Developmental hyperbilirubinemia and CNS toxicity in mice humanized with the *UDP glucuronosyltransferase 1* (*UGT1*) locus

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High levels of unconjugated bilirubin (UCB) in newborn children is associated with a reduction in hepatic UDP glucuronosyltransferase (UGT) 1A1 activity that can lead to CNS toxicity, brain damage, and even death. Little is known regarding those events that lead to UCB accumulation in brain tissue, and therefore, we sought to duplicate this condition in mice. The human *UGT1* locus, encoding all 9-*UGT1A* genes including *UGT1A1*, was expressed in *Ugt1*$^{-/-}$ mice. Because the most common clinical condition associated with jaundice in adults is Gilbert's syndrome, which is characterized by an allelic polymorphism in the *UGT1A1* promoter, hyperbilirubinemia was monitored in humanized *UGT1* mice that expressed either the Gilbert's *UGT1A1*28* allele [*Tg(UGT1*^{A1*28}*)Ugt1*$^{-/-}$ mice] or the normal *UGT1A1*1* allele [*Tg (UGT1*^{A1*1}*)Ugt1*$^{-/-}$ mice]. Adult *Tg(UGT1*^{A1*28}*)Ugt1*$^{-/-}$ mice expressed elevated levels of total bilirubin (TB) compared with *Tg(UGT1*^{A1*1}*) Ugt1*$^{-/-}$ mice, confirming that the promoter polymorphism associated with the *UGT1A1*28* allele contributes to hyperbilirubinemia in mice. However, TB accumulated to near toxic levels during neonatal development, a finding that is independent of the Gilbert's *UGT1A1*28* promoter polymorphism. Whereas serum TB levels eventually returned to adult levels, TB clearance in neonatal mice was not associated with hepatic UGT1A1 expression. In ~10% of the humanized *UGT1* mice, peak TB levels culminated in seizures followed by death. UCB deposition in brain tissue and the ensuing seizures were associated with developmental milestones and can be prevented by enhancing regulation of the *UGT1A1* gene in neonatal mice.

Ugt1 knockout | kernicterus | bilirubin | *UGT1A1*28* | Gilbert's Syndrome

UDP-glucuronosyltransferase 1A1 (UGT1A1) is one of nine transferases encoded on the *UGT1* locus. Serum unconjugated bilirubin (UCB) requires glucuronidation by hepatic UGT1A1 before biliary secretion of the glucuronide can take place (1). Hyperbilirubinemia or jaundice is a common malady in healthy newborn children and results from inadequate expression of hepatic UGT1A1, showing that developmental regulation of the *UGT1A1* gene is an important process that is needed to achieve normal levels of serum total bilirubin (TB). Although the outcome of neonatal hyperbilirubinemia is usually benign, infants with extremely high and untreated levels of TB can develop signs of acute bilirubin encephalopathy (2). In preterm infants, bilirubin toxicity remains a significant problem, regardless of the advances that have been made in treating severe forms of hyperbilirubinemia. A recent report by the March of Dimes titled "White Paper on Preterm Birth" estimated that nearly 13 million children are born prematurely each year, resulting in more than 4 million deaths. If severe hyperbilirubinemia is not treated in the early stages of neonatal development, as often seen in preterm births, the condition can lead to kernicterus, which is severe neurologic damage resulting from bilirubin toxicity (3). Developmentally induced bilirubin toxicity leads to a paradigm of toxic responses in newborns that becomes clinically apparent as lethargy, ophthalmoplegia (ocular muscle paralysis), high-pitch crying, opisthotonus (bowed body and rigid extremities or dystonia), and seizures as well as mental retardation, long-term physical impairment, and often death (2, 4). In addition, severe hyperbilirubinemia can be brought on by infection, ischemia, biliary obstruction, and breastfeeding with inadequate intake as well as a metabolic deficiency in glucose-6-phosphate dehydrogenase (G6PD) activity (5, 6). Among the most severe of the genetic deficiencies, Crigler–Najjar type 1 (CN1) disease is characterized by a complete inactivation of UGT1A1-dependent bilirubin glucuronidation activity (7). The complete loss of UGT1A1 activity in CN1 is often fatal, resulting from the progression of hyperbilirubinemia to CNS toxicity (8).

Gilbert's syndrome is the most common inheritable condition resulting in transient unconjugated hyperbilirubinemia (9, 10); it is considered benign, because it does not lead to chronic hepatic destruction. The cause of Gilbert's syndrome results from the nucleotide TA insertion into the TATA box-like sequence of the promoter region of the *UGT1A1* gene (11), which generates $A(TA)_7$ TAA (*UGT1A1*28* allele) instead of the normal $A(TA)_6$ TAA sequence (*UGT1A1*1* allele). In adults, the *UGT1A1*28* promoter variant leads to a reduction in transcriptional activity and bilirubin glucuronidation (11). Recent findings have confirmed that the nuclear protein binding complex that associates with the TATA box-like sequence binds at a weaker affinity to the $A(TA)_7$TAA sequence than to the normal $A(TA)_6$TAA sequence (12). Individuals that inherit the Gilbert's phenotype are predisposed to potential drug-induced toxicities resulting from inadequate glucuronidation of therapeutic agents by hepatic UGT1A1 (9, 13).

