

Paraoxonase polymorphisms and self-reported chronic ill-health in farmers dipping sheep

A. C. Povey¹, M. I. Mackness², P. N. Durrington², M. Dippnall¹, A. E. Smith¹, B. Mackness²
and N. M. Cherry³

Background Serum paraoxonase (PON1) provides protection against organophosphate induced toxicity. Recently we reported that the frequency of paraoxonase polymorphisms in sheep dippers with self-reported chronic ill-health differed from that in dippers with a similar dipping history but no ill-health. As these analyses may have included subjects with conditions unrelated to organophosphate exposure, the aim of this study was to examine whether the risk associated with *PON1* polymorphisms varied using a more homogenous case and referent population.

Methods Each subject completed a detailed symptom questionnaire and their general practitioner was asked whether there was any history of neurological disease that could be confused with the effects of organophosphate poisoning. Subjects were then excluded both on clinical grounds and where identified as atypical on discriminant analysis.

Results Risk associated with the *PON1* 192 and 55 genotypes altered little with these changes in the population.

Conclusions These findings are consistent with the hypothesis that organophosphates contribute to the self-reported ill-health of sheep dippers.

Key words Paraoxonase; PON1; organophosphates; sheep dipping.

Introduction

Health problems associated with acute organophosphate (OP) toxicity are well defined but, ill-health induced by chronic exposures to OPs remains controversial [1]. A substantial number of sheep farmers complain of chronic ill-health which they attribute to repeated exposure to OPs. Those complaining of ill-health that they attribute to repeated OP exposure report a wide range of symptoms, often severe, that includes headache, limb pains, fatigue, sleep disturbance, poor concentration, mood changes and suicidal thoughts [2]. A recent study reported an association between psychiatric disorders (particularly depression) and musculoskeletal disorders (particularly myalgia) and short-term and long-term exposure to OPs [3]. However, there are no widely accepted diagnostic criteria for chronic ill-health associated with OP exposure. Investigation of current workers

shows, at most, slight changes in cognitive function following repeated exposure [4]. Although acute poisoning can result in significant neuropsychological abnormalities [5], the symptoms (neurobehavioural and cognitive complaints, affective disturbances, chronic fatigue and neurological complaints) [2] reported by sheep farmers do not correspond closely to the type of subclinical effect that has been documented following frank poisoning or repeated exposure [1]. Moreover, symptoms appear to occur only in a small proportion of dippers whose characteristics are poorly understood.

If OPs were associated with chronic ill-health then individuals with specific defects in OP metabolism might be expected to be at greater risk of ill-health following exposure [6]. The hydrolysis of OP pesticides by serum paraoxonase (PON1) is a major factor determining their toxicity to vertebrates including man [7–9]. PON1 has an amino acid polymorphism at position-192 [10] that results in two proteins (the R form with arginine at 192 and Q with glutamine), which differ in their hydrolytic activity towards paraoxon, an active metabolite of the organophosphate, parathion. Paraoxon is hydrolysed at a higher rate by the R than the Q form [10]. The Q form hydrolysed diazoxon, an active form of the organophosphate diazinon, and the nerve gases sarin and soman

¹Centre for Occupational and Environmental Health, University of Manchester, Manchester, UK.

²University Department of Medicine, Manchester Royal Infirmary, Oxford Road, Manchester, UK.

³Department of Public Health Science, University of Alberta, Canada.

Correspondence to: A. C. Povey, Centre for Occupational and Environmental Health, University of Manchester, Oxford Road, Manchester M13 9PL, UK.

faster than the R form *in vitro* [11]. A second *PON1* polymorphism at position 55 involving a leucine (L)- methionine (M) substitution has been shown to have an independent effect on serum PON1 activity and concentration with MM homozygotes having the lowest levels [12,13].

Previously we have shown that farmers with self-reported chronic ill-health differed from healthy farmers with a similar exposure history in the frequency of human serum paraoxonase (*PON1*) polymorphisms [14,15]. *PON1* polymorphisms have also been associated with risk of coronary heart disease and Parkinson's disease [16,17], but *PON1* activity and concentration may be more important in coronary heart disease risk than genotype [18]. Low serum *PON1* activity has been associated with Persian Gulf War Veterans self-reporting Gulf War syndrome [19] and the *PON1* Q alloenzyme associated with neurological symptom complexes in Gulf War Veterans [20].

