

Mucin Glycoprotein Content of Human Pigment Gallstones

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Mucin glycoproteins, a secretory product of the gallbladder, are thought to contribute to the matrix or nucleus of gallstones. Human black pigment stones originate in the gallbladder and have as their major constituent calcium bilirubinate, as well as inorganic salts and small amounts of cholesterol. The object of this study was to estimate the amount of glycoprotein in black pigment stones and to isolate gallbladder mucin from dissolved stones. Black pigment stones containing 18 to 65% calcium bilirubinate were first dissolved in 12.5 mM EDTA/0.1 N NaOH and decolorized, then subjected to glycoprotein assay. The mean glycoprotein content of eight stones was 12.4%. In separate experiments, pigment stones were partially dissolved by brief exposure to EDTA/NaOH to minimize glycoprotein breakdown, and the glycoproteins isolated by gel filtration and ultracentrifugation. Pigment stones contained two glycoprotein fractions on Sepharose 4B; a high molecular weight mucin glycoprotein in the void volume and a lower molecular fraction in the included volume. Mucin was further purified by density gradient ultracentrifugation in cesium chloride. Three separate mucin fractions had an average buoyant density of 1.48 gm per ml which is typical for these glycoproteins. Bile pigment was associated with high molecular weight mucin even after extensive dialysis, gel filtration, and density gradient ultracentrifugation. The identity of mucin was further established by β -elimination of glycoproteins in alkaline borohydride which yielded galactosaminitol from cleavage of *O*-glycosidic bonds.

Our results indicate that mucin glycoproteins are present in significant concentrations in human black pigment stones and can be purified from stones solubilized in EDTA/NaOH. The association of bile pigment with gallbladder mucin, even after extensive purification, is consistent with the hypothesis that mucin contributes to the matrix of pigment gallstones.

A major organic component of human black pigment gallstones is calcium bilirubinate, which accounts for 30 to 75% of stone weight (1). Other measured components in pigment stones (calcium, phosphate, and carbonate, and small amount of cholesterol) do not account for the remaining stone weight. Thus, approximately 25 to 70% of pigment stone weight remains unmeasured. A potentially important "unmeasured" constituent of gallstones is gallbladder mucus. High molecular weight mucus glycoproteins are secreted in increased concentrations in patients with lithogenic bile (2) and are also found in experimental (3) and human (4) pigment gallstones. Furthermore, mucus glycoproteins are capable of binding

calcium (5) and lipids (6), and appear to form a matrix or nidus in human pigment gallstones (7).

The present study was undertaken to analyze the glycoprotein content of human black pigment gallbladder stones. Mucin glycoproteins were isolated by gel chromatography and density gradient ultracentrifugation, and further analyzed by alkaline hydrolysis and oligosaccharide analysis. Our results indicate that high molecular weight mucin is present in significant concentrations in black pigment gallstones, where it appears to be complexed with bile pigments.

MATERIALS AND METHODS

Black pigment gallbladder stones [as classified by the recent NIH sponsored Workshop on Pigment Gallstone Disease (8)] obtained from human cholecystectomy specimens were washed carefully in deionized distilled water to remove adherent bile and debris, dried to constant weight in a dessicator, and ground to a fine powder.

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PROTEIN AND GLYCOPROTEIN ANALYSIS

Direct measurement of protein or glycoprotein concentration of gallstones is impossible because of aqueous insolubility of the stone powder. We therefore used two separate methods of stone dissolution to prepare aqueous extracts suitable for colorimetric assays. In the first method stone powders were solubilized by prolonged exposure to EDTA and NaOH, whereas in the second stone powders were exposed only briefly in EDTA/NaOH, then subjected to column chromatography on Sepharose 4B-C1.

METHOD 1: EDTA/NaOH SOLUBILIZATION

Black pigment stone powder (0.5 mg) was dissolved in 1.0 ml of 12.5 mM EDTA in 0.1 N NaOH with continuous shaking and exposure for decolorization to long wavelength UV light (20 hr, 4°C) from a Blak-Ray Model B-100A lamp supplied by Ultraviolet Products, San Gabriel, Calif. The glycoprotein concentration of the resulting clear solution was measured according to the PAS method of Mantle and Allen (9), using bovine submaxillary mucin (Sigma Chemical Co., St. Louis, Mo.) as the standard. In each run, a standard containing known concentrations of bovine submaxillary mucin was also extracted in EDTA/NaOH as described above, in order to correct for losses of protein secondary to alkaline hydrolysis. The reactivity of extracted standards was approximately 50% of that obtained with unextracted bovine submaxillary mucin.

