Pathophysiologica conditions promoting mixed “black” pigment plus cholesterol gallstones in a ΔF508 mouse model of cystic fibrosis

Folke Frederiksen,1,2 Monika R. Leonard,2 Shou-An Liu,2 Jonathan N. Glickman,3 and Martin C. Carey1,2

1Department of Medicine, Harvard Medical School and Harvard Digestive Diseases Center; 2Department of Medicine, Gastroenterology Division, Brigham and Women’s Hospital, and 3Pathology Department, Harvard Medical School and Brigham and Women’s Hospital, Boston, Massachusetts

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Pathophysiologica conditions promoting mixed “black” pigment plus cholesterol gallstones in a ΔF508 mouse model of cystic fibrosis. Am J Physiol Gastrointest Liver Physiol 299: G205–G214, 2010. First published April 29, 2010; doi:10.1152/ajpgi.00341.2009.—Gallstones are frequent in patients with cystic fibrosis (CF). These stones are generally “black” pigment (i.e., Ca bilirubinate) with an appreciable cholesterol admixture. The pathophysiology and molecular mechanisms for this “mixed” admixture in CF are unknown. We investigate in a CF mouse model with no overt liver or gallbladder disease whether pathophysiologica changes in the physical chemistry of gallbladder bile might predict the occurrence of “mixed” cholelithiasis. Employing a ΔF508 mouse model with documented increased fecal bile acid loss and induced enterohepatic cycling of bilirubin (Am J Physiol Gastrointest Liver Physiol 294: G1411–G1420, 2008), we assessed gallbladder bile chemistry, morphology, and microscopy in CF and wild-type mice, with focus on the concentrations and compositions of the common biliary lipids, bilirubins, Ca2+, and pH. Our results demonstrate that gallbladder bile of CF mice contains significantly higher levels of all bilirubin conjugates and unconjugated bilirubin with lower gallbladder bile pH values. Significant elevations in Ca bilirubinate ion products in bile of CF mice increase the likelihood of supersaturating bile and forming black pigment gallstones. The risk of potential pigment cholelithogenesis is coupled with the increased bile salt hydrophobicity indexes, consistent with a proclivity to cholesterol phase separation during pigment gallstone formation. This is an initial step toward unraveling the molecular basis of CF gallstone disease and constitutes a framework for investigating animal models of CF with more severe biliary disease, as well as the human disease.

cystic fibrosis transmembrane conductance regulator; hepatobiliary disease; enterohepatic cycling; bilirubin; gallbladder

Cystic fibrosis (CF) is a common inherited disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, with >1,500 examples documented to date (23, 54, 57). Dysfunction of CFTR, an apical plasma membrane Cl− channel regulated by cAMP, impairs Cl−/HCO3− exchange via anion exchanger-2 on apical plasma membranes of specific parenchymal cells, most notably lung, pancreas, and small intestine. In the hepatobiliary system, CFTR is located on the apical plasma membranes of large cholangiocytes and cholecystocytes, but not hepatocytes (18, 20, 35). Approximately 10–25% of pediatric CF patients (16, 28, 50) and 30–72% of adult patients with CF (42, 43) display hepatobiliary abnormalities, most notably “biliary sludge” and gallstones (1, 43). Earlier authoritative publications (19, 58, 67) assumed that cholesterol stones form uniquely in CF because of the frequent bile salt malabsorption in the disease (58, 67), analogous to regional ileitis (19). However, in CF the gallstones are known to be insoluble with ursodeoxycholic acid therapy (21), and microscopy of CF gallbladder bile aspirated by duodenal drainage shows an absence of cholesterol crystals (1). In contrast to most common “black” pigment gallstones, the stones in CF are radiolucent, suggesting little, if any, crystalline calcium carbonate or calcium phosphate and/or high cholesterol content (1). The pathophysiological basis for the molecular pathogenesis of these stones is unknown.

The aim of this study was to investigate in a mouse model carrying the ΔF508 mutation (68) whether chemical changes that might presage the pathophysiology of such “mixed” gallstones occur in gallbladder bile. Not only is the ΔF508 mutation the most common CFTR gene mutation (~70%) in CF patients (57), but the pathophysiology of the intestinal tract and the liver in this mouse model has been well described (30, 68). Moreover, gross and direct light microscopy of the hematoxylin-and-eosin (H&E)-stained gallbladders of a small number of very young ΔF508 mice revealed histopathology that was indistinguishable from the gallbladders of wild-type (WT) mice (68). In studies of other CF mouse models, several gallbladders were reported to be distended with “black bile” (33), suggesting that a physical chemical change and precipitation had occurred. Patch-clamp studies on epithelial cells from ΔF508 CF mice reveal that the number of functional CFTR channels is ~1% of normal (29), which seems to be sufficient to prevent gross hepatobiliary disease in these mutants. Nonetheless, a high percentage of animals develop meconium ileus and growth retardation (30, 68).

