Human and Ecological Risk Assessment: Vol. 8, No. 1, pp. 31-43 (2002)

Genetic Factors in Susceptibility: Serum PON1 Variation Between Individuals and Species

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ABSTRACT

In mammals, serum paraoxonase (PON1) is tightly associated with high-density lipoprotein (HDL) particles. In human populations, PON1 exhibits a substrate dependent activity polymorphism determined by an Arg/Gln (R/Q) substitution at amino acid residue 192. The physiological role of this protein appears to be involvement in the metabolism of oxidized lipids. Several studies have suggested that the $PON1_{R192}$ allele may be a risk factor in coronary artery disease. PON1 also plays an important role in the metabolism of organophosphates including insecticides and nerve agents. The $PON1_{R192}$ isoform hydrolyzes paraoxon rapidly, but diazoxon, soman and sarin slowly compared with the $PON1_{Q192}$ isoform. Both PON1 isoforms hydrolyze phenylacetate at approximately the same rate, while $PON1_{R192}$ hydrolyzes chlorpyrifos oxon slightly faster than PON_{Q192}. Animal model studies involving injection of purified rabbit PON1 into mice clearly demonstrated the ability of PON1 to protect cholinesterases from inhibition by OP compounds. The consequence of having low PON1 levels has been addressed with toxicology studies in *PON1* knockout mice. These mice showed dramatically increased sensitivity to chlorpyrifos oxon, diazoxon and some increased sensitivity to the respective parent compounds. These observations are consistent with earlier studies that showed a good correlation between high rates of OP hydrolysis by serum PON1 and resistance to specific OP compounds. They are also consistent with the observations that newborns have an increased sensitivity to OP toxicity, due in part to their not expressing adult PON1 levels for weeks to months after birth, depending on the species. Together, these studies point out the importance of considering the genetic variability of $PON1_{192}$ isoforms and levels as well as the developmental time course of PON1 appearance in serum in developing risk assessment models.

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1080-7039/01/$.50

Key Words: paraoxonase, PON1, parathion, chlorpyrifos, diazinon, soman, sarin, genetic variability, environmental genome, functional genomics, organophosphate poisoning.

INTRODUCTION

Advances in genetics have made it possible to address the question of the effects of genetic variability on susceptibility to diseases and different environmental agents. There is currently an effort underway to understand genetic differences in humans that govern responses to environmental exposures and to map susceptibility genes. This effort is referred to as the Environmental Genome Project (Kaiser 1997). Experiments aimed at understanding the function of specific genes are referred to as functional genomics. This contribution explores the consequences of genetic variation in the serum paraoxonase (*PON1*) gene (Clendenning *et al.* 1996; Primo-Parmo *et al.* 1996; Sorenson 1995a). The protein product of the *PON1* gene is a serum enzyme found tightly associated with high density lipoprotein (HDL) particles (Mackness and Walker 1983; Blatter *et al.* 1993). The normal physiological role of PON1 appears to be the metabolism of oxidized, biologically active lipid molecules (Mackness *et al.* 1991, 1993; Watson *et al.* 1995). The association of this gene with susceptibility to vascular disease (reviewed in Laplaud *et al.* 1998; Mackness *et al.* 1998) is an active subject of current interest.

This contribution will, however, focus on the role of PON1 in the metabolism of xenobiotics, particularly toxic organophosphorus compounds and the question of whether PON1 status may serve as an important biomarker for susceptibility. Figure 1 shows the two-step enzymatic pathway for the bioactivation and hydrolysis of organophosphate compounds metabolized through the cytochrome P450/PON1 pathway. Nerve agents are directly hydrolyzed by PON1 (Davies *et al.* 1996). With respect to actual OP exposures, it is generally thought that field foliar residues are primarily composed of the parent compounds. However, a recent survey of reported values for the ratio of oxon to parent compound shows that there are often very high levels of oxon found in the field (Yuknavage *et al.* 1997).

