Genetic variation of iron-induced uroporphyria in mice

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Iron overload causes inhibition of hepatic uroporphyrinogen decarboxylase (UROD) and uroporphyria in C57BL/10ScSn but not DBA/2 mice [Smith, Cabral, Carthew, Francis and Manson (1989) Int. J. Cancer **43**, 492–496]. We have investigated the induction of uroporphyria in 12 inbred strains of mice 25 weeks after iron treatment (600 mg/kg) to determine if there was any correlation with the *Ah* locus. Under these conditions, inhibition of UROD occurred to varying degrees in Ah^d mice (SWR and AKR) as well as nominally Ah^{b-1} (C57BL/6J, C57BL/10ScSn and C57BL/10-cc) and Ah^{b-2} strains (BALB/c and C3H/HeJ). Five other Ah^b or Ah^d strains (C57BL/Ks, A/J, CBA/J, LP and DBA/2) were unaffected. Thus there appeared to be no correlation with the *Ah* phenotype and this illustrated that some other variable inherited factors are involved. Comparisons between another susceptible strain, A2G, and the congenic A2G-*hr*/+ strain (carrying the recessive *hr* gene) showed a modulating influence associated with the *hr* locus. In contrast with individual mice of inbred strains, which showed consistent responses to iron, those of the outbred MF1 strain showed a spectrum of sensitivities as might be expected for a heterogeneic stock. The rate of porphyria development was accelerated by administration of 5-aminolaevulinic acid (5-ALA) in the drinking water, but this did not overcome strain differences. Among four strains the order of susceptibility was SWR > C57BL/10ScSn > C57Bl/6J > DBA/2 (the last strain was completely resistant). With degrees of iron loading greater than 600 mg of Fe/kg (1200–1800 mg of Fe/kg) C57BL/10ScSn mice (after 20 weeks) and SWR mice (after 5 weeks which included 4 weeks of 5-ALA treatment) had less inhibition of UROD and a lower uroporphyric response, showing that there was an optimum level of liver iron concentration. Studies on selected microsomal enzyme activities associated with cytochrome *P*-450 showed no correlation with the propensities of strains to develop porphyria. These activities included the NADPH-dependent oxidation of uroporphyrinogen I to uroporphyrin I.

INTRODUCTION

The disease, sporadic porphyria cutanea tarda (PCT) occurs in some patients with moderate hepatic damage associated with chronic exposure to alcohol, oestrogenic drugs and other toxic insults [1,2]. Another important predisposing factor in the majority of patients seems to be an unknown aspect of iron metabolism. Hepatic siderosis is frequently observed and treatment of the disease entails phlebotomy or desferrioxamine infusion to reduce iron stores [1–6]. The disease is characterized biochemically by a depression in the hepatic activity of the haem biosynthetic enzyme uroporphyrinogen decarboxylase [EC 4.1.1.37] (UROD) leading to the accumulation of uroporphyrinogens and their partially decarboxylated products together with the corresponding porphyrins formed by oxidation [1,2]. Sporadic PCT is distinguished from the familial form of PCT, in which there are mutations of the decarboxylase gene and the depression of enzyme activity in erythroid tissue as well as in the liver [2]. Although currently there is no evidence that in sporadic PCT there are inherited differences in UROD activity, some kind of genetic predisposition seems likely since the disease only occurs in a minority of patients at risk [1,2]. Because of the evidence for the frequent involvement of iron, one possibility is that there are genetic variations in the response to iron or its metabolism predisposing certain individuals to the disease.

Experimentally, some polyhalogenated chemicals that are poorly metabolized, such as hexachlorobenzene (HCB), polyhalogenated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), cause a uroporphyria in rodents and humans which is very similar to sporadic PCT [1,2,7]. Since these chemicals are all chronic inducers of the cytochrome *P*-4501A subfamily a great deal of effort over many years has concentrated on the involvement of CYP1As in the development of this disease [1,2]. Indeed under some conditions, non-chlorinated inducers of the CYP1A subfamily, such as 3-methylcholanthrene and β-naphthoflavone, also cause porphyria [8,9]. In mice, responses to these chemicals vary between strains and in some studies appear to correlate with the *Ah* locus. This locus is thought to be the structural gene encoding the *Ah* receptor which mediates CYP1A1 induction. Strains with Ah^b and Ah^d alleles express receptors with high affinity and low affinity respectively for potential agonists [10]. Mice of the C57 family which carry the Ah^b allele are responsive to CYP1A1 induction and develop porphyria, whereas some strains such as DBA/2 carrying the Ah^d allele are non-responsive and are resistant to developing porphyria [11–13]. Unfortunately, despite many studies it is not yet clear exactly what role CYP1A1 or CYP1A2 play, if any, in the development of uroporphyria.

In parallel with human PCT, there is also considerable evidence in mice for the role of iron in the experimental uroporphyrias [1,14]. Lowering liver iron stores has been reported to protect C57BL/6J mice from both the uroporphyric and hepatotoxic effects of TCDD [15,16], whereas iron overload will potentiate its action even in strains which are not very susceptible [17]. In addition, iron overload greatly potentiates the action of weaker agents such as HCB and 3-methylcholanthrene and this has proved to be a useful experimental system [8,9,12,13,18–21].

The most compelling evidence for the role of iron has emerged from recent long-term studies. Uroporphyria occurred 3–6 months after iron administration (a longer period than employed in previous experiments) in C57BL/10ScSn mice without the influence of any other exogenous agent [14,22–25]. With

Abbreviations used: PCT, porphyria cutanea tarda; UROD, uroporphyrinogen decarboxylase; HCB, hexachlorobenzene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 5-ALA, 5-aminolaevulinic acid; EROD, resorufin ethyl ether deethylase; BROD, resorufin benzyl ether dealkylase.

C57BL/6J mice this process can be speeded up by including the haem precursor 5-aminolaevulinic acid (5-ALA) in the drinking water [26]. As in studies with xenobiotic inducers of CYP1A, DBA/2 mice are not susceptible [22,26]. However, it is important to note that in these experiments no ligand for the Ah receptor and inducer of CYP1A production was administered.

In the present work we have examined in more detail the induction of uroporphyria in mice after iron treatment. We have particularly focused on the comparison between the development of uroporphyria and the recent reclassification of some *Ah*-responsive and non-responsive inbred mouse strains depending on their carrying one of three, not two, *Ah* alleles.