Our prior publication (30) was a systematic evaluation of the secretory, i.e., production, rates of the common biliary lipids and lipopigments carried out without appreciable perturbation of the enterohepatic circulation. We showed (30) that hepatic bile of these CF mice was rendered more cytotoxic to cholangiocytes by events that occurred in the distal small and large intestines. Specifically, we demonstrated significantly reduced ileal pH levels, as well as increased fecal bile acid loss (30), leading to “hyperbilirubinemia” (increased secretion of conjugated bilirubins into bile) from induced enterohepatic cycling (EHC) of unconjugated bilirubin (UCB) (64). We also found significantly increased secretion rates of all common biliary lipids (30), especially cholesterol, in CF mice. We now propose that the same scenario sets in place biochemical and pathophysiological changes in bulk gallbladder bile that render
it potentially lithogenic, i.e., a "pre-stone" stage for Ca bilirubinate and cholesterol phase separation. We found significant increases in the lithogenic potential for biliary cholesterol and Ca bilirubinates that would allow these molecules to eventually precipitate from gallbladder bile if the observed levels were to become further elevated. This study forms a framework for understanding the pathophysiological and molecular mechanisms underlying gallstone disease in animal models with a more severe CF phenotype, as well as in the human disease.

METHODS

Animals. Heterozygous breeding pairs of ΔF508 mice (68) on a 75% C57BL/6-25% 129SvEv background were donated by Dr. Marie Egan (Yale University School of Medicine, New Haven, CT). Breeding pairs of G551D mice (25) were obtained from Dr. Gerald Pier (Channing Laboratory, Brigham and Women's Hospital and Harvard Medical School). Of the two murine CF models [ΔF508 (68) and G551D (25)] that were evaluated in preliminary studies, both displayed bile acid malabsorption (G551D > ΔF508). However, ΔF508 mice exhibited hepatic lipopigment secretory data consistent with EHC of bilirubin (30), whereas G551D mice did not (F. Freudenberg and M. C. Carey, unpublished observations). This was due to chronic diarrhea in the G551D, but not the ΔF508, mice, thereby curtailing colonic residence times, and is analogous to our previous observations in Asbt-null mice (24, 31). ΔF508 mice and WT controls were housed in the animal facility in the Thorn Research Building (Brigham and Women's Hospital). To minimize the risk of potentially lethal meconium ileus, all animals were housed on corn cob bedding (The Andersons, Maumee, OH), and for the same reason, a polyethylene glycol-3350-electrolyte solution (Golytely, Braintree Laboratories, Braintree, MA) was added to their drinking water. Mice were fed a diet that contained 11% fat and was replete in nutrients, vitamins, and minerals (Mouse Diet 5015, Labdiet, Richmond, IN). Housing illumination consisted of normal 12:12-h light-dark cycles employing conventional fluorescent lamps. All surgeries were performed at the same time of day on age-matched (unless otherwise noted, 3–14 mo of age) and sex-matched homozygous CF and WT mice that had been fasted for 4–8 h. Each mouse was genotyped as follows. Tails were clipped at 3 wk of age, and DNA was isolated using DNeasy kits (Qiagen, Valencia, CA). For PCR amplification, we used AmpliTag Gold Master Mix (Applied Biosystems, Foster City, CA) with the primer sequences GAG TGT TTT CTT GAT GTG and ACC TCA ACC AGA AAA ACC AG (Mg-optimized using the enzyme Rsal (Applied Biosystems) and separated by agarose gel electrophoresis. Mice were weighed prior to surgery. Unless stated otherwise, all analyses were performed on individual bile samples. Humane protocols for induction of anesthesia, laparotomy, cholecystectomy, and mouse euthanasia were approved by the Harvard University Medical Area Standing Committee on Animals.

Gallbladder bile volume, pH, and microscopy. After induction of anesthesia (intrapерitoneal injection of ketamine, xylazine, and atropine in 0.15 M NaCl), laparotomy was performed (30), and gallbladders were examined under direct and transmitted illumination (SZ40 Stereo Zoom Microscope, Olympus America, Chelmsford, MA). Cholecystectomy was then performed by standard technique for mice. Subsequently, the mouse was euthanized with an overdose of ketamine and xylazine, and bilateral thoracotomy was performed. After emptying gallbladder bile completely into tared 200-μl collection tubes, we measured bile volumes gravimetrically by numerically equating weight and volume. Immediately thereafter, pH values were measured on individual bile tubes by microelectrode (Thermo Electron, Beverly, MA). Gallbladder histopathology of prepared sections stained with H&E was assessed as described previously (30). Gallbladder bile was examined by direct and polarizing light microscopy for insoluble mucin gel, phase-separated amorphous and crystalline precipitates, and pigment and cholesterol gallstones. In addition, intact gallbladders of nonfasted mice (4 female and 2 male CF, 5 female and 3 male WT) were fixed in 20-fold volumes of formalin solution, dehydrated serially with ethanol and H2O2, embedded, and sectioned along the longitudinal axis to expose maximum luminal volumes and mucosa. For each gallbladder, separate slides were stained with Alcian blue to detect acidic mucins and with periodic acid-Schiff (PAS) to detect neutral mucins. All sections were coded and read blindly by three of the investigators in conjunction with an experienced rodent pathologist. We analyzed and scored stained sections as follows: 0, no mucin on apical columnar cells or within the gallbladder lumen; 1, a few wisps of mucin within the lumen and a uniformly thin layer of mucin adherent to the apical columnar cells; 2, a mucin layer of variable thickness adherent to apical columnar cells, small aggregates of mucin within the gallbladder lumen; 3, a mucin layer of variable thickness on epithelial cells, moderate amounts of mucin within the gallbladder lumen; 4, a few agglomerated mucin spherules within the gallbladder lumen, moderate-to-large amount of mucin within the lumen; and 5, many stained globules or mucin spherules within the gallbladder lumen and/or nonagglomerated mucin filling the gallbladder lumen.

