

Paraoxonase status and plasma butyrylcholinesterase activity in chlorpyrifos manufacturing workers

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Chlorpyrifos is an organophosphorus (OP) anticholinesterase insecticide. Paraoxonase (PON1) is an enzyme found in liver and plasma that hydrolyzes a number of OP compounds. PON1 polymorphisms include a glutamine (Q)/arginine (R) substitution at position 192 (PON1_{Q192R}) that affects hydrolysis of OP substrates, with the PON1_{192Q} allotype hydrolyzing chlorpyrifos oxon less efficiently than the PON1_{192R} allotype, a variation potentially important in determining susceptibility to chlorpyrifos. We studied 53 chlorpyrifos workers and 60 referents during 1 year and estimated chlorpyrifos exposure using industrial hygiene and employment records and excretion of the chlorpyrifos metabolite 3,5,6-trichloro-2-pyridinol (TCP). Plasma butyrylcholinesterase (BuChE) activity, which may be inhibited by chlorpyrifos exposure, was measured monthly. In addition, plasma samples were assayed for paraoxonase (PONase), diazoxonase (DZOase), and chlorpyrifosoxonase (CPOase) activity to determine PON1 status (inferred genotypes and their functional activity). Linear regression analyses modeled BuChE activity as a function of chlorpyrifos exposure and covariates. We postulated that the level of CPOase activity and the inferred PON1₁₉₂ genotype (together reflecting PON1 status) would differ between groups and that PON1 status would modify the models of chlorpyrifos exposure on BuChE activity. Chlorpyrifos workers and referents had a 100-fold difference in cumulative chlorpyrifos exposure. Contrary to our hypotheses, mean CPOase activity was similar in both groups ($P = 0.58$) and PON1_{192Q} showed a slight overrepresentation, not an underrepresentation, in the chlorpyrifos group compared with referents (PON1_{192QQ}, 51% chlorpyrifos, 40% referent; PON1_{192QR}, 43% chlorpyrifos, 40% referent; PON1_{192RR}, 6% chlorpyrifos, 20% referent, $P = 0.08$). In our models, BuChE activity was significantly inversely associated with measures of interim chlorpyrifos exposure, but the biological effects of chlorpyrifos exposure on BuChE activity were not modified by PON1 inferred genotype or CPOase activity.

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Introduction

The effects of acute organophosphorus (OP) insecticide poisoning are well known relative to our understanding of the consequences of long-term, low-dose OP exposures that do not produce acute cholinergic toxicity (Hernandez et al., 2006). Recent studies of the effects of chronic occupational exposure to chlorpyrifos (*O,O*-diethyl-*O*-[3,5,6-trichloro-2-pyridyl]-phosphorothioate) during the manufacturing process at levels sufficient to produce biological effects on butyrylcholinesterase (BuChE) activity found no clinically evident or subclinical adverse neurological effects at baseline or after 1 year of additional exposure (Albers et al., 2004b, 2007).

It is believed that metabolism rates of various OP compounds influence susceptibility to OP toxicity, and that individual differences in xenobiotic metabolism may modify the adverse effects from OP exposure (Hernandez et al., 1999; Costa et al., 2003, 2005). A number of OP compounds are metabolized in part through bioactivation of the parent compound by the cytochrome P450 systems, followed by hydrolysis of the resulting aryl ester (oxon) by paraoxonases (Furlong et al., 2000b). Paraoxonase 1 (PON1) is an arylesterase expressed chiefly in liver and secreted into plasma that hydrolyzes *in vitro* a number of OP compounds and aryl esters, including chlorpyrifos oxon and paraoxon, the active metabolites of chlorpyrifos and parathion, respectively (Costa et al., 2005; Furlong et al., 2006). The catalytic efficiency of hydrolysis is one of the most important factors in preventing or modulating OP-induced neurotoxicity (Hegele, 1999; Costa et al., 2005; Fu et al., 2005; Khersonsky and Tawfik, 2005). The PON1 gene includes two common coding region amino acid substitutions (polymorphisms), one involving a methionine/leucine substitution at amino acid position 55 and the other involving a glutamine (Q)/arginine

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(R) substitution at position 192 (PON1_{Q192R}) (Hassett et al., 1991). Unlike the polymorphisms at position 55, the polymorphisms at position 192 influence the catalytic efficiency of hydrolysis of some OP substrates, thereby representing a genetic variation potentially important in determining susceptibility to these compounds (Aviram et al., 1998; Costa et al., 1999; Furlong et al., 2000b).

The potential implication of the PON1₁₉₂ alloforms is complicated, because some OP substrates are hydrolyzed with greater catalytic efficiency by the PON1_{R192} alloform than by the PON1_{Q192} alloform, whereas other substrates show an opposite activity (Billecke et al., 2000; Amitai et al., 2006). For example, the PON1_{192R} alloform hydrolyzes paraoxon *in vitro* with greater catalytic efficiency than does the PON1_{192Q} alloform, whereas the PON1₁₉₂ alloforms hydrolyze diazinon oxon (diazoxon) with nearly equivalent catalytic efficiencies (Haley et al., 1999; Furlong et al., 2000b; Lee et al., 2003). In each example, the PON1_{192QR} genotype shows an intermediate activity relative to the PON1_{192RR} and PON1_{192QQ} alloforms. Chlorpyrifos oxon (the active metabolite of chlorpyrifos) is hydrolyzed more efficiently by the PON1_{192R} alloform than by the PON1_{192Q} alloform, and individual differences in susceptibility to chlorpyrifos are likely explained by a combination of the PON1₁₉₂ genotype and by the activity or level of the plasma PON1 alloform(s)—PON1 status (Furlong et al., 1988, 2000a, b). This notion is supported by *in vivo* studies showing that PON1-knockout mice are conferred better protection against chlorpyrifos oxon exposure by intraperitoneal injection of PON1_{192R} alloform than of PON1_{192Q} (Li et al., 2000). PON1 status includes both the amount of PON1 (e.g., the level of activity of plasma chlorpyrifosoxonase [CPOase] in the metabolism of chlorpyrifos oxon) and the efficiency of hydrolysis (e.g., the functional PON1₁₉₂ genotype, PON1_{192QQ}, PON1_{192QR}, PON1_{192RR}) (Li et al., 1993; Furlong et al., 2006; Holland et al., 2006). Unexpectedly, human and mouse PON1 do not appear to protect against paraoxon exposure. Moreover, PON1 knockout mice are not more sensitive to paraoxon exposure than wild-type mice, and injection of human PON1_{R192} into knockout mice does not provide protection against dermal paraoxon exposure (Li et al., 2000).

