Cytogenetic Effects from Exposure to Mixed Pesticides and the Influence from Genetic Susceptibility

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Exposure to pesticides remains a major environmental health problem. Health risk from such exposure needs to be more precisely understood. We conducted three different cytogenetic assays to elucidate the biological effects of exposure to mixed pesticides in 20 Costa Rica farmers (all nonsmokers) compared with 20 matched controls. The farmers were also exposed to dibromochloropropane during the early employment years, and most of them experienced sterility/fertility problems. Our data show that the farmers had consistently higher frequencies of chromosome aberrations, as determined by the standard chromosome aberration assay, and significantly abnormal DNA repair responses (p < 0.05), as determined by the challenge assay, but no statistically significant differences in the tandem-probe fluorescence in situ hybridization (FISH) assay (p > 0.05). Genotype analysis indicates that farmers with certain "unfavorable" versions of polymorphic metabolizing genes (cytochrome P450 2E1, the glutathione S-transferases mu and theta, and the paraoxonase genes) had significantly more biological effects, as determined by all three cytogenetic assays, than both the farmers with the "favorable" alleles and the matched controls. A unique observation is that, in individuals who had inherited any of the mentioned "unfavorable" alleles, farmers were consistently underrepresented. In conclusion, the Costa Rican farmers were exposed to genotoxic agents, most likely pesticides, which expressed the induction of biological and adverse health effects. The farmers who had inherited "unfavorable" metabolizing alleles were more susceptible to genotoxic effects than those with "favorable" alleles. Our genotypic data suggest that the well-recognized "healthy worker effect" may be influenced by unrecognized occupational selection pressure against genetically susceptible individuals.

Key words: chromosome aberrations, cytogenetic effects, FISH, pesticide exposure, susceptibility.

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Pesticides are some of the most frequently released toxic chemicals into the environment. Although the use of pesticides enhances crop productivity, humans also pay a price for the benefits. Around the world, approximately 3 million acute poisonings and 220,000 deaths from pesticide exposure have been reported annually (1–3). In addition, farmers with prolonged exposure to low doses of pesticides eventually develop health effects similar to those seen in acute high-dose exposures, for example, neurobehavioral abnormalities (4–6), adverse reproductive outcomes (7), and increased cancer incidence (e.g., leukemia, non-Hodgkin lymphoma, and multiple myeloma) (8,9). Therefore, chronic exposure to pesticides can cause serious health effects in farmers who do not have a history of acute poisoning.

In many Latin-American countries, banana farming is a main agricultural activity for which multiple pesticides are used extensively, for example, dibromochloropropane (DBCP) in the recent past. Unfortunately, DBCP exposure has been linked to reduced sperm counts and infertility among workers (10). Other health effects have also been indicated in banana workers, for example, dermatitis (11) and cancer (12). With regard to the potential health hazards associated with pesticides, biomarker monitoring of exposed workers should be conducted to determine whether current exposure levels to pesticides are safe or not and whether serious health risk can be precisely predicted.

In Costa Rica, many banana workers have been exposed to DBCP in the past, and some of them have expressed DBCP-related sterilization effects based on clinically documented azoospermia and on the low number of offspring. With continued employment, they have additional exposure to other pesticides. The farmers were concerned about additional health risks from their exposure to pesticides. We were therefore invited to conduct cytogenetic monitoring of these workers.

Cytogenetic assays such as the chromosome aberration assay can be used as a reliable biomarker for cellular damage and adverse health risk (13,14). Therefore, this biomarker can be used as a mechanistic link between exposure and disease outcome. Increases in chromosome aberrations are frequently detected in lymphocytes of farmers who were exposed to mixed pesticides. Given sufficient exposure, the increase is consistently observed if adequate sample sizes are included (15) and if highly sensitive techniques are used, for example, chromosome banding (16) and the tandem-probe fluorescence in situ hybridization (FISH) assay (17). Therefore, some of these highly relevant cytogenetic assays were used in our study. Specifically, we conducted the standard chromosome aberration assay, the challenge assay to determine abnormal DNA repair response (18), and the tandem-probe FISH assay (17,19) using blood lymphocytes from the farmers and their matched controls.