Quantitation of Ca2+ activities. Immediately after measurement of gallbladder bile pH values, we quantified Ca2+ activities. To adjust for the ion strength of bile and to prepare standard solutions, Na+ activities were measured in a preliminary experiment (Clinical Chemistry Core Laboratory of Children’s Hospital, Boston, MA) using a Roche/Hitachi cobas 6000 analyzer (Roche Diagnostics, Indianapolis, IN) with a calibrated ion-selective electrode. Employing pooled samples (n = 4; necessitated by the volumetric requirement of the analyzer) of mouse gallbladder bile diluted fivefold (to protect the analyzer’s electrode from bile salt detergent), we determined a range of 190–205 mM Na+ in murine gallbladder bile. Accordingly, we adjusted all CaCl2 standards to an ion strength of 200 mM NaCl. Semilogarithmic calibration curves were generated from five stock solutions ranging from 10−1 to 10−5 M CaCl2 prepared by serial dilution of a 0.1 M CaCl2 standard solution (Microelectrodes). Ca2+ activities were measured in millivolts using a pH meter (Orion model 720A, Thermo Fisher Scientific, Wallingford, CT) and an ion-specific microelectrode (MI-600, Microelectrodes, Bedford, NH) by introduction of the Ca2+ and reference microelectrodes into individual gallbladder bile samples. Between measurements, Ca2+ and reference electrodes were rinsed with deionized, ultrfiltered H2O (Fisher Scientific) and stored in 0.1 M CaCl2 solution. Bile sample readings were interpolated on the standard curve to obtain millimolar Ca2+ concentrations. Ion products were calculated as the product of molar concentrations of Ca2+ and unconjugated bilirubin (UCB) or monoconjugated bilirubin.

Bilirubin molecular species. To separate and quantify bilirubin molecular species in bile, we injected a 10-μl aliquot of individual bile samples onto a reverse-phase HPLC precolumn-and-column assembly (59) within 5 min of bile collection. Prior to HPLC, samples were maintained in darkness to preserve bile pigments from actinic degradation. Concentrations of the two bilirubin monoglucuronoside (BMG) isomers in bile, the principal biliary conjugates in the mouse; bilirubin diglucuronosides (BDG); all other minor bilirubin mono- and diconjugates (BMX and BDX); and UCB are reported in micromolar concentrations. All injections of sample were performed using a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA). HPLC was performed on a Beckman HPLC apparatus equipped with a Model 152 pump, a 150-μl flow rate, and a Model 168 refractive index detector. The 250-μl sample loop was used for injection. The mobile phase was 0.05 M sodium acetate and 0.05 M sodium phosphate at pH 3.6. At 3.6, the mobile phase is 80% aqueous and 20% acetonitrile. Separation was achieved on a 4.6 × 250-mm 5-μm Waters CN reversed-phase column at a 1 ml/min flow rate. The column was eluted with a gradient of 100% water (60 min) for 20 min followed by 10% acetonitrile for 40 min. The column was then reequilibrated for 15 min. Quantitation of bilirubin molecular species was achieved using a Waters 486 spectrophotometric detector set at 552 nm.

Molecular species of individual bile lipids in gallbladder bile. Molecular species of individual bile salts in gallbladder bile were separated and quantified by HPLC (56). Concentrations of total bile salts were assayed by the 3α-hydroxysteroid dehydrogenase method (62), biliary phospholipids were assayed as inorganic phosphorus (2), and biliary cholesterol was extracted (40) prior to HPLC assay (63). Bile salt hydrophobicity indexes were quantified using the method of Heuman (36). The major biliary lipids are reported as millimolar concentrations. We calculated cholesterol saturation indexes (CSI) of gallbladder bile samples using critical tables (11), with the initial assumption that all bile salts present...
are Na taurocholate. Then “urso-correction” factors (12) were employed to adjust the initial CSI values on the basis of the percent total muricholate plus ursodeoxycholate conjugates in each bile sample.