Our initial analyses included all cases and referents recruited for the study [14,15]. As cases had a range of different conditions, some of which may not have been caused by OP exposure and in addition some referents may be symptomatic without invoking OP as a cause, there may have been some misclassification of outcome. The aim of this study was then to examine whether the risk associated with specific *PON1* polymorphisms varied after the exclusion of subjects who on increasingly strict assumptions contributed no information to the comparison of cases and referents.

Methods

Study design and population has been described previously [14,15]. In brief, sheep dippers ('cases'), who believed that their chronic ill-health was a result of exposure to sheep dip, were recruited by advertisement. Each sheep dipper who volunteered was asked to name up to three other sheep dippers (referents) whom they believed to be in good general health, living in their locality, but not blood relatives, who were of similar age (± 10 years) and had a similar pattern of dipping.

Cases and referents were sent health and exposure questionnaires to be completed in advance of a visit from a nurse who reviewed the questionnaire with the farmer, collected more exposure information and took venous blood samples, from which DNA was extracted. Consent to approach the general practitioner was sought from each respondent. General practitioners were then approached and asked whether there was any history of neurological disease that could be confused with the effects of organophosphate poisoning. The study was approved by the Central Manchester Research Ethics Committee and all subjects gave informed consent for participation in the study.

Subjects were asked in detail (using a detailed symptom questionnaire based upon that used for the Gulf War study [21] carried out in Manchester), about their health during the previous month, indicating on an adjacent visual analogue scale ranging from 'not at all' to 'very seriously', how much they had been troubled by each of the 95 symptoms. They were also asked whether they felt their health at the time of completing the questionnaire had been affected by using sheep dip. Health questionnaire responses on each 10 cm visual analogue scale were allocated as a symptom score to 1–20 equally spaced segments. Subjects who provided usable answers to at least 90 of the 95 symptoms were included in the main analysis. The subject's mean response to all other symptoms was assigned where five or fewer symptoms had been missed [21].

Analysis of *PON1-55* and 192 genotypes were determined by polymerase chain reaction amplification and restriction enzyme digestion as previously described [10].

Statistical analysis

The initial analyses previously published included all cases and referents recruited for the study [14,15]. Additional analyses were then carried out for those with not more than five missing symptoms. A stepwise approach was used with subjects being excluded first on clinical grounds (see below) and, subsequently, from the results of a discriminant analysis. Discriminant analysis was carried out using a stepwise method with, at each step, the variable that minimized the unexplained variance (Wilk's Lambda) being entered. The minimum F to enter was set at 3.84 and the maximum F to remove at 2.71. The subjects excluded fell into four groups.

Group 1 comprised cases and referents with a chronic condition attributed by their general practitioner to a clearly defined event (e.g. trauma, stroke), causal agent (e.g. paralytic poliovirus) or congenital condition (e.g. cerebral palsy). Subjects to be excluded were identified blind to case status, using answers to questions about these specific conditions provided on the GP questionnaire.

Group 2 comprised cases and referents with established neurological disease (e.g. multiple sclerosis, Parkinson's disease, Guillain-Barré syndrome, Alzheimer's disease) for which there are objective diagnostic criteria, but uncertain aetiology. Subjects to be excluded were identified blind to case status, using answers to questions about these specific conditions provided on the GP questionnaire.

Group 3 comprised subjects, excluding those in group 1, who were identified by discriminant analysis as having symptoms atypical of their classification.

Group 4 comprised subjects, excluding those in groups 1 and 2, identified by discriminant analysis as having symptoms atypical of their classification.

The associations between case status and *PON1-192*, *PON1-55* polymorphisms were initially calculated for the whole population ('full analysis') and then also for subpopulations defined by excluding groups 1–4 in a series of analyses as follows:

Stage 1 analysis: all subjects excluding those with more than five unusable responses to the symptom question

Stage 2 analysis: all subjects except subject group 1

Stage 3 analysis: all subjects except subject groups 1 and 2

Stage 4 analysis: all subjects except subject groups 1 and 3

Stage 5 analysis: all subjects except subject groups 1, 2 and 4

Results

Four hundred and nine farmers were interviewed (175 cases and 234 controls). Subjects were recruited from throughout the United Kingdom. Sixty-five per cent of cases lived in England, 22% in Wales and 13% in Scotland and Northern Ireland. All subjects were caucasian.

Of the 409 subjects in the initial study 12 gave responses to less than 90 symptoms and were excluded

from the analyses reported here. Information from family physicians was obtained for 402 subjects. Those with no physician information (seven subjects) were assumed not to have a group 1 or group 2 condition and were included in the analysis.