Because individual responses to environmental toxicants are influenced by the metabolic capability of the individual, inheritance of variant polymorphic metabolizing genes may alter the pharmacokinetics and thus the biological and health outcome resulting from exposure (20,21). In this regard, we have characterized the inheritance patterns of the cytochrome P450 2E1 (CYP2E1), the glutathione S-transferases mu and theta (GSTM1 and GSTT1), and the paraoxonase (PON) genes in this population. The selection of these polymorphic genes is based on their role in the metabolism of pesticides, which can influence their toxicity (22–24). Inheritance of "unfavorable" versions of these genes have been shown to be involved with increased activation and/or decreased detoxification/elimination of environmental mutagens and to be associated with serious disease outcome (25–28).

The population size for our study is adequate for a cytogenetic analysis (29,30). Although the population size is small for genotype analysis in a molecular epidemiologic investigation, we expected that the combination of genotype analysis and cytogenetic study would provide some insight into the genetic susceptibility to pesticides, as we have noticed previously in cigarette...
smokers (19). Furthermore, the extensive pesticide exposure conditions for the farmers may increase our chance of detecting an association between genetic susceptibility and biological effects.

The results from our study indicate that the farmers had increased chromosome aberrations and significantly abnormal DNA repair responses. Furthermore, increased abnormalities were frequently associated with farmers who had inherited “unfavorable” versions of the monitored polymorphic metabolizing genes as compared with farmers who had inherited “favorable” genes and with controls. A unique observation is that, in individuals who had inherited “unfavorable” metabolizing genes, farmers were consistently underrepresented as compared to controls, therefore suggesting the existence of unrecognized occupational selection against susceptible individuals.

Materials and Methods

Selection of study populations. A group of plantation workers in Costa Rica with long-term employment in banana farming constituted the exposed group. Among these farmers, many of them expressed reduced sperm counts and fertility problems. These problems were considered to be related to work exposure as described above and were not used as an exclusion criteria for our study. Each of the farmers was interviewed with the Spanish version of our established questionnaire (31). Farmers were asked for information on age, sex, employment history, exposure to pesticides, health history, exposure to cytotoxic therapeutic drugs and radiation, cigarette smoking habits, and number of children. Only healthy farmers who were nonsmokers and who had no previous exposure to cytotoxic therapeutic drugs and radiation were selected for participation in our cytogenetic and genotype analyses.

The information from the selected farmers was used to recruit matched controls who were referred by the workers. Potential controls were interviewed using the same questionnaire as used for the farmers. The controls were matched with the farmers on a one-to-one basis with respect to age (± 5 years). The only exceptions were that the controls were not exposed to pesticides occupationally and did not have sperm reduction/fertility problems.

Matching pairs of farmers and controls were scheduled to visit a designated clinic on three specific days. On these days, a phlebotomist collected peripheral blood samples from both farmers and controls into vacutainers containing sodium heparin as an anticoagulant. These samples were then coded by our study coordinator. Samples were collected in the afternoon for personal delivery via commercial airlines to our laboratory in Galveston, Texas. The collected blood samples were aliquoted for both the cytogenetic and genotype analyses. The laboratory procedures were initiated within 24 hr of sample collection.

Techniques for cytogenetic analyses. Whole blood cultures were set up for the standard chromosome aberration and the challenge assays as previously reported (31). The bromodeoxyuridine (BrdU) incorporation protocol was used so that chromosome damage was recorded only from metaphase cells that had completed one DNA synthesis cycle in vitro. For the challenge assay, lymphocyte cultures were irradiated with gamma-rays in vitro at the 24th hr after the initiation of cell culture, corresponding to the G1 phase of the cell cycle. BrdU was added to the cultures immediately after the irradiation. After cytological preparations were made, bright field microscopy was used to analyze 150 metaphase cells/donor to determine the frequency of chromosome aberrations for the standard cytogenetic assay and 75 cells/donor for the challenge assay.