**Statistics.** Values for most measurements are means ± SE. For these comparisons between mutant ΔF508 and WT groups, statistical significance was assessed using an unpaired, two-tailed Student’s t-test, corrected with Welch’s correction factor in cases of unequal variance. However, data for the Ca bilirubinate ion products show skewness and kurtosis. To evaluate these nonparametric data, we used the Mann-Whitney test (also known as Wilcoxon’s rank sum test) to report medians and Mann-Whitney U and P values. For all statistics, P < 0.05 is considered significant.

**RESULTS**

**General.** As we described earlier (30), not only does breeding these CF mice yield fewer-than-expected offspring on the basis of Mendelian predictions, but in the pre- and postweaning periods a higher proportion of ΔF508 CF offspring die spontaneously, usually from unavoidable intestinal obstruction. This occurs despite corncob bedding and GoLYTELY in the drinking water. There is also a significant weight difference in age-matched, mature mice (Fig. 1A): CF mice (n = 21) are ~34% lighter than WT mice (n = 20, P < 0.0001).

**Gallbladder volumes, microscopy, and mucin scores.** Gallbladders are markedly larger in CF than WT mice, whether expressed as absolute volumes (Fig. 1B; CF 23% greater than WT, P = 0.04) or normalized to body weight (Fig. 1C; CF 79% larger than WT, P < 0.0001, n = 21 and 20, respectively). All biles are bright yellow and translucent. By direct light microscopy, neither yellow precipitates of phase-separated Ca bilirubinates nor black gall-sand or black gallstones are observed in the gallbladders or found in gallbladder biles of CF (n = 7) or WT (n = 5) mice. Under direct and polarized light microscopy, we sometimes noted one or two narrow, birefringent crystalline objects of unknown chemistry per high-power field in CF and WT gallbladder biles. There are no phase-separated liquid crystals, cholesterol monohydrate crystals, inorganic Ca carbonate/Ca phosphate crystals, or amorphous bilirubinate precipitates in the gallbladders and bile samples of CF and WT mice. By direct light microscopy, gallbladders of CF mice invariably contain a mild-to-moderate amount of insoluble mucin gel. Some gallbladders of WT mice are completely isotropic, although moderate amounts of mucin gel are found in the gallbladders of two 8- and 9-mo-old WT mice. We further assessed epithelial-adherent and luminal gallbladder mucins semiquantitatively by Alcian blue and PAS stains in 3- to 18-mo-old male and female CF and WT mice (n = 6 and 8, respectively). PAS scores confirm those of sections stained with Alcian blue, being identical in all but one case. Blinded mucin scores (see METHODS) range from 0 to 4 for WT mice and from 1 to 4 for CF mice, and mean scores for both genotypes are essentially identical (CF mucin score = 2.4, WT mucin score = 2.5); no sex differences are found. Although a trend toward increasing scores with age is observed in CF mice, this is not the case with WT mice.

**Gallbladder morphology.** The gross appearances of gallbladders from CF (6–19 mo old) and WT (4–9 mo old) mice, despite marked differences in size (Fig. 1B), are identical; in particular, no yellow or black staining of the CF gallbladders is noted, and, by transillumination, no phase-separated precipitates are observed. The H&E-stained gallbladder tissues were read by two independent pathologists with different backgrounds who were blinded as to their origin. After examination of 12 CF and 13 WT mouse gallbladders, no significant histopathological difference could be documented to distinguish gallbladders of CF from WT mice. Specifically, one gastrointestinal histopathologist, a specialist in human tissues, scored the histology as 0 to 2+ inflammation, with no difference between CF and WT genotypes. A dedicated murine pathologist scored WT and CF sections as within normal limits for the laboratory mouse.

**Gallbladder bile pH values.** As displayed in Fig. 1D, pH values of gallbladder bile are significantly (P = 0.004) less alkaline in ΔF508 mutant CF (7.32 ± 0.04, n = 21) than WT (7.53 ± 0.06, n = 20) mice.

