

# Differential Expression of the Two Human Arginase Genes in Hyperargininemia

## Enzymatic, Pathologic, and Molecular Analysis

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### Abstract

Previous studies in our laboratory and others have demonstrated in humans and other mammals two isozymes of arginase (AI and AII) that differ both electrophoretically and antigenically. AI, a cytosolic protein found predominantly in liver and red blood cells, is believed to be chiefly responsible for ureagenesis and is the one missing in hyperargininemic patients. Much less is known about AII because it is present in far smaller amounts and localized in less accessible deep tissues, primarily kidney. We now report the application of enzymatic and immunologic methods to assess the independent expression and regulation of these two gene products in normal tissue extracts, two cultured cell lines, and multiple organ samples from a hyperargininemic patient who came to autopsy after an unusually severe clinical course characterized by rapidly progressive hepatic cirrhosis. AI was totally absent (< 0.1%) in the patient's tissues, whereas marked enhancement of AII activity (four times normal) was seen in the kidney by immunoprecipitation and biochemical inhibition studies. Immunoprecipitation-competition and Western blot analysis failed to reveal presence of even an enzymatically inactive cross-reacting AI protein, whereas Southern blot analysis showed no evidence of a substantial deletion in the AI gene. Induction studies in cell lines that similarly express only the AII isozyme indicated that its activity could be enhanced severalfold by exposure to elevated arginine levels. Our findings suggest that the same induction mechanism may well be operative in hyperargininemic patients, and that the heightened AII activity may be responsible for the persistent ureagenesis seen in this disorder. These data lend further support to the existence of two separate arginase gene loci in humans, and raise possibilities for novel therapeutic approaches based on their independent manipulation.

### Introduction

Arginase (L-arginine ureahydrolase; EC 3.5.3.1) is the fifth and final enzyme of the urea cycle, catalyzing the hydrolysis of

arginine to ornithine and urea. Hyperargininemia, resulting from deficiency of this enzyme, is the least frequently described of the heritable defects of ureagenesis, characterized clinically by progressive mental impairment, progressive spasticity, growth retardation, and periodic episodes of hyperammonemia (1–3). Whereas the principal ureagenic enzyme activity (AI)<sup>1</sup> is most abundant in normal mammalian liver (4–6) and absent in the patients (2, 7, 8), studies of rat and human tissues have revealed a second form of arginase in several other organs: most prominently in kidney, but also found to a lesser extent in brain, gastrointestinal tract, and lactating mammary gland. Although this second isozyme (AII) exhibits some similar kinetic and physicochemical properties, it can be distinguished from AI by its lack of cross-reactivity with anti-liver arginase antibody (8), its different electrophoretic mobility in polyacrylamide gels (6), its quantitatively different requirement for divalent cation ( $Mn^{2+}$ ) activation, and its differential inhibition by proline and isoleucine (9). In addition, it is localized to the mitochondrial matrix, whereas AI is cytoplasmic (10). Immunoprecipitation studies have indicated that AI comprises about 98% of the high arginase activity in liver (as well as in red blood cells), but accounts for only 50% of the much lower arginase activity found in kidney and other organs, the remainder being produced by AII (8).

It has been suggested that the existence of this second form of arginase, predominantly in extrahepatic tissues, might be responsible for the persistent near-normal urea production and relatively more mild clinical course in hyperargininemia as compared to other urea cycle disorders. Indeed, although hyperargininemic patients have been shown to lack enzymatically active liver (and red blood cell) arginase (2, 7, 11, 12), a single kidney biopsy in one patient revealed abundant (possibly elevated) residual activity of AII (8).

Taken together, these biochemical and immunoenzymatic data suggest the existence of two distinct structural gene loci encoding the arginase isozymes in man. The recent cloning of cDNA probes for rat (13, 14) and human (15, 16) liver arginase and the finding that these probes fail to cross-hybridize with AII sequences by Southern analysis, Northern analysis, or hybrid-select translation (15–17) lends support to this hypothesis. The liver arginase-deficient patients serve as valuable natural experimental models, and a detailed study of their residual arginase activity is helping to elucidate the differential regulation of the two arginase gene loci. Until now, however, the infrequency of the disease, the generally nonfatal outcome in well-cared-for patients, and the inaccessibility of isozyme AII (found to a significant extent only in brain and deep viscera,

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1. Abbreviations used in this paper: AI, liver arginase; AII, kidney arginase; CRM, cross-reacting material; HEK, human embryonic kidney (cell line).

primarily kidney) has restricted access to only a small kidney biopsy on one of our patients, and the minute amount of tissue obtained limited the extent of the studies that could be performed. Recently, however, we have had access to several deep tissues obtained at autopsy on a hyperargininemic patient who succumbed after an unusually severe clinical course. We report here a pathologic, biochemical, and molecular analysis of this material, along with relevant tissue culture models, and demonstrate that this patient's rather substantial residual arginase activity consisted entirely of the kidney isozyme, AII, supporting the two-gene hypothesis and suggesting unusual mechanistic and therapeutic considerations for this rare in-born error of metabolism.

