent who was recruited by the specimen-providing worker. When we later determined that the proportion of non-smokers was larger in the exposed group compared to the referent group, we began to preferentially select non-smoking referents. None of the participants refused our invitation to participate in this component of the study. Because participants were not involved in the selection process, self-selection bias was not judged to be present. A total of 31 workers and 27 referents provided biological specimens for marker measurement. The study protocol was approved by the NIOSH Human Subjects Review Board and the University of Miami Institutional Review Board. Informed consent was obtained from all participants.

Information on worker and referent health status was collected through a comprehensive set of standardized interviews and medical examinations. A lifetime medical and occupational history was elicited from each participant using interviewer-administered questionnaires. Duration of each job and duration of occupational exposure to many specific substances were recorded beginning with the participant's 18th birthday. Detailed information on fumigation industry employment was also obtained.

Blood was collected to obtain lymphocytes for the *hprt* mutation assays and the micronucleus assays. In addition, oropharyngeal cells were collected by swabbing the subject's oropharynx with a hydrated wooden spatula. A smear was made by applying the spatula to a precleaned slide. The spatula was then immersed in a vial containing RPMI 1640 media with 25 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco). These vials were then placed on ice and shipped with the prepared slides and blood to the laboratory where the assays were performed (University of Texas Medical Branch, Galveston, TX). The biological specimens arrived at the laboratory within 24 h of collection. Spot urine samples were obtained from all participants and analyzed for total bromide by specific ion chromatography (American Scientific Products, McGaw Park, IL). A University of Miami laboratory in Miami, FL analyzed the urine specimens within 2 weeks of collection and stored them at 4°C until analysis. All laboratory personnel were blind to the exposure status (worker or referent) of the participant.

2.2. Lymphocyte isolation and freezing

Lymphocytes and plasma were isolated from whole blood by density centrifugation on Histopaque (Sigma, St. Louis, MO). The blood plasma was stored at −20°C. The lymphocytes were washed, resuspended in RPMI 1640 (Sigma), counted, and an aliquot of 4×10^6 cells was removed for the lymphocyte micronucleus assay. The remaining cell suspension was centrifuged, resuspended at 1×10^7 cells/ml in RPMI 1640 with 10% dimethyl sulfoxide (Sigma) and 50% heat-inactivated fetal bovine serum (Hyclone), and frozen in 1.2 ml cryotubes.

2.3. Autoradiographic hprt assays

Detailed methods for the autoradiographic *hprt* mutant lymphocyte assay have been described elsewhere [18–20]. In summary, thawed lymphocytes were washed twice and resuspended in growth medium at a cell density of 1.1×10^6 cells/ml. Next, 4.5 ml aliquots were added to each of three to eight 25 cm^2 culture flasks. A labeling index (LI) flask received 0.5 ml of pH adjusted medium, and the other flasks received 0.5 ml of 2×10^{-3} M 6-thioguanine (TG; Sigma). The cultures were incubated 24 h at 37°C. Following this incubation, 25 μCi of tritiated thymidine (ICN, Costa Mesa, CA) was added to each flask and the cells were incubated for an additional 18 h. Cultures were terminated by releasing free nuclei with the addition of chilled 0.1 M citric acid. Nuclei from the TG-treated flasks were combined into one or two 50-ml centrifuge tubes, washed with 20 ml fixative, and resuspended in 0.25 ml of fixative. Later the fixed nuclei were triturated, counted and added to 18 mm × 18 mm coverslips previously attached to microscope slides. An aliquot of about 0.2×10^6 nuclei from the LI culture was

distributed evenly on an 18 mm × 18 mm coverslip on a separate slide.

All slides were stained with aceto-orcein (Gurr) and autoradiographed using NTB-2 emulsion (Kodak). Developed slides were scanned microscopically, and a count was made of all labeled cells on the slides made from the TG-containing cultures. For the LI slide, a random differential count of 3000 cells, both labeled and unlabeled, was made. This LI count provided, for each subject, an estimate of the proportion of normal, non-mutant lymphocytes that were able to grow in culture. The *hprt* variant frequency (Vf) is calculated by dividing the number of labeled (mutant) cells derived from the TG-containing cultures by the total number of cells recovered from the TG cultures multiplied by the LI.

