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Comments on Genetic and Developmental Variability of Sensitivity to Chlorpyrifos/Chlorpyrifos oxon Exposure as Modulated by Plasma Paraoxonase (PON1)

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The following comments relate to differences in sensitivity to chlorpyrifos/chlorpyrifos oxon exposures modulated by genetic and developmental variability of the polymorphic human serum enzyme paraoxonase (PON1).

Background

PON1 is found in the plasma (or serum) tightly associated with high density lipoprotein particles (Blatter et al, 1993). It is also found in the liver (Aldridge 1953a, Furlong et al. 2000). Recent evidence from the mouse model system indicates that the same gene encodes both proteins (Shih et al, 1998; Li et al., 1999; Furlong et al., 2000). In humans, PON1 is encoded as a 355 amino acid protein (Hassett et al. 1991). It has a signal sequence on the amino-terminal end of the protein that directs it to be secreted from the liver cells into the plasma where it is assembled into the HDL particles. Only the amino terminal methionine is cleaved from the protein following secretion, unlike other proteins that have the entire signal sequence (approximately 25 amino acids) removed following secretion (Hassett et al. 1991). The hydrophobic signal sequence of PON1 is involved in binding to the HDL particles (Sorensen et al. 1999).

The major physiological function of PON1 appears to be metabolism of harmful, oxidized lipids (Watson et al. 1995). However, PON1 also hydrolyzes the toxic metabolites (oxon forms) of several commonly used insecticides such as chlorpyrifos, diazinon and parathion (Davies et al. 1996). There are two common polymorphisms (or amino acid substitutions) known to exist in human populations, a leucine (L)/methionine (M) substitution at position 55 and a glutamine(Q)/arginine(R) substitution at position 192 (Hassett et al. 1991) The latter has a major affect on the rates of hydrolysis of different organophosphates (Humbert et al. For example, the PON_{192} arginine isoform ($PON1_{R192}$) hydrolyzes paraoxon rapidly, but diazoxon, soman and especially sarin more slowly than the $PON1_{192}$ glutamine isoform ($PON1_{Q192}$) as determined with *in vitro* assays. The $PON1_{R192}$ isoform hydrolyzes chlorpyrifos oxon more efficiently *in vivo* than the $PON1_{Q192}$ isoform (Li et al, 2000).

These rate differences for the two isoforms are such that if the rates of hydrolysis of diazoxon are plotted against the rates of hydrolysis of paraoxon with serum or plasma samples from a number of individuals in a population, a two-dimensional distribution of activities is seen (Davies et al. 1996; Richter and Furlong 1999). From this distribution, it is possible to accurately infer the genotype at codon 192 of an individual in the population and, in addition, know the rate at which their plasma PON1 hydrolyzes specific OP compounds (Davies et al. 1996; Richter and Furlong 1999; Jarvik et al. 2000). An individual's PON1 level is quite stable over time (Zech and Zürcher 1974) once that mature levels are reached, probably between 1-2 years of age Augustinsson and Barr 1963). (Recent experiments with recombinant PON1 have clearly shown that plasma PON1

and arylesterase is indeed the same enzyme, validating the earlier time courses of appearance of PON1/arylesterase in plasma.). The two-dimensional enzyme analysis/plot using the substrate pair diazoxon and paraoxon provides the best resolution to date of the different $PON1_{192}$ genotypes of the currently available enzymatic approaches to phenotype resolution (Davies et al. 1996; Richter and Furlong 1999; Jarvik et al. 2000).

The data linking the level and position 192-phenotype of PON1 with resistance to specific insecticides comes from a number of different studies. Initially, it was observed that animals with high PON1 activities against the toxic metabolites of a specific OP were more resistant to that OP than were other animals with low PON1 activity Furlong et al. 1989; Brealey et al. 1980; Costa et al. 1987). Experiments where purified or partially purified PON1 was injected into rats or mice showed that increasing PON1 in the plasma led to an increase in resistance to specific OPs Li 1999; Main 1956; Costa et al 1990; Li et al. 1993; Li et al. 1995). The most recent and convincing evidence for the relationship of plasma PON1 to resistance to OPs comes from toxicology experiments done with mice that have no PON1 (*PON1* knockout mice) (Shih et al. 1998). These mice exhibited very high sensitivity to chlorpyrifos oxon (Shih et al. 1998) as well as diazoxon (Li et al. 2000). Surprisingly, they were not more sensitive than wild type mice to paraoxon (Li et al. 2000). The *PON1* knockout mice also exhibited a slight increase in sensitivity to the parent compounds chlorpyrifos (Shih et al 1998) and diazinon (Li et al. 2000).

