Journal of Exposure Science and Environmental Epidemiology **(2009) 19, 634–642**

www.nature.com/jes

Cholinesterase inhibition in chlorpyrifos workers: Characterization of biomarkers of exposure and response in relation to urinary TCPy

DAVID H. GARABRANT[a,b], LESA L. AYLWARD[c], STANLEY BERENT[d], QIXUAN CHEN[e], CHARLES TIMCHALK[f], CAROL J. BURNS[g], SEAN M. HAYS[h] AND JAMES W. ALBERS[i]

[a]*Risk Science Center, School of Public Health, University of Michigan, Ann Arbor, MI, USA*
[b]*Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI, USA*
[c]*Summit Toxicology, LLP, Falls Church, VA, USA*
[d]*Department of Psychiatry, University of Michigan School of Medicine, Ann Arbor, MI, USA*
[e]*Department of Biostatistics, School of Public Health, University of Michigan, Ann Arbor, MI, USA*
[f]*Battelle Pacific Northwest Division, Richland, WA, USA*
[g]*The Dow Chemical Company, Midland, MI, USA*
[h]*Summit Toxicology, LLP, Lyons, CO, USA*
[i]*Department of Neurology, University of Michigan School of Medicine, Ann Arbor, MI, USA*

The objective of this study was to evaluate the quantitative relation between measured red blood cell acetylcholinesterase (RBC AChE) and plasma butyrylcholinesterase (BuChE) activities with exposure to chlorpyrifos (CPF) as assessed by measurement of urinary 3,5,6-trichloro-2-pyridinol (TCPy) in a study group of workers occupationally exposed in the manufacture of CPF and a referent group of chemical manufacturing workers. Measures of plasma BuChE and RBC AChE activity and urinary TCPy concentration collected over a year-long study (1999–2000) in CPF-exposed workers ($n=53$) and referents ($n=60$) were analyzed using linear mixed models to characterize exposure–response relationships. Intraindividual variability in cholinesterase measures was compared between CPF-exposed workers and referents. Urinary TCPy concentrations in CPF workers were substantially elevated compared to referents, with median and 95th percentile concentrations during typical employment conditions 10-fold and more than 30-fold higher, respectively, than corresponding measures in the referents. Intraindividual variability in cholinesterase activities was substantial, with 17% of unexposed referents experiencing one or more plasma BuChE measures more than 20% below baseline over a year of repeated, periodic measurements. RBC AChE activity, an early biomarker of effect, was unrelated to urinary TCPy concentration over the entire range of exposure, up to 1000 μg TCPy/g creatinine (Cr). Plasma BuChE activity, a non-adverse biomarker of exposure, was negatively related to urinary TCPy concentrations above approximately 110 μg TCPy/g Cr. No-effect levels for inhibition of plasma BuChE and RBC AChE corresponding to absorbed doses of CPF of approximately 5 and greater than 50 μg/kg/day, respectively, were identified. These findings are consistent with previous no-effect level determinations for ChE inhibition in humans and suggest that general population CPF exposure levels are substantially below the identified no-effect levels. The dose–response relationships observed in this study are consistent with predictions from the previously published physiologically based pharmacokinetic/pharmacodynamic model for CPF. Intraindividual variability in measured cholinesterase activities in referents was substantial, suggesting that ongoing monitoring programs may have a substantial rate of false positives.
Journal of Exposure Science and Environmental Epidemiology (2009) **19,** 634–642; doi:10.1038/jes.2008.51; published online 20 August 2008

Keywords: *cholinesterase inhibition, chlorpyrifos, urinary 3;5;6-trichloro-2-pyridinol, dose–response assessment.*

Introduction

Chlorpyrifos (CPF) and related organophosphate insecticides can inhibit B-esterases, resulting in neurological effects at exposures sufficient to substantially inhibit brain acetylcholinesterase (AChE) activity (reviewed in Zhao et al., 2006). Inhibition of red blood cell (RBC) AChE activity is recognized as an early biological marker of this critical effect in numerous current risk assessments of CPF, with substantial inhibition of RBC AChE activity occurring at exposures below those resulting in inhibition of brain AChE activity (ATSDR, 1997; USEPA, 2000; van Gemert et al., 2001; Health Canada, 2003; UK ACP, 2003; WHO, 2004; Zhao et al., 2005, 2006). Plasma butyrylcholinesterase (BuChE) activity, which constitutes 99% of human plasma B-esterase activity (van Gemert et al., 2001), can be inhibited at exposures below those required to inhibit RBC AChE, but no adverse biological effects are causally associated with the inhibition of plasma BuChE (Nolan et al., 1984; Lotti, 1995; Albers et al., 2007). However, changes in cholinesterase (ChE) activity can occur in response to exposure to other compounds as well, so monitoring ChE activity is not specific to CPF.

1. Address all correspondence to: Dr. Lesa L. Aylward, Summit Toxicology, LLP, 6343 Carolyn Drive, Falls Church, VA 22044, USA. Tel.: +1 703 349 3515. Fax: +1 303 747 0286.
E-mail: laylward@summittoxicology.com
Received 2 June 2008; accepted 25 July 2008; published online 20 August 2008

Monitoring of ChE activity through measurement of RBC AChE activity is recommended by the American Conference of Governmental Industrial Hygienists (ACGIH) for workers potentially exposed to ChE inhibitors and is required in certain states (see, for example, Washington State Department of Labor and Industries, 2008, requirement for plasma BuChE monitoring) for individuals who mix, load or apply organophosphate pesticides including CPF. The action levels for BuChE inhibition in Washington state trigger risk management activities to evaluate and reduce routes of exposure (20% reduction from baseline) or remove the worker from exposure (40% reduction from baseline).

