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Non-Ahr Gene Susceptibility Loci for Porphyria and Liver Injury Induced by the Interaction of 'Dioxin' with Iron Overload in Mice

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ABSTRACT

Among the actions of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) in mice is the induction of hepatic porphyria. This is similar to the most common disease of this type in humans, sporadic porphyria cutanea tarda (PCT). Evidence is consistent with the actions of dioxin being mediated through binding to the aryl hydrocarbon receptor (AHR) with different *Ahr* alleles in mouse strains apparently accounting for differential downstream gene expression and susceptibility. However, studies of dioxin-induced porphyria and liver injury indicate that the mechanisms must involve interactions with other genes, perhaps associated with iron metabolism. We performed a quantitative trait locus (QTL) analysis of an F_2 cross between susceptible C57BL/6J (Ahr^{b1} allele) and the highly resistant DBA/2 (Ahr^{d} allele) strains after treatment with dioxin and iron. For porphyria we found QTLs on chromosomes 11 and 14 in addition to the *Ahr* gene (chromosome 12). Studies with C57BL/6.D2 Ahr^{d} mice confirmed that the Ahr^{d} allele alone did not completely negate the response. SWR mice are syngenic for the Ahr^{d} allele with the DBA/2 strain but are susceptible to porphyria after elevation of hepatic iron. Analysis of SWR×D2 F_2 mice treated with iron and dioxin showed a QTL on chromosome 11, as well as finding other loci on chromosomes 1 (and possibly 9), for both porphyria and liver injury. These findings show for the first time the location of genes, other than *Ahr*, that modulate the mechanism of hepatic porphyria and injury caused by dioxin in mice. Orthologous loci may contribute to the pathogenesis of human sporadic PCT.

The aryl hydrocarbon receptor (AHR), a member of the basic helix-loop-helix–periodicity/ARNT/simple-minded family of transcription factors, is the primary target for the environmental agent 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin). Mechanisms that lead to transcription of a few genes, such as *CYP1A1,* have been well studied (Gu et al., 2000), but the pathogenesis of many of the large variety of toxic and carcinogenic disturbances in man and experimental systems remain unclear (Pohjanvirta and Tuomisto, 1994; Gu et al., 2000). In mice, polymorphism of the *Ahr* gene with a resulting 10-fold difference in affinity for ligands is apparently associated with high susceptibility of the C57BL/6J (*b1* allele) and marked resistance of the DBA/2 strain (*d* allele) (Poland and Glover, 1980). The *Ahr null* mouse seems to be intractably resistant to environmental chemicals of the dioxin type (Fernandez-Salguero et al., 1996)). On the other hand, there is considerable tissue and species variability in response to dioxin that cannot be ascribed simply to polymorphisms of the *Ahr* gene (Pohjanvirta and Tuomisto, 1994; Geyer et al., 1997). This suggests that other modulating genes have profound effects on AHR-mediated toxicity. In skin carcinogenesis, the ability of dioxin to act as a promoter is dependent not only on the Ahr^{b1} genotype but also on the *Hr* locus (Knutson and Poland, 1982).

One of the actions of dioxin in rodents is to produce a malfunction of hepatic heme synthesis (Fig. 1) (De Matteis, 1998) similar to that seen in the human liver disorder sporadic porphyria cutanea tarda (PCT), which has no known cause (Elder, 1998; Anderson et al., 2001). In rodents exposed to dioxin and in human PCT patients, hepatic uroporphyrinogen decarboxylase (UROD) activity becomes markedly inhibited by an undetermined mechanism, leading to massive accumulation of uroporphyrin in the liver, and there is some association with hepatic toxicity (Pohjanvirta and Tuomisto, 1994; De Matteis, 1998; Elder, 1998; Smith et al., 1998). In mice, CYP1A2 seems essential for the UROD defect, porphyria and aspects of the liver injury (Smith et al., 2001). Over the years, it has become clear that iron metabolism is also

ABBREVIATIONS: AHR, aryl hydrocarbon receptor; dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCT, porphyria cutanea tarda; UROD, uroporphyrinogen decarboxylase; ARNT, aryl hydrocarbon receptor nuclear translocator; QTL, quantitative trait locus; ALT, alanine aminotransferase; AST aspartate aminotransferase; URO, uroporphyrin; PCR, polymerase chain reaction; PCA, principle component analysis; LOD, logarithm of odds, the common logarithm of likelihood ratio for the observed segregation frequency at a pair of loci; ANOVA, analysis of variance.

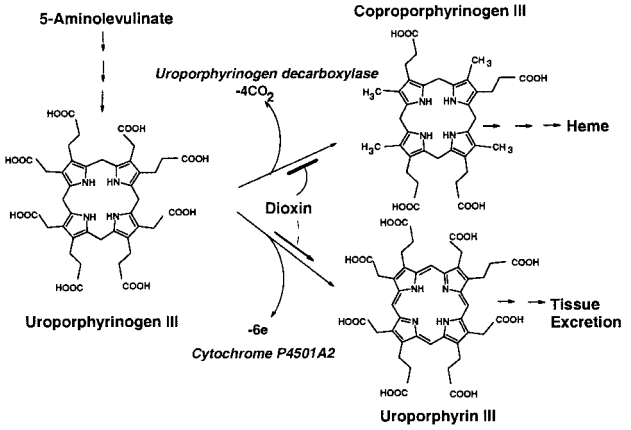

Fig. 1. Disruption by dioxin of heme synthesis leading to accumulation and excretion of uroporphyrin. A dual action of dioxin causing both depressed uroporphyrinogen decarboxylase (UROD) activity and induced *Cyp1a2* expression. The former causes the accumulation of uroporphyrinogens and some partial decarboxylation products whereas the latter enhances oxidation of uroporphyrinogens I and III to unmetabolizable uroporphyrins I and III. Severe hepatic porphyria is associated with liver injury as is the similar human disorder PCT (Elder, 1998).

implicated. First, depletion of liver iron partly protects against the porphyrinogenic and toxic action of dioxin in the livers of C57BL/6J mice (Sweeney et al., 1979; Jones et al., 1981), whereas iron overload maximizes the porphyria and liver injury (Smith et al., 1998). Second, the response to weak AHR ligands or the marked resistance of some strains with the Ahr^d allele (e.g., SWR but not DBA/2) can be overcome by elevation of iron stores (Greig et al., 1984; De Matteis, 1998; Smith et al., 1998). In fact, iron itself will eventually induce porphyria in some strains of mice (Smith and Francis, 1993; Philips et al., 2001). Thus uroporphyria in mice can be viewed as a genetically determined disorder induced by iron that is enhanced by chemicals, most potently by dioxin.

