Increased Influence of Genetic Variation on PON1 Activity in Neonates

Jia Chen,[1,2,3] Madhu Kumar,[4] Wendy Chan,[2] Gertrud Berkowitz,[2] and James G. Wetmur[4,5]

[1]The Derald H. Ruttenberg Cancer Center, and Departments of [2]Community and Preventive Medicine, [3]Pediatrics, [4]Microbiology, and [5]Human Genetics, Mount Sinai School of Medicine, New York, New York, USA

PON1 (paraoxonase-1) detoxifies organophosphates by cleavage of active oxons before they have a chance to inhibit cholinesterases. The corresponding gene *PON1* has common polymorphisms in both the promoter (–909, –162, –108) and the coding region (L55M, Q192R). The five *PON1* genotypes were determined for maternal blood (n = 402) and cord blood (n = 229) as part of a study of the effects of organophosphate pesticide exposure on infant growth and neurodevelopment. PON1 enzymatic activities were determined for a majority of subjects. The population contained Caucasians, Caribbean Hispanics, and African Americans. PON1 activity was strongly dependent upon the promoter alleles in both maternal and cord blood. For example, PON1 activities for position –108*CC*, *CT*, and *TT* mothers were 146, 128, and 109 arylesterase U/mL (analysis of variance, $p < 0.0001$), whereas the same PON1 activities for the respective cord bloods were 49.0, 32.4, and 23.2 U/mL ($p < 0.0001$). Compared with adults, neonates had lower PON1 activity, implying reduced capacity to detoxify organophosphates. In addition there was a larger difference in activity between genotype groups in neonates than in adults. Because the five polymorphisms in *PON1* occur in a short stretch of DNA, they were tested for linkage disequilibrium (LD). Significant LD was found among all three promoter polymorphisms as well as between promoter polymorphisms and L55M, with the strongest LD for Caucasians and the weakest for African Americans. The Caribbean Hispanics fall between these two groups. Surprisingly, significant LD also was observed between the promoter polymorphisms and C311S in *PON2*. LD between the promoter polymorphisms and Q192R was not significant. ***Key words:*** **children, chlorpyrifos, linkage disequilibrium, neonate, pesticide, polymorphism, PON1, pregnancy. *Environ Health Perspect* 111:1403–1410 (2003). doi:10.1289/ehp.6105 available via *http://dx.doi.org/* [Online 7 May 2003]**

Chlorpyrifos, an insecticide implicated as a developmental neurotoxin in animals (Shih et al. 1998), has been used routinely in the homes of urban populations in the United States. The biochemical contributions of metabolizing enzymes to the neurotoxic potential of chlorpyrifos and other organophosphates remain subjects of active investigation (Sams et al. 2000; Costa et al. 1999; Furlong et al. 2000). Specifically, after activation of chlorpyrifos to the reactive oxon, the oxon may be detoxified by PON1 (paraoxonase-1) before it has the chance to inactivate acetylcholinesterase in the peripheral nervous system (PNS) and central nervous system (CNS). PON1, which is associated with high-density lipid lipoprotein (HDL), also functions to cleave oxidized lipids in low-density lipid lipoprotein (LDL) as well as in HDL (Aviram et al. 1998, 2000). PON1 also has an arylesterase activity (Gan et al. 1991). HDL levels and PON1 activities are somewhat elevated during pregnancy (Roy et al. 1994). As part of the continuing effort of the Mount Sinai Children's Environmental Health Center to prospectively assess the neurodevelopmental risks associated with chlorpyrifos exposure in an inner-city population in New York City, we investigated the genetic determinants of chlorpyrifos metabolism in a population of ethnically diverse mothers and their neonates. In this study, we examined the hypothesis that genetic factors may be more crucial in determining risk in neonates than in adults of various ethnicities.

The *PON1* gene has been fully characterized (Clendenning et al. 1996) and has been found to be a member of a multigene family of three linked genes, *PON1* (MIM 168820), *PON3* (MIM 602720), and *PON2* (MIM 602447) (OMIM 2002), in that order (Primo-Parmo et al. 1996; Entrez Chromosome Map 2002). Human *PON1* had long been recognized to be polymorphic based both on large differences in serum PON1 activity (Geldmacher-von Mallinckrodt et al. 1983) and on the phosphodiesterase/arylesterase activity ratio for different substrates, including organophosphate oxons derived from pesticides and direct-acting cholinesterase inhibitors such as the nerve gas sarin (Davies et al. 1996). The purified isozymes retained these differences in organophosphate hydrolysis activity normalized to arylesterase activity (Smolen et al. 1991). *PON1* contains two common polymorphisms in the coding region, Q192R (Adkins et al. 1993; Humbert et al. 1993) and L55M (Garin et al. 1997), and three common polymorphisms in the promoter at –909, –162 and –108 (Leviev and James 2000; Brophy et al. 2001a, 2001b). Promoter variants in *PON1* affect the level of expression by more than 2-fold (Boright et al. 1998; Brophy et al. 2001a, 2001b). Q192R affects the relative rate of hydrolysis of certain organophosphate substrates compared with phenyl acetate by as much as an order of magnitude but has only a small effect on chlorpyrifos oxon/phenylacetate rate of hydrolysis (Davies et al. 1996). The L55M polymorphism has been found to affect lipid peroxidation (Malin et al. 2001) and PON1 protein stability (Leviev et al. 2001), although the recent work on promoter mutations has suggested that the apparent effect of the L55M polymorphism on enzyme concentration also may be due to linkage disequilibrium (LD) with one of the promoter variants (Brophy et al. 2001b).