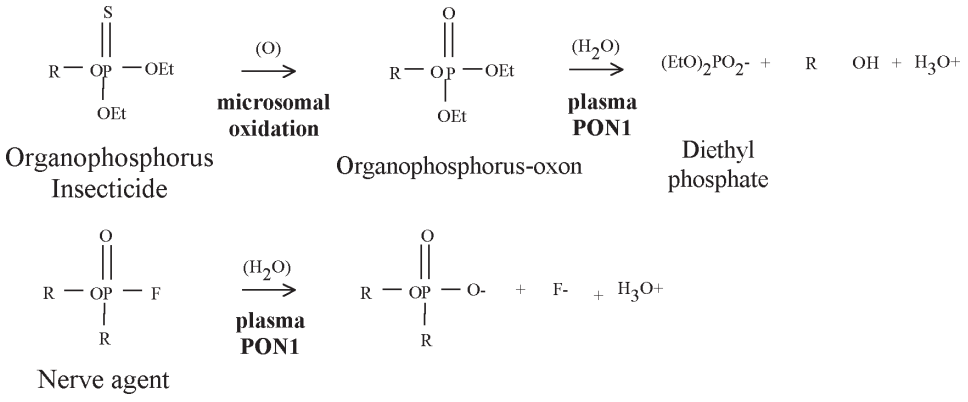

Figure 1. Cytochrome P450/PON1 detoxication of OP compounds.

GENETIC VARIABILITY OBSERVED IN HUMAN POPULATIONS

It has been known since the experiments of Krisch (1968), Geldmacher-v. Mallinckrodt *et al.* (1973) and Playfer *et al.* (1976) that human PON1 activity is multimodally distributed in human populations (reviewed in Geldmacher v. Mallinckrodt and Diepgen 1988). Many laboratories subsequently developed different assays to use in investigating the PON1 polymorphism (Ortigoza- Ferrado *et al.* 1984). There was a question as to whether the population distribution of enzyme activity was bi- or trimodal. As described below, subsequent research has shown why this was a confusing issue. In 1991, Hassett *et al.* cloned and sequenced the cDNAs that encoded human and rabbit PON1s. They described two different polymorphisms in the characterized human sequences, a Leu/Met (L/M) polymorphism at amino acid residue 55 and a Gln/Arg (Q/R) polymorphism at amino acid residue 192. Finally, in 1993 Humbert *et al.* reported that the molecular basis for the observed activity polymorphism was due to the Q/R polymorphism at amino acid residue 192. These findings were independently confirmed by Adkins *et al.* (1993).

The effect of the polymorphism is substrate dependent (Davies *et al.* 1996). The $PON1_{R192}$ isoform hydrolyzes paraoxon rapidly, but is virtually inactive in hydrolyzing the nerve agent sarin. It also hydrolyzes diazoxon and the nerve agent soman less rapidly than the $PON1_{Q192}$ isoform. Both isoforms hydrolyze phenylacetate at the same rate. $PON1_{R192}$ hydrolyzes chlorpyrifos oxon slightly faster than $PON1_{Q192}$. In addition to the qualitative differences in rates of hydrolysis of different substrates by these two PON1 isoforms, there is a large interindividual variability in the levels of PON1 protein circulating in serum (La Du *et al.* 1986; Davies *et al.* 1996; Furlong *et al.* 1993). The serum PON1 level in a given individual is stable over time (Zech and Zucher 1974).

Figure 2 shows why the early histograms or graphs that plotted number of individuals against paraoxonase levels did not resolve the three $PON1_{192}$ phenotypes, ($PON1_{QQ192}$, $PON1_{QR192}$ and $PON1_{RR192}$). In a one-dimensional histogram of paraoxonase activities, a large percentage of the individuals homozygous for $PON1_{R192}$ were "hidden" under the heterozygotes. The two-dimensional enzyme analysis (using the substrate pairs diazoxon and paraoxon, Figure 2) clearly resolves all three phenotypes and shows why there was so much confusion generated in the early population distribution histograms based on a single enzyme assay. This procedure is amenable to high throughput formats and allows for an accurate inference of $PON1_{192}$ genotype (Richter and Furlong 1999).

The polymorphism at position 55 has not been demonstrated to affect the ratios of turnover numbers for different substrates, but has been linked to lower PON1 levels (Blatter Garin *et al.* 1997) as well as lower levels of PON1 mRNA (Leviev *et al.* 1997). While this is in general statistically true, determining the $PON1_{55}$ genotype of a given individual does not allow for the prediction of PON1 levels in that individual (Richter *et al.* unpublished data). PON1 status is best determined by the two-dimensional enzyme assay shown in Figure 2 (Richter and Furlong 1999).