EXPERIMENTAL

Chemicals

Uroporphyrins I and III and pentacarboxyporphyrin I were purchased from Porphyrin Products (Logan, UT, U.S.A.). Resorufin ethyl ether (ethoxyphenoxazone or ethoxyresorufin) was synthesized as previously described [27]. Resorufin benzyl ether (benzyloxyresorufin) was purchased from Boehringer. Iron–dextran (Imferon) and the dextran base were from Fisons Pharmaceuticals, U.K. All other reagents were from Sigma.

Mice and protocols

Inbred male mice (6–10-weeks-old) were obtained from the following sources: C57BL/6J, C57BL/10ScSn, C57BL/10-cc, BALB/c, C3H/HeJ, A2G-*hr*/+ and A2G-*hr*/*hr* strains were bred in these laboratories. C57BL/10-cc mice were created by seven generations of backcross of NZW on to the C57BL/10ScSn strain to introduce the albino gene (M. Festing, personal communication). The well-established recessive *hr* locus [28] was introduced into the A2G strain by six generations of backcross with an outbred *hr* strain (M. Festing, personal communication). Homozygous mice (A2G-*hr*/*hr*) develop normal coats but these are soon lost with hyperkeratosis of the upper hair canals [28]. C57BL/Ks, CBA/J, A/J, SWR (for 25-week-long studies), LP and AKR mice were obtained from the National Institute of Medical Research, U.K. A2G, SWR (for 5-ALA studies) and DBA/2 mice were purchased from Harlan Olac Ltd., Oxon, U.K. Outbred MF1 mice were bred on site from stock purchased originally from Harlan Olac Ltd. Mice were fed Breeder Diet No. 3 expanded (Special Diet Services, Eltham, Essex, U.K.). Iron, as Imferon (50 mg of Fe/ml and 200 mg of dextran/ml) was given by subcutaneous injection (12 ml/kg; 600 mg of Fe/kg). Mice received either tap water or water containing 5-ALA dihydrochloride (2 mg/ml) which was prepared twice weekly as described by Urquhart et al. [9]. Animals were killed by cervical dislocation and livers analysed while fresh (where microsomes were prepared) or frozen in liquid N_2 and stored at −70 °C until analysed. Microsomes were prepared as described previously after initial homogenization of livers in 0.25 M sucrose/50 mM Tris/HCl buffer (1:4, w/v) at pH 7.5 [29]. For most experiments livers were homogenized in 0.25 M sucrose (w/v) and part of the homogenate centrifuged at 40000 ***g*** for 30 min to give a supernatant which was used for assay of UROD activity [19].

Analyses

UROD activity in supernatants was assayed using 5 μM pentacarboxyporphyrinogen I as substrate in 0.1 mM EDTA/3 mM dithiothreitol/50 mM sodium succinate buffer (pH 5.4) under N_2 in the dark at 37 °C for 10 min and is expressed as pmol of coproporphyrinogen I formed/min per mg of protein [19]. The inhibitory properties of heat-treated extracts of livers towards UROD were determined as described previously using C57BL/10ScSn mouse liver supernatant from untreated mice with pentacarboxyporphyrinogen I (5 μM) or uroporphyrinogen III (5 μM) as substrates [21]. Total liver porphyrin levels in homogenates were determined by spectrofluorimetry and are expressed in terms of uroporphyrin [30]. Porphyrins in urine samples were estimated by reverse-phase h.p.l.c. [24] using a Spherisorb 5 μm ODS-1 column (25 cm × 4.6 mm internal diam.) and related to creatinine concentrations measured with Sigma Diagnostic kit 555A. Analysis of liver porphyrins was also by reverse-phase h.p.l.c. using a gradient of 1 M ammonium acetate, pH 5.15, in acetonitrile (15–38 %) [31]. Non-haem iron concentrations of homogenates were estimated by adaptation of the method of Torrance and Bothwell [23,32]. Protein concentration was determined using BSA as the standard [33]. Microsomal total cytochrome *P*-450, resorufin ethyl ether deethylase (EROD) and resorufin benzyl ether dealkylase (BROD) activities at 25 °C and NADPH-cytochrome *P*-450 reductase at 25 °C with cytochrome *c* (as the substrate) were determined by previously published methods [29,34]. The oxidation of uroporphyrinogen I by microsomes in the presence of NADPH was performed as described by Smith et al. [29] based on the method of Jacobs et al. [35]. SDS/PAGE of microsomes and immunoblotting using polyclonal antibodies to rat CYP1A1 and CYP1A2 were performed as described previously [29,36].

RESULTS

Influence of *Ah* variance

A number of inbred mouse strains have been categorized based on their carrying different allelic forms of the Ah receptor gene i.e. Ah^{b-1}, Ah^{b-2} and Ah^{d} [37,38]. Differences in the receptors depend on affinity of ligand binding, apparent molecular mass and heat stability. This classification broadly agrees with the original categories of *Ah* responsive and non-responsive strains based on inducibility of arylhyrocarbon hydroxylase activity, but with the former strains being split into the two groups Ah^{b-1} and Ah^{b-2}. From these lists two Ah^{b-1} strains (C57BL/6J, C57BL/Ks), four Ah^{b-2} strains (BALB/c, C3H/HeJ, A/J, CBA/J) and four Ah^{d} strains (SWR, AKR, LP, DBA/2) were selected from those available to us to determine whether there was any correlation with iron-induced uroporphyria. In addition, two other strains C57BL/10ScSn and C57BL/10-cc were selected as probably carrying the Ah^{b-1} allele, since this has been true for all members of the C57 and C58 family (descended from a common parent [39]) so far examined [38]. Mice received dextran or iron–dextran and hepatic UROD activity and porphyrin levels were examined after 25 weeks (Table 1). Markedly depressed UROD activity in iron-dosed mice relative to controls with accompanying accumulation of porphyrins was observed with C57BL/10ScSn, C57BL/10-cc, BALB/c and SWR mice, with milder effects in the C57BL/6J and AKR strains. One-way analysis of variance confirmed that there was no apparent correlation with *Ah* grouping despite the fact that the results with C57BL/10ScSn and DBA/2 alone suggested a link with *Ah* responsiveness. Although some interstrain differences in control UROD levels were observed, these were not considered to be of biological significance. In repeat experiments we consistently observed that C57BL/6J mice did not respond as well as the C57BL/10ScSn strain. Although non-haem iron levels after 25 weeks showed considerable variation this did not seem to be the reason for strain variation in the activity of UROD, either in controls or iron-dosed mice. H.p.l.c. of the liver porphyrins from six of the