Toxicity of chlorpyrifos is inversely related to serum PON1 activity. Intravenous injection of PON1 before challenge by chlorpyrifos is protective in the rat (Costa et al. 1990). *PON1* knockout mice were more susceptible to chlorpyrifos toxicity (Shih et al. 1998), and the toxicity was alleviated by intraperitoneal injection of purified PON1, with the 192RR (homozygous) enzyme providing better protection than 192QQ (Li et al. 2000). Using the rat model, Attenberry et al. (1997) have demonstrated that the age-related decrease in susceptibility to chlorpyrifos CNS toxicity is not due to either the rate of activation to the oxon or to the reactivity of the oxon in the CNS but is entirely due to age-related differences in the detoxification of the oxon. Developing human fetuses have much lower protective PON1 activities than do adults, with the level in cord blood being severalfold lower (Mueller et al. 1983). Furthermore, both chlorpyrifos and chlorpyrifos oxon can cross the placenta. Subcutaneous delivery of a high dose of chlorpyrifos to pregnant rats

Address correspondence to J.G. Wetmur, Microbiology, Box 1124, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029-6574 USA. Telephone: (212) 241-7685. Fax: (212) 534-1684. E-mail: james.wetmur@mssm.edu

This work was supported by grants ES09584 and ES11643 from the National Institute of Environmental Sciences and R827039 from the U.S. Environmental Protection Agency. J.C. was supported by a Career Development Award CA81750 from the National Cancer Institute.

The authors declare they have no conflict of interest.

Received 12 November 2002; accepted 7 May 2003.

led to significant (30–49%) inhibition of fetal acetylcholinesterase activity (Chanda and Pope 1996). Thus, both fetuses and very young children may be more susceptible to pesticide exposure compared with adults. Lower PON1 activities may reflect increased risk after acute or chronic exposure to chlorpyrifos *in utero* and in the neonatal period.

Using maternal and cord blood, we have carried out a systematic study of the origin and extent of variation in PON1 activities in our population of three racial/ethnic groups comprising the majority of many inner-city populations: Caucasians, African Americans, and Caribbean Hispanics. Specifically, we address genotype–phenotype relationships for all five polymorphisms and note differences among these three racial/ethnic groups, highlighting the differences between mothers and neonates. We also address LD among the five polymorphisms, noting differences among of three racial/ethnic groups that will affect inference of haplotypes.

Subjects and Methods

Study Population

This study is part of an ongoing prospective study of the effect of pesticide exposure on infant growth and neurodevelopment conducted at the Mount Sinai School of Medicine. The study protocol was approved by the institutional review board. A total of 479 pregnant women scheduled to deliver at Mount Sinai Hospital and their neonates were enrolled in the study. Seventy-seven of these women have been excluded because of medical complications, terminations of pregnancy, very premature births, failure to collect specimens from the women before they gave birth, delivery of an infant with birth defects, change of residence, or refusal to continue to participate. A total of 402 maternal blood samples were obtained from individuals self-identified as being Caucasian (n = 82), African American (n = 117), or Hispanic (n = 203, predominantly of Caribbean origin). Maternal blood was collected in heparin-treated Vacutainer tubes (Becton, Dickinson and Co., Franklin Lakes, NJ) at about 26–30 weeks of gestation. At birth, a sample of umbilical cord blood was obtained for 229 infants (Caucasian n = 56, African American n = 66, Caribbean Hispanic n = 107). Cord blood was collected with the same anticoagulant. Although EDTA is a better anticoagulant for long-term DNA storage and polymerase chain reaction (PCR), it cannot be used because it inhibits and destabilizes PON1. Plasma was separated immediately (within 24 hr) by two cycles of centrifugation. Three aliquots of maternal plasma and three aliquots of cord blood plasma were frozen at –70°C, one for determination of PON1. The buffy coat was separated from the red blood cells, and DNA was extracted and purified using a QIAamp blood kit (Qiagen, Valencia, CA) as described by the manufacturer.

Genotyping

All PCR reactions were assembled using standard precautions in an ultraviolet-irradiated hood in a room dedicated to this purpose to prevent carryover contamination. The PON1 –909*G*→*C*, L55M, and Q192R and the *PON2* C311S genotypes were determined by the clamp-dependent allele-specific PCR (CD-ASPCR) method of Germer and Higuchi (1999). Real-time PCR methods was selected because it is a single-tube assay and offers high throughput. The primer sequences for genotyping were as follows:

–909*G*→*C*: G (sense), 5′-TGCCTCTGTACAACCATGTC–3′; C (sense), 5′-CGGCGGGGGCGGTGCCTCTGTACAACCATGTG-3′; reverse, 5′-CATGGAGCAAATCATTCACAG-3′.

L55M: M, 5′-GGCAGAAACTGGCTCTGAAGACA-3′; L, 5′-CGGCGGGGGCGCGGCAGAAACTGGCTCTGAAGACT; reverse, 5′-CACTCACAGAGCTAATGAAAGCC-3′.

Q192R: Q, 5′-TATTTTCTTGACCCCTACTTACA-3′; R, 5′-CGGCGGGGGCGGGGCTTTCTTGACCCCTACTTACG-3′; reverse, 5′-CTTCACTTGGACTATAGTAGACAA-3′.

PON2 C311S: S, 5′-CGGCGGCGGCCGCATCCA-3′; C, 5′-TCCGCATCCA-GAACATTCTATG-3′; reverse, 5′-ACACTGAGGCTACAGAACTTCC-3′.

The –108*C*→*T* and –162*A*→*G* genotypes were determined by restriction fragment length polymorphism analysis of PCR products (Brophy et al. 2001a). For –108*C*→*T*, heminested PCR was performed first with the primers 5′-TGGGCGCAGACACCGACGGG-3′ and 5′-GCCCAGCTAGCTGCCGACCC-3′ and after 1,000-fold dilution with 5′-TGGGCGCAGACACCGACGGG-3′ and 5′-CTAGCTGCCGACCCGGCCCAGAGGGG-3′. The products were digested with *Bst*XI. For –162*A*→*G*, the primers were 5′-CCAGGTGCACAGAAGGCG-3′ and 5′-GCTCCTGCGGTGGGGGCTGA-3′, and the restriction endonuclease was *Bst*UI. For each coded sample, genotypes were entered into the laboratory database and verified by a second investigator.