3,5,6-Trichloro-2-pyridinol (TCPy) is a metabolite of CPF and chlorpyrifos methyl. Its presence in urine is regarded as a specific marker for exposure to these compounds in the occupational setting, although in the general population it is also a marker for direct exposure to TCPy, mostly from TCPy in food (Morgan et al., 2005). Urinary TCPy is used to assess exposure to CPF in the population at large (Barr et al., 2005; CDC, 2005) and among applicators and their families (Alexander et al., 2006). Urinary TCPy has been measured in epidemiological studies of children (Berkowitz et al., 2004; Eskenazi et al., 2004, 2007), pesticide applicators (Steenland et al., 2000) and manufacturers (Albers et al., 2004a). None of the studies, however, has analyzed the relationship between urinary TCPy and ChE activity, measurements that represent a range of biomarkers of exposure and effect for CPF.

There exists a continuum between external exposure to a chemical and an eventual resulting clinical effect (Figure 1; NRC, 2006). Biological monitoring can provide information regarding internal doses or early biological effects (which are most informative as biomarkers of exposure) or of biological responses corresponding to altered structure or function preceding an adverse effect (biomarkers of response).

In the context of the continuum between external exposure and adverse effects as presented in the NRC report on biological monitoring (Figure 1), inhibition of plasma BuChE activity is best regarded as an early biological response, which falls within the category of biomarkers of exposure because no adverse effects are associated with inhibition of BuChE. RBC AChE inhibition, in contrast, is regarded as an early precursor event to the critical effect of interest, inhibition of brain AChE activity. As such, RBC AChE can best be regarded as an early biomarker of effect, and in this context has been recognized as an appropriate and conservative basis for establishment of a point of departure for risk assessments of CPF and other AChE inhibitors.

A longitudinal study of neurological end points in CPF workers has been previously reported (Burns et al., 2006; Albers et al., 2004a, b, c, 2007). In addition to the comprehensive neurological examination components that were the focus of the study, the researchers compiled a unique data set including measures of plasma BuChE and RBC AChE activities and urinary TCPy excretion collected repeatedly over the course of a year in a population with documented occupational exposures and a reference population. These data provide an opportunity to assess the quantitative relationship between two biomarkers of exposure: plasma BuChE activity, which is used in the occupational environment, and urinary TCPy concentration, which is often measured in general population biomonitoring efforts (CDC, 2005). In addition, this robust data set provides real-world human exposure–response data for RBC AChE inhibition, allowing a direct comparison to the human no-effect levels established in previous controlled clinical studies using small numbers of individuals. Finally, these data provide a basis for comparison with and evaluation of the predictions of the previously developed physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model developed by Timchalk et al. (2002b).

Methods

Study Population

The study population was previously described in publications by Burns et al. (2006) and Albers et al. (2004a, b, c, 2007). Briefly, the study group consisted of 53 CPF manufacturing workers and a referent group of 60 workers

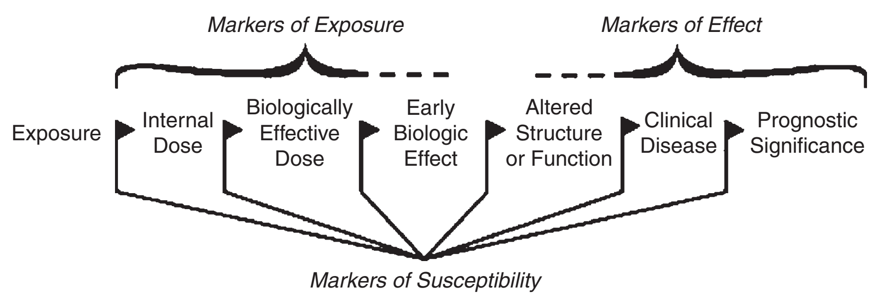

Figure 1. Continuum of biological markers of exposure and effect. Measures of red blood cell acetylcholinesterase (RBC AchE) activity fall into the category of early biomarkers of effect relevant to clinical outcomes, whereas measures of plasma butyrylcholinesterase (BuChE) activity fall into the category of biomarkers of exposure. Figure from NRC (2006), reprinted with permission from the National Academies Press, Copyright 2006, National Academy of Sciences.

Table 1. Comparison of gender, age, anthropometric and other data for CPF ($n = 53$) and referent ($n = 60$) subjects at the baseline examination (from Albers et al., 2004c).

Variable	CPF % ($n = 53$)	Referent % ($n = 60$)	*P*-value
Gender			0.62
Female	22.6	26.7	
Male	77.4	73.3	
Body mass index >29.0	50.9	43.3	0.45
	Mean (SD)	Mean (SD)	*P*-value
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
Smoking (pack years)	8.2 (15.8)	5.9 (9.5)	0.37

in the Saran manufacturing process with no occupational exposure to organophosphate pesticides or ChE inhibitors (Table 1). All participants underwent physical examinations approximately 1 year apart in the fall of 1999 and the fall of 2000. As part of the physical examinations, first morning void urine samples were collected and analyzed for TCPy.

At these two examinations, blood samples were collected and analyzed for AChE activity in all participants, and for BuChE activity in referents. In CPF workers, BuChE activities were monitored approximately monthly in a separate, ongoing biological monitoring program. This program also included a preexposure measurement of plasma BuChE activity (a "baseline" measurement) for each individual employed on the CPF process. In the referents, BuChE activities were also measured approximately monthly between the fall 1999 and fall 2000 examinations.

In addition to the two measurements made during the physical examinations, TCPy measurements were also made on first morning void urine samples collected during the spring of 2000, a time period when investigators hypothesized that pesticide use may have occurred outside the occupational environment (e.g., in agriculture). TCPy was also measured in urine samples collected during the annual fall 2000 plant shutdown and maintenance period (called "turnaround") under the assumption that atypical exposures could occur during such activities.

Analytical Methods

Urinary 3,5,6-Trichloro-2-pyridinol: First morning void urine samples were collected by each test subject, using a container provided by The Dow Chemical Company (Dow). Samples were stored in a $-20°C$ freezer until analysis and then were analyzed for TCPy and creatinine (Cr) by personnel at Dow using GC/MS and HPLC/UV methods according to Dow method AL no. 2000-7. In addition, spiked samples, spanning a TCPy concentration range of 0.026–0.260 μg/ml, submitted by the University of Michigan, were analyzed for stability determination. The limit of quantitation for this method was defined as 0.002 μg/ml.