Table 1 Effect of iron overload on UROD activity in the livers of different inbred mice

Male mice received dextran solution (2.4 g/kg) or iron–dextran (600 mg of Fe/kg), i.e. −Fe and +Fe groups respectively, and were fed control diet for 25 weeks. Hepatic UROD activities were estimated as described in the Experimental section using pentacarboxyporphyrinogen I as substrate. Mouse strains were classified as possessing Ah^{b-1} and Ah^{b-2} (*Ah*-responsive) or Ah^{d} (*Ah*-non-responsive) alleles based on Poland and Glover [37,38]. Results are means ± S.E.M. of three or mainly four mice per group and were analysed by one-way analysis of variance between strains. The difference between iron- and non-iron-dosed mice within a strain was tested by Student's *t*-test. $^{*}P < 0.05$; significantly different from respective non-iron-dosed group.

Strain	UROD activity (pmol/min per mg of protein)			Total porphyrins (nmol/g of tissue)			Non-haem iron (μg/g of tissue)	
	−Fe	+Fe	+Fe activity as a percentage of −Fe activity (%)	−Fe	+Fe	Increase (fold)	−Fe	+Fe
Ah^{b-1}								
C57BL/10ScSn	34.0 ± 0.8	1.4 ± 0.2*	4.1	0.9 ± 0.2	225.8 ± 16*	266	29 ± 5	1599 ± 96*
C57BL/10-cc	36.1 ± 0.4	4.8 ± 1.6*	13.2	0.6 ± 0.1	74.6 ± 23*	124	75 ± 15	2130 ± 135*
C57BL/6J†	37.0 ± 0.9	28.2 ± 1.6*	76.7	0.4 ± 0.1	2.5 ± 0.8*	6	76 ± 23	1739 ± 119*
C57BL/Ks	36.9 ± 0.4	39.7 ± 0.7*	107.6	0.3 ± 0.1	0.3 ± 0.1	1.1	35 ± 5	1995 ± 80*
Ah^{b-2}								
BALB/c	33.7 ± 0.4	8.9 ± 2.3*	26.4	0.5 ± 0.1	53.6 ± 37.1	109	59 ± 6	1640 ± 128*
C3H/HeJ	41.0 ± 0.7	36.6 ± 1.4*	89.3	0.8 ± 0.2	1.1 ± 0.3	1.3	99 ± 23	1382 ± 39*
A/J	35.6 ± 0.6	33.9 ± 0.9	95.2	0.5 ± 0.1	0.4 ± 0.2	–	54 ± 6	1854 ± 240*
CBA/J	36.2 ± 0.8	37.4 ± 1.2	103.3	0.6 ± 0.2	0.7 ± 0.1	1.1	73 ± 8	1917 ± 96*
Ah^{d}								
SWR	34.6 ± 0.9	1.2 ± 0.1*	3.4	1.2 ± 0.2	132 ± 31*	107	87 ± 7	1131 ± 122*
AKR	38.3 ± 1.0	25.2 ± 7.4	65.8	0.4 ± 0.1	10.9 ± 6.7	27	132 ± 10	1220 ± 66*
LP	42.0 ± 0.9	44.0 ± 1.5	104.8	0.4 ± 0.1	1.4 ± 0.5	3.3	65 ± 2	1266 ± 53*
DBA/2	33.3 ± 0.3	36.5 ± 1.0*	109.6	0.4 ± 0.1	0.5 ± 0.1	–	118 ± 5	985 ± 147*

† Experiments on this strain were repeated on two occasions with percentage activities of UROD relative to control of 80% and 73% ($n = 4$ per study).

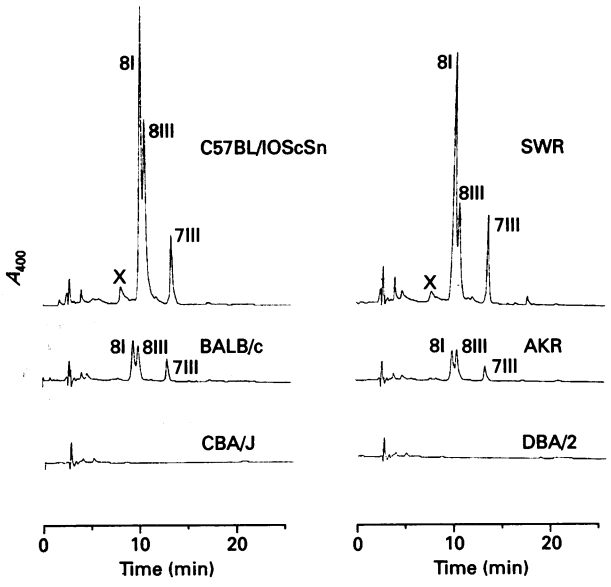

Figure 1 H.p.l.c. of porphyrins in livers from six strains of mice (C57BL/10ScSn, BALB/c, CBA/J, SWR, AKR and DBA/2) 25 weeks after administration of iron

Mice were injected with Imferon (12 ml/kg) and fed a control diet for 25 weeks. Treatment of liver samples and h.p.l.c. conditions were as described in the Experimental section. Peak 8I, uroporphyrin I; peak 8III, uroporphyrin III; peak 7III, heptacarboxyporphyrin III; peak X, unknowns.

strains are shown in Figure 1 (three Ah^{b}, C57BL/10ScSn, BALB/c and CBA/J; three Ah^{d}, SWR, AKR and DBA/2). All four strains with elevated total porphyrin levels showed that this was mostly due to uroporphyrins I and III and heptacarboxyporphyrin III. Small unknown peaks, more hydrophilic than uroporphyrins, were seen in all porphyric samples. The proportion of uroporphyrin I relative to uroporphyrin III was greater in the more highly porphyric samples (C57BL/10ScSn and SWR) than in the strains (BALB/c and AKR) with a much lower degree of porphyria. One interpretation of this finding is that uroporphyrinogen III synthase may become inhibited subsequent to the primary effects on UROD, as suggested by a previous experiment [19], perhaps by the accumulating uroporphyrinogens.