Recently, we modeled an inactivating mutation in exon 4 of the murine *Ugt1* locus (14). *Ugt1*$^{-/-}$ mice developed severe hyperbilirubinemia within hours of birth. Although newborn *Ugt1*$^{-/-}$ mice originally seemed normal, this mutation led to death within 1 week. We predicted that the fatal outcome of hyperbilirubinemia in *Ugt1*$^{-/-}$ mice mimicked the irreversible CNS damage that occurs in diseases such as CN1, which culminate in UCB-initiated encephalopathy that is linked to the kernicterus sequelae. Development of *Ugt1*$^{-/-}$ mice served as a unique animal model to humanize these mice with the human *UGT1* locus. In this report, we examine the contribution of the Gilbert's *UGT1A1*28* and the normal *UGT1A1*1* alleles to hyperbilirubinemia in neonatal and adult mice. The generation of humanized *UGT1* mice that express either the *UGT1A1*28* or *UGT1A1*1* allele were investigated to examine the clearance of UCB during the neonatal period and the resulting CNS abnormalities from UCB-induced toxicities.

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Results

Humanized *UGT1* Mice Recover from Developmental Lethality Observed in *Ugt1* Null Mice. Inactivation of the murine *Ugt1* locus leads to neonatal lethality ~7 days after birth (14). The $Ugt1^{-/-}$ mice exhibit a 50- to 60-fold increase in UCB that exceeds 12–13 mg/dL within several days after birth (Fig. 1). Severe hyperbilirubinemia leads to a metabolic syndrome that ultimately results in early neonatal death (14). As UCB accumulates in $Ugt1^{-/-}$ mice, it deposits in brain tissue, which is shown by intense yellow staining in this tissue (Fig. 2*B*). Healthy littermates do not accumulate UCB (Fig. 2*A*). This abnormal accumulation of UCB can be classified as kernicterus.

In humans, the most common genetic form of hyperbilirubinemia results from inheritance of the Gilbert's *UGT1A1*28* allele, which leads to mild elevations in UCB in adults (9). When BAC clones that encoded the *UGT1* locus, including either the *UGT1A1*28* or *UGT1A1*1* allele, were expressed in the $Ugt1^{-/-}$ background, expression of the *UGT1A1* gene rescued the early neonatal lethality observed in $Ugt1^{-/-}$ mice. Adult $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice were mildly hyperbilirubinemic with levels of serum TB that ranged between 0.8 and 1.2 mg/dL compared with undetectable levels in $Tg(UGT1^{A1*1})Ugt1^{-/-}$ and wild-type mice. In Gilbert's syndrome, the promoter variant linked to the *UGT1A1*28* gene is speculated to lead to reduced synthesis of UGT1A1 RNA and protein in hepatic tissue (11), resulting in inadequate UCB glucuronidation and mild hyperbilirubinemia. Using tissues from adult $Tg(UGT1^{A1*28})Ugt1^{-/-}$ and $Tg(UGT1^{A1*1})Ugt1^{-/-}$ mice, gene-expression patterns were determined for the human *UGT1* locus by RT-PCR analysis (Fig. 3). Examining the expression patterns from liver tissue, similar RNA levels were noted for liver-specific expression of UGT1A3, -1A4, -1A6, and -1A9. In contrast, there was more than a 7-fold increase in UGT1A1 RNA expression in $Tg(UGT1^{A1*1})Ugt1^{-/-}$ mice as determined by quantitative (Q) RT-PCR (Fig. 3*A*), indicating that the promoter polymorphism associated with the *UGT1A1*28* allele is linked to reduced expression in $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice. Interestingly, the expression levels of UGT1A1 in extrahepatic tissues, such as the small intestine, are very similar in $Tg(UGT1^{A1*28})Ugt1^{-/-}$ and $Tg(UGT1^{A1*1})Ugt1^{-/-}$ mice (Fig. 3*B*), showing that the promoter polymorphism dictates altered expression of UGT1A1 only in hepatic tissue. The reduced levels of UGT1A1 protein in liver from $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice correlated with lower expression levels of UGT1A1 in human liver microsomes prepared from individuals that were genotyped to express the *UGT1A1*28* gene (Fig. 3*C*). The differences in UGT1A1 protein expression in $Tg(UGT1^{A1*1})Ugt1^{-/-}$ and $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice and the corresponding serum TB values represent a unique example of an altered phenotype associated with a human polymorphism that can be accurately reproduced in animals through expression of the allelic variants.