PON1 Assay

The hydrolysis of phenylacetate to acetic acid and phenol, measured by absorbance maximum A_{270}, was used to determine human plasma PON1 activity using the standard definition of units (Furlong et al. 1989). The molar extinction coefficient of phenol is 1,310. On the order of 5% of the PON1 activity in plasma may be due to serum cholinesterase and serum albumin (Gan et al. 1991). This contribution was minimized by performing the assays at high pH. A cholinesterase inhibitor, in this case eserine sulfate, is also necessary for specificity. The assay was first performed as described in a Hewlett-Packard diode array spectrophotometer (Agilent, Palo Alto, CA) equipped with a cell holder equipped with a thermostat. The assay was then adapted to 96-well format for high throughput and replication. A buffer solution (1.2×) was prepared as 12 mM Tris–HCl (pH 8.0), 1.2 mM $CaCl_2$, 6 µM EDTA, and 4.8 µM eserine sulfate. Individual plasma samples were diluted 333-fold by addition of 3 µL plasma per milliliter phosphate-buffered saline. Phenylacetate was dissolved in water at 36 mM (10×) immediately before use and mixed with nine parts buffer. Two hundred microliters of this mixture were added to each well. Twenty-microliter aliquots of diluted plasma were placed in individual wells. The plates were incubated at 37°C in a Bio-Tek PowerwaveX microplate reader (Bio-Tek, Winooska, VT). The increase in A_{270} was followed over time. The slope of the kinetic curve determined by the instrument software was used to calculate the rate of hydrolysis of phenylacetate. Blanks included both samples lacking plasma and samples with plasma but lacking substrate. The measurements of individual samples were reproducible within 10%, mostly limited by the serum dilution step.

Statistical Analysis

The genetic influence of *PON1* polymorphisms on PON1 activity was analyzed by analysis of variance. Because of limited serum availability for enzymatic activity determinations, a total of 390 maternal and 189 neonate data sets were analyzed. Log transformation of the enzyme activity was performed to improve normality. Linear regression was used to assess age-adjusted trends in genotypic effects on PON1 phenotypes. These relationships were examined separately in mothers and neonates as well as by race/ethnicity. LD was calculated as D' based on Lewontin, which ranges from 0 (no LD) to 1 or –1 (complete LD) (Lewontin 1988). The EH (estimating

haplotype-frequency) linkage utility program (Terwilliger and Ott 1994) was used to determine chi-square statistics and p-values for tests of allelic association between polymorphic markers. To assess independent and group effects of polymorphisms on PON1 activity, linear and multivariable regression models were used to determine the independent and group contributions of the coding and promoter polymorphisms to phenotypic expression based on Brophy et al. (2001b). All p-values are two-sided. Statistical tests were performed using SAS (SAS Institute 2001).

Results

PON Allele Frequencies

Single nucleotide polymorphism (SNP) frequencies were obtained for the five common *PON1* polymorphisms and for one common *PON2* polymorphism (Table 1). The promoter alleles are named in the sense direction. Although the allele frequencies varied substantially among race/ethnicity groups, each of the six polymorphisms has a minor allele frequency of at least 11% in all of the ethnic groups.

Table 1. Allele frequencies of *PON* genotypes.

Gene, position and allele	Mother: Caucasian (n = 82)	Mother: African American (n = 117)	Mother: Hispanic (n = 203)	Neonate: Caucasian (n = 56)	Neonate: African American (n = 66)	Neonate: Hispanic (n = 107)
PON1						
–909						
G	0.38	0.84	0.61	0.42	0.88	0.65
C	0.62	0.16	0.39	0.58	0.12	0.35
–162						
A	0.20	0.42	0.26	0.18	0.42	0.25
G	0.80	0.58	0.74	0.82	0.58	0.75
–108						
C	0.38	0.85	0.65	0.44	0.89	0.67
T	0.62	0.15	0.35	0.56	0.11	0.33
55						
L	0.54	0.79	0.71	0.58	0.81	0.78
M	0.46	0.21	0.29	0.42	0.19	0.22
192						
Q	0.73	0.37	0.54	0.76	0.30	0.50
R	0.27	0.63	0.46	0.24	0.70	0.50
PON2						
311						
S	0.80	0.79	0.77	0.75	0.73	0.77
C	0.20	0.21	0.23	0.25	0.27	0.23

PON1 Genotypes and PON1 Activity

The maternal geometric mean PON1 activity was independent of race/ethnicity, with mean values of 123, 131, and 130 U/mL for African Americans, Caucasians, and Caribbean Hispanics, respectively (p = 0.08). Effects of maternal genotypes and race/ethnicity on maternal PON1 activity are summarized in Table 2. Overall, a statistically significant ($p \leq 0.02$) difference in PON1 activity was found among genotypes of each of the three promoter polymorphisms as well as L55M. The Q192R polymorphism did not influence PON1 activity with phenylacetate. When examined by race/ethnicity independently, L55M was not a significant predictor (p = 0.14) in Caucasian mothers, whereas the promoter polymorphisms were not significant predictors in African-American mothers ($p \geq 0.16$). All four polymorphisms remained significant predictors of PON1 activity in Caribbean-Hispanic mothers ($p \leq 0.05$).

For neonates, the geometric mean PON1 activity was dependent on race/ethnicity: 44.4 (referent), 33.3 ($p < 0.002$), and 25.4 ($p < 0.0001$) U/mL for African Americans, Caribbean Hispanics, and Caucasians, respectively. Effects of neonate genotypes and race/ethnicity on neonate PON1 activity are summarized in Table 3. Similar to the mothers, the three promoter polymorphisms as well as L55M (but not Q192R) were significantly associated with PON1 activity ($p < 0.0001$). Unlike the mothers, these associations were

Table 2. Maternal *PON1* polymorphism frequencies, enzyme activities, and p-values for trend.

