

Diabetes Insipidus in Uricase-Deficient Mice: A Model for Evaluating Therapy with Poly(Ethylene Glycol)-Modified Uricase

SUSAN J. KELLY,* MARIELLE DELNOMDEDIEU,‡ MICHAEL I. OLIVERIO,†
L. DAVID WILLIAMS,|| MARK G. P. SAIFER,|| MERRY R. SHERMAN,||
THOMAS M. COFFMAN,† G. ALLAN JOHNSON,‡ and MICHAEL S. HERSHFELD*||
*Divisions of *Rheumatology and †Nephrology, Department of Medicine, ‡Center for In Vivo Microscopy,
Department of Radiology, and §Department of Biochemistry, Duke University School of Medicine, Durham,
North Carolina, and ||Mountain View Pharmaceuticals, Inc., Menlo Park, California.*

Abstract. Uricase-deficient mice develop uric acid nephropathy, with high mortality rates before weaning. Urate excretion was quantitated and renal function was better defined in this study, to facilitate the use of these mice as a model for evaluating poly(ethylene glycol)-modified recombinant mammalian uricases (PEG-uricase) as a potential therapy for gout and uric acid nephropathy. The uric acid/creatinine ratio in the urine of uricase-deficient mice ranges from 10 to >30; on a weight basis, these mice excrete 20- to 40-fold more urate than do human subjects. These mice consistently develop a severe defect in renal concentrating ability, resulting in an approximately sixfold greater urine volume and a fivefold greater fluid requirement, compared with normal mice. This nephrogenic

diabetes insipidus leads to dehydration and death of nursing mice but, with adequate water replacement, high urine flow protects adults from progressive renal damage. Treatment of uricase-deficient mice with PEG-uricase markedly reduced urate levels and, when initiated before weaning, preserved the renal architecture (as evaluated by magnetic resonance microscopy) and prevented the loss of renal concentrating function. PEG-uricase was far more effective and less immunogenic than unmodified uricase. Retention of uricase in most mammals and its loss in humans and some other primates may reflect the evolution of renal function under different environmental conditions. PEG-uricase could provide an effective therapy for uric acid nephropathy and refractory gout in human patients.

Urate oxidase (uricase, EC 1.7.3.4), an enzyme found in liver peroxisomes of most mammalian species, converts uric acid to a more soluble and easily excreted compound, *i.e.*, allantoin. However, the uricase gene (*Uox*) has undergone mutational silencing during the evolution of humans, hominoid apes, and some New World monkeys (1,2). A selective evolutionary advantage resulting from the loss of uricase has been postulated (3,4), but disadvantages resulting from the low solubility of urate include a predisposition to gouty arthritis and to uric acid renal stones (5). During chemotherapy to treat leukemia or lymphoma, a marked increase in the excretion of uric acid derived from the nucleic acids of malignant cells can obstruct renal tubules, causing acute renal failure (“tumor lysis syndrome”) (6–8). Uric acid nephropathy and gout are also complications of the overproduction or impaired excretion of urate in patients with the inherited disorders hypoxanthine-guanine phosphoribosyltransferase deficiency, phosphoribosylpyro-

phosphate synthase overactivity, and type I glycogen storage disease (5,9).

Blockage of urate synthesis by xanthine oxidase inhibition or promotion of uric acid excretion is usually effective for the treatment of urate-induced disorders (5,10,11). Indeed, no new therapies for controlling hyperuricemia have been introduced in the United States since allopurinol was introduced in the 1960s. In the past four decades, the prevalence of gout has been increasing (12) and an often-aggressive form of gout has been recognized as a complication of cyclosporine therapy among recipients of kidney and other organ transplants (13–15). Hyperuricemia has proved difficult to control in some patients (5,16,17). Multiple factors may contribute to this problem, including hypersensitivity to allopurinol, renal insufficiency, coexisting chronic diseases in elderly patients, azathioprine therapy in organ transplant recipients, and marked uric acid overproduction in patients with inherited metabolic disorders. “Replacement” therapy with a long-acting form of uricase is a potential alternative for such patients.

Daily infusion of uricase derived from *Aspergillus flavus* has been used to control acute hyperuricemia during chemotherapy of malignancies (18,19). However, use of the fungal enzyme for chronic therapy of gout has been limited by rapid clearance, which requires daily infusion, and by potential immunogenicity (19–21). Very low solubility at physiologic pH is an additional barrier to therapy with unmodified mammalian urate

Received July 25, 2000. Accepted October 5, 2000.

Correspondence to Dr. Michael S. Hershfield, Box 3049, Duke University Medical Center, Durham, NC 27710. Phone: 919-684-4184; Fax: 919-684-4168; E-mail: msh@biochem.duke.edu

1046-6673/1205-1001

Journal of the American Society of Nephrology

Copyright © 2001 by the American Society of Nephrology

oxidases (22). Covalent linkage of poly(ethylene glycol) (PEG) to exposed amino groups has been demonstrated to enhance solubility, prolong circulating life, and reduce the immunogenicity of several proteins in animals (23). PEG-modified bovine adenosine deaminase, injected once or twice per week, has been well tolerated and effective as chronic replacement therapy for inherited adenosine deaminase deficiency (24,25).

We have produced PEG-modified, recombinant, mammalian urate oxidases and tested their efficacy in a strain of mice homozygous for a targeted disruption of the *Uox* gene (*Uox*^{-/-}). These mice have been demonstrated to develop obstructive nephropathy, akin to that associated with tumor lysis in human patients; most died by 4 wk of age, unless they were treated with allopurinol (26). Renal function in these mice was not studied. Here we report that untreated *Uox*^{-/-} mice have a profound defect in the ability to concentrate urine, resulting in nephrogenic diabetes insipidus (DI). This condition can be largely prevented by treatment with PEG-uricase. These findings provide a “proof of principle” for the clinical use of PEG-uricase; they also suggest factors that underlie the evolutionary loss or retention of uricase among species.

Materials and Methods

Urate Oxidase-Deficient Mice

Mice heterozygous for a urate oxidase transgene disrupted by a neomycin resistance gene insert, on a C57BL/6J-129Sv hybrid genetic background (26), were provided by Dr. M. Wakamiya, Baylor University. These *Uox*^{+/-} mice were interbred to generate *Uox*^{-/-} homozygotes. Mice were maintained on a standard diet with free access to water. All experiments involving mice were performed at Duke University and were approved by the Duke University Institutional Animal Care and Use Committee.