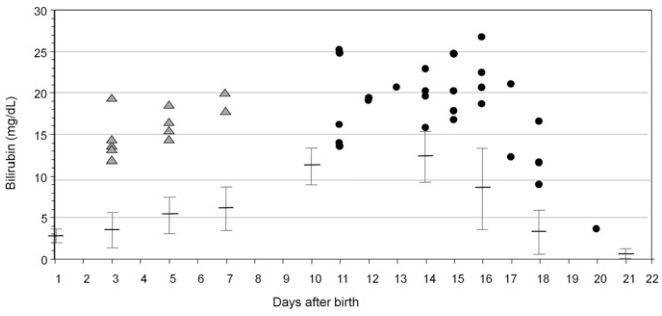

Fig. 1. Serum TB levels in developing *Ugt1* null mice and humanized *UGT1* mice. At different days after birth, serum TB levels were measured from $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice. Triangles represent measurements taken from $Ugt1^{-/-}$ mice. The circles are measurements taken from mice that showed signs of bilirubin toxicity as evident by seizure patterns (Movies S1, S2, S3, and S4). Normal ranges for TB levels in $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice are shown at different ages (n = 30–50 mice).

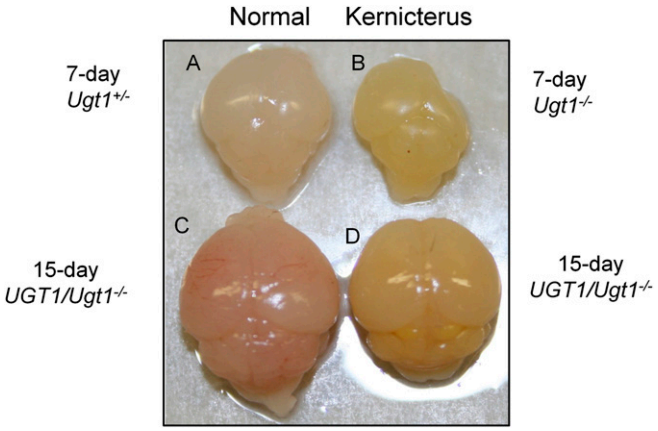

Fig. 2. Bilirubin accumulation in the brain resulted from hyperbilirubinemia. The brain from a 7-day-old $Ugt1^{+/-}$ heterozygous mouse (*A*) is shown compared with a $Ugt1^{-/-}$ littermate (*B*). Also displayed are a brain from a 15-day-old $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mouse that showed no behavioral phenotype (*C*) and a brain from a mouse that was developing seizures (*D*; Movies S1, S2, S3, and S4).

Neonatal Hyperbilirubinemia and UGT1A1 Expression in the Gastrointestinal Track. Hyperbilirubinemia in humans is more pronounced in neonatal children than in adults and is associated with a lag in expression of UGT1A1. Within 8 hours of birth, serum TB levels increased to 2.5–3.0 mg/dL (Fig. 1) in both $Tg(UGT1^{A1*1})Ugt1^{-/-}$ and $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice. This increase in TB continued through 14 days, reaching values that sometimes exceeded 15 mg/dL. Remarkably, between 14 and 21 days of birth, the TB values declined rapidly to adult levels. During the neonatal developmental

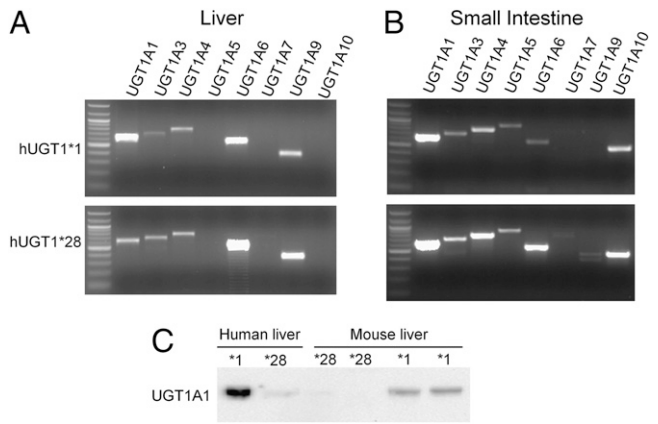

Fig. 3. UGT1A gene and protein expression patterns in livers and small intestine from humanized UGT1 mice. (*A*) Liver RNA from humanized $Tg(UGT1^{A1*1})Ugt1^{-/-}$ and $Tg(UGT1^{A1*28})Ugt1^{-/-}$ liver was used in RT-PCR studies, and the isoform-specific patterns were identified in ethidium bromide-stained agarose gels. (*B*) Comparative values of *UGT1A* gene expression by RT-PCR using small-intestine RNA from $Tg(UGT1^{A1*1})Ugt1^{-/-}$ and $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice are shown. (*C*) The panel shows an immunoblot of microsomal protein from a pool of human liver microsomes (5 μg/lane) that were genotyped as either *UGT1A1*1* (*1) or *UGT1A1*28* (*28) along with liver microsomes (20 μg/lane) from $Tg(UGT1^{A1*1})Ugt1^{-/-}$ (*1) and $Tg(UGT1^{A1*28})Ugt1^{-/-}$ (*28) mice. Detection of human UGT1A1 was identified using a specific human anti-UGT1A1 antibody.