In previous studies with porphyrogenic organic chemicals, an inhibitor of UROD, more hydrophilic than uroporphyrins, has been detected in heat-treated extracts of porphyric liver [21,40,41]. So far its identity and any role in the development of uroporphyria have not been established. This inhibitory activity (measured as inhibition of pentacarboxyporphyrinogen I decarboxylation by a control enzyme source) was also present in livers from C57BL/10ScSn mice 25 weeks after receiving iron, but not in similarly treated DBA/2 animals (C57BL/10ScSn controls 31.1 ± 0.7, plus iron 5.3 ± 0.7; DBA/2 controls 30.6 ± 0.7, plus iron 31.0 ± 0.4 pmol/min per mg of protein). The same extracts from iron-treated C57BL/10ScSn mice were also capable of inhibiting control UROD activity when uroporphyrinogen III was the substrate (coproporphyrinogen III formation was < 25% of that with extracts from controls).

Effect of *hr* locus

A2G and the A2G-*hr*/+ congenic strain (carrying the recessive *hr* hairless locus) appear to be *Ah* responsive, probably having an Ah^{b} allele [17,42]. However, in previous studies with A2G-*hr*/+ heterozygotes and A2G-*hr*/*hr* homozygotes the *hr*/*hr* group were slightly more susceptible than the *hr*/+ group to the hepatic porphyrogenicity and toxicity of TCDD, suggesting an additional influence associated with the *hr* locus [42]. In the

Table 2 Influence of the *hr* gene on iron-induced porphyria in A2G mice

A2G mice and a congenic strain carrying the *hr* gene (*hr*/+ heterozygotes and *hr*/*hr* homozygotes) received dextran (2.4 g/kg) or iron–dextran (600 mg of Fe/kg) and then were kept on a control diet for 25 weeks. Hepatic UROD activity, porphyrin levels and non-haem iron contents were estimated as described in the Experimental section. Number of mice per group is given in parentheses. Results are means ± S.E.M. The results were analysed by two-way analysis of variance for genotype and iron treatment. UROD activity and porphyrins in iron-treated A2G, A2G *hr*/+ and A2G *hr*/*hr* were significantly different from each other ($P < 0.01$). All iron-treated mice were significantly different from their non-iron-dosed controls (*$P < 0.01$).

Strain	Iron treatment	UROD activity (pmol/min per mg of protein)	Total porphyrins (nmol/g)	Non-haem iron (μg/g)
A2G (4)	–	33.9 ± 0.5	0.5 ± 0.1	52 ± 19
A2G *hr*/+ (4)	–	32.6 ± 1.7	0.6 ± 0.1	50 ± 3
A2G *hr*/*hr* (4)	–	35.4 ± 0.6	0.8 ± 0.1	43 ± 13
A2G (4)	+	1.2 ± 0.2*	140 ± 15*	1053 ± 62*
A2G *hr*/+ (9)	+	10.4 ± 3.1*	31.6 ± 11.0*	1488 ± 164*
A2G *hr*/*hr* (9)	+	22.9 ± 2.0*	15.3 ± 12.5*	1604 ± 182*

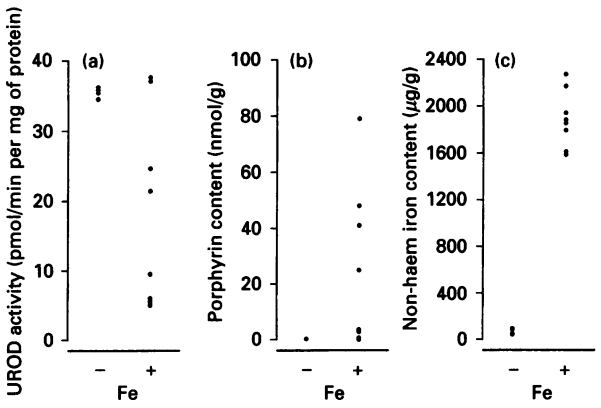

Figure 2 Iron-induced uroporphyria in outbred MF1 mice

Four mice received dextran and eight mice iron–dextran (600 mg of Fe/kg) and were then fed a control diet for 25 weeks. Livers were analysed for (**a**) UROD activity, (**b**) porphyrin content and (**c**) non-haem iron levels as described in the Experimental section.

present work, the induction of uroporphyria by iron in *hr*/+ and *hr*/*hr* mice was compared with the parent A2G strain. In contrast with uroporphyria caused by TCDD in homozygous mice, the *hr*/*hr* group was apparently less susceptible than *hr*/+ animals to iron and the parent strain was even more susceptible (Table 2). This would suggest that in iron-induced uroporphyria, without the administration of TCDD, the presence of the *hr* locus is associated with a net negative or protective influence.

Uroporphyria in outbred MF1 mice

MF1 mice are an outbred albino strain bred by Harlan Olac Ltd. from a mixture of Swiss-type mice from which the SWR strain is also descended [43]. A group of eight of these mice was administered iron–dextran and examined for uroporphyria after 25 weeks (Figure 2). In contrast with the inbred strains (Table 1), considerable variation in response was observed in both UROD activity and porphyrin content, probably reflecting the heterozygosity of the stock. Although some variation in non-haem iron levels was found this did not correlate with response and was no greater than found within groups of inbred strains.

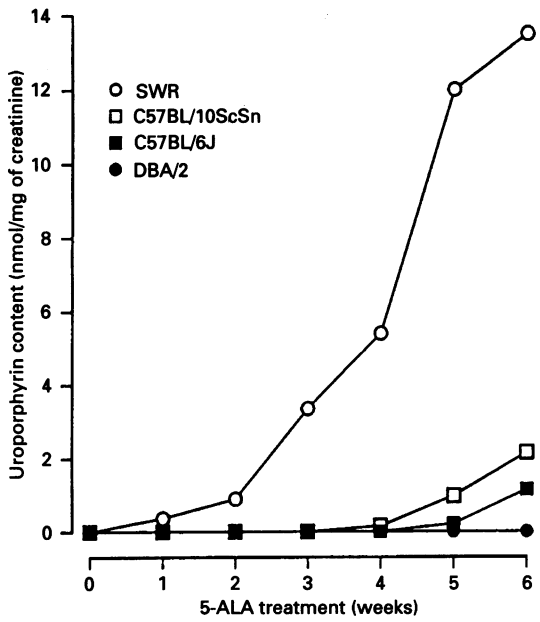

Figure 3 Urinary uroporphyrin in iron-loaded mice administered 5-ALA

Groups of C57BL/6J (■), C57BL/10ScSn (□), SWR (○) and DBA/2 (●) mice (four per group) received iron–dextran (600 mg of Fe/kg) and after 3 days were administered 5-ALA in their drinking water (2 mg/ml). Urine samples from mice were obtained once a week and combined for each group. They were analysed for uroporphyrin content by h.p.l.c. as described in the Experimental section. Levels were normalized against creatinine concentrations. Mice which did not receive 5-ALA showed no elevation of urinary uroporphyrin levels at this time.

