methods

A sandwich enzyme-linked immunosorbent assay for human serum paraoxonase concentration

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Abstract **Serum paraoxonase (PON) is associated with plasma high density lipoproteins, and prevents the oxidative modification of low density lipoproteins. We have developed a sensitive sandwich enzyme-linked immunosorbent assay (ELISA), using two monoclonal antibodies against PON, to measure serum PON concentration. The concentration of PON in healthy Japanese subjects was 59.3 ± 1.3 μg/mL (mean ± SEM; n = 87). Serum PON concentrations in relation to the PON 192 genetic polymorphism were: 69.5 ± 2.9 μg/mL in the QQ genotype; 63.0 ± 1.9 μg/mL in the QR genotype; and 52.8 ± 1.7 μg/mL in the RR genotype. Concentrations were significantly lower in the RR than in the QQ genotype ($P < 0.01$). Serum paraoxonase specific activity was higher in RR than in QQ subjects (18.6 ± 0.40 vs. 2.56 ± 0.05 nmol/min/μg, $P < 0.01$), but arylesterase specific activity was unrelated to genotype. PON concentration was positively associated ($P < 0.001$) with both serum arylesterase activity and, after adjusting for the effect of the position 192 polymorphism, with serum paraoxonase activity. Subjects with angiographically verified coronary heart disease had significantly lower PON concentrations than the healthy controls (52.0 ± 2.3 μg/mL; n = 35, $P < 0.01$). This association was independent of the position 192 genotype. Our new ELISA should be of value for epidemiologic and clinical studies of serum PON concentration.—** Kujiraoka, T., T. Oka, M. Ishihara, T. Egashira, T. Fujioka, E. Saito, S. Saito, N. E. Miller, and H. Hattori. **A sandwich enzyme-linked immunosorbent assay for human serum paraoxonase concentration. *J. Lipid Res.* 2000.** 41: **1358–1363.**

Supplementary key words low density lipoproteins • high density lipoproteins • phospholipids • coronary heart disease

Paraoxonase (PON, aryldialkylphosphatase, EC 3.1.8.1) is a calcium-dependent esterase that is a component of plasma high density lipoproteins (HDLs), and hydrolyzes organophosphates (e.g., paraoxon, diazoxon) and arylesters (e.g., phenyl acetate) (1). Although its natural substrate is not certain, studies have indicated that PON hydrolyzes phospholipid peroxides in both HDLs and low density lipoproteins (LDLs) (2), and that this is one mechanism by which HDLs prevent the oxidative modification of LDLs (3). Two major genetic polymorphisms of PON have been described, due to glutamine or arginine at position 192 and methionine or leucine at position 55 (4). The paraoxonase activity of the Gln-192 (Q allele) isoform has been reported to be lower than that of the Arg-192 (R allele) isoform (5). Nevertheless, PON in QQ genotype subjects appears to be more effective than that in RR genotype subjects in protecting LDLs from oxidation (6). It has been suggested that the active site in PON that protects LDLs differs from the active sites for its paraoxonase and arylesterase activities (7). Low serum paraoxonase activity has been observed in patients with myocardial infarction (8), hypercholesterolemia (9), and diabetes mellitus (9), and there is evidence that the position 192 polymorphism may be a risk factor for coronary heart disease (CHD) in some populations (10). Such findings have indicated that PON plays an important role in lipid metabolism, and have created a need for reliable immunoassays of serum PON concentration for clinical and epidemiologic studies. In the present work we raised two monoclonal antibodies against PON purified from human plasma, and used them to develop a sandwich enzyme-linked immunosorbent assay (ELISA). The assay was then used to examine the relationships of serum PON concentration to serum paraoxonase activity, serum arylesterase activity, the PON 192 genotype, and angiographically verified CHD.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid; CHD, coronary heart disease; EDTA, ethylenediamine-*N,N,N′,N′*-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; LCAT, lecithin–cholesterol acyltransferase; PAF-AH, platelet-activating factor acetylhydrolase; PON, paraoxonase.