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period, levels of TB accumulation were found to be similar in male and female *Tg(UGT1^{A1*1})Ugt1$^{-/-}$* and *Tg(UGT1^{A1*28})Ugt1$^{-/-}$* mice, indicating that neonatal hyperbilirubinemia in humanized mice is not linked to the *UGT1A1*28* promoter mutation. Because expression of the human *UGT1A1* gene rescues *Ugt1$^{-/-}$* neonatal lethality, these studies confirmed that the human *UGT1A1* gene plays a key role in the early developmental stages of bilirubin metabolism and is an essential target gene for regulation during late neonatal development.

During the neonatal period, analysis of UGT1A1 expression in liver tissue by Western blot showed no detectable protein (Fig. 4*A*). It seemed that hepatic *UGT1A1* gene expression in neonates did not correlate with the clearance of TB, because there was a sharp reduction in TB after 14 days of birth that was not mirrored with increases in hepatic UGT1A1. However, when we examined extrahepatic tissues, there was a dramatic developmental increase in *UGT1A1* gene expression and protein between 14 and 21 days in the small intestine (Fig. 4). The increases in small intestine UGT1A1 were concordant with the reductions observed in serum TB (Fig. 1) during the same developmental period. The changes in small-intestine UGT1A1 expression were independent of the promoter genotype, because comparable levels of gene expression in this tissue were noted in both *Tg(UGT1^{A1*1})Ugt1$^{-/-}$* and *Tg(UGT1^{A1*28})Ugt1$^{-/-}$* mice during development (Fig. 4*B*). These findings indicated that UGT1A1-dependent intestinal glucuronidation may play an important role in TB clearance during the neonatal stages in humanized *UGT1* mice.

Developmental Onset of Kernicterus and Seizures in Humanized *UGT1* Mice. During neonatal development, ~10% of the humanized *UGT1* mice exhibited exaggerated levels of TB that exceeded those of their littermates by 20–30% and peaks in levels at ~14 days after birth (Fig. 1, closed circles). Those mice with severely elevated TB levels developed CNS toxicity that was displayed by a characteristic seizure pattern. As toxicity developed, their gait became initially awkward, because the mice appeared to suffer from a balance problem (Movie S1). This was followed by seizures (Movie S2), evident by exhaustive and uncontrollable running. This seizure pattern was also induced by a simple startle reflex as shown in Movie S3. The final stage of the seizure paradigm was dystonia, which was represented by stiffness of the limbs (Movie S4). The mice often regressed back to the seizure paradigm after what appeared to be a resting phase in dystonia. This process lasted up to 24 hours but always results in death.

Humanized *UGT1* mice that developed normally showed no signs of brain toxicity (Fig. 2*C*), even at 14 days when we observed the highest concentrations of serum TB. However, humanized *UGT1* mice that developed seizures all showed intense yellow staining in brain tissue (Fig. 2*D*), similar to the patterns observed in *Ugt1$^{-/-}$* mice. There were also developmental milestones that played an important role in providing resistance to the potentially harmful accumulation of serum UCB. Healthy humanized *UGT1* mice accumulated TB levels by day 14 that were comparable in serum concentration with those values achieved in only 5–7 days in *Ugt1$^{-/-}$* mice (Fig. 1). Unlike humanized *UGT1* mice, the early accumulation of UCB in *Ugt1$^{-/-}$* mice led to the development of kernicterus (Fig. 2*B*). Thus, expression of the *UGT1* locus in humanized mice delayed the accumulation of TB. Because the serum levels of TB in *Ugt1$^{-/-}$* mice approximated the levels in healthy humanized *UGT1* mice at 14 days, it seems that early brain development is highly sensitive to increasing concentrations of UCB, which was observed in *Ugt1$^{-/-}$* mice.

To determine whether increasing concentrations of serum TB negatively impact brain development, we treated *Tg(UGT1^{A1*28})Ugt1$^{-/-}$* mice at different ages with phenylhydrazine (PHZ), a compound that induces hemolysis and spontaneously increases serum TB levels (Fig. 5). *Tg(UGT1^{A1*28})Ugt1$^{-/-}$* mice at 7, 11, 13, and 15 days of age were challenged by an i.p. injection of PHZ (10 or 20 mg/kg), and serum TB levels were measured each day after treatment (Fig. 5). At each age group, PHZ treatment dramatically induced TB levels well above the average serum levels for neonatal *Tg(UGT1^{A1*28})Ugt1$^{-/-}$* mice. For the youngest age group treated, 100% of the mice developed seizures and died. When 11-day-old mice were treated with PHZ, 25% of those mice survived, indicating that they start to develop resistance to UCB-induced brain injury as they mature during development. At 13 days of age, PHZ treatment was lethal in only 25% of the mice, whereas at 15 days of age, only 1 of 9 treated mice died. In wild-type mice, the treatment