METHOD 2: COLUMN CHROMATOGRAPHY OF EXTRACTS

Stone powder (20 mg) was suspended in 80 ml of 12.5 mM EDTA in 0.1 N NaOH and stirred at 20°C for 5 min. Extracts were protected from light during this and all subsequent steps. The stone extract was then titrated to pH 8 with HCl and centrifuged at $500 g \times 10$ min. The sediment was reextracted in 25 ml of EDTA/NaOH solution according to the same procedure. The combined supernatants were dialyzed for 72 hr against deionized water which was changed twice daily. The dialyzed extract, which was deeply pigmented, was then lyophilized and stored at -20°C until further analysis. The efficiency of solubilization was calculated by expressing the dry weight of sediments remaining after centrifugation as a percentage of starting dry weight (assuming no loss of volatile components).

Lyophilized stone extracts were resuspended in 0.01 M Tris, 0.2 M NaCl buffer (pH 9) containing 0.02% sodium azide and loaded onto a 80- x 0.9-cm column of Sepharose 4B-C1 (Pharmacia Co., Piscataway, NJ) equilibrated with the same buffer. The column was wrapped in aluminum foil to exclude light and eluted at 4°C with Tris buffer at a rate of 5 ml per hr. Fractions of 2 ml were collected in the dark and analyzed for glycoprotein concentration according to the PAS method (9) and protein concentration according to the method of Lowry et al. (10) or optical absorbance at 280 nm.

In order to correct for bilirubin absorbance at O.D.₅₅₅ in the PAS assay, we subtracted a blank for each column

fraction assayed. This blank tube was identical to the sample tube except that the PAS dye was omitted in the blank. The O.D.₅₅₅ in the blank tubes contributed from 10 to 36% of total O.D.₅₅₅ in the PAS assay of column fractions. We also compared the O.D.₅₅₅ of equal amounts (10 to 100 µg) of submaxillary mucin and purified bovine gallbladder mucin, in order to validate the use of submaxillary mucin in our assays. These two mucin glycoproteins gave absorbances within 10% of each other in the PAS assay. Column fractions were monitored for bile pigment by measuring absorbance at 420 nm (11). In order to ascertain whether extraction in EDTA/NaOH caused destruction of mucin glycoprotein, we exposed purified bovine gallbladder mucin labeled in sialic acid residues with tritiated borohydride (12) to EDTA/NaOH as described above. In the control sample, NaOH was neutralized to pH 7 prior to addition of the mucin, while a duplicate sample was extracted in NaOH for 5 min then neutralized. The ³H-mucin samples were then chromatographed on Sepharose 4B as described above.

CHEMICAL ANALYSES

Calcium bilirubinate and carbonate were measured on aliquots of stone powder by quantitative infrared spectroscopy (13). Calcium was measured by atomic absorption spectroscopy (14), phosphate according to the method of Fiske and Subbarow (15), and cholesterol according to the method of Abell et al. (16). Data are expressed as percentage of stone weight (w/w).

DENSITY GRADIENT ULTRACENTRIFUGATION

High molecular weight glycoprotein fractions from the Sepharose 4B-C1 column were brought to 60% cesium chloride concentration by addition of dry CsCl₂ (density gradient grade; Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, NY). These mixtures were then subjected to ultracentrifugation as described by Starkey et al. (17). Eight 1-ml fractions from the gradient were analyzed for density, optical absorbance at 280 nm (protein) and 420 nm (bile pigment), and glycoprotein content by the PAS technique (9).

