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Dubin-Johnson-like Syndrome in Golden Lion Tamarins (*Leontopithecus rosalia rosalia*)

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Abstract. On routine blood screens, persistent conjugated hyperbilirubinemia was discovered in two groups of closely related adult female golden lion tamarins (*Leontopithecus rosalia rosalia*, $n = 8$). Bromosulfophthalein (BSP) retention tests were performed on four hyperbilirubinemic and three control tamarins. BSP excretion was delayed in hyperbilirubinemic tamarins as compared with controls. Grossly, liver of affected tamarins was dark brown to black, with a prominent reticulated pattern. Histologic examination revealed abundant intrahepatic pigment, primarily in a centrilobular and midzonal distribution. Most of the pigment did not react with Perls' Prussian blue method for iron, Hall's method for bilirubin, or the Armed Forces Institute of Pathology acid-fast method for lipofuscin but was positive with Fontana and lipofuscin-ferric ferricyanide reduction techniques. Liver from control golden lion tamarins had intrahepatocellular Perls' iron-positive pigment diffusely throughout the lobule with a small amount of Fontana method-positive pigment. Ultrastructurally, hepatocytes from a hyperbilirubinemic tamarin contained pleomorphic electron-dense structures within lysosomes. Transport studies demonstrated secretion of fluorescein isothiocyanate-labeled glycocholic acid, a fluorescent bile acid analog, into bile canaliculi and no secretion of carboxydichlorofluorescein diacetate, a non-bile acid organic anion, by liver from a hyperbilirubinemic tamarin. In contrast, control liver secreted carboxydichlorofluorescein diacetate readily into bile canaliculi. The clinicopathologic presentation of this syndrome in golden lion tamarins is similar to that described for Dubin-Johnson syndrome of human beings.

Key words: Dubin-Johnson syndrome; golden lion tamarins; hyperbilirubinemia; liver; pigment.

In human beings, Dubin-Johnson syndrome is an autosomal recessively transmitted disorder characterized by an inherited hepatic defect in the transfer of non-bile acid organic anions from hepatocytes into the bile. This defect results in intermittent, chronic nonhemolytic, predominately conjugated hyperbilirubinemia and a black liver. Although this disease is benign in human beings, its recognition and understanding are important to exclude more serious liver diseases and to gain insight into hepatic transport mechanisms.

Mutant Corriedale sheep, TR^- rats, and EHBR rats have similar hepatic excretionary abnormalities and are animal models for Dubin-Johnson syndrome;[4–7,11,13] however a nonhuman primate, being closer phylogenetically, may be a better animal model in which to study a human disease. The golden lion tamarin (*Leontopithecus rosalia rosalia*) is an endangered nonhuman primate that is being bred in captivity and gradually reintroduced into its natural habitat, the South American Atlantic costal forests.[16] This is the first report of a Dubin-Johnson-like syndrome with persistent conjugated hyperbilirubinemia and pigmented liver in nonhuman primates.

Materials and Methods

As part of an ongoing preventive healthcare program, golden lion tamarins bred at the National Zoological Park (Washington, DC) for reintroduction into South America routinely have blood collected for analysis and undergo physical examinations upon entering and exiting the zoo, as well as periodically during their stay. Persistent hyperbilirubinemia was detected in eight asymptomatic adult female golden lion tamarins (Nos. 1–8) on routine blood screens and subsequent blood samples. These animals were designated for further study. Three healthy adult golden lion tamarins (two female and one male; Nos. 9, 10, 11 respectively) without hyperbilirubinemia served as controls.

Golden lion tamarins at the National Zoological Park are housed in different areas of the zoo and fed similar diets of canned marmoset diet (Hill's Pet Products, Topeka, KS) supplemented with various fruits and vegetables. Three affected

(Nos. 1, 2, 4) and one control (No. 10) tamarin had given birth; four affected (Nos. 1, 2, 4, 8) and the two female control (Nos. 9, 10) tamarins had been implanted with melengesterol acetate contraceptives.

Direct and indirect serum bilirubin concentrations were determined in seven of the eight tamarins with hyperbilirubinemia. In addition, bromosulfophthalein (BSP) retention tests were conducted in four of the eight hyperbilirubinemic and all control animals. Animals fasted for 6 to 12 hours prior to testing and were anesthetized with ketamine HCl. BSP (5 mg/kg) was injected into the saphenous vein, and blood samples were drawn 60 and 120 minutes later from the femoral vein.

Pedigree information from the International Golden Lion Tamarin Studbook[1] was used to determine the genealogy of the eight hyperbilirubinemic and three control tamarins.