GENE STRUCTURE AND PON1 SEQUENCES IN DIFFERENT SPECIES

The structure and organization of the *PON1* gene have been determined in humans (Clendenning *et al.* 1996) and mice (Sorenson *et al.* 1995a). In both species,

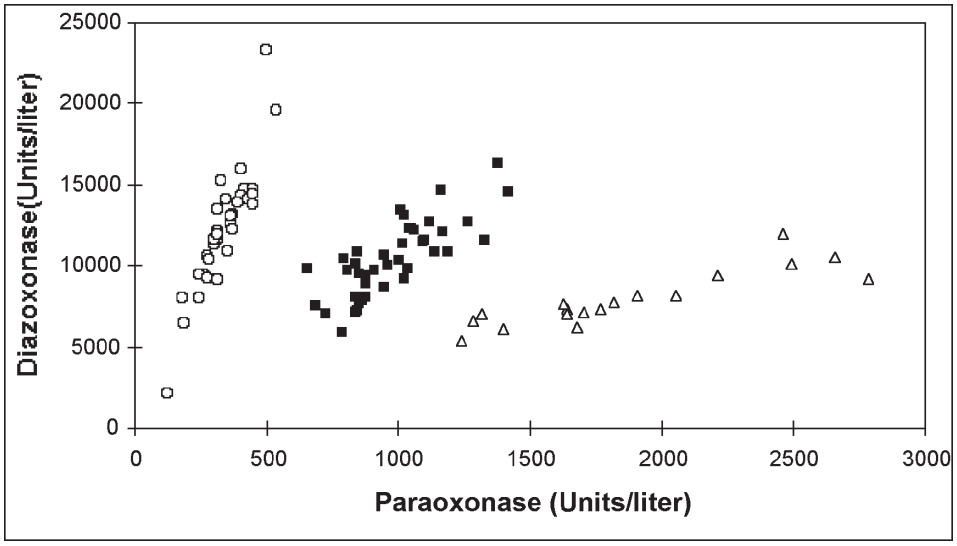

Figure 2. A two-dimensional enzyme analysis of PON1 activity distributions in a population of farm workers [reproduced from Figure 2c, Davies *et al.* (1996), with permission). The rates of hydrolysis of diazoxon are plotted against the rates of hydrolysis of paraoxon for each individual in the population sample. (Units = μmol/min).

PON1 has nine exons and includes approximately 26 kb of genomic sequence. *PON1* maps to chromosome 7 in humans (Humbert *et al.* 1993) and to an analogous region of chromosome 6 in mice (Sorenson *et al.* 1995a; Shih *et al.* 1996; Li *et al.* 1997). Related genes, *PON2* and *PON3* have been described (Primo-Parmo *et al.* 1996), however, their role in OP metabolism has yet to be established.

The PON1 cDNAs have been characterized for human, rabbit and mice. A comparison of the deduced and determined amino acid sequences of PON1s from several different species is shown in Figure 3. It is clear from this comparison that there is a high degree of conservation of sequence, especially for several different domains. It is also interesting that PON1 retains its signal sequence, with only the amino terminal methionine removed (Furlong *et al.* 1991; Hassett *et al.* 1991).

PON1 Levels in Different Animal Species

The variability of PON1 levels in different mammals was investigated by Aldridge (1953) using paraoxon as a substrate. He found that of the animals investigated, rabbits had by far the highest activity in their sera and mice the lowest. Birds were found to have very low serum paraoxonase activity (Brealey *et al.* 1980). PON1 activities for hydrolysis of paraoxon, diazoxon and chlorpyrifos oxon are listed for several species in Table 1, along with LD_{50} values for the respective parent compounds and oxon forms, where available.

Correlation of PON1 Levels with Resistance to OP Toxicants

Published data indicate a reasonable correlation between rates of hydrolysis of OPs by PON1 and resistance to the specific OP compound (Table 1). The variability of hydrolytic activities in animals was found to correlate with LD_{50} values (McCollister *et al.*

Figure 3. Comparison of human (Hassett *et al.* 1991), mouse (Li *et al.* 1997; see also Sorenson *et al.* 1995b; Shih *et al.* 1996); rabbit (Hassett *et al.* 1991) and partial dog (Primo-Parmo *et al.* 1996) PON1 deduced amino acid sequences.

1974; Costa *et al.* 1987; Furlong *et al.* 1989). For example, rabbit serum PON1 has very high rates of hydrolysis of chlorpyrifos oxon contributing to an LD_{50} value for chlorpyrifos of greater than 2000 mg/kg. Birds, which were found to have very low serum paraoxonase levels, were very sensitive to the toxicity of OP compounds (Brealey *et al.* 1980). A study of PON1 levels in pesticide applicators who sprayed primarily chlorpyrifos found that there were no "low hydrolyzers" of chlorpyrifos oxon in the group of 90 individuals whose plasma was assayed (Furlong *et al.* unpublished results). Fish, which have very low PON1 activity, are very sensitive to OP insecticides (Table 1). The increased sensitivity of *PON1* knockout mice to specific OP compounds is discussed in the next section.