**Fig. 1.** Mouse weight, gallbladder volume, and bulk pH of ΔF508 [cystic fibrosis (CF)] and control (WT) mice (n = 21 and 20, respectively). *Statistically significant differences between ΔF508 CF and WT mice. A: mouse weights at time of cholecystectomy are significantly lower in CF than WT mice (23.8 ± 0.9 vs. 36.1 ± 1.8 g, P < 0.0001). B: gallbladder bile volumes are significantly (P = 0.04) larger in CF than WT mice (36.4 ± 2.7 vs. 29.6 ± 1.6 μL). C: normalized gallbladder bile volumes are markedly different in the same direction (153.2 ± 10.4 and 85.4 ± 5.3 μL/100 g body wt for CF and WT mice, respectively, P < 0.0001). D: individual gallbladder bile pH values measured by micro-electrode immediately following bile expression from gallbladders. The pH values are significantly (P = 0.004) lower in CF than in WT mice (7.32 ± 0.04 vs. 7.53 ± 0.06).
Concentrations of bilirubin molecular species in gallbladder bile. Figure 2 shows significantly higher concentrations of all bilirubin molecular species, including UCB, in gallbladder bile of CF mice. The differences between CF (n = 21) and WT (n = 20) mice are marked and highly significant (P < 0.0001) for all conjugates and at the P = 0.0002 level for UCB concentrations: 38.2 ± 3.0 and 23.3 ± 1.6 μM BDG for CF and WT, respectively (Fig. 2A); 47.0 ± 3.5 and 29.1 ± 1.9 μM BDG + BDX for CF and WT, respectively (Fig. 2B); 92.3 ± 6.6 and 58.0 ± 3.7 μM BMG for CF and WT, respectively (Fig. 2C); 95.4 ± 6.8 and 59.5 ± 3.7 μM BMG + BMX for CF and WT, respectively (Fig. 2D); 142.3 ± 10.1 and 88.6 ± 5.6 μM total of conjugated bilirubin species for CF and WT, respectively (Fig. 2E); and 1.5 ± 0.2 and 0.7 ± 0.1 μM UCB for CF and WT, respectively (Fig. 2F). Within each mouse genotype, we found sex differences in the concentrations of individual bilirubin species, with higher levels in female (n = 7/group) than male (n = 6/group) mice; these differences are significant in WT mice. However, all molecular species of bilirubins are significantly elevated in CF females compared with WT females and in CF males compared with WT males (comparisons not shown).

Common biliary lipids in gallbladder bile. Absolute concentrations of bile salts, phospholipids, and cholesterol in individual gallbladder biles of CF and WT mice (n = 10 and 8, respectively, for both bile salts and phospholipids; n = 6 and 7 for cholesterol) plus their respective total lipid concentrations (n = 6 and 7) are delineated in Fig. 3, A–D. Bile salt concentrations are decreased significantly (P = 0.02) in CF compared with WT mice (132.3 ± 5.7 vs. 155.1 ± 6.5 mM; Fig. 3A). Although slightly depressed in CF mice, no significant differences in biliary phospholipid levels are noted between CF and WT mice (18.6 ± 1.3 and 20.7 ± 1.9 mM, respectively, P = 0.38; Fig. 3B).

Cholesterol concentrations are appreciably greater in bile of CF than WT mice (Fig. 3C), but the mean value marginally misses statistical significance (3.23 ± 0.56 vs. 1.77 ± 0.18 mM, respectively, P = 0.056). However, molar percentages of cholesterol are doubled in CF compared with WT mice (2.06 ± 0.34 vs. 0.99 ± 0.14 mol%, P = 0.01). Total lipid concentrations were calculated for the CF and WT samples for which sufficient bile was available to measure all three common biliary lipids in the same sample, with values of 8.8 ± 0.5 and 10.3 ± 0.5 g/dl for CF and WT mice, respectively. Comparison of the means barely misses statistical significance (P = 0.057; Fig. 3D).

Cholesterol saturation indexes. Figure 3E shows the CSI values (uncorrected for percent muricholates and ursodeoxycholates in murine bile). CSI values are significantly higher (P = 0.003) in gallbladder biles of CF (0.45 ± 0.05, n = 6) than WT (0.23 ± 0.04, n = 7) mice. When appropriate urso-correction factors (12) tailored to the total percent muricholate and ursodeoxycholate conjugates in individual bile samples are applied, these values remain statistically significant, increasing to 0.56 ± 0.05 for CF mice and 0.32 ± 0.05 for WT mice (P = 0.01; Fig. 3F).

Fig. 2. Concentrations of bilirubins in individual (10-μl aliquots) gallbladder bile samples of WT (n = 20) and CF (n = 21) mice measured by HPLC immediately following cholecystectomy. *Statistically significant differences between groups. A: bilirubin diglucuronoside (BDG) concentrations ([BDG]). B: concentrations of bilirubin monogluconjugates ([BDG + BDX]), which include pure and mixed conjugates with xylose and glucose (BDX). C: bilirubin monoglucuronoside concentrations ([BMG]). D: concentrations of bilirubin monogluconjugates, including conjugates with xylose and glucose (BMG + BMX). E: concentrations of total conjugated bilirubins ([cBR]) in gallbladder bile. F: unconjugated bilirubin concentrations ([UB]). In the case of the conjugates (A–E) and UCB (F), concentrations are significantly higher (P < 0.0001 and 0.0002, respectively) in CF than WT mice. As inferred visually from these plots (means ± SE), all bilirubin levels in gallbladder bile of CF mice are 1.5- to >2-fold greater than in WT mice.
Bile salt molecular species and hydrophobicity indexes. Figure 4, A–G, shows the bile salt molecular species, calculated as percentage of total bile salt concentration, in gallbladder biles of CF and WT mice (n = 17 per group). Significantly lower proportions of tauro-β-muricholate (P = 0.01) are present in CF than WT mice (34.2 ± 1.6 vs. 42.3 ± 2.5% bile salt; Fig. 4B). Similarly, tauroursodeoxycholate is decreased in CF compared with WT mice (3.5 ± 0.4 vs. 5.1 ± 0.4, P = 0.006; Fig. 4D). In contrast, the percent taurocholate is significantly (P = 0.02) greater in CF than WT mice (51.1 ± 2.4 vs. 42.3 ± 2.7%). We found no significant differences between ΔF508 CF and WT mice in terms of percent bile salt concentration for tauro-α-muricholate, taurochenodeoxycholate, or taurodoxycholate. As shown in Fig. 4H, hydrophobicity indexes are significantly increased (i.e., less negative and more hydrophobic) in CF compared with WT mice (−0.35 ± 0.02 vs. −0.41 ± 0.02, respectively, n = 17 per group, P < 0.05).