As part of a prospective longitudinal study of the neurological and neurobehavioral effects associated with occupational exposure to chlorpyrifos, we measured plasma CPOase activity and determined the distribution of PON1₁₉₂ inferred functional genotypes from among 53 chlorpyrifos-manufacturing workers and 60 referents. In the present study, we attempted to determine as part of a *post hoc* analyses if PON1 status, as measured by plasma CPOase activity and PON1₁₉₂ inferred genotypes, differed among workers with chronic and ongoing exposure to chlorpyrifos relative to referents workers who had no current or recent occupational chlorpyrifos exposure during the study interval.

We postulated that CPOase activity would be higher and that the PON1_{192Q} alloform would be underrepresented among chlorpyrifos manufacturing workers relative to referents who had no ongoing occupational exposure to chlorpyrifos. This hypothesis was based on the assumption that individuals with low CPOase activity and the PON1_{192Q} alloform would be susceptible to chlorpyrifos and unlikely to sustain employment associated with ongoing chlorpyrifos exposure.

We also postulated that PON1 status would modify the effect of chlorpyrifos exposure on BuChE activity. In the present analyses, we modeled BuChE activity as a function of measures of chlorpyrifos exposure over a 1-year interval. Plasma BuChE can serve as a detoxifying enzyme that acts as a stoichiometric “suicide trap” for specific toxic OP compounds (Furlong, 2000). After BuChE reacts with an OP molecule, the OP is no longer available to inhibit nervous system acetylcholinesterase (AChE), but the BuChE molecule is inactivated in the process (Furlong et al., 2000b). This reduction in BuChE activity is the basis for considering it as an effect of exposure to chlorpyrifos in the analyses we now report. After modeling the relationship between BuChE activity and various measures of chlorpyrifos exposure and potential confounders, we entered CPOase activity and the PON1₁₉₂ inferred functional genotype into the final models to determine whether these indicators of PON1 status acted as effect modifiers. From our previous analyses, we knew that the average BuChE activities differed significantly between the chlorpyrifos workers and referents and that many of the chlorpyrifos workers showed appreciable inhibition of this enzyme. In contrast, measures of red blood cell AChE levels were similar for both groups, indicating that there was no substantial inhibition of AChE associated with work in the chlorpyrifos exposed areas compared with the referent plant (Albers et al., 2004b).

Materials and methods

The study design, subject description, exposure measures, and statistical analyses have been described previously (Albers et al., 2004b) and are briefly summarized below. Full details are provided for PON1 status and the current statistical analyses, which were not described in the previous report.

Study Design

We evaluated two subject groups: (1) chlorpyrifos manufacturing workers who had known and measurable exposure to chlorpyrifos; and (2) Saran (clear plastic film) manufacturing workers who had no current or recent occupational exposure to chlorpyrifos. Examinations occurred on two occasions, approximately 1 year apart. The subjects were all chemical workers at the Dow Chemical Company in Midland, Michigan, USA. No subjects had occupational exposure to

other OP insecticides or to other known or suspected neurotoxicants. Examiners were masked to group membership and to exposure histories. The study was approved by the University of Michigan Institutional Review Board for Human Subject Research and the Dow Human Subject Review Board.

Subjects

Dow employees were eligible for participation if they were between 18 and 65 years of age and had no condition that made them unable to complete the protocol. All eligible chlorpyrifos manufacturing workers and a random sample of Saran manufacturing workers were asked to participate. All subjects provided written consent indicating their willingness to participate. We evaluated 113 subjects, including 53 of 66 eligible chlorpyrifos workers (80%) and 60 of 74 randomly chosen Saran workers (81%).

Exposure Assessment

Ambient chlorpyrifos exposure was estimated using air monitoring data and internal dose was estimated using biological measures. We used industrial hygiene records to estimate chlorpyrifos exposure during the year between the baseline (beginning of study) and 1-year examinations (interim chlorpyrifos exposure) and chlorpyrifos exposure from the time of initial employment to the baseline examination (historic chlorpyrifos exposure). Historical personal air sampling results of chlorpyrifos exposure were compiled for similarly exposed groups (SEGs) of workers, and geometric mean exposure levels were calculated for each SEG and used to estimate historic chlorpyrifos exposure. Interim and historic chlorpyrifos exposures were calculated by multiplying the exposure estimate for each SEG by the number of days worked in the job and summing these products across jobs during the relevant intervals. Interim chlorpyrifos exposure was assessed biologically by urinary excretion of 3,5,6-trichloro-2-pyridinol (TCP), a metabolite of chlorpyrifos, as a weighted average of four overnight collections obtained during the year, expressed as a function of creatinine (Cr) excretion as interim TCP/Cr ($\mu\text{g/g}$), with the weights chosen to reflect the number of months each measurement represented. The sample taken during the annual 2-week fall maintenance period was given a weight of 0.5 months. The spring sample was given a weight of 1 month because chlorpyrifos is typically used on home gardens and farms during a 1-month period in the spring when crops emerge. The samples taken during the baseline and 1 year physical examinations were each given a weight of 5.75 months (Burns et al., 2006).