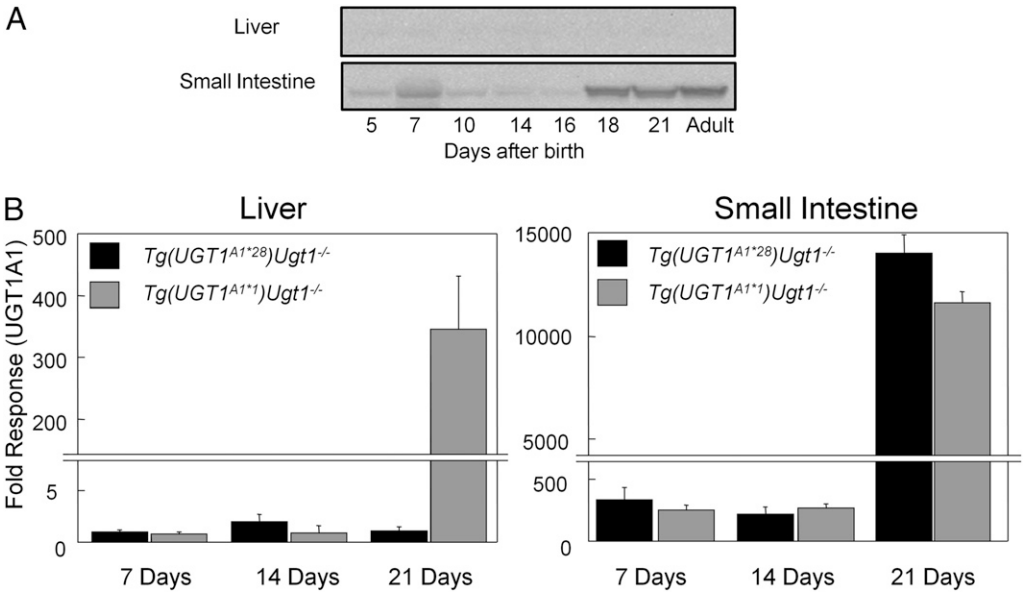

Fig. 4. Expression of UGT1A1 in neonatal liver and small-intestine tissues from humanized UGT1 mice. (*A*) An immunoblot of UGT1A1 expression in liver and small intestine from *Tg(UGT1^{A1*28})Ugt1$^{-/-}$* mice during the neonatal period is shown. (*B*) *UGT1A1* gene-expression patterns in the liver and small intestine from *Tg(UGT1^{A1*1})Ugt1$^{-/-}$* and *Tg(UGT1^{A1*28})Ugt1$^{-/-}$* mice are quantitated by Q-RT-PCR at 7, 14, and 21 days after birth. The response is based on Ct values that are normalized to liver expression at 7 days. The values at 7 days are set to a 1-fold response.

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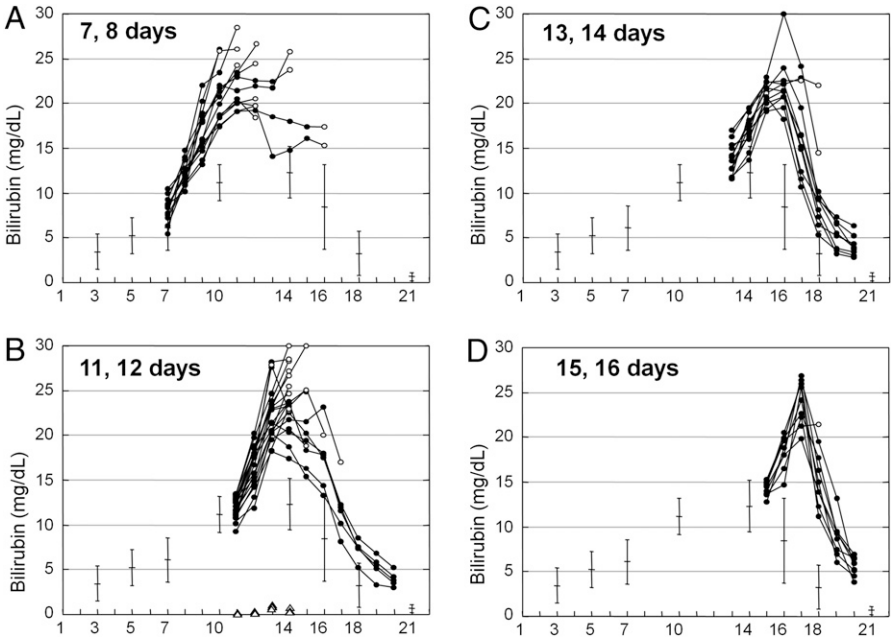