Six participants (four workers, two referents) had sufficiently low total lymphocyte counts and/or low LI (below 2%) to preclude adequate *hprt* Vf determinations. In addition, one participant (a worker who was a tobacco smoker) had an exceptionally high Vf of 135.87×10^{-6} which was considered an outlier value, possibly due to clonal expansion of a single mutant cell [21]. Analytic results were essentially unchanged when this participant with a high Vf was included vs. excluded from the Vf analyses. Therefore, the Vf analyses that we report exclude the participant with an exceptionally high Vf and the six participants with low lymphocyte counts and/or low LI.

2.4. Lymphocyte micronucleus assays

The cytokinesis-block micronucleus assay was performed using methods previously described [22,23], with minor modifications. For each sample, 4×10^6 lymphocytes were used to set up standard cultures with phytohemagglutinin as the mitogen. The cells were incubated in two 15-ml tubes at 37°C for 44 h. Cytochalasin B (Sigma) was added, and the cultures were incubated for an additional 24 h. After harvest, the cells were treated with 0.075 M potassium chloride for 7 min, smeared onto microscopic slides, fixed in chilled 90% methanol for 20 min and stored at −20°C.

Antibody labeling procedures were adapted from methods described by Eastmond and Tucker [24]. The slides were thawed and placed in 0.1% Tween 20 in phosphate buffered saline (PBS) for 5 min. After draining, 50 μl of an anti-kinetochore solution, diluted 1:1 with the 0.1% Tween 20–PBS, was placed on the slides, which were then loosely coverslipped and incubated at 37°C for 1.5 h. After rinsing three times in 0.1% Tween 20–PBS, a 50-μl aliquot of fluoresceinated goat anti-human IgG (Chemicon) diluted 1:120 with PBS was added and the slides were incubated for an additional 1.5 h. After another three rinses, two drops of 4′-6′-diamidino-2-phenylindole (DAPI), (12.5 μg/ml in 9:1 (glycerol:PBS) containing 10 mg/ml *p*-phenylenediamine, pH 8.0) were dropped onto the slide. The stained slides were reviewed using 1000 diameter magnification, and excitation wavelengths of 488 and 355 nm. For each subject, 1000 binucleated cells were scored and the number of kinetochore positive and kinetochore negative micronuclei were recorded.

2.5. Oropharyngeal micronucleus assay

The oropharyngeal cell samples were resuspended in 9 ml of 0.075 M potassium chloride and incubated for 35 min at 37°C. After centrifugation the cells were smeared on microscope slides and fixed in chilled 90% methanol for 20 min. The slides were stored at −20°C, along with the extra, freshly smeared slide that had been prepared at the time of sampling. Thawed slides were stained using the same procedures as described for the lymphocyte micronucleus assay. Affinity pure fluorescent goat anti-human IgG (DTAF; Chemicon) was used in an attempt to stain kinetochores in the micronuclei. The DTAF, however, appeared to penetrate and stain only about 20% of the cells in each sample. Cells with micronuclei were scored, initially, using 1000 diameter magnification and excitation wavelengths of 355 and 488 nm. Final scoring of the cells was based only on the DAPI DNA staining at 355 nm because of inadequate DTAF penetration of the oropharyngeal cells. A total of 1000 oropharyngeal cells were scored for most participants; however, the range in the number of cells scored was 324 to 1000.

For both the lymphocytes and the oropharyngeal cells, the following criteria were used to evaluate each potential micronucleus: (a) having comparable texture to the main nucleus, (b) shape: round to oval with distinct boundary, (c) positive staining for DNA,

(d) micronuclei positioned within the boundary of the cytoplasm of the cell, but distinct from the main nucleus, and (e) micronuclei approximately 10% or less in size compared to the main nucleus. The frequency of micronuclei per 1000 cells was reported.

2.6. Analysis of data

Student's *t*-tests were used to compare continuous demographic characteristics and outcomes between the workers and referents. Chi-square (χ^2) statistics were used to compare categorical demographic characteristics. Vf were log-transformed values to improve normality. Comparisons were also made between workers and referents by cigarette smoking status (current smokers and current non-smokers). Current smokers included those who reported cigarette consumption during the past year and those who reported smoking more than one marijuana cigarette per week during the past year. The two participants who reported current tobacco chewing (one worker, one referent) were removed from all analyses of oropharyngeal micronuclei. In addition, the analyses of the oropharyngeal micronuclei data were performed both by including and excluding the two former tobacco chewers (referents who claimed that they last chewed 3 months before sampling).