### Case report

Patient D.L. was a 6-mo-old male, the only child of first cousin parents of Cambodian descent who reported no family history of metabolic disease. He was the 3,410-g product of his 26-yr-old mother's first pregnancy, which was uncomplicated except that the mother's serum was noted to be positive for hepatitis B surface antibody (but negative for hepatitis B surface antigen, hepatitis B core antigen, and hepatitis B core antibody). At 24 h of age the patient was noted to be jaundiced, with a total bilirubin of 11.0 mg/dl (direct 1.1) which increased to 18.5 mg/dl (direct 2.3) within 3 d. Other liver function parameters at this time included alkaline phosphatase 326 IU/liter (normal, 35–100 IU/liter), aspartate transaminase 56 IU/liter (normal 8–42 IU/liter), alanine transaminase 25 IU/liter (normal, 3–30 IU/liter), total protein 4.6 g/dl (normal, 6.0–8.0 g/dl), and albumin 2.7 g/dl (normal, 2.9–5.5 g/dl). TORCH titers were negative, while the hepatitis profile was the same as the mother's and felt to be passively acquired.

Despite phototherapy, bilirubin levels remained elevated and hepatomegaly was noted. In addition, a blood ammonia level of 126  $\mu$ g/dl (normal, 20–55  $\mu$ g/dl) was recorded. At 3 wk of age a percutaneous liver biopsy was performed, revealing nonspecific cholestasis and periportal fibrosis. Quantitative serum amino acids were measured and revealed an arginine level of 1305  $\mu$ mol/liter (normal, 10–114  $\mu$ mol/liter). Red blood cell arginase activity was undetectable (< 1% of normal). The patient was placed on a protein-restricted diet supplemented with sodium benzoate, which resulted in a return of ammonia levels to the normal range.

On the 57th day of life, he contracted a viral respiratory illness and became lethargic. His studies at this time showed a prothrombin time of 80.3 s, partial prothrombin time of > 120 s, ammonia 446  $\mu$ g/dl, albumin 3.7 g/dl, total bilirubin 6.2 mg/dl (direct 2.9), alanine transaminase 68 IU/liter,  $\gamma$ -glutamyl transferase 191 IU/liter (normal, 15–85 IU/liter), and arginine 500  $\mu$ mol/liter (normal, 21–151  $\mu$ mol/liter). Despite adjustments in the patient's diet and sodium benzoate supplementation, the liver function continued to worsen, xanthomas developed, and the patient required repeated hospital admissions for lethargy, respiratory infections, and hyperammonemic episodes. Chromosome analysis showed 46,XY q+, a normal male karyotype variant. At the 500–600-band level, no evidence of a deletion in the region of 6q23, the proposed arginase gene locus (17), was detected. His final admission for anorexia, fever, and dehydration was marked by refractory acidosis, hyperammonemia (447  $\mu$ g/dl), deteriorating neurologic status, and heart failure, terminating in death by bronchopneumonia and cardiac arrest at age 6 mo.

## Methods

**Clinical material.** All therapeutic and diagnostic clinical procedures, including postmortem examination, were performed at Children's Medical Center of Dallas, except for the initial red blood cell arginase assay which was done at Baylor College of Medicine, Houston (laboratory of Dr. A. L. Beaudet), and the cytogenetic study which was done at the University of Texas Health Science Center at Dallas. All studies conformed to the guidelines established by Institutional Review Boards at the respective institutions. Tissue sections for light microscopy were fixed in 10% buffered formalin, embedded in paraffin, cut at 5  $\mu$ m, and stained with hematoxylin and eosin. Tissue samples for biochemical analysis at UCLA were quick-frozen at  $-70^{\circ}\text{C}$  within 2 or 4 h after death and maintained at this temperature until homogenization. Control tissues were obtained from the surgical pathology and autopsy services of UCLA Hospital and treated in a similar manner.

**Chemicals.** Arginine, isoleucine, and all buffers were obtained from Sigma Chemical Co., St. Louis, MO, and were of the highest grade available. Guanidino[ $^{14}\text{C}$ ]arginine (53  $\mu\text{Ci}/\mu\text{mol}$ ) was purchased from Amersham-Searle, Chicago, IL. Staphylococcal protein A (IgG-sorb, in 10% cell suspension) was obtained from The Enzyme Center, Boston, MA.

**Tissue extracts.** Organ tissues were thawed, minced, and homogenized in 1–2 vol of water or 0.01 M Tris-HCl, pH 7.4, using a Polytron (Brinkmann Instruments Co., Westbury, NY). The homogenates were centrifuged at 30,000 g for 60 min and the resulting supernatants used for all subsequent studies.

**Cell cultures.** Propagation of cell cultures and preparation of their extracts was as previously described (18, 19). Cell lines used in this study were H4-II-E-C3 (H4), a rat hepatoma line, and human embryonic kidney cells (HEK) transformed with sheared adenovirus type 5 DNA (20).

**Enzyme assay.** Arginase activity was determined using our previously described modification (21) of the urease method of Schimke (22). Some tissue and cell extracts were first diluted appropriately to place their activity within the linear range of the assay. A unit of enzyme activity is defined as 1  $\mu\text{mol}$  arginine hydrolyzed per mg total protein in 30 min. Protein was determined by the method of Bradford (23), with bovine serum albumin used as the standard. Coomassie blue reagent was obtained from Bio-Rad Laboratories, Richmond, CA.

