Spectrophotometric Determination of Plasma and Red Blood Cell Cholinesterase Activity of 53 Fruit Farm Workers Pre- and Post-Exposed Chlorpyrifos for One Fruit Crop

Anamai THETKATHUEK,[a,b] Matthew KEIFER,[c] Wijitr FUNGLADDA,[d] Jaranit KAEWKUNGWAL,[d] Chantana PADUNGTOD,[e] Barry WILSON,[f] and Samlee MANKHETKORN*,[b]

[a] *Department of Industrial Hygiene and Safety, Faculty of Public Heath, Burapha University; Bangsaen, Chonburi 20131 Thailand:* [b] *Laboratory of Physical Chemistry, Molecular and Cellular Biology, Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiangmai University; Chiangmai 50200, Thailand:* [c] *Department of Environmental and Occupational Health Sciences, Washington University; Box 357234 Seattle, WA. 98195-7234, U.S.A.:* [d] *Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University; 420/6 Rajvithi Rd, Bangkok 10400, Thailand:* [e] *Bureau of Occupational and Environmental Disease, Ministry of Public Health; Nontaburi 11000, Thailand: and* [f] *Deparment of Animal Science and Environmental Toxicology, California-Davis University; Oneshield Avenue, Davis CA 95616, U.S.A.* Received August 23, 2004; accepted January 6, 2005

We sought to investigate the early biological effects of chlorpyrifos among 53 Thai fruit farm workers by measuring the plasma cholinesterase (PChE) and red blood cell cholinesterase (AChE) activities, a biomarker of organophosphate (OPs) pesticide during one fruit crop. The ChE activity (V_m/K_m) was spectrophotometrically analyzed before and after exposing to chlorpyrifos. The V_m/K_m values of both non-spraying and spraying seasons are found as normal distribution pattern. The median PChE and AChE activities among farm workers in the non-spraying season were 2.3×10^{-3} s^{-1} and 7.26×10^{-5} s^{-1}, respectively. The median PChE and AChE activities of the farm workers in the spraying season were 2.02×10^{-3} s^{-1} and 5.95×10^{-5} s^{-1}, respectively. The mean V_m/K_m values of PChE shifted left (*t*-test, $p=0.013$), indicating a decrease in PChE activity in the farm workers exposed to chlorpyrifos. However, the V_m/K_m values of AChE in nonspraying season and in the spraying season were not different (*t*-test, $p=0.246$). We propose that PChE activity can be used as a biomarker for monitoring early toxicity induced by chlorpyrifos insecticide.

Key words chlorpyrifos; plasma cholinesterase (PChE); red blood cell cholinesterase (AChE); fruit farm worker, biomarker

Chlorpyrifos (*O*,*O*-diethyl 0-(3,5,6-trichloro-2-pyridyl) phosphorothioate) belongs to the organophosphate insecticide (OPs) family, and is widely used to kill aphids in fruit farms. The demand for this insecticide is increasing. To our knowledge, there is no biological monitoring data for fruit-farm worker exposure to chlorpyrifos insecticide is available in Thailand. Biological monitoring is an essential component of any comprehensive assessment of exposure. In fact, these farm workers were at high risk of direct exposure to pesticides while mixing, loading, or spraying pesticide.[1—4] It has been reported that chlorpyrifos insecticide is rapidly absorbed by skin contact and/or oral administration, metabolized and excreted by mammals.[5] The primary mechanism of action of organophosphorus (OP) insecticides like chlorpyrifos involves the inhibition of acetylcholinesterase (AChE), resulting in a wide range of neurotoxic effects in humans, such as sensory and motor neuropathy with permanent paralysis. The inhibition of cholinesterase enzyme (ChE) may cause acetylcholine accumulation at the synaptic cleft, causing the stimulation of autonomic receptors and depolarizing blocks of neuromuscular junction receptors in the human body. In addition, it was proposed that both long-term exposure to subclinical doses of OP, and after acute intoxication, could be responsible for chronic OP-induced neuropsychiatric disorders.[6—9]

ChE can be used as an index of cholinergic function and changes in enzyme activity may indicate alterations in the availability of acetylcholine at the receptor level.[10—14] *In vivo*, ChE activity reflecting the functional quantities of the enzyme can be assessed using blood, plasma, and red blood cells. Inhibition of ChE leads to a down-regulation of cholinergic receptors. The levels of ChE appear to be controlled by the interaction of acetylcholine with its receptors, with enhanced interaction increasing the levels of ChE.[15]

This study aims to investigate any changes in ChE activity reflecting the cholinergic function of fruit farm workers exposed to the neurotoxin, chlorpyrifos insecticide, during one fruit crop. For this purpose, the blood ChE activities of 53 farm workers on fruit farms in Rayong Province, Thailand, were collected and analyzed.