Biochemical and Physiologic Studies

Uricase activity was determined spectrophotometrically, at 23 to 25°C, by monitoring the decrease in absorbance at 292 nm in a reaction mixture containing 0.1 M sodium borate (pH 8.6) and 0.1 mM uric acid (1 unit was defined as a decrease of 1 μ mol of uric acid/min) (27). Commercial kits were used to determine the concentrations of uric acid (Sigma kit 685-10) and creatinine (Sigma kit 557) in serum and urine. Protein was determined by the Lowry or bicinchoninic acid (Pierce, Rockford, IL) method, with bovine serum albumin as the standard. Some serum samples were analyzed for urea nitrogen, creatinine, calcium, phosphorus, albumin, and urate levels by AniLytics, Inc. (Gaithersburg, MD).

Blood was obtained from the retro-orbital plexus using methoxyflurane anesthesia. In some studies of mice treated with uricase, *ex vivo* degradation of uric acid was prevented by rapid mixing of blood with an equal volume of 2 N perchloric acid on ice; after centrifugation (2 min at 4°C at 11,000 \times g), the supernatant was neutralized with 3.0 M KOH/1.0 M KHCO₃. The serum urate concentration was calculated from the concentration in the neutralized extract, assuming a hematocrit value of 0.44. Spontaneously voided urine was collected from a clean surface. Mice were placed in metabolic cages for quantitation of water intake and urine output. Urine osmolality was determined with a vapor pressure osmometer (Westcor Instruments, Logan, UT).

PEG-Uricase

Standard methods were used to amplify, clone, and sequence cDNA. Reverse transcription-PCR primers for pig and baboon liver urate oxidases were based on published sequences (1,28). A pig-baboon chimeric (PBC) uricase cDNA, consisting of pig codons 1 to 225 joined in-frame to baboon codons 226 to 304, was constructed. The PBC cDNA encodes 30 lysines, one more than either “parental” uricase cDNA, because of the presence of lysine codons replacing Asn-103 in the baboon cDNA and Arg-291 in the pig cDNA. Other cDNA based on the pig coding sequence but possessing a Lys-291 codon were subsequently constructed; proteins derived from the latter are referred to collectively as PigK uricase. Uricase cDNA were overexpressed in *Escherichia coli* BL21(DE3)pLysS, using pET vectors (Novagen, Madison, WI), or in other expression systems not described here. Purified preparations of the recombinant pig, PBC, and PigK uricases had specific activities of 3 to 7 U/mg. Similarly prepared recombinant baboon uricase was four- to fivefold less active; baboon uricase purified from liver is also less active than pig liver uricase (29). These recombinant uricases were soluble and stable in carbonate buffer (pH 10.2) but, like the mammalian urate oxidases purified from tissues (22), were sparingly soluble at pH 7.4.

PBC uricase purified to >95% homogeneity (Kelly SJ, Hershfield MS, unpublished procedure) was provided to Mountain View Pharmaceuticals, Inc. (Menlo Park, CA), where it was modified by covalent attachment of monomethoxy-PEG (*i.e.*, PEGylated), using a modification of a method described by Sherman *et al.* (30). Approximately 60 kD of PEG was coupled to each uricase subunit, determined as described (31). The resulting PEG-uricase had specific activity ranging from 2.3 to 5.6 U/mg; it was soluble and stable for at least 6 mo at 4°C in phosphate-buffered saline (PBS) (pH 7.4). A PEGylated recombinant PigK uricase with similar properties was prepared by Drs. J. R. Hartman and S. Mendelovitz of Bio-Technology General Ltd. (Rehovot, Israel).

Uricase Replacement Therapy

Serum uricase activity was observed to peak 20 to 24 h after intraperitoneal injection of *Uox*^{-/-} mice with PEG-uricase; the circulating half-life was approximately 78 h. In experiments involving repeated intraperitoneal administration, the 24-h postinjection serum uricase activity was used as an index of the rate of enzyme clearance (as in analogous studies of PEG-modified *E. coli* purine nucleoside phosphorylase) (32). The concentration of uric acid in urine provided a noninvasively measured index of the capacity to catabolize urate. Use of this index reduced the need for blood sampling and the associated risk of death resulting from anesthesia and blood volume depletion.

Enzyme-Linked Immunosorbent Assay for Anti-Uricase Antibody

Microtiter plates (Immulon 2HB; Dynatech, Chantilly, VA) were treated overnight at 4°C with 1 μ g/well of purified recombinant uricase in 0.1 M sodium carbonate (pH 10.2) or with buffer alone. After blocking with 2% goat serum (Life Technologies BRL, Gaithersburg, MD) in PBS, the plate was washed with PBS containing 0.1% Tween 20 (PBST). Aliquots (0.1 ml) of mouse serum (diluted in PBS with 2% goat serum) were added to adjacent rows of uricase-coated and antigen-free wells. After 1 h at 37°C, plates were washed with PBST and then incubated for 45 min at 37°C with a goat anti-mouse Ig-peroxidase conjugate (Calbiochem, San Diego, CA). After washing with PBST, 0.1 ml/well of *o*-phenylenediamine peroxidase substrate solution (Calbiochem) was added. After 25 min at 25°C, 0.1 ml/well

of 1 N HCl was added, and absorbance at 490 nm was measured with a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The A_{490} values for uricase-free wells were subtracted to determine bound antigen-specific mouse Ig. Results are expressed in enzyme-linked immunosorbent assay units ($A_{490}/10 \mu\text{l}$ of serum), as defined by Chaffee *et al.* (33).

Magnetic Resonance Microscopy

Mice were euthanized by barbiturate overdose. The blood supply to the left kidney was ligated immediately, to maintain blood in the organ [as a negative contrast agent for magnetic resonance (MR) imaging]. The body was perfused with 0.9% saline solution and then with 10% neutral buffered formalin-glutaraldehyde solution. The perfused (right) kidney was used for histologic analyses. The nonperfused (left) kidney was placed in a cylindrical container and immersed in Fomblin (perfluoro-polyether; Ausimont, Morristown, NJ) to limit magnetic susceptibility variations at the tissue surface. MR imaging was performed immediately, using a custom-designed 11-mm solenoid radiofrequency coil. MR images were acquired at 9.4 T with a Bruker CSI system (Fremont, CA) equipped with actively shielded gradients (34). A spin-echo pulse sequence was used (repetition time, 500 ms; echo time, 10 ms; number of excitations, 2). Spatial encoding was accomplished using three-dimensional Fourier transform, which allowed the simultaneous imaging of 128 contiguous planes (each 39 μm thick) through the specimen. The 10-mm field of view was reconstructed on a 256×256 matrix, leading to a voxel size of (39 μm)³. Images were analyzed on a Silicon Graphics workstation (Reality Engine 2; SGI, Mountain View, CA), using VoxelView software (Vital Images, Fairfield, IA).

Histopathologic Analyses

Formalin/glutaraldehyde-fixed kidneys were embedded in paraffin, sectioned (3 μm) in the axial plane (corresponding to the axial MR images), and stained with hematoxylin and eosin. Histologic evaluation was performed without knowledge of genotype or the MR microscopy results.