Laparotomies were performed on all eight affected and three control tamarins to obtain liver biopsy samples. Sections of liver were immediately placed in Trump-McDowell's solution and processed for light microscopic examination. Six-micrometer sections were embedded in paraffin and stained with hematoxylin and eosin, Fontana, Fontana after bleaching, Perls' Prussian blue method for iron, Hall's method for bilirubin, the Armed Forces Institute of Pathology acid-fast method for lipofuscin,[19] and lipofuscin-ferric ferricyanide reduction techniques.[20] Liver specimens from a hyperbilirubinemic tamarin (No. 4) and a control tamarin (No. 9) were further post-fixed in 1% osmium tetroxide, dehydrated in a series of graded alcohols, embedded in epon, sectioned on a ultramicrotome, and stained with 4% uranyl acetate in 35% methanol with Renolds lead citrate. Ultrathin sections were examined with a Zeiss EM 10 electron microscope.

Additional liver samples obtained from tamarin Nos. 8 (hyperbilirubinemic) and 11 (control) were prepared for investigation of hepatobiliary transport of carboxydichlorofluorescein diacetate (CFDA), a non-bile acid organic anion, and fluorescein isothiocyanate-labeled glycocholic acid (FITC-GC), a bile acid. After excision, liver biopsies were immediately flushed to remove blood by inserting an 18-gauge needle into the large vessels exposed on the cut surface and perfusing with 10 ml of heparinized buffer (5 mM KCl, 0.3 mM KH_2PO_4, 137.9 mM NaCl, 0.3 mM Na_2PO_4, 5.5 mM D-glucose, 10 mM Hepes [pH 7.4]) warmed to 37 C and saturated with 100% O_2. A thin section of the biopsy was used for transport studies in liver slices, and the remaining tissue was minced into 1-mm cubes for isolation of hepatocyte doublets.

Liver cubes were incubated in 0.05% collagenase in L-15 medium (GIBCO, Grand Island, NY) for 15 to 20 minutes and continuously shaken. The cell suspension was rinsed in L-15, centrifuged, and plated in 35-mm culture dishes on glass coverslips in modified Eagle medium containing 10% fetal calf serum and incubated at 37 C in air/5% CO_2 for 4 to 8 hours. Thirty minutes prior to confocal microscopic examination, FITC-GC (0.5 μm) or CFDA (0.2 μm) were added to the culture dishes. The coverslips were assembled in a Dvorak-Stotler cell chamber, and doublets were examined using a confocal microscope.[14] Biliary secretion in liver slices was studied by injecting the fluorescent probe, CFDA (0.1 μm) dissolved in Krebs-Henseleit Buffer (pH = 7.4) containing heparin 500 U/ml, into the large exposed vessels. The control liver slice was injected with CFDA (0.1 μm). Two liver slices from the hyperbilirubinemic tamarin were available for injection with both CFDA and FITC-GC (0.2 μm). Slices were placed on ice, and confocal microscopic examination was performed within 30 to 90 minutes. Hepatobiliary secretion in hepatocyte doublets and liver slices was examined by use of a confocal laser scanning microscope (MRC-500, Bio-Rad) with a 100× plan objective (Zeiss) operating in the epifluorescent mode with light of 488 nm wavelength.

Results

Persistent hyperbilirubinemia was documented in eight nonanemic adult female golden lion tamarins (Table 1). Elevated serum bilirubin concentrations ranged from 1.0 to 8.4 mg/dl (normal mean total serum bilirubin ± standard deviation = 0.4 ± 0.15 mg/dl based on blood values from healthy adult male and female golden lion tamarins at the National Zoological Park Clinical Laboratory). There was an elevation in direct (conjugated) bilirubin. Six of eight animals had icteric serum on at least one occasion. Three of eight tamarins had normal bilirubin concentrations as juveniles and young adults (<6 years old); but once they developed hyperbilirubinemia, bilirubin concentrations tended to remain elevated, only rarely returning to the normal range.

Bromosulfophthalein (BSP) retention was prolonged in hyperbilirubinemic animals as compared with control animals (Table 2). BSP retention was less than 2% at 60 and 120 minutes post-injection in control animals. In hyperbilirubinemic tamarins, BSP retention was always greater than 2.4% and averaged 6.7% at 120 minutes after injection.