In the present study, a significant decrease in plasma (PChE), but not red blood cell ChE (AChE) activity after exposure to chlorpyrifos in the spraying season, compared with the non-spraying season, was found. The results suggest an interaction between chlorpyrifos and monoaminergic-cholinergic neurotransmission that leads to a decrease in the functional enzyme available to degrade acetylcholine. We propose that PChE activity can be used as a biomarker for monitoring early toxicity induced by chlorpyrifos insecticide. Determination of baseline red blood cell and plasma cholinesterase activity among applicators in the non-spraying season is very important, before identifying the effects of these pesticide exposures.

Experimental

Farm Sites and Farm Worker Participants This study was conducted in the period May—December, 2003. The fruit farms in this study are located in Rayong Province, in the east of Thailand. Farm selection was based on the following eligibility criterion: farms mainly using chlorpyrifos to control aphids during that period.

The farm workers (53 persons) recruited was Thai men and women who were only exposed to chlorpyrifos due to their agricultural occupations, whose ages ranged between 16—60 years. The insecticide was applied using conventional sprayer tanks. Applicators in these situations wear chemical-

* To whom correspondence should be addressed. e-mail: samlee@chiangmai.ac.th

resistant boots and gloves. Participants were asked about occupational pesticide use and the frequency of both residential and agricultural pesticide use in and around the home during the past 6 months.

All procedures involving human subjects were reviewed and approved by the University of Washington, U.S.A., and the Human Subjects Review Committee of Mahidol University, Thailand, before the study began.

Chemicals All reagents used were analytical grade and used without any purification. Acetylthiocholine iodide (ATCh), 5,5′dithiobis-2-nitrobenzoic acid (DTNB), purified cholinesterase (Chase, EC 3.1.1.7) were from Sigma Aldrich, Sigma Chemical Co., U.S.A.

Blood Sample Collection Blood collection was done regularly for the same 53 farm workers at least 30 d before (non-spraying season) and at least 30 d after (in-spraying season) exposure to chlorpyrifos. Whole blood samples (5 ml) were collected in green-topped Vacutainer tubes containing heparin, and the samples were mixed immediately by turning them upside down 3—5 times to ensure mixture with the anticoagulant. Samples were kept in an icebox before transportation, within 2 h, from the field to the laboratory. Hematocrit counts were performed. Blood samples were centrifuged at $1200\times\boldsymbol{g}$ for 5 min, and the plasma was collected in 1.5 ml centrifuge tubes and stored at −20 °C prior to analysis. The pallets of erythrocytes were washed twice with phosphate buffer solution (PBS) pH 7.0. Physiological normal saline solution (0.9% NaCl) with the same volume of plasma withdrew was added to the erythrocytes and the product was kept at −20 °C until analysis.

Measurment of ChE Activity in Plasma and Red Blood Cells ChE activity was analyzed by Ellman's method[13] with some modification. ChE activity to catalyze ATCh hydrolysis may be summarized in reactions 1 and 2.

$$\text{acetylthiocholine} \xrightarrow{\text{ChE}} \text{acetate}+\text{thiocholine} \quad (1)$$

$$\text{thiocholine}+\text{DTNB} \longrightarrow \text{TNB-(yellow color)} \quad (2)$$

ChE activity may be measured spectrophotometrically by following the formation of the hydrolysis product, yellow colored 5-thio-2-nitrobenzoic acid (TNB) compound, at 412 nm. The reactions were performed in a spectrophotometer (Hewlett Packard HP 8435). Experiments were conducted in a 1-cm quartz cuvette containing 3 ml of solution under continuous stirring. The temperature was controlled at 37 °C using a Peltier model 89090A temperature-controlled cell holder. Absorbance at 412 nm was registered as a function of time. The initial rate of ATCh hydrolysis (V_i) in the presence of ChE can be determined by the slope of the tangent to the curve of $\text{Abs}_{(412\,\text{nm})}=f(t)$. The molar extinction coefficient (ε) at 412 used is equal to $13600\,\text{M}^{-1}\cdot\text{cm}^{-1}$.