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MATERIALS AND METHODS

Materials

Cibacron blue F3GA-agarose type 3000-CL was purchased from Sigma (St. Louis, MO), DEAE-Sepharose from Pharmacia (Uppsala, Sweden), and Nonidet P-40 from Nacalai Tesque (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Purification of PON

Human PON was purified by a modification of the procedure described by Gan et al. (11). Briefly, 1 M $CaCl_2$ was added to heparinized plasma to yield a final concentration of 10 mM. After stirring at room temperature for 2 h, the insoluble precipitate was removed by centrifugation (5000 *g*, 20 min) at 4°C. The supernatant was mixed with an equal volume of Cibacron blue F3GA-agarose type 3000-CL, equilibrated with 50 mM Tris-HCl (pH 8.0) containing 1.0 mM $CaCl_2$, 5 μM EDTA, and 3 M NaCl (blue buffer). The volume was adjusted to 2 L with the same buffer. The mixture was then filtered by suction in a Buchner funnel (18.5-cm diameter) through a Whatman (Clifton, NJ) No. 3 filter paper. The gels were washed 5 times with 2 L of blue buffer and 3 times with 2 L of blue buffer without NaCl (to reduce the ionic strength), and the gel slurry was then transferred to a C26/40 column (Pharmacia). The PON was eluted with blue buffer containing a 0–0.1% deoxycholate linear gradient (500 mL each).

The partially purified fraction thus obtained was mixed with an equal volume of 20 mM Tris-HCl (pH 8.0) containing 0.2% Nonidet P-40, 40% glycerol, 1 mM $CaCl_2$, and 5 μM EDTA. The mixture was then mixed with an equal volume of DEAE-Sepharose CL-6B equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.1% Nonidet P-40, 20% glycerol, 1 mM $CaCl_2$, and 5 μM EDTA (DEAE buffer). After the suspension had been centrifuged (1000 *g*, 1 min) at room temperature, the gel slurry was transferred to the C16/40 column. The PON was eluted with DEAE buffer containing a 0–0.35 M NaCl linear gradient (250 mL each).

The eluate obtained by DEAE chromatography was diluted with an equal volume of DEAE buffer without NaCl. The suspension was then applied to the DEAE-Sepharose CL-6B column (C10/10; Pharmacia). PON was eluted with DEAE buffer containing a 0–0.35 M NaCl linear gradient (100 mL each), dialyzed against 10 mM Tris-HCI (pH 8.0) containing 0.1% Nonidet P-40, 20% glycerol, 1 mM $CaCI_2$, and 5 μM EDTA, and stored at 4°C. The paraoxonase activity, arylesterase activity, and immunoreactivity of the final PON preparation were stable for at least 6 months. The purity of the enzyme was verified by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein mass was quantified with a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL), using bovine serum albumin as standard.

Preparation of monoclonal antibodies against PON

Female BALB/c mice were immunized four times by intraperitoneal injection of 25 μg of purified PON mixed with Freund's complete (first injection) or incomplete (subsequent injections) adjuvant. Spleen cells from immunized mice were fused with SP2 murine myeloma cells, and cultured in hypoxanthine–aminopterin–thymidine selection medium. Positive hybridoma cells were screened by an ELISA, employing a microtiter plate coated with 0.5 μg of purified PON. After repeat cloning by limiting dilution techniques, positive hybridoma cells were cloned. The monoclonal hybridoma cells were passaged in Pristane-primed BALB/c mice for ascites fluid production, and the monoclonal IgG was isolated on a protein A-Sepharose CL4B column (Pharmacia) according to the manufacturer instructions. The IgG was then dialyzed against phosphate-buffered saline (PBS) at 4°C, and stored in aliquots at −80°C. The specificity of monoclonal antibodies was analyzed by Western blotting, using purified PON and serum samples.