Serum aspartate aminotransferase (AST) and alkaline phosphatase (ALP) concentrations in all control and hyperbilirubinemic tamarins were within normal ranges (normal AST = 38–258 IU and normal ALP = 22–138 IU for adult golden lion tamarins at the National Zoological Park Clinical Laboratory). Serum alanine aminotransferase (ALT) concentrations were within normal range (normal ALT = 4–155 IU for adult golden lion tamarins at the National Zoological Park Clinical Laboratory) for all control and five of eight hyperbilirubinemic tamarins. Tamarin No. 4 had a single elevated ALT concentration of 160 IU out of seven samples over 4 years. Tamarin No. 6 also had one elevated ALT concentration of 276 IU out of five samples over 9 months, and tamarin No. 8 had two abnormal ALT concentrations of 439 IU and 296 IU out of 11 samples over 6 years.

Urinalysis could not be performed because of the small amount of urine produced by the tamarins, fecal contamination, and group housing.

Examination of the genealogy revealed that the hy-

Table 1. Age-specific bilirubin values in 11 golden lion tamarims.

Tamarin No.	Age at Sampling (years)	Bilirubin (mg/dl)		
		Total	Direct	Indirect
1	14.7	1.8		
	14.8	1.2		
	15.0	0.7		
2	4.1	0.4		
	4.9	0.4		
	6.0	0.3		
	7.7	6.0		
	7.8	3.9		
	8.0	2.5		
	8.0	5.4	3.5	1.9
	8.5	2.4		
3	5.1	5.2		
	5.1	3.8		
	5.3	2.7	1.3	1.4
4	0.6	0.2		
	1.3	0.6		
	1.9	1.0		
	3.8	0.2		
	4.3	5.2		
	4.4	3.2	1.5	1.7
	5.2	8.4		
5	0.7	0.1		
	1.4	0.3		
	2.5	0.7		
	3.8	8.0		
	3.9	6.6	4.2	2.4
6	10.6	2.2		
	10.6	2.4		
	11.3	2.9		
	11.4	2.1		
	11.4	1.8		
	13.3	1.6	0.7	0.9
7	4.9	1.8		
	7.2	6.4		
	7.2	7.0		
	7.5	8.4		
	9.2	8.3	3.6	4.7
8	4.9	4.4		
	5.6	2.8		
	6.2	5.6		
	6.3	5.4		
	6.3	4.5		
	6.3	4.7		
	7.2	6.0		
	7.3	5.2		
	7.8	5.8		
	8.1	4.6	2.6	2.0
	8.5	4.7		
9	2.8	0.4		
	3.1	0.3		
	4.1	0.5		
	5.5	0.4		
	6.3	0.3		
10	6.1	0.3		
	8.2	0.5		
	10.5	0.3		
11	4.2	0.4		
	4.3	0.4		
	5.0	0.1		
	5.4	0.5		

perbilirubinemic animals represented two groups of closely related animals: 1) a mother, her three daughters, and her granddaughter, and 2) an aunt and two nieces (Fig. 1). All affected tamarins have many unaffected siblings and several also have many half siblings. Many of these animals are housed at other zoos. Because of these complexities, a diagrammatic depiction of the full pedigree and a detailed study of the inheritance of this syndrome is beyond the scope of this report. Control tamarin No. 10 is the paternal grandmother of affected tamarin No. 5, and the other two control tamarins are more distantly related (data not shown).

Liver from affected tamarins was grossly dark brown to black with a prominent reticulated pattern and was mildly enlarged. Control tamarin liver was diffusely brown.

Histologic examination of hematoxylin and eosin (HE)-stained sections of hyperbilirubinemic tamarin

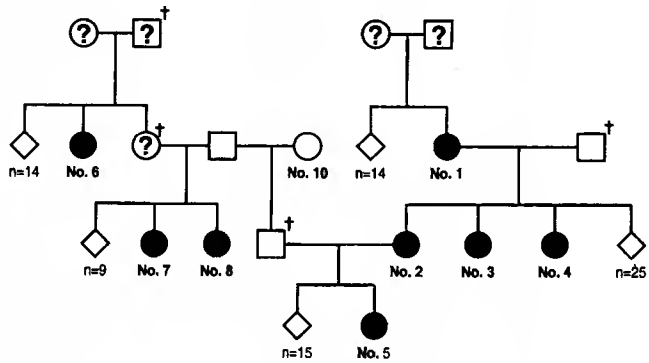

Fig. 1. Pedigree illustrating direct interrelationships of golden lion tamarins identified as having Dubin-Johnson-like syndrome. ○ = female with normal serum bilirubin concentrations; □ = male with normal serum bilirubin concentrations; ● = female with Dubin-Johnson-like syndrome; ⓘ = female with unknown bilirubin status; ⓘ = male with unknown bilirubin status; ◇ = variable number of normal bilirubinemic/untested siblings; n = number; and † = dead.