Ionized Ca and ion products for Ca bilirubinates. Ca$^{2+}$ levels in gallbladder bile samples of CF and WT mice (n = 13 for each group, 3–11 mo old) are 0.42 ± 0.04 and 0.45 ± 0.06 mM, respectively (data not shown). Clearly, Ca$^{2+}$ concentration ([Ca$^{2+}$]) did not differ between groups, as was also the case when males and females were compared: 0.40 ± 0.03 and 0.56 ± 0.09 mM for CF and WT males, respectively (n = 6 per group, P = 0.15), and 0.44 ± 0.07 and 0.37 ± 0.06 mM for CF and WT females, respectively (n = 7 per group, P = 0.45). Differences in [Ca$^{2+}$] between sexes for each genotype are at the P = 0.62 and 0.10 levels for CF and WT mice, respectively.

Figure 5 displays scatter plots of the negative logarithms (base 10) of the ion products of Ca$^{2+}$ with WT biles (n = 13 per group). Nontransformed median ion products for [Ca$^{2+}$][HUCB$^{10}$], [BMG$^{10}$][BMX$^{10}$] (dianionic form of UCB) ion products are 5.6 ± 0.33 and 2.2 ± 0.48, respectively (Fig. 5A). Corresponding values for [Ca$^{2+}$][HUCB$^{10}$][HUCB$^{10}$] are 6.0 ± 0.30 and 2.5 ± 0.48, respectively (Fig. 5B). For [Ca$^{2+}$][BMG$^{10}$][BMX$^{10}$] ion products, the CF values are 6.5 ± 0.31 and 2.7 ± 0.49, respectively (Fig. 5C). For [Ca$^{2+}$][BMG$^{10}$][BMX$^{10}$] ion products, the WT values are 4.9 ± 0.32 and 1.9 ± 0.46, respectively (Fig. 5D).

Fig. 3. Concentrations of common biliary lipids in gallbladder biles of WT (n = 7 or 8) and CF mice (n = 6 or 10). *Statistically significant differences between CF and WT mice. A: bile salts are decreased significantly (P = 0.02) in gallbladder bile of CF mice compared with WT mice. B: no significant differences in total phospholipid concentrations are noted between WT and CF mice. C: cholesterol concentrations are markedly increased in CF compared with WT gallbladders, but the values miss statistical significance at the P = 0.056 level. D: total biliary lipid concentrations in gallbladder bile, despite the marked trend, are not statistically different in CF mice compared with WT mice (P = 0.057). E: cholesterol saturation index (CSI) of murine biles based on biliary taurocholate only. These estimates in CF biles are approximately double those in WT biles (P = 0.003). F: with the “urso-corrected” values [i.e., corrected individually for percent muricholates + percent ursodeoxycholate in each bile (12)], CSI values remain significantly elevated in CF compared with WT biles (P = 0.01).
In the present work, our aim was to obtain the putative molecular “fingerprints” underlying gallstone disease in CF, employing this mouse model of the commonest human mutation (H9004F508) with documented bile acid malabsorption. We were interested in the integrated biliary lipid and lipopigment compositions of gallbladder bile and how they represent secretory data (30) averaged over a short (6 ± 2 h) fasting period with obligatory gallbladder modifications. Although in humans the ΔF508 mutation is known to be associated with a relatively high incidence of liver disease (3, 28, 43) and gallstones (1, 27, 60), despite 99% CFTR ablation (29), the ΔF508 mouse acquires neither clinically significant liver disease (30) nor gallstones (68). In preliminary studies, we tested the same hypothesis in another CF mouse model (G551D) that also exhibits no hepatobiliary disease (25). Despite highly significant bile acid malabsorption in G551D CF compared with WT mice, we did not observe hyperbilirubinemia but did document higher biliary cholesterol secretory rates. We determined that the lack of induced EHC of UCB occurred because these animals suffer from chronic diarrhea analogous to the Asbt (Slc10a2)-null mouse (24), where insufficient colonic residence time curtails UCB reabsorption (F. Freudenberg and M. C. Carey, unpublished observations; 31). Accordingly, the