Table 3 Hepatic porphyria in iron-loaded mice after 5-ALA administration

C57BL/6J, C57BL/10ScSn, SWR and DBA/2 mice (four per group) received iron–dextran (600 mg of Fe/kg) and after 3 days were given water or administered 5-ALA in the drinking water (2 mg/ml) for 6 weeks as shown in Figure 3. Livers were analysed for UROD activity and porphyrin content as described in the Experimental section. By two-way analysis of variance each strain after treatment was significantly different ($P < 0.05$) from the others and the C57BL/6J, C57BL/10ScSn and SWR mice were significantly different from their controls not given 5-ALA.

Strain	5-ALA treatment	UROD activity (pmol/min per mg of protein)	Total porphyrins (nmol/g)
DBA/2	–	37.2 ± 0.7	1.3 ± 0.2
	+	36.7 ± 0.5	0.8 ± 0.1
C57BL/6J	–	35.3 ± 0.6	0.9 ± 0.1
	+	15.8 ± 0.8	7.8 ± 1.4
C57BL/10ScSn	–	35.6 ± 0.8	0.7 ± 0.2
	+	8.1 ± 0.9	52.3 ± 11.1
SWR	–	34.7 ± 0.7	1.8 ± 0.3
	+	1.6 ± 0.6	307 ± 59

Influence of 5-ALA

To determine whether differences between any strains could be explained by variations in 5-ALA availability, C57BL/6J, C57BL/10ScSn, SWR and DBA/2 were administered iron and then allowed 5-ALA in their drinking water, a procedure which considerably enhances the rate of porphyria development [9,26]. Urine samples were monitored by h.p.l.c. for uroporphyrin content over 6 weeks. The relative differences in the 25-week study between DBA/2, C57BL/6J and C57BL/10ScSn mice were maintained in this experiment (Figure 3). In contrast, SWR mice responded much more quickly than the other strains, with

Table 4 Influence of iron dose on induction of uroporphyria in C57BL/10ScSn mice

Mice (three per group) received dextran or iron–dextran (600 mg of Fe/kg) as one, two or three doses on weeks 0, 2 and 4 respectively. Groups not receiving iron at weeks 2 and 4 were given dextran. After 20 weeks, livers were analysed for UROD activity, porphyrin content and non-haem iron levels as described in the Experimental section.

Iron dose	UROD activity (pmol/min per mg of protein)	Total porphyrins (nmol/g)	Non-haem iron (μg/g)
0	38.7±0.4	0.4±0.1	38±13
1	5.2±0.6	154±25	1906±36
2	30.7±1.4	0.6±0.3	3729±148
3	29.5±0.1	0.5±0.1	4312±156

Table 5 Effect of iron loading on induction of porphyria in SWR mice administered 5-ALA

SWR mice (three or four per group) were given dextran (no dose), iron–dextran (200 mg of Fe/kg, i.e. one-third of a dose) or 600 mg of Fe/kg (one dose) and after 3 days administered 5-ALA in the drinking water (2 mg/ml) for 4 weeks. A further group of mice given one dose of iron received iron with commencement of 5-ALA and again after 10 days (i.e. three doses). Livers were analysed for UROD activity, porphyrin content and non-haem iron levels as described in the Experimental section.

Iron dose	UROD activity (pmol/min per mg of protein)	Total porphyrins (nmol/g)	Non-haem iron (μg/g)
0	36.9±0.4	6.8±1.2	213±15
1/3	10.0±1.7	103±32	1172±72
1	2.6±0.9	294±85	2849±224
3	10.9±3.0	23.9±9.9	5343±575

detectable elevated uroporphyrin levels after one week of 5-ALA treatment. These findings were confirmed by analyses of the livers for UROD activity and total porphyrin levels with a consistent difference between the two C57 strains (Table 3). Thus it seems unlikely that strain differences are the result of variation in 5-ALA synthetase activities induced by iron but depend on some other inherited variable factor(s).

Variation in iron loading

Although no correlation between small variations in iron status and development of porphyria has been observed, the influence of markedly different degrees of iron loading was examined. C57BL/10ScSn mice received 0, 1, 2 or 3 doses of iron–dextran (600 mg of Fe/kg) and livers were examined after 20 weeks (Table 4). Surprisingly, considerably less inhibition of UROD activity and accumulation of porphyrins occurred at the 2- or 3-dose levels than after the single dose usually employed. Non-haem iron concentrations, however, were consistently increased by larger doses. In another study, SWR mice received one-third, 1 or 3 doses of iron–dextran and then were administered 5-ALA for 4 weeks. The lowest dose of iron (200 mg of Fe/kg) caused more than 70% inhibition of the decarboxylase. Although the standard dose of 600 mg of Fe/kg was even more effective, three doses caused the least inhibition of the enzyme and especially the accumulation of porphyrins (Table 5). Histological examination of the liver showed dose-related toxicity manifested as focal inflammatory lesions with necrosis.