Results

Uric Acid Excretion, Water Balance, and Renal Concentrating Ability

The uric acid/creatinine molar ratio in the urine of $Uox^{-/-}$ mice was 30- to 100-fold higher than that for heterozygotes ($Uox^{+/-}$). For both genotypes, the highest ratio occurred in the second week of life (Figure 1). For $Uox^{-/-}$ mice, the uric acid/creatinine ratio peaked at approximately 35 and then decreased to a stable level of approximately 10 after weaning. Total 24-h urinary uric acid excretion by 4-mo-old $Uox^{-/-}$ mice was 6 ± 2.4 mg ($35 \pm 14 \mu\text{mol}$) per 20-g mouse (mean \pm SD, $n = 10$). On a body weight basis, this value is 20- to 40-fold higher than that for human subjects. Crystals appeared rapidly as urine from $Uox^{-/-}$ mice cooled (data not shown). Bladders of some adults were dilated and contained uric acid stones.

Most adult $Uox^{-/-}$ mice were similar in appearance to $Uox^{+/-}$ or $Uox^{+/+}$ mice of the same C57BL/6J-129Sv hybrid genetic background. However, we noticed that $Uox^{-/-}$ mice as young as 10 to 12 d of age produced copious pale urine. This led to studies of water balance and renal concentrating function. When animals were given free access to water,

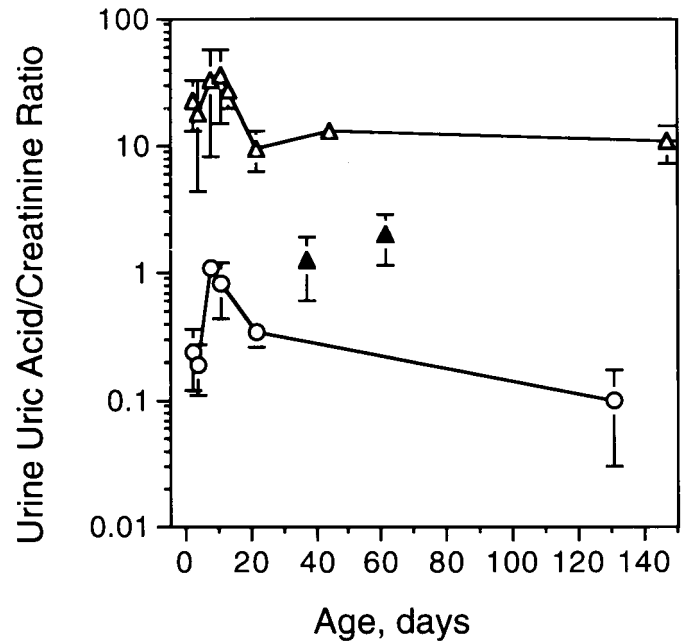


Figure 1. Uric acid excretion by uricase-deficient and heterozygous mice. At the indicated ages, the uric acid/creatinine molar ratio (mean \pm SD) was determined in urine samples from three to seven $Uox^{+/-}$ mice (\circ) and three to 12 $Uox^{-/-}$ mice (Δ). Data for six $Uox^{-/-}$ mice that had been treated with weekly intraperitoneal injections of pig-baboon chimeric (PBC) poly(ethylene glycol)-uricase (PEG-uricase) (see legend to Figure 5) are also presented (\blacktriangle).

the mean water intake and urine output for 44-d-old $Uox^{-/-}$ mice ($n = 4$) were 15.7 and 6.9 ml/24 h, respectively, normalized to a body weight of 20 g, or approximately fivefold more water ingested and sixfold more urine voided, compared with normal mice. The urine osmolality for the $Uox^{-/-}$ mice ranged from 520 to 630 mosmol/kg, compared with 900 to >2500 mosmol/kg for normal mice with access to water. The ability of $Uox^{-/-}$ mice to concentrate urine when deprived of water was severely impaired (Figure 2); the maximal urine osmolality for five $Uox^{-/-}$ mice deprived of water for 12 h averaged approximately 680 mosmol/kg and did not exceed 885 mosmol/kg, whereas heterozygotes increased urine osmolality to approximately 3500 mosmol/kg.

Except for moderate azotemia, we did not observe signs of renal insufficiency in adult $Uox^{-/-}$ mice. Thus, when evaluated at 50 to 60 d and 9 to 13 mo of age, the serum urea nitrogen concentrations for $Uox^{-/-}$ mice were 56 ± 20 and 43 ± 10 mg/dl (mean \pm SD), respectively, or 1.5- to 2-fold higher than for heterozygotes. BP and serum creatinine, calcium, phosphorus, and albumin levels were normal (data not shown), and ascites was never observed.

Treatment with PEG-Uricase

Although it is stable and active at high pH, unmodified (native) recombinant uricase was not effective *in vivo* in $Uox^{-/-}$ mice. Thus, serum uricase activity remained undetectable (<0.005 U/ml) at 4 and 24 h after intraperitoneal injection of 1 unit of native uricase (diluted 20-fold, to 0.23 mg/ml, in

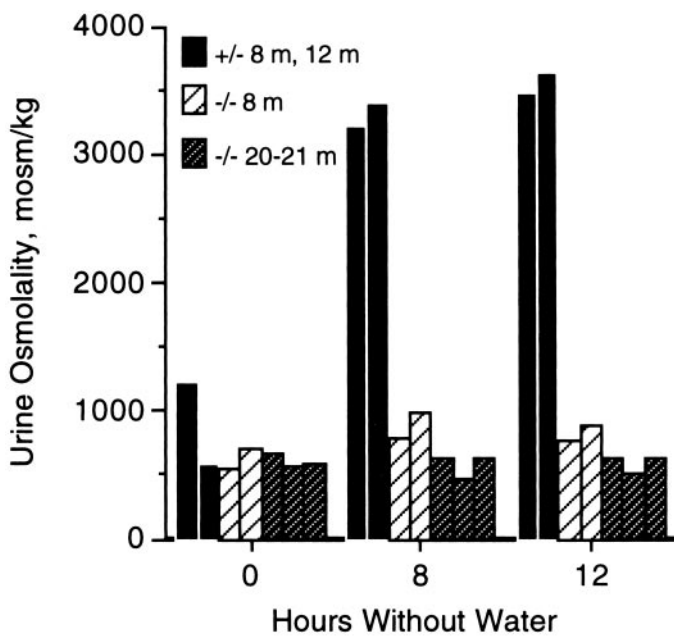


Figure 2. Urine-concentrating ability during water deprivation. Urine osmolality was measured in samples obtained before and 8 and 12 h after withdrawal of water. Each vertical bar represents one mouse. The mice studied included two *Uox* heterozygotes (+/-), which were 8 and 12 mo of age, and five *Uox* homozygotes (-/-), of which two were 8 mo and three were 20 to 21 mo of age, as indicated.