The observation that the knockout mice were not more sensitive to paraoxon led us to examine the *in vivo* efficiency of each of the purified human $PON1_{192}$ isoforms into *PON1* knockout mice and examined their ability to protect against a dermal exposure to each of these oxons. We also kinetically characterized each purified human PON1 isoform under assay conditions closer to physiological salt levels (we had used high salt conditions in the assays to "move the "R/R" data points out of the heterozygote data points). The results explained our observations (Li et al. 2000). The kinetic characterization agreed well with the ability of each isoform to protect against the corresponding oxon. Despite the fact that the $PON1_{R192}$ isoform was nearly ten-times more efficient at hydrolyzing paraoxon than the $PON1_{Q192}$ isoform, its catalytic efficiency was not sufficient to provide protection against a paraoxon exposure. This observation agreed with the experiments showing that the knockout mice were not more sensitive to paraoxon than wild type mice and that wild type mice expressing a high level of the human $PON1R_{192}$ isoform did not show increased resistance to paraoxon.

The observations on resistance to diazoxon were also interesting. The knockout mice were dramatically more sensitive to diazoxon than were the wild type mice. Based on the velocities of diazoxon hydrolysis in a population distribution (Davies et al. 1996; Richter and Furlong 1999), we had expected the $PON1_{Q192}$ isoform to provide better protection against an exposure to diazoxon than the $PON1_{R192}$ isoform. When we injected each isoform and tested them under *in vivo* conditions in mice, we found that both isoforms provided essentially equal protection. These observations were explained by the data from the kinetic characterization of the two purified $PON1_{192}$ isoforms. While the $PON1_{R192}$ isoform has a lower maximal rate of diazoxon hydrolysis (Vm) than the $PON1_{Q192}$ isoform, it has a higher affinity. The catalytic efficiencies (Vm/Km) for the two isoforms are nearly identical accounting for the equal protection observed in the *in vivo* protection assays.

The *in vitro* characterization of chlorpyrifos oxon hydrolysis also correlated well with the *in vivo* protection provided by the injected $PON1_{192}$ isoforms. The catalytic efficiency of chlorpyrifos oxon hydrolysis *in vitro* was better with the $PON1R_{192}$ isoform than with the $PON1Q_{192}$ isoform. This agreed with the greater degree of protection observed when the $PON1_{R192}$ isoform was injected into the knockout mice. In short, the more efficiently PON1 hydrolyzes a given OP, the

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better is the protection provided *in vivo*. Also, the higher the affinity for the substrate, the more capable is PON1 of hydrolyzing that substrate at lower, physiological concentrations. The catalytic efficiencies are conveniently expressed as the maximal velocity (Vm) divided by the affinity constant (or Km value). These observations are important in that approximately half of populations of Northern European origin are homozygous for $PON1_{Q192}$ while approximately 36% of individuals of Hispanic origin are homozygous for $PON1_{Q192}$ (Davies et al. 1996)

Thus, the available evidence indicates that individuals with low plasma PON1 status will be much more sensitive to exposures to chlorpyrifos oxon and diazoxon than individuals with high PON1 levels and somewhat more sensitive to the respective parent compounds. Both position 192 genotype and PON1 expression level influence sensitivity to chlorpyrifos oxon, whereas plasma level of PON1 is the best indicator of sensitivity to diazoxon.

The sensitivity to the oxon forms is not insignificant. A survey of various oxon forms present in foliar pesticide residues indicates that the oxon forms can account for a very significant percentage of the total OP residues (Yuknavage et al. 1997). The survey did not report values for chlorpyrifos oxon, however recent surveys of ambient air monitoring of chlorpyrifos and chlorpyrifos oxon indicate significant percentages of the oxon form present (Report 1996). Since rates of cholinesterase inhibition by chlorpyrifos oxon are approximately 1,000 times faster than by chlorpyrifos Huff et al. 1994), even small percentages of oxon in an exposure are very significant.

As mentioned above, in addition to the Q192R polymorphism that affects the catalytic efficiency of PON1, other 5' regulatory region polymorphisms, developmental dependence of expression, environmental factors and other, as yet undetermined, genetic background factors affect the plasma levels of PON1.

