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INVITED REVIEW

Cover: scanning electron micrograph of endocardial endothelial cells lining left ventricle of perfusion-fixed rat heart. From D. L. Brutsaert and L. J. Andries. The endocardial endothelium. *Am. J. Physiol.* 263 (*Heart Circ. Physiol.* 32): H985–H1002, 1992.

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EDITORIAL REVIEW

SPECIAL COMMUNICATION

RAPID COMMUNICATION

Fibronectin in human gallbladder bile: cholesterol pronucleating and/or mucin "link" protein?

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Miquel, Juan Francisco, Christoph Von Ritter, Reginald Del Pozo, Volker Lange, Dieter Jüngst, and Gustav Paumgartner. Fibronectin in human gallbladder bile: cholesterol pronucleating and/or mucin "link" protein? *Am. J. Physiol.* 267 (*Gastrointest. Liver Physiol.* 30): G393–G400, 1994.—Some biliary proteins (pronucleators) seem to be essential factors for cholesterol crystal formation and crystal growth in bile. A recent study suggests that fibronectin is such a pronucleator in bile. Fibronectin also seems to closely interact with intestinal mucin. Since biliary mucin plays an important role in gallstone formation, such an interaction in bile may be of relevance in cholesterol gallstone formation. To more clearly elucidate the role of fibronectin in cholesterol gallstone disease, we measured the concentration of fibronectin in native bile of cholesterol gallstone patients and checked its influence on the cholesterol nucleation time of model bile. We further looked for a molecular interaction between biliary fibronectin and gallbladder mucin. We found that fibronectin is present in gallbladder bile of gallstone patients in low concentrations (2.6 ± 1.2 μg/ml). Bile fibronectin did not interact with gallbladder mucin. Moreover, in a wide range of concentrations fibronectin had no influence on the nucleation time of model bile. We conclude that fibronectin does not seem to play a major role in cholesterol gallstone disease.

biliary proteins; cholesterol gallstone disease

IT IS NOW WELL ACCEPTED that multiple factors are involved in the development of cholesterol gallstones in the human gallbladder (28). Cholesterol supersaturation alone does not explain the formation of stones. Measurement of "crystal detection time" or "nucleation time" (NT) allows a clear separation of bile samples from patients with and without cholesterol gallstones, and it, therefore, appears that cholesterol nucleation is a critical step in the formation of cholesterol gallstones (16, 28). Biliary proteins seem to play an important role in the regulation of the nucleation process (10, 36). In recent years, major efforts have been undertaken by different research groups to characterize biliary proteins that favor precipitation of cholesterol in model bile solutions (3, 5, 14, 25), to understand the molecular mechanism of interaction between these proteins and biliary lipids (12, 22, 25), and to define the relative importance of different known pronucleating proteins (26).

In a recent study (7), it has been suggested that fibronectin, a well-characterized multifunctional extracellular matrix and plasma glycoprotein (33), is a cholesterol pronucleating protein. The pronucleating effect of fibronectin on model bile was dose dependent in a range of concentrations found by the authors in native bile samples. It was also found that the concentrations of biliary fibronectin were significantly higher in patients with cholesterol gallstones compared with stone-free patients. However, no correlation between fibronectin concentrations and NT was found in the native bile samples.

It is now well accepted that mucin is an important contributor to cholesterol gallstone formation (21, 36). In addition to its cholesterol pronucleating effect, the viscoelastic features of mucin seem to be relevant for its role in lithogenesis. The high viscosity of mucin may increase the residence time of lithogenic bile in the gallbladder lumen (21, 36). The integrity of the macromolecular structure of mucin is essential for its rheological features. There have been major methodological difficulties in characterizing the macromolecular structure of this complex, large, and densely glycosylated glycoprotein (38). One view holds that an integral low-molecular-mass protein links mucin subunits to form a large polymer ("windmill" model). A "link protein" was isolated from purified human gallbladder mucin (29) but not from bovine gallbladder mucin (37). Slomiany et al. (35) have recently postulated that fibronectin closely interacts with intestinal mucin. It was shown that a concanavalin A (ConA)-binding fragment of fibronectin (118 kDa) is immunologically and chemically identical to the integral "118-kDa link protein" component found in purified mucin in previous studies (8, 24, 29).