We used the procedure for the tandem-probe FISH assay described by Rupa et al. (17), which we previously used successfully (19). We used fluorescence microscopy to analyze 1,500 interphase nuclei/donor for the presence of chromosome aberrations.

Techniques for genotype analyses. Lymphocytes were isolated from whole blood using the standard Ficol-Histopaque procedure and DNA samples were isolated from the lymphocytes using the standard salting out method (32). We used the DNA samples to characterize the inheritance patterns of the polymorphic CYP2E1, GSTM1, and GSTT1 genes, as previously reported (27). The PON gene was characterized according to the procedure of Schmitz and Lindpaintner (33). Briefly, we conducted two allele-specific polymerase chain reactions (PCR) for each DNA sample, one reaction containing a sense primer specific for the A allele (192 glumatic acid) and one containing the sense primer specific for the B allele (192 arginine). A common antisense primer was used for both reactions, resulting in a 117-base pair (bp) PCR product. As a positive control, the cytochrome P4501A1 gene was co-amplified in both reactions, resulting in a 312-bp product. The PCR products were electrophoresed on 2.5% ethidium bromide-stained gels and the individual genotypes determined by the visualization of product bands over ultraviolet light. All the primers used for this study were synthesized by the National Institute of Environmental Health Sciences Molecular Biology Facility Core in the Sealy Center for Molecular Science at The University of Texas Medical Branch.

We classified individuals having “favorable” or “unfavorable” genotypes, as previously reported (27). According to our classification, the “unfavorable” alleles for each gene are as follows: CYP2E1 is represented by a base substitution at the transcriptional region causing overexpression of the gene for activation of small organic chemicals; GSTM1 and GSTT1, are represented by gene deletion, and PON is represented by a mutation that causes reduced detoxification capacity of the enzyme. Individuals with the “unfavorable” alleles are expected to have increased body burdens of reactive metabolites by either increasing bioactivation or decreasing detoxification of xenobiotics. Therefore, individuals with these genes may be susceptible to environmental mutagens, resulting in increased chromosome aberrations and adverse health effects following exposure.

Statistical analyses. All analyses were performed using the computer software program ABStat release 1.90 (Anderson-Bell Corp., Arvada, CO). All variables are expressed as mean ± standard error (SE). Statistical significance was determined by one-way analysis of variance (ANOVA), followed by independent t-test if the overall F-test was significant. For testing the differences in genotype distribution between the study subjects, the Fisher’s exact test was used. Significant levels (p-values) correspond to two-sided tests. An alpha error (p) of < 0.05 was considered significant.

Results

Study population. From information collected during the interviews, we selected 20 farmers and 20 matched controls. The selected farmers had been employed as farm hands and/or sprayers, primarily on banana plantations since the late 1960s and early 1970s. Like the typical exposure conditions for farm hands and sprayers in developing countries with year-round warm weather, they were exposed to pesticides routinely, on a year-round basis, and without protective equipment. They reported previous exposure to DBCP and current exposure to other pesticides at work (e.g., chloryphosphos, imidazol, thiabendazole, gramozone, terbufos, and fenamiphos). From a review of the U.S. Environmental Protection Agency Genetic Activity Profiles database (34), none of the six pesticides have been reported to cause chromosome aberrations, although chlorpyrifos and terbufos have some genotoxic activities. The farmers did not report any exposure to cytotoxic medication or to
excessive diagnostic radiation. They did not have any illness that required radiation therapy. None of the selected farmers were cigarette smokers and they drank alcohol only lightly (less than 2 drinks/day). The mean age (± SE) of the farmers was 43.8 ± 1.9 years. Among these farmers, 15 (75%) had excessive apy. have any illnesses that required radiation therapy (lessthan2drinks/day). The mean age was 43.8 ± 1.9 years. The offspring of each farmer was 0.7 ± 0.2. Otherwise, the farmers were healthy.