Biological Effects of Chlorpyrifos Exposure

Red blood cell AChE activity was measured twice, from venipunctures taken at baseline and 1 year later, by LabCorp, Burlington, NC, USA, using an automated analysis based on

the Ellman method (Ellman et al., 1961). Plasma BuChE activity was measured monthly from finger pricks taken on most subjects in both groups during the examination period, over an interval of approximately 15 months. Assays were done by MidMichigan Medical Center, Midland, MI, USA, using an automated procedure based on a solid-state variation (Cattozzo et al., 1993) of the Ellman et al. (1961) method. The BuChE measurements used in our analyses began 2 months after their initial evaluation (so as not to represent a possible effect of exposure that occurred before study onset). Most subjects had 12 BuChE measurements, and 106 subjects had at least six BuChE measurements. Not all subjects participated as scheduled, however, and the total number of BuChE measures ranged from 2 to 18. The higher number represented BuChE measures obtained beyond routine monitoring, taken because of a suspected chlorpyrifos over exposure. For each subject, the measures of BuChE activity obtained during the study year were expressed in three forms; the *mean BuChE* (mU/ml) as an averaged of all results; the *lowest BuChE* (mU/ml) recorded during study year (mU/ml); and the *BuChE ratio* of lowest BuChE during study year to the pre-exposure BuChE. The pre-exposure BuChE activity for chlorpyrifos subjects was based on the historic BuChE activity measured for each subject before beginning work in an area associated with potential chlorpyrifos exposure.

PON1 Inferred Genotype and Chlorpyrifosoxonase Activity

Blood samples were obtained at baseline by venipuncture into either lithium-heparin or sodium-heparin Vacutainer tubes (2.5 ml). Plasma was separated from red blood cells by centrifugation, aliquotted into 2 ml cryocontainers, and frozen on dry ice (-78°C) before express shipping for analyses (Dr. Clement Furlong, Department of Medicine, Division of Medical Genetics, University of Washington, Seattle, WA, USA). Plasma samples were thawed and thoroughly mixed by vortexing in the original tube. A 0.5-ml aliquot was removed and placed in a 1.5 ml labeled Eppendorf tube. The mixed 0.5 ml aliquots were centrifuged at room temperature for 2 min at 14,000 rpm. The samples were then stored on ice or in the refrigerator at 4°C .

Plasma samples were assayed in duplicate in a Molecular Devices SpectraMax PLUS microplate reader for PONase, diazoxonase (DZOase), and CPOase. The plate reader data in OD/min were converted to U/L using a conversion factor derived from a graphic comparison of Beckman DU-70 spectrophotometer-determined rates versus rates determined on the plate reader. Plasma standards from three individuals whose PON1 status had been determined regularly over a 7–18-year period were used as internal standards on every assay plate. These standards showed less than 8% variability between independent assays. An additional 26 samples from a previously determined PON1 status frequency distribution

of known phenotypes and genotypes (Jarvik et al., 2000) were included as additional standards for CPOase assays. There was a mean 4.4% variation between assays for these 29 samples. The assay results for the three substrates permitted separation of activity levels, providing determination of the inferred genotype of each subject's PON1 (Furlong et al., 1989; Davies et al., 1996; Richter and Furlong, 1999). PON1 inferred genotypes were determined from two-dimensional enzyme plots showing the rates of hydrolysis of chlorpyrifos oxon and of diazoxon plotted against the rates of hydrolysis of paraoxon for each subject's plasma.

Statistical Analyses and Model Structure

All data were double entered or hand checked against the original records for accuracy. Data were managed in Microsoft Access and Excel, and analyses were carried out in SAS, version 8.2 (SAS Institute, Cary, NC, USA). All linear regression analyses modeled BuChE activity as a function of interim chlorpyrifos exposure, interim TCP/Cr, historic chlorpyrifos exposure, age, sex, height, weight, and alcohol exposure (drinks/day). Using a backward stepwise selection procedure, covariates that had a P -value <0.15 were included in the model. Interaction terms between each of the covariates and each of the exposure measures were evaluated, and those having a P -value <0.05 were kept in the final model. We examined all covariates for correlations to insure that the linear regression models did not suffer from problems due to inclusion of highly correlated variables. None of the covariates was correlated highly, with the exception of weight and BMI, $r=0.86$. The correlations between the two exposure variables were not sufficiently high to suggest collinearity issues. The analyses were repeated using the lowest BuChE and the ratio of the lowest BuChE during the study year to the pre-exposure BuChE as the outcome effect. For each outcome variable (interim chlorpyrifos exposure and TCP/Cr), the "best" model was selected based on the lowest value of the Akaike Information Criterion. We repeated the analyses to determine whether there was an interaction between PON1 status (PON1 inferred functional genotype and CPOase activity) and TCP/Cr. These analyses were carried out to determine whether PON1 status modified the effect of chlorpyrifos exposure on BuChE activity. In terms of the PON1 inferred functional genotype, we investigated the two-way interaction of PON1_{192QQ} subjects versus PON1_{192QR} and PON1_{192RR} subjects combined.

Results

Subjects

Descriptive characteristics are shown in Table 1. Chlorpyrifos subjects and referents were comparable in terms of most

Table 1. Descriptive statistics for the subject population at baseline (Albers et al., 2004a).

Variable	Chlorpyrifos (<i>n</i> = 53) Mean ± SD	Referent (<i>n</i> = 60) Mean ± SD	<i>P</i> -value ^a
<i>Gender</i>			
Female	22.6	26.7	0.62
Male	77.4	73.3	
	Mean ± SD	Mean ± SD	<i>P</i> -value ^b
Age (years)	41.2 ± 7.5	41.3 ± 8.4	0.92
Height (m)	1.74 ± 0.1	1.75 ± 0.1	0.94
Weight (kg)	88.1 ± 15.0	88.3 ± 20.3	0.96
Body mass index (kg/m ²)	29.0 ± 4.2	29.0 ± 6.0	0.99
Alcohol use (drinks/day)	0.8 ± 0.9	1.3 ± 1.4	0.19

^a P -value determined by Fisher exact test.

^b P -value determined by t -test for difference in means (adjusted for unequal variance between groups when indicated) or Wilcoxon two-sample test.

factors known to be important to PON1 status, including racial distribution (primarily Caucasian subjects in both groups) (Catano et al., 2006). This was a healthy cohort with few physician-diagnosed disorders (two subjects had diabetes, one in each group). No subject was eliminated because of a physician-diagnosed condition.

