SERUM PARAOXONASE (PON1) ISOZYMES: THE QUANTITATIVE ANALYSIS OF ISOZYMES AFFECTING INDIVIDUAL SENSITIVITY TO ENVIRONMENTAL CHEMICALS

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ABSTRACT:
In a recent study on Gulf War veterans who developed delayed neurotoxicity symptoms, we found their levels of serum paraoxonase (PON1) isozyme type Q to be significantly lower than in the control, unaffected veteran group. These results were obtained in 25 ill veterans and 20 well control subjects, of which 10 were deployed and 10 were nondeployed battalion members who remained in the United States during the Gulf War. The blood samples were also assayed for serum butyrylcholinesterase in our laboratory, and more recently in Dr. C. Broomfield’s laboratory for somanase and sarinase activities. The cholinesterase activities showed no significant correlation with the PON1 isozyme levels or the severity of the clinical symptoms, but the somanase and sarinase levels ran parallel to the PON1 type Q isozyme concentrations. Although there is no direct evidence that these Gulf War veterans were directly exposed to or encountered either of these nerve gases, they may have been exposed to some environmental or chemical toxin with a similar preference for hydrolysis by the PON1 type Q isozyme. The number of subjects is relatively small, but the results should encourage other investigators to examine both the individual phenotypes and the levels of PON1 isoforms in other groups exhibiting neurological symptoms.

Recent technical advances have greatly simplified the methods for determining individual genotypes and phenotypes, and these methods are now being widely used in epidemiological investigations and surveys of large numbers of people with various medical conditions. The aim of most of these studies is to identify particular genetic markers and/or isoforms that are closely associated with hereditary disorders or may be responsible for unusual individual sensitivities to certain environmental chemicals. The polymorphic forms of a human serum enzyme, paraoxonase (PON1)1, have become a conspicuous example of such correlative studies because one of its two isoformic forms (Q or R) has been reported by some investigations (but not others) to be associated with several types of cardiovascular disease (Serrato and Marian, 1995; Antikainen et al., 1996; Herrmann et al., 1996; see Navab et al., 1996; Suehiro et al., 1996; Heinecke and Lusis, 1998; Mackness et al., 1998), Parkinson’s disease (Kondo and Yamamoto, 1998; Akhmedova et al., 1999), diabetic neuropathy (Ikeda et al., 1998; Kao et al., 1998), and central retinal vein occlusion (Murata et al., 1998), as well as greater toxicity after exposure to certain organophosphate compounds (Costa et al., 1998, 1999). Most, but not all of these studies have been confined to either genotyping by DNA analysis, or to only phenotyping by different substrate activity ratios. It is important to point out the advantages and greater usefulness of measuring the levels of the PON1 isoformic components as well as knowing the genotypes for the sample population being surveyed. Advantages of combining the enzyme levels with genotypic information will be illustrated by extending our recent studies on the characteristics of the serum PON1 of some Gulf War veterans (Haley et al., 1999).

Polymorphic Variation in Human Paraoxonase Activity

Human serum PON1 contains two common polymorphic sites; one is at position 55 (Leu/Met), and affects the level of PON1 activity, with the Leu55 isozyme associated with about 30% higher activity, on average, than the Met55 isozyme (Garin et al., 1997). To date, no evidence has been presented that the 55 (Leu/Met) polymorphism influences to any significant degree, the quality of the PON1 enzymatic activity. In contrast, the second common polymorphic site at position 192 (Arg/Gln) determines qualitative properties of the enzyme, the particular substrate specificity patterns of each of the two isoforms, and their distinctive affinities for very different substrates. Exactly how the Arg/Gln isoforms exert such a pronounced effect on the catalytic properties of the active center of this enzyme is still to be determined. The different substrate preferences cannot be anticipated, and these properties must be determined experimentally by direct testing. In the presence of 1 M NaCl and at a pH of 10.5, the Arg192 isozyme shows nearly 10 times as much paraoxonase activity as the same concentration of the Gln192 isozyme (Eckerson et al., 1983). Although these are not physiological conditions, they bring out the distinct features useful for phenotyping and help distinguish between homozygous type R individuals and heterozygous (type Q/R) subjects.

1 For the PON1 codon 192 (glutamine or arginine) polymorphism, we generally use A or B, respectively, when we determined phenotype by the paraoxonase/arylesterase activity ratio (Eckerson et al., 1983). When DNA analyses are made (Humbert et al., 1993), we use Q and R to indicate the respective genotypes. Generally these two designations are equivalent (A = Q, B = R), but we have found a few interesting exceptions.
In contrast, several organophosphates are much better substrates for the Gln192 isozyme; these include diazoxon, soman, and sarin (Davies et al., 1996). A few substrates, like phenyl acetate and chlorpyrifos oxon, are hydrolyzed at about the same velocities by both the Arg192 and the Gln192 isozymes.

