Genetic loci that affect aristolochic acid-induced nephrotoxicity in the mouse

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Rosenquist TA. Genetic loci that affect aristolochic acid-induced nephrotoxicity in the mouse. Am J Physiol Renal Physiol 300: F1360–F1367, 2011.—Aristolochic acids (AA) are plant-derived nephrotoxins and carcinogens found in traditional medicines and herbal remedies. AA causes aristolochic acid nephropathy (AAN) and is a suspected environmental agent in Balkan endemic nephropathy (BEN) and its associated upper urothelial cancer. Approximately 5–10% of individuals exposed to AA develop renal insufficiency and/or cancer; thus a genetic predisposition to AA sensitivity has been proposed. The mouse is an established animal model of AAN, and inbred murine strains vary in AA sensitivity, confirming the genetic predisposition. We mapped quantitative trait loci (QTL) correlated with proximal tubule dysfunction after exposure to AA in an F2 population of mice, derived from breeding an AA-resistant strain (C57BL/6J) and an AA-sensitive strain (DBA/2J). A single main QTL was identified on chromosome 4 (Aanq1); three other interacting QTLs, (Aanq2–4) also were detected. The Aanq1 region was also detected in untreated mice, raising the possibility that preexisting differences in proximal tubule function may affect the severity of AA-elicited toxicity. This study lays the groundwork for identifying the genetic pathways contributing to AA sensitivity in the mouse and will further our understanding of human susceptibility to AA found widely in traditional medicines.

Chinese herb nephropathy; Balkan endemic nephropathy; tubule interstitial disease; albuminuria; C57BL/6J mice; DBA/2J mice

Aristolochic acids (AA) are nitrophenanthrene carboxylic acids found in Aristolochia species used throughout the world in preparing traditional herbal remedies (29). Efforts to exploit the anti-inflammatory and antitumor properties of AA were halted after it was shown to be mutagenic and carcinogenic (2, 41). Aristolochic acid nephropathy (AAN) was first described among clients of a Belgian weight loss clinic inadvertently treated with an herbal mixture containing extracts of A. fangchi (4), the anti-inflammatory and antitumor properties of AA were demethylate AAI, the most potent form of AA, to generate AA1a, an inactive form (53). Gene-targeted mouse models confirm the role of hepatic cytochrome P-450 enzymes in detoxifying AA in vivo (69–71). In the mouse, the primary CYP that metabolizes AAI is CYP1A2 (48), and human CYP1A2 is kinetically competent to metabolize AAI as well. Although a pilot genetic study found a potential link of the Cyp3A5*1 allele with BEN (6), CYP3A5 does not metabolize AA in vitro (48, 53). Other studies have failed to establish a genetic link between known functional alleles in several CYPs, nitroreductases, or phase II genes and BEN (5, 6, 64). Among other risk factors in BEN families are small kidneys, microalbuminuria, and increased urinary β2-microglobulin (20). Hypertension is elevated in some families with BEN (4, 21), but the significance of this is not clear since hypertension is also found generally in the endemic regions (4).

The mouse is an established animal model for studying the effects of AA. The pattern of renal damage in the mouse resembles that in humans (40). AA target the epithelial cells of the proximal convoluted tubules in the outer kidney cortex. The mouse also accumulates promutagenic, AA-derived aristolactam-DNA adducts in DNA extracted from kidneys of patients with AAN (45). The renal histopathology of AAN, marked by proximal tubule atrophy and cortical hypocellular interstitial fibrosis, is quite similar to that of Balkan endemic nephropathy (BEN) (15), a regional nephropathy affecting thousands of rural farmers in the Danube River basin. BEN often affects multiple members of households after residence in the endemic region for two to three decades and is also associated with a high incidence of UUC. AA poisoning (31), ochratoxin, or lignites from coal field runoff, have been proposed as environmental agents that may cause BEN (reviewed in Ref. 54). Several lines of evidence, including the finding of AA-derived aristolactam-DNA adducts in kidney DNA of BEN patients, have solidified the consensus that BEN is a form of AAN (26). Cases of AAN are now recognized around the world, especially in Asian countries with prevalent practice of traditional medicines (18). In Taiwan, a country with a high rate of renal pelvic transitional cell carcinoma (11), a positive association of ingesting AA in herbal remedies with end-stage renal disease, and with UUC incidence, have recently been demonstrated (34, 61).