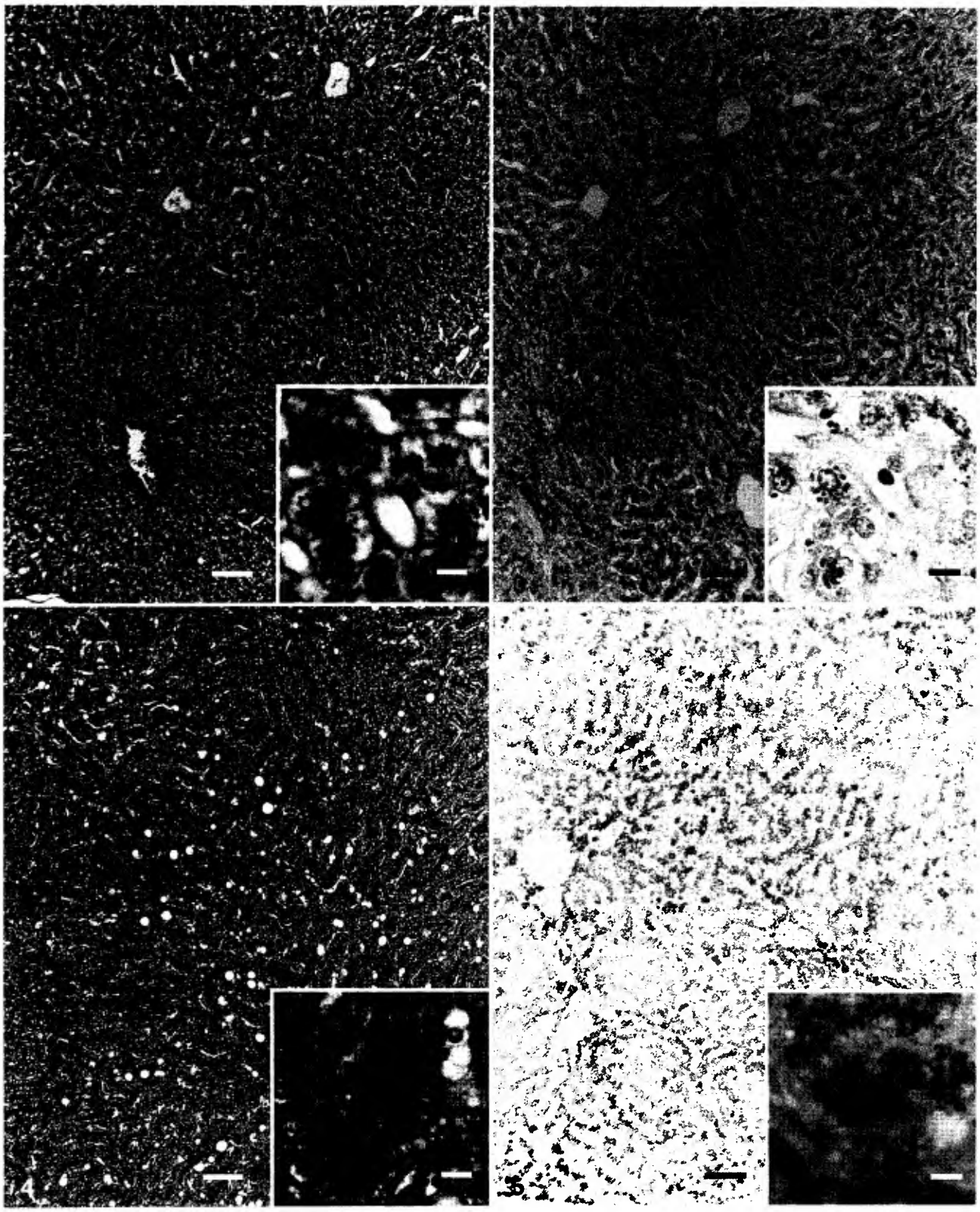

Fig. 2. Liver; hyperbilirubinemic tamarin No. 4. Note the prominent greenish brown, granular to globular pigment located in centrilobular to midzonal hepatocytes. HE. Bar = 100 μm. *Inset:* Higher magnification. Bar = 12.5 μm.