CARBOHYDRATE COMPOSITION OF PIGMENT STONE GLYCOPROTEIN

The carbohydrate composition of pigment stone glycoprotein could not be measured directly by gas-liquid chromatography (18) of column fractions or density gradient fractions owing to considerable interference by pigment. This problem was partially circumvented by performing β-elimination of pigment stone powder using the method of Herzberg et al. (19). Pooled pigment stone powder (800 mg) from five patients was suspended in 10.0 ml of 1.0 M sodium borohydride/0.1 N NaOH and incubated at 37°C for 24 hr. The mixture was centrifuged at $1,000 g \times 20$ min into an optically clear supernatant which was removed and a brownish black sediment which was reextracted under the same conditions. The combined supernatants were brought to pH 4.0 with acetic acid, and excess BH₄ was converted to boric acid by addition of 10-fold excess of 0.1 N formic acid. This material was loaded on a 2.5- x 5.0-cm column of Dowex

50-X 8 (H+ form), and oligosaccharides eluted with 5 column volumes of 0.01 *N* formic acid. Borate was removed by evaporation after the addition of methanol, and the oligosaccharide fraction was lyophilized. The greenish brown powder was then resuspended in 5 ml of a 1:1 mixture of benzene:water, vigorously shaken for 10 min, and centrifuged at 1,000 *g* × 30 min. The upper phase and insoluble interphase were separated from the light brown lower phase. Lyophilization of the latter yielded 12.9 mg of brownish powder which was subjected to gas-liquid chromatography for monosaccharides as described above. A galactosaminitol hexaacetate pure standard (Supelco, Bellefonte Pa.) was used to identify galactosaminitol produced by β -elimination of mucin-type glycoproteins.

RESULTS

COMPOSITION OF BLACK PIGMENT GALLSTONES

Analysis of eight pigment gallstones (Table 1) revealed a preponderance of bilirubin (mean 30.7%) and only small amounts of cholesterol (4.1%). The relative amounts of calcium, phosphate, and carbonate varied widely from 2.8 to 44.6%, with a mean of 19.1%. The glycoprotein contents of pigment stones in Table 1 were obtained by direct assay of stones solubilized in NaOH/EDTA for 20 hr (Method 1). The mean glycoprotein content was 12.4%. By using these values, we calculated the unmeasured portion of these eight black pigment stones to average 33.6%.

The glycoprotein composition of pigment stones, using direct colorimetric assay of solubilized stones (Method 1) vs. assay of Sepharose column fractions (Method 2), is given in Table 2. Direct assay of solubilized pigment stones yielded a mean glycoprotein content of 12.4%, approximately 3-fold higher than the value of 3.8% obtained by assay of Sepharose 4B column eluates. The results of these two methods were not well correlated for a given stone. Furthermore, the amount of total eluted glycoprotein did not correlate with the percentage of stone powder solubilized by this method. High molecular weight glycoprotein, as measured by PAS assay of void

TABLE 2. COMPARISON OF GLYCOPROTEIN CONTENT OF PIGMENT GALLSTONE EXTRACTS (METHOD 1) AND SEPHAROSE 4B COLUMN FRACTIONS (METHOD 2)^a

Stone	Method 1		Method 2	
	EDTA/NaOH extracts		Sepharose 4B column fractions	
	Glycoprotein (%)	Solubilized (%)	Total eluted glycoprotein (%)	High molecular weight mucin (%)
1	8	65.0	1.4	1.0
2	7	91.6	1.7	0.3
3	20	94.4	2.0	0.2
4	10	74.8	3.0	0.2
5	20	73.1	3.3	2.0
6	3	78.4	5.2	0.5
7	11	95.6	5.9	1.1
8	20	86.8	7.8	5.1
Mean	12.4	82.5	3.8	1.3

^a Glycoprotein concentrations (expressed here as percentage of dry weight) were determined on EDTA/NaOH extracts and Sepharose 4B column fractions as described in "Materials and Methods." The glycoprotein in the entire Sepharose 4B column eluate is given as total eluted glycoprotein, and glycoprotein in the void volume (see Figure 1) is given as high molecular weight mucin. These percentages are not corrected for the amount of stone powder not solubilized under these conditions.

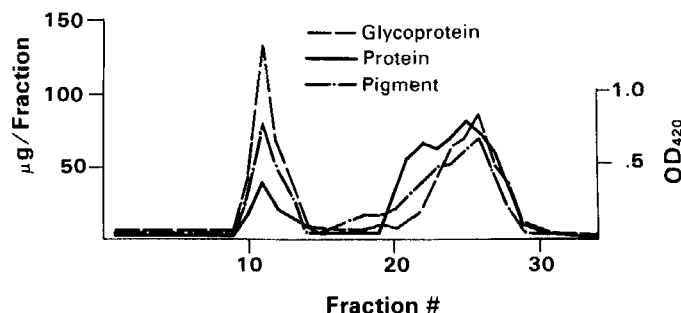


FIG. 1. Sepharose 4B column chromatography of dissolved pigment gallstone. Pigment stone (no. 7, Table 2) was partially dissolved in EDTA/NaOH, dialyzed, lyophilized, and then suspended in 2 ml of buffer and loaded onto a column of Sepharose 4B as described in "Materials and Methods." Each fraction was analyzed for glycoprotein and protein concentration (microgram per fraction) and for pigment (O.D.₄₂₀).

volume fractions of the Sepharose column (Figure 1), comprised on average one-third of the total eluted glycoprotein.