Exposure Assessment

The descriptive statistics for the industrial hygiene exposure variables and the TCP/Cr results are shown in Table 2. The industrial hygiene data showed a wide range of chlorpyrifos exposures, with significant group differences for all exposure measures. Six referents had past chlorpyrifos exposure ranging from 3.1 to 15.98 mg/m³ × days; none had any identifiable occupational chlorpyrifos exposure during the observation period.

Biological Effects of Chlorpyrifos Exposure

At baseline, the average AChE was 6923 mU/ml (SD, 828) among chlorpyrifos subjects and 6967 mU/ml (SD, 766) among referents ($P=0.77$). One year later, the average AChE was 7148 mU/ml (SD, 751) among chlorpyrifos subjects and 7253 mU/ml (SD, 811) among referents ($P=0.48$). These results indicate that there was no substantial inhibition of AChE associated with work in the chlorpyrifos exposed areas, compared with work in the Saran plant. The descriptive statistics for biological outcomes for chlorpyrifos workers and referents are shown in Table 3. The biological measures of chlorpyrifos exposure reflected a wide range of exposures, with significant group differences for all measures of BuChE activity (mean BuChE, lowest BuChE, and BuChE ratio). Among chlorpyrifos subjects, urine TCP excretion was significantly related to inhibition of BuChE ($R^2=0.3842$) (Figure 1) but unrelated to inhibition of AChE ($R^2=0.000006$), suggesting that the measures of internal

Table 2. Descriptive statistics for exposure variables (Albers et al., 2004a).

Exposure Measure	Chlorpyrifos (<i>n</i> = 53) Mean ± SD (range)	Referent (<i>n</i> = 60) Mean ± SD (range)	<i>P</i> -value ^a
Historic cumulative chlorpyrifos (mg/m ³ × days)	64.16 ± 59.97 (0.00–220.36)	0.69 ± 2.51 (0.00–23.71)	<0.0001
Duration of work in exposed areas (years)	9.72 ± 6.12 (0–23.71)	0.10 ± 0.37 (0–2.12)	<0.0001
Interim cumulative chlorpyrifos (mg/m ³ × days)	6.13 ± 3.70 (0.00–15.37)	0.00 ± 0.00 (0.00–0.00)	<0.0001
Urine TCP (μg/ml)	0.251 ± 0.399 (0.006–1.894)	0.008 ± 0.004 (0.001–0.017)	<0.0001
TCP/Cr (μg/g-Cr)	192.2 ± 311.8 (8.8–1536.5)	6.2 ± 2.2 (1.4–12.1)	<0.0001

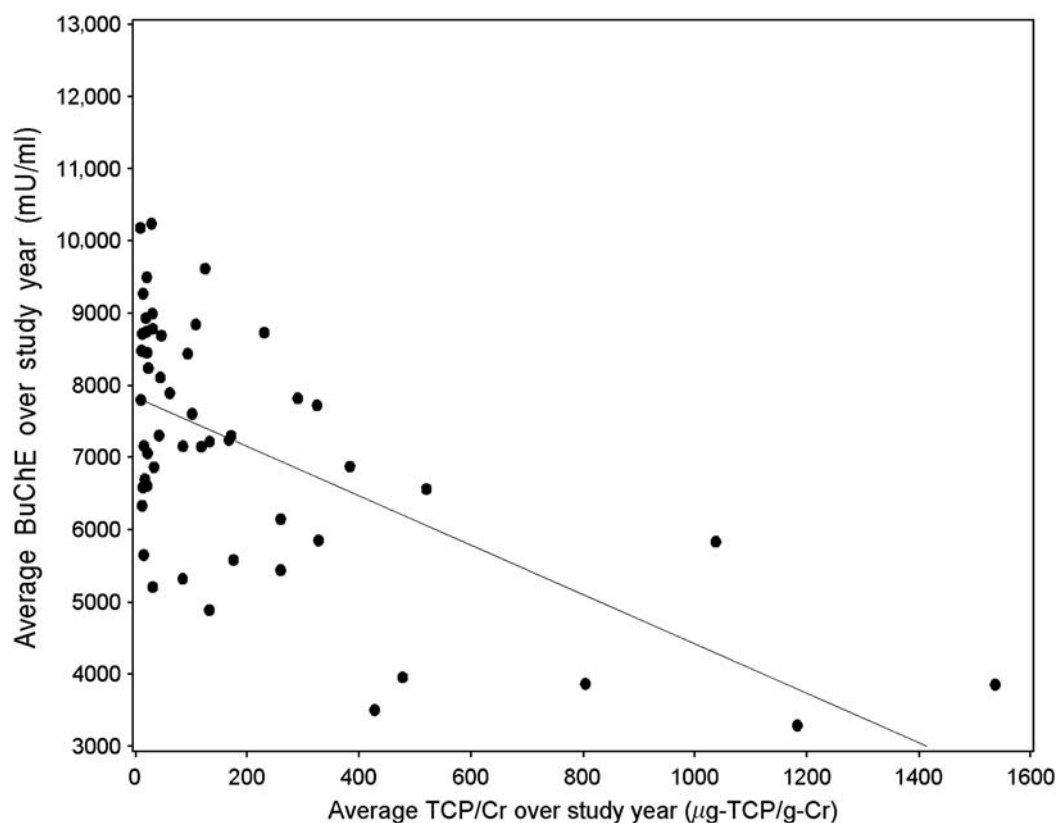
^a*P*-value determined by *t*-test for difference in means (adjusted for unequal variance between groups when indicated) or Wilcoxon two-sample test.

Table 3. Descriptive statistics for the biological outcomes.