Fig. 3. Liver; hyperbilirubinemic tamarin No. 4. The majority of the centrilobular to midzonal pigment does not react positively for iron, but retains its greenish brown color. Perls' iron technique. Bar = 100 μm. *Inset:* Higher magnification. Bar = 12.5 μm.

liver demonstrated abundant green-brown finely granular to globular intracytoplasmic pigment diffusely within centrilobular and midzonal hepatocytes. In severe cases, pigmentation was diffuse throughout the hepatic lobule. In sections stained with Perls' iron technique, a small amount of the hepatocellular pigment stained blue, but most retained its unstained green-brown color (Figs. 2, 3). Most pigment reacted positively with the Fontana method and the lipofuscin-ferric ferricyanide reduction technique. There was minimal to no positive reaction of this pigment with acid-fast technique for lipofuscin, with Fontana after bleaching, or with Hall's method for bilirubin. Multifocally, primarily within portal areas, small aggregates of macrophages contained globular dark green-brown intracytoplasmic pigment and were admixed with a few lymphocytes and plasma cells. This pigment stained positively with Perls' method for iron and negatively with the other special stains used. There was diffuse mild hepatocellular microvacuolization and mild Ito cell hyperplasia.

HE staining of control liver sections resulted in diffuse moderate green-brown to brown finely granular hepatocellular pigmentation. Most of this pigment was positive with Perls' method for iron (Figs. 4, 5), and a small percentage stained positively with the Fontana method. This pigment did not stain with the Fontana method after bleaching, Hall's method for bilirubin, acid-fast stain for lipofuscin, and lipofuscin-ferric ferricyanide reduction. There was also hepatocellular microvacuolization, Ito cell hyperplasia, and portal aggregates of granular green-brown pigment-laden macrophages, with lymphocytes and plasma cells similar to that in liver of hyperbilirubinemic animals. The macrophage pigment had staining characteristics identical to those of the macrophage pigment of affected animals.

Ultrastructural examination of affected liver revealed multiple pleomorphic moderately to intensely electron-dense single membrane-bound particles scattered throughout the cytoplasm of the hepatocytes (Fig. 6). Also, occasional intracytoplasmic lipid droplets were present. Control liver contained multiple pleomorphic uniformly electron-dense intracytoplasmic particles and occasional lipid globules.

Liver slices from the hyperbilirubinemic tamarin (No. 8) did not secrete carboxydichlorofluorescein diacetate into the bile canaliculi (Fig. 7a), whereas, liver slices from control tamarin No. 11 did (Fig. 7b). Hyperbilirubinemic tamarin liver readily secreted the fluorescent bile acid analog (fluorescein isothiocyanate-labeled glycocholic acid) in liver slices and hepatocyte doublet preparations. Because of a limited amount of tissue, these studies could not be performed in tissue from the control tamarin.

Table 2. Results of bromosulfophthalein (BSP) retention test in seven golden lion tamarins.

Tamarin No.	Status	BSP Retention (%)	
		60 minutes	120 minutes
1	Hyperbilirubinemic	4.8	2.4
2	Hyperbilirubinemic	11.9	9.5
3	Hyperbilirubinemic	5.9	3.8
5	Hyperbilirubinemic	15.2	11.2
9	Control	1.3	1.6
10	Control	1.6	1.3
11	Control	1.7	2.0

Discussion

The hyperbilirubinemic golden lion tamarins were clinically normal. Elevated serum bilirubin concentrations were found on routine screening. Dubin-Johnson patients are also generally asymptomatic, although they may have mild hepatomegaly, abdominal discomfort, fluctuating jaundice, and dark urine.[2,3] However, Dubin-Johnson-like syndrome in mutant Corriedale sheep can be fatal because of failure to excrete phylloerythrin, a non-bile acid organic anion, properly, with consequent photosensitivity.[7]

Based on elevated total and direct serum bilirubin concentrations, mutant tamarins appear to be capable of hepatic uptake and conjugation of bilirubin but are unable to excrete bilirubin conjugates efficiently into the bile. Dubin-Johnson patients, mutant Corriedale sheep, and mutant (TR^-) albino Wistar rats have a predominately conjugated hyperbilirubinemia. The excretion failure results from a defect in a non-bile acid organic anion transporter on the bile canaliculus.[2,3,5,13–15] In TR^- rats, a bile canalicular ATP-dependent non-bile acid organic anion transport system is functionally absent.[15]

Bromosulfophthalein (BSP) excretion kinetics are

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Fig. 4. Liver; control tamarin No. 10. Greenish brown to brown granular intracytoplasmic pigment is present in hepatocytes throughout the lobule and also in small aggregates of macrophages. HE. Bar = 100 μm. *Inset:* Higher magnification. Bar = 12.5 μm.