Sandwich ELISA for serum PON concentration

Each well of a microtiter plate was coated with 0.25 μg of monoclonal antibody 5-10D, and incubated at 4°C overnight. After blocking the plate with Block Ace™ (Dainihon Pharmacy, Osaka, Japan) for 2 h at room temperature, standard and serum samples (diluted in PBS containing 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid [CHAPS]) were added to wells, and incubated for 2 h at room temperature. After washing with PBS containing 0.1% Tween 20, 100 μL of biotinylated monoclonal antibody 4C-1 (0.5 μg/mL) was added, and the plate was incubated for 2 h at room temperature. After washing the plate, avidin–horseradish peroxidase conjugate (0.2 μg/mL) in PBS containing 0.1% Tween 20 was added to each well, and incubated for 1 h at room temperature. *o*-Phenylenediamine dihydrochloride (0.4 mg), dissolved in 50 mM citrate buffer (pH 5.0) containing 0.012% H_2O_2, was then added to each well. After incubation for 30 min at room temperature, the reaction was terminated by addition of 20 μL of 2 N H_2SO_4, and absorbance was read at 492 nm in a microplate reader. Serum concentration of PON was determined by reference to a standard curve constructed with purified PON.

Assay of paraoxonase and arylesterase activities

Paraoxonase and arylesterase activities were determined according to Eckerson et al. (5). Briefly, paraoxonase activity was measured by using as substrate 1.0 mM paraoxon in 50 mM glycine–NaOH (pH 10.5), 1 mM $CaCl_2$, and 1 M NaCl. The generation of *p*-nitrophenol at 25°C was monitored at 412 nm in a continuously recording spectrophotometer (DU-640; Beckman, Fullerton, CA). Enzyme activity was calculated with a molar extinction coefficient of 18,290 M^{-1} cm^{-1}. One unit of paraoxonase activity produced 1 nmol of *p*-nitrophenol per minute. Arylesterase activity was measured by using as substrate phenyl acetate in 20 mM Tris-HCl (pH 8.0) containing 1 mM $CaCl_2$. The rate of hydrolysis was determined spectrophotometrically at 270 nm. Enzyme activity was calculated with a molar extinction coefficient of 1,310 M^{-1} cm^{-1}. One unit of arylesterase activity corresponded to 1 μmol of phenyl acetate hydrolyzed per minute.

Analysis of PON 192 genotype

The PON 192 genotype was determined by the restriction fragment length polymorphism described by Humbert et al. (12). Genomic DNA (0.5 μg) was amplified by polymerase chain reaction (PCR), using paired primers 5′-TAT TGT TGC TGT GGG ACC TGA G-3′ (sense primer) and 5′-CTT GCC ATC GGG TGA AAT GTT G-3′ (antisense primer). Amplification was carried out for 30 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 90 sec. The 193-bp products were digested with 1 U of restriction endonuclease *Alw*I (New England BioLabs, Beverly, MA) at 37°C overnight. The digested products were separated by electrophoresis on a 3% agarose gel, and visualized with ethidium bromide.

Subjects and clinical procedures

Blood from apparently healthy volunteers who had fasted overnight was collected at the BML Clinical Reference Laboratory (Saitama, Japan). Blood from patients with chest pain on exertion and angiographically verified CHD was collected at the Second Department of Internal Medicine (Nihon University Hospital, Toyko, Japan). An angiogram was considered to be positive when at least one vessel had a lumenal narrowing of

TABLE 1. Clinical details of the study subjects

	Healthy Controls	CHD Cases
Number of subjects (M/F)	87 (56/31)	35 (27/8)
Age (years)	61.8 ± 3.4	60.4 ± 1.8
Plasma cholesterol (mg/dL)	182 ± 19.5	193. ± 7.9
Plasma triglycerides (mg/dL)	117 ± 12.7	151 ± 14.1
HDL cholesterol (mg/dL)	60 ± 1.6	45 ± 3.4

Lipid concentrations are means ± SEM.

75% or more. The CHD patients were not taking any drugs, nor did they have any diseases known to affect HDL concentration. They had significantly lower HDL cholesterol concentrations (mean difference, −25%, $P < 0.001$) than the controls, but there were no significant differences between the two groups in terms of plasma triglycerides or total cholesterol. Clinical details are summarized in **Table 1**.

The study was approved by the Institutional Review Board and subjects gave informed consent.