Measure	Chlorpyrifos (<i>n</i> = 52) ^a Mean ± SD (range)	Referent (<i>n</i> = 60) Mean ± SD (range)	<i>P</i> -value ^b
Butyrylcholinesterase (BuChE) activity averaged over study year (mU/ml)	7155 ± 1729 (3286–10237)	8183 ± 1631 (4605–12848)	0.002
Lowest BuChE activity during study year (mU/ml)	5820 ± 2055 (240–9735)	7168 ± 1501 (3878–11562)	0.0002
BuChE ratio of lowest BuChE during study year to pre-exposure BuChE	0.75 ± 0.26 (0.03–1.21)	0.88 ± 0.09 (0.64–1.00)	0.0008
Red blood cell cholinesterase (RBC-AChE) activity at baseline (mU/ml)	6923 ± 828 (4940–8713)	6967 ± 766 (4366–8403)	0.77
RBC-AChE activity at 1 year (mU/ml)	7149 ± 751 (5450–9281)	7253 ± 811 (4917–9208)	0.48

^aAlthough there were 53 chlorpyrifos workers, one was no longer active in the manufacturing process and had no interval BuChE measurements.

^b*P*-value determined by *t*-test for difference in means (adjusted for unequal variance between groups when indicated) or Wilcoxon two-sample test.

**Figure 1.** Relationship between average BuChE over the study year and average TCP/Cr over the study year for chlorpyrifos subjects.

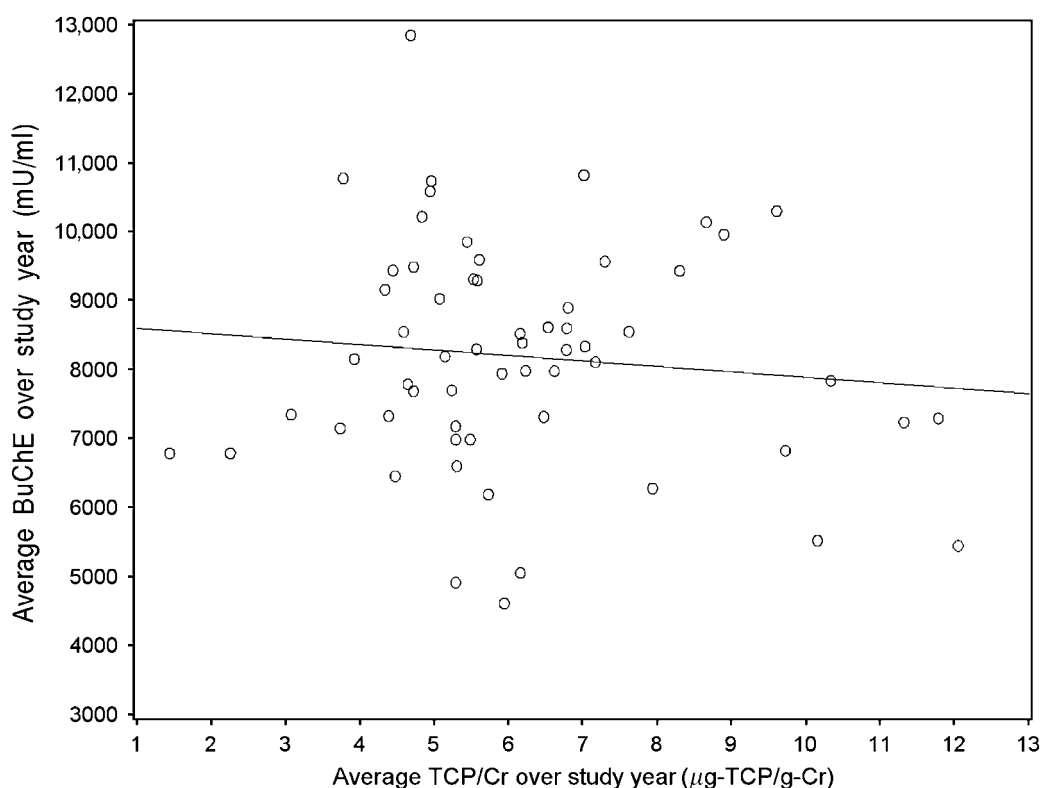


Figure 2. Relationship between average BuChE over the study year and average TCP/Cr over the study year for referent subjects.

dose were in the range where appreciable inhibition of BuChE occurs but below the range where a physiologic effect on AChE exists. The urine TCP was unrelated to inhibition of BuChE ($R^2=0.0111$) among referents (Figure 2). The average AChE activity did not differ between chlorpyrifos subjects and referents at the baseline or 1-year examination, indicating there was no substantial inhibition of AChE associated with work in the chlorpyrifos exposed areas, compared with work in the Saran plant.

PON1 Inferred Genotype and Chlorpyrifosoxonase Activity

Two-dimensional plots of DZOase activity versus PONase activity and of CPOase activity versus PONase activity are presented in Figure 3 for chlorpyrifos subjects (filled symbols) and for referent subjects (open symbols). Although the catalytic efficiency of diazoxon hydrolysis by the two PON1₁₉₂ alloforms is nearly equivalent (Richter and Furlong, 1999; Li et al., 2000), the DZOase activity of the PON1_{192R} isoform was selectively inhibited by high salt to separate the RR data points from the QR data points. In this way, the two-dimensional plot was used to assign the inferred functional genotypes to each subject. As can be seen, subjects distribute into readily identified clusters in the diazoxonase versus paraoxonase plot. These clusters correlate with the PON1_{192Q/R} genotype and allow determination of the

inferred genotype. This method has been shown to be a highly reliable predictor of genotype; moreover, it provides functional information about the protein product, that is, the enzymatic activity for hydrolysis of OP substrates (Furlong et al., 2000b).