Position and genotype	Caucasian (n = 78): % (n)	Caucasian: Mean PON1 enzyme activity (SD)	Caucasian: p-Value (trend)[a]	African American (n = 112): % (n)	African American: Mean PON1 enzyme activity (SD)	African American: p-Value (trend)[a]	Hispanic (n = 200): % (n)	Hispanic: Mean PON1 enzyme activity (SD)	Hispanic: p-Value (trend)[a]	Combined (n = 390): % (n)	Combined: Mean PON1 enzyme activity (SE)[b]	Combined: p-Value (trend)[a]
–909												
GG	15 (12)	160.5 (16.3)		72 (80)	129.0 (28.5)		38 (76)	148.4 (34.6)		43 (168)	143.7 (2.7)	
CG	45 (35)	135.0 (33.2)	p = 0.0009	24 (27)	123.0 (29.0)	p = 0.28	47 (93)	134.1 (32.6)	$p < 0.0001$	40 (155)	130.6 (2.7)	$p < 0.0001$
CC	40 (31)	122.5 (29.1)		4 (4)	120.8 (40.9)		16 (31)	109.7 (33.8)		17 (66)	111.4 (4.1)	
–162												
AA	8 (6)	159.5 (17.2)		21 (23)	128.5 (31.4)		7 (13)	146.6 (42.6)		10 (42)	140.7 (5.2)	
AG	24 (19)	139.5 (26.6)	p = 0.02	43 (48)	130.1 (27.2)	p = 0.35	39 (78)	139.9 (33.1)	p = 0.05	37 (145)	136.4 (2.9)	p = 0.02
GG	68 (53)	129.0 (33.5)		36 (40)	122.5 (29.8)		55 (109)	131.4 (36.6)		52 (202)	127.5 (2.4)	
–108												
CC	14 (11)	161.6 (16.6)		72 (81)	129.1 (28.3)		44 (88)	150.9 (34.6)		46 (180)	145.9 (2.6)	
CT	46 (36)	134.0 (27.8)	p = 0.001	25 (28)	122.4 (28.8)	p = 0.16	42 (84)	129.9 (28.7)	$p < 0.0001$	38 (148)	127.9 (2.7)	$p < 0.0001$
TT	40 (31)	124.0 (35.1)		3 (3)	113.9 (46.9)		14 (28)	105.7 (35.8)		16 (62)	109.3 (4.1)	
55												
LL	33 (26)	139.8 (28.6)		58 (65)	134.0 (27.5)		51 (101)	149.3 (31.7)		49 (192)	143.5 (2.4)	
LM	42 (33)	133.3 (45.5)	p = 0.14	39 (44)	118.8 (27.7)	p = 0.001	43 (85)	123.9 (35.2)	$p < 0.0001$	42 (162)	124.2 (2.6)	p = <0.0001
MM	24 (19)	126.9 (34.3)		3 (3)	96.5 (32.3)		7 (14)	110.1 (29.8)		9 (36)	114.6 (5.4)	
192												
QQ	55 (43)	138.1 (33.4)		14 (16)	116.8 (32.5)		29 (57)	134.1 (42.1)		30 (116)	131.7 (3.2)	
QR	35 (27)	130.6 (31.0)	p = 0.22	44 (49)	133.2 (31.2)	p = 0.67	51 (101)	137.0 (33.9)	p = 0.55	45 (177)	134.2 (2.7)	p = 0.74
RR	10 (8)	122.7 (24.7)		42 (47)	124.1 (23.7)		21 (42)	134.8 (31.7)		25 (97)	129.4 (3.6)	

[a] p-Value for trend on log-transformed values. [b] Least-squares means adjusted for mother's race/ethnicity.

apparent in all three race/ethnicity groups ($p = 0.02$).

Figure 1 compares the genetic influences of the three promoter and L55M polymorphisms on PON1 activity in mothers and their neonates (all race/ethnicities combined). There was a striking difference in the PON1 activities, with the neonates having a geometric mean 4.1-fold lower. In addition, the genetic influence in neonates appears to be stronger, with no polymorphisms affecting maternal PON1 activity by more than 35% but with both –108 (and –909) and L55M polymorphisms affecting PON1 activity by more than 2-fold in neonates.

PON Linkage Disequilibrium: Important Alleles Affecting PON1 Activity

Pairwise LD calculations for the five polymorphisms and three race/ethnicities, reported as D' and chi-square with associated p-values, are presented in Table 4. In all three race/ethnicities, the promoter polymorphisms are in nearly complete LD with one another ($D' \geq 0.9$, $p < 0.0001$). The promoter polymorphisms are also in significant LD with L55M, but more so for Caucasians than for African Americans. L55M and Q192R are in LD in Caucasians but not in African Americans. On the other hand, the promoter polymorphisms –108*C*→*T* and –909*G*→*C* are in significant LD with Q192R in African-Americans but not in Caucasians. Interestingly, strong LD is observed between *PON2* C311S and the *PON1* promoter polymorphisms in all race/ethnicities, with the strongest linkage in Caucasians. In all of these cases, values for Caribbean Hispanics fall between those for Caucasians and African Americans.

Analysis of conditional variance (adjusted for the other polymorphisms based on Brophy et al. 2001b) showed that maternal –108*C*→*T* and L55M polymorphisms contributed 12 and 4%, 1 and 9%, and 17 and 12% of the total variance, respectively, for Caucasians, African Americans, and Caribbean Hispanics. A similar analysis of conditional variance for neonates showed that the –108*C*→*T* and L55M polymorphisms contributed 53 and 44%, 8 and 3%, and 19 and 10% of the total variance, respectively, for Caucasians, African Americans, and Caribbean Hispanics.

Table 5 shows the maternal PON1 activities for the nine possible –108*C*→*T* and L55M genotypes. Using –108*CC*, 55LL as referent, all three L55M genotypes of –108*TT* were significantly different, with decreasing PON1 activity trend from 55LL (126.6 U/mL) to 55LM (116.1 U/mL) to 55MM (105.1 U/mL). With the same referent, only the –108*TT* genotype of 55MM was significantly different, although the sample sizes were low (7 and 9) for the other –108 genotypes.