**Antibodies.** Rabbit anti-human liver arginase was prepared as described previously (8). Goat anti-rabbit immunoglobulin-horseradish peroxidase complex was obtained from Bio-Rad Laboratories.

**Immunoprecipitation of arginase in tissue extracts.** Immunoprecipitation procedures were essentially as described previously (8, 24), with minor modifications. Tissue extracts, diluted (if necessary) to a final activity of 0.2–5.0 U per 50 or 100  $\mu\text{l}$ , were incubated with varying amounts of antiarginase serum in a constant volume for 30 min at  $37^{\circ}$  and then overnight at  $4^{\circ}\text{C}$ . Total protein in the mixtures was held constant by addition of appropriate amounts of control (preimmune) rabbit serum. Antigen-antibody complexes were precipitated by adding 50 or 100  $\mu\text{l}$  IgG-sorb, incubating at  $4^{\circ}\text{C}$  for 10 min, and centrifuging in a microfuge at top speed for 5 min. Triplicate 50 or 100  $\mu\text{l}$  aliquots of the supernatants were then assayed for arginase activity in the usual manner.

**Immunoprecipitation-competition assays.** To assay for the presence of immunologically cross-reacting material (CRM) in patient D.L., his tissue extracts were mixed with those of normal controls at ratios ranging from 1:5 to 7:1 (milligram protein per milligram protein). A fixed amount of antiarginase serum (previously determined adequate to precipitate > 95% of the arginase activity in a normal liver extract), was added to the tubes, and immunoprecipitation and arginase assay performed as above.

**Competitive inhibitor studies.** Differential competitive inhibition of arginase activity, reported to be a distinguishing characteristic of the AI and AII enzymes (9), was performed by assaying arginase activity in tissue extracts in the presence of varying amounts (0–20 mM) of iso-

leucine, while holding arginine and all other components of the assay constant.

**Western analysis.** To confirm the presence or absence of CRM in patient D.L., tissue extracts were electrophoresed in SDS-10% polyacrylamide gels, blotted by electrotransfer on to nitrocellulose filters, treated with anti-liver arginase antibody, and probed with goat anti-rabbit immunoglobulin-horseradish peroxidase complex according to published procedures (24). Detection was via a colorimetric reaction, using 4-chloro-1-naphthal as substrate.

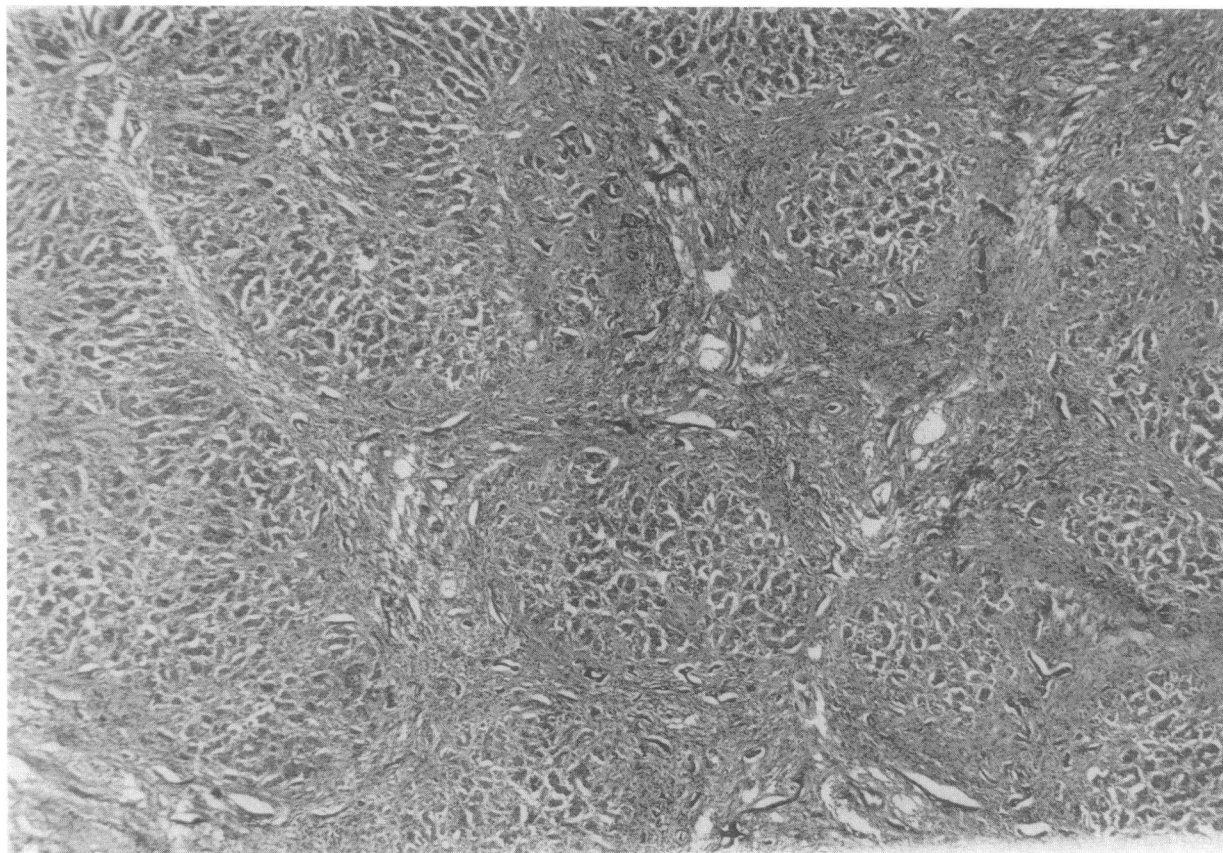
**Induction studies.** Arginine at various concentrations (0–6 mM) was added to the culture media of HEK and H4 cells. After 5 d of incubation, cell extracts were prepared and the arginase activity assayed by the usual procedure.