In both familial and sporadic forms of PCT, there is also considerable evidence for a crucial role of iron that is potentiated by exogenous agents (Elder, 1998). For some groups of PCT patients, the C282Y and H63D hemochromatosis gene (*HFE*) mutations are significant risk factors (Roberts et al., 1997; Bonkovsky et al., 1998; Sampietro et al., 1998; Bulaj et al., 2000).

Most of the actions of AHR as a regulator of gene expression are thought to occur by heterodimerization with ARNT, a related transcription factor that can also form a partnership with HIF1α (Gu et al., 2000). The three transcription factors are members of the periodicity/ARNT/simple-minded superfamily. The evidence that dioxin in the liver may act in an oxidative mechanism (Shertzer et al., 1998; Smith et al., 1998), the role of the pro-oxidant iron, and the relationship of AHR with ARNT and HIF1α, which are implicated in oxygen signaling pathways, may indicate that these are all interrelated in the development of porphyria and liver injury. Here, we have searched for quantitative trait loci (QTL) determining susceptibility to dioxin toxicity by undertaking genetic analysis of crosses of C57BL/6J and SWR mice with DBA/2 mice, all of which received the same doses of iron and dioxin. The identification of genes that markedly modify the actions of dioxin would be of considerable interest in understanding the mechanism of how AHR ligands bring about pathological changes. In addition, because we are dealing with an important transcription factor family and iron metabolism, elucidation of such gene interactions and novel polymorphisms might be important for understanding some human disease processes.

Materials and Methods

Mice and Treatments. C57BL/6J, DBA/2, C57BL/6×DBA/2 F_1 and SWR mice were purchased at 6 weeks of age from Harlan UK Ltd (Bicester, Oxfordshire, UK). C57BL/6.D2-*Ahr* ^{d} mice were obtained from the Jackson Laboratories (Bar Harbor, ME). F_2 crosses of C57BL/6J or SWR mice with DBA/2 mice were bred in the University of Leicester. All mice had free access to water and rat and mouse diet no. 3 (Special Diet Services, Witham, UK) and were housed in negative pressure isolators maintained at 21°C with a 12-h light/dark cycle.

Male mice at approximately 8 weeks of age received iron-dextran (800 mg iron/kg of body weight) by subcutaneous injection; then, after 1 week, they received 75 μg/kg dioxin in corn oil by oral administration (40 ml/kg) (Smith et al., 1998). Mice were monitored up to a further 5 weeks then blood was obtained by cardiac puncture under terminal anesthesia. Liver and thymus were weighed. Where possible, tissue and blood were obtained from mice that were culled earlier when appearing morbid. All experiments were conducted under Home Office regulations. Liver and plasma were stored at −80°C for analysis.

Phenotypic Analyses. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities as indicators of hepatic damage, were measured using kits (Sigma-Aldrich, Poole, Dorset, UK). Porphyrin liver contents, expressed as nanomoles of uroporphyrin per gram, were estimated by spectrofluorometry (Grandchamp et al., 1980). Uroporphyrin (URO) levels and plasma ALT and AST were converted to log values for analysis. Cytosolic UROD activity was measured as in (Smith and Francis, 1987) using pentacarboxyporphyrinogen I as substrate. Hepatic microsomal

TABLE 1

Effects of dioxin and iron on porphyria and plasma ALT in mice with different Ahr genotypes

Mice received a single dose of iron-dextran (800 mg of Fe/kg) by subcutaneous injection and then after 1 week they received dioxin (75 μg/kg) by oral administration as described under *Materials and Methods.* The experiments were terminated after 5 weeks. Values are means ± S.D. ALT data for SWR taken from Smith et al. (1998). Values for URO and ALT in untreated mice or in those exposed to iron alone are in Smith et al. (1998). In general, they resembled those recorded here for DBA/2 mice.

Strain	*N*	Ahr Alleles	URO	ALT
			nmol/g	*U/L*
C57BL/6J	5	*b1/b1*	653 ± 203	616 ± 144
SWR	5	*d/d*	139 ± 138	446 ± 153
DBA/2	5	*d/d*	1.8 ± 1.9	58 ± 18
C57 × DBA F_1	20	*b1/d*	140 ± 206	101 ± 45
			(0.7–446)	(47–201)
C57 × DBA F_2	204	*b1/b1, b1/d, d/d*	371 ± 446	409 ± 392
			(0.2–3332)	(12–2264)
SWR × DBA F_2	242	*d/d*	63 ± 121	209 ± 192
			(0.2–981)	(30–1027)