Genetic studies of BEN have focused on common alleles of candidate biotransformation genes and familial risk factors. In vitro studies have shown that nitroreductases, such as NAD(P)H:quinone oxidoreductase (60), SULT1A1 (39), cytochrome P-450 reductase (57, 59), and prostaglandin H synthase (56) can activate AA to the ultimate mutagen, proposed to be an aristolactam N-acyl nitrenium ion that reacts with DNA to form DNA adducts. Microsomal CYP450 1A1/2 also can demethylate AAI, the most potent form of AA, to generate AA1a, an inactive form (53). Gene-targeted mouse models confirm the role of hepatic cytochrome P-450 enzymes in detoxifying AA in vivo (69–71). In the mouse, the primary CYP that metabolizes AAI is CYP1A2 (48), and human CYP1A2 is kinetically competent to metabolize AAI as well. Although a pilot genetic study found a potential link of the Cyp3A5*1 allele with BEN (6), CYP3A5 does not metabolize AA in vitro (48, 53). Other studies have failed to establish a genetic link between known functional alleles in several CYPs, nitroreductases, or phase II genes and BEN (5, 6, 64). Among other risk factors in BEN families are small kidneys, microalbuminuria, and increased urinary β2-microglobulin (20). Hypertension is elevated in some families with BEN (4, 21), but the significance of this is not clear since hypertension is also found generally in the endemic regions (4).
QTL software mouse genotype and phenotypic data were maintained and merged in Excel (Microsoft, Redmond, WA.). QTL mapping and genetic model testing was done in the R/QTL suite of programs (10).

RESULTS

AAI-sensitive and -resistant strains of mice. To efficiently study AA genetics in mice, a streamlined protocol for determining AA sensitivity was developed. Although the histopathology of end-stage AAN is complex, the proximal tubule is the primary renal target of AA in exposed people and in rodent models of AAN (reviewed in Ref. 18). Subsequently, AAN progresses to end-stage renal disease through development of interstitial fibrosis and tubule atrophy, even after withdrawal from AA exposure (38). Thus urinary markers of proximal tubule function and injury in 8-wk-old male mice given a single dose of AAI were measured. AAI has been determined to be the most toxic form of AA (7, 52). Overnight urine samples were collected before treatment and again on the fourth night after treatment. To monitor proximal tubule cell function, urinary protein and glucose, which are normally reabsorbed from the renal filtrate in the proximal tubule, were measured. As an indicator of proximal tubule injury, the activity of the proximal tubule lysosomal enzyme NAG, which is released into the pro-urine in the proximal tubule lumen from damaged tubule cells, was measured. Each of these indicators of proximal tubule cell toxicity increase 3 days after AAI treatment, peak between days 4 and 5 after treatment, and gradually return to baseline values over the following week (Fig. 1). Sato et al. (49) described the inbred strains C3H/He and BALB/c mice as sensitive to the acute nephrotoxic effects of AA and the strain C57BL/6J as relatively resistant. Additionally, in this protocol the strains DBA/2J, A/J, NOD/ShiLtJ, C57BL/10J, SM/J, and C57BR/cdJ are AA sensitive; the strains CAST/EiJ and BTBR T+ tf/J are resistant; and the strains NZW/LacJ, KK/HJ, and SWR/J have intermediate resistance to AA-induced acute nephrotoxicity (supplementary data; all supplementary material for this article is available online at the journal web site.).