SEPARATION OF MUCIN GLYCOPROTEINS

Aqueous extraction of pigment stones was not successful in solubilizing significant amounts of the stone matrix. Therefore, we performed extraction in alkaline EDTA which solubilized a mean of 82.5% of these stones. After exhaustive dialysis, these aqueous extracts remained deeply pigmented. Sepharose 4B-Cl chromatography of dialyzed stone extracts from eight patients revealed a heterogeneous elution pattern of proteins and glycoproteins. However, in all samples, a PAS-positive high molecular weight glycoprotein eluted in the void volume of Sepharose 4B-Cl where mucin glycoproteins would be expected to elute. A typical elution pattern on

TABLE 1. COMPOSITION OF HUMAN BLACK PIGMENT STONES^a

Stone	% glycoprotein	% bilirubin	% cholesterol	% calcium, phosphate, and carbonate	% unmeasured
1	8	65.0	13.8	5.1	8.1
2	7	28.0	11.4	2.8	50.8
3	20	37.8	0.7	14.7	26.8
4	10	25.6	1.7	18.5	44.2
5	20	23.0	0.2	19.0	37.8
6	3	21.2	1.4	23.2	51.2
7	11	25.7	1.8	44.6	15.1
8	20	18.9	1.9	24.5	34.7
Mean	12.4	30.7	4.1	19.1	33.6

^a Each component is expressed as percentage (w/w) based on starting weight of desiccated stone powder. The percentage unmeasured was obtained by subtracting the sum of the other components from 100. Glycoprotein was measured on stone powders extracted for 20 hr in NaOH/EDTA as described in "Materials and Methods."

Sephacryl 4B-C1 is shown in Figure 1. In addition to the high molecular weight glycoprotein, a second broader PAS-reactive peak appeared in the included volume of the column. Both PAS-reactive peaks also contained significant amounts of protein (O.D.₂₈₀) and pigment (O.D.₄₂₀).

Further purification of mucin glycoproteins was carried out by density gradient ultracentrifugation on cesium chloride (Figure 2). Previous studies of gastric (20), small intestinal (21), and colonic mucus (22) have indicated that mucin glycoproteins can be separated by this technique from DNA and proteins which are often bound to mucin. Density gradient centrifugation (Figure 2) of mucin glycoproteins from the Sepharose 4B void volume revealed a major mucin fraction with an average density of 1.48 gm per ml (mean of four separate stones). This value agrees well with reported buoyant densities for gastric (20), intestinal (21), and respiratory (6) mucin glycoproteins.

The *O*-glycosidic bond of mucin-type glycoproteins is known to be sensitive to alkaline cleavage. We therefore reasoned that solubilization of pigment stones in alkaline EDTA (pH 11.5), even for 5 min, might significantly degrade gallbladder mucin. To estimate the extent of degradation in alkaline conditions, we exposed ³H-mucin from bovine gallbladder to our solubilization procedure, then chromatographed the dialyzed extract on Sepharose 4B. As shown in Figure 3, the mucin sample exposed to alkaline conditions was partially degraded to smaller components, which appeared in the included volume of the column in contrast to native (unextracted) mucin which eluted primarily in the void volume of this column. Following exposure to alkaline EDTA and dialysis as described in "Materials and Methods," we found an overall loss of 41.4% (mean of three experiments) of starting radioactivity, presumably from loss of small (<10,000 M.W.) radiolabeled glycopeptides from the dialysis bag.