The association between the outcomes and methyl bromide exposure during the 14 days prior to sampling was also examined. Workers were categorized into two groups based on a summary measure calculated using self-reported information obtained from the questionnaire (those who performed activities having potential methyl bromide exposure for 4 h or more over the 2-week period, and those who performed such activities for less than 4 h). Although one of the components used to calculate this summary measure had poor agreement between worker self-reports and company-recorded data, it was the only available measure of methyl bromide exposure during the 2-week time period [25]. Each group of workers was compared to the referent group using Student's *t*-tests. Similar analyses stratified by smoking were also performed.

To evaluate the association between methyl bromide exposure and each of the four genotoxicity measures of interest (Vf, oropharyngeal micronuclei, kinetochore-positive lymphocytic micronuclei, kinetochore-negative micronuclei) linear regression analyses were also performed. A log transformation was applied to Vf to help normalize the residuals and/or equalize the variances. The potential confounders that were included in the regression analyses were selected based on biological relevance. The potential confounders were history of current smoking, age, and years of education (12 or more years vs. less than 12 years). All models were checked for assumptions of normality of residuals and model fit.

In the regression analyses, the effect of exposure to methyl bromide was assessed using a dichotomous exposure variable (defined as being a fumigation worker or a referent). For Vf and lymphocyte micronuclei, the effect of exposure to methyl bromide was also assessed using duration of occupational exposure to methyl bromide. The duration of methyl bromide exposure for each worker was calculated as the product of his self-reported years of fumigation industry employment and his self-reported lifetime proportion of fumigation jobs which used methyl bromide. Workers were stratified into two groups based on the median years of methyl bromide exposure among these workers: high methyl bromide-years and low methyl bromide-years. Finally, for all outcomes, methyl bromide exposure was also assessed using the 2-week summary measure for methyl bromide described above. Because no evidence was found for increasing genotoxicity with increasing exposure in any of the regression models containing years or hours of methyl bromide exposure, only the models containing the dichotomous exposure variable (worker vs. referent) will be reported. All reported *p*-values are two-sided. A *p*-value of 0.05 or less was considered statistically significant. All analyses were carried out using SAS procedures (SAS Institute, Cary, NC).

3. Results

Descriptive information on the study participants is provided in Table 1. There were no significant differences or consistent pattern of differences between workers and referents for any demographic characteristics, except that mean years of education was higher among the referents ($p = 0.02$). Among

Table 1
Characteristics of the study participants who contributed specimens for biological marker measurement

	Worker ($n = 31$)	Referent ($n = 27$)
Mean age (years)	33.9	32.9
(Range)	(21.4–67.8)	(20.7–50.8)
Race		
White	13 (42%)	10 (37%)
Black	8 (26%)	7 (26%)
Hispanic	10 (32%)	10 (37%)
Smoking status[a]		
Current smoker	17 (55%)	14 (52%)
Current non-smoker	14 (45%)	13 (48%)
Mean pack years[a]	13	10
(Range)	(0–70)	(0–105)
Alcohol status[b]		
Current drinker	22 (71%)	20 (74%)
Current non-drinker	9 (29%)	7 (26%)
Mean alcohol years[b]	15	21
(Range)	(0–60)	(0–150)
Mean years of education	11	12
(Range)	(5–19)	(0–16)
Gender		
Male	100%	100%
Median years of employment in the fumigation industry	6	0
(Range)	(0.5–32)	
Median years of methyl bromide exposure	3	0
(Range)	(0.3–22)	
Median hours of methyl bromide exposure during the 2 weeks preceding examination	4	0
(Range)	(0–145)	
Mean urine bromide concentration (mg/l)	131	110
(Range)	(40–247)	(32–254)

[a]Current smoker = those who reported cigarette consumption during the past year and those who reported smoking more than one marijuana cigarette per week during the past year. Pack years = average number of packs of cigarettes smoked per day multiplied by the number of years cigarettes were smoked.
[b]Current alcohol drinker = those who consumed 12 or more alcoholic drinks during the past year. Alcohol years = average number of alcoholic drinks consumed per day multiplied by the number of years alcohol was consumed.

workers, the median duration of fumigation industry employment was 6 years. The median number of years of methyl bromide exposure was 3 years (range 0.3–22 years). Although workers had a higher mean urine bromide concentration compared to referents, the difference was not statistically significant.