Fig. 5. Effects of phenylhydrazine treatment on serum bilirubin levels in neonatal humanized mice. Neonatal humanized *UGT1* mice were administered PHZ by the i.p. route at 7 and 8 days (10 mg/kg; *A*), 11 and 12 days (10 mg/kg; *B*), 13 and 14 days (10 mg/kg; *C*), or 15 and 16 days (20 mg/kg; *D*). At 11 and 12 days of age, PHZ was also administered to wild-type mice (triangles). UCB levels were determined before the first injection and were followed by analysis every 24 h. In comparison, the escalating TB values for each treated mouse are shown by the closed circles. Open circles represent values taken from mice that progressed into seizures and died. The number of mice that escaped bilirubin-induced toxicity in each treatment group is shown. For example, in *A*, 13 humanized *UGT1* mice were challenged with PHZ when they were 7 days old, and all of the mice eventually developed seizures resulting from the formation of kernicterus.

of neonatal mice with PHZ also increased the serum levels of TB but at levels that had no impact on initiating seizures (Fig. 5*B*). These results indicate that the development of severe hyperbilirubinemia (>15 mg/dL) is a prelude to CNS damage within the first 2 weeks of life, leading us to speculate that brain tissue matures and is capable of combating the impact of elevating serum TB in the later stages of neonatal development.

Regulation of UGT1A1 During Neonatal Development Leads to a Reduction in Serum TB. The human *UGT1A1* gene is regulated by various nuclear receptors, including the aryl hydrocarbon receptor (AhR) (15). In attempts to regulate neonatal UGT1A1 expression, lactating $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice were administered an inducing dose of 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) 1 day after birth. Serum TB levels were monitored in the neonatal mice at 14 days after birth (Fig. 6), and it was shown that TCDD treatment led to a decline in TB levels. Murine *Cyp1a1* gene expression, which is regulated in response to activation of the AhR, was induced in the treated group. Maternal TCDD treatment led to the induction of the neonatal *UGT1A1* gene and protein expression in the liver and small intestine. Thus, regulation of the *UGT1A1* gene during early neonatal development can efficiently lower TB levels. Because these humanized mice expressed the *UGT1A1*28* allele, induction of UGT1A1 during the neonatal period, which resulted in lowering of serum TB levels, was not influenced by the *UGT1A1* promoter polymorphism.

Discussion

Hyperbilirubinemia is most prevalent in children that are born prematurely, which is defined as birth before 37 weeks of gestation (40 weeks being the normal gestation period). If left unattended or if the condition worsens, the abnormally high levels of UCB can

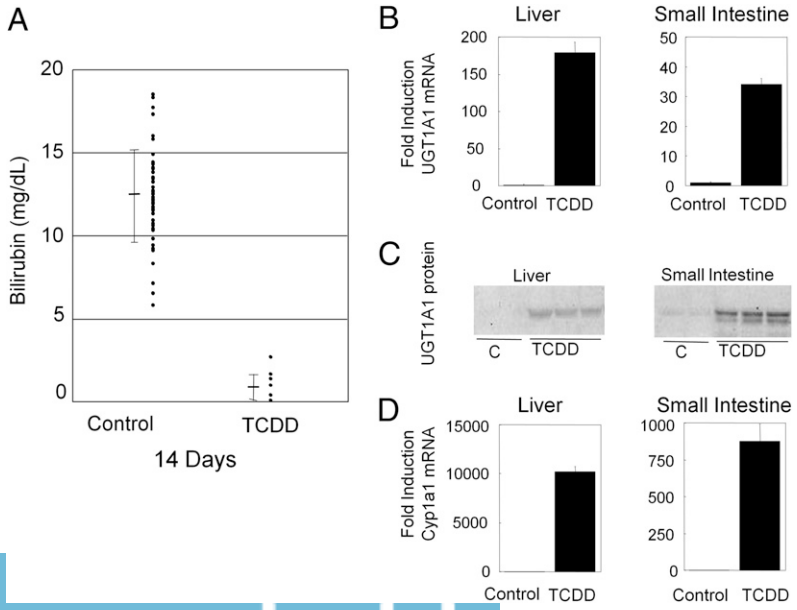

Fig. 6. TCDD exposure through lactational treatment and the clearance of serum bilirubin. Maternal humanized *UGT1* mice were injected by the i.p. route with 16 μg/kg TCDD shortly after giving birth. Neonatal mice were allowed to nurse freely in the cages until 14 days of age. (*A*) Blood TB values at 14 days are shown for the nursing mice. In comparison, the normal UCB range values recorded from our panel of untreated humanized *UGT1* neonatal mice at 14 days of age are shown. (*B*) cDNA was synthesized from RNA collected from livers and small intestine from unexposed and TCDD-exposed 14-day-old mice. It was used for analysis of RNA content by Q-RT-PCR. The expression level of UGT1A1 was normalized by mouse CPH. (*C*) Expression of UGT1A1 in the liver and small intestine from microsomal preparations taken from untreated and TCDD-exposed mice was determined by Western blot analysis using an anti-human UGT1A1 antibody. (*D*) RNA from livers and small intestine was used in Q-RT-PCR experiments to measure *Cyp1a1* gene expression.