For this QTL study, C57BL/6J and DBA/2J mice were used as resistant and sensitive strains, respectively. Each strain has a complete genomic sequence available, and the genetic basis of differences in their ability to respond to xenobiotics is extensively studied (reviewed in Ref. 8). The peak (day 4)
response to AAI in mice from these strains, and F1 mice produced from intercrossing them (BDF1 mice), is shown in Table 1. Urinary albumin, glucose, and NAG activity were measured; each parameter was normalized to creatinine. Albumin was used instead of total protein as a specific measure of protein reuptake as it has been shown that while AA treatment induces tubular proteinuria it also decreases the expression of major urinary proteins in the mouse (30). For each parameter measured, the response to AA treatment for the DBA/2J strain was significantly higher than for the C57BL/6J strain. The AA-treated F1 mice had urinary NAG and glucose levels intermediate between C57BL/6J and DBA/2J, but the F1 albumin response was similar to that for DBA/2J. It has been previously reported that the DBA/2J and F1 mice have higher baseline levels of urinary albumin than C57BL/6J mice (51). This study confirmed that and found that DBA/2J mice also had higher baseline urinary NAG activity than C57BL/6J mice (Table 1).

**QTL detection.** BDF1 mice were intercrossed to generate a population of F2 mice. Two hundred 8-wk-old male F2 mice were subjected to the AAI protocol. Figure 2 shows the distribution of responses to AAI in F2 mice, as determined by urinary albumin (Fig. 2A), glucose (Fig. 2B), or NAG activity (Fig. 2C). To combine each measure of AA-induced proximal tubule impairment, each mouse was ranked for each phenotype and the rankings were summed. This AA-induced toxicity score was used as a phenotype for QTL mapping. For comparison, a similar score was generated for the mice using urinary values obtained before AA treatment. Mice were genotyped using the medium-density SNP array from Illumina. In this array, 885 SNPs proved informative. Complete phenotype data were obtained for 191 mice, and all were successfully genotyped for >99% of the SNPs. The genotyping accuracy was confirmed by regenotyping the complete set of F2 mice with eight microsatellite markers on chromosome 2 (data not shown).

The genotypes of each mouse and phenotypic values were analyzed with the QTL package of programs (10) in the R statistical computing environment. The genetic map of the SNP markers for each chromosome was compared with the mouse genome, Build 37.1, to rule out any errors. To detect main effect QTLs, a one-dimensional scan of the genome was conducted using the program Scanone with Haley-Knott regression (28). To determine significance cutoff levels, 1,000 permutations of the data were generated and analyzed. Only a single main effect QTL on chromosome 4 was detected using the composite score, as shown in Fig. 3A. Chromosome 4 also contains a main effect QTL when just the albumin or NAG values are used (Fig. 3, B and C). No significant main effect QTL was detected using glucose values alone (not shown). Figure 3D shows that the same location on chromosome 4 houses the QTL found using the AA-induced proximal tubule dysfunction/injury score as the AA-induced albuminuria and NAG QTLs, implying that all three analyses detected the same locus. We have designated this QTL Aanq1, for *aristolochic acid nephrotoxicity 1*.

To screen for interacting QTLs, we used the function Scantwo using the composite score as a phenotype. Once again, significance thresholds were established with 1,000 permutations of the data. Three additional QTL were revealed (Table 2) on chromosomes 3 (Aanq2), 9 (Aanq3), and 6 (Aanq4). Aanq2 and Aanq3 interact. The DBA allele of Aanq3

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**Table 1. Phenotypic values for mice in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Albumin, μg/mg Cr</th>
<th>Glucose, mg/mg Cr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0 n</td>
<td>Day 4 n</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>64.7 ± 8.9†</td>
<td>72.4 ± 10.8†</td>
</tr>
<tr>
<td>DBA/2</td>
<td>101.3 ± 2.7‡</td>
<td>175.1 ± 9.2‡</td>
</tr>
<tr>
<td>B6D2F1</td>
<td>86.9 ± 10.4‡</td>
<td>125.3 ± 9.9‡</td>
</tr>
<tr>
<td>B6D2F2</td>
<td>108.3 ± 2.7‡</td>
<td>188.3 ± 6.3‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. Each value is normalized to urinary creatinine (Cr). NAG, N-acetyl-β-D-glucosaminidase. *P < 0.05 vs. C57BL/6J. †P < 0.05 vs. DBA/2J. ‡P < 0.05 vs. day 0 value.