In order to measure ChE activity in plasma (PChE) and red blood cells (AChE), a series of experiments with similar conditions was performed. A plasma sample (10 μl) was added to a 1-cm quartz cuvette containing 3 ml of 0.1 M phosphate buffer, pH 8 and 3×10^{-4} M DTNB. The reaction was initiated by addition of varied concentrations of ATCh as substrate, ranging from 2 to 6×10^{-5} M. To measure AChE, red blood cells were thawed and diluted using normal saline solution, as follows; 10 μl of red blood cells in 2990 μl of normal saline solution and 80 μl of diluted red blood cell solution, were used for analysis. We checked that using 80 μl of diluted red blood cell solution did not disturb the measurement system. The Michaelis–Menten's constant, K_m, of each sample was determined using a Lineweaver–Burke plot for plasma and red blood cells.

The ChE activity is reported as V_m/K_m in s^{-1} units, which signify the ability of ChE to catalyze acetylthiocholine hydrolysis per turnover. As V_m is dependent to amount of enzyme, the PChE activity value was normalized by unit volume (L) and that of AChE activity by concentration of hemoglobin. Indeed, V_m/K_m is a specific indicator that reflects ChE activity, and/or the quantity of ChE, in both plasma and red blood cells.

Statistical Analysis Descriptive data, and T-test analyses were performed with SPSS software version 10. The paired t-test was used for comparisons of mean PChE and AChE activities between the non spraying-season and the spraying season.

Results

Measurement of Cholinesterase Activity in Plasma and Red Blood Cells The validation of the spectrophotmetric technic for measurement of kinetic parameters of ChE catalysed acetylthiocholine hydrolysis was performed using purified AChE (EC 3.1.1.7). The kinetic parameters of ChE, such as K_m, V_m and V_m/K_m, the ability of the AChE to catalyze ATCh hydrolysis per turnover are equal to 0.116 ± 0.003 mM, $3.8\pm0.4\,\text{mM}\cdot\text{s}^{-1}$, and $32.7\,\text{s}^{-1}$, respectively.

Figure 1 demonstrates that the V_m/K_m value distribution pattern among the farm workers in this study is normal dis-

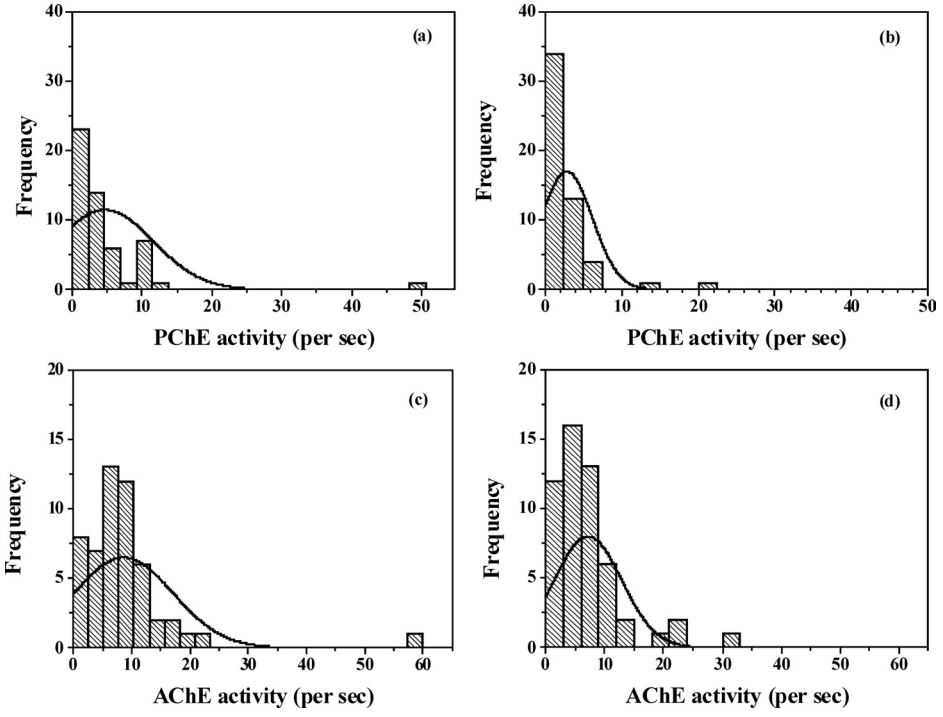

Fig. 1. Distribution of ChE Activity among 53 Farm Workers (a) PChE Activity in the Non-spraying Season; (b) PChE in Spraying Season; (c) AChE Activity in Non-spraying Season; and (d) AChE Activity in Spraying Season

The ChE activity is reported as V_m/K_m in s^{-1} units multiplied with a coefficient of 10^3 and 10^5 for PChE and AChE activity, respectively.