Three groups have examined the effects of 5' regulatory region polymorphisms on the levels of PON1 in adult plasma Leviev et al. 2000; Suehiro et al. 2000; Brophy et al. 2002). The largest contribution to level differences appears to be the C-108T polymorphism. Individuals homozygous for the position -108C allele produce on average twice as much PON1 as individuals homozygous for the -108T allele. While the 5' regulatory region polymorphisms are predictive of high or low plasma PON1, other factors such as the individuals' genetic background also contribute in as yet undetermined ways. The best analysis of an individual's PON1 status (genotype/phenotype) is still the two-dimensional enzyme assay analysis with the substrate pair diazoxon and paraoxon (Davies et al. 1996; Jarvik et al. 2000. An example of such an analysis is shown in Fig. 1. PCR analysis will provide only genotype and will not, for example, pick up a non-functional allele. It also provides little information on an individual's PON1 level that is critical for determining sensitivity to an exposure.

The contribution of developmental regulation to variation in PON1 levels is shown in Fig. 2 (Richter et al. unpublished results). To our knowledge, PON1 is responsible for virtually all of the hydrolysis of diazoxon, paraoxon and chlorpyrifos oxon in human plasma (Davies et al. 1996). The unpublished data shown in Fig. 2 are consistent with other earlier data published on the time course of appearance of arylesterase (A-esterase) in human plasma determined with phenylacetate as a substrate (Ecobichon and Stephens 1972; Augustinsson and Barr 1963) or in one case with -naphthylacetate as a substrate (Burlina et al 1977). Our most recent data indicate that there is a baby to baby variability in the time that their plasma activity reaches or approaches a plateau, with some babies reaching a plateau at about 6 months of age and others at about 15 months or later. As noted above, once the plateau level is reached, there will be at least a 13-fold

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variability in plasma levels among individuals and also different catalytic efficiencies of hydrolysis of chlorpyrifos oxon, depending on $PON1_{192}$ genotype.

The PON1 knockout mice provided important information on the role of PON1 in OP sensitivity. To obtain a better estimate of the function of the human PON1 192 isoforms under physiological conditions, the human PON1Q192R isoforms were introduced as transgenes on the PON1 knockout background, i.e., the mouse PON1 gene was replaced with either human $PON1_{Q192}$ or $PON1_{R192}$. These constructs will allow characterization of the protective role of the human $PON1_{192}$ isoforms under physiological conditions in an animal model system.

Summary and recommendations

The factors to consider in trying to estimate the total variability in sensitivity of human populations to chlorpyrifos oxon exposure are: 1) PON1 status (position 192 genotype (QQ, QR or RR), 2) the level of plasma PON1 and 3) the age of the individual. The variability of PON1 levels in plasma is approximately 13-fold among adults (Davies et al. 1996). Newborns, on average, have about 3-4-fold lower PON1 levels at birth (and presumably yet lower levels before birth) (Mueller et al. 1982) and reach adult levels between 1-2 years of age. Thus, the individual in an adult population with the lowest PON1 level would have had 3-4-fold lower level of PON1 as a newborn. This would suggest an approximate 50-fold variability in absolute levels of PON1 protein, not considering the differences in catalytic efficiency. Our current approach to get at this question is to use the humanized PON1 transgenic mice for generating dose response curves. Our preliminary results comparing mice expressing only human $PON1_{R192}$ with mice expressing only human $PON1_{Q192}$ (at the same plasma PON1 protein levels as the R 192 mice) and with PON1 knockout mice indicated that the female mice expressing human $PON1_{Q192}$ were nearly as sensitive to chlorpyrifos oxon as the PON1 knockout mice (Furlong et al. 2002). We had expected to see an intermediate phenotype (chlorpyrifos oxon sensitivity) based on the catalytic efficiencies determined in the *in vitro* assays. The *in vivo* analysis, however, is more reliable, since it assesses PON1 function *in vivo* under actual physiological conditions. Accurate dose response curves need to be carried out for the three strains of mice (PON1 knockouts ($PON1^{-/-}$), $hPON1^{-/-}{}_{R192}$ and $hPON1^{-/-}{}_{Q192}$).

The data available to date indicate that the PON1 genetic variability is reflected mainly in differential sensitivity to the oxon component of an exposure. The oxon component is not insignificant in many exposures. Thus, safety studies should include percentages of the oxon form of an OP that are compatible with levels encountered in actual exposures. The lower levels of PON1 in the very young are most likely reflected in increased sensitivity. One serious concern would be the consequence of exposure of a fetus of a mother with very low PON1 status.

These comments do not address the variability in sensitivity governed by other enzymes or pathways. These would include at least 1) carboxylesterases and cholinesterases which react with the oxon forms stoichiometrically to inactivate them and 2) the cytochromes P450 which both bioactivate the parent organophosphorothioate compounds as well as inactivate them as well as the oxon forms. The role of the glutathione transferases in protecting against OP exposures and the effects of genetic variability of these enzymes have not yet been clearly established.

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