The unadjusted and stratified results for the *hprt* mutant lymphocyte assays are shown in Table 2A and Table 3A. Among current non-smokers, mean Vfs were only marginally elevated when all workers were compared to referents ($p = 0.22$; Table 2A). Non-smoking workers with 4 h or more of recent methyl bromide exposure had Vfs that were higher when compared to non-smoking referents ($p = 0.06$; Table 3A) The difference was statistically significant ($p = 0.03$, Student's *t*-test) when a non-smoking referent who had been employed for approximately 10 years in a plant that produced neutron triggers for nuclear weapons was excluded. This exclusion may be appropriate because there is some evidence that exposure to even relatively low levels of ionizing radiation may be associated with persistent increases in hprt mutant frequencies [26–28]. Using linear regression analysis, Vf was positively associated with smoking, but a significant association was not observed between methyl bromide exposure and Vf (Table 4A).

The unadjusted and stratified results for oropharyngeal micronuclei are shown in Table 2B and Table 3B. Workers overall were found to have an

Table 2
Crude and stratified analysis results for lymphocytic *hprt* Vfs and micronuclei among workers and referents

(A) Lymphocyte *hprt* Vf	Workers		Referents		
	N	Geometric mean (GSD) ($\times 10^{-6}$)	*N*	Geometric mean (GSD) ($\times 10^{-6}$)	Two-sided *p*-value
All	26	5.18 (2.39)	25	4.76 (2.31)	0.73
Current smoker	14	5.84 (2.31)	13	7.38 (2.10)	0.45
Current non-smoker	12	4.49 (2.51)	12	2.96 (1.96)	0.22
(B) Micronucleated cells/1000 oropharyngeal cells	*N*	Mean (SD)	*N*	Mean (SD)	Two-sided *p*-value
All	30	2.00 (1.49)	26	1.31 (1.41)	0.08
Current smokers	17	1.65 (1.41)	14	1.21 (1.53)	0.42
Current non-smokers	13	2.46 (1.51)	12	1.42 (1.31)	0.08
(C) Kinetochore-negative micronucleated cells/1000 lymphocytes					
All	31	10.48 (5.25)	27	10.41 (4.61)	0.95
Current smokers	17	10.76 (5.07)	14	10.71 (4.14)	0.98
Current non-smokers	14	10.14 (5.63)	13	10.08 (5.22)	0.98
(D) Kinetochore-positive micronucleated cells/1000 lymphocytes					
All	31	10.81 (6.12)	27	10.44 (6.99)	0.83
Current smokers	17	12.65 (7.29)	14	8.07 (5.17)	0.06
Current non-smokers	14	8.57 (3.37)	13	13.00 (7.96)	0.08

GSD = geometric standard deviation, SD = standard deviation.

Table 3
Geometric mean lymphocyte Vf and mean micronucleus frequencies among workers stratified by hours of recent methyl bromide exposure and referents