The present study was designed to more clearly elucidate the role of fibronectin in cholesterol gallstone disease. We *1*) measured the concentrations of fibronectin in native bile of cholesterol gallstone patients, *2*) defined the molecular mass and immunological status of fibronectin in bile, *3*) investigated the influence of fibronectin on the nucleation time of a supersaturated model bile, and *4*) looked for a possible molecular interaction between biliary fibronectin and gallbladder mucin.

MATERIALS AND METHODS

Chemicals and reagents. Rabbit polyclonal anti-human fibronectin was obtained fron Calbiochem (San Diego, CA). The monoclonal anti-human fibronectin antibody was purchased from Boehringer Mannheim (Mannheim, Germany). This antibody belongs to the immunoglobulin (Ig) G1 subclass and reacts with human fibronectin and with proteolytic fragments that bind to heparin (17, 30). Monoclonal anti-human gallbladder mucin antibodies (GBM 59) were generated in the laboratories of Dr. A. K. Groen (Amsterdam, Netherlands). This mouse monoclonal antibody belongs to the IgM class. It specifically reacts with human gallbladder mucin with no cross-reactivity to pig gastric mucin, bovine submaxillar mucin, or ConA-binding biliary glycoproteins. The epitope recognized is probably the core sequence of the mucin carbohydrate

chain. Sepharose 4B-Cl and Sepharose 2B were obtained from Pharmacia (Uppsala, Sweden). CsCl was purchased from Beckman Instruments (Palo Alto, CA). All other chemicals were of analytical grade and were obtained from either Sigma Chemical (St. Louis, MO) or Merck (Darmstadt, Germany).

Bile collection. Gallbladder bile samples obtained from 10 cholesterol gallstone patients undergoing elective laparoscopic cholecystectomy were included in the study. At the beginning of the operation, before any surgical manipulation of the gallbladder and under visual control, gallbladder bile was completely aspirated into a sterile syringe by transcutaneous puncture with a 14-gauge needle. The samples were mixed with proteinase inhibitors phenylmethylsulfonyl fluoride, EDTA, *N*-ethylmaleimide, and NaN_3 at final concentrations of 1 mM, 5 mM, 10 mM, and 0.02%, respectively. The bile was immediately transported to the laboratory for further processing. Absence of blood contamination in the samples was checked by microscopic inspection of bile sediment obtained by centrifugation of 2 ml bile at 18,000 *g* for 10 min. Blood-contaminated bile samples ($n = 2$) were excluded from the study. Aliquots for biochemical determinations and fibronectin quantification were flushed with N_2 and stored at −30°C and −80°C, respectively. All patients except one had functional gallbladders as defined by a total lipid concentration >5 g/dl (9). Cholesterol stones were defined as stones with >50% cholesterol by weight and presence of multiple cholesterol monohydrate crystals at direct microscopic inspection of bile (18). After centrifugation of native bile at 18,000 *g* for 10 min at 37°C and subsequent microfiltration over 0.2 μm sterile Minisart filter (Sartorius, Göttingen, Germany), the NT of bile was measured as described by Holan et al. (16).

Gallbladder mucosal scrapings. In some of these patients ($n = 7$), the fresh closed gallbladder was also obtained. Within 30 min, the gallbladder was opened and gently washed with 0.9% NaCl (4°C). The epithelial surface was scraped with a glass slide to remove both epithelial cells and adherent mucus gel (37). The scraping was homogenized on ice in a Potter/Elvehjem tube for 3 min in 10 mM