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lead to irreversible brain damage. The detoxification of UCB from the blood requires transport into liver hepatocytes followed by UGT1A1-dependent glucuronidation (16), which is proceeded by transport of the glucuronide through the apical surface of the hepatocytes into the biliary canaliculi for deposition into the gastrointestinal tract (1, 16–20). The rate-limiting step in UCB clearance is efficient UGT1A1-dependent glucuronidation, an enzymatic process that is often not fully matured in newborns and dependent on developmental factors that are not currently understood. Since the *UGT1A* genes are regulated as a function of development, humanized *UGT1* mice were employed to examine the influence of neonatal development on human UGT1A1 expression and the impact of tissue-specific control on the status of hyperbilirubinemia. In addition, humanized *UGT1* mice provide the unique opportunity to investigate the underlying mechanisms that might lead to CNS damage and eventually, the development of kernicterus.

When UCB exceeds its binding capacity to serum proteins, it is displaced and accumulates in tissues like the brain (21). This irreversible accumulation of UCB is evident from intense and diffuse yellow staining of the brain tissue. In humans, kernicterus is defined by selective yellow staining of discrete brain regions (22), and the blood–brain barrier plays a crucial role in protection from elevated levels of UCB. In $Ugt1^{-/-}$ mice, UCB accumulates rapidly and reaches peak levels within 5 days. Although UCB is accumulating at a slower rate in humanized *UGT1* mice, the mice ultimately develop seizures and are predisposed to the kernicterus sequelae if TB exceeds 15 mg/dL before 14 days of age. We speculate that the diffuse deposition of UCB in brain tissue from $Ugt1^{-/-}$ and humanized *UGT1* mice results from a combination of excessive albumin-free UCB and premature development of the blood–brain barrier. Escalating the TB levels in humanized *UGT1* mice after exposure to PHZ confirmed that bilirubin-induced toxicities were refractory in mice after 14 days of age. Based on the age of the animal, our findings indicate that TB levels can be a predictor of kernicterus. Serum levels found to exceed 8–10 mg/dL in the first week after birth (as in $Ugt1^{-/-}$ mice) or those that exceed 15 mg/dL by the second week of age (as in humanized *UGT1* mice) are positive indicators that brain toxicity will develop. Although it has been a challenge to predict the onset of neurologic dysfunction in humans based simply on serum UCB levels (21, 22), the humanized *UGT1* mice provide an opportunity to examine the direct impact of elevated UCB levels on the onset of neurologic disease.

The rapid decline in TB levels that occurs 2 weeks after birth in humanized *UGT1* mice is mirrored by the developmental onset of UGT1A1 expression in the gastrointestinal tract. During neonatal development, regulation of the *UGT1A1* gene in hepatic tissue is negligible. Because human UGT1A1-dependent glucuronidation is essential in recovering UCB-induced lethality in humanized *UGT1* mice, alternative mechanisms linked to UCB glucuronidation are being used in humanized *UGT1* mice to lower serum levels of TB. When we analyzed the expression patterns of UGT1A1 in extrahepatic tissues, the temporal increase in expression of UGT1A1 in the small intestine occurred 2 weeks after birth and continued to increase throughout adulthood. The programmed increase in small-intestine UGT1A1 correlated well with the decline in serum UCB during the neonatal period. The importance of the small intestine for bilirubin metabolism is supported by in vivo studies showing that transplantation of small intestine from Wister rats to Gunn rats, which are genetically deficient in the *UGT1* locus, resulted in a decrease in serum bilirubin levels (23). Combined with these results, our findings support the possibility that the developmental changes in UGT1A1 expression in the small intestine play an important role in bilirubin glucuronidation in humanized *UGT1* mice.

It has been estimated that Gilbert's syndrome may be inherited in up to 10% of Caucasians, establishing that mutations in the *UGT1A1* promoter are not rare events (9). Individuals with Gilbert's syndrome have gained recent attention because of the growing list of therapeutics that is metabolized by UGT1A1 (9). Insufficient UGT1A1-dependent drug metabolism coupled with competition for glucuronidation between drug and UCB in Gilbert's syndrome result in delayed clearance of the therapeutics, leading to drug toxicities and hyperbilirubinemia. Expression of human UGT1A1 in liver samples taken from individuals with the *UGT1A1*28* (**28/*28*) genotype displayed greatly reduced expression levels compared with those that were *UGT1A1*1* (**1/*1*) (24). From Western blot analysis (Fig. 3), these differences are 8- to 10-fold. Comparable differences in hepatic UGT1A1 expression were noted between adult $Tg(UGT1^{A1*1})Ugt1^{-/-}$ and $Tg(UGT1^{A1*28})Ugt1^{-/-}$ mice. Differences in hepatic UGT1A1 expression between the humanized *UGT1* mouse lines show that liver-specific control of the Gilbert's promoter polymorphism associated with the *UGT1A1* gene is regulated in a fashion that is comparable with its expression in human liver.