Cases were more likely than referents to report that they had been troubled by ill-health during the past month and more likely than referents to report that they felt that their health now had been affected by using sheep dip (data not shown). Cases and referents differed markedly in their symptom reporting. Cases were more likely to have a severity score greater than the median symptom score of the population (data not shown).

The study population was refined first by the exclusion of subjects on clinical grounds. A total of 20 subjects (of which eight were cases) had a chronic condition attributed by their general practitioner to a clearly defined event (that is falling into group 1). Of these seven had a stroke and thirteen trauma. A total of 21 subjects had established neurological disease of which 19 were cases. These included seven cases of multiple sclerosis, two of Alzheimer's disease and two of Parkinson's disease.

After exclusion of study group 1 ($n = 20$), discriminant analysis of the study population correctly identified 83.8% of cases. Variables in the analysis at the final (10th step) are shown in Table 1. A total of 61 subjects were identified as belonging to subject group 3, of which 42

Table 1. Symptoms remaining in the final discriminant analysis step

| Group | Question | Symptom | F to remove | Coefficient |
|----------------|------------------------|--------------------------|-------------|-------------|
| 3 ^a | 18 | Fits or convulsions | 7.36 | 0.21 |
| | 22 | Difficulty concentrating | 38.06 | 0.51 |
| | 36 | Difficulty standing up | 13.51 | 0.30 |
| | 40 | Fainting | 6.53 | -0.21 |
| | 46 | Ringing sounds | 11.24 | 0.27 |
| | 64 | Ear infections | 7.33 | -0.23 |
| | 77 | Smell of chemicals | 18.25 | 0.34 |
| | 91 | Tingling under the skin | 7.33 | 0.23 |
| 4 ^b | 18 | Fits or convulsions | 3.95 | 0.15 |
| | 22 | Difficulty concentrating | 16.61 | 0.47 |
| | 26 | Headaches | 5.55 | -0.22 |
| | 34 | Waking up tired | 6.83 | 0.27 |
| | 42 | Losing balance | 4.05 | -0.21 |
| | 46 | Ringing sounds | 13.89 | 0.30 |
| | 58 | Clumsiness | 9.85 | 0.33 |
| | 64 | Ear infections | 5.41 | -0.19 |
| | 77 | Smell of chemicals | 18.56 | 0.35 |
| | 80 | Shortness of breath | 6.82 | 0.25 |
| 83 | Incapable of decisions | 13.87 | -0.39 | |
| 87 | Mood change | 4.87 | 0.23 | |

^aAnalysis carried out on 377 subjects (161 subjects and 216 referents) who had completed 90 or more symptom questions and who had not been identified by their family physician as having a chronic disease with a clearly defined cause. Symptoms remaining in the final, 10th step.

^bAnalysis carried on 356 subjects (142 cases and 212 referents) who had completed 90 or more symptom questions and who had not been identified by their family physician as having either a chronic disease with a clearly defined cause or a clearly defined syndrome with uncertain cause. Symptoms remaining in the final, 12th step.

were cases predicted as being referents and 19 referents predicted as cases. After exclusion of subject groups 1 and 2 ($n = 41$), discriminant analysis of the study population correctly identified 86.8% of cases. Variables in the analysis at the final (12th step) are shown in Table 1. A total of 47 subjects were identified as belonging to subject group 4, of which 35 were cases predicted as being referents and 12 referents identified as cases.

The risk associated with the *QR* or *RR* genotype remained elevated (>2 -fold) when compared with the *QQ* genotype even after exclusion of subjects on clinical grounds or by discriminant analysis (Table 2). Risk associated with the *QR* genotype (*versus QQ*) remained similar, but that of the *RR* genotype (*versus QQ*) was reduced when subjects were excluded by discriminant analysis. The risk associated with the *LL* genotype remained elevated when compared with the *LM* or *MM* genotype even after subject exclusion (Table 3). The decreased risk associated with the *MM* genotype was lower than that of the *LM* genotype.

Discussion

Results from this study indicate that there were differences in the case and referent population in the frequency of *PON1* genotypes even after exclusion of subjects on both clinical grounds and after discriminant analysis. Subjects were excluded so as to provide a more homogenous case and referent population. This

refinement made very little difference to the risk associated with *PON1* genotype suggesting that the original analysis was robust; the missing symptom data on 12 subjects and physician information on seven subjects is unlikely to influence this conclusion.