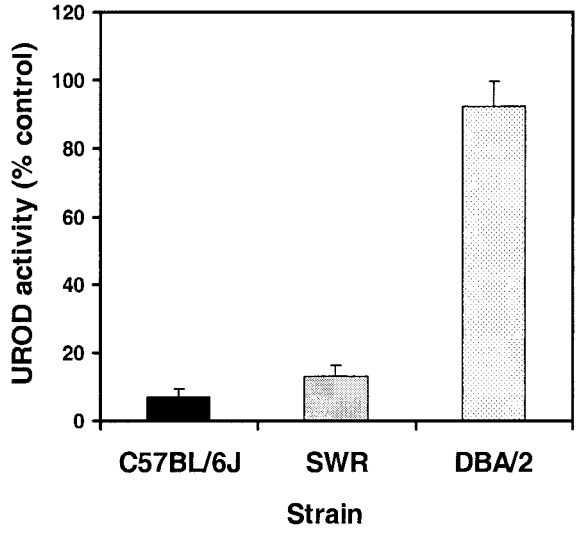

Fig. 2. Depression of UROD activity by dioxin is strain dependent in mice. Activity was measured in iron-loaded mice 5 weeks after a single dose of dioxin (75 μg/kg). Activity is expressed as a percentage of untreated controls as estimated in Smith and Francis (1987). Results are means ± S.D. (four mice per group).

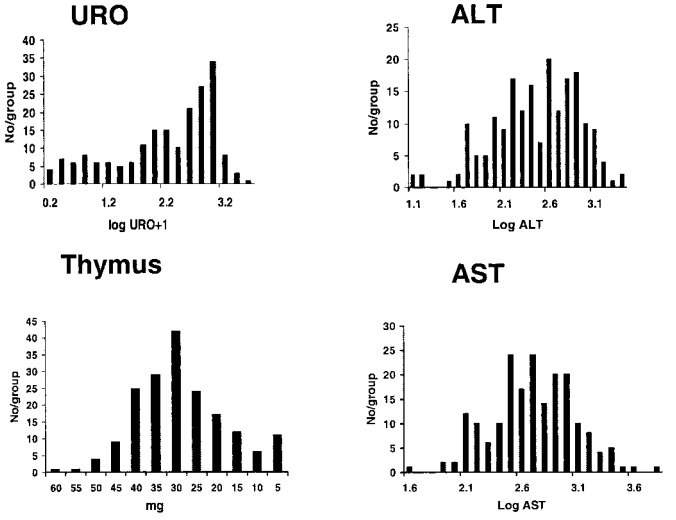

Fig. 3. Distribution patterns of hepatic uroporphyrin levels, thymus weights, and plasma ALT and AST responses in iron-loaded C57BL/6×DBA/2 F_2 mice 5 weeks after dioxin. URO and plasma enzyme levels are plotted as log values because of the wide range of upper extreme values.

dealkylations catalyzed by CYP1A isoforms and Western blotting (antibody from Gentest, Corp. MA) were performed as described previously (Sinclair et al., 1990; 1998; Smith et al., 2001). In the presence of both induced isoforms the microsomal dealkylation of ethoxyresorufin and methoxyresorufin are mediated predominantly by CYP1A1 and CYP1A2, respectively (Sinclair et al., 1998; Smith et al., 2001). Blots with chemiluminescence detection were extensively analyzed using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Genotyping by Microsatellite Analysis. Genome-wide scans of both the C57BL/6JxDBA/2 and the SWR×DBA/2 F_2 crosses were performed using polymerase chain reaction (PCR) amplification of microsatellite DNA markers. Initially, high- and low-responding mice were selected on the basis of uroporphyrin levels. Marker locations and appropriate polymorphism data for the C57BL/6J×DBA/2 cross were determined from the Whitehead Institute/MIT (Cambridge, MA; http://www-genome.wi.mit.edu/). Polymorphism data for the SWR strain is not readily available in the databases; therefore, a number of markers were tested for polymorphisms. SWR polymorphism data were also kindly supplied by N. Drinkwater (McArdle Laboratory for Cancer Research, Madison, WI) and R. A. McIndoe (CuraGen Corp., New Haven, CT) (McIndoe et al., 1999). Fluorescently labeled PCR primers were obtained from Applied Biosystems (Warrington, Cheshire, United Kingdom). Nonlabeled primers were synthesized by the Protein and Nucleic Acid Laboratory in the University of Leicester. PCR reactions contained 200 ng of genomic DNA extracted from tail or liver samples, 10 pmol/μl each primer, 10 mM Tris, 50 mM KCl, 1.5 mM Mg Cl_2, 0.2 mM dNTPs, and 0.5 U of *Taq* polymerase (PerkinElmer Life Sciences, Boston, MA). Samples were cycled in a PTC-225 DNA Engine Tetrad (MJ Research, Watertown, MA) using a touchdown program with an annealing temperature of 66 to 56°C with a final 35 cycles at 55°C annealing temperature. PCR products were analyzed on an ABI 377 sequencer using GeneScan and Genotyper (Applied Biosystems). Data from these scans for the high and low responding groups were analyzed using the χ^2 test, which showed any deviation from the 1:2:1 allelic ratio assumed for random inheritance and by principle component analysis (PCA). From these we chose chromosomes with potential QTLs for further analysis.

For the C57BL/6J×DBA/2 F_2 cross, all individual progeny were genotyped for the *Trp53* (chromosome 11) and *Ahr* (chromosome 12) polymorphisms (Schmidt et al., 1993; Yang et al., 1999) and for further markers on chromosomes 11, 12, and 14. All mice of the SWR×DBA/2 F_2 cross were further typed with markers on chromosomes 1, 7, 9, and 11. Linkage analysis was performed on the resultant data using MAPMAKER/EXP and MAPMAKER/QTL (Lander and Green, 1987; http://www-genome.wi.mit.edu/genome_software/) to obtain peak LOD scores for each of the selected chromosomes for a number of quantitative traits (i.e., hepatic uroporphyrin levels, plasma ALT and AST levels, and thymus atrophy). Suggestive linkage was defined as a LOD score of 2.8 or above and significant linkage as a LOD score of 4.3 or above (Lander and Kruglyak, 1995). Gene positions are those stated by UniGene (http://www.ncbi.nlm.nih.gov/UniGene/Mm.Home.html). Ranges of QTLs in centimorgans were estimated from 1 LOD score below the peak.