Fig. 4. Bile salt molecular species, as a percentage of total moles of bile salts, and “hydrophobicity indexes” in gallbladder bile samples. *Statistically significant differences between groups. A–G: individual bile salt molecular species for WT and CF mice (n = 17 per group). Percent tauro-β-muricholate (B) and percent taurocholate (D) are significantly lower (both P ≤ 0.01) in gallbladder bile of CF than WT mice, whereas percent taurocholate (E) is significantly higher (P = 0.02) in ΔF508 CF than WT mice. Percent tauro-α-muricholate (A), total tauromuricholates (C), taurochenodeoxycholate (F), and taurodeoxycholate (G) are not different between genotypes. H: bile salt hydrophobicity indexes (36) are significantly more hydrophobic (i.e., less negative; P < 0.05) in gallbladder bile of CF than WT mice.
served in CF patients (27), fecal bile acid loss is increased significantly in the ∆F508 homozygous mice (~2-fold) compared with WT controls (5, 30), a feature seen in the human disease, where ~30% of CF patients exhibit bile salt malabsorption (49, 67). We documented earlier (6, 7) that when increased spillage of bile acids into the colon occurs and provided colonic residence time is sufficient (31), EHC of UCB is induced and leads to hyperbilirubinemia (30, 64); moreover, upregulated bile acid synthesis leads to increased hepatic availability of cholesterol and its hypersecretion into bile. While these factors contribute to cytotoxicity of bile to cholangiocytes (30), we now propose that the same scenario sets in place the physical-chemical preconditions for mixed black pigment plus cholesterol gallstones in the gallbladder.

We found that CF gallbladder bile is indeed richer in all conjugated and unconjugated bilirubins by a factor of ~2 (Fig. 2). This is in line with the evidence that secretion rates of all bilirubin molecular species are elevated in hepatic biles of the same model (30). Moreover, we anticipated that the Cftr mutation on cholecystocytes, in addition to cholangiocytes, would lead to an appreciable decrease in gallbladder pH values, as shown in Fig. 1D. It is reasonable to speculate that enzymatic deconjugation of bilirubin conjugates by biliary endogenous β-glucuronidase, with its pH optimum of ~5, is active at pH ~7 (37–39) and would be augmented by nonenzymatic hydrolysis in the setting of significantly lower pH in CF gallbladder bile. Indeed, gallbladder bile UCBC was significantly elevated (Fig. 2F), but UCBC also forms more easily from BMG, the principal murine conjugate, than from BDG (9). One can dismiss direct secretion of UCBC into bile in this setting (26) since UCBC has no affinity for the canalicular transporter MRP2 (ABCC2) (32). It is generally believed that UCB, which at neutral biliary pH is in the monoanionic (HUCB-) form (9), would precipitate as insoluble calcium hydrogen bilirubinate salts. However, all Ca monobilirubinate ion products are elevated in CF compared with WT mice (Fig. 5).

It has been demonstrated that, in the black pigment gallstones of the hemolytic nb/nb mouse model (61), Ca salts of bilirubin monoconjugates also separate in solution from addition to Ca(HUCB)2. The experimental solubility products (dimensionless) for Ca(HUCB)2 and CaUCB in 50 mM Na taurocholate are reported to be $6.5 \times 10^{-13}$ and $1.8 \times 10^{-8}$, respectively (9). Using these values as approximate estimates for murine gallbladder bile [since muricholate and ursodeoxycholate solubilization of Ca bilirubinates is minimal (M. D. Berman and M. C. Carey, unpublished observations) and biliary phosphatidylcholine decreases UCBC solubility (14)], we find that the ion products (Fig. 5) are unsaturated in murine bile by ~2 orders of magnitude. Because microscopic examination of a sizable cohort of ∆F508 gallbladder biles of all ages did not reveal any Ca bilirubinate precipitates, it is likely that, in this study, CF mouse gallbladder biles are unsaturated with these Ca bilirubinate salts (Fig. 5). Furthermore, we did not observe any evidence of CaCO3 crystals when gallbladder biles of ∆F508 and WT mice were examined microscopically. Moore and Vérine (46, 47) demonstrated that the formation constants of Ca(HCO3)2 and CaCO3 are also promoted by gallbladder acidification of hepatic bile; hence, in CF it is likely that these salts could contribute to black pigment gallstone formation (9).
In the face of small differences in absolute bile salt and phospholipid concentrations between CF and WT mice, gallbladder bile of CF mice was more enriched in cholesterol (Fig. 3). Even as the cholesterol results typified hepatic bile (30), the gallbladder compositions of bile salts and phospholipids showed a reverse trend compared with their increased secretion rates in hepatic bile of CF compared with WT mice. The 2.8-fold increase in bile salt secretion in CF compared with WT mice that we found in our previous work (30) may be accounted for, in part, by hepatic bile flow that was nearly doubled in CF compared with WT mice (30). It is likely that CFTR-ablated function at the level of the cholecystocytes is also partly responsible for the decrease in bile salt concentration found in gallbladders of CF compared with WT mice. Gallbladder phospholipid concentrations were decreased in CF compared with WT mice by −2.1 mM, a fact that is not surprising given the gallbladder’s selective absorption of biliary phosphatidylcholine in addition to cholesterol (22). The markedly higher cholesterol levels (Fig. 3C) and CSI values (Fig. 3E), which were nearly twofold greater in CF than WT mice, are only slightly diminished when the values were “urso-corrected” (see METHODS; Fig. 3F). It would be feasible to present these mice, whose genetic background (see METHODS) contains multiple Lith genes (41, 44), with powerful lithogenic challenges, such as a cholesterol lithogenic diet (52) and bacterial β-glucuronidase per os, to induce EHC of UCB from the upper small intestine. However, we did not believe it was within the scope of the present work to perform these chronic epidemiological experiments. Nonetheless, on the basis of analogy (13), if a percent elevation in CSI occurred in CF humans similar to that found in the ∆F508 CF mice (Fig. 3E), the CSI values, normally falling ~0.8–1.2 in human bile, would increase to 1.4–1.6.