Experiments in animals have shown that *PON1* is important in protecting against OP toxicity [22] and that *PON1-192* genotype is a major determinant of the rate of hydrolysis of OPs *in vitro* [11], but not necessarily *in vivo* [23]. The *PON1-55* polymorphism affects the capacity of *PON1* to hydrolyse OPs *in vitro* [12]. *PON1* also metabolizes a range of substrates other than OPs [24, 25] so that it is conceivable that the reported associations in this study may result from exposure to another substrate. However, at the current time there would appear to be no other plausible candidate agent. Physiological substrates for *PON1* include lipid peroxidation products [25] and accordingly associations between heart disease and *PON1* genotype/phenotype have been described but not consistently [26]. Selection bias may thus result from only selecting referents in good health. However, it should be noted that, exclusion of those patients with known heart disease did not alter the risk estimates [13]. Furthermore, selection bias did not appear to be a problem as the genotype distribution in the referent group in this study population was consistent with previously published UK datasets [12,27].

In summary, results from the study are consistent with the *a priori* hypothesis that organophosphates contribute

Table 2. Associations between *PON1-192* genotype and case status

| Analysis stage | Total <i>N</i> | Case | | Referent (%) | | OR (95%CI) <i>QR</i> or <i>RR</i> <i>versus QQ</i> |
|----------------|----------------|------------------------------|---------------------|------------------------------|---------------------|---|
| | | <i>QQ/QR/RR</i> (<i>n</i>) | <i>QQ/QR/RR</i> (%) | <i>QQ/QR/RR</i> (<i>n</i>) | <i>QQ/QR/RR</i> (%) | |
| Full | 409 | 69/90/16 | 39/51/9 | 140/81/13 | 60/35/5 | 2.25 (1.49–3.42) |
| 1 | 397 | 67/86/16 | 40/51/9 | 137/78/13 | 60/34/6 | 2.29 (1.53–3.44) |
| 2 | 377 | 64/81/16 | 40/50/10 | 129/74/13 | 60/34/6 | 2.25 (1.48–3.41) |
| 3 | 356 | 53/75/14 | 37/53/10 | 128/73/13 | 60/34/6 | 2.50 (1.62–3.87) |
| 4 | 316 | 48/63/8 | 40/53/7 | 115/69/13 | 58/35/7 | 2.08 (1.31–3.30) |
| 5 | 309 | 39/61/7 | 36/57/7 | 118/71/13 | 58/35/7 | 2.45 (1.51–3.97) |

Table 3. Associations between *PON1-55* genotype and case status

| Analysis stage | Total <i>N</i> | Case | | Referent (%) | | OR (95%CI) <i>LL</i> <i>versus LM</i> or <i>MM</i> |
|----------------|----------------|------------------------------|---------------------|------------------------------|---------------------|---|
| | | <i>LL/LM/MM</i> (<i>n</i>) | <i>LL/LM/MM</i> (%) | <i>LL/LM/MM</i> (<i>n</i>) | <i>LL/LM/MM</i> (%) | |
| Full | 409 | 86/75/14 | 49/43/8 | 74/124/36 | 32/53/15 | 1.92 (1.26–2.93) |
| 1 | 397 | 84/71/14 | 50/42/8 | 73/122/33 | 32/54/14 | 2.10 (1.39–3.16) |
| 2 | 377 | 80/67/14 | 50/42/8 | 69/116/31 | 32/54/14 | 2.10 (1.38–3.21) |
| 3 | 356 | 71/60/11 | 50/42/8 | 68/115/31 | 32/54/14 | 2.15 (1.39–3.32) |
| 4 | 316 | 55/55/9 | 46/46/8 | 64/104/29 | 33/53/14 | 1.79 (1.12–2.85) |
| 5 | 309 | 52/48/7 | 49/45/6 | 66/108/28 | 33/54/13 | 1.95 (1.21–3.15) |

to the reported ill-health of people who dip sheep. If so, further work is required to determine whether this reported ill-health is associated more with acute high-dose exposure or chronic low-dose exposure.

Acknowledgements

The study was funded by the UK Health & Safety Executive (3837/R79.002). The authors would like to thank Stuart Thomson, Janet Schofield, Elizabeth Smallshaw and Caroline Fitzgerald for interviewing participants, Priscilla Appelbe for coordinating the field work and Stephanie Middleton for expert technical assistance.

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