Plasma BuChE Activity: Blood was collected in a capillary tube by finger-stick and the analyses were performed at Mid-Michigan Regional Medical Center, Midland, MI. Analysis of plasma BuChE was performed using the VITROS CHE Slides and the VITROS Chemistry Products Calibrator Kit 6 on VITROS Chemistry Systems (Johnson & Johnson). The VITROS CHE Slide is a multilayered, analytical element coated on a polyester support. A drop of patient sample is deposited on the slide and is evenly distributed by the spreading layer to the underlying layers. ChE hydrolyzes butyrylthiocholine to thiocholine. The liberated thiocholine reduces potassium hexacyanoferrate III (potassium ferricyanide) to potassium hexacyanoferrate II (potassium ferrocyanide). The rate of color loss is monitored by reflectance spectrophotometry. The rate of change in reflection density is proportional to the ChE activity in the sample.

RBC AChE Activity: Venous blood (5 ml) was drawn into a lavender top Vacutainer tube preserved with EDTA and shipped on ice to Labcorp, 1447 York Street, Burlington, NC 27215 for analysis. AChE activity was measured in a hemolysate of washed RBCs. Enzyme activity was measured using an acetylthiocholine ester as the substrate. Released thiocholine reacts with Ellman's reagent (dithiobis nitrobenzoic acid), releasing 5-mercapto-2-nitro benzoic acid, which was measured photometrically.

Statistical Analysis

Intraindividual Variation in BuChE Activity: A primary interest in monitoring BuChE activity in this study was to assess the intrasubject variation (fluctuations of ChE measurements within the same subject). The intrasubject variance was calculated for each participant. Then, the mean, standard deviation and coefficient of variation (CV) of the intrasubject variation were calculated within the CPF and referent groups and were compared between groups. An assessment was made of how often subjects experienced appreciable declines in BuChE activity from baseline over the year of testing for each group. Kaplan–Meier survival curves were calculated for referent and CPF workers, with failure defined as a 20% or greater decrease from the individual's baseline BuChE activity.

Plasma BuChE in Relation to Urinary TCPy: Each subject had a series of measurements of BuChE activity and TCPy excretion over time, as well as fixed covariates: age, sex, body mass index, smoking habits, drinking habits, examination time (fall 1999, spring 2000 and fall 2000), paraoxonase 1 (PON1) phenotype and chlorpyrifos oxonase activity. To assess the relationship between CPF exposure and BuChE activity, each subject's measurements of TCPy and BuChE that were taken within 7 days of each other were selected. This time period reflects the recognition that TCP is eliminated in urine with a half time of approximately 27 h, so that measurement of urinary TCP on any given day reflects exposures occurring within the previous 3–5 days (Nolan et al., 1984). Similarly, plasma BuChE activity recovers from inhibition with a half time for recovery of approximately 8–9 days, with relatively complete recovery from acute inhibition expected within approximately a month (Nolan et al., 1984; Timchalk et al., 2002b).

The outcome of interest was the ratio of measured BuChE to preexposure baseline BuChE (in CPF workers) or to the first measure of BuChE (in the referent workers), referred to subsequently as "BuChE ratio". The marginal association between the BuChE ratio and ln(TCPy) was examined using scatter plots, which suggested that the BuChE ratio was constant as TCPy increased to approximately 100 μg/g Cr, then decreased as TCPy increased further (Figure 5). To properly describe this relationship, piecewise linear mixed models were used. As the piecewise model demonstrated a slight, non-significant positive relationship between the BuChE ratio and ln(TCPy) at low ln(TCPy) values, and there was no biological explanation for this, we forced a flat slope at low values and allowed the piecewise regression to estimate the slope and cutpoint that best fit the data (measured by the smallest Aikake's Information Criterion). Forward stepwise selection of covariates was used, with the criterion of $P<0.05$ for retention of covariates in the model. Statistical analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC, USA). The fall 2000 turnaround samples were omitted from linear mixed models because of the dynamic nature of potential exposures during this period compared to the more consistent exposures during normal operations during the rest of the year.

RBC AChE in Relation to Urinary TCPy: RBC AChE activities were measured at two time points directly coincident with the measurement of urinary TCPy concentrations (during the two physical examinations) in all study participants. Linear mixed models were used to estimate the change in AChE activity over time in relation to urinary TCPy excretion while controlling for covariates (as described above). Because no "baseline" AChE measures were collected for CPF workers, the modeling was conducted on the raw activity data.

Estimation of Absorbed CPF Dose: Under steady-state CPF exposure conditions, and with the assumption that all TCPy in urine is derived from exposure to CPF, the amount of TCPy excreted daily in the urine is equal (on a molar basis) to the moles of CPF absorbed systemically. To estimate the absorbed daily dose of CPF in μg/kg bodyweight per day associated with a given urinary concentration of TCPy (μg/g Cr), the following equation can be used:

$$\text{CPF daily dose} = \text{TCP} \times \frac{\text{CPF}_{MW}}{\text{TCP}_{MW}} \times CR \times \frac{1}{BW} \qquad (1)$$

where CPF_{MW} and TCP_{MW} are the molecular weights of CPF (350.6) and TCPy (198.4), respectively, CR the daily Cr excretion rate in g/day, and BW bodyweight in kg.

Predicted dose–response relationships for the inhibition of plasma BuChE, RBC AChE and brain AChE as a function of urinary TCPy excretion under conditions of steady-state exposure were generated using the previously developed PBPK/PD model for CPF from Timchalk et al. (2002b). The model was run to steady state at a range of absorbed doses from 0.03 to 3000 μg/kg/day, and the percent inhibition of each ChE as a function of urinary TCPy excretion at each steady-state dose rate was recorded.