Discussion

PON1 Allele Frequencies

In this study we have investigated the effect of genotype, as reflected by all of these five common *PON1* polymorphisms, on phenotype, as reflected by PON1 activity. The subjects were pregnant women and their neonates at risk of prenatal exposure to chlorpyrifos in the home. The subjects included Caucasians, African Americans, and Caribbean Hispanics. The observed frequencies (Table 1) for the 192R allele among Caucasians and African Americans were consistent with previous studies [e.g., 0.28 for Caucasians compared with 0.27–0.31 in several previous studies (Davies et al. 1996; Leviev and James 2000; Brophy et al. 2001b)]. The allele frequencies for all five *PON1* SNPs and for *PON2* S311C among Caribbean Hispanics in New York City fell between the values for Caucasians and African Americans, with allele frequencies consistent with similar Caucasian and African-American derivation. This study was the first to include all of these polymorphisms and these race/ethnicities in a study of PON1 activity in mothers and their neonates.

PON1 Genotypes and PON1 Activity: Increased Genotypic Influence in Neonates

It has been known for many years that neonates have much lower PON1 activities than adults (Mueller et al. 1983). We have confirmed this observation for each of the race/ethnicities (Tables 2 and 3, Figure 1). Specifically, the mean PON1 activity in maternal blood was 4.6, 3.6, and 2.6 times greater than the mean PON1 activity in cord blood for Caucasians, Caribbean Hispanics, and African Americans, respectively. Again, the data for Caribbean Hispanics fall between Caucasians and African Americans. For all groups, however, the results suggest a potentially greater

Table 3. Neonate *PON1* polymorphism frequencies, enzyme activities, and *p*-values for trend.

	Caucasian (*n* = 47)			African American (*n* = 51)			Hispanic (*n* = 91)			Combined (*n* = 189)		
Position and genotype	% (*n*)	Mean PON1 enzyme activity (SD)	*p*-Value (trend)[a]	% (*n*)	Mean PON1 enzyme activity (SD)	*p*-Value (trend)[a]	% (*n*)	Mean PON1 enzyme activity (SD)	*p*-Value (trend)[a]	% (*n*)	Mean PON1 enzyme activity (SE)[b]	*p*-Value (trend)[a]
–909												
GG	15 (7)	41.8 (12.4)		75 (38)	52.4 (23.9)		40 (36)	42.8 (14.9)		43 (81)	47.1 (2.2)	
CG	53 (25)	32.2 (18.2)	$p < 0.0001$	25 (13)	36.4 (12.8)	$p = 0.005$	51 (46)	37.6 (23.9)	$p = 0.006$	44 (84)	35.8 (2.1)	$p < 0.0001$
CC	32 (15)	18.5 (7.7)		—	—		10 (9)	37.6 (23.9)		13 (24)	35.8 (2.1)	
–162												
AA	2 (1)	63.1		18 (9)	51.8		7 (7)	45.7		8 (17)	49.7	
AG	32 (15)	38.5 (20.5)	$p = 0.0004$	48 (24)	53.2 (29.3)	$p = 0.08$	41 (37)	41.3 (15.9)	$p = 0.02$	40 (76)	44.3 (2.3)	$p < 0.0001$
GG	66 (31)	23.7 (10.7)		34 (17)	40.2 (11.4)		52 (47)	34.9 (24.1)		50 (95)	32.3 (2.1)	
–108												
CC	17 (8)	50.5 (23.1)		76 (39)	51.8 (23.8)		48 (43)	46.3 (23.5)		48 (90)	49.0 (2.0)	
CT	51 (24)	28.8 (11.0)	$p < 0.0001$	24 (12)	36.8 (13.3)	$p = 0.01$	41 (37)	33.2 (13.9)	$p = 0.0004$	39 (73)	32.4 (2.2)	$p < 0.0001$
TT	32 (15)	18.5 (7.7)		—	—		11 (10)	37.6 (23.9)		13 (25)	35.8 (2.1)	
55												
LL	32 (15)	41.0 (21.1)		71 (36)	52.0 (22.5)		60 (55)	42.8 (21.9)		56 (106)	45.6 (1.9)	
LM	51 (24)	27.5 (9.1)	$p < 0.0001$	25 (13)	40.7 (21.7)	$p = 0.004$	35 (32)	33.0 (15.3)	$p = 0.0007$	36 (69)	32.6 (2.4)	$p < 0.0001$
MM	17 (8)	12.5 (5.6)		4 (2)	29.6 (15.3)		4 (4)	19.0 (19.4)		7 (14)	17.2 (5.3)	
192												
QQ	64 (30)	28.4 (14.9)		8 (4)	47.5 (29.8)		25 (23)	40.3 (20.5)		30 (57)	39.2 (2.9)	
QR	30 (14)	30.9 (21.7)	$p = 0.50$	43 (22)	48.5 (27.6)	$p = 0.59$	53 (48)	39.1 (22.5)	$p = 0.33$	44 (84)	39.4 (2.3)	$p = 0.08$
RR	6 (3)	30.0 (8.2)		49 (25)	48.2 (17.0)		22 (20)	34.1 (14.9)		25 (48)	36.6 (3.1)	

[a] *p*-Value for trend on log-transformed values. [b] Least-squares means adjusted for neonate's race/ethnicity.

degree of susceptibility of a fetus or newborn to the toxic effects of organophosphates.

Effects of functional polymorphisms in the coding region of a gene (e.g., L55M and Q192R) normally remain constant throughout the life of an individual. Polymorphisms in the promoter region of a gene (e.g., –909, –168, –108) can affect the level of transcription. We hypothesized that transcription factor interactions with the promoter may vary throughout life. PON1 activity differed among genotypes for promoter polymorphisms, as exemplified by –108*C*→*T*, and the functional polymorphism L55M. A significant effect of Q192R on PON1 activity was found in one large recent study (Brophy et al. 2001b). Our results for all race/ethnicities individually as well as combined agree with a second recent study showing no significant effect (Leviev and James 2000), but this lack of effect may be due to smaller sample size. In our combined maternal population, the –108*CC* and –108*TT* homozygotes as well as the 55LL and 55MM homozygotes had approximately 15% greater and lesser activities than the corresponding heterozygotes, respectively. The effect of the promoter polymorphisms in African-American mothers did not reach the level of significance, probably because of sample size considering the lower minor allele frequency. Another study found 20 and 12% for the –108 and L55M polymorphisms, respectively (Leviev and James 2000), and a third study found about 27 and 20%, respectively (Brophy et al. 2001b). All of these results are similar and are strikingly different from our results with cord blood.