(A) Lymphocyte *hprt* Vf	Workers with ≥ 4 h of recent methyl bromide exposure*			Workers with < 4 h of recent methyl bromide exposure*			Referents	
	N	Geometric mean (GSD) ($\times 10^{-6}$)	Two-sided *p*-value†	*N*	Geometric mean (GSD) ($\times 10^{-6}$)	Two-sided *p*-value†	*N*	Geometric mean (GSD) ($\times 10^{-6}$)
All	13	7.23 (2.40)	0.16	13	3.71 (2.13)	0.37	25	4.76 (2.31)
Current smokers	6	8.08 (2.06)	0.80	8	4.58 (2.41)	0.20	13	7.38 (2.10)
Current non-smokers	7	6.56 (2.81)	0.06	5	2.64 (1.41)	0.73	12	2.96 (1.96)
(B) Micronucleated cells/1000 oropharyngeal cells	*N*	Mean (SD)	Two-sided *p*-value†	*N*	Mean (SD)	Two-sided *p*-value†	*N*	Mean (SD)
All	14	2.07 (1.64)	0.13	16	1.94 (1.39)	0.16	26	1.31 (1.41)
Current smokers	7	2.14 (1.77)	0.23	10	1.30 (1.06)	0.88	14	1.21 (1.53)
Current non-smokers	7	2.00 (1.63)	0.40	6	3.00 (1.26)	0.03	12	1.42 (1.31)
(C) Kinetochore-negative micronucleated cells/1000 lymphocytes								
All	15	10.07 (5.97)	0.85	16	10.88 (4.63)	0.75	27	10.41 (4.61)
Current smokers	7	10.00 (4.58)	0.72	10	11.30 (5.56)	0.77	14	10.71 (4.14)
Current non-smokers	8	10.13 (7.30)	0.99	6	10.17 (2.79)	0.97	13	10.08 (5.23)
(D) Kinetochore-positive micronucleated cells/1000 lymphocytes								
All	15	9.73 (4.45)	0.72	16	11.81 (7.37)	0.55	27	10.44 (6.99)
Current smokers	7	11.29 (5.50)	0.20	10	13.60 (8.47)	0.06	14	8.07 (5.17)
Current non-smokers	8	8.38 (3.02)	0.13	6	8.83 (4.07)	0.25	13	13.00 (7.96)

* Hours of recent methyl bromide exposure = number of hours spent performing activities having potential methyl bromide exposure during the 2 weeks that preceded the examination.
† Compared to the referent group.
GSD = geometric standard deviation, SD = standard deviation.

Table 4
Parameter estimates for adjusted linear regression analyses for Vf, oropharyngeal micronuclei kinetochore-negative lymphocytic micronuclei, and kinetochore-positive lymphocytic micronuclei

Outcome	Fumigation worker (Yes = 1, No = 0)	Current cigarette smoker (Yes = 1, No = 0)	Age (33 years or greater = 1, all else = 0)	Years of education (12 years or more = 1, < 12 years = 0)	R^2 of the model *	Number of observations
(A) Vf (log transformed)	−0.04 ($p = 0.86$)	0.55 ($p = 0.02$)	0.10 ($p = 0.68$)	−0.34 ($p = 0.19$)	0.16	51
(B) Oropharyngeal micronuclei	0.50 ($p = 0.24$)	−0.58 ($p = 0.15$)	−0.19 ($p = 0.62$)	−0.54 ($p = 0.23$)	0.12	56
(C) Kinetochore-negative lymphocytic micronuclei	−0.44 ($p = 0.74$)	−0.009 ($p = 0.99$)	3.87 ($p = 0.003$)	−1.46 ($p = 0.29$)	0.17	58
(D) Kinetochore-positive lymphocytic micronuclei	0.92 ($p = 0.62$)	−0.39 ($p = 0.82$)	2.70 ($p = 0.13$)	1.49 ($p = 0.44$)	0.06	58

* The R^2 for a particular outcome corresponds to the model containing the dichotomous exposure variable (fumigation worker or a referent), history of current cigarette smoking, age, and years of education.

elevated frequency of oropharyngeal micronuclei ($p = 0.08$; Table 2B). The differences were increased ($p = 0.04$) when the two former tobacco chewers were removed from the analysis. The regression analyses found little evidence for an association between oropharyngeal micronuclei and methyl bromide exposure (Table 4B), even when the two former tobacco chewers were removed from the analyses (data not shown).

No statistically significant differences were observed between workers and referents for mean kinetochore-negative lymphocyte micronuclei, even when stratifying by smoking status (Table 2C and Table 3C). The findings were similar for kinetochore-positive lymphocytic micronuclei (Table 2D and Table 3D). The regression analyses found that neither of the lymphocyte micronuclei measures were significantly associated with methyl bromide exposure (Table 4C and D). Kinetochore-negative micronuclei were found to have a positive association with age (Table 4C).