Comparisons of microsomal uroporphyrinogen oxidation

Some studies of uroporphyria implicate microsomal oxidation of uroporphyrinogen to uroporphyrin [35,44,45]. DBA/2, C57BL/6J, C57BL/10ScSn and SWR mice were examined to determine whether any susceptibility between strains could be accounted for by differential uroporphyrinogen oxidation either in controls or after iron overload (Table 6). No differences between strains were observed to account for the propensities to develop porphyria. Activities were consistently lower with microsomes from mice 4 weeks after loading with iron as was the level of EROD (as a measure of CYP1A activity) and to a lesser extent total cytochrome *P*-450 and cytochrome *P*-450 reductase. In contrast, BROD (as a possible estimate of CYP2B1 among other isoenzymes) was apparently elevated after iron treatment in C57BL/10ScSn, SWR and especially DBA/2 mice (Table 6). Microsomes from these four strains, with or without prior iron treatment, were also subjected to PAGE followed by

Table 6 Comparison between microsomal activities of DBA/2, C57BL/6J, C57BL/10ScSn and SWR mice 4 weeks after iron treatment

Mice received dextran (2.4 g/kg) or iron–dextran (600 mg of Fe/kg) and then were kept on a control diet for 4 weeks. Microsomal activities were estimated as described in the Experimental section. Results are means±S.E.M. for three mice per group and were analysed by two-way analysis of variance for strain and iron treatment. Although a variety of statistically significant differences between strains were observed most were not consistent with the large variation between strains in the development of porphyria after iron treatment. The one exception was the greater increase in BROD levels in iron-dosed DBA/2 mice. *$P < 0.05$; significantly different from corresponding non-iron-dosed group by Student's *t* test.

Strain	Iron treatment	Cytochrome *P*-450 (nmol/mg of protein)	Cytochrome *P*-450 reductase (nmol/min per mg of protein)	EROD activity (pmol/min per mg of protein)	BROD activity (pmol/min per mg of protein)	Uroporphyrinogen oxidation (pmol/min per mg of protein)
DBA/2	−	1.32±0.03	257±14	91±2	71±8	68±6
DBA/2	+	1.22±0.11	243±37	65±1*	232±15*	26±3*
C57BL/6J	−	1.14±0.09	430±41	123±3	76±13	67±3
C57BL/6J	+	0.90±0.05	306±12*	86±4*	110±13	34±3*
C57BL/10ScSn	−	1.03±0.10	481±36	142±18	92±3	65±3
C57BL/10ScSn	+	0.92±0.03	381±8	85±9*	155±18*	35±1*
SWR	−	1.42±0.13	328±14	150±8	67±8	69±2
SWR	+	1.31±0.03	271±25	73±5*	104±8*	48±4*

immunoblotting using polyclonal antibodies to rat CYP1A1 and CYP1A2. No differences between the strains could be detected, which could account for the wide range of propensities to develop uroporphyria (results not shown).

DISCUSSION

Despite some evidence that the induction of uroporphyria in mice caused by TCDD and HCB with or without iron overload is predominantly influenced by the *Ah* locus [11–13], additional evidence has indicated a role for other genes which as yet are unknown [17,46]. The recent demonstrations that inhibition of UROD by iron alone in the susceptible Ah^b C57BL/6J and C57BL/10ScSn inbred strains but not in the Ah^d strain, DBA/2 [22–26], would seem to confirm a relationship with the *Ah* locus although no ligand for the Ah receptor was administered. However, our experiments shown in Table 1 clearly demonstrate no correlation with Ah^{b-1} or Ah^{b-2} phenotypes [37,38] in iron-induced UROD inhibition and indicate that some other factors are involved. Indeed, the Ah^d strain SWR appeared to be the most sensitive. In previous studies, a moderate response to HCB-induced porphyria in SWR iron-loaded mice was observed when the *Ah* responsive strains CBA/J and A/J were refractory [21]. For the present, it is not clear whether there are more than two alleles or loci implicated in iron-induced uroporphyria but this would seem likely since some strains such as BALB/c and AKR were intermediate between C57BL/10ScSn and DBA/2 mice in their propensity for UROD inhibition. Previous work indicated that the presence of the *hr* gene enhanced the hepatic toxicity (including uroporphyria) of TCDD as well as its dermal effects [42], but as far as iron-induced inhibition of UROD is concerned the reverse seems to be true. Interestingly, the spread of sensitivity of individual outbred MF1 mice to iron mirrors the problem of variability of response frequently reported for the induction of porphyria caused by HCB in outbred rats (e.g. [47]) and demonstrates the importance of the use of inbred strains to obtain consistent group results. The variability of the MF1 mice can also be compared with the development of PCT in only a few of those patients at risk.

Compatible with the lack of correlation between the propensities of strains to develop porphyria and the *Ah* locus, the present findings seem to show no correlation with the activity (EROD) of the Ah receptor-mediated product CYP1A1 or by immunoblotting for CYP1A1 and CYP1A2 either at a constitutive level or after iron overload. It is probably worth mentioning here that in previous studies of EROD induction after β-naphthoflavone treatment of 12 *Ah*-responsive or non-responsive mice, a tremendous variation of inducibility was observed which did not completely segregate with the *Ah*-phenotype or induction of uroporphyria with HCB [21]. As might be predicted with iron [48], EROD activity was depressed under the influence of iron both in the short-term (4 weeks) and long-term (25 weeks; A. G. Smith, unpublished work). Although total cytochrome *P*-450 and cytochrome *P*-450 reductase levels were also depressed, albeit to a less significant extent, we consistently observed stimulation by iron of BROD activity, as reported in a previous study [24]. We have been unable to determine whether this is due to induction of a particular isoenzyme of cytochrome *P*-450 or to changes in microsomal conditions leading to increased metabolism of this resorufin ether specifically, under the assay conditions.

The mechanisms of human sporadic PCT and the experimental uroporphyrias are frequently the source of speculation and may be read in detail elsewhere [1,2,9,14,21,35,44,45,49,50]. There is strong circumstantial evidence for a role for the cytochrome *P*-450 system, especially the CYP1A subfamily in experimental uroporphyrias [14,35]. *In vitro*, CYP1A2 will oxidize uroporphyrinogen to non-metabolizable uroporphyrin [35,51] although, so far, mouse-strain differences in oxidation have not been observed ([52] and Table 3). Also, this does not account for the inhibition of UROD observed in experiments *in vivo* [1,2,8,9,12,19,25] and formation of a heat-stable inhibitory factor [21,40,41], as shown here; although the significance of this latter finding for the development of uroporphyria is not yet clear. Most importantly, the involvement of the cytochrome *P*-450 system alone does not explain the strongly potentiating effects of iron observed *in vivo* [8,9,12,17–21]. To explain this aspect, theories have centred on iron-catalysed hydroxyl radical formation from reactive oxygen species possibly produced by the microsomal system [9,14,18,19,44,45,49,50,53]. As a consequence a modified uroporphyrinogen might be produced [21,44,54], such as an hydroxylated analogue [55], which could act as a potent inhibitor of UROD [54] or cause an active-site-directed inactivation of the enzyme [5]. Interestingly, there is evidence for genetic variation in mice of NADPH-dependent, iron-catalysed, microsomal lipid peroxidation [19,56]. It should be stressed that iron not only potentiates the porphyria caused by TCDD and HCB in mice [9,12,17,18,25] but also the general hepatic toxicity and liver carcinogenicity [16,17,22,23,57], so that these effects have to be accounted for in any mechanistic theory.