The matched controls were healthy individuals, not exposed to pesticides or cytotoxic agents. All the selected controls were nonsmokers and only light alcohol drinkers. They were employed as either accountants, computer operators, electricians, laboratory technicians, or office workers. Each control was matched with one farmer based on age of the farmer ± 5 years. The mean age (± SE) of the selected controls was 39.8 ± 1.4 years. The mean number of offspring was 2.2 ± 0.5 per control. All subjects participated in our study voluntarily. All of them provided oral consent (in Spanish) before blood samples were drawn from them. A registered nurse was employed to draw blood specimens.

**Standard chromosome aberration assay.** Cytological preparations were analyzed microscopically for the presence of chromatin-type abnormalities. From each individual, 150 metaphase cells showing the first cell cycle staining pattern were analyzed from coded slides. The summary from this analysis is shown in Table 1. Farmers had higher frequencies of aberrant cells (containing any types of chromosome abnormalities) compared with the controls (2.4 ± 0.3 vs. 1.9 ± 0.3 per 150 cells analyzed; mean ± SE) and higher frequencies of cells with chromatid breaks (2.2 ± 0.4 vs. 1.8 ± 0.3). Although the increase was consistent in the two chromosome categories, neither difference between the farmers and controls was statistically significant (p > 0.05).

**Challenge assay.** In this assay, lymphocytes were assayed for their repair of radiation-induced DNA damage. Increased chromosome aberrations were used as indicators of abnormal DNA repair responses. From each individual, 75 metaphase cells showing a first metaphase staining pattern were analyzed from coded slides for the presence of chromosome aberrations. The data from the farmers and the controls are summarized in Table 1. Among the four recorded categories, the workers had consistently more abnormalities than the controls in terms of the frequency of aberrant cells (25.4 ± 0.9 vs. 23.2 ± 0.9 per 75 cells analyzed, mean ± SE; p < 0.05), frequency of chromatid breaks (2.7 ± 0.5 vs. 1.7 ± 0.3; p = 0.05), frequency of chromosome deletions (14.4 ± 1.2 vs. 13.3 ± 0.9; p > 0.05), and frequency of dicentrics (14.4 ± 0.8 vs. 13.3 ± 0.7; p > 0.05). As indicated, the difference in DNA repair response between the two groups was significant for the frequency of aberrant cells and chromatid breaks.

**Tandem-probe FISH assay.** Cytological preparations of lymphocyte cultures were stained with fluorescence-tagged probes that hybridized to the classical and alaphid sequences near the centromere of chromosome 1. We recorded aberrations resulting in spatial dissociation at this region of chromosome 1. We observed 1,500 nuclei from each individual. As shown in Table 1, cells from the farmers had fewer chromosome aberrations and fewer chromatid breaks as compared with the controls (2.7 ± 0.3 breaks/1,500 cells analyzed vs. 3.5 ± 0.6 and 1.7 ± 0.3 breaks/1,500 cells analyzed vs. 2.7 ± 0.5, respectively; mean ± SE). Although the trend of the observed biological effect is inconsistent with those from the other two cytogenetic assays, the difference between them is not significant (p > 0.05).

**Genotypes and relationship with cytogenetic effects.** DNA samples from the 20 farmers and the 20 controls were characterized for their inheritance of the polymorphic CYP2E1, GSTM1, GSTT1, and PON genes. The association between inheritance of “unfavorable” alleles and expression of the observed biological effects were evaluated using independent two-tailed t-tests on the collected data. Although the sample sizes for each of the comparison groups were small, the overall outcome was that the inheritance of “unfavorable” alleles was frequently associated with increased biological effects. The opposite effect, increased biological effects associated to “favorable” genotypes, was not observed from our data. The highlights of these comparisons are shown in Table 2. As shown in Table 2, a statistically significant increase in chromosome aberrations was observed in the farmers with the homozygous deletion of GSTM1 as compared to the controls carrying the same genotype (25.46 ± 1.32 vs. 21.93 ± 0.86, mean ± SE; p = 0.028). Inheritance of the CYP2E1 mutant allele in the farmers was significantly associated with increased challenge breaks (4.5 ± 0.50 vs. 1.60 ± 0.51; p = 0.022) as compared to controls who had the same allele. The number of challenge dicentrics was significantly increased in the farmers who inherited the low detoxifying allele of the PON gene (PON A/A) relative to controls with the same allele (17.03 ± 1.48 vs. 13.23 ± 0.95, p = 0.040). Among the farmers, inheritance of the PON A/A genotype was associated with a significantly increased number of challenge dicentrics as compared to farmers who inherited either the heterozygous PON A/B or the high detoxifying PON B/B alleles (17.03 ± 1.48 vs. 13.32 ± 0.88, p = 0.037).