RESULTS

Purified PON

The purified PON showed one 45-kDa band after silver staining of SDS-polyacrylamide gels (**Fig. 1A**). Gel scanning showed that this represented more than 90% of total protein.

Monoclonal antibodies

Several clones of cells secreted antibody to purified PON coated on a microtiter plate. The immunoreactivities of all antibodies (with and without biotinylation) were further tested against human serum samples. In Western blots each reacted with a single 45-kDa serum protein (Fig. 1B). No antibody inhibited the paraoxonase activity or arylesterase activity of the purified PON (data not shown). After a series of experiments using biotinylated antibodies and PON-coated microtiter plates, two antibodies were selected on the basis of absence of competition for PON (data not shown). These were 4C-1 for capture, and 5-10D for detection. They reacted equally with PON from QQ and RR genotype subjects (data not shown). Both are available from BML.

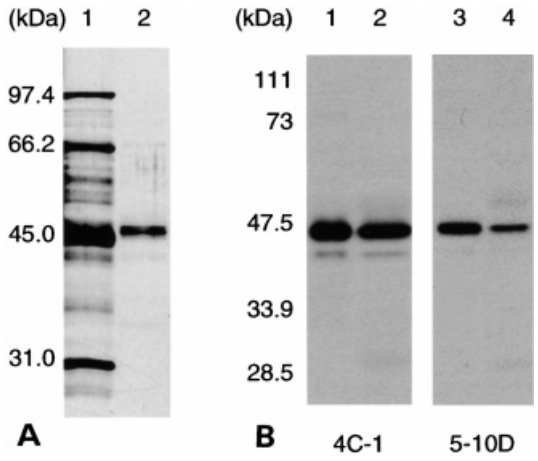

Fig. 1. Characterization of PON and monoclonal antibodies by SDS-PAGE. (A) Purified PON visualized by silver staining. Lane 1, molecular mass markers; lane 2, 0.5 μg of purified PON. (B) Immunoblots of 0.5 μg of purified PON (lanes 1 and 3) and 0.1 μL of serum (lanes 2 and 4) obtained with monoclonal antibodies 4-C1 (lanes 1 and 2) and 5-10D (lanes 3 and 4).

Standardization of the ELISA

For calibration of the ELISA purified PON was used as a primary standard. The protein concentration of the PON solution was typically 0.5–1.0 mg/mL. A calibration curve obtained with the ELISA, using concentrations of purified PON ranging from 0.3125 to 20 ng/mL, is shown in **Fig. 2**. We tested several types of detergent for sample dilution, including Tween-20, Triton X-100, and CHAPS. Serum samples diluted with PBS containing 0.1% CHAPS gave the highest absorbance values, but those for the primary standard did not differ. Therefore, 0.1% CHAPS was used routinely. To avoid potential nonlinearity caused by low or high absorbance, serum samples were assayed at several dilutions, and the lowest dilution ($1:10^4$) that gave an absorbance value of 0.5–1.0 was chosen.

Precision and accuracy of the assay

Replicated assays (n = 5) gave interassay coefficients of variation of 11.7, 6.8, 5.2, and 4.9%, and intraassay coefficients of variation of 6.1, 3.7, 4.6, and 2.2%, at PON concentrations of 12.5, 25, 50, and 100 μg/mL, respectively. Intraassay coefficients of variation for serially diluted serum samples were 5.1, 4.1, 4.2, and 3.3% at final measured concentrations of 15.8, 32.2, 62.0, and 115.6 μg/mL, respectively.