In particular, in our combined neonate population, the –108*CC* and –108*TT* homozygotes as well as the 55LL and 55MM homozygotes had approximately 51 and 40% greater and 28 and 47% lesser activities than the corresponding heterozygotes, respectively. Thus, not only is the average potential risk to a fetus or newborn from organophosphate exposure greater than in the adult, the range of protection afforded by PON1 is more dependent on genotype than in the adult, with the –108*TT* and the 55MM homozygotes having PON1 activities 5.8- and 7.8-fold lower than the average adult, respectively.

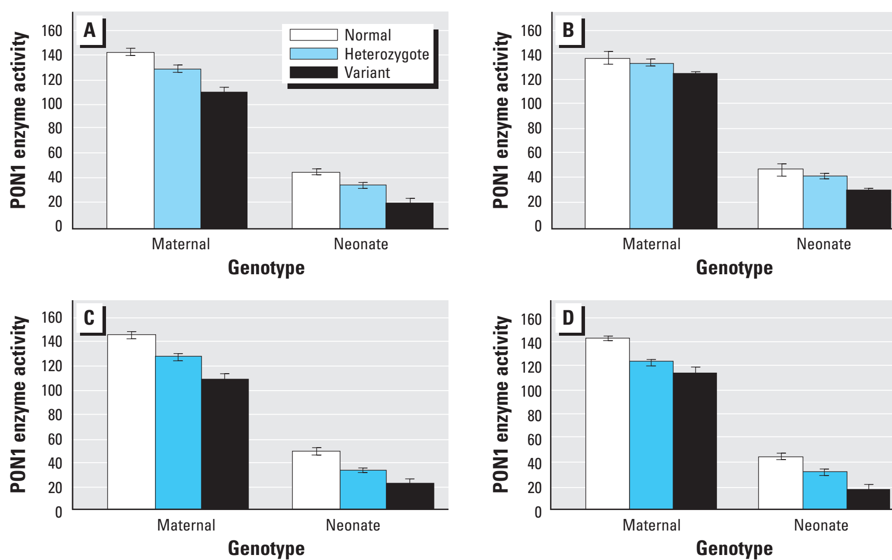

Figure 1. PON1 activity by genotype, all race/ethnicities combined for mothers and neonates. Normal refers to the allele with the highest PON1 activity. Genotypes: (*A*) –909*G*→*C*; (*B*) –162*A*→*G*; (*C*) –108*C*→*T*; (*D*) L55M. (*A, C, D*) Maternal and neonate *p*-value for trend < 0.0001. (*B*) Maternal *p*-value for trend = 0.02; neonate *p*-value for trend < 0.0001.

PON Linkage Disequilibrium: Important Alleles Affecting PON1 Activity

As previously observed (Leviev and James 2000; Brophy et al. 2001b), the promoter polymorphisms were in significant LD (Table 4). Examination of maternal PON1 activity and genotypes confirmed that nearly all of the contribution of these polymorphisms to PON1 activity could be determined by analyzing the –108 polymorphism.

The LD extends to L55M and –108*C*→*T*, but is less than among the promoter polymorphisms, with *D*′ varying from 0.42 to 0.51 depending on race/ethnicity. After correction for LD, Leviev and James (2000) and Brophy et al. (2001b) found that the –108*CT* polymorphism accounted for 24.7 and 22.8%, respectively, of the variance in PON1 for Caucasians. They also found that L55M accounted for 4.4–4.6 or 4.1% of the

Table 4. Tests of LD between maternal *PON* polymorphisms: *D*′, chi-square, and associated *p*-values.

Position:	*PON1* –909		–162		55		192		*PON2* –311	
	D′	Chi-square (*p*)	*D*′	Chi-square (*p*)	*D*′	Chi-square (*p*)	*D*′	Chi-square (*p*)	*D*′	Chi-square (*p*)
Caucasian										
–108	0.92	119.38 ($p < 0.0001$)	–1.00	51.44 ($p < 0.0001$)	0.50	12.71 ($p < 0.001$)	0.16	0.40 (p = NS)	–0.82	32.08 ($p < 0.0001$)
–909			–1.00	52.83 ($p < 0.0001$)	0.60	18.82 ($p < 0.0001$)	0.02	0.01 (p = NS)	–0.88	38.89 ($p < 0.0001$)
–162					–1.00	30.99 ($p < 0.0001$)	–0.77	5.88 ($p < 0.02$)	0.37	14.04 ($p < 0.001$)
55							–0.93	33.85 ($p < 0.0001$)	–0.43	4.30 ($p < 0.05$)
192									–0.02	0.00 (p = NS)
African American										
–108	0.97	126.06 ($p < 0.0001$)	–0.90	20.73 ($p < 0.0001$)	0.42	11.45 ($p < 0.001$)	–0.43	7.49 ($p < 0.01$)	–1.00	7.05 ($p < 0.01$)
–909			–1.00	28.23 ($p < 0.0001$)	0.35	8.41 ($p < 0.01$)	–0.34	4.99 ($p < 0.05$)	–0.99	7.81 ($p < 0.01$)
–162					–0.44	4.71 ($p < 0.05$)	–0.11	1.06 (p = NS)	0.10	0.36 (p = NS)
55							–0.18	1.72 (p = NS)	0.03	0.14 (p = NS)
192									–0.25	3.23 (p = NS)
Hispanic										
–108	0.90	213.47 ($p < 0.0001$)	–1.00	61.33 ($p < 0.0001$)	0.51	38.22 ($p < 0.0001$)	–0.29	8.76 ($p < 0.01$)	–0.87	25.38 ($p < 0.0001$)
–909			–1.00	73.61 ($p < 0.0001$)	0.47	24.77 ($p < 0.0001$)	–0.22	5.02 ($p < 0.05$)	–0.95	42.09 ($p < 0.0001$)
–162					–0.72	15.24 ($p < 0.0001$)	–0.15	1.23 (p = NS)	0.36	20.21 ($p < 0.0001$)
55							–0.50	18.72 ($p < 0.0001$)	–0.25	1.21 (p = NS)
192									–0.04	0.07 (p = NS)

NS, not significant.