TABLE 2
Comparison of *Ahr b1/b, b1/d* and *d/d* genotypes with extreme response cohorts of C57BL/6J (Ahr^{b1}) × *DBA/2* (Ahr^{d}) F_2 mice by χ^2 analysis

Phenotype	Response[a]	Ahr Genotype			χ^2	p
		b1/b1	*b1/d*	*d/d*		
URO	H	20	30	0	18.00	<0.001
	L	4	26	20	10.32	0.006
ALT	H	15	30	5	6.00	0.049
	L	11	22	17	2.16	0.340
AST	H	23	25	2	17.64	<0.001
	L	4	19	27	24.04	<0.001
Thymus	H	22	23	5	11.88	0.003
	L	5	21	24	15.72	<0.001

[a] For each parameter, H = 50 highest responding mice and L = 50 lowest responding mice (i.e., URO, 3332–600 nmol/g and 26.3–0.2 nmol/g; ALT, 2264–576 and 57–12 U/L; AST, 6264–768 and 264–32 U/L; thymus, 0–22 and 34–68 mg, respectively).

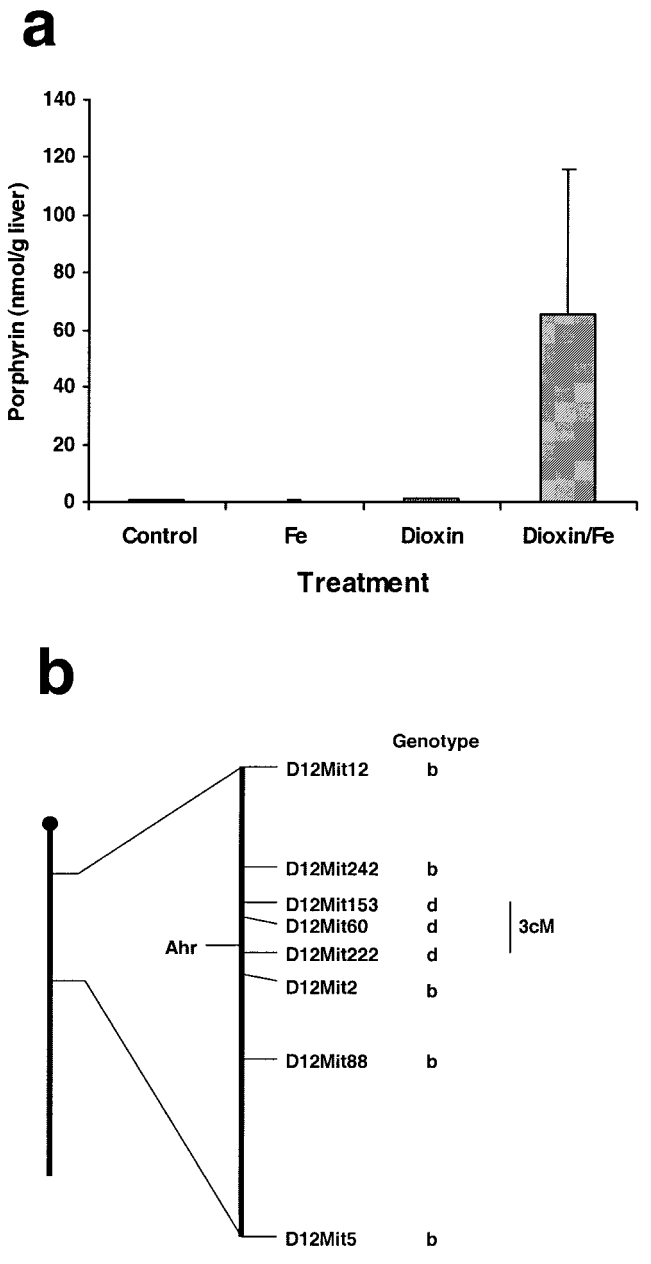

Fig. 4. Porphyric response of C57BL/6. D2-*Ahr*^{d} congenic mice after dosing with iron and dioxin as described in Table 1 (a). This demonstrates that iron pretreatment partially overcame the resistance of mice with the *Ahr*^{d} allele to dioxin when present in a C57BL/6J background. Data are means of five mice per group ± S.D. The congenic region around the *Ahr* gene on chromosome 12 was estimated in case other genes of potential influence are eventually found nearby (b). The *Ahr* gene lies close to D12 Mit222 in a region approximately 3 to 5 centimorgans long .

Results

Response of Mice to Iron-Dioxin. The response of parent strains to treatment with iron plus dioxin is shown in Table 1. Not only were there marked differences in elevation of hepatic porphyrin and plasma ALT levels but also in the depression of UROD in the liver (Fig. 2). Previous work has shown that prior administration of iron will maximize the hepatic response of C57BL/6J (*Ahr*^{b1} allele) mice to dioxin observed 5 weeks later. Such mice show massive uroporphyrin accumulation, moderate to severe inflammation, hepatocyte loss, and biliary proliferation together with elevated plasma ALT (Smith et al., 1998). In contrast, DBA/2 (*Ahr*^{d} allele) mice treated similarly show no porphyria, only mild lipid accumulation and hepatocyte hypertrophy, and low elevation of plasma ALT. However, iron will overcome to a significant degree the porphyric and injury response to dioxin of another *Ah*^{d} strain, SWR (Poland and Glover, 1990; Smith et al., 1998).