PBS, pH 7.4, just before injection, to maintain solubility). Serum urate levels for these mice ($n = 12$) decreased from 10.2 ± 2.5 mg/dl before treatment to 6.3 ± 1.8 mg/dl at 4 h after injection but returned to baseline values by 24 h. The urinary uric acid concentration transiently decreased from 164 ± 19 mg/dl before injection to 139 and 146 mg/dl at 5 and 9 h after injection, respectively, but no decrease was observed at 3, 19, or 30 h. With weekly administration, anti-uricase antibody appeared in all 12 mice after the second injection and levels increased after the third and fourth injections (Figure 3, A to C).

Compared with the native enzyme, PEG-uricase exhibited greater bioavailability and was far more effective in decreasing urate levels, including results after repeated injections. In one study, 4-mo-old *Uox*^{-/-} mice ($n = 10$) received intraperitoneal injections of 0.34 U of PEG-uricase every 5 d. Each injection produced a marked reduction in the urinary level of uric acid; reductions were greatest after 24 h but were evident throughout the period between injections (Figure 4). In urine obtained 5 d after the 11th injection, the uric acid/creatinine ratio was 1.7 ± 2.5 , compared with 10.6 ± 1.5 before treatment. Sera obtained 9 d after the 11th injection exhibited detectable uricase activity (0.053 ± 0.025 U/ml) and lacked significant anti-uricase antibody (Figure 3D); the mean serum urate concentration was 1.3 ± 2.1 mg/dl, compared with 7.3 ± 0.8 mg/dl before treatment. After the 10th PEG-uricase dose, we evaluated renal concentrating ability. During a 12-h period of water deprivation, urine osmolality for the treated mice increased by 623

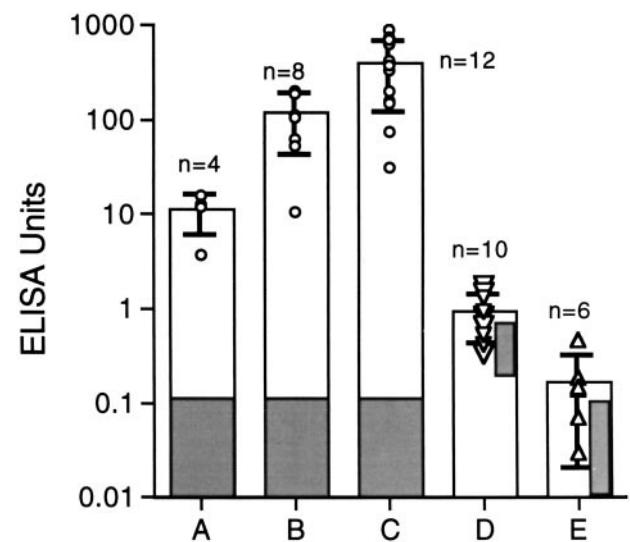


Figure 3. Immunogenicity of unmodified uricase and PEG-uricases. The development of anti-uricase antibody was assessed in three experiments examining the efficacy of intraperitoneally injected uricases in *Uox*^{-/-} mice (see Results section). (A to C) Twelve mice (2.5 to 5 mo of age) each received four injections of unmodified PBC uricase (0.23 mg) at 6- to 7-d intervals. Enzyme-linked immunosorbent assays (ELISA) were performed after injections 2 (A), 3 (B), and 4 (C) for 4, 8, and all 12 mice, respectively. (D) Ten 4-mo-old mice received 0.08 mg of PEGylated PigK uricase every 5 d. ELISA were performed 9 d after the 11th injection. (E) Beginning at 3 to 10 d of age, six mice received weekly injections of PEGylated PBC uricase (0.04 mg until 6 wk of age and then 0.08 mg). ELISA were performed after the 10th injection. The open bars and error bars represent the mean \pm SD response, in ELISA units (see Materials and Methods section); the symbols within the bars represent the responses of individual treated mice. The stippled areas within the bars indicate the range of ELISA values for sera from untreated *Uox*^{-/-} mice, assayed using as immobilized antigen the unmodified uricase corresponding to the PEG-uricase under study (PBC uricase for A, B, C, and E, $n = 16$ serum samples; PigK uricase for D, $n = 10$ serum samples).

± 364 mosmol/kg, compared with an increase of 319 ± 236 mosmol/kg before treatment ($P < 0.05$). The residual concentrating defect may have been attributable to irreversible structural renal damage (see below).

PEG-uricase therapy initiated early in life was effective in preventing the development of renal injury. We determined this by studying two groups of six *Uox*^{-/-} mice, one of which was given a series of 10 or 11 intraperitoneal injections of PEG-uricase and the other of which was given saline solution, administered at 4- to 10-d intervals (mean, 6.7 d) between the first 10 d and the 10th week of life (0.095 U/dose until 6 wk of age and then 0.19 U/dose). After 10 injections, none of the PEG-uricase-treated mice exhibited detectable anti-uricase antibody (Figure 3E). When measured at 6 to 9 wk of age, the mean urinary uric acid/creatinine ratio for the six PEG-uricase-treated *Uox*^{-/-} mice was 1.2 to 2, which was much lower than that for the untreated *Uox*^{-/-} mice (Figure 1). At 10 wk, the treated mice demonstrated significantly greater renal concentrating

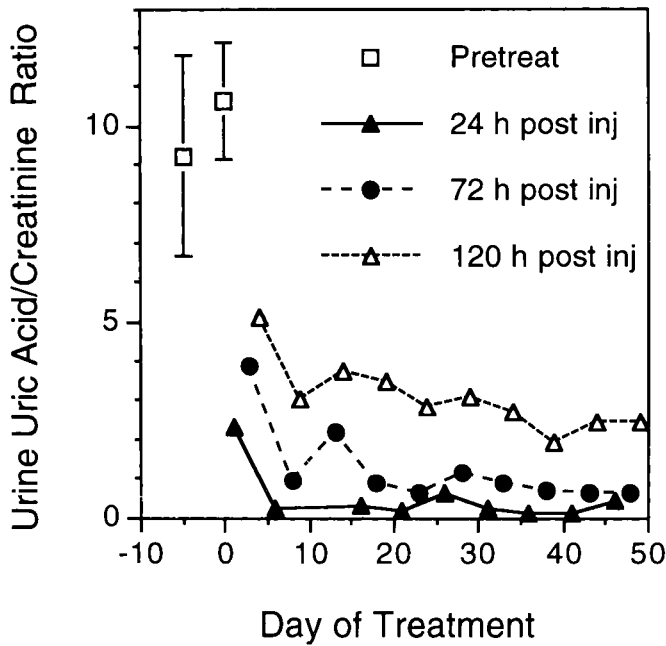


Figure 4. Effect of treatment with PEG-uricase on uric acid excretion. Ten 4-mo-old *Uox*^{-/-} mice were given intraperitoneal injections of 0.34 U of PigK PEG-uricase every 5 d. The uric acid/creatinine molar ratio was assessed in urine samples from all mice on two occasions before treatment (Pretreat; bars, SD). During treatment, the uric acid/creatinine ratio was assessed for all mice in urine samples obtained approximately 24, 72, or 120 h after injection (post inj) (for clarity, error bars are not shown for these data points).

ability than did the untreated *Uox*^{-/-} control mice, with nearly normal fluid balance. Thus, after 12 h of water deprivation, the untreated control animals exhibited a mean urine osmolality of approximately 750 mosmol/kg; the treated mice exhibited a mean basal urine osmolality of approximately 1850 mosmol/kg, which increased to 2700 mosmol/kg (Figure 5A). With free access to water, the untreated mice ingested 4.4-fold more water and produced 6.3-fold more urine than did normal (*Uox*^{+/+}) mice, compared with 1.7-fold more water and 1.4-fold more urine for the treated mice (Figure 5B).