Clearly from the experiments reported here iron alone will also cause hepatic uroporphyria with a polymorphism which does not apparently depend on any large differential constitutive cytochrome *P*-450 activities that we have been able to detect (although there could be some others not investigated). This demonstrates the importance of iron in this syndrome, but also indicates an unknown genetic variability. One possibility is that iron enhances 5-ALA synthase activity [9] preferentially in some strains, as observed with some porphyria-inducing drugs [58]; however, this seems unlikely due to the maintenance of strain differences after administration of 5-ALA (Table 3) [26]. As all the four strains responded differently this would suggest that more than one variable is involved. Since the 5-ALA treatment greatly accelerates the onset of porphyria, this illustrates the importance that an enlarged haem precursor pool might have on UROD inhibition, perhaps by providing more uroporphyrinogen to be oxidized to an inhibitor [44,54]. Genetic variations of mice in 5-ALA dehydratase [59,60] and 1-hydroxymethylbilane synthase [61] are known but do not correlate with the susceptibility of the strains in our studies. Whether the results can be partly explained by genetic variations in UROD requires study, although there is no evidence so far to support this possibility.

As an alternative to genetic variations in haem biosynthesis or cytochrome *P*-450 isoenzymes, the propensities of strains for iron-induced uroporphyria may depend on interstrain differences in various aspects of iron metabolism. Susceptibility to free-radical-induced toxicity due to genetic variations in iron metabolism would be of general interest and we are exploring this possibility.

Despite the lack of correlation with levels of some cytochrome *P*-450 isoenzymes, none of the findings in the present work is incompatible with a role for cytochrome *P*-4501A1/2 isoenzymes in the mechanism of experimental uroporphyrias, especially after induction by agents such as HCB and TCDD. Somehow the chemicals also cause uroporphyria which can be potentiated by iron and vice versa. Polymorphism in the expression of CYP1A1, in conjunction with the variations after iron treatment reported here, may account for some of the complex inheritance of TCDD-induced porphyria reported previously [17,46].

Finally, it was surprising to us that uroporphyria was greatly diminished at much higher iron doses than we have employed previously, suggesting an optimum iron level in hepatocytes. Heterozygosity for the haemochromatosis gene [62] has been suggested as a reason for the mild to moderate hepatic siderosis found in Caucasian PCT patients [63–65]. An optimum hepatocyte iron level for the development of uroporphyria might explain why the disease is not more prevalent than it is in homozygous patients displaying overt haemochromatosis [63,64].

We thank Dr. M. Festing for help and advice with statistical evaluations, Dr. C. R. Wolf for cytochrome *P*-450 antibodies, and Dr. P. Carthew for histological examinations.