Table 5. *PON1* genotypes and maternal PON1 activity [(–108*C*→*T* and L55M) all race/ethnicities combined (n = 390)].

	PON1: L55M		
PON1: –108	LL	LM	MM
CC	11,731 SD = 2,409 (n = 128)	10,366 SD = 3,006 (n = 45)	10,446 SD = 2,836 (n = 7)
CT	11,044 SD = 2,439 (n = 48)	9,940* SD = 2,148 (n = 91) p < 0.0001[a]	10,897 SD = 2,014 (n = 9)
TT	10,125 SD = 2,308 (n = 16) p = 0.02[a]	9,284* SD = 3,422 (n = 26) p < 0.0001[a]	8,410* SD = 2,500 (n = 20) p < 0.0001[a]

[a]p-Value for contrasts and estimates comparing to *PON1*: –108*CC*/55LL genotype. *Scheffe's multiple comparisons procedure: p < 0.05 compared to *PON1*:–108*CC*/55LL genotype.

variance, respectively. Our data for Caucasian mothers were qualitatively consistent, with conditional variances attributable to –108*C*→*T* being greater than that attributable to L55M, at 12 and 4%, respectively, after adjustment for the other polymorphisms. In Table 5, PON1 activity for all three L55M genotypes combined with –108*TT* were significantly different from the referent (55LL, –108*CC*, p < 0.02) and decreased from 126.6 (55LL) to 116.1 (55ML) to 105.1 (55MM) U/mL. Although the number of neonates was smaller than the number of mothers, the conditional variance attributable to genes was greater, and both polymorphisms again contributed significantly. Taken together, these data suggest that analysis of both –108*C*→*T* and L55M *PON1* genotypes is required to infer phenotype from genotype.

LD for Q192R depended strongly on race/ethnicity, with D' to –108*C*→*T* being –0.93 (p < 0.0001) in Caucasians and –0.18 (not significant) in African Americans. The reverse was seen for L55M and Q192R, with D' being –0.43 (p < 0.01) in African Americans and 0.16 (not significant) in Caucasians. As expected, D' values for Caribbean Hispanics fell between those for Caucasians and African Americans. Although Q192R had no significant effect on PON1 activity as measured using phenyl acetate as the substrate, this polymorphism affects substrate specificity and thus affects the enzymatic activity with other substrates, such as chlorpyrifos oxon, both *in vitro* and *in vivo* (Furlong et al. 2000). Therefore, for other substrates, Q192R genotype must also be determined to infer phenotype from genotype, and the race/ethnicity dependence of LD must be taken into account.

Linkage disequilibrium depends on local recombination rate (Taillon-Miller et al. 2000), population structure, and the number of generations since the mutation (polymorphism) arose (Slatkin 1999). There has been a significant debate over the size of the DNA blocks exhibiting LD (Reich et al. 2001; Dunning et al. 2000; Abecasis et al. 2001). In this study we also looked at LD between the *PON1* polymorphisms and *PON2* C311S located 97.2 kb away from Q192R and more distant from the other *PON1* polymorphisms. *PON2* C311S exhibits significant LD only in Caucasians for *PON1* L55M (D' = –0.43, p < 0.05) and no linkage for *PON1* Q192R. However, *PON2* C311S was highly linked to *PON1* –108*C*→*T* in all three race/ethnicities. It is of interest to note that both *PON1* (Imai et al. 2000) and *PON2* (Sanghera et al. 1998) polymorphisms have been linked to cardiovascular disease, although a mechanism for PON2 action has not been demonstrated (Mackness et al. 1999). The LD demonstrated in this work may facilitate similar epidemiologic analyses.

References

Abecasis GR, Noguchi E, Heinzmann A, Traherne JA, Bhattacharyya S, Leaves NI, et al. 2001. Extent and distribution of linkage disequilibrium in three genomic regions. Am J Hum Genet 68:191–197.

Adkins S, Gan KN, Mody M, La Du BN. 1993. Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B allozymes. Am J Hum Genet 52:598–608.

Atterberry TT, Burnett WT, Chambers JE. 1997. Age-related differences in parathion and chlorpyrifos toxicity in male rats: target and nontarget esterase sensitivity and cytochrome P450-mediated metabolism. Toxicol Appl Pharmacol 147:411–418.

Aviram M, Hardak E, Vaya J, Mahmood S, Milo S, Hoffman A, et al. 2000. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. Circulation 101:2510–2517.

Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL, La Du BN. 1998. Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. J Clin Invest 101:1581–1590.

Boright AP, Connelly PW, Brunt JH, Scherer SW, Tsui LC, Hegele RA. 1998. Genetic variation in paraoxonase-1 and paraoxonase-2 is associated with variation in plasma lipoproteins in Alberta Hutterites. Atherosclerosis 139:131–136.

Brophy VH, Hastings MD, Clendenning JB, Richter RJ, Jarvik GP, Furlong CE. 2001a. Polymorphisms in the human paraoxonase (PON1) promoter. Pharmacogenetics 11:77–84.

Brophy VH, Jampsa RL, Clendenning JB, McKinstry LA, Jarvik GP, Furlong CE. 2001b. Effects of 5′ regulatory-region polymorphisms on paraoxonase-gene (PON1) expression. Am J Hum Genet 68:1428–1436.

Chanda SM, Pope CN. 1996. Neurochemical and neurobehavioral effects of repeated gestational exposure to chlorpyrifos in maternal and developing rats. Pharmacol Biochem Behav 53:771–776.

Clendenning JB, Humbert R, Green ED, Wood C, Traver D, Furlong CE. 1996. Structural organization of the human PON1 gene. Genomics 35:586–589.