As assessed by MR microscopy (Figure 6), the renal architecture was largely preserved in the PEG-uricase-treated mice, in contrast to the scarred, irregularly shaped, hydronephrotic kidneys of untreated *Uox*^{-/-} mice. Histologic sections of kidneys from untreated mice exhibited cortical foci of fibrosis and tubular atrophy, with apparent crowding of glomeruli (Figure 7). In both the cortex and medulla, collecting ducts were markedly dilated, possibly because of elevated tubular flow. In contrast, kidneys from treated mice exhibited no gross irregularities. Atrophy of the inner medulla was absent, tubules were packed tightly, and glomeruli exhibited normal distribution.

Discussion

DI Attributable to Uricase Deficiency

We have observed that mice that lack uricase develop a severe, persistent, renal concentrating defect that results from

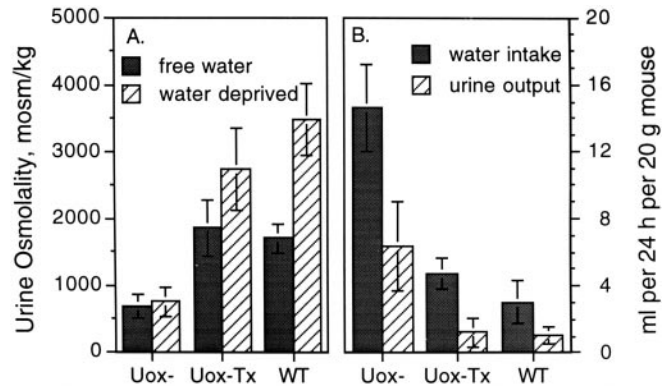


Figure 5. Effect of treatment with PEG-uricase on the development of diabetes insipidus. (A) Urine-concentrating ability. (B) Water balance. As described in Results, beginning at 3 to 10 d of age, two groups of six uricase-deficient (*Uox*⁻) mice were given a series of 10 or 11 weekly intraperitoneal injections of either saline solution or PBC PEG-uricase (Tx). At the end of the treatment period, the ability to concentrate urine after 12 h of water deprivation (A) and the amounts of water ingested and urine produced in 24 h (B) were analyzed. These results are compared with data obtained separately for wild-type (WT) C57BL/6J-129Sv hybrid mice.

renal damage induced by excretion of uric acid in amounts that exceed its solubility in urine. The magnitude of uric acid overexcretion is remarkable. The uric acid/creatinine ratio in urine was >30 in young mice and approximately 10 in adults. By comparison, urinary uric acid/creatinine ratios of 3 to 5 have been observed in children with hypoxanthine-guanine phosphoribosyltransferase deficiency, and uric acid/creatinine ratios of >1 have been associated with acute uric acid nephropathy in human patients (35,36). In comparison with body weight, we estimate that uricase-deficient mice excrete 20- to 40-fold more uric acid/d than do human subjects.

The DI of uricase-deficient mice resembles the persistent concentrating defect in Gunn rats, which lack hepatic glucuronyl transferase; the defect was reversible if unconjugated bilirubinemia was corrected in young animals, before permanent structural renal damage occurred (37). The DI is also related mechanistically to the transient DI that may follow renal tubular injury caused by ischemia, toxins, or obstruction (e.g., postobstructive diuresis). This transient DI might be a manifestation of injury or of the recovery of tubular epithelium. It can result in severe volume depletion, but it may also serve a protective function, i.e., to reduce the concentration of an offending toxin or obstructing agent. The persistent DI of uricase-deficient mice may be lethal in nursing animals but essential for the survival of adults.

Structural kidney damage and DI are evident in *Uox*^{-/-} mice by 2 wk of age, when mortality (26) and uric acid excretion (Figure 1) are highest. Adult *Uox*^{-/-} mice with severe DI exhibited little evidence of progressive renal insufficiency, and many exhibited normal lifespans. However, before our recognition of their fivefold greater water requirement, some died as a result of dehydration after exhausting water supplies adequate for normal mice. The high mortality rate