The PON1_{Q192R} inferred functional genotypes and alleles for all subjects are shown in Table 4. Among chlorpyrifos workers, 51% were PON1_{192QQ}, 23% were PON1_{192QR}, and 6% were PON1_{192RR} compared with referents who were 40% PON1_{192QQ}, 40% PON1_{192QR}, and 20% PON1_{192RR}. The group distributions of inferred functional genotypes were not significantly different ($P=0.08$), although there were only three PON1_{192RR} chlorpyrifos workers (6%) compared with 12 PON1_{192R} referents (20%). Allele frequencies for PON1_{192Q} were 73% (chlorpyrifos) and 60% (referents), and for PON1_{192R} were 27% (chlorpyrifos) and 40% (referents) ($P=0.05$; Yates'-corrected $P=0.06$). Group differences of inferred allele frequencies were borderline significant. Collectively, these findings were contrary to our hypothesis that the PON1_{192Q} allele or PON1_{192QQ} alloform would be underrepresented among chlorpyrifos manufacturing workers relative to referents. Moreover, the CPOase activity (mean $\pm$ SD, U/L) was 9733 ± 1918 among chlorpyrifos workers and 9536 ± 1853 among referents. These means were not significantly different ($P=0.58$). Similarly, and also contrary to our hypothesis, the distributions of

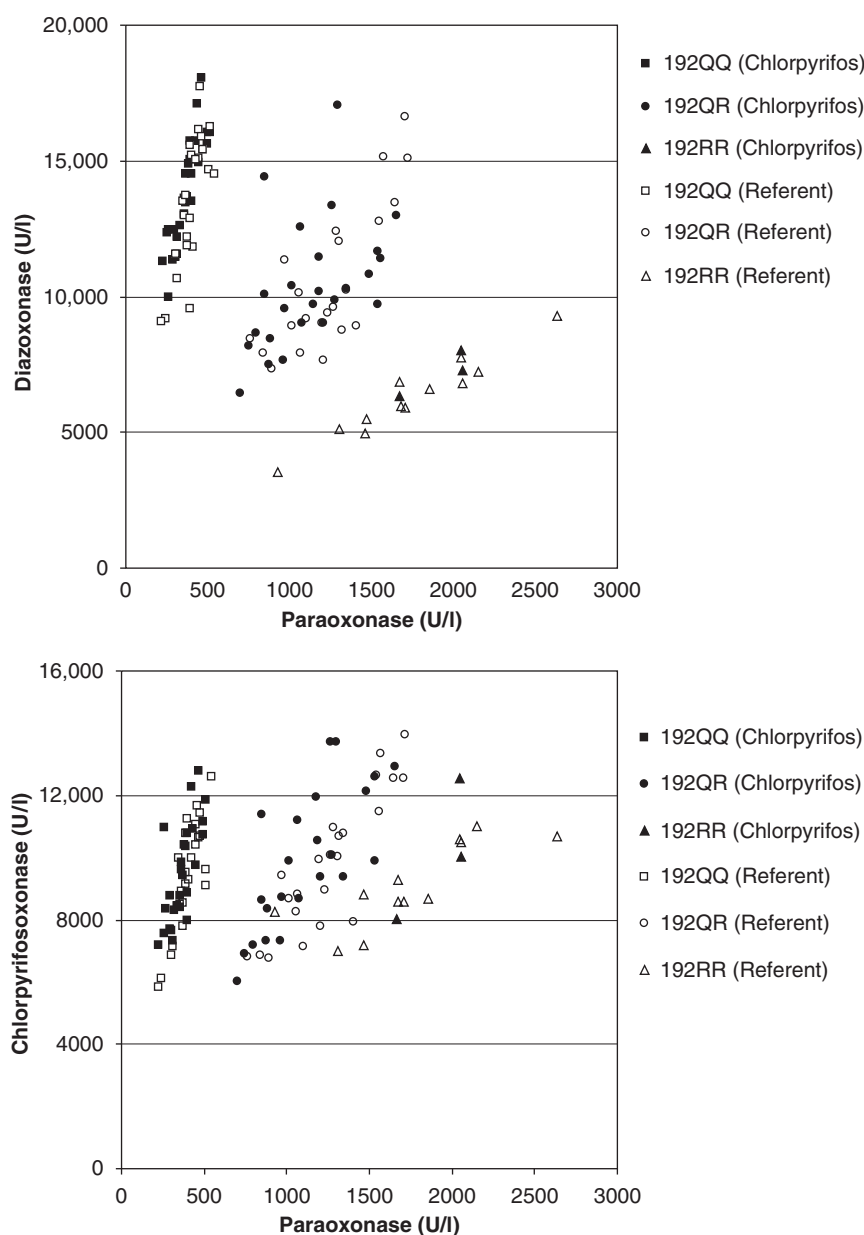


Figure 3. Diazoxonase activity versus paraoxonase (PONase) activity (upper plot) and chlorpyrifosoxonase (CPOase) activity versus PONase activity (lower plot) for chlorpyrifos subjects (filled symbols) and referent subjects (open symbols). Inferred genotypes were derived from the clustering observed in the diazoxonase versus PONase plot (Furlong et al., 2000b; Jarvik et al., 2003).

CPOase activity for the different inferred functional genotypes depicted in Figure 3 showed no significant difference in terms of the mean CPOase activity for the three groups (Table 4).

Results of Linear Regression Models

The results of the regression analyses are shown in Table 5. Interim TCP/Cr level was the only covariate that entered significantly into all of the BuChE effect models at a $P < 0.0001$ level. The interim chlorpyrifos exposure (mg/

$\text{m}^3 \times \text{days}$) based on the industrial hygiene estimations also entered into all of the models at a $P < 0.05$ level, but the average interim urinary excretion of TCP/Cr provided the best fit. Historic chlorpyrifos exposure entered into only the model of the lowest BuChE activity during the study year to the pre-existing BuChE activity ($P = 0.02$). Height, weight, or BMI entered into all of the models, with a larger BMI and larger weight and shorter height associated with a positive effect (higher BuChE). Sex entered into two of the models, with male sex associated with a negative effect (lower

Table 4. PON1 ^{Q192R} functional inferred genotype and allele distribution and chlorpyrifosoxonase (CPOase) activity among chlorpyrifos workers and referents.

	Group			CPOase Activity (mU/ml)		
	Chlorpyrifos	Referent	<i>P</i> -value ^a	Chlorpyrifos	Referent	<i>P</i> -value ^b
All subjects	52 (100%) ^c	60 (100%)		9733	9536	0.58
Genotypes						
QQ	26 (50%)	24 (40%)	0.08	9528	9451	0.87
QR	23 (44%)	24 (40%)		9901	9839	0.92
RR	3 (6%)	12 (20%)		10,219	9,099	0.28
Alleles						
Q	75 (72%)	72 (60%)	0.05 (0.06)			
R	29 (28%)	48 (40%)				

^a*P*-value from Chi-square test; *P*-value with Yates' correction shown in parentheses.