**Southern blot analysis.** Genomic DNA was isolated from frozen tissue pieces of D.L.'s liver by pulverizing in liquid N<sub>2</sub> and extraction with phenol-chloroform according to published protocols (25). Control DNA from peripheral blood leukocytes of normal individuals was prepared by standard methods (26). Restriction enzyme digestion, agarose gel electrophoresis, capillary blotting onto nylon membranes (GeneScreen Plus, New England Nuclear, Boston, MA), and hybridization with a <sup>32</sup>P-labeled, 1400-bp human liver arginase cDNA probe (16) was performed as described (27, 28).

## Results

**Pathologic findings.** Postmortem examination was performed 90 min after death. Major findings at autopsy were limited to the respiratory and gastrointestinal tracts; examination of the brain was excluded by parental consent. The proximate cause

of death appeared to be a confluent bronchopneumonia; although no viral inclusions were seen on microscopic examination, cytomegalovirus was cultured from lung tissue. The liver weighed 350 g and demonstrated micronodular cirrhosis. Microscopic examination of the liver and three pre-mortem percutaneous liver biopsies demonstrated the evolving consequences of chronic cholestasis. On the 20th and 57th days of life the hepatic lobular architecture and bile ducts were intact and only a moderate accumulation of bile pigment was noted within the hepatocytes and canaliculi. A minimal lymphocytic infiltrate accompanied by a mild reactive fibrosis was present in the portal tracts. Tissue obtained on the 153rd day of life and at autopsy (Fig. 1) demonstrated moderate cholestasis, ballooning degeneration of hepatocytes, focal destruction of bile ducts, marked chronic inflammation of the portal tracts, striking proliferation of cholangioles, and extensive portal fibrosis with extension along the perilobular septa. In none of the tissue samples was acute inflammation or fatty change evident. Electron microscopic studies demonstrated the progressive accumulation of amorphous, granular, and laminated forms of cholestatic pigment. Dilated smooth endoplasmic reticulum and normal numbers of mitochondria, lipid droplets and a-rosettes were seen in all biopsies. In the day 153 biopsy, the Golgi cisterns were prominent and tortuous, and intercellular collagen was conspicuous. Since the pathologic features were similar to those observed in cases of extrabiliary obstruction, and because chronic cholestasis was not observed in the only other arginase-deficient patient whose liver was examined



**Figure 1.** Histopathology of liver in fatal hyperargininemia. At autopsy the liver demonstrated cholestasis, portal fibrosis, chronic inflammation, and proliferation of cholangioles. (Hematoxylin and eosin,  $\times 100$ .)

ultrastructurally (2), patency of the bile ducts was carefully confirmed at autopsy. Findings in other organ systems included acute renal tubular necrosis, skeletal muscle atrophy, and lipid vacuolization of myocardial fibers.

**Enzyme assays.** Frozen postmortem tissue samples on patient D.L. were available of liver, kidney, heart, and spinal cord. Assayed arginase activity in extracts of these tissues and, where applicable, corresponding normal tissues, is listed in Table I. As expected in light of the earlier diagnostic red blood cell arginase assay, arginase activity in liver tissue of D.L. was extremely low, about 3% of that in normal control liver. In contrast, the arginase activity in kidney extract of D.L. was abundant and actually somewhat elevated above that in control—a finding in concert with the augmented activity found in the only other hyperargininemic patient in whom kidney tissue was assayed (8). As shown in the table, D.L.'s kidney extract had double the total activity, in raw units, of control kidney. However, as will be substantiated below, the activity in D.L.'s kidney was composed entirely of isozyme AII, whereas half the activity in a normal kidney is due to AI (8). Thus, the activity of arginase AII in D.L.'s kidney was actually elevated four times above normal, a figure in agreement with the three- to fivefold enhancement seen in our earlier kidney biopsy study (8). Although not controlled side-by-side in the present study, the miniscule amounts of active arginase found in D.L.'s heart and spinal cord tissue are consistent with previously observed levels in organs other than liver and kidney from both rats and humans (5, 6, 22).