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**Fig. 2.** Distribution of AA-induced albuminuria (A), glucosuria (B), and urinary NAG activity (C) in 200 F2 mice 4 days after treatment with 2 mg/kg AAI. Each parameter was normalized to creatinine concentration.
increases the phenotype in a recessive manner, but this effect is decreased by a C57BL/6J allele at Aanq2. The Aanq4 locus interacts additively with Aanq1. A model incorporating these interactions explains 39% of the variance in the phenotype. Aanq1 accounted for 15.1% of the variance, the additive interaction of Aanq1 and Aanq4 accounted for 5.8%, and the interaction of Aanq2 and Aanq3 accounted for 18.2%. Further attempts to detect more QTLs by adding all potential QTLs individually, or in pairs, to this model did not detect any other significant interactions.

Baseline urinalysis QTLs. Since untreated DBA/2J, C57BL/6J, and F1 mice exhibit significant differences in urinary NAG and albumin levels (Table 1), a composite score using the urinary parameters from the untreated F2 mice was compiled and used for QTL mapping as described previously for the posttreatment urine values. A single significant QTL was detected (Fig. 4A) that localized to the same region as Aanq1 on chromosome 4 (Fig. 4B). However, unlike the AA-treated mice, a two-dimensional search using the untreated mouse data did not reveal any interacting QTLs.

The baseline urine parameter QTL and Aanq1 may be the same locus. However, as shown in Fig. 5, the copy number effect of the DBA/2J allele is different for baseline kidney function and AA-induced toxicity. This DBA/2J chromosome 4 region is additive for the baseline urine parameter score but is fully dominant for AA-induced toxicity.

Finally, to test whether the Aanq1 QTL is present in other AA-sensitive strains, the ability of AA to induce tubular proteinuria or injury was compared between C57BL/6J mice and mice consomic for the A/J chromosome 4 on the C57BL/6J genetic background. Indeed, after AA treatment, mice carrying the A/J chromosome 4 had 25% more urinary albumin than mice with the C57BL/6J chromosome 4 (P < 0.01, n = 4). Similarly, the A/J Chr4 mice had 31% more urinary NAG.

Table 2. AAN QTL in (B6xD2)F2 mice

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Name</th>
<th>Chromosome</th>
<th>Peak,* MB</th>
<th>CI,† MB</th>
<th>High Allele</th>
<th>Gene Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAI-induced nephropathy</td>
<td>Aanq1</td>
<td>4</td>
<td>63.3</td>
<td>56.5–70.7</td>
<td>DBA</td>
<td>Dominant</td>
</tr>
<tr>
<td></td>
<td>Aanq2</td>
<td>3</td>
<td>121.3</td>
<td>116.2–126.1</td>
<td>DBA</td>
<td>Additive‡</td>
</tr>
<tr>
<td></td>
<td>Aanq3</td>
<td>9</td>
<td>118</td>
<td>113.2–121.8</td>
<td>DBA</td>
<td>Recessive‡</td>
</tr>
<tr>
<td></td>
<td>Aanq4</td>
<td>6</td>
<td>127.9</td>
<td>125–134.2</td>
<td>DBA</td>
<td>Dominant§</td>
</tr>
<tr>
<td>Baseline renal function</td>
<td>Aanq1</td>
<td>4</td>
<td>62.4</td>
<td>46.2–71.8</td>
<td>DBA</td>
<td>Additive</td>
</tr>
</tbody>
</table>