The above-mentioned pronounced differences in isozymic preferences for the substrates (paraoxon versus phenyl acetate) were used to identify individual phenotypes [formerly called isozymes A, AB, and B by Eckerson et al. (1983)] before the structural basis of the Arg/Gln isozymes became known. The ratio obtained by dividing paraoxonase activity (in the presence of 1 M NaCl) by the arylesterase activity (using phenyl acetate) was used to identify the three phenotypes, based on the observed ratios of about 1.2 for the A phenotype, about 4.5 for the AB heterozygous group, and about 9 or 10 for the B phenotype. These phenotype ratios corresponded perfectly to the Q, QR, and R genotypes, respectively, when the latter could be determined by DNA sequencing of the nucleotide regions responsible for the amino acid (Gln or Arg) at position 192 (Adkins et al., 1993). Family pedigree analyses also showed the expected concordance between the A/B phenotypes and the Q/R genotypes. Several alternative typing methods have subsequently been suggested, such as the ratio of chlorpyrifos-oxonase activity versus paraoxonase, or diazoxon hydrolysis versus paraoxonase (see Richter and Furlong, 1999).

All of these phenotyping methods take advantage of the different isozymic preferences for substrates, and enhanced effects by salts and selective inhibitors on the human serum Q and R PON1 isozymes. It should be noted, however, that although the phenotype classes distinguished by plasma enzyme assays and genotypes are regularly found to be in complete agreement, rare exceptions have been observed in our laboratory. It is important to distinguish between phenotypes, based on properties of the blood PON1 enzyme, and the PON1 genotype, determined by direct analysis of the patient’s DNA (Humbert et al., 1993).

Analyses of PON1 genotypes by DNA analysis, alone, give much less information than measuring both the level of the individual isozymic esterase activities, and the genotype (see Richter and Furlong, 1999). Having both pieces of information permits a calculation of the contribution of each isozymic type of activity. This is regularly done with homozygous individuals, but it can also be extended to heterozygous individuals. The mixture of two isozymes in heterozygous individuals may vary considerably from a 50:50 ratio. Heterozygous subjects can also be used to determine whether one isozyme is much more important than the other in determining the sensitivity (or resistance) to a toxic substrate from the environment. This should be particularly evident in instances where there will be relatively few individuals homozygous for the less common allele in the sample population (Playfer et al., 1976). This is the situation for the less frequent R allele (<10% homozygous) in Caucasian populations of Northern Europe and the United States. Interestingly, the Q allele is

### Table 1

Relative hydrolytic rates and kinetic constants of purified human PON1 types Q and R with select organophosphates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; / K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>Q</th>
<th>R</th>
<th>QR</th>
<th>Q</th>
<th>R</th>
<th>QR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>355</td>
<td>38</td>
<td>9.34</td>
<td>329</td>
<td>68</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Soman&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2,143</td>
<td>992</td>
<td>2.16</td>
<td>195</td>
<td>124</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Diazoxon&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12,318</td>
<td>7,948</td>
<td>1.55</td>
<td>1225</td>
<td>2667</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Phenyl acetate&lt;sup&gt;c&lt;/sup&gt;</td>
<td>138</td>
<td>145</td>
<td>0.95</td>
<td>1225</td>
<td>2667</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos oxon&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.48</td>
<td>9.79</td>
<td>0.76</td>
<td>0.94</td>
<td>7.8</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Paraoxon&lt;sup&gt;c&lt;/sup&gt;</td>
<td>328</td>
<td>1,769</td>
<td>0.19</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Broomfield and Ford, 1991.
<sup>b</sup> Davies et al., 1996.
<sup>c</sup> Smolen et al., 1991.

**FIG. 1.** The hydrolysis of sarin versus total Type Q PON1 arylesterase activity in a sample of Gulf War veterans (see Haley et al., 1999). The circled subjects were those with neurological symptoms. ND = nondeployed veterans.
the rarer one (<10% homozygous) in Oriental and African populations (see La Du, 1992).