Results

The age, gender distribution and anthropometric characteristics of the CPF and referent groups were similar (Table 1). The measured urinary TCPy concentrations at each time period in the CPF and referent workers are presented in Table 2. The median TCPy concentrations in CPF workers ranged between 37 and 59 μg/g Cr over the four time points, whereas the 95th percentiles of non-turnaround TCPy concentrations ranged from 346 to 987 μg/g Cr. TCPy concentrations measured during the plant turnaround period included measurements that were far higher, with the 95th percentile at 7403 μg/g Cr. Urinary TCPy measures in referent workers were relatively consistent over the course of the study, with median values between 3.2 and 6.6 μg/g Cr

Table 2. Creatinine-adjusted urinary TCPy concentrations (μg TCP/g Cr in first morning void samples).

Group	TCPy collection period	*n*	Mean (SD)	Percentiles		
				5th	50th	95th
CPF	Fall 1999	53	108.6 (145.7)	6.7	37.5	432.6
	Spring 2000	53	110.6 (180.7)	5.0	40.7	346.3
	Turnaround[a]	50	1290.9 (3684.4)	9.7	59.2	7403.8
	Fall 2000	52	196.9 (387.0)	8.9	51.5	987.2
Referent	Fall 1999	60	4.3 (3.8)	1.0	3.2	13.9
	Spring 2000	57	5.0 (2.7)	1.6	4.8	11.2
	Turnaround	57	7.1 (3.0)	2.8	6.6	13.0
	Fall 2000	58	7.6 (4.4)	1.8	6.3	15.5

[a]Annual plant shutdown and maintenance period.

Table 3. Descriptive statistics for serum BuChE activity by group.

Group	Baseline		Intrasubject variation		
	Mean	SD	Mean	SD	CV (%)
CPF	7914	1631	733,333	555,412	76
Referent	8113	1737	427,670	297,277	70

and 95th percentiles all below 16 μg/g Cr at the four time points.

Multiple measures of BuChE activity over the study year were available for nearly all participants. Among CPF workers, the median number of measurements was 12 (25–75th percentile, 8–14 measurements). Among referents, the median number of measurements was 11 (25–75th percentile, 9.5–12 measurements). The BuChE activity was similar between the CPF and referent groups at baseline (Table 3). However, on average, the CPF workers had greater intrasubject variation than the referent workers (mean intrasubject variation = 733,333 for CPF *versus* 427,670 for referents). In addition, the standard deviation of the intrasubject variation was larger among the CPF workers (555,412) than among the referents (297,277). Among the referents, the intraindividual variation represents the test variation in the absence of exposure, which is similar across individuals. In contrast, in the CPF workers the intraindividual variation was larger because response to CPF exposure also contributed to the observed variation. The distribution of BuChE activity in referent workers is of particular interest because there is little published data on variation in BuChE activity within subjects among working populations not exposed to ChE-inhibiting chemicals; these data provide an opportunity to understand the inherent intrasubject variation in this measurement.

Kaplan–Meier survival plots for referent and CPF subjects indicate that over the year of follow-up, 17% of referent workers had at least one measured BuChE that was lower than the baseline measure by 20% or more (Figure 2). This indicates that even in the absence of any known exposure to ChE-inhibiting agents, a substantial proportion of subjects had substantial changes in measured BuChE activity, adequate to require employers to take protective actions in some jurisdictions. Of the 53 CPF workers, 3 workers either had no BuChE measurements or obtained only their initial measurement during the year of observation. For 13 of the remaining 50 CPF workers, the initial measurement during the year of observation was 20% or more below their baseline. Of the remaining 37 CPF workers, 43% experienced a measured BuChE activity that was 20% or more below baseline during the year of observation, a substantially greater proportion than among the referent workers.

The PBPK/PD model predictions of inhibition of plasma BuChE, RBC AChE and brain AChE activities as a function of urinary TCPy excretion at steady-state exposure in humans are presented in Figure 3. The model predicts that no inhibition of RBC AChE activity will be observed until exposure levels sufficient to produce substantial inhibition of plasma BuChE activity (greater than 50% inhibition) are attained. Inhibition of brain AChE activity occurs at higher exposure levels, with inhibition predicted to begin to occur only at doses at which complete inhibition of plasma BuChE activity is observed.

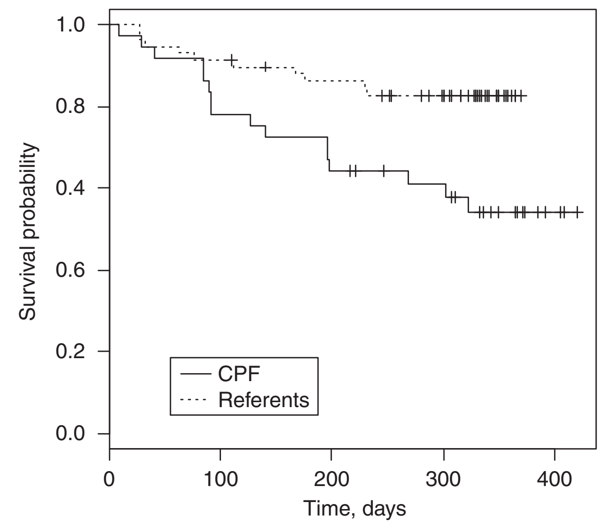

Figure 2. Kaplan–Meier survival plot of plasma butyrylcholinesterase (BuChE) activity during a year of follow-up with repeated measurements for chlorpyrifos (CPF) and referent workers. Failure events are defined as a measured plasma BuChE 20% or more below baseline.