**Immunoprecipitation studies.** In order to ascertain whether the residual enzymatic activity in D.L.'s liver was due to the small amount of AII isozyme normally present in that tissue as opposed to a mutated AI enzyme of very low activity, and also to determine the relative contributions of AI and AII to his high kidney extract activity, these tissue extracts were subjected to immunoprecipitation with our rabbit anti-human liver arginase antibody. Previous studies have verified that this antibody does not cross-react with the mitochondrial isozyme (8). Fig. 2 shows the arginase activity remaining in the supernatants of liver and kidney extracts from D.L. and normal controls after incubation with increasing amounts of anti-serum. As expected, the activity in normal liver was precipitated by anti-AI antibody almost quantitatively, and the activity in normal kidney by about half, consistent with the 98% and 50% contributions of AI to total arginase activity in these two tissues, respectively. In contrast, none of the enzyme activity in either the liver or kidney extracts from the patient was precipitated, even at very high levels of anti-AI antibody.

Table I. Arginase Activity in Tissue Extracts

Tissue	Activity	
	Normal	Patient D.L.
	U	
Liver	28.90	0.98
Kidney	0.74	1.43
Heart	ND	0.01
Spinal Cord	ND	0.02

ND, not determined.

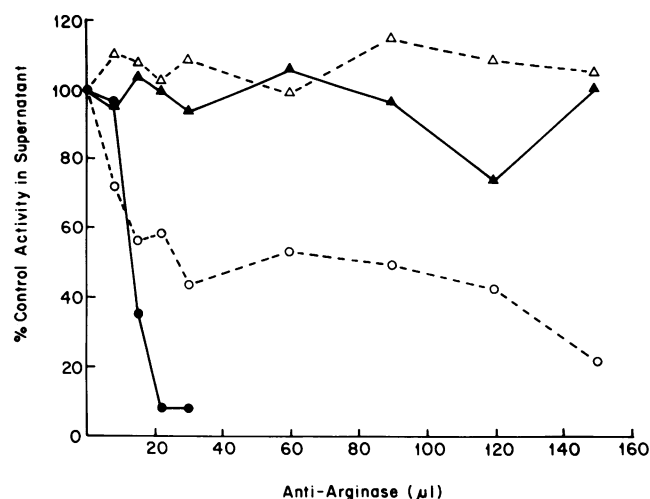
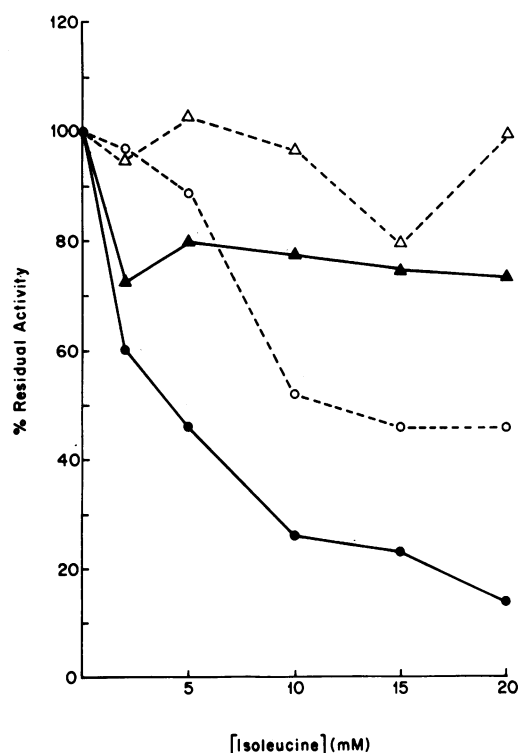


Figure 2. Reaction of anti-human liver arginase antibody with arginase in tissue extracts of normals and patient D.L.: normal liver (●), normal kidney (○), D.L. liver (▲), D.L. kidney (△). Each point represents the average of triplicate determinations from one experiment. Each tissue was tested at least twice.

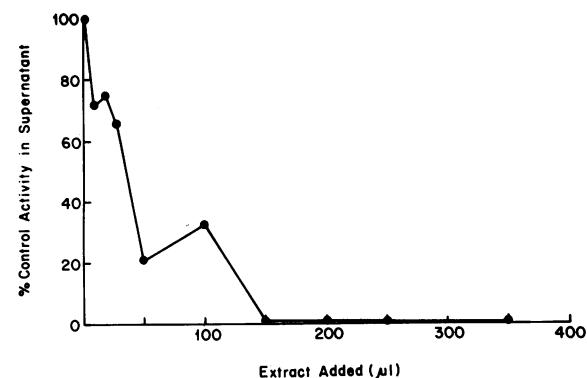
**Enzyme inhibition study.** While the above results seemed to indicate that all of D.L.'s residual active arginase in both his liver and kidney were the AII isozyme, the possibility remained that the residual activity in liver and perhaps a portion of that in kidney were due to a mutant AI enzyme possessing not only abnormally low catalytic activity but also poor antigenic reactivity with anti-AI antibody. Distinguishing the liver and kidney isozymes by other than immunologic methods is not a trivial matter, since the two enzymes behave identically by almost all biochemical criteria tested, including  $K_m$  for arginine,  $K_i$  for lysine and ornithine, heat stability at 68°C and 81°C, pH optimum, pH stability, and requirement for heat activation (6). However, we and others have observed differential inhibition of the two forms in rat by proline and branched-chain amino acids (9, 29). The most dramatic effect was seen with isoleucine, which markedly inhibited activity of the liver enzyme but only slightly diminished AII activity (9). Therefore, we assayed D.L.'s residual arginase activity in the presence of increasing isoleucine concentrations (from 0 to 20 mM) and compared the results to those using normal tissue extracts. As shown in Fig. 3, normal liver extract was markedly inhibited by isoleucine, while the effect in normal kidney was only partial, owing to the 50% contribution of noninhibitable AII enzyme to total arginase activity in this tissue. However, neither of D.L.'s tissue extracts demonstrated such inhibition. While it is still formally possible that D.L.'s AI mutation affected isoleucine inhibition as well, these results are again consistent with the absence, in both his liver and kidney, of the AI isozyme.