4. Discussion

This is the first study to evaluate in vivo genotoxicity of methyl bromide exposure among humans. Because prior evidence suggested that methyl bromide may be a point mutagen and a clastogen, test systems were selected which could measure these different types of genetic damage. The *hprt* mutations are useful for detecting point mutations that may follow exposure to toxic agents. The *hprt* is an enzyme involved in the regulation of purine synthesis. The *hprt* gene is found on the X chromosome. Presence of the mutation is determined by assessing the cell's resistance to TG. Unlike normal cells, cells with *hprt* mutations are able to survive in a medium containing TG [29]. The alterations responsible for this mutation can include deletions of DNA, frameshifts, and base substitutions [30].

The *hprt* assay is non-specific and has been shown to respond to a wide variety of exposures. However, there is evidence to suggest that detection of *hprt* mutations is dependent on the time interval between exposure and measurement of Vfs, as well as on the intensity of exposure [30]. The importance of the time interval has been demonstrated by the studies of Ammenheuser et al. [20,31,32]. These investigators found that multiple sclerosis patients who were treated with cyclophosphamide had elevated lymphocytic Vfs, compared to their pre-treatment levels [31]. However, within 13 weeks after the cessation of cyclophosphamide treatment, the Vfs for all subjects had returned to near pre-treatment levels, suggesting that many of the thioguanine-resistant mutant cells were selectively removed from circulation. Furthermore, cancer patients receiving radiotherapy had 5- to 15-fold increased Vfs during the 3rd and 4th weeks of treatment compared to pre-radiotherapy levels [32]. Once radiotherapy had ceased for 6–32 weeks, the Vfs had declined to levels only 2-fold higher than the pre-treatment values. Also, a 3- to 4-fold increase in *hprt* Vf detected in tobacco smokers and in marijuana smokers [19,20] was not evident in former smokers who had quit at least 1 year before being tested for *hprt* mutations. These reductions in mutant frequencies over time suggest that recent exposures may be more predictive of Vf compared to chronic past exposures. As for the importance of exposure intensity, elevations in lymphocyte mutant frequencies have been observed among workers with high-level exposure to ethylene oxide [33], but not among workers with low-level exposure to ethylene oxide [34]. In addition, because smoking is strongly associated with Vf [20,31], the effects on Vf of genotoxic agents are more likely to be detectable in non-smokers [35]. The findings from this study are consistent with these various characteristics of Vf. We found elevations among non-smoking exposed workers, especially among those with recent methyl bromide exposures.

Micronuclei are formed as a result of chromosomal (DNA) breakage or as a result of damage to the proteins of the spindle apparatus [36,37]. If the spindle apparatus is damaged, micronuclei would consist largely of whole chromosomes including the kinetochore (kinetochore-positive micronuclei), whereas the majority of micronuclei formed from chromosome breaks would consist of chromosomal fragments without the kinetochore (kinetochore-negative micronuclei). Use of an anti-kinetochore antibody to mark the presence of this structure in the micronucleus allows one to distinguish the mechanism of micronucleus formation. The anti-kinetochore antibody was used in the analyses of lymphocytic mi-

cronuclei but we were unsuccessful in our attempts to use it in our analyses of oropharyngeal micronuclei.

Measurement of micronuclei in oropharyngeal mucosal cells had the advantage of ready accessibility (they can be collected by rubbing the back of the throat with a cotton swab). Furthermore, because of the hydrophilic properties of methyl bromide, the major part of an inhaled dose may be absorbed by mucosal cells in the nose and oropharynx prior to passage to the lung, making these cells useful for measurement of micronuclei. Because these mucosal cells have an average lifespan of approximately 14 days, the oropharyngeal micronucleus assay was particularly useful for assessing genotoxicity from recent methyl bromide exposures. Our unadjusted and smoking-adjusted data provided some evidence to suggest that methyl bromide exposure may be associated with increased frequencies of micronuclei in oropharyngeal cells (Table 2B). Although there was little evidence of an exposure–response relationship, this finding may not be unexpected. Lack of an exposure–response relationship involving exfoliated cell micronuclei may arise if exposure to the genotoxic agent produces a decrease in cell proliferation (proliferation is needed for micronucleus production), or if it produces high levels of cytotoxicity [38]. Unfortunately, we are unable to determine if either of these factors were responsible for the limited exposure–response findings observed in this study for oropharyngeal micronuclei.