^b*P*-value from t-test of difference in means.

^cAlthough there were 53 chlorpyrifos workers, one had no genotype or CPOase measured.

Table 5. Results of linear regression models.^a

Variable	Mean BuChE activity over study year (mU/ml) Parameter estimate (<i>P</i> -value)	Lowest BuChE during study year (mU/ml) Parameter estimate (<i>P</i> -value)	Ratio of lowest BuChE during study year to pre-exposure BuChE Parameter estimate (<i>P</i> -value)
Interim TCP/Cr level (μg/g Cr)	−2.8 (0.0002)	−3.2 (<0.0001)	−0.00047 (<0.0001)
Interim chlorpyrifos exposure (mg/m ³ × days)	−88.5 (0.04)	−138.8 (0.002)	−0.022 (0.001)
Historic chlorpyrifos exposure (mg/m ³ × days)			0.0012 (0.02)
Sex (M = 1, F = 0)	−1478.4 (<0.0001)	−1687.2 (<0.0001)	−0.084 (0.06)
Body mass index (kg/m ²)	63.3 (0.01)		
Weight (kg)		8.2 (0.03)	
Height (m)		−104.7 (0.05)	−0.0095 (0.08)
Model adjusted <i>R</i> ²	0.41	0.46	0.43

^aFor each measure, the model having the lowest AIC is presented.

BuChE). Neither age nor alcohol use entered any of the models. The model that accounted for the greatest variance was the model of interim TCP/Cr and the ratio of the lowest BuChE during study year to pre-exposure BuChE. In this model, significant covariates included interim TCP/Cr level, historic chlorpyrifos exposure, sex, and BMI.

In the interaction analyses, we modeled each of the BuChE summary variables as a function of covariates and the interaction of TCP/Cr and CPOase activity as well as TCP/Cr and PON1 inferred functional genotype (PON1_{192QQ} subjects versus combined PON1_{192QR} and PON1_{192RR} subjects). No model showed a significant interaction between the TCP/Cr (the exposure measure) and CPOase activity or PON1 inferred genotype (e.g., mean BuChE activity over study year and TCP/Cr-CPOase, *P* = 0.27; lowest BuChE during study year to pre-exposure BuChE and TCP/Cr-CPOase, *P* = 0.50; ratio of lowest BuChE during study year to pre-exposure BuChE and TCP/

Cr-CPOase, *P* = 0.79; mean BuChE activity over study year and PON1 inferred genotype, *P* = 0.76; lowest BuChE during study year to pre-exposure BuChE and PON1 inferred genotype, *P* = 0.45; ratio of lowest BuChE during study year to pre-exposure BuChE and PON1 inferred genotype, *P* = 0.57).

Discussion

Evaluation of PON1 status may be important for determining individual susceptibility to increased risk of exposure to OP insecticides, including chlorpyrifos (Furlong et al., 2006). In the present analyses, we sought to determine whether PON1 status differed among individuals who have high occupational exposure to a specific OP insecticide, chlorpyrifos, relative to a referent group representative of the chemical workers who do not have occupational exposure to

chlorpyrifos. These analyses used data collected during a prospective evaluation of chlorpyrifos manufacturing workers and referents comprising chemical workers similar in all ways aside from chlorpyrifos exposure.

We reported earlier that the same chlorpyrifos workers had no measurable adverse neurological symptoms (e.g., numbness, weakness, memory loss) or effects (e.g., neuropathy, mental status change, or nerve conduction abnormalities) attributable to historic or interval chlorpyrifos exposures (Albers et al., 2004a, b). In the present analyses, we found no significant differences in the distribution of PON1_{Q192R} inferred genotype, alleles, or CPOase activity between the chlorpyrifos manufacturing workers or referents. The hypothesis that PON1 status would differ between these two groups was based on the presumption that those with PON1_{192QQ} allotype would be more sensitive to chlorpyrifos exposure and that they would avoid jobs associated with chlorpyrifos exposure. In other words, PON1 status would be reflected in a self-selection of subjects, so that those with “poor” PON1 status (e.g., PON1_{192QQ} and/or low overall CPOase activity) would not remain in jobs associated with chlorpyrifos exposure. This premise of self-selection presumes the presence of some adverse effect of chlorpyrifos exposure (e.g., headache, nausea, irritability) that would eventually lead an individual away from the specific job involving chlorpyrifos exposure exceeding background exposures. On the basis of this “healthy worker effect,” workers who are less sensitive to chlorpyrifos exposure remain in chlorpyrifos-related activities, whereas those who are more sensitive to chlorpyrifos exposure eventually seek activities that do not involve chlorpyrifos exposure. There is no assumption that the adverse effect would be recognized by the individual worker as being related to chlorpyrifos exposure. Failure to identify a significant difference in the distribution is consistent with the conclusion that there was nothing about chlorpyrifos exposures at levels associated with the manufacturing process that was perceived as being sufficiently adverse to produce a change in job location. This conclusion is also consistent with the Monte Carlo simulation reported by Timchalk et al. (2002), which predicted that PON1 status would not be influential at low chlorpyrifos exposure levels. Alternatively, PON1_{192Q} may not be a valid indicator of sensitivity, or the differences in CPOase activity we found may be insufficient to include those who are the most sensitive. Although we did not address the question directly, it is possible that some workers experience subjective discomfort because of their increased sensitivity but elect to remain on the job despite such perceptions.