Experiments were performed to determine the recoveries of PON mass and arylesterase activity, when serum samples with different PON concentrations and enzyme activities were mixed in equal volumes. For this purpose sera

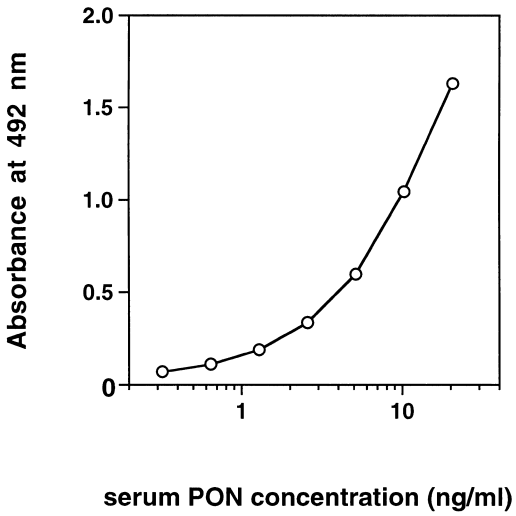

Fig. 2. Standard curve for purified PON concentration by ELISA. Each point is the mean of measurements made in triplicate.

TABLE 2. Recoveries of PON mass and arylesterase activity when serum samples were mixed

	PON Concentration			Arylesterase Activity		
	Measured	Expected	Recovery	Measured	Expected	Recovery
	μg/mL	*μg/mL*	%	*μmol/min/mL*	*μmol/min/mL*	%
Serum A	54.1			116.8		
Serum B	53.1			110.5		
Serum C	41.5			88.8		
Serum D	5.3			7.2		
Serum A plus B	53.7	53.9	99.6	112.0	113.7	98.5
Serum A plus C	48.6	47.8	101.7	103.7	102.8	100.9
Serum A plus D	29.3	29.7	98.6	62.7	62.0	101.1

Sera were mixed in equal volumes. Serum D was from a patient with liver failure. Results are the means of triplicates.

were used from three normal subjects and from one subject with an extremely low PON concentration due to liver failure. The results obtained with the mixtures were in good agreement with those predicted (**Table 2**).

Serum PON concentrations

Results obtained with serum samples from the healthy subjects are presented in **Table 3**. PON concentration was lowest in RR genotype subjects, highest in the QQ genotype subjects, and intermediate in the QR genotype subjects. Both serum paraoxonase activity and paraoxonase specific activity were highest in the RR subjects, lowest in the QQ subjects, and intermediate in the QR subjects. Serum arylesterase activity showed the opposite trend, while arylesterase specific activity was unrelated to genotype. Within each genotype serum PON concentration was positively correlated with the serum enzyme activities (**Fig. 3**). Within each genotype PON concentration was also positively correlated with HDL cholesterol, although in none did this achieve significance ($r = 0.19–0.31$, $P = 0.29–0.38$).

In the CHD patients the RR genotype was again associated with the lowest PON concentrations (**Table 4**). Patients of each genotype had lower mean concentrations of PON than did healthy subjects of the same genotype. This was significant for the RR subjects ($P < 0.05$) and for all genotypes combined ($P < 0.01$) (Table 4). Mean serum paraoxonase and arylesterase activities were also lower in the CHD patients than in the controls, these differences achieving statistical significance in comparisons of QR subjects ($P < 0.05$) and RR subjects ($P < 0.01$) and, in the case of arylesterase activity, also in all genotypes combined ($P < 0.001$). Within the genotypes, PON concentration was positively correlated with both paraoxonase activity and arylesterase activity (Fig. 3). Within each genotype positive associations were also found between PON concentration and HDL cholesterol, but only in the case of QR subjects was this statistically significant ($r = 0.72$, $P < 0.01$).

DISCUSSION

We have developed a sandwich ELISA for serum PON concentration, using two monoclonal antibodies to PON purified from human plasma. The specificity of the antibodies was confirmed by Western blotting. Monoclonal antibodies 4C-1 and 5-10D each reacted with a single protein in human serum of about 46 kDa in molecular mass, which is the same as that previously reported for serum PON (13). The antibodies did not compete for purified PON. Reactivity with serum from QQ genotype subjects was identical to that with serum from RR genotype subjects, indicating that the glutamine/arginine polymorphism of PON had no effect on the assay. We used a solution of the cation–anion detergent CHAPS for dilution of serum samples, as this produced greater reactivity than other mild nonionic detergents.