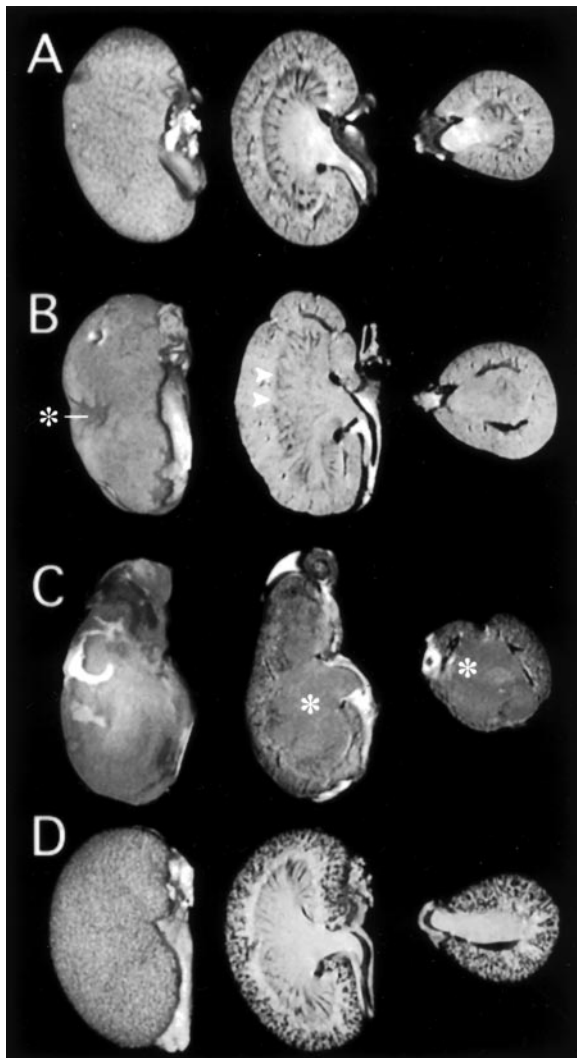


Figure 6. Nephropathy resulting from uricase deficiency and the effect of treatment with PEG-uricase. Selected magnetic resonance (MR) microscopy images are shown for nonperfused left kidneys from four mice. (A) A 51-d-old heterozygote. (B and C) Two untreated *Uox*^{-/-} mice, 72 and 65 d of age, respectively. (D) A 68-d-old *Uox*^{-/-} mouse that had received 10 intraperitoneal injections of PBC PEG-uricase between 3 and 65 d of age (see Results). For each mouse, three MR images are shown, *i.e.*, a three-dimensional surface-rendering (left), a midcoronal section (center), and a midaxial section (right) (voxel size, $(39 \mu\text{m})^3$). Although differing somewhat in textural detail, the three-dimensional surface-rendered MR images from the heterozygote (A) and the PEG-uricase-treated *Uox*^{-/-} mouse (D) both show a smooth uniform pattern corresponding to underlying cortical components (medullary rays and cortical labyrinth). This contrasts with the irregular shape and surface scarring evident to varying degrees on kidneys from two untreated *Uox*^{-/-} mice (B, asterisk). The coronal and axial MR sections from A and D show well defined morphologic details because of blood retained in the organ, which provided a negative contrast agent for the observation of blood vessels, cortex, medulla, and papilla. The same sections from B and C show an effaced corticomedullary junction and atrophic inner medulla (B, arrowheads). The featureless gray areas in the axial and coronal MR images for these kidneys (C, asterisks) are attributable to fluid in cysts and in the dilated pelvocalyceal region.

before weaning is probably attributable to the inadequacy of maternal milk as the only source of fluid replacement. Death resulting from dehydration was similarly attributed to polyuria in nursing mice in which the gene for the NaK2Cl cotransporter had been disrupted (38). However, after weaning, a sixfold elevated urine volume, compensated by adequate water intake, may limit further deposition of uric acid in renal tubules, preventing progressive renal failure. Such a protective role for nephrogenic DI is consistent with the finding that high tubular fluid flow attributable to inherited pituitary DI prevented acute uric acid nephropathy in a rat model in which the serum urate concentration was increased to 20 mg/dl by the infusion of both urate and oxonic acid (an inhibitor of uricase) (39). Maintenance of high urine volume is used clinically to treat uric acid renal stones and acute uric acid nephropathy in human patients (5,6).

Relevance to Evolution

Discussions of uricase evolution have often speculated on advantages derived from loss of the enzyme, such as increased longevity resulting from the radical-scavenging ability of urate (3). A less anthropocentric view might consider the benefit of uricase to other species for which renal water conservation has been an essential adaptation to seasonal drought or arid climates. The avoidance of uric acid precipitation in maximally concentrated urine would strongly favor the retention of uricase; its loss might lead to lethal nephropathy, as in uricase-knockout mice. In contrast, ancestors of New World monkeys, hominoid apes, and humans, in which *Uox* gene mutations became fixed (2), may have lived in rain forests. With abundant water, maintenance of relatively dilute urine may have been better tolerated and favored as an adaptation to environmental toxins, as well as to the loss of uricase. Other protective mechanisms might have been in place or selected subsequently, *e.g.*, efficient proximal tubular reabsorption to limit the access of uric acid to collecting ducts, where maximal concentration and acidification occur, or shorter renal papillae to limit distal water reabsorption. The reduced ability of the neonatal kidney to concentrate and acidify urine may protect human infants from uric acid nephropathy at a time when urate clearance is considerably higher than that in adults (40).

PEG-Uricase as a Potential Therapy for Refractory Gout

Fungal and pig liver uricases, PEGylated in various ways, have been studied in uricase-expressing animals (41–43). Where reduced immunogenicity has been achieved, it has been at levels of PEG coupling that cause substantial loss of catalytic activity. PEGylated *Candida* and *Arthrobacter* uricases were demonstrated to decrease serum urate concentrations in a few patients with malignancies, but neither preparation was subsequently produced for clinical trials (44,45).

We have taken several steps aimed at producing a PEG-uricase more suitable for long-term therapy. (1) We chose porcine uricase, which has >85% identity with the deduced amino acid sequence of human uricase, rather than a microbial enzyme with <40% identity or baboon uricase, which has