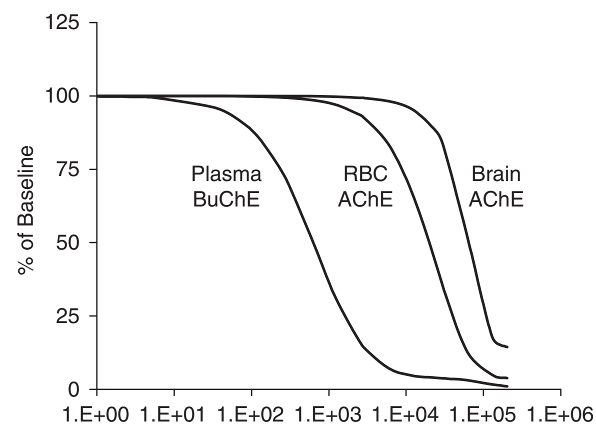

Figure 3. Predicted dose–response for inhibition of plasma butyrylcholinesterase (BuChE), red blood cell acetylcholinesterase (RBC AchE) and brain AChE from the Timchalk et al. (2002a, b) pharmacokinetic and pharmacodynamic (PBPK/PD) model under steady-state exposure conditions as a function of urinary 3,5,6-trichloro-2-pyridinol (TCPy) concentration.

RBC AChE Activity in Relation to Urinary TCPy

As previously reported, there were no statistically significant differences in RBC AChE activity between CPF and referent

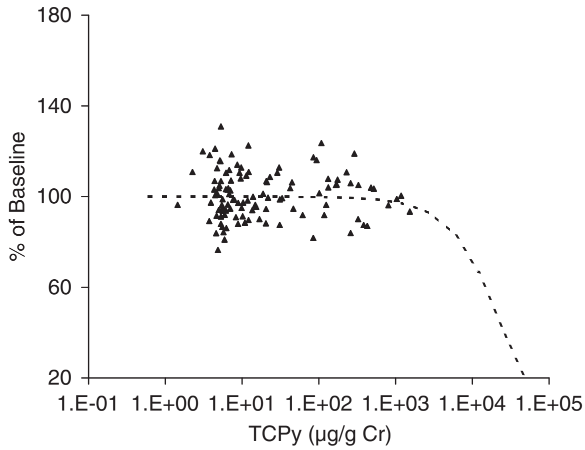

Figure 4. Paired acetylcholinesterase (AchE) scatter plot with pharmacokinetic and pharmacodynamic (PBPK/PD) predicted dose–response (dashed line) under steady-state exposure conditions. Measured AChE data are plotted as a ratio of the second to first measure for comparison to PBPK/PD model predictions, although regression modeling was conducted on raw AChE activity data.

workers at either examination (Albers et al., 2004c). No relation between urinary TCPy concentration and RBC AChE activity was observed over the entire exposure range, nor was there any evidence of a cutpoint where AChE declined with increasing TCPy (Figure 4). The linear mixed model found no relationship between AChE and ln(TCPy) ($P = 0.91$). Small but statistically significant effects of exam date (fall 2000 was 344 mU/ml higher than fall 1999, $P = 0.0004$) and current smoking (smokers were 399 mU/ml higher than non-smokers, $P = 0.01$) were identified in regression analyses, but none of the other covariates examined were significantly related to RBC AChE activity.

Plasma BuChE Activity in Relation to Urinary TCPy

Various time windows ranging from 5 to 14 days for paired BuChE and TCPy samples were examined. Requiring sample collections for the two parameters within 7 days of one another resulted in an optimal combination of sample size (161 paired measures: 84 derived from 44 CPF workers and 77 derived from 49 referent workers) and model performance (results not shown). The ratio of current BuChE to baseline BuChE was inversely related to the natural logarithm transformed TCPy (ln(TCPy); Table 4). The intercept was 0.97 (SE = 0.02), and there was no statistically significant relationship between TCPY and BuChE ratio up to the cutpoint of ln(TCPy) = 4.7 (corresponding to 110 μg TCPy/g Cr). Above this cutpoint, for each unit increase in ln(TCPy) the BuChE ratio decreased by 21% ($P < 0.0001$). There was a small effect of season, such that the BuChE ratio was 0.04 ($P = 0.06$) and 0.05 ($P = 0.01$) units higher in fall 2000 and fall 1999, respectively, than in spring 2000.

Table 4. Linear mixed model for plasma BuChE ratio as a function of ln(TCPy) and covariates.

Parameter	Parameter estimate	SE	*P*-value
Intercept	0.97	0.02	<0.0001
Ln(TCPy) (>cutpoint)[a]	−0.21	0.02	<0.0001
Season (fall 2000)[b]	0.04	0.02	0.06
Season (fall 1999)[b]	0.05	0.02	0.01

[a]Cutpoint occurred at urinary TCPy concentrations of 110 μg/g Cr (ln TCPy = 4.7).
[b]Spring 2000 as reference.

The measured urinary TCPy concentrations in this study range up to approximately 1000 μg/g Cr. Using Eq. (1) and the method of Mage et al. (2004) to estimate Cr excretion rates, and assuming that all urinary TCPy is due to CPF exposure, the estimated absorbed daily dose rate of CPF associated with a measured TCPy concentration under steady-state conditions can be estimated. On the basis of this relationship and the average height and weight of individuals in this study, a measured urinary TCPy concentration of 1000 μg/g Cr would correspond to an absorbed CPF dose of approximately 50 μg/kg/day; and in general, an estimate of approximately 1 μg/kg/day absorbed dose of CPF would be attributable to each increment of 20 μg/g Cr urinary TCPy.

Discussion

The data regarding ChE activity and urinary TCPy concentration collected as part of a comprehensive longitudinal study of neurological end points in CPF and referent workers and analyzed here present an opportunity to evaluate the exposure–response relationships between ChE activity and urinary TCPy as a marker of exposure to CPF (Burns et al., 2006; Albers et al., 2004a, b, c, 2007). No relationship between urinary TCPy and RBC AChE inhibition was observed over the entire range of exposures encountered in this occupationally exposed population, despite the substantial sample size, the collection of repeated measures of AChE activity and urinary TCPy, and the substantial CPF exposures experienced by portions of the study population. The range of urinary TCPy excretion measured here indicates absorbed doses of CPF up to approximately 50 μg/kg/day in this population over the course of the study period under the normal plant operating conditions; higher exposures were encountered during the plant turnaround activities, as indicated by higher urinary TCPy measures. The lack of any observed relation between urinary TCPy and RBC AChE inhibition in the exposure range up to 50 μg/kg/day encountered during the normal plant operations is consistent with the previous small controlled human studies identifying 100 μg/kg/day of CPF as a no-effect level for RBC AChE inhibition (reviewed in van Gemert et al., 2001). Similarly, a no-effect level for RBC AChE in dogs following chronic dietary exposure has also been estimated at 100 μg/kg/day (Mattsson et al., 2001). In turn, the lack of effect on RBC AChE activity observed in this population is also consistent with the finding of no differences in neurological function related to exposure in the exposed workers compared to the referents (Albers et al., 2004a, b, c, 2007).