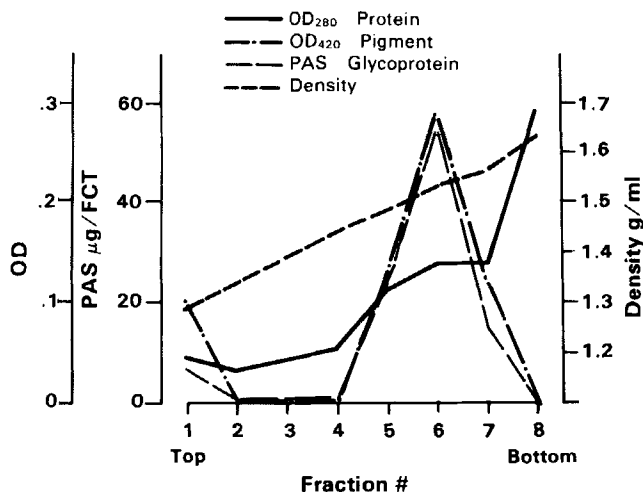


FIG. 2. Density gradient ultracentrifugation in cesium chloride of mucin fraction from Sepharose 4B column (Fig. 1). After 24 hr of centrifugation at 65,000 rpm (300,000 *g*), each 1-ml fraction was analyzed for protein (O.D.₂₈₀), pigment (O.D.₄₂₀) glycoprotein (microgram per fraction) and density (gram per milliliter).

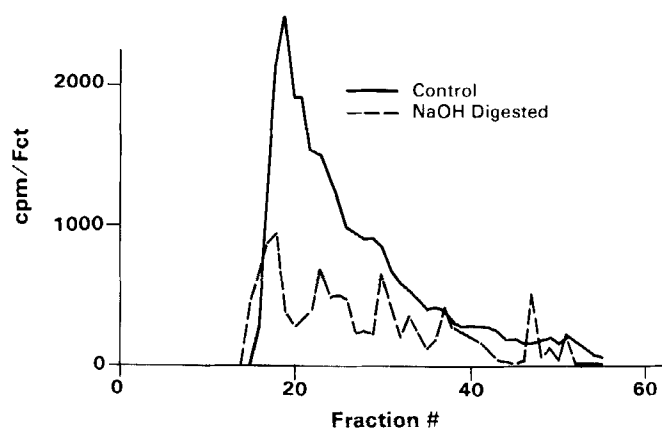


FIG. 3. Sepharose 4B column chromatography of undigested or EDTA/NaOH digested tritiated bovine gallbladder mucin. After exposure to EDTA/NaOH for 5 min, tritiated mucin was dialyzed for 48 hr and then subjected to Sepharose 4B chromatography. Column conditions were identical to those described in Fig. 1 except fractions of 1.5 ml were analyzed for radioactivity in a scintillation counter.

MONOSACCHARIDE COMPOSITION OF GALLSTONE GLYCOPROTEINS

Mucin glycoproteins typically contain 70 to 80% carbohydrate by weight, specifically fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid (20-22). In contrast, serum and membrane glycoproteins contain mannose or glucose, while glycosaminoglycans are characterized by the presence of glucuronic or iduronic acid (23). We therefore measured the monosaccharide content of glycoprotein fractions isolated from pigment stone extracts. Considerable difficulty was encountered in our initial attempts at gas-liquid chromatography of trimethylsilyl derivatives of either the Sepharose 4B void volume or cesium chloride gradient fractions, probably because of pigment interference. Numerous peaks were observed on the chromatograms, but unfortunately the retention times differed substantially from those of pure monosaccharide standards. Mixing pigmented glycoprotein fractions with known monosaccharide standards prior to gas-liquid chromatography produced considerable quenching and alteration of retention time of all monosaccharides (data not shown).

In order to obtain qualitative analysis of the protein-bound carbohydrates in pigment stones, we employed alkaline hydrolysis in the presence of borohydride (β -elimination) followed by isolation of oligosaccharides by Dowex chromatography and gas-liquid chromatography. This analysis (Figure 4) revealed the presence of fucose, galactose, *N*-acetylglucosamine, *N*-acetylneuraminic acid, and galactosaminitol. A prominent unidentified peak emerged from the column just before the internal standard mannitol.