Other consistent but not statistically significant differences were also observed in the different combinations of genotypes and biological effects. These associations are also summarized in Table 2. An increase in challenge dicentrics was observed in the farmers with the GSTM1 null as compared to the controls carrying the same genotype (14.68 ± 1.18 vs. 12.60 ± 0.66, mean ± SE; p = 0.110). Farmers harboring the null GSTT1 had an increase in challenge dicentrics as compared to controls (16.30 ± 1.94 vs. 13.5 ± 0.43; p = 0.122). Farmers with the GSTT1 null genotype had an increase in chromatid breaks, as detected by FISH, as compared to farmers who had inherited the wild-type GSTT1 (3.00 ± 1.08 vs. 1.81 ± 0.22; p = 0.096). Inheritance of the CYP2E1 mutant allele in the farmers was associated with a marginally significant

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**Table 1. Cytogenetic effects among Costa Rican farmers and controls.**

| Subjects | No. aberrant cells (mean ± SE) | No. chromatid breaks (mean ± SE) | No. chromosome deletions (mean ± SE) | No. dicentrics (mean ± SE) |
|----------|--------------------------------|---------------------------------|------------------------------------|--------------------------|
| Standard chromosome aberration assay (150 cells/person analyzed) | Control: 1.9 ± 0.3 | 1.8 ± 0.3 | NA | NA |
| | Farmers: 2.4 ± 0.3 | 2.2 ± 0.4 | NA | NA |
| Challenge assay (75 cells/person analyzed) | Control: 23.2 ± 0.9 | 1.7 ± 0.3 | 13.3 ± 0.9 | 13.3 ± 0.7 |
| | Farmers: 25.4 ± 0.9** | 2.7 ± 0.5*** | 14.4 ± 1.2 | 14.4 ± 0.8 |
| Tandem-probe FISH assay (1,500 cells/person analyzed) | Control: 3.5 ± 0.6 | 2.9 ± 0.6 | NA | NA |
| | Farmers: 2.7 ± 0.3 | 2.0 ± 0.3 | NA | NA |

Abbreviations: FISH, fluorescence in situ hybridization; NA, not applicable; SE, standard error.

*Twenty farmers and 20 matched controls.

* p < 0.05 and ** p = 0.05 based on two-tailed t-test.
increase in chromosomal aberrations (3.00 ± 1.00 vs. 1.41 ± 0.24; p = 0.062) as compared to controls who had the same allele. An increase in challenge dicentrics was observed for exposed individuals lacking both GSTM1 and GSTT1 genes as compared to controls carrying the same genotype (16.30 ± 1.94 vs. 13.20 ± 0.37; p = 0.120). In addition, exposed farmers with these two null alleles demonstrated an increased number of breaks as detected by FISH, as compared to farmers with at least one of the two wild-type alleles (3.00 ± 1.08 vs. 1.81 ± 0.22; p = 0.096). Importantly, no statistically significant opposite effect was detected.

**Genotype distribution study.** The distribution of the “favorable” and “unfavorable” versions of the polymorphic genes among the farmers and the controls were analyzed and are summarized in Figure 1. Fewer farmers had the “unfavorable” versions of these genes than did matched controls: CYP2E1, 10 vs. 25%；GSTM1, 50 vs. 75%；GSTT1, 20 vs. 30%；PON, 30 vs. 64%. Among these comparisons, the difference for PON, which is responsible for metabolism of organophosphate insecticides, shows the strongest significant level (p = 0.056).