With respect to plasma BuChE inhibition, the no-effect level or cutpoint of 110 μg TCPy/g Cr identified in the modeling is consistent with an absorbed steady-state CPF dose of approximately 5 μg/kg/day (see Figure 5). This result is consistent with an early human clinical experiment, in which a no-effect level for plasma BuChE inhibition of 7 μg CPF/kg/day oral dose was estimated based on a repeated dosing study in human volunteers (Coulston et al., 1972). Assuming 70% absorption by the oral route of exposure (Nolan et al., 1984), this dose rate corresponds to approximately 5 μg/kg/day absorbed dose, essentially identical with the cutpoint identified in the modeling here.

Finally, the dose–response data presented here for both plasma BuChE and RBC AChE display striking consistency with the predictions of the PBPK/PD model of Timchalk et al. (2002b) (see Figures 4 and 5). These data can be regarded as providing validation for the PBPK/PD model in the exposure ranges encountered in this study, and support use of the model in other contexts.

Substantial inter- and intraindividual variability in measured AChE and BuChE activity among referent workers not occupationally exposed to CPF was observed in this study. The ACGIH Biological Exposure Index for AChE inhibitors sets a threshold of 30% reduction from baseline of RBC AChE in the occupational environment, noting that no adverse neurological consequences are expected until inhibition of RBC AChE exceeds that benchmark and that variation of up to that degree can occur as a result of normal variability (ACGIH, 2001). The data in this study are consistent with that degree of intraindividual variability. On the basis of data presented here for the referent workers, it appears that plasma BuChE exhibits similar variability, with 17% of the referents experiencing one or more measured plasma BuChE values 20% or more below baseline over the course of a year of repeated, periodic measurements. This suggests that use of a threshold of 20% inhibition of plasma BuChE activity from baseline to indicate elevated occupational exposures will result in a substantial rate of false positives over time.

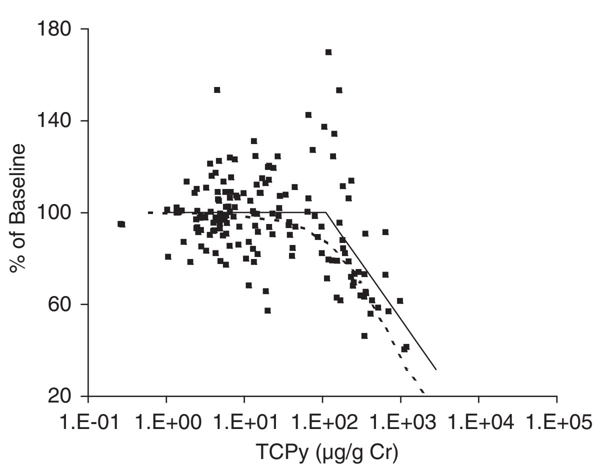

Figure 5. Paired butyrylcholinesterase (BuChE) scatter plot with pharmacokinetic and pharmacodynamic (PBPK/PD) predicted dose–response (dashed line) and linear mixed model (solid line) results.

The lack of any relationship between RBC AChE and urinary TCPy concentrations over the range of exposures in this study is relevant to the interpretation of general population biomonitoring data. Urinary TCPy excretion in the general population is being measured as part of the ongoing CDC National Exposure Reports (CDC, 2005). In the most recent report, the upper 95th percentile of urinary TCPy concentration in adults was approximately 10 μg/g Cr in urinary samples collected during 2001–2002. No relation between urinary TCPy and RBC AChE was observed in the current study up to urinary TCPy concentrations of approximately 1000 μg/g Cr. Thus, these results suggest a margin of exposure of 100 or more between current CPF exposures in the general population and exposures resulting in no effect on RBC AChE. However, the actual margin of exposure between general population exposure and the upper bound exposures to CPF (with no effect on RBC AChE) in this study is likely to be substantially higher due to non-CPF contributions to urinary TCPy in the general population.

Urinary TCPy is regarded as a relatively specific marker of exposure to CPF and chlorpyrifos methyl (Barr and Angerer, 2006). However, urinary excretion of TCPy can also occur following exposure to TCPy itself, because TCPy occurs in foods and the environment as a relatively stable degradate of CPF, but is not the moiety responsible for ChE inhibition. A recent study quantified exposures to CPF and TCPy from diet and inhalation and dermal contact in a group of preschool-aged children (Morgan et al., 2005). The authors found that preschool-aged children had aggregate exposure of 3 ng/kg/day of CPF and 38 ng/kg/day of TCPy from all sources combined, with the majority of aggregate exposure occurring due to dietary exposures. In addition, the authors found that these combined sources of CPF and TCPy exposures could only account for approximately 30% of the daily TCPy excreted in urine (Morgan et al., 2005). Recent experimental work in rats has confirmed that TCPy can be directly absorbed and excreted in urine unchanged (Timchalk et al., 2007). Taking this information into account, Barr et al. (2005) assumed conservatively that 20% of urinary TCPy in the general population was due to CPF exposure (although the Morgan et al. (2005) data suggest this proportion may be as low as 3–10%). Using this assumption, Barr et al. (2005) estimated that 95th percentile intakes of CPF in general population children, adolescents and adults based on the NHANES data sets were 0.06, 0.034 and 0.036 μg/kg/day, respectively. These exposures are 1000-fold or more below the upper end of estimated exposures in this study, exposures that were associated with no inhibition of RBC AChE.