Development of an Animal Model System for Directly Addressing the Role of Serum PON1 in OP Metabolism

Early experiments by Main (1956), where partially purified rabbit PON1 was injected into rats, showed that the injected enzyme provided protection against

Table 1. Paraoxonase kinetic values for different substrates and OP sensitivity of several species.

Organism	POase		DZOase		CPOase		LD_{50} Values (mg/kg)					
	Vm[a]	Km[b]	Vm	Km	Vm	Km	PS	PO	CPS	CPO	DZS	DZO
Sheep	241[1] 300[2] 267[2]	1.63[1]	1290[2]	--	4243[2]	--	--	--	800[10]	--	1000[9]	--
Rat	61[1] 167[1]-267[2]	2.2[1]	6774[2]	--	3992[2]	--	2[3]	>0.5-0.6[8] (ip)	95-270[3]	118-245[4]	200[1]	--
Rabbit	694 2439[1] 3183[2] 2372[5]	0.23[5] 5.25[1]	22523[5]	0.17[5]	27779[5]	0.12[5]	10[10]	>2[7] (ip)	2000[4]	--	140-350[1]	--
Chicken	ND 0-11.2[2]		0-193[2]	--	143.8[6]	--	2.1 (ducks)[11]		32[4]	--	3-15[1]	--
Mouse (WT)	54[1] 232♂[5] 269♀[5]	0.34[5]	3569♂[5] 4211♀[5]	0.26[5]	1922♂[5] 1863♀[5]	0.032[5]	5-25[10]	>0.5[5] (dermal)	60[10]	>0.6[5] (dermal)	300[3]	>0.4[5] (dermal)
Mouse (PON1-/-)	ND[5]	NA	ND[5]	NA	75-81[5]	--	--	>0.5[5] (dermal)		<3[5] (dermal)		<2[5] (dermal)
Human $PON1_{QQ192}$[c]	121-544[6]	0.36[5]	2174-23316[6]	0.52[5] 0.39[6]	2415-11101[7]	0.105[5]	--	--	--	--	--	--
Human $PON1_{RR192}$[d]	711-2513[6]	0.42[5]	2025-10158[6]	0.13[5] 0.2[6]	7480-13540[7]	0.084[5]	--	--	--	--	--	--
Trout	11.1[2]	--	ND[2]	--	162[2]	--	1.6 mg/l (LC_{50})[11]	--	0.009-0.098 mg/l (LC_{50})[12]	--	2.6-3.2 mg/l (LC_{50})[13]	--

a. Km values are in μM.
b. Vmax values are expressed as nmol/min/ml plasma (or serum).
c. $PON1_{QQ}$ = individuals homozygous for Gln at position 192
d. $PON1_{RR}$ = individuals homozygous for Arg at position 192
ND = not detectable
NA = not applicable
-- = data not available

1. Brealey *et al.* (1980)
2. Furlong *et al.* (2000)
3. Material Safety Data Sheet
4. McCollister *et al.* (1974)
5. Li (1999)
6. Richter and Furlong (1999)
7. Davies *et al.* (1996)
8. Costa *et al.* (1987)
9. Machin *et al.* (1976)
10. EXTONET, (1989)
11. Johnson and Finley (1980)
12. USEPA (1986)
13. Kidd and James (1991)

paraoxon toxicity. In recent years, we have confirmed and extended his observations. Our initial observations that were carried out with rats (Costa *et al.* 1990) confirmed Main's observations. However, we decided to switch to mice for two main reasons, (1) mice are much smaller and more experiments can be carried out with a limited amount of purified PON1, and (2) the mouse system is more convenient for genetic manipulation.