**Immunoprecipitation-competition studies.** All of the studies presented thus far have monitored the presence and behavior only of enzymatically active arginase in patient D.L.'s tissues, as quantitated by standard arginase assay. These studies indicated the absence of any arginase activity recognizable by our normal anti-human liver arginase antibody. However, if D.L.'s tissues contained an immunologically precipitable but inactive enzyme, the above studies would not have detected it. To check this possibility, we wished to see whether the D.L.



**Figure 3.** Effect of isoleucine on activity of arginase in tissue extracts from normals and patient D.L.: normal liver (●), normal kidney (○), D.L. liver (▲), D.L. kidney (Δ). Each point represents the average of triplicate determinations from one experiment. Each tissue was tested at least twice.

extracts contained some enzymatically silent molecule with the ability to inhibit immunoprecipitation of normal liver arginase activity by competing for anti-AI antibody. The results of such a study are presented in Fig. 4. D.L.'s liver extract was added over a wide range (1:5 to 7:1) of total protein/total protein ratios to normal human liver extract, and an immunoprecipitation of arginase activity performed in the usual manner. Because D.L.'s cirrhotic liver contained much fibrous

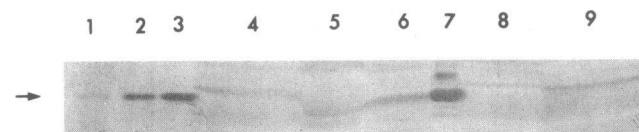


**Figure 4.** Immunoprecipitation-competition experiment. Extract from normal liver was incubated with increasing amounts of liver extract from patient D.L. and enough anti-human liver arginase antibody to precipitate > 95% of the AI activity. Residual activity in the supernatant after immunoprecipitation was assayed as a measure of competition for antibody binding as described in Methods.

tissue in place of normal hepatocytes, extractable protein was quite low and necessitated going to larger than usual reaction volumes to achieve the high competition ratios we desired. Also, final enzyme activity remaining in the supernatants after immunoprecipitation had to be corrected for the addition of the small amount of residual activity already present in D.L.'s liver extract. As shown in the figure, even after correcting for these factors, there was no evidence of inhibition of normal AI immunoprecipitation by anything in the D.L. extract, even when the latter was added to the antigen-antibody reaction in very high amounts (up to 7× excess). Thus, patient D.L.'s liver was found to contain neither an enzymatically active nor an immunologically recognizable translation product of the AI gene.

**Western analysis.** The apparent absence of CRM in patient D.L. was further confirmed by Western blot of his tissue extracts. As illustrated in Fig. 5, anti-AI antibody probing of the resultant filters failed to detect the presence of a protein band at the usual position of normal human liver arginase.

**Tissue culture studies.** The deduced enzymatic profile in patient D.L.'s kidney is fortuitously echoed in one of our tissue culture models, HEK: unlike normal adult kidney, which expresses equal parts of the AI and AII isozymes, this cell line expresses the AII form exclusively, as demonstrated by its lack of precipitability with anti-AI antibody (19). However, D.L.'s kidney activity additionally differs from normal in that it appears to be elevated about fourfold over control levels (see above). To test the hypothesis that this "induction" might be caused by the high circulating arginine levels experienced in this disorder, we attempted to see whether the same phenomenon could be mimicked in the HEK cell culture model. Fig. 6 shows the effect on total arginase activity levels in HEK extracts produced by exposure of the cells to increasing concentrations of arginine (from 0 to 6 mM). Arginase activity clearly increased up to fourfold over baseline at high arginine concentration, approximately the same magnitude of induction calculated for D.L.'s kidney extract. In contrast, no induction by arginine was seen for the AI enzyme activity in H4 cell extracts (broken line), an interesting result considering that liver arginase (AI) is probably expressed constitutively in vivo (10).



**Figure 5.** Western blot analysis of D.L. liver and kidney extracts using anti-human liver arginase. Lane 1, purified rat liver arginase; lanes 2 and 3, different amounts of purified human liver arginase; lane 4, D.L. liver extract; lanes 5, 6, and 7, normal human liver extract at progressively advanced steps of the arginase purification procedure; lane 8, D.L. kidney extract; lane 9, normal human kidney extract. Position of liver arginase band indicated by arrow. Note that D.L.'s liver extract exhibits no CRM at the position of normal liver arginase, although a faint unrelated contaminating band of larger molecular weight present in normal kidney extract is seen in D.L.'s tissue extracts as well. The AI band in normal crude liver extract (lane 5) migrates ahead of the arrow due to distortion from the large amount of other proteins present, but D.L.'s extracts exhibit no cross-reacting species at this position either.