Costa LG, Li WF, Richter RJ, Shih DM, Lusis A, Furlong CE. 1999. The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism. Chem-Biol Interact 119–120:429–438.

Costa LG, McDonald BE, Murphy SD, Omenn GS, Richter RJ, Motulsky AG, et al. 1990. Serum paraoxonase and its influence on paraoxon and chlorpyrifos-oxon toxicity in rats. Toxicol Appl Pharmacol 103:66–76.

Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, Furlong CE. 1996. The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. Nat Genet 14:334–336.

Dunning AM, Durocher F, Healey CS, Teare MD, McBride SE, Carlomagno F, et al. 2000. The extent of linkage disequilibrium in four populations with distinct demographic histories. Am J Hum Genet 67:1544–1554.

Entrez Chromosome Map. 2002. Available: http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?, expanded to see PON1, PON3, PON2 [accessed 5 November 2002].

Furlong CE, Li WF, Richter RJ, Shih DM, Lusis AJ, Alleva E, et al. 2000. Genetic and temporal determinants of pesticide sensitivity: role of paraoxonase (PON1). Neurotoxicology 21:91–100.

Furlong CE, Richter RJ, Seidel SL, Costa LG, Motulsky AG. 1989. Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. Anal Biochem 180:242–247.

Gan KN, Smolen A, Eckerson HW, La Du BN. 1991. Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. Drug Metab Dispos 19:100–106.

Garin MC, James RW, Dussoix P, Blanche H, Passa P, Froguel P, et al. 1997. Paraoxonase polymorphism Met-Leu54 is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. J Clin Invest 99:62–66.

Geldmacher-von Mallinckrodt M, Diepgen TL, Duhme C, Hommel G. 1983. A study of the polymorphism and ethnic distribution differences of human

serum paraoxonase. Am J Phys Anthropol 62:235–241.

Germer S, Higuchi R. 1999. Single-tube genotyping without oligonucleotide probes. Genome Res 9:72–78.

Humbert R, Adler DA, Disteche CM, Hassett C, Omiecinski CJ, Furlong CE. 1993. The molecular basis of the human serum paraoxonase activity polymorphism. Nat Genet 3:73–76.

Imai Y, Morita H, Kurihara H, Sugiyama T, Kato N, Ebihara A, et al. 2000. Evidence for association between paraoxonase gene polymorphisms and atherosclerotic diseases. Atherosclerosis 149:435–442.

Leviev I, Deakin S, James RW. 2001. Decreased stability of the M54 isoform of paraoxonase as a contributory factor to variations in human serum paraoxonase concentrations. J Lipid Res 42:528–535.

Leviev I, James RW. 2000. Promoter polymorphisms of human paraoxonase PON1 gene and serum paraoxonase activities and concentrations. Arterioscler Thromb Vasc Biol 20:516–521.

Lewontin RC. 1988. On measures of gametic disequilibrium. Genetics 120:849–852.

Li WF, Costa LG, Richter RJ, Hagen T, Shih DM, Tward A, et al. 2000. Catalytic efficiency determines the in-vivo efficacy of PON1 for detoxifying organophosphorus compounds. Pharmacogenetics 10:767–779.

Mackness B, Durrington PN, Mackness MI. 1999. Polymorphisms of paraoxonase genes and low-density lipoprotein lipid peroxidation. Lancet 353:468–469.

Malin R, Laine S, Rantalaiho V, Wirta O, Pasternack A, Jokela H, et al. 2001. Lipid peroxidation is increased in paraoxonase L55 homozygotes compared with M-allele carriers. Free Radic Res 34:477–484.

Mueller RF, Hornung S, Furlong CE, Anderson J, Giblett ER, Motulsky AG. 1983. Plasma paraoxonase polymorphism: a new enzyme assay, population, family, biochemical, and linkage studies. Am J Hum Genet 35:393–408.

OMIM. 2002. Online Mendelian Inheritance in Man. Available: http://www.ncbi.nlm.nih.gov/Omim/ (for PON1 [MIM 168820], PON2 [MIM 602447], PON3 [MIM 602720]) [accessed 5 November 2002].

Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. 1996. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. Genomics 33:498–507.

Reich DE, Cargill M, Bolk S, Ireland J, Sabeti PC, Richter DJ, et al. 2001. Linkage disequilibrium in the human genome. Nature 411:199–204.

Roy AC, Loke DF, Saha N, Viegas OA, Tay JS, Ratnam SS. 1994. Interrelationships of serum paraoxonase, serum lipids and apolipoproteins in normal pregnancy. A longitudinal study. Gynecol Obstet Invest 38:10–13.

Sams C, Mason HJ, Rawbone R. 2000. Evidence for the activation of organophosphate pesticides by cytochromes P450 3A4 and 2D6 in human liver microsomes. Toxicol Lett 116:217–221.

Sanghera DK, Aston CE, Saha N, Kamboh MI. 1998. DNA polymorphisms in two paraoxonase genes (PON1 and PON2) are associated with the risk of coronary heart disease. Am J Hum Genet 62:36–44.

SAS Institute. 2001. SAS/STAT Guide for Personal Computers, Version 8. Cary, NC:SAS Institute, Inc.

Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, et al. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature 394:284–287.

Slatkin M. 1999. Disequilibrium mapping of a quantitative-trait locus in an expanding population. Am J Hum Genet 64:1764–1772.

Smolen A, Eckerson HW, Gan KN, Hailat N, La Du BN. 1991. Characteristics of the genetically determined allozymic forms of human serum paraoxonase/arylesterase. Drug Metab Dispos 19:107–112.

Taillon-Miller P, Bauer-Sardina I, Saccone NL, Putzel J, Laitinen T, Cao A, et al. 2000. Juxtaposed regions of extensive and minimal linkage disequilibrium in human Xq25 and Xq28. Nat Genet 25:324–328.

Terwilliger J, Ott J. 1994. Handbook of Human Genetic Linkage. Baltimore, MD:Johns Hopkins University Press.