DISCUSSION

The major result of our study was the quantitative estimation of the glycoprotein composition of human black pigment stones, and the isolation from stone extracts of a high molecular weight glycoprotein with typical chromatographic and compositional features of high

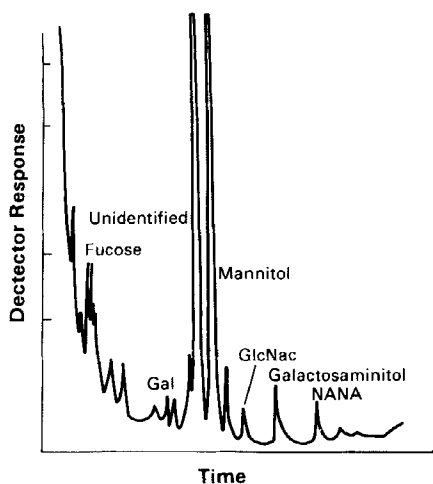


FIG. 4. Gas-liquid chromatography of combined pigment stone ($n = 3$) glycoproteins subjected to β -elimination and Dowex 50-X 8 chromatography prior to hydrolysis. Mannitol eluted at 3,927 sec after injection, and galactosaminitol at 5,369 sec. These values are within 2% of authentic standards run separately under identical conditions. Gal, galactose; GlcNac, *N*-acetylglucosamine; NANA, *N*-acetylneuraminic acid.

molecular weight mucin. These findings confirm and extend previous qualitative reports of the presence of mucin glycoproteins in cholesterol and carbonate stones of man and experimental animals. Lee et al. (4) reported the presence of monosaccharides in human pigment gallstones using gas-liquid chromatography but did not specifically identify gallbladder mucin glycoproteins as the source of these carbohydrates. In the present study, we have identified a high molecular weight glycoprotein which behaved, in gel chromatography (Figure 1) and density gradient ultracentrifugation studies (Figure 2), in a fashion similar to mucin from stomach (20), colon (22), and bovine gallbladder (Smith, B. F., et al., *Gastroenterology* 1982; 82:1245, Abstract). Moreover, the identity of mucin in pigment stones was further confirmed by the finding of galactosaminitol after β -elimination of gallstone glycoproteins in the presence of borohydride (Figure 4). Galactosaminitol is released only when the *O*-glycosidic linkage of mucin-type glycoproteins is cleaved in alkaline conditions, and thus serves as a convenient biochemical fingerprint for the presence of mucin (24).

The glycoprotein (Table 2) of pigment stones measured by the PAS assay of undialyzed alkaline extracts (Method 1) was considerably higher than that measured by the same assay of Sepharose 4B column eluates (Method 2). This result was not surprising in view of the fact that the preparation of the samples for Sepharose chromatography involved prolonged dialysis of the alkaline extract, resulting in loss of small glycopeptides. Furthermore, a mean of 82.5% of stone powders were solubilized after brief exposure to EDTA/NaOH (Table 2), leaving an insoluble residue of 17.5% which was not subjected to glycoprotein analysis. Although brief alkaline solubilization followed by dialysis probably underestimates glycoprotein content, this technique has the advantage of allowing further separation of intact or only partially degraded (Figure 3) mucin glycoproteins.

Bile pigment appeared to comigrate with high molecular weight mucin during Sepharose 4B chromatography (Figure 1) and also during analytical ultracentrifugation (Figure 2). Although the identity of this yellow pigment absorbing at 420 nm was not studied further, it may be bilirubin. Mucin from other organs has been shown to bind strongly to various lipid classes. For example, human tracheobronchial mucin remains strongly bound to neutral lipids, glycolipids, and phospholipids even after gel filtration and cesium bromide density ultracentrifugation (25). Hydrophobic binding sites on mucin probably reside in the protein core of the molecule rather than in the hydrophobic oligosaccharide chains. A cluster of hydrophobic amino acid residues occurs in the carboxy terminal region of bovine cervical mucin (26) which could provide a binding site for lipids.

A number of experimental and clinical observations suggest that gallbladder mucin may contribute to gallstone formation by providing a nidus or matrix for precipitation of lipid components (17-19). In mice with congenital hemolytic anemia, microscopic pigment-mucin concretions first arise in gallbladder neck glands, and are then extruded into the lumen where they grow to become pigment stones (3, 30). Hypersecretion of gallbladder mucin occurs in cholesterol-fed prairie dogs prior to stone formation (31); moreover, stone formation can be completely prevented in this animal model by aspirin, a potent inhibitor of mucin secretion (32). The initial steps of black pigment stone nucleation in humans are unknown. However, the presence of mucin glycoproteins in these stones, and the association of pigment with mucin even after stone dissolution, dialysis and purification are both consistent with the hypothesis that mucin glycoproteins may contribute to the organic matrix of these stones.

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