Enzyme injection experiments with mice demonstrated that PON1 could be injected iv, ip, or im. Injection of rabbit PON1 into mice resulted in an approximate 40-fold increase of rates of hydrolysis of chlorpyrifos oxon over the basal level. The brain and diaphragm cholinesterases were protected nearly 100% from inhibition of a dermal exposure to chlorpyrifos oxon, compared with an inhibition of approximately 80% in control animals not receiving injected PON1. Injected PON1 also protected against exposure to the parent compound chlorpyrifos. It was also shown that injection of enzyme 30 min post-exposure provided good protection of brain and diaphragm cholinesterases with some protection still observable if injected 3 h post-exposure. Injection of enzyme 24 h prior to exposure also provided protection against chlorpyrifos toxicity. Thus, for at least the case of chlorpyrifos and its highly toxic oxon, high levels of circulating PON1 provided protection against toxicity from a dermal exposure (Li *et al.* 1993, 1995).

Recent experiments have addressed the consequences of low PON1 levels. *PON1* knockout mice are very sensitive to exposures to chlorpyrifos oxon (Furlong *et al.* 1998; Shih *et al.* 1998) and diazoxon (Li 1999). Exposures at levels that show no symptoms in the parent strain killed the knockout mice. The *PON1* knockout mice also show an increased sensitivity to the respective parent compounds chlorpyrifos (Shih *et al.* 1998) and diazinon (Li 1999), although not as dramatic as the increase in sensitivity to the respective oxons. The paradoxical observation that *PON1* knockout mice are not more sensitive to paraoxon, while injected rabbit PON1 provides protection against paraoxon is explained by the much better catalytic efficiency of rabbit PON1 compared with mouse PON1 (Li 1999).

Developmental Time Course of PON1 Levels in Serum.

Early studies by Augustinsson and Barr (1963) and Ecobichon and Stephens (1972) showed that the aryl- or 'A'-esterase (now known to be PON1; Gan *et al.* 1991; Furlong *et al.* 1993) levels in the serum of humans had not reached adult levels by 5 to 6 months of age, in agreement with our later observation of low paraoxonase levels in newborns (Mueller *et al.* 1983). Our studies on the development of PON1 levels in rats and mice showed that adult enzyme levels are reached at 3 weeks of age (Li *et al.* 1997). The low PON1 levels observed in young animals are consistent with their observed increased susceptibility to OP toxicity (Brodeur and Du Bois 1963; Lu *et al.* 1965; Gagné and Brodeur 1972; Benke and Murphy 1975; Harbison 1975; Murphy 1982; Pope *et al.* 1991; Virgo 1984; Mortensen *et al.* 1995, 1996).

Distribution of Paraoxonase in Different Tissue Compartments.

Aldridge's early studies (1953) compared paraoxonase levels in different tissues of different species. It was found that rabbits have very high paraoxonase levels in the serum and lower levels in the liver. Rats, on the other hand, have much lower

paraoxonase levels in serum, and levels approximately equal to the rabbit in liver. Recent studies by Zech *et al.* (1999) in cattle show that they have a much higher proportion of PON1 in their livers compared with serum, just the opposite of the distribution in rabbits. The compartmental distribution of PON1 activity may be worth considering when trying to evaluate PON1 status in a given species. It is still not known how human PON1 variability in serum is reflected in PON1 tissue distribution.

CONCLUSIONS

The mouse model system that we have been developing in collaboration with Dr. Lusis' research group (Shih *et al.* 1998) is ideal for answering questions related to the *in vivo* role of PON1 in OP detoxication and for developing more reliable PBPK models. By replacing the mouse PON1 gene with each of the human PON1 alleles, it should be possible to generate "humanized" populations of mice that closely mimic human populations with respect to the genetic variability of serum PON1 isoforms, levels and activities. The important parameters of Km and Vm and catalytic effeciency (Vm/Km) can be addressed with the mouse model.

An excellent example of the importance of considering the catalytic efficiency of PON1 for hydrolysis of OP substrates is that seen in comparing *PON1* knockout mice with normal mice and mice injected with rabbit PON1. While the *PON1* knockout mice are much more sensitive to chlorpyrifos oxon (Furlong *et al.* 1998; Shih *et al.* 1998) and diazoxon (Li 1999), they do not show increased sensitivity to paraoxon, the substrate for which the enzyme was named (Li 1999). This is particularly surprising in light of the experiments demonstrating that injected rabbit PON1 provides protection against paraoxon toxicity (Main 1956; Costa *et al.* 1990). The differences in efficacy of the PON1s from different species can be explained by differences in catalytic efficiency. Thus, it is important to consider the catalytic efficiency of a given PON1 (from a specific species or for a given isoform within a species) for specific substrates (Li 1999) in developing PBPK models. These questions can be addressed *in vivo* with the mouse model system, either by generating the appropriate transgenic strains or by direct injection of purified or recombinant PON1s.