Table 1. ChE Activity in Plasma (PChE) and in Red Blood Cells (AChE) of 53 Farm Workers in the Off-Spraying and In-Spraying Seasons

Cholinesterase	*n*	Median	Min	Max
PChE				
Off-season	53	2.31×10^{-3}	0.64×10^{-3}	48.3×10^{-3}
In season	53	2.02×10^{-3}	0.66×10^{-3}	21.3×10^{-3}*
AChE				
Off-season	53	7.26×10^{-5}	0.57×10^{-5}	59.2×10^{-5}
In season	53	5.95×10^{-5}	0.33×10^{-5}	30.1×10^{-5}**

Chase activity is reported as V_m/K_m in s^{-1} units, $*p<0.01$ (T-test=2.582, df=52, confidence interval 95% ($\alpha=0.05$)), $**p<0.246$ (T-test=1.175, df=52) compared with the off-spraying season.

tribution in both non-spraying and in-spraying season. The median PChE and AChE activity of farm workers in non-spraying season are equal to $2.3\times10^{-3}\,s^{-1}$ and $7.6\times10\times5\,s^{-1}$ and in-spraying season are equal to 2.02×10^{-3} and $5.95\times10^{-5}\,s^{-1}$, respectively. However, the mean V_m/K_m value was shift to left site (t-test, $p=0.013$) indicating a decrease in PChE activity in the farm workers with exposure to chlorpyrifos. Table 1 shows the significance (p-value) of PChE between the non-spraying season and in-spraying season (paired t-test, $p=0.013$). However, AChE was not significantly different between the non-spraying and the in-spraying season (paired t-test, $p=0.246$).

Discussion

The organophosphate insecticides mediate toxicity *via* inhibition of neuropathy target esterase (NTE) and AChE, which cause delayed neuropathy and acute neurotoxicity, respectively. ChE, such as butyrylcholinesterase (EC 3.1.1.8) and acetylcholinesterase (EC 3.1.1.7), has been used as a biomarker for exposure to organophosphates and carbamates in the field.[10,11,16—18] However, a panel of experts who met to discuss the data available on chlorpyrifos, both human and animal, concluded that inhibition of BuChE is not an adverse effect, and the reference dose for chlorpyrifos should be based on AChE inhibition.[19] Moreover, Chen *et al.* reported that the inhibition of AChE activity is consistently exhibited at lower dosages of chlorpyrifos than those required to result in clinical symptoms of OP toxicity, or alterations in cognitive functional responses. The inhibition of red blood cell AChE activity is 12- to 14-fold more sensitive as an indicator of chlorpyrifos exposure than the AChE in the most sensitive relevant neurological tissues (brain or retina). These authors proposed that the inhibition of AChE activity is an appropriate surrogate measurement of chlorpyrifos exposure, and provides a conservative endpoint for establishing appropriate margins of safety for both adults and infants.[20] Contrary to the report by Chen *et al.*, the main finding of the present study was a significant decrease in PChE, but not in AChE, activity of 53 farm workers after regular exposure to chlorpyrifos in field sprays compared with the non-spraying season for one fruit crop. However, they had sufficiently high chlorpyrifos exposure to significantly depress PChE activity. Indeed, it is clearly necessary to collect pre-exposure blood samples as a baseline to determine ChE activity. Our results are consistent with those of a study of 39 Australian pest-control operators exposed to a termiticide containing chlorpyrifos, and 34 unexposed control subjects. The authors noted AChE activity ranged from 30.50—46.50 nmol/min/mg Hb in 32 control subjects, and the mean (37.04±0.63) did not differ from 35 chlorpyrifos-exposed workers (37.5±0.80; ANOVA $p>0.05$), while the mean serum ChE was 52% of control activity.[21] Nolan *et al.* also found that in six healthy male subjects, after administration of 0.5 mg/kg chlorpyrifos, PChE was depressed by 15% from pre-administration, while there was no inhibition of red blood cell ChE activity.[22] Other investigators also studied ChE activities among pest-control workers who used chlorpyrifos occupationally, and the result showed that mean PChE and AChE activities after pesticide exposure were significantly lower than pre-exposure values ($p<0.05$); 16 and 40% of the pest-control workers had PChE and AChE levels below 50% of the pre-exposure values, respectively.[23]

We propose that farm-worker exposure to OPs, such as chlorpyrifos, may be monitored by measuring the activity of plasma cholinesterase (PChE), but not AChE. Therefore, from a biological point of view, it may be used as a potential biomarker in monitoring pesticide exposure in the field.