Arylesterase activity measured by the rate of hydrolysis of phenyl acetate is about the same per milligram of enzyme protein for both the Q-type and R-type PON1 isozymes. This permits simple allocation of the total aryelsterase activity in heterozygous serum, by interpolation, based on the ratio of paraoxonase/arylesterase activity found for that person. The average type Q and R homozygous types show ratios of about 1.2 and 9.0, respectively, so an equal mixture of the two isozymes would fall about half-way between (La Du and Eckerson, 1984). Thus, a Q/R heterozygous person with a ratio of 5.3 should have a mixture containing about half type Q and half type R. If one isozyme is critical concerning sensitivity to a particular toxic agent, there should be a dependence on the level of that particular isozyme in heterozygous individuals. The requirements for such a relationship are: 1) that the other isozyme contributes very little, so the latter could be given a value of zero, and homozygotes would lack activity of the critical type. 2) There would be a reasonably high correlation between the level of the critical isozyme and the degree of protection, that is, quantitative differences in the level of the important isozyme confer different degrees of resistance. A recent review of several toxicological studies has provided critical experimental data supporting the quantitative requirements for PON1, and, to some degree, the effect of different isozymes against organophosphate damage (Costa et al., 1999). The availability of mice lacking PON1 also provides a useful model to study the role of different isozymes and their quantitative requirements for different organophosphates and other substrates (Shih et al., 1998).

**Kinetic Evidence of Isozymic Differences in Organophosphate Inactivation**

The $K_m$ and $V_{max}$ properties of the human serum PON1 isozymes with different ester substrates (Smolen et al., 1991) suggested that one isozyme is likely to be more critical than another for the metabolism of many other compounds, and that has been found to be true for a number of the organophosphate substrates (Table 1). Clearly, isozyme R appears to be much more important for paraoxon metabolism than type Q. In contrast, the two isozymes are about equally effective in hydrolyzing chlorpyrifos oxon. Thus, chlorpyrifos oxon, like phenyl acetate, can be used to measure total aryelsterase activity for both isozymes. However, other organophosphate compounds, such as soman and particularly sarin, are hydrolyzed much better by the Q isozyme. Whether a comparison is made based on the relative rates of hydrolysis of these substrates in Table 1 under standard assay conditions, or from the ratios of their $V_{max}/K_m$ values, there is a nearly a 50-fold difference between the preferences for paraoxon and sarin. Whether the in vivo comparisons would be as great is not known, but appreciable differences in isozymic preference would be anticipated.

One might predict that people homozygous for R-type PON1 activity would be more susceptible to the acute effects of sarin and might also be subject to delayed effects of this agent. They might be less efficient in completely clearing sarin from the blood and tissues initially and be at increased risk from chronic delayed effects from this agent. On the other hand, agricultural workers, including sprayers and pesticide manufacturer workers, who are homozygous for the R-isozyme might show greater tolerance to paraoxon. These hypotheses may be supported or rejected in the future by experiences in accidental organophosphate exposures, if individual PON1 genotypes and serum enzyme levels can be obtained and compared with the severity of their clinical symptoms.

Some modifying features that would affect such a simple interpretation must be considered. For example, the liver and possibly other tissue sources of PON1 may not be parallel to the serum levels, so measuring the serum PON1 enzymatic activity may not be a reliable index for the total PON1 enzymatic activity in the body. Activation of the agents generally used as organophosphate insecticides requires conversion to the oxon forms by microsomal systems that may be induced and at variable levels in the population. It is not clear how much of the derived active organophosphate circulates in the blood and tissues after activation from the precursor compounds. Some authorities have claimed in the past that very little of it circulates in the blood, or escapes from the liver via the blood and thence to the brain. Nonetheless, these simple relationships can be tested and critical thresholds for the Q- or R-type PON1 activities may be predicted that represent minimal protective concentrations, below which toxicity would be expected. Fortunately, although there exists wide individual variation in the levels of PON1 among different ethnic groups around the world, each person maintains not only his inherited genotype but also his PON1 activity level, which remains relatively constant over the years (Furlong, 2000).
Direct Assays of Somanase and Sarinase Activities of Gulf War Veteran Plasma Samples

From the kinetic considerations given in Table 1, it would be predicted that the reduced level of type Q-PON1 arylesterase activity found in the more severely affected Gulf War veterans with neurological symptoms, should also have a reduced level of sarinase and somanase activities in these plasmas. Dr. C. Broomfield kindly did such assays, and the results are illustrated in Figs. 1 and 2. Although the numbers are small, there is obviously a direct relationship between the total type Q activity and the somanase and sarinase activities in these individuals. Dr. Broomfield has obtained somanase and sarinase measurements in a much larger group of control subjects obtained in another investigation (Davies et al., 1996), and these will be compared with the Gulf War veteran values in another report now being written.

In summary, the neurological symptoms experienced by the Gulf War veterans that we studied were more severe in those with reduced levels of serum PON1 type Q, sarinase, and somanase activities. This suggests that these soldiers may have been made chronically ill by exposure to one or more toxic compounds that are preferentially hydrolyzed by the Q-type isozyme. This possibility needs to be investigated further.

References