AAN, aristolochic acid nephropathy; QTL, quantitative trait loci. A DBA allele at Aanq4 increases the effect of Aanq1. Aanq4 does not interact significantly with Aanq1 for baseline renal function. *Chromosomal position (in megabases) from Build 37.1 of the C57BL/6J genome. †Confidence interval (in megabases) determined by a 1 logarithm of the odds (LOD) drop support interval. ‡Aanq2 and Aanq3 interact, heterozygosity at Aanq2 decreases the effect of a DBA allele at Aanq3. §Aanq4 and Aanq1 interact for AAI-induced nephropathy.
activity than C57BL/6J mice after AA treatment (P < 0.05, n = 4). Because, in isolation, the Aanq1 locus accounts for 15% of the AA response, a dose of 5 mg/kg was used to elicit a large enough response to detect a significant difference in a small set of mice. At this dose, the peak urine concentration of albumin after AA treatment was 8.2 mg/mg creatinine for the A/J mice and 6.1 mg/mg creatinine for C57BL/6J mice. Although this result is potentially confounded by the higher dose of AA used to elicit a significant response, and/or by the presence of the entire A/J chromosome 4 in the consomic mice, it appears that at least one chromosome 4 locus, probably Aanq1, is responsible for AA susceptibility in A/J mice.

DISCUSSION

Four QTLs that influence the response to AA in the mouse kidney have been identified. A single main effect QTL, Aanq1, was detected. The Aanq1 region also harbors a QTL that may influence normal proximal tubule function or injury as reflected in baseline differences between DBA/2J and C57BL/6J mice.

The possibility exists that Aanq1 and the baseline tubule function QTL are the same. The loci differ in their copy number effect, however. A single DBA/2J Aanq1 allele conferred sensitivity to AAI, while, for baseline function, the gene effect is additive. It is possible this difference is due to the effect of the Aanq4 locus, which interacts with Aanq1 to increase AAI-induced toxicity but has no effect on normal function.

The Aanq1 region on proximal chromosome 4 has been implicated in impaired renal function in other studies. A QTL, Alby6, that contributes to differences in microalbuminuria between these strains, maps also to proximal chromosome 4 (51). In addition, three separate studies that mapped modifiers in polycystic kidney disease mutations in the genes pcy (68), jck (33), and cpk (43) detected modifier loci on proximal chromosome 4. Each of these studies involved either the DBA/2 or C57BL/6J strain or both strains (33). At this time, we cannot say whether these loci are the same as Aanq1. Typically, polycystic kidney disorders affect distal tubule segments, while AA affects primarily proximal tubules. A kinesin-encoding gene in this region, kif12, has been proposed to be the modifier of cpk, a congenital polycystic kidney disease gene (43). There is a unique five-amino acid deletion in the C57BL/6J KIF-12 protein, and the expression of KIF12 is restricted to renal tubules (43). However, results of sequencing studies (not shown) established that the Kif-12 genes in AA-resistant (Cast/EiJ and B6BTBR^tff/J) and AA-sensitive strains (DBA/2J, C3H/HeJ, A/J) lack this deletion.

Recently, albuminuria in the SM/J mouse strain has been mapped to a QTL on chromosome 4, and the Tlr12 gene has been proposed to be the causative locus (27). Although Tlr12 maps distal to the Aanq1 confidence region, a related gene, Tlr4, that has been implicated in modulating tubular atrophy and interstitial fibrosis after injury (47) does map within the Aanq1 region. However, the Tlr4 gene lacks coding differences between C57BL/6J and DBA/2J mice. Also, a search of the
WebQTL (67) microarray database that includes kidney and liver gene expression comparisons between the C57BL/6J and DBA/2J strains, as well as several recombinant inbred strains, failed to reveal a significant difference in expression.

Other candidate genes in the Aanq1 region include several genes encoding for lipocalins, a family of small (~20–40 kDa) proteins that bind and transport hydrophobic molecules. Lipocalin genes in this region include the major urinary protein (MUPs) gene family (37) and orosomucoids (ORM1,2,3) (13). Humans lack a functional MUP gene. However, the mouse Mup gene family is large with many pseudogenes and strain differences in expression (44). Expression differences in Mup genes between the strains C57BL/6J and DBA/2J have not been exhaustively analyzed. Among the various Mup genes, the Mup5 locus differs at one amino acid between the strains. The chicken ORM protein has been shown to bind AA (72), but the mouse or human ORM proteins have, to date, not been tested for AA binding. A search of SNP differences between the DBA/2J and C57BL/6J strains in the WebQTL database (67) and the Jackson Laboratory informatics databases did reveal coding differences in the Orm genes between the strains.