To investigate the reasons for susceptibility differences between strains, F_2 intercrosses of C57BL/6J and SWR mice with DBA/2 mice were given dioxin and iron for determination of QTLs. Ranges of hepatic URO and plasma ALT levels in these crosses are shown in Table 1. Of the 204 mice of the C57BL/6×DBA/2 cross, 179 survived to 5 weeks. Among those that had to be culled prematurely, a significant proportion (16 mice) showed signs of peritoneal, pulmonary, or facial edema. Plasma ALT and AST were measured as markers of liver injury. Each showed the expected wide range of response (Table 1 and Fig. 3) with a mean AST/ALT ratio of activity of 3.78. Interestingly, mice recorded as having observable edema had ALT values in the normal range but highly elevated AST levels (ratio 24.6). A possible explanation is that this reflected cardiac injury that may be the cause of dioxin-induced edema (Pohjanvirta and Tuomisto, 1994; Walker and Catron, 2000).

No morbidity occurred with the SWR×DBA/2 F_2 cross and there was a wide range of URO and ALT responses. As expected (Smith et al., 1998), these were of an overall lower intensity than seen with the C57BL/6J×DBA/2 cross (Table 1).

Ahr Genotype of C57BL/6J×DBA/2 Cross. All mice of the C57BL/6J×DBA/2 cross were genotyped for the *Ahr*^{b1} and *Ahr*^{d} alleles. Those observed with edema were either of the *b1/b1 or b1/d* genotype. The extreme cohorts for each of the parameters (Fig. 3) were analyzed using the χ^2 test (Table 2) for correlation with *Ahr* alleles. Uroporphyrin and AST showed highly significant correlations with the *Ahr*^{b1} alleles but ALT was equivocal. Thymus atrophy, an established, nonhepatic effect of dioxin (Poland and Glover, 1980; Pohjanvirta and Tuomisto, 1994) was highly correlated with the possession of an *Ahr*^{b1} allele. However, for all four parameters there were approximately as many resistant mice with the *b1/d* genotype as there were with the sensitive phenotype. This finding suggests that although the *Ahr*^{b1} gene allele played a major role in determining the susceptibility to dioxin for porphyric and other responses, functional polymorphisms in other genes as well as subtle environmental factors might be as important for these endpoints.

The hypothesis that genes other than the *Ahr* locus contributing to susceptibility to iron-dioxin treatment was confirmed by the development of a degree of porphyria in C57BL/6.D2-*Ahr*^{d} congenic mice (Fig. 4a). In contrast, the DBA/2 strain is highly resistant to this regime (Table 1 and Greig et al., 1984; Smith et al., 1998). A similar response of C57BL/6.D2-*Ahr*^{d} mice has been observed with hexachlorobenzene (Hahn et al., 1988). The DBA/2 region on chromosome 12 in the congenic mice was mapped to approximately 3 centimorgans between D12 Mit153 (12 centimorgans) and D12 Mit222 (15.3cM). With these markers, *Ahr* lies close to D12 Mit 222 (Fig. 4b). Thus, we cannot currently exclude the possibility that some other genes close to *Ahr* within the 3-centimorgan region are also associated with toxicity.

TABLE 3

Peak LOD scores for fully mapped chromosomes

A LOD score of 2.8 was taken to be statistically suggestive and 4.3 as significant (Lander and Kruglyak, 1995). Regression analysis was used to quantify the percent of variance of URO by the most statistically significant (as estimated by a one-way ANOVA) genetic markers. The ratio in the C57BL/6 × DBA/2 F_2 cross was estimated as 1:1.75:0.88 for D11Mit179, Ahr, and D14Mit60 respectively. In the SWR × DBA/2 F_2 cross, 1:0.08:1.21 for D1Mit14, D9Mit15, and D11Mit70 respectively.

Cross	Phenotype	Chromosome		
		11	12	14
C57 × DBA	URO	5.46	6.02	2.94
	ALT	1.33	1.54	0.75
	AST	0.66	5.45	0.41
	Thymus	0.93	5.70	0.59
		1	9	11
SWR × DBA	URO	6.89	2.51[a]	11.84
	ALT	5.82	3.06	2.84
	Thymus	1.15	0.69	1.02

[a] Although just lower than LOD 2.8, D9Mit15 and D9Mit17 gave significant linkage ($p < 0.05$) with URO by χ^2 analysis.

Mapping of QTLs in C57BL/6J×DBA/2 Cross. To determine the location of susceptibility genes, the extreme phenotype cohorts (48 each) were initially genotyped for 44 markers spread across the genome at an average of about 20 centimorgans apart. As well as chromosome 12 (presumably the *Ahr* gene), PCA and χ^2 analyses showed significant ($p < 0.05$) associations between URO phenotypes and markers on other chromosomes, in particular 11 and 14. Consequently, all mice were genotyped with six to nine markers for each of these chromosomes. LOD scores by MAPMAKER for URO, plasma enzymes, and thymus weights are shown in Table 3. Mapping of significant QTLs for URO on chromosomes 11 and 12 with peak LOD scores above 4.3 (Lander and Kruglyak, 1995) are shown in Fig. 5. The QTL for porphyria on chromosome 11 at approximately 45 to 60 centimorgans had a peak LOD score similar to that for the *Ahr* gene on chromosome 12. It was distal to *Trp53* (39cM) that in preliminary studies showed linkage by PCA of a combination of all phenotype parameters including body and liver weight (Yang et al., 1999). A possible QTL for URO on chromosome 14 was also detected at 20 to 30 centimorgans (LOD score, 2.94) (Table 3). No interaction between any of these loci was found using a two-way general linear model ANOVA. For thymus atrophy and plasma AST, only one QTL, on chromosome 12 in the region encompassed by D12 Mit242, *Ahr,* and D12 Mit88, was detected (Table 3). Although no QTLs above LOD score 2.8 could be detected on chromosomes 11 and 14 for ALT and AST, there was significant association by PCA with