Several limitations are inherent in this study. First, because evaluation of the relationship between ChE activity and urinary TCPy was not the primary goal of this study, the study design did not call for simultaneous collection of blood for plasma BuChE measures and urine samples. However, the samples for measurement of RBC AChE activity were collected on the same day as the corresponding urine samples. Second, because the study population included only workers from the CPF or Saran manufacturing processes, no children, elderly or otherwise infirm individuals were included in the study, and thus, no assessment of potentially increased sensitivity in such populations could be made. The study design could not address all sources of potential interindividual differences in sensitivity to CPF-induced ChE inhibition, although at least one factor was included in this analysis. Previous research has indicated a relationship between PON1 phenotype and sensitivity to CPF-induced ChE inhibition (reviewed in Furlong 2007), indicating a marker for potentially sensitive subpopulations, but in these analyses PON1 phenotype was not a significant covariate in the regression models. This result is consistent with previous analyses using the PBPK/PD model, which suggested little impact of PON1 phenotype on responses to CPF at the exposure levels observed in this study (Timchalk et al., 2002a). Third, no baseline RBC AChE measures were collected for the CPF workers, so no direct evaluation of a change from preemployment baseline on an individual basis could be made. However, the inclusion of a referent population and evaluation of the relationship between absolute RBC AChE activity and urinary TCPy across both the exposed and referent populations mitigates this limitation. Finally, estimations of daily CPF doses corresponding to the measured TCPy concentrations presented here are approximate due to the use of spot urine samples, estimates of average Cr excretion and the assumption of steady-state exposures.

The data presented here, collected prospectively over time from a real-world occupational population that includes a substantial proportion of women, provide a robust data set confirming a lack of biologically relevant response to CPF at exposures substantially above general population exposures. Although this data set does not include children, elderly or other potentially sensitive subpopulations, the substantial margin of exposure suggested here based on biological monitoring indicates that general population exposures to CPF are likely to be below exposures associated with inhibition of RBC AChE (the basis of current risk assessments for CPF), and by extension, below exposures associated with inhibition of brain AChE, even in such subpopulations. These data also provide a quantitative relation between monitored BuChE activity and absorbed CPF dose in the occupational environment at elevated CPF exposures, confirming the use of BuChE as a biomarker of exposure in this context.

Acknowledgements

Additional investigators on this project included Richard P. Garrison, Brenda Gillespie, Bruno Giordani, Jonathon Raz (deceased), Sarah S. Cohen, Camelia Sima and other members of the Neurobehavioral Toxicology Program Chlorpyrifos Study team including Jennifer N. Baughman, Nathan Bradshaw and Zhuolin Li. We acknowledge the Dow and Dow AgroSciences employees who assisted at various points in supporting this research. Finally, we are indebted to the Dow employees who volunteered their time as subjects in this study.

Research support: This study was financially supported by Dow Agrosciences, Indianapolis, Indiana, with additional support from The Dow Chemical Company and Dow Chemical Company Foundation, which included a SPHERE (Supporting Public Health and Environmental Research Efforts) Award. Model simulations were supported by grants R01 OH003629 and R01 OH008173 from the Centers for Disease Control and Prevention (CDC). The contents of this article are solely the responsibility of the authors and have not been subject to review by CDC and therefore do not necessarily represent the official view of CDC, and no official endorsement should be inferred.

Disclosure

Some of the authors have received research support and at times been retained as consultants or served as expert witnesses in litigation for firms or companies, including Dow and Dow AgroSciences, concerned with the manufacture or use of insecticides. Support of these activities has included both personal and institutional remuneration.

References

Agency for Toxic Substances and Disease Registry (ATSDR). *Toxicological Profile for Chlorpyrifos*. US Department of Health and Human Services, Public Health Service, Atlanta, GA, 1997.

Albers J.W., Berent S., Garabrant D.H., Giordani B., Schweitzer S.J., Garrison R.P., *et al.* The effects of occupational exposure to chlorpyrifos on the neurologic examination of central nervous system function: a prospective cohort study. *J Occup Environ Med* 2004a: 46(4): 367–378.

Albers J.W., Garabrant D.H., Mattsson J.L., Burns C.J., Cohen S.S., Sima C., *et al.* Dose–effect analyses of occupational chlorpyrifos exposure and peripheral nerve electrophysiology. *Toxicol Sci* 2007: 97(1): 196–204.

Albers J.W., Garabrant D.H., Schweitzer S., Garrison R.P., Richardson R.J., and Berent S. Absence of sensory neuropathy among workers with occupational exposure to chlorpyrifos. *Muscle Nerve* 2004b: 29(5): 677–686.

Albers J.W., Garabrant D.H., Schweitzer S.J., Garrison R.P., Richardson R.J., and Berent S. The effects of occupational exposure to chlorpyrifos on the peripheral nervous system: a prospective cohort study. *Occup Environ Med* 2004c: 61(3): 201–211.

Alexander B.H., Burns C.J., Bartels M.J., Acquavella J.F., Mandel J.S., Gustin C., *et al.* Chlorpyrifos exposure in farm families: results from the farm family exposure study. *J Expo Sci Environ Epidemiol* 2006: 16(5): 447–456.

American Conference of Governmental Industrial Hygienists (ACGIH). *Acetylcholinesterase Inhibiting Pesticides. Documentation of the BEIs*. ACGIH, Cincinnati, OH, 2001.

Barr D.B., Allen R., Olsson A.O., Bravo R., Caltabiano L.M., Montesano A., *et al.* Concentrations of selective metabolites of organophosphorus pesticides in the United States population. *Environ Res* 2005: 99(3): 314–326.

Barr D.B., and Angerer J. Potential uses of biomonitoring data: a case study using the organophosphorus pesticides chlorpyrifos and malathion. *Environ Health Perspect* 2006: 114(11): 1763–1769.