Fig. 5. Liver; control tamarin No. 10. The vast majority of the hepatocellular and histiocytic pigment reacts positively for iron. Perls' iron technique. Bar = 100 μm. *Inset:* Higher magnification. Bar = 12.5 μm.

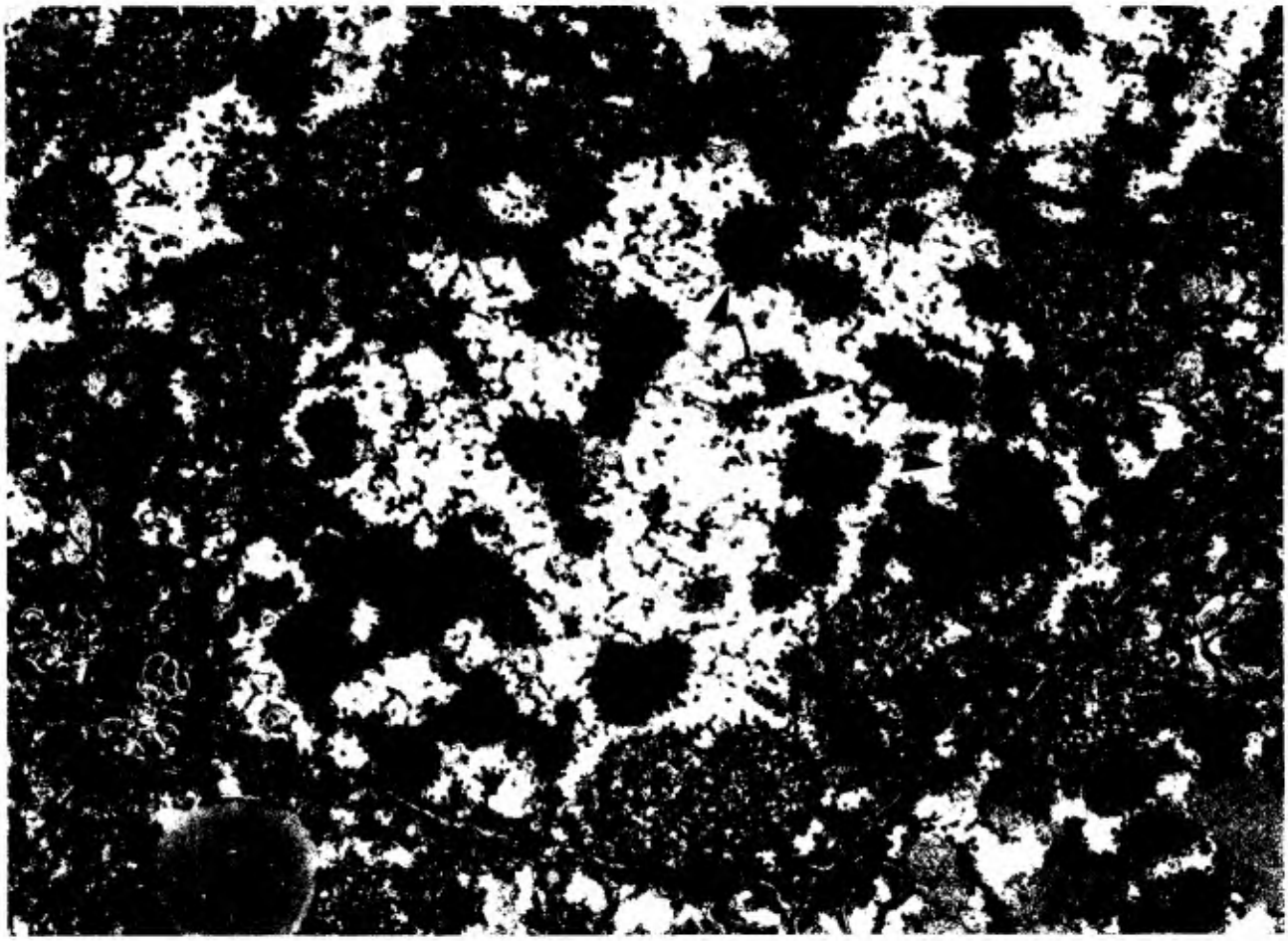

Fig. 6. Electron micrograph. Liver; hyperbilirubinemic tamarin No. 4. Note many pleomorphic variably electron-dense membrane-bound structures within the cytoplasm (arrowheads) and bile canaliculi (arrows). Bar = 0.5 μm.