Serum PON concentration has been measured by others with a competitive ELISA using a monoclonal antibody (14–16). In that method a plate is precoated with HDLs,

TABLE 3. Serum PON concentration, serum paraoxonase and arylesterase activities, and paraoxonase and arylesterase specific activities, in apparently healthy subjects according to PON 192 genotype

	PON 192 Genotype			
	QQ	QR	RR	Total
Number of subjects	13	34	40	87
PON concentration (μg/mL)	69.5 ± 2.9	63.0 ± 1.9	52.8 ± 1.7[a]	59.3 ± 1.3
Paraoxonase activity (nmol/min/mL)	177 ± 7.7	556 ± 15[a]	968 ± 27[a]	689 ± 34
Arylesterase activity (μmol/min/mL)	147 ± 6.2	136 ± 3.6	114 ± 2.9[a]	127 ± 2.5
Paraoxonase specific activity (nmol/min/μg)	2.56 ± 0.05	8.95 ± 0.23[a]	18.6 ± 0.40[a]	12.4 ± 0.69
Arylesterase specific activity (μmol/min/μg)	2.12 ± 0.05	2.18 ± 0.05	2.19 ± 0.05	2.18 ± 0.03

Results are means ± SEM. Comparisons with QQ subjects by Student's *t*-test.
[a] $P < 0.01$.

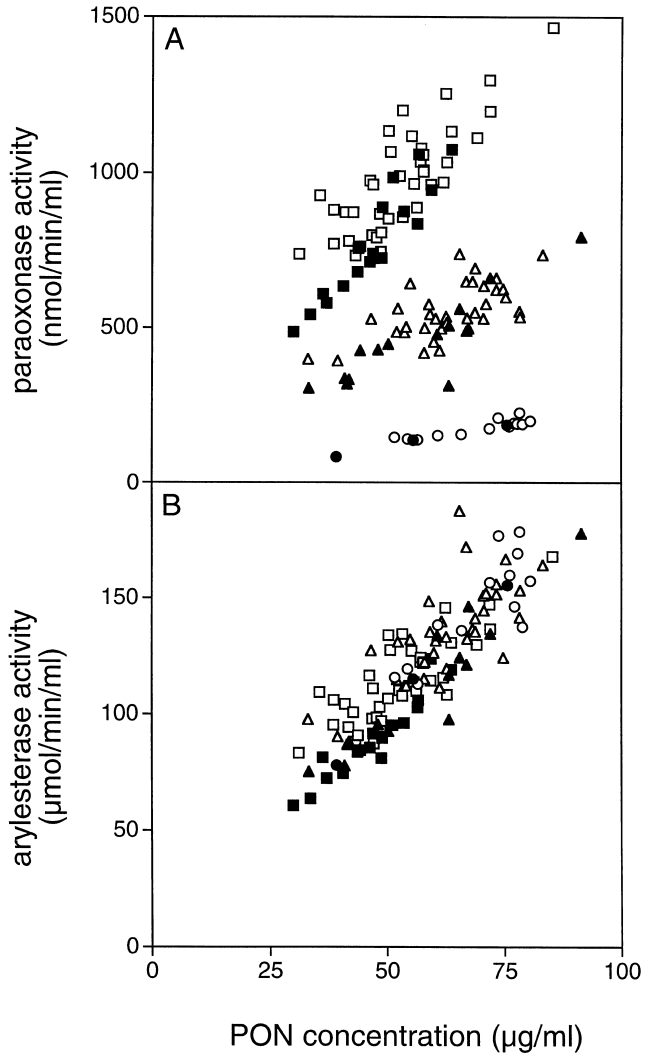

Fig. 3. Correlations between serum PON concentration and paraoxonase (A) and arylesterase (B) activities in serum samples from apparently healthy controls (open symbols) and CHD patients (closed symbols). QQ genotype (open and closed circles); QR genotype (open and closed triangles); RR genotype (open and closed squares). The correlations between PON concentration and paraoxonase activity within the three genotypes of the controls were as follows: QQ genotype, $r = 0.89$; QR genotype, $r = 0.65$; and RR genotype, $r = 0.81$ (all P values < 0.001). In the pooled data from all controls the correlation between PON concentration and arylesterase activity was $r = 0.87$ ($P < 0.001$).

previously isolated by preparative ultracentrifugation. The assay is carried out without a detergent. The PON concentrations observed in British subjects (59.9 ± 24.1 μg/mL, mean ± SD) by this assay were similar to those that we have obtained in healthy Japanese subjects (14, 15). In contrast, Swiss subjects had higher serum PON concentrations (14–16). Mackness et al. (17) found significantly lower PON concentrations in Belfast (median, 56.3 μg/mL) than in Toulouse (71.0 μg/mL).