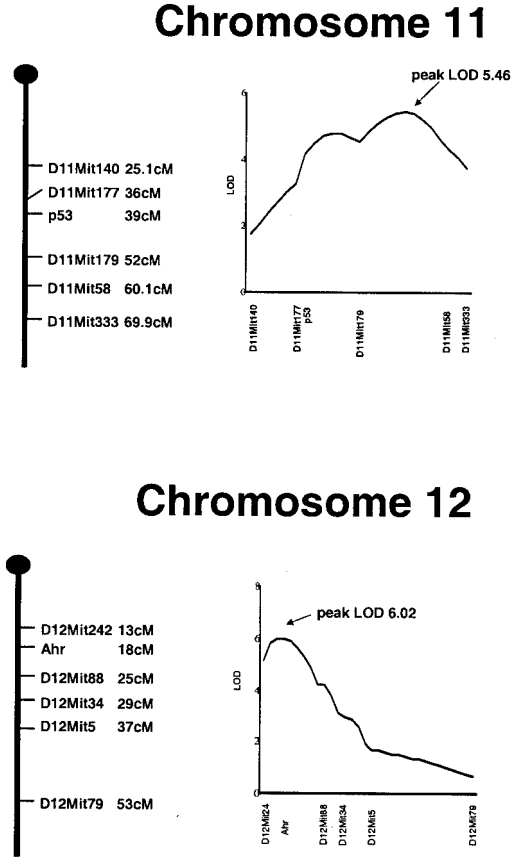

Fig. 5. QTL plots for porphyria on chromosomes 11 and 12 of C57BL/6×DBA/2 F_2 mice after dioxin and iron. The QTL on chromosome closely correlated with that for the *Ahr* gene.

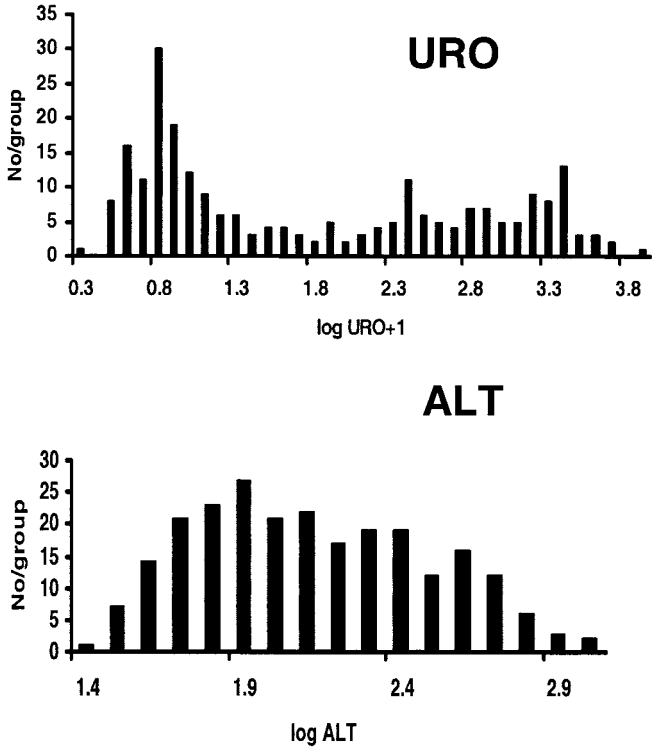

Fig. 6. Distribution of hepatic uroporphyrin levels and plasma ALT responses in iron loaded SWR×DBA/2 F_2 mice 5 weeks after dosing with dioxin. Data are plotted as log values.

extreme phenotypes. Similarly, PCA gave significant linkage between extreme ALT values and chromosome 12 (D12 Mit242) genotype.

Mapping of QTLs in SWR×DBA/2 cross. To investigate further possible loci other than *Ahr*, a genome scan (42 markers) was conducted on the extreme cohorts (35–47 per phenotype) for URO and ALT of the SWR×DBA/2 cross (Fig. 6). Significant association by PCA and one-way ANOVA or χ^2 analysis was observed between response and potential loci on chromosomes 1, 9, and 11, but not with chromosome 12 (even with D12 Mit88 at 19.7 centimorgans) because the parent strains are syngenic for the *Ahr* gene. All mice were subsequently genotyped for 9 to 12 markers on chromosomes 1,9 and 11. This confirmed the C57BL/6JxDBA/2 result indicating a highly significant QTL for uroporphyria on chromosome 11 at approximately 40 to 60 centimorgans (Fig. 7; Table 3). In addition, significant loci for both porphyria (Fig. 7) and plasma ALT were found on chromosome 1 (Table 3) at approximately 27 to 57 centimorgans. Two-way ANOVA for URO levels showed a significant interaction between the loci on chromosomes 1 and 11, although these seemed to control uroporphyria more than ALT. That is to say, the largest differences in URO levels among genotypes at the D1 Mit21 locus were seen when there was an SWR allele at the D11 Mit288 locus. There seemed to be a second locus on chromosome 1 for uroporphyrin (closest marker, D1 Mit110) with a peak LOD score 4.4 at 86 to 100 centimorgans (Fig. 4), but unlike the most significant locus on this chromosome, it showed no significant correlation with ALT (LOD 2.1). A possible QTL was also detected on chromosome 9 (closest marker D9 Mit15 at 60 centimorgans) but distal to *Cyp1a2*. Although weak, this locus seemed to be linked particularly with ALT and less so with uroporphyrin but this may just reflect the limitation of the analyses. In this cross, a QTL was not detected for thymus weight (Table 3).