abnormal in human beings and animals with this syndrome.[2–7,9,11,12,15,21,22] In Dubin-Johnson patients, there is normal hepatic uptake and conjugation of the BSP, but because of abnormal transhepatic transport, conjugated BSP diffuses back into the circulation, producing a characteristic secondary rise in the BSP curve.[2,3,9,12,21] In the rat and sheep models, BSP excretion is delayed, but a secondary rise has not been documented.[4–7,11,15] To derive kinetics of BSP excretion in human beings, several blood samples are drawn over time. Unfortunately, the small size of the tamarins (adult body weight is 360–710 g) limits blood sampling, and a detailed curve could not be constructed; however, BSP excretion was clearly prolonged in hyperbilirubinemic tamarins relative to that of normal tamarins. In addition to bilirubin and BSP, transhepatic transport of other non-bile acid organic anions, such as phylloerythrin and iodopanoic acid, in Dubin-Johnson-like tamarins are also observed in Dubin-Johnson human patients and mutant Corriedale sheep. The pigment responsible for this gross change was originally described as a lipochromelike pigment.[8] Currently, the pigment is described as melaninlike based on the experimental incorporation of [^{3}H] epinephrine and electron spin resonance spectroscopy.[2,3,7] Lack of pigmentation in the mutant TR$^-$ albino Wistar rat is believed to be related to an inability of albinos to produce melaninlike pigments.[15]

Similar to pigment seen in Dubin-Johnson patients and in mutant Corriedale sheep, pigment in hyperbilirubinemic tamarins primarily accumulates in centrilobular hepatocytes. The histochemical staining for Dubin-Johnson pigment is usually summarized as melaninlike, with few references discussing specific staining results. The pigment is reported to be negative for the Stein test and the Gmelin reaction for bilirubin and the Gömöri iron stain and positive for periodic acid-Schiff (PAS) reaction;[8] the pigment also has been reported not to stain with Perls' method for iron and oil red O but to react positively with the Fontana method.[18] Lipofuscin is variably positive with PAS reaction, oil red O, acid-fast method for lipofuscin, and the lipofuscin-ferric ferricyanide reduction technique.[19] The pigment in Corriedale sheep is classified as melaninlike

based on electron spin resonance and elemental analysis.[4,7] The results of the battery of stains applied to the hyperbilirubinemic tamarin liver indicate the melaninlike nature of the hepatic pigment; it is not positive for iron or bilirubin, stains minimally for lipofuscin, and is positive with the Fontana method. Positive reaction with the lipofuscin-ferric ferricyanide reduction technique indicates the presence of a reducing substance, such as melanin. Moderate iron storage in normal captive tamarins and marmosets is a common finding,[17] accounting for the iron-positive pigment present in both affected and control tamarin liver.

The ultrastructural findings are consistent with what has been described in Dubin-Johnson syndrome, mutant Corriedale sheep, and TR$^-$ rats. Variably dense pleomorphic cytoplasmic structures are primarily found in lysosomes.[2,3,5,6,12,13,18] However, the particles in the tamarin hepatocytes were scattered throughout the cytoplasm of the hepatocytes and were not primarily pericanalicular, as has been described for human beings, sheep, and rats with Dubin-Johnson or Dubin-Johnson-like syndrome.[2,7,12,13,18] Because the ultrastructural morphologic appearance of hemosiderin and Dubin-Johnson pigment is similar,[2,3,10,12,13] the use of special histologic stains is helpful in separating these pigments.

The limited number of cases and a large complicated pedigree precludes, in this preliminary report, specific identification of the mode of inheritance. However, genetics clearly plays a major role, as indicated by the fact that affected individuals belong to two immediate families and were housed in different environments. Although affected tamarins were identified in three successive generations, there is no way at present of determining heterozygosity, further preventing the determination of recessive versus dominant modes of inheritance at this time. Determination of inheritance may be further complicated by variable penetrance. All of the tamarins with Dubin-Johnson-like syndrome were female. In human beings, female patients are often asymptomatic until they become pregnant or start using oral contraceptives,[2,3] suggesting that female sex hormones may increase production of bilirubin and/or depress transhepatic transport. To control breeding, adult female golden lion tamarins are often implanted with melengesterol acetate contraceptives; however, there is no correlation between pregnancy or melengesterol acetate implants and hyperbilirubinemia in these tamarins. Perhaps the Dubin-Johnson-like trait is inherited in a mild, asymptomatic form and acquired factors such as female sex hormones or other as yet unidentified factors are required to produce hyperbilirubinemia. Dubin-Johnson syndrome in human beings and the Dubin-Johnson-like syndrome in mutant rats and sheep are inherited in an autosomal recessive manner.[2,3,4–7,11,12,15,21,22]