Acknowledgements The authors are grateful to Dr. Chutchanok Leotchutinat, Faculty of Sciences, Burapha University and Daniel Arrieta, University of California Davis, U.S.A. for their valuable comments and technical help. This project was partly supported by grant from the faculty of Public Health, Burapha University, Thailand, and ISOEH, U.S.A.

References

1) Wolf T. M., Gallander K. S., Downer R. A., Hall F. R., Fraley R. W., Pompeo M. P., *Toxicol. Lett.*, **105**, 31—38 (1999).
2) de Cock J., Heederik D., Kromhout H., Boleij J. S., Hoek F., Wegh H., Tjoe Ny E., *Am. Ind. Hyg. Assoc. J.*, **59**, 166—172 (1998).
3) Dosemeci M., Alavanja M. C., Rowland A. S., Mage D., Zahm S. H., Rothman N., Lubin J. H., Hoppin J. A., Sandler D. P., Blair A., *Ann. Occup. Hyg.*, **46**, 245—260 (2002).
4) Coronado G. D., Thompson B., Strong L., Griffith W. C., Islas I., *Environ. Health Perspect.*, **112**, 142—147 (2004).
5) Griffin P., Mason H., Heywood K., Cocker J., *Occup. Environ. Med.*, **56**, 10—13 (1999).
6) He F., Xu H., Qin F., Xu L., Huang J., He X., *Exp. Toxicol.*, **17**, 40—45 (1998).
7) Karalliedde L., Senanayake N., *Br. J. Anaesth.*, **6**, 736—750 (1989).
8) Meggs W. J., *Toxicol. Clin. Toxicol.*, **41**, 883—886 (2003).
9) Mattingly J. E., Sullivan J. E., Spiller H. A., Bosse G. M., *J. Emerg. Med.*, **25**, 379—381 (2003).
10) Rama D. B., Jaga K., *Sci. Total Environ.*, **122**, 315—319 (1992).
11) Magnotti R. A., Jr., Dowling K., Eberly J. P., McConnell R. S., *Clin. Chim. Acta*, **176**, 315—332 (1988).
12) McConnell R., Cedillo L., Keifer M., Palomo M. R., *J. Occup. Med.*, **34**, 34—37 (1992).
13) Ellman G. L., Courtney D. K., Anders V., Featherstone R. M., *Biochem. Pharmac.*, **7**, 88—95 (1961).
14) Carmona G. N., Baum I., Schindler C. W., Goldberg S. R., Jufer R., Cone E., Slaughter E., Belendiuk G. W., Gorelick D. A., *Life Sci.*, **59**, 939—943 (1996).
15) Koelle G. B., *J. Appl. Toxicol.*, **14**, 105—109 (1994).
16) Panemangalore M., Dowla H. A., Byers M. E., *Int. Arch. Occup. Environ. Health*, **72**, 84—88 (1999).
17) Lopez-Carillo L., Lopez-Cervantes M., *Arch. Environ. Health*, **48**, 359—363 (1993).
18) Soogarun S., Wiwanitkit V., Suwansaksri J., *J. Trop. Med. Public Health*, **34**, 687—689 (2003).
19) van Gemert M., Dourson M., Moretto A., Watson M., *Regul. Toxicol. Pharmacol.*, **33**, 110—116 (2001).
20) Chen W. L., Sheets J. J., Nolan R. J., Mattsson J. L., *Regul. Toxicol. Pharmacol.*, **29**, 15—22 (1999).
21) Dyer S. M., Cattani M., Pisaniello D. L., Williams F. M., Edwards J. W., *Toxicology*, **169**, 177—185 (2001).
22) Nolan R. J., Rick D. L., Freshour N. L., Saunders J. H., *Toxicol. Appl. Pharmacol.*, **73**, 8—15 (1984).
23) Lakew K., Mekonnen Y., *Ethiop. Med. J.*, **36**, 175—184 (1998).