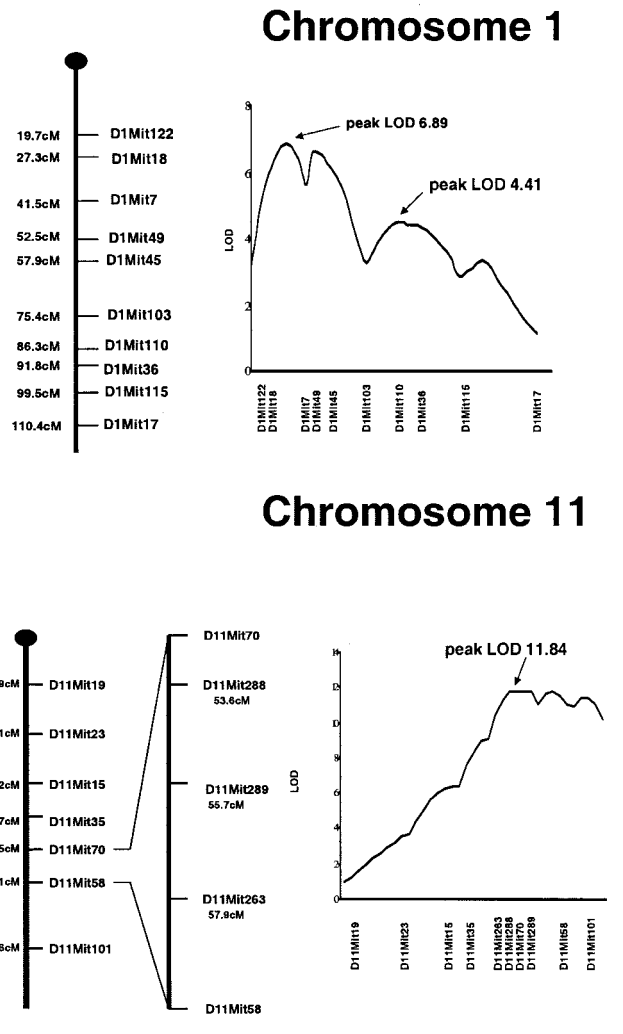

Fig. 7. QTL plots for porphyria on chromosome 1 and 11 of the SWR×DBA/2 F_2 cross after dioxin and iron using all 242 mice. At least one QTL was present on chromosome 1.

Expression of CYP1A2. From studies with *Cyp1a2* (−/−) mice, it is clear that CYP1A2 is essential for the development of uroporphyria in mice and some aspects of liver injury caused by dioxin (Sinclair et al., 1998; Smith et al., 2001). Despite this, there was no evidence that polymorphism of the *Cyp1a2* gene (chromosome 9 at 31 centimorgans) accounted for a major proportion of the susceptibility of C57BL/6J mice compared with the resistant DBA/2 strain. However, expression of *Cyp1a2* under these conditions is controlled by the AHR so that the polymorphism of the *Ahr* gene might contribute to the differences in susceptibility. The dose of dioxin was greater than that known to achieve maximum induction of CYP1A isoforms in Ahr^{b1} and Ahr^{d} mice (Pohjanvirta and Tuomisto, 1994). Marked induction of CYP1A2 protein and activity was observed in both strains and their intercross and did not seem to correlate with sensitivity (Fig. 8). The SWR and DBA/2 strains are not polymorphic for the *Ahr* gene (Poland and Glover, 1990) and we did not find any significant linkage with D9 Mit31 (33 centimorgans) close to the *Cyp1a1/2* genes. In addition, there was no marked difference in CYP1A2 expression in the extreme porphyria phenotypes of the SWRxDBA/2 cross (Fig. 8).

Discussion

This study has demonstrated QTLs, in addition to *Ahr*, that have a marked effect on porphyria and some aspects of liver injury caused by dioxin when interacting with iron. There are at least three QTLs on chromosomes 1, 11, and 14, and probably one on chromosome 9, besides *Ahr*, that are responsible for the differences in susceptibility between DBA/2 mice and the C57BL/6J and SWR strains. Thus, as far as liver injury is concerned, the view that resistance to dioxin in mice is completely dominated by the Ahr^{d} allele is misleading, as has been argued previously (Greig et al., 1984; Pohjanvirta and Tuomisto, 1994; Geyer et al., 1997). It seems likely that at least one QTL will be linked with liver iron mobilization and metabolism and that interactions with expressions mediated by the AHR lead to depression of UROD activity, porphyria, and some aspects of hepatic injury, which can result in elevated plasma ALT and AST levels. The SWR×DBA/2 QTL for both ALT and porphyria on chromosome 1 is in the same region as some genes associated with iron metabolism (e.g., ferroportin). In addition, although weak, the QTL on chromosome 9 at approximately 60 centimorgans is also in approximately the same region as some genes for iron metabolism (e.g., lactotransferrin, transferrin, and ceruloplasmin). These iron metabolism genes may not be involved in the porphyria response, but other potential target genes may be associated with them. Iron has the most

marked effect on porphyria development in SWR mice (Smith et al., 1998). So far the QTL on chromosome 11 seems to have no strong candidate, yet accounts for much of the susceptibility of C57BL/6J and SWR strains to iron/dioxin synergism and in the latter may interact with a gene on chromosome 1 in SWR mice. The QTL found on chromosome 14 in the C57BL/6×DBA/2 F_2 cross is in a similar region to *Hcbip1* found previously in a C57BL/10ScSn×DBA/2 F2 cross of susceptibility to porphyria induced by hexachlorobenzene-iron synergism (Akhtar and Smith, 1998) but is centromeric to the *Hr* gene at (39cM). No evidence for this locus was found in the SWR×DBA/2 cross. No linkage was detected in either study between phenotype and markers that are close to *Urod* on chromosome 4 (D4 Mit352 and D4 Mit57) or known positions of other heme synthesis genes. However, an important possibility is that one QTL corresponds to alleles of *Alas1*, the controlling gene of heme synthesis (Roberts and Elder, 2001), but polymorphic alleles and the chromosomal location of this gene in the mouse have not been reported.