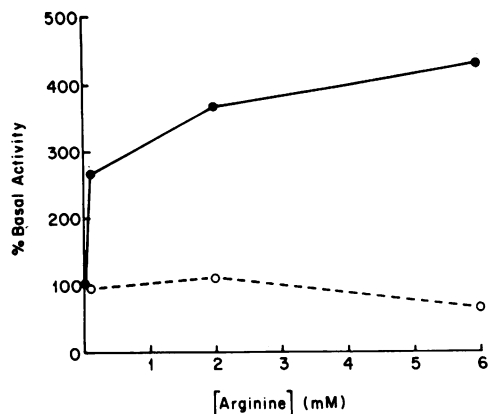


Figure 6. Effect of increased arginine levels on arginase activity in tissue culture extracts of HEK cells (●) and H4 cells (○). Enzyme activity was measured 5 d after adding varying amounts of arginine to the culture media.

**Southern blot analysis.** Hybridization studies of DNA isolated from D.L.'s liver tissue, digested with a number of restriction enzymes and probed with our nearly full-length human liver arginase cDNA clone (16) revealed no differences in band pattern on Southern blots as compared to normal human control DNA (Fig. 7). Despite the absence of CRM, these results indicate that patient D.L.'s lack of liver arginase activity was not the result of a substantial deletion in the AI gene.

## Discussion

Although rare, arginase deficiency is now a well-recognized disorder with relatively uniform signs and symptoms in the patients described to date (1, 2, 3). When suspicions are raised

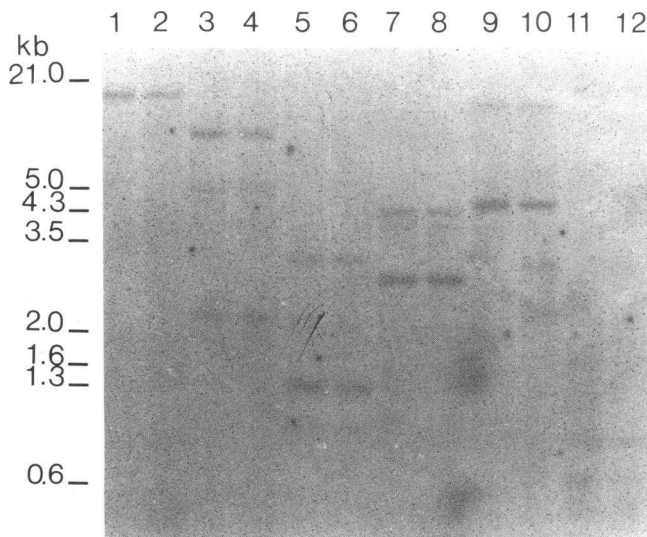


Figure 7. Southern blot analysis of genomic DNA from patient D.L. (even-numbered lanes) compared to normal human control DNA (odd-numbered lanes), digested with the following restriction enzymes and probed with a 1,400-bp,  $^{32}$ P-labeled human liver arginase (AI) cDNA (16): lanes 1 and 2, *EcoRI*; lanes 3 and 4, *BglII*; lanes 5 and 6, *HindIII*; lanes 7 and 8, *TaqI*; lanes 9 and 10, *Pst I*; lanes 11 and 12, *RsaI*.

by the discovery of elevated arginine levels on urine or plasma amino acid screening, the diagnosis is usually confirmed by assaying arginase activity in the patient's red blood cells. The erythrocyte line is considered to be a secondary (perhaps fortuitous) expressor of the AI isozyme predominantly found in liver, the main site of ureagenesis; earlier kinetic and immunologic studies had indicated an identity between the arginases of these two cell types (30–32). However, direct confirmation of the inferred enzyme deficiency in liver in hyperargininemia has taken somewhat longer since it requires liver biopsy, a procedure not typically mandated for diagnostic or therapeutic purposes in these patients. Liver biopsies with confirmatory tissue arginase assays have been reported in only two previous patients (2, 7), and there have been no documented autopsy studies. Assessment of the even more elusive kidney isozyme and its possible role in mitigating the impact of deficient hepatic ureagenesis has been even harder to come by, with kidney biopsy having been performed in only a single case previously (2).