REFERENCES

1 Sweeney, G. D. (1986) Clin. Biochem. **19**, 3–15
2 Kappas, A., Sassa, S., Galbraith, R. A. and Nordmann, Y. (1989) In The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), 6th edn, pp. 1305–1365, McGraw Hill, New York
3 Lundvall, O., Weinfeld, A. and Lundin, P. (1970) Acta Med. Scand. **188**, 37–53
4 Felsher, B. F., Jones, M. L. and Redeker, A. G. (1973) J. Am. Med. Assoc. **226**, 663–665
5 Elder, G. H., Urquhart, A. J., De Salamanca, R. E., Munoz, J. J. and Bonkovsky, H. L. (1985) Lancet **II**, 229–234
6 Rocchi, E., Gilbertini, P., Cassanelli, M., Peitrangela, A., Borghi, A., Pantoleoni, M., Jensen, J. and Ventura, E. (1986) Br. J. Dermatol. **114**, 621–629
7 Sweeney, G. D. (1985) Clin. Dermatol. **3**, 125–143
8 Francis, J. E. and Smith, A. G. (1987) Biochem. Biophys. Res. Commun. **146**, 13–20
9 Urquhart, A. J., Elder, G. H., Roberts, A. G., Lambrecht, R. W., Sinclair, P. R., Bement, W. J., Gorman, N. and Sinclair, J. A. (1988) Biochem. J. **253**, 357–362
10 Landers, J. P. and Bunee, N. J. (1991) Biochem. J. **276**, 273–287
11 Jones, K. G. and Sweeney, G. D. (1980) Toxicol. Appl. Pharmacol. **53**, 42–49
12 Smith, A. G. and Francis, J. E. (1983) Biochem. J. **214**, 909–913
13 Hahn, M. A., Gasiewicz, T. A., Linko, P. and Goldstein, J. A. (1988) Biochem. J. **254**, 245–254
14 Smith, A. G. and De Matteis, F. (1990) Xenobiotica **20**, 865–877
15 Sweeney, G. D., Jones, K. G., Cole, F. M., Basford, D. and Krestynski, F. (1979) Science **204**, 332–335
16 Jones, K. G., Cole, F. M. and Sweeney, G. D. (1981) Toxicol. Appl. Pharmacol. **61**, 74–78
17 Greig, J. B., Francis, J. E., Kay, S. J. E., Lovell, D. P. and Smith, A. G. (1984) Toxicol. Appl. Pharmacol. **74**, 17–25
18 Rizzardini, M., Graziani, A., Carugo, C. and Cantoni, L. (1988) J. Biochem. Toxicol. **3**, 33–46
19 Smith, A. G., Francis, J. E., Kay, S. J. E., Greig, J. B. and Stewart, F. P. (1986) Biochem. J. **238**, 871–878
20 Sinclair, P. R., Benent, W. J., Lambrecht, R. W., Jacobs, J. M. and Sinclair, J. F. (1989) Biochem. Pharmacol. **34**, 4341–4344
21 Smith, A. G. and Francis, J. E. (1987) Biochem. J. **246**, 221–226
22 Smith, A. G., Cabral, J. R. P., Carthew, P., Francis, J. E. and Manson, M. M. (1989) Int. J. Cancer **43**, 492–496
23 Smith, A. G., Francis, J. E. and Carthew, P. (1990) Carcinogenesis **11**, 437–444
24 Smith, A. G., Francis, J. E., Walters, D. G. and Lake, B. G. (1990) Biochem. Pharmacol. **40**, 2564–2568
25 Siersema, P. D., van Helvoirt, R. P., Ketelaars, D. A. M., Cleton, M. I., de Bruijn, W. C., Wilson, J. H. P. and van Eijk, H. G. (1991) Hepatology **14**, 1179–1188
26 Deam, S. and Elder, G. H. (1991) Biochem. Pharmacol. **41**, 2019–2022
27 Smith, A. G., Francis, J. E. and Bird, I. (1986) J. Biochem. Toxicol. **1**, 105–117
28 Green, M. C. (1989) in Genetic Variants and Strains of the Laboratory Mouse (Lyon, M. F. and Searle, A. G., eds.), 2nd edn, pp. 12–403, Oxford University Press, Oxford
29 Smith, A. G., Francis, J. E., Green, J. H., Greig, J. B., Wolf, C. R. and Manson, M. M. (1990) Biochem. Pharmacol. **40**, 2059–2068
30 Granick, S., Sinclair, P., Sassa, S. and Grieninger, G. (1975) J. Biol. Chem. **250**, 9215–9225
31 Lim, C. K. and Peters, T. J. (1984) Clin. Chim. Acta **139**, 55–63
32 Torrance, J. D. and Bothwell, T. H. (1968) S. Afr. J. Med. Sci. **3**, 9–11
33 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
34 Lubet, R., Nims, R., Mayer, R., Cameron, J. and Schechtmann, L. (1985) Mutat. Res. **142**, 127–131
35 Jacobs, J. M., Sinclair, P. R., Bement, W. J., Lambrecht, R. W., Sinclair, J. F. and Goldstein, J. A. (1989) Biochem. J. **258**, 247–253
36 Wolf, C. R., Sedman, S., Oesch, F., Mayer, R. T. and Burke, M. D. (1986) Biochem. J. **240**, 27–33
37 Poland, A., Glover, E. and Taylor, B. A. (1987) Mol. Pharmacol. **32**, 471–478
38 Poland, A. and Glover, E. (1990) Mol. Pharmacol. **38**, 306–312
39 Festing, M. F. W. (1979) Inbred Mouse Strains in Biomedical Research, MacMillan Press Ltd., London
40 Rios de Molina, M. D. C., Wainstock de Calmanovici, R. and San Martin de Viale, R. (1980) Int. J. Biochem. **12**, 1027–1032
41 Cantoni, L., Dal Fuime, D., Rizzardini, M. and Ruggieri, R. (1984) Toxicol. Lett. **20**, 211–217
42 Greig, J. B., Francis, J. E., Kay, S. J. E., Lister, T., Ray, D. E., Seawright, A. A. and Smith, A. G. (1987) Arch. Toxicol. **60**, 350–354
43 Rice, M. C. and O'Brien, S. J. (1980) Nature (London) **283**, 157–161
44 De Matties, F. (1988) Mol. Pharmacol. **33**, 463–469
45 Mukerji, S. K. and Pimstone, N. R. (1990) Arch. Biochem. Biophys. **281**, 177–184
46 Greig, J. B., Francis, J. E. and Smith, A. G. (1990) in Organohalogen Compounds, vol. 1, 'Dioxin' 90 EPRI-Seminar, pp. 177–180, Ecoinforma Press, Bayreuth
47 Wainstok de Calmanovici, R., Billi, S. C., Aldonatti, C. A. and San Martin de Viale, C. (1986) Biochem. Pharmacol. **35**, 2399–2405
48 Bacon, B. R. and Britton, R. S. (1990) Hepatology **11**, 127–137
49 De Matteis, F. (1988) Semin. Hematol. **25**, 321–329
50 De Matteis, F. (1991) in Hepatotoxicology (Meeks, R. G., Harrison, S. D. and Bull, R. J., eds.), pp. 437–479, CRC Press, Florida
51 Lambrecht, R. W., Sinclair, P., Gorman, N. and Sinclair, J. F. (1992) Arch. Biochem. Biophys. **294**, 504–510
52 Jacobs, J. M., Sinclair, P. R., Lambrecht, R. W., Sinclair, J. F. and Jacobs, N. J. (1990) J. Biochem. Toxicol. **5**, 193–197
53 De Matteis, F. and Stonard, M. (1977) Semin. Hematol. **14**, 187–192
54 Francis, J. E. and Smith, A. G. (1988) FEBS Lett. **233**, 311–314
55 Guo, R., Rideout, J. M. and Lim. C. K. (1989) Biochem. J. **264**, 293–295
56 Cheeseman, K. H., Proudfoot, K. A., Maddix, S. P., Collins, M. M., Milia, A. and Slater, T. F. (1985) FEBS Lett. **184**, 343–346
57 Faux, S. P., Francis, J. E., Smith, A. G. and Chipman, J. K. (1992) Carcinogenesis **13**, 247–250
58 Gross, S. R. and Hutton, J. J. (1971) J. Biol. Chem. **246**, 606–614
59 Hutton, J. J. and Coleman, D. L. (1969) Biochem. Genet. **3**, 517–523
60 Bishop, T. R., Cohen, P. J., Boyer, S. H., Noyes, A. N. and Freilin, L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5568–5572
61 Meisler, M. H. and Carter, M. L. C. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2848–2853
62 Bothwell, T. H., Charlton, R. W. and Motulsky, S. G. (1989) in The Metabolic Basis of Inherited Disease (Seriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), 6th edn, pp. 1433–1462, McGraw-Hill, New York
63 Kushner, J. P., Edwards, C. W., Dadone, M. M. and Skolnick, M. H. (1985) Gastroenterology **88**, 1232–1238
64 Edwards, C. Q., Griffen, L. M., Goldgar, D. E., Skolnick, M. H. and Kushner, J. P. (1989) Gastroenterology **97**, 972–981
65 Bottomley, S. S. (1987) Boll. Ist. Dermatol. S. Galliacano **13**, 237–240

Received 20 August 1992/16 October 1992; accepted 22 October 1992