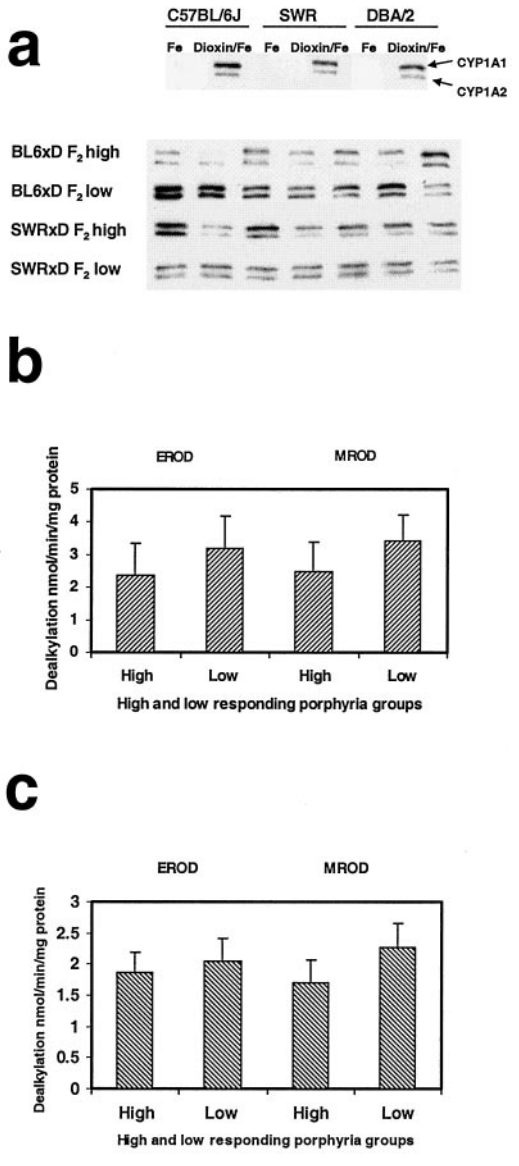

Fig. 8. Hepatic microsomal CYP1A2 expressions and activities in extreme porphyria responses of F_2 crosses after dioxin and iron. a, Western blotting of CYP1A1 and CYP1A2 in C57BL/6J, SWR, and DBA/2 iron-loaded mice 5 weeks after dioxin and representative C57BL/6×DBA/2 F_2 high- (990–3332 nmol/g) and low- (0.8–1.7 nmol/g) responding mice and SWR×DBA/2 F_2 high- (297–981 nmol/g) and low- (0.2–0.4 nmol/g) responding mice for uroporphyrin levels. No differences were detected between high- and low-responding groups of the SWR×DBA/2 F_2 cross after extensive quantitation of three blots with different samples (ImageQuant). Levels were reduced in high responding C57BL/6×DBA/2 F_2 mice compared with low responders perhaps reflecting liver damage. b, dealkylations by highest and lowest responding mice (see Table 2) of C57BL/6×DBA/2 F_2 cross (50 per group) and were statistically different whether *b1b1* or *b1d* genotypes. Ethoxyresorufin dalkylation (EROD) is particularly carried out by CYP1A1 and methoxyresorufin dealkylation (MROD) by CYP1A2. c, dealkylations by highest (282–981 nmol/g) and lowest (0.2–0.4 nmol/g) responding mice of SWR×DBA/2 F_2 mice (16 per group).

One of the interesting findings from the C57BL/6×DBA2 F_2 study is the association of a QTL with the *Ahr* gene, yet this did not seem to correlate with CYP1A2 expression that is essential for the porphyric response as shown by studies with *Cyp1a2* (−/−) mice (Sinclair et al., 1998; Smith et al., 2001). Although recent studies of caffeine metabolism in mice have shown a QTL on chromosome 9, close to the *Cyp1a2* gene that might be related to variable CYP1A2 levels (Casley et al., 1999), this did not seem to be linked in our experiments. It is possible that genes in addition to *Cyp1a2* controlled by the AHR are important for porphyria and some aspects of liver injury in C57BL/6J mice and are still differentially expressed when *Cyp1* genes are maximally induced. This also illustrates that null mice may demonstrate the necessity of a particular gene to a pathological process but do not show whether it is a candidate susceptibility gene. Of course as far as levels of CYP1A2 are concerned, we compared activity and protein at the termination of the experiments. It is possible that levels are critical at earlier times in the porphyrinogenic process in this model although we have no real supportive evidence for this from the QTL or previous studies (Smith et al. 1998).

The development of hepatic porphyria by dioxin has many similarities with human sporadic PCT (Elder, 1998; Anderson et al., 2001). Both seem to involve interactions with iron metabolism (Elder, 1998). Identification of the genes responsible for the QTLs in the present studies may be important to our understanding the mechanism of human sporadic PCT as well as being pertinent to other disorders. Of course, identifying the genes responsible for the QTLs is not an easy undertaking given the large regions of chromosomes identified, and this may entail more specific crosses for finer mapping resolution. However, the number of potential candidate genes is growing rapidly and with the draft of the mouse genome, progress should be far quicker than might have been envisaged only a few years ago. The only susceptibility genes found for some groups of sporadic PCT patients have been identified as mutants of *HFE* (Roberts et al., 1997; Bonkovsky et a1., 1998; Sampietro et al., 1998; Bulaj et al., 2000). Interestingly, penetrance for hemochromatosis by *HFE* is thought to depend on alleles of other genes (Whitfield et al., 2000; Fleming et al., 2001). It is now recognized that many diseases are complex interactions of susceptibility genes and environmental factors. Chemically induced porphyria and sporadic PCT are unlikely to be any different. The influence of modifier genes in other actions of dioxin also requires investigation.

In summary, although gene expression controlled by variants of the AHR is usually considered to be the major gene manifesting toxicity of dioxin, the development of porphyria

and elevation of plasma enzymes as indicators of liver injury have shown the presence and location of other susceptibility genes. It seems possible that some of the genes constituting the QTLs are also of significance in the development of PCT and perhaps other human disorders.

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