Bilirubin has been proposed to serve as a physiologic antioxidant when bound to serum proteins such as albumin (25), and it provides a large proportion of the antioxidant potential in serum. After it is internalized in cells, bilirubin can be oxidized to biliverdin, where it serves as a substrate for biliverdin reductase (BVR) and is immediately reduced back to bilirubin (26). This antioxidant cycle generated by BVR (26, 27) provides another mechanism for providing protection from oxidative-generated damage to intracellular lipids. High serum bilirubin levels within the upper limits of the normal range have also been associated with lower cancer mortality in men and the antioxidant activity of bilirubin (28). Individuals with Gilbert's syndrome were found to have a lower risk of cardiovascular disease, and it is predicted that the inverse correlation of high bilirubin and heart protection are linked to bilirubin's antioxidant potential (29). Recent findings have indicated that the decreasing susceptibility of LDL oxidation by elevated levels of UCB in Gilbert's syndrome may play a role in protection against atherogenesis (30). The elevated levels of serum bilirubin in adult $Tg(Ugt1^{A1*28})Ugt1^{-/-}$ mice and the dramatic increase in serum bilirubin that we observe during the neonatal period may provide unique opportunities to investigate the antioxidant potential of bilirubin to oxidative-directed tissue damage and disease.

In conclusion, a unique animal model has been developed; it displays consistent and reproducible changes in serum UCB levels that can result in CNS toxicity during neonatal development. Although other animal models, such as the icterus Gunn rat, have been used to study hyperbilirubinemia and kernicterus (31), humanized *UGT1* mice offer the advantage of investigating the regulatory implications of the human *UGT1A1* gene both in neonatal jaundice and adult hyperbilirubinemia. In addition, the spontaneous onset of seizures and the accumulation of UCB in brain tissue will allow future work exploring the impact of UCB on those cellular and molecular mechanisms that lead to disease. It will also be possible to take advantage of current findings linking the roles of nuclear and xenobiotic receptor activation of the *UGT1A1* gene (32–35) to explore future initiatives designed to identify appropriate therapies or nutritional supplements; these findings could be used in translational medicine to reduce the accumulation of serum UCB and limit its potential toxicity in humans.

Materials and Methods

Reagents. The mouse anti-human UGT1A1 antibody was a gift from Joseph K. Ritter (Richmond, VA), and the anti-human UGT1A antibody was supplied by Chantal Guillemette (Quebec, Canada). Human liver microsomes were purchased from BD Gentest (BD Biosciences).

Humanization of $Ugt1^{-/-}$ Mice with the UGT1 Locus. Human BAC that encoded the Gilbert's *UGT1A1*28* allele (32) or the normal *UGT1A1*1* allele as part of the entire *UGT1* locus were placed into mice as transgenes. The transgenic mice

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were crossed with heterozygous *Ugt1*$^{+/-}$ mice (14), creating litters that contained mice carrying the human transgenic DNA in a heterozygous *Ugt1*$^{+/-}$ background. These mice were then backcrossed and further interbred to generate humanized *Tg(UGT1*^{A1*28}*)Ugt1*$^{-/-}$ and *Tg(UGT1*^{A1*1}*)Ugt1*$^{-/-}$ mice. The *UGT1* locus in *Tg(UGT1*^{A1*28}*)Ugt1*$^{-/-}$ mice encodes UGT1A8, -1A10, -1A9, -1A7, -1A6, -1A5, -1A4, -1A3, and -1A1. However, *Tg(UGT1*^{A1*1}*)Ugt1*$^{-/-}$ mice are missing the *UGT1A8* gene but express the other eight *UGT1A* genes.

Genotyping. Genomic DNA was isolated from tail biopsies and used as a template for PCR. For genotyping the *UGT1* transgenes, we used the same PCR method described previously to detect the 366-bp band (32). For genotyping the *Ugt1*$^{-/-}$ mice, the following PCR primers and conditions were used: sense, 5′-GGG CAT CTG ACA TGG AAA A-3′ and antisense, 5′-TGT AAG ACA ATC TTC TCC TCT-3′; 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min for 35 cycles.

Bilirubin Measurements. Blood was obtained from the submandibular vein and centrifuged at 2,000 × *g* for 5 min. Serum samples (20 μL) were immediately measured for total bilirubin using a Unistat Bilirubinometer (Reichert, Inc.).

Real-Time RT-PCR. Total RNA from whole tissues was isolated using TRIzol reagents according to the manufacturer's instructions (Invitrogen). One microgram of RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (BioRad). Real-time Q-PCR was performed with qPCR MasterMix Plus for SYBR (Eurogentec), and the reactions were run in a Mx4000 Multiplex QPCR System (Stratagene) as previously described (33). The mouse cyclophilin B (CPH) gene was used as a reference gene for normalization. For analysis of gene transcript activity by RT-PCR, synthesized cDNA was used for amplifications of human UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A9, and UGT1A10 as described previously (32).

SDS/PAGE and Immunoblotting. Microsomal fractions were prepared as previously described (36) with 20 μg of protein separated on 4–12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen). Immunoblots with antibodies to UGT1A1 were performed as previously outlined (33).

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