**Discussion**

Our data indicate that the banana farmers in Costa Rica had a consistent increase in chromosome abnormalities in all measured categories, as determined by the standard chromosome aberration assay, and significantly abnormal DNA repair responses, as determined by the challenge assay, as compared to the matched controls (Table 1). The observation was, however, not consistent with the data from the tandem-probe FISH assay, although the difference between the farmers and the controls was not statistically significant. The difference in the results between the FISH assay and the other two assays may be due to several factors. One possibility is that the use of the FISH assay was not appropriate in this study. In this assay, the tandem FISH probes hybridize to a small region of chromosome 1, which has been shown to be prone to breaks by mutagens (17). It is possible that the pesticides in our study did not cause enough damage to the chromosome 1 region in the lymphocytes of the farmers to be detectable by this assay. Therefore, the frequency of chromosome damage between the two groups in this specific region was not statistically different from each other.

The data from the standard chromosome aberration and the challenge assays are consistent with each other and suggest that the farmers have been exposed to genotoxic agents, most likely to mixed pesticides. Like most studies on farmers, the target populations have been exposed to mixed pesticides. The observed chromosome effects can be considered the sum of the interactions among the pesticides. Furthermore, we observed that farmers with “unfavorable” alleles had significantly more biological effects as compared to controls having the same “unfavorable” alleles

| Subjects (n) | Genotypes | Associated with         | p-Value* |
|-------------|-----------|-------------------------|----------|
| Farmers (10) | GSTM1/0/0 | Challenge aberrations | 0.028    |
| Controls (15) | GSTM1/0/0 | Challenge aberrations | 0.028    |
| Farmers (2)  | CY2P2E1m* | Challenge breaks       | 0.022    |
| Controls (5) | CY2P2E1m* | Challenge dicentrics   | 0.040    |
| Farmers (6)  | PON/A/A   | Challenge dicentrics   | 0.037    |
| Farmers (14) | PON AB-AB | Challenge dicentrics   | 0.100    |

Abbreviations: FISH, fluorescence in situ hybridization; GSTM1, glutathione S-transferase mu gene; GSTT1, glutathione S-transferase theta gene; PON, paraoxonase gene.

*Values based on two-tailed t-test. **Both genes are absent; null genotypes (+/−). ***Having presence of one or both genes (+/-, −/+ or +/+).

![Figure 1](image_url) Distribution of "unfavorable" metabolizing genes among Costa Rican farmers and controls. Abbreviations: CYP2E1, cytochrome P450 2E1 gene; GSTM1, glutathione S-transferase mu gene; GSTT1, glutathione S-transferase theta gene; PON, paraoxonase gene.

*P = 0.056 based on Fisher's exact test.

(Table 2). The results indicate that the genotoxic agents the farmers were exposed to were absent from the environment of the matched controls. It has been reported that enzymes from the cytochrome P450, glutathione S-transferases, and PON genes are responsible for metabolism of pesticides (22,23,35), suggesting that the “unfavorable” alleles could have caused increased body burden of reactive genotoxic agents in the farmers. This indication is confirmed by comparing the effects among the farmers: those with the “unfavorable” alleles exhibited significantly increased genotoxicity compared to those with the “favorable” alleles. It should be reemphasized that, based on our analyses, no significant opposite association was detected.

A study with some resemblance to ours was reported by Scarpato et al. (36). They investigated the associations between two detoxifying genes (GSTM1 and GSTT1) and one biomarker (chromosome breakage) among pesticide-exposed farmers. In this investigation, cigarette smokers were not excluded from the study populations. As a result, Scarpato et al. (36) did not observe any association between pesticide exposure and elevated frequencies of chromosome aberrations. In addition, the association between the GST null genotypes and chromosome aberrations was found only with cigarette smoking. With the selection of nonsmokers in our study, we were able to demonstrate the biological effects of pesticide exposure and their association with inheritance of “unfavorable” alleles.

A unique observation regarding the distribution of genotypes among the farmers and the matched controls in our population is that the farmers were consistently under-represented in the number of carriers of the “unfavorable” versions of the four polymorphic genes (CYP2E1, GSTM1, GSTT1, and

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may drastically impact the current practice on exposure control/disease prevention in the workplace.

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