The bile salt pool is significantly more hydrophobic in CF than WT mice (Fig. 4H). We reasoned earlier (6, 30) that the increased hydrophobicity is secondary to increased spillage of bile salts into the colon with augmented fecal bile acid loss. Although, in theory, the necessity for GoLYTELY in the drinking water to prevent small intestinal obstruction could have contributed to bile acid loss, our WT controls were hydrated in an identical manner and displayed approximately half the bile acid wastage as did the CF mice. Interestingly, the high levels of secondary bile salts from colonic anaerobic catabolism of primary bile salts are not fully “corrected” hepatically in CF mice. The significant shift in hydrophobicity index (Fig. 4H), which in gallbladder bile is accompanied by an increase in UCB levels (Fig. 2F), should render bile more cytotoxic to cholecystocytes (8, 10, 15, 51). Paralleling the significant taurocholate elevation in ∆F508 mice (Fig. 4E), studies in CF patients show that cholate conjugates constitute a higher percentage of total bile salts, with chenodeoxycholate conjugates, from which muricholate conjugates are derived in the mouse, constituting a lower percentage (4, 60). Both changes likely reflect upregulated bile salt synthesis via the classic pathway because of increased fecal bile acid loss (5, 30).

Gallbladders are also significantly enlarged in ∆F508 mice compared with WT mice (Fig. 1, B and C), and, in mouse models of CF in contrast to humans with CF, distended gallbladders are the rule (33, 34). Although not tested here, larger gallbladders in CF mice might be a surrogate index of decreased motility, but in our CF mice, they are not a result of mucin gel accumulation (see RESULTS) (65, 66). It is, however, possible that cholecystocytic (and smooth muscle cell) absorption of elevated levels of cholesterol (17) and UCB (53) from gallbladder bile plus T cell activation (45) may compromise gallbladder motility by ablating smooth muscle function. In CF humans, the common ∆F508 mutation causes more severe disease, with gallstones forming early within a highly viscous gallbladder mucin gel and the ensuing chronic cholecystitis leading to fibrosis and scarring (27).

This systematic study of gallbladder bile chemistry and early pathophysiology in a large cohort of ∆F508 CF and WT mice is likely to presage the earliest events in human gallbladder disease secondary to CF. These mice exhibit neither gross liver nor biliary disease yet display bile acid malabsorption, which is the only pathophysiological abnormality they share with CF humans. Nonetheless, the preponderance of the evidence based on the current data and earlier work (30) suggests that mild bile acid malabsorption is sufficient to perturb bile pigment and bile lipid chemistry at the level of the gallbladder and likely provides the mechanistic setting that promotes mixed stone formation in humans with CF disease (1, 21). Our studies will obviously need to be confirmed noninvasively in humans and extended to animal models of severe CF that acquire gallstones (55). If these findings are translatable to humans with higher CSI values from the beginning, it could explain the high cholesterol admixture in the black pigment stones of CF patients found by Angelico et al. (1). It is hoped that this work will lead to therapeutic targets and, perhaps, new options for preventing and treating this common hepatobiliary complication in CF patients.

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Present addresses: F. Freudenberg, Division of Pediatric Gastroenterology and Hepatology, Dr. von Haunersches Kinderspital, Ludwig Maximilians University, Lindwurmsstrasse 4, 80337 Munich, Germany; J. N. Glickman, GI Pathology Services, Caris Diagnostics, Newton, MA.

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DISCLOSURES

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