The Dubin-Johnson-like syndrome of golden lion tamarins closely parallels the Dubin-Johnson syndrome in human beings in many ways. Affected tamarins have intermittent to persistent predominately conjugated hyperbilirubinemia and prolonged BSP re-

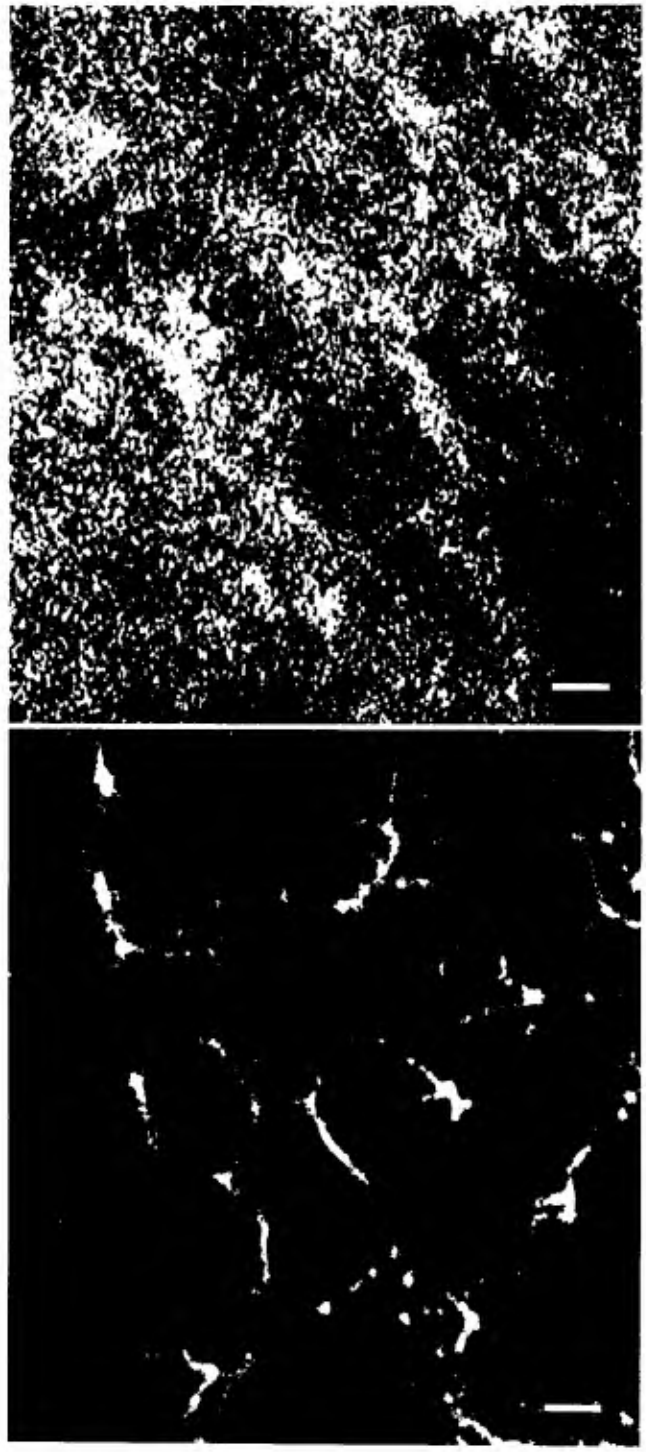

Fig. 7. Confocal fluorescent micrographs. **Fig. 7a.** Liver; hyperbilirubinemic tamarin No. 8. Note the absence of carboxydichlorofluorescein diacetate (CFDA) in the bile canaliculi surrounding hepatocytes. Bar = 5 μm. **Fig. 7b.** Liver; control tamarin No. 11. The bile canaliculi contain CFDA (broad white lines) and surround hepatocytes. Bar = 5 μm.

tention, are clinically asymptomatic, have black livers, and have defective secretion of non-bile acid organic anions from the hepatocyte into the bile. Histopathologic examination of the liver reveals abundant intrahepatocellular pigment that reacts positively with the Fontana method and not for iron. Ultrastructurally, this pigment is pleomorphic, variably electron dense, and membrane bound. Study of these tamarins, which are more closely related to human beings than are other Dubin-Johnson animal models, may improve our understanding of this syndrome and of hepatic transport systems.

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