Berkowitz G.S., Wetmur J.G., Birman-Deych E., Obel J., Lapinski R.H., Godbold J.H., *et al. In utero* pesticide exposure, maternal paraoxonase activity, and head circumference. *Environ Health Perspect* 2004: 112(3): 388–391.

Burns C.J., Garabrant D., Albers J.W., Berent S., Giordani B., Haidar S., *et al.* Chlorpyrifos exposure and biological monitoring among manufacturing workers. *Occup Environ Med* 2006: 63(3): 218–220.

Centers for Disease Control (CDC). *Third National Report on Human Exposure to Environmental Chemicals*. National Center for Environmental Health, Division of Laboratory Sciences, Atlanta, Georgia, 30341-3724, NCEH Pub. No. 05-0570 2005.

Coulston F., Goldberg L., and Griffin T. *Safety Evaluation of Dowco 179 in Human Volunteers*, Unpublished report from the Institute of Experimental Pathology and Toxicology, Albany Medical College. Albany, NY. MRID No. 00030754, 00095175 1972.

Eskenazi B., Harley K., Bradman A., Weltzien E., Jewell N.P., Barr D.B., *et al.* Association of *in utero* organophosphate pesticide exposure and fetal growth and length of gestation in an agricultural population. *Environ Health Perspect* 2004: 112(10): 1116–1124.

Eskenazi B., Marks A.R., Bradman A., Harley K., Barr D.B., Johnson C., *et al.* Organophosphate pesticide exposure and neurodevelopment in young Mexican-American children. *Environ Health Perspect* 2007: 115(5): 792–798.

Furlong C.E. Genetic variability in the cytochrome P450-paraoxonase 1 (PON1) pathway for detoxication of organophosphorus compounds. *J Biochem Mol Toxicol* 2007: 21(4): 197–205.

Health Canada. *Proposed Acceptability for Continuing Registration: Phase 2 of the Re-evaluation of Chlorpyrifos*. Health Canada, Ottawa, Canada,PACR2003-03 2003.

Lotti M. Cholinesterase inhibition: complexities in interpretation. *Clin Chem* 1995: 41(12 Part 2): 1814–1818.

Mage D.T., Allen R.H., Gondy G., Smith W., Barr D.B., and Needham L.L. Estimating pesticide dose from urinary pesticide concentration data by creatinine correction in the Third National Health and Nutrition Examination Survey (NHANES-III). *J Expo Anal Environ Epidemiol* 2004: 14(6): 457–465.

Mattsson J.L., Holden L., Eisenbrandt D.L., and Gibson J.E. Reanalysis with optimized power of red blood cell acetylcholinesterase activity from a 1-year dietary treatment of dogs to chlorpyrifos. *Toxicology* 2001: 160(1–3): 155–164.

Morgan M.K., Sheldon L.S., Croghan C.W., Jones P.A., Robertson G.L., Chuang J.C., *et al.* Exposures of preschool children to chlorpyrifos and its degradation product 3,5,6-trichloro-2-pyridinol in their everyday environments. *J Expo Anal Environ Epidemiol* 2005: 15(4): 297–309.

National Research Council (NRC). *Human Biomonitoring for Environmental Chemicals. Committee on Human Biomonitoring for Environmental Toxicants, Board on Environmental Studies and Toxicology, Division on Earth and Life Studies*. National Academies Press, Washington, DC, 2006.

Nolan R.J., Rick D.L., Freshour N.L., and Saunders J.H. Chlorpyrifos: pharmacokinetics in human volunteers. *Toxicol Appl Pharmacol* 1984: 73(1): 8–15.

Steenland K., Dick R.B., Howell R.J., Chrislip D.W., Hines C.J., Reid T.M., *et al.* Neurologic function among termiticide applicators exposed to chlorpyrifos. *Environ Health Perspect* 2000: 108(4): 293–300.

Timchalk C., Busby A., Campbell J.A., Needham L.L., and Barr D.B. Comparative pharmacokinetics of the organophosphorus insecticide chlorpyrifos and its major metabolites diethylphosphate, diethylthiophosphate and 3,5,6-trichloro-2-pyridinol in the rat. *Toxicology* 2007: 237(1–3): 145–157.

Timchalk C., Kousba A., and Poet T.S. Monte Carlo analysis of the human chlorpyrifos-oxonase (PON1) polymorphism using a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model. *Toxicol Lett* 2002a: 135: 51–59.

Timchalk C., Nolan R.J., Mendrala A.L., Dittenber D.A., Brzak K.A., and Mattsson J.L. A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate insecticide chlorpyrifos in rats and humans. *Toxicol Sci* 2002b: 66(1): 34–53.

UK ACP (United Kingdom Advisory Committee on Pesticides) Chlorpyrifos human health review: evaluation of further papers requested by the ACP, York, UK, ACP 6 (299/03) 2003.

USEPA (United States Environmental Protection Agency). *Human Health Risk Assessment: Chlorpyrifos*. Health Effects Division, Office of Pesticide Programs, Washington, DC, June 8 2000.

van Gemert M., Dourson M., Moretto A., and Watson M. Use of human data for the derivation of a reference dose for chlorpyrifos. *Regul Toxicol Pharmacol* 2001: 33(2): 110–116.

Washington State Department of Labor and Industries. *Cholinesterase Monitoring — Employer Information*, www.lni.wa.gov/Safety/Topics/AtoZ/Cholinesterase/EmployerInfo.aspAccessed 9 May 2008 2008.

WHO (World Health Organization). *Chlorpyrifos in Drinking Water: Background Document for Development of WHO Guidelines for Drinking-Water Quality*. World Health Organization, Geneva, WHO/SDE/WSH/03.04/87 2004.

Zhao Q., Dourson M., and Gadagbui B. A review of the reference dose for chlorpyrifos. *Regul Toxicol Pharmacol* 2006: 44(2): 111–124.

Zhao Q., Gadagbui B., and Dourson M. Lower birth weight as a critical effect of chlorpyrifos: a comparison of human and animal data. *Regul Toxicol Pharmacol* 2005: 42(1): 55–63.