Our regression analyses showed that BuChE activity, as a biological measure of chlorpyrifos exposure, was significantly related to average TCP/Cr excretion averaged over the year of observation (a quantitative biological measure chlorpyrifos dose), a finding consistent with our previous report (Burns et al., 2006). We recognize that the relationship

between measures of BuChE activity and TCP/Cr excretion is dynamic and that averaging the values for each measure throughout the year could possibly obscure or blunt the relationship between the two measures. Ideally, we would have used paired BuChE and TCP/Cr obtained in close temporal proximity for each subject. We had a range of BuChE measures over the year of study, which varied from 2 to 18 measurements among subjects. We averaged the intra-individual BuChE activity to use all measurements and to reduce the random variation of BuChE activity within each subject. We also used a weighted average of the four intra-individual TCP/Cr measurements over the year of study. The regression analyses examined the relationship between average TCP/Cr and average BuChE activity. Although the chlorpyrifos exposure causes rapid inhibition of BuChE activity, the average BuChE inhibition in relation to the average TCP/Cr correctly reflects this relationship, assuming that the measures are a random sample of both distributions. In support of our methodology, we have previously reported additional analyses in which we identified all BuChE and TCP/Cr measurements taken within 7 days of each other for a given subject (Garabrant et al., 2009). Using this subset of the data, we analyzed the relationship between TCP/Cr and BuChE activity using linear mixed models, which correctly account for both intra-subject and inter-subject variation, while controlling for covariates. These models (results not shown) gave results comparable to those presented in Table 5. There was no relationship between TCP/Cr and BuChE at low exposure levels (TCP/Cr less than 90 $\mu\text{g/g Cr}$), and there was a highly significant inverse relationship above that level with a slope similar to that presented in Table 5 for mean BuChE. Further, in the linear mixed models, neither CPOase activity nor PON1 inferred genotype was a significant predictor of BuChE activity. These results agree with the findings presented in this paper; namely, subjects with high CPOase activity do not respond significantly differently to chlorpyrifos exposure than do subjects with low CPOase activity, and PON1 inferred genotype does not influence the relationship between measures of chlorpyrifos exposure and BuChE activity.

We also found that BuChE activity was significantly related to sex and anthropometric measures of BMI, weight, or height. The negative influence of male gender related to low BuChE levels likely reflects the fact that most of the chlorpyrifos exposed workers were males. Studies exist in which simple correlational analyses associated BuChE activity with sex and a number of other factors related to coronary artery disease, but step-wise multiple regression analysis did not show independent correlations with BuChE activity (Alcantara et al., 2002). Cross-sectional evaluations of the possible role of BuChE activity in Alzheimer disease (as related to the introduction of anticholinesterase treatments) and cardiovascular disease found that BuChE activity was inversely associated with age, positively associated with

being overweight and obese (e.g., BMI), and positively associated with serum albumin, cholesterol, and triglyceride levels (Calderon-Margalit et al., 2006). Although we did not have access to measures of serum lipids, the positive associations we found between BuChE activity and larger BMI, heavier weight, and shorter height are consistent with the associations with overweight reported above. The positive associations between BuChE activity and BMI and weight or stature have been attributed to a possible genetic relation between BuChE alleles and anthropometric characteristics (Souza et al., 2005). What we did not find was evidence that BuChE activity was significantly modified by the PON1₁₉₂ inferred genotype or CPOase activity. This negative result parallels our initial finding involving a lack of indication of self-selected job exclusion among this group of chlorpyrifos workers, in that biological measures of chlorpyrifos exposure were not influenced by PON1 status, at least not at the exposure levels that we studied.

One strength of our study was the ability to evaluate a group of chemical manufacturing workers who had measurable and substantial exposure to a single OP insecticide, chlorpyrifos, and a group of referent chemical workers comparable aside from chlorpyrifos exposure. This is in contrast to evaluation of other workers, such as insecticide applicators, who are exposed occupationally to multiple OP and other insecticides over time, not just chlorpyrifos. Unlike many occupational studies and most environmental studies of OP insecticides, we had excellent exposure measures, including biological measures of TCP excretion, a metabolite of chlorpyrifos, averaged over the study year. The chlorpyrifos exposure levels we evaluated covered a wide range. The daily TCP/Cr excretion among chlorpyrifos workers (192.2 $\mu\text{g/g}$; range, 8.8–1536.5) suggested an estimated daily chlorpyrifos exposure of about 576–627 $\mu\text{g/day}$, equivalent to approximately 30% (range, 0–250%) of the internal dose that would be received by a hypothetical subject exposed during a working day at the TLV of 200 $\mu\text{g/m}^3$. The duration of exposure among chlorpyrifos workers averaged almost a decade, and there was no indication from our study of movement of workers from chlorpyrifos-related jobs.

Aside from its strengths, our study had limitations. Despite the wide range of chlorpyrifos exposure, levels were insufficient in amount to affect AChE, as there was no group effect on AChE activity. Although we recruited a high percentage of available chlorpyrifos-exposed workers, the number of chlorpyrifos exposed subjects was relatively small, and the number of subjects in the highest exposure group may have been too small to show the effects we studied. The assumption that subjects with the PON1_{192Q} allele had high vulnerability to chlorpyrifos relative to subjects with the PON1_{192R} allele may have been incorrect, or at least not applicable to this group of subjects. Namely, the distributions of CPOase activity for the different inferred functional genotypes depicted in Figure 3 show no difference in terms of

the mean CPOase activity for the three groups, a finding that differs from previous reports (Costa et al., 1999). If the disparity in findings between ours and those reported by others reflects some undetected aspect of self-selection, it is difficult to explain why workers with the PON1_{192RR} inferred genotype showed a lower mean CPOase activity than expected from previous studies. Most likely, the small number of subjects classified as PON1_{192RR} in our study simply showed a lower CPOase activity than expected based on chance. In terms of CPOase levels, although there was a substantial range of activity, it is possible that the chlorpyrifos dose was insufficient to produce the adverse effects that would remove a subject from ongoing exposure. Another limitation of our study is the possibility that over time some chlorpyrifos workers had moved into activities of less chlorpyrifos exposure. Although we were unable to determine specifically whether chlorpyrifos workers had moved to other jobs, within the plant or elsewhere, there was no evidence to suggest that this had occurred and the long exposure durations averaging more than 9 years as shown in Table 2 argue against the possibility.

In summary, the biological effects of chlorpyrifos exposure on BuChE activity were not modified by PON1 inferred genotype or CPOase activity, indicating that there remained adequate capacity to metabolize chlorpyrifos and chlorpyrifos oxon independent of these factors over the range of occupational exposure levels that occurred in our study.

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Disclosure

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