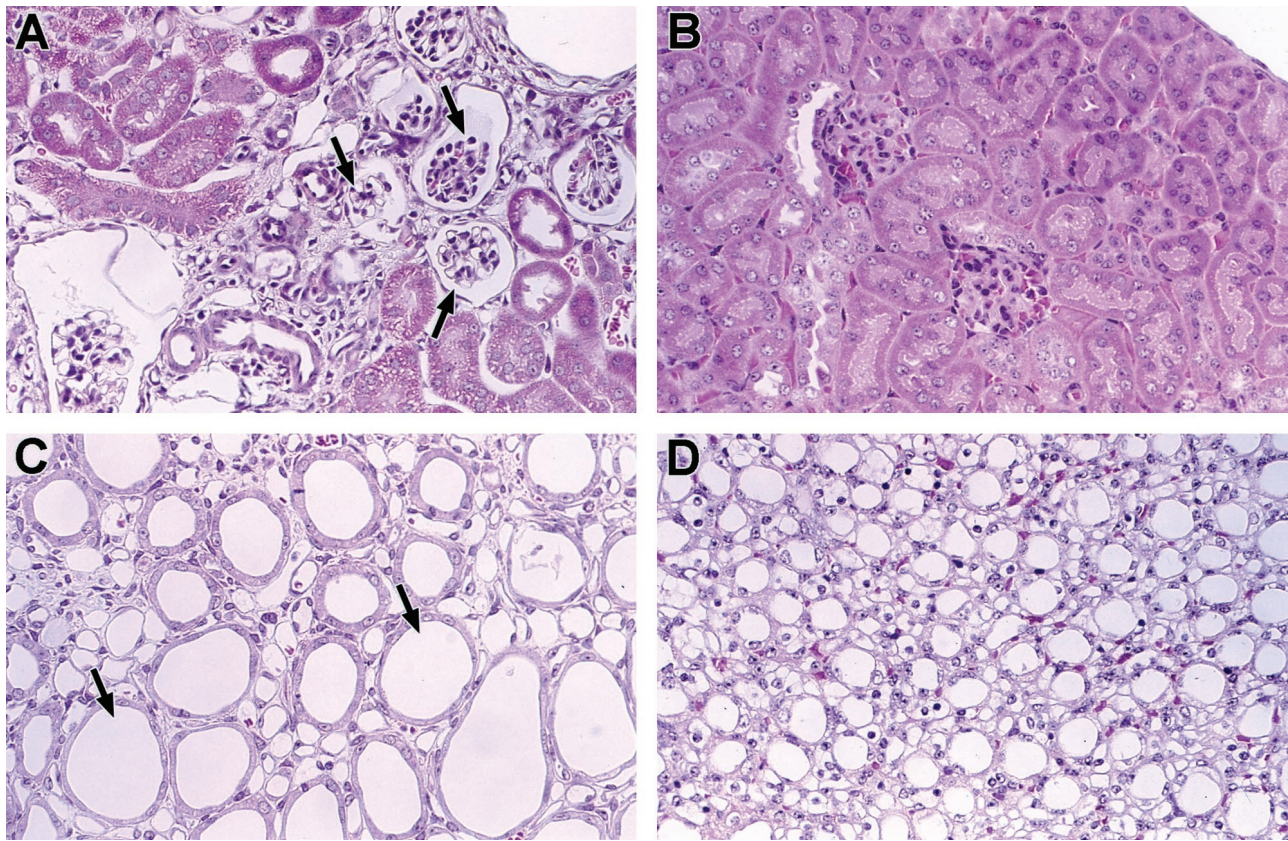


Figure 7. Kidney histomorphologic features. Representative sections of the renal cortex (A and B) and inner medulla (C and D) are shown for kidneys from a 72-d-old, untreated, uricase-knockout (*Uox*^{-/-}) mouse (A and C) (MR images of the companion kidney are shown in Figure 6B) and from a 68-d-old *Uox*^{-/-} mouse that had received 10 intraperitoneal injections of PBC PEG-uricase between 3 and 65 d of age (B and D) (MR images of the companion kidney are shown in Figure 6D). Magnification, $\times 100$. The cortex of the untreated kidney shows crowding of glomeruli, with an enlarged Bowman's space (A, arrows) attributable to focal tubular dropout and fibrosis extending from the kidney surface. These changes are absent in the kidney of the treated animal (B). The inner medulla of the kidney from the untreated *Uox*^{-/-} mouse shows markedly dilated collecting ducts (C, arrows), whereas collecting ducts from the PEG-uricase-treated mouse are normal in diameter and appearance (D).

slightly greater sequence similarity but is much less active. We considered “reactivating” human uricase by eliminating both known nonsense mutations but concluded that it would be difficult to identify and “correct” any missense mutations acquired during evolution. (2) Addition of potential PEG attachment sites (lysine residues) enhanced the ability of PEGylation to reduce the immunogenicity of a bacterial enzyme in mice (32). This presumably resulted from better epitope masking. We applied this strategy to pig uricase by changing Arg-291 to lysine. The resulting PBC and related enzymes are fully active and possess one to four more lysines than other cloned mammalian uricases (mouse, rat, rabbit, and baboon uricases). (3) We optimized PEGylation to achieve a PEG-uricase that retains the activity of the native enzyme but is substantially more soluble at physiologic pH and is much less immunogenic in mice.

Because of their extraordinary excretion of uric acid and the resulting renal damage, *Uox*^{-/-} mice provide an excellent model for testing efficacy. Injections of PEG-uricase at 5- to 7-d intervals controlled hyperuricemia and markedly reduced urate excretion in uricase-knockout mice and, when initiated

before weaning, prevented the development of nephropathy. This treatment elicited minimal antibody response. In contrast to PEG-uricase, unmodified recombinant mammalian uricase was ineffective in decreasing uric acid levels and was highly immunogenic. These results suggest that this form of PEG-uricase could be effective in treating both acute and chronic hyperuricemia and uricosuria. In addition to use in the acute setting of chemotherapy for malignancies, PEG-uricase could provide a much-needed therapy for controlling chronic hyperuricemia in patients with inherited metabolic disorders and in patients with severe gout who are allergic or unresponsive to conventional therapy.

Acknowledgments

We acknowledge the expert technical assistance of Stephan Toutain, Kyle Pollard, K. T. Park, and John A. French and the cooperation of M. L.-M. D. Drs. Kelly and Hershfield also acknowledge many useful discussions with Emma Jakoi. This work was supported by Grants 2-R42-DK48529 and P41-RR05959 from the National Institutes of Health, by a grant from the United States-Israel

Binational Industrial Research and Development Foundation, and by Bio-Technology General Corporation.