Another lipocalin gene of note in the Aanq1 region is the AMBP gene. The AMBP gene encodes two distinct proteins, the lipocalin α1-microglobulin (A1M) (3) and the urinary trypsin inhibitor bikunin (24). Although we have not found any coding differences or RNA expression differences in the AMBP gene between the C57BL/6J and DBA/2J strains, the AMBP gene is noteworthy because tubular low-molecular-weight proteinuria, in particular, urinary A1M, is a hallmark of BEN, an environmental disease resulting from chronic dietary exposure to AA (26). The elevation of urinary A1M has been proposed as a specific biomarker of early stage BEN (17, 22, 55). Elevated urinary A1M is believed to be symptomatic of proximal tubule dysfunction. However, diagnoses relying on A1M measurements should be reconsidered in future testing of this candidate chromosomal region for association with BEN.

Biotransformation systems are important in the detoxification and elimination of xenobiotics. C57BL/6J mice exhibit enhanced induction of many antixenobiotic activities relative to DBA/2J mice due to differences in the arylhydrocarbon receptor (23, 25, 63). However, the Ahr gene, which encodes this receptor, is on chromosome 12 in the mouse genome and not included in the QTL regions mapped here. Similarly, no genes known to produce enzymes that are implicated in the metabolism or activation of AA map in the QTL regions. These include NAD(P)H dehydrogenase, quinone 1 (Nqo1) (58), prostaglandin-endoperoxide synthase 2 (Ptgs2) (56), CYP1A1 and 2 (Cyp1a1, Cyp1a2) (50, 57), or Cyp3a5 (6). Thus differences in metabolic activity by the products of these genes may not result in differential sensitivity of C57BL/6J and DBA/2J mice.

The human chromosomal region of synteny orthologous to the Aanq1 region is Chr9p31–33. An autosomal dominant hypertensive nephropathy, HNP1, described in a four-generation African American pedigree, has been mapped to 9q31–32 (12). We did not measure hypertension in this study, but prior studies have described hypertension QTLs Bbg3 and Bbg10, which are concordant with Aanq1 (9, 62). The Bbg3 locus was mapped in a cross between A/J mice, an AA-sensitive strain, and C57BL/6J mice (62). The Bbg10 locus was mapped in a cross between DBA/2J and C57BL/6J mice, and the authors proposed that Bbg3 and Bbg10 shared the same locus (9). The potential role of hypertension as a risk factor in BEN, an environmental disease resulting from chronic dietary exposure to AA (26), has been postulated since hypertension is prevalent in the rural Balkans (4) and within families affected by BEN (4, 21). However, the connection of hypertension and BEN has not been confirmed (55).

A search of human genetic nephropathies in OMIM (1) did not find any candidate genes or phenotypes that map to positions orthologous to Aanq2, Aanq3, or Aanq4, i.e., those limited to the kidney and characterized by tubular atrophy and interstitial fibrosis. Additionally, the human orthologs have not been detected in recent genome-wide association studies designed to detect loci affecting kidney function (32, 46). This is not surprising as Aanq2–4 were detected as interacting, not main, QTLs. This level of analysis usually is not possible in GWAS population studies or family pedigree analysis.

In the United States, the prevalence of early-stage chronic kidney disease is estimated to be 5% in individuals over 20 yr old (14, 36). Our results indicate that a major genetic determinant of AA sensitivity in the mouse may also influence baseline kidney function. Among humans, it is estimated that only a small fraction (~5%) are highly sensitive to AA (18); thus, AAN is likely to result from an intersection of exposure and genetic sensitivity. Possibly, monitoring of renal function may identify susceptible individuals living in endemic regions of Balkan countries and in other countries where the use of herbal medicines incorporating Aristolochia are prevalent (34).

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES
1. Online Mendelian Inheritance in Man, OMIM (TM): McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD).
F1366  QTL FOR ARISTOLOCHIC ACID NEPHROTOXICITY