Although oropharyngeal micronucleus frequencies were found to be higher among non-smokers than smokers (Table 2B), other studies also failed to find a clear association with smoking [39,40]. In contrast, one study found smoking-associated increases in oropharyngeal micronuclei [41].

Measurement of micronuclei in T-lymphocytes is advantageous because these cells are in general circulation, have a long life span (months to years) and have generally low repair rates allowing damage to accumulate over many years. Our data provided little evidence of an association between methyl bromide exposure and an increase in either kinetochore-negative or kinetochore-positive micronucleus formation. The general lack of an association in the lymphocyte micronuclei analyses may be due to the insensitivity of the micronucleus assay among those with relatively low exposure to chemical agents that produce mainly non-clastogenic mutations [22,42].

We did not observe an association between cigarette smoking and lymphocyte micronucleus formation. Our findings are consistent with two studies that examined the association between cigarette smoking and lymphocyte micronucleus formation [42,43]. However, other studies reported that smokers had a significant elevation in micronuclei [44–46], and another study reported a non-significant elevation in micronuclei among smokers [23].

Our study found that kinetochore-negative lymphocyte micronucleus frequencies were positively associated with age. Other studies have observed similar age-related increases [47,48]. However, in contrast to our study, these other studies also found age-related increases for kinetochore-positive lymphocyte micronucleus frequencies.

This study had limitations involving exposure and sample size. The study had three limitations related to exposure. First, we measured workplace methyl bromide levels experienced by fumigation workers, but found that the recommended analytic method for measuring low-level methyl bromide exposures was imprecise, and that a sensitive alternative method does not exist [49]. However, our measurements suggested that the fumigation workers we studied may have been periodically exposed on a short-term basis to peak levels exceeding 80 mg/m^3 (the current OSHA permissible exposure limit [50]). Second, because we had little useful quantitative data to estimate exposure intensity, self-reported data were used to assess methyl bromide exposure. Although we previously found adequate agreement between self-reported and company-recorded information for several measures of exposure (e.g., duration of exposure) [25], one of the components used to calculate the 2-week exposure variable was found to have relatively poor agreement. Therefore, findings using the 2-week exposure variable should be interpreted cautiously. In addition, although we found no statistically significant difference in mean urine bromide concentration between workers and referents, there is no literature to suggest that this measurement is a useful marker of intensity of methyl bromide exposure. Third, our findings are limited by a recent industry-wide reduction in methyl bromide usage. Although methyl bromide exposure was ongoing

among the workers we examined, its use as a structural fumigant was being phased out at the time of our study. As such, workers reported a recent reduction in the proportion of jobs that used methyl bromide. During the 2 weeks preceding the examination, these workers reported that methyl bromide was used on 39% of fumigation jobs, whereas over the entire time they were employed in the fumigation business this proportion was 70%. Sulfuryl fluoride was used on the other structural fumigation jobs.

Another limitation was the small sample size of this study. The statistical power of future studies may be improved by targeting individuals with absent or low activity of glutathione transferase activity for compounds containing one or two carbon atoms. The absence of this enzyme may be associated with increased susceptibility to the cytogenetic toxicity of methyl bromide [51]. It is estimated that 25% of the population lacks this enzyme activity. Unfortunately, we did not measure the glutathione transferase activity of our participants.

It is unlikely that sulfuryl fluoride is responsible for the observed findings. For one thing, we were unable to find published reports on the genotoxicity of sulfuryl fluoride. In addition, the structure of sulfuryl fluoride (a halogenated inorganic acid derivative without any electrophilic leaving groups), suggests that significant genotoxicity would not be expected. Agents that are mutagenic and carcinogenic generally give rise to electrophilic reactants [52]. Furthermore, chronic inhalation studies have not demonstrated evidence of carcinogenicity [53]. Therefore, we think it is reasonable to attribute to methyl bromide the genotoxicity findings observed in our study.

In conclusion, the present study provides some evidence that methyl bromide exposure may be associated with genotoxic effects in lymphocytes and oropharyngeal cells. However, further study is needed involving larger sample sizes, improved assessment of methyl bromide exposure, and use of measures of susceptibility.

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