With the present case having come to autopsy, the consequent availability of multiple internal organ samples frozen soon after death has provided a unique opportunity to address these questions. This case also differed from most of those reported previously by virtue of its unusually severe clinical course, characterized most conspicuously by progressive hepatic cirrhosis and failure, leading to death at the age of 6 mo. Such features have not usually been associated with arginase deficiency or indeed any of the urea cycle disorders, for although the enzymes of this pathway are located in the liver, the major consequence of their deficiency, hyperammonemia, exerts its toxic effect principally on the brain. However, ornithine transcarbamylase deficiency may display variable non-specific signs of liver pathology such as microvesicular steatosis and stellate portal scarring (1), and severe liver fibrosis has been reported in one case of argininosuccinate lyase deficiency (33). Recently, Jorda et al. (34) reported a case of arginase deficiency with a rapidly fatal clinical course similar to ours and exhibiting a moderate degree of portal fibrosis. They also observed prominent macrovesicular steatosis and giant mitochondria, neither of which were present in our case. Heterogeneity in the mutation responsible for arginase deficiency has been suggested (3, 10) and may be responsible for variations in the clinical features. Still, the presence of hepatitis antibodies in both mother and patient suggests another plausible source (possibly synergistic) of the liver pathology in our case.

Our patient demonstrated markedly reduced arginase activity in premortem red blood cell assays and postmortem liver tissue. By kinetic and immunologic studies we have shown that the small amount of residual arginase activity present in the liver did not arise from either a catalytically normal AI enzyme greatly reduced in quantity or a mutant AI enzyme of low catalytic activity. Rather, the residual activity was due solely to presence of the AII enzyme which contributes about 2% of the total arginase activity in a normal liver (4, 8). These studies support the concept of clinical hyperargininemia resulting from some structural or regulatory mutation at the AI gene locus, causing loss of activity of the liver isozyme. Indeed, immunoprecipitation-competition and Western blot studies in this patient revealed complete absence of even an inactive AI protein, as shown by the lack of CRM. This finding is mirrored in one of our earlier patients (3) but not in others (8), suggest-



ing the existence of mutational heterogeneity at the AI locus. Southern blot analysis of genomic DNA from both these patients using our cloned human liver arginase cDNA as probe has failed to detect any substantial deletion of the AI gene, suggesting the existence of either a regulatory mutation lying outside the AI structural gene, or a point mutation leading to rapid degradation of the AI enzyme or its antigenic sites.

The enzymatic profile of D.L.'s kidney extract is of interest with regard to the speculated physiologic function of the AII enzyme in hyperargininemia and because this tissue has never before been accessible to extensive study in this disorder, the only previous material consisting of a 0.26 g kidney biopsy in one of our patients (8). The normal function of the AII isozyme is not known with certainty. With kidney arginase activity about 100-fold lower than that in liver (6), it is not felt to play a major role in ureagenesis in normals. Some workers have theorized that its chief metabolic function may be in the synthesis of ornithine as a precursor of proline and glutamate (35), with the latter then feeding into important neurotransmitter pathways in the nervous system. At any rate, it seems likely that for it to be of use in supplementing deficient ureagenesis in hyperargininemia, its activity level would have to be augmented to some degree. We have now demonstrated such augmentation (by about fourfold) in two patients, and have suggested by companion tissue culture studies that this induction is most likely brought about by elevated plasma arginine levels. Interestingly, such a phenomenon has already been documented in vivo in an animal model, albeit a nonmammalian one. Birds are considered non-ureotelic organisms, but they do utilize a mitochondrial kidney arginase to degrade dietary arginine, and the urea thus produced appears in their excreta. Chu and Nesheim (36) have demonstrated that the activity of this arginase can be increased up to fourfold by elevated plasma arginine levels (produced by infusing arginine into wing veins). Considering that the mammalian kidney arginase is also mitochondrial, it may well be a more ancient enzyme than the mammalian liver form and possibly rather closely related evolutionarily to the chicken enzyme, particularly with regard to its inducible recruitment for arginine hydrolysis under hyperargininemic conditions.

Our finding of markedly enhanced kidney arginase (AII) activity in a patient with absent liver arginase (AI) strongly supports the idea that these two isozymes are specified by separate and distinct genetic loci which are subject to independent regulatory control. Indeed, our patient studies and tissue culture data (HEK vs. H4) suggest that in at least one regulatory parameter, inducibility by changing substrate concentrations, the two enzymes are quite different. They must also be quite different structurally, as indicated by their failure to cross-react immunologically and by the lack of detectable cross-hybridization on northern blots between our cloned human liver arginase cDNA and the abundant kidney arginase mRNA in HEK cell extracts (15, 16). All of these findings render alternative possibilities such as posttranslational modification of the product of a single genetic locus highly unlikely. Still, ultimate proof of the two-gene hypothesis must be deferred until the kidney arginase gene, too, has been cloned, sequenced, and mapped. This undertaking is now in progress in our laboratory.

No clinical disorder involving deficiency of the kidney arginase has yet been recognized. The reason for this could be either that its effects are too subtle to be noticed or, at the other

extreme, that the effects are so severe as to be lethal in early embryonic life. One possible explanation may be that any deficiency in kidney arginase is quite well compensated for by the vastly greater activity of liver arginase or that its abnormalities are not defined by hyperargininemia. Conversely, in light of our findings reported here, the question of whether kidney arginase could ever fully compensate for absence of the liver enzyme in hyperargininemia becomes an intriguing one. We have shown that its activity is already somewhat enhanced in these patients, probably induced by increased substrate concentration. Further study of the kidney isozyme and its genetic locus may illuminate new strategies for enhancing its activity still further, possibly setting the stage for a novel therapeutic approach to this disorder.

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