References

1. Wu X, Lee CC, Muzny DM, Caskey CT: Urate oxidase: Primary structure and evolutionary implications. *Proc Natl Acad Sci USA* 86: 9412–9416, 1989
2. Wu X, Muzny DM, Lee CC, Caskey CT: Two independent mutational events in the loss of urate oxidase. *J Mol Evol* 34: 78–84, 1992
3. Ames BN, Cathcart R, Schwiers E, Hochstein P: Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. *Proc Natl Acad Sci USA* 78: 6858–6862, 1981
4. Hooper DC, Spitsin S, Kean RB, Champion JM, Dickson GM, Chaudhry I, Koprowski H: Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis. *Proc Natl Acad Sci USA* 95: 675–680, 1998
5. Becker MA, Roessler BJ: Hyperuricemia and gout. In: *The Metabolic and Molecular Bases of Inherited Disease*, 7th Ed., edited by Scriver CR, Beaudet AL, Sly WS, Valle D, New York, McGraw-Hill, 1995, pp 1655–1677
6. Conger J: Acute uric acid nephropathy. *Med Clin North Am* 74: 859–871, 1990
7. Jones DP, Mahmoud H, Chesney RW: Tumor lysis syndrome: Pathogenesis and management. *Pediatr Nephrol* 9: 206–212, 1995
8. Steele TH: Hyperuricemic nephropathies. *Nephron* 81[Suppl 1]: 45–49, 1999
9. Rossiter B, Caskey CT: Hypoxanthine-guanine phosphoribosyltransferase deficiency: Lesch-Nyhan syndrome and gout. In: *The Metabolic and Molecular Bases of Inherited Disease*, 7th Ed., edited by Scriver CR, Beaudet AL, Sly WS, Valle D, New York, McGraw-Hill, 1995, pp 1679–1706
10. Emmerson BT: The management of gout. *N Engl J Med* 334: 445–451, 1996
11. Hershfield MS: Gout and uric acid metabolism. In: *Cecil Textbook of Medicine*, 21st Ed., edited by Goldman L, Bennett JC, Philadelphia, WB Saunders, 1999, pp 1541–1548
12. Roubenoff R: Gout and hyperuricemia. *Rheum Dis Clin North Am* 16: 539–550, 1990
13. Burack DA, Griffith BP, Thompson ME, Kahl LE: Hyperuricemia and gout among heart transplant recipients receiving cyclosporine. *Am J Med* 92: 141–146, 1992
14. Baethge BA, Work J, Landreneau MD, McDonald JC: Tophaceous gout in patients with renal transplants treated with cyclosporine A. *J Rheumatol* 20: 718–720, 1993
15. Ben Hmida M, Hachicha J, Bahloul Z, Kadour N, Kharrat M, Jarraya F, Jarraya A: Cyclosporine-induced hyperuricemia and gout in renal transplants. *Transplant Proc* 27: 2722–2724, 1995
16. Arellano F, Sacristán JA: Allopurinol hypersensitivity syndrome: A review. *Ann Pharmacother* 27: 337–343, 1993
17. Rosenthal AK, Ryan LM: Treatment of refractory crystal-associated arthritis. *Rheum Dis Clin North Am* 21: 151–161, 1995
18. Masera G, Jankovic M, Zurlo MG, Locasciulli A, Rossi MR, Uderzo C, Recchia M: Urate-oxidase prophylaxis of uric acid-induced renal damage in childhood leukemia. *J Pediatr* 100: 152–155, 1982
19. Pui C-H, Relling MV, Lascombes F, Harrison PL, Struxiano A, Mondesir J-M, Ribeiro RC, Sandlund JT, Rivera GK, Evans WE, Mahmoud HH: Urate oxidase in prevention and treatment of hyperuricemia associated with lymphoid malignancies. *Leukemia* 11: 1813–1816, 1997
20. Mourad G, Cristol JP, Chong G, Andary M, Mion C: Role of precipitating anti-urate oxidase antibodies in urate oxidase-resistant hyperuricemia [Letter]. *Presse Med* 13: 2585, 1984
21. Montagnac R, Schillinger F: Anaphylactic complication tied to intravenous injection of urate oxidase in a dialysis patient. *Nephrologie* 11: 259, 1990
22. Conley TG, Priest DG: Purification of uricase from mammalian tissue. *Prep Biochem* 9: 197–203, 1979
23. Harris JM, Zalipsky S: *Poly(Ethylene Glycol) Chemistry and Biological Applications*, Washington DC, American Chemical Society, 1997
24. Hershfield MS, Buckley RH, Greenberg ML, Melton AL, Schiff R, Hatem C, Kurtzberg J, Markert ML, Kobayashi RH, Kobayashi AL, Abuchowski A: Treatment of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase. *N Engl J Med* 316: 589–596, 1987
25. Hershfield MS: Adenosine deaminase (ADA) deficiency: Clinical expression, molecular basis, and therapy. *Semin Hematol* 35: 291–298, 1998
26. Wu X, Wakamiya M, Vaishnav S, Geske R, Montgomery CM Jr, Jones P, Bradley A, Caskey CT: Hyperuricemia and urate nephropathy in urate oxidase-deficient mice. *Proc Natl Acad Sci USA* 91: 742–746, 1994
27. Priest DG, Pitts OM: Reaction intermediate effects on the spectrophotometric uricase assay. *Anal Biochem* 50: 195–205, 1972
28. Lee CC, Wu X, Gibbs RA, Cook RG, Muzny DM, Caskey CT: Generation of cDNA directed by amino acid sequence: Cloning of urate oxidase. *Science (Washington DC)* 239: 1288–1291, 1988
29. Christen P, Peacock WC, Christen AE, Wacker WEC: Urate oxidase in primate phylogenesis. *Eur J Biochem* 12: 3–5, 1970
30. Sherman MR, Williams LD, Saifer MGP, French JA, Kwak LW, Oppenheim JJ: Conjugation of high molecular weight poly(ethylene glycol) to cytokines: Granulocyte-macrophage colony-stimulating factors as model substrates. In: *Poly(Ethylene Glycol) Chemistry and Biological Applications*, edited by Harris JM, Zalipsky S, Washington DC, American Chemical Society, 1997, pp 155–169
31. Kunitani M, Dollinger G, Johnson D, Kresin L: On-line characterization of polyethylene glycol-modified proteins. *J Chromatogr* 588: 125–137, 1991
32. Hershfield MS, Chaffee S, Koro-Johnson L, Mary A, Smith AA, Short SA: Use of site-directed mutagenesis to enhance the epitope shielding effect of covalent modification of proteins with polyethylene glycol. *Proc Natl Acad Sci USA* 88: 7185–7189, 1991
33. Chaffee S, Mary A, Stiehm ER, Girault D, Fischer A, Hershfield MS: IgG antibody response to polyethylene glycol-modified adenosine deaminase (PEG-ADA) in patients with adenosine deaminase deficiency. *J Clin Invest* 89: 1643–1651, 1992
34. Delnomdedieu M, Hedlund LW, Johnson GA, Maronpot RR: Magnetic resonance microscopy: A new tool for the toxicologic pathologist. *Toxicol Pathol* 24: 36–44, 1996
35. Kaufman JM, Greene ML, Seegmiller JE: Urinary uric acid/creatinine ratio: A screening test for inherited disorders of purine metabolism. *J Pediatr* 73: 583–592, 1968
36. Kelton J, Kelley WN, Holmes EW: A rapid method for the diagnosis of acute uric acid nephropathy. *Arch Intern Med* 138: 612–615, 1978

37. Call NB, Tisher CC: The urinary concentrating defect in the Gunn strain of rats: Role of bilirubin. *J Clin Invest* 55: 319–329, 1975
38. Takahashi N, Chernavvsky DR, Gomez RA, Igarashi P, Gitelman HJ, Smithies O: Uncompensated polyuria in a mouse model of Bartter's syndrome. *Proc Natl Acad Sci USA* 97: 5434–5439, 2000
39. Conger JD, Falk SA: Intrarenal dynamics in the pathogenesis and prevention of acute urate nephropathy. *J Clin Invest* 59: 786–793, 1977
40. Raivio K: Neonatal hyperuricemia. *J Pediatr* 88: 625–630, 1976
41. Chen RH-L, Abuchowski A, van Es T, Palczuk NC, Davis FF: Properties of two urate oxidases modified by the covalent attachment of poly(ethylene glycol). *Biochim Biophys Acta* 660: 293–298, 1981
42. Tsuji J, Hirose K, Kasahara E, Naitoh M, Yamamoto I: Studies on the antigenicity of the polyethylene glycol-modified uricase. *Int J Immunopharmacol* 7: 725–730, 1985
43. Caliceti P, Schiavon O, Veronese FM: Biopharmaceutical properties of uricase conjugated to neutral and amphiphilic polymers. *Bioconjugate Chem* 10: 638–646, 1999
44. Davis S, Park YK, Abuchowski A, Davis FF: Hypouricaemic effect of polyethylene glycol modified urate oxidase. *Lancet* 2: 281–283, 1981
45. Chua CC, Greenberg ML, Viau AT, Nucci M, Brenckman WD Jr, Hershfield MS: Use of polyethylene glycol-modified uricase (PEG-uricase) to treat hyperuricemia in a patient with non-Hodgkin lymphoma. *Ann Intern Med* 109: 114–117, 1988

**Access to UpToDate on-line is available for additional clinical information
at <http://www.jasn.org/>**