For risk assessment in humans and for establishing an individual's PON1 status, the high throughput two-dimensional enzyme assays are probably the most useful protocol at the present time. This analysis provides both an accurate inference of $PON1_{192}$ genotype as well as the equally important phenotype, or level of PON1 in an individual's serum. The two-dimensional enzyme assay that plots rates of hydrolysis of diazoxon vs. paraoxon for each individual in a population (Figure 2) has proven to be the most useful procedure for determining PON1 status of individuals. This assay divides the population into three phenotypes, individuals homozygous for $PON1_{Q192}$, heterozygotes and individuals homozygous for $PON1_{R192}$. It also indicates the level of PON1 in the serum of each individual. Genotyping alone provides no information about PON1 levels, which can vary up to at least 13-fold between individuals (La Du *et al.* 1986; Furlong *et al.* 1989; Davies *et al.* 1996). Inference of $PON1_{192}$ genotype from the enzyme analyses has proven to be 100% accurate in our laboratory, as verified by PCR genotyping of the individuals (Richter and Furlong 1999).

Evaluation of the $PON1_{55}$ polymorphism, on the other hand, can only be done by PCR, allele specific probe analysis or other DNA analytical procedures, since this amino acid substitution does not affect the enzymatic activity of PON1. However, while the $PON1_{55}$ polymorphism has been reported to affect levels of PON1 and PON1 mRNA (Leviev *et al.* 1997), it should be pointed out that the $PON1_{R192}$ allele is linkage disequilibrium. Nearly all individuals examined to date with the $PON1_{R192}$ allele have the $PON1_{L55}$ allele, whereas individuals with the $PON1_{Q192}$ allele can have $PON1_{L55}$, or $PON1_{M55}$. Further, genotyping a given individual for the position 55 polymorphism does not provide any information about PON1 levels in that individual (Richter *et al.* unpublished data). Thus, the analysis illustrated in Figure 2 provides the most valuable information for an individual's PON1 status, an accurate estimation of $PON1_{192}$ genotype as well as the level of plasma PON1 in that individual (Costa *et al.* in press; Richter and Furlong 1999). Since PON1 levels appear to be very stable once that adult levels are reached, it is probably not necessary to determine PON1 status for a given individual more than once, unless they develop a disease that would affect the levels of enzymes produced by the liver.

Special attention should be paid to the developmental time course of PON1 in humans. As noted above, newborns have very low PON1 levels and do not develop maximal levels until sometime after 6 months of age (Augustinsson and Barr 1963; Ecobichon and Stephens 1972). It is clear that high PON1 levels provide protection against specific OP compounds (Main 1956; Costa *et al.* 1990; Li *et al.* 1993, 1995) and low PON1 levels result in increased sensitivity to specific OP compounds in animal model systems (Furlong *et al.* 1998; Shih *et al.* 1998; Li 1999). For a given individual, at a given age, the two-dimensional enzyme analysis should provide the information necessary to estimate the contribution of PON1 to the predicted sensitivity or resistance to a given OP compound. This will require the development and validation of PBPK models. The validation of the models will be facilitated by the availability of the *PON1* knockout mouse model, where the human $PON1_{192}$ isoforms may be introduced by direct injection or by transgenic manipulation. Recent experiments by Li (Li 1999) demonstrate the importance of examining the *in vivo* efficacy of specific PON1s. The accumulation of *in vivo* data will provide the information necessary for carrying out reliable risk assessments related to the genetic variability seen with the PON1 polymorphism.

In summary, it is clear that there are large inter- and intra-species differences in susceptibility to OP compounds. Variation in the sequence and expression of the PON1 gene in humans and animals probably plays a major role in determining sensitivity to specific environmental OP exposures. Additional *in vivo* experiments with the mouse model system will contribute important information for developing risk assessment models.

ACKNOWLEDGMENTS

This research was supported in part by NIH Grant 2PO1HL30568-16 (to AJL); American Heart Association Grant in Aid No. 1133-GI2 (to DMS); UW Center Grant Number P30 ES 07033 from the NIEHS and a grant to the UW Center for Child Environmental Health Risks Research from NIEHS (1 PO1 ES09601-01) and EPA (EPA-R826886-01-0). Although the research described in this article has been funded

in part by the United States Environmental Protection Agency through a grant, it has not been subjected to the Agency's required peer and policy review. Therefore, it does not necessarily reflect the views of the Agency, and no official endorsement should be inferred.

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