Blatter Garin et al. (18) observed a relationship between serum PON concentration and the Met–Leu 55 polymorphism of PON, higher concentrations being associated with the L allele. The PON 192 polymorphism appeared to have a much weaker effect, although the Q allele was associated with higher concentrations than was the R allele in subjects of the LL genotype for the position 55 polymorphism. However, as that study was limited to patients with non-insulin-dependent diabetes mellitus the results might not apply to normal subjects. In our healthy controls the QQ genotype was associated with significantly higher concentrations of PON than was the RR genotype, and a similar nonsignificant trend was observed in the CHD patients. Leviev and James (16) described associations between serum PON concentration and three polymorphisms of the promoter region of the PON gene.

We found that serum PON concentration was correlated with both serum paraoxonase activity and serum arylesterase activity. As the PON 192 polymorphism had no effect on arylesterase specific activity, the association between PON concentration and arylesterase activity was present in the combined data from all subjects. However, the association between PON concentration and serum paraoxonase activity was evident only within the same genotype, owing to a major effect of the position 192 polymorphism on paraoxonase specific activity. Others have also presented evidence that the Gln-192 isoenzyme of PON has lower activity toward paraoxon than the Arg-192 isoenzyme (5, 15).

In subjects with angiographically verified CHD serum PON concentration, paraoxonase activity and arylesterase activity were all lower than those in the apparently healthy subjects. In the case of PON concentration the difference between controls and CHD patients was significant for subjects of the RR genotype and for subjects of all geno-

TABLE 4. Serum PON concentration, serum paraoxonase and arylesterase activities, and paraoxonase and arylesterase specific activities, in patients with angiographically verified CHD according to PON 192 genotype

	PON 192 Genotype			
	QQ	QR	RR	Total
Number of subjects	3	15	17	35
PON concentration (μg/mL)	56.8 ± 10.5	56.7 ± 4.0	46.9 ± 2.3[a]	52.0 ± 2.3[b]
Paraoxonase activity (nmol/min/mL)	136 ± 30.2	461 ± 35.6[a]	773 ± 43.3[b]	585 ± 42.6
Arylesterase activity (μmol/min/mL)	116 ± 22.5	110 ± 7.6[b]	89 ± 4.2[c]	100 ± 4.5[c]

Twenty-seven of the subjects were males. Results are means ± SEM. Comparison with healthy controls of the same genotype (Table 3) by Student's t-test.

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

types combined. Although low serum paraoxonase activity has been reported in CHD patients (8), to our knowledge these are the first data on serum PON concentration in subjects with angiographically verified disease. Our findings strengthen the hypothesis that PON may have a significant impact on atherogenesis (19).

The case-control difference in HDL cholesterol in the present study was proportionately greater than that for PON concentration (25 vs. 12%). As PON exists in serum exclusively as a component of HDLs (13, 15), it was of interest to examine the associations between PON concentration and HDL cholesterol concentration. Positive associations were observed in all six groups of subjects, but only in the case of CHD patients of the QR genotype was this significant. This is compatible with previous reports of weak positive correlations between PON concentration and HDL cholesterol (15, 16) and between serum PON and apolipoprotein A-I (apoA-I) concentrations (15), as well as with the demonstration that PON is carried by only a minor subclass of apoA-I-containing particles (13, 15). Overall our findings suggest that the low mean PON concentration in CHD patients was independent of HDL cholesterol. However, large prospective epidemiologic studies will be needed to clearly define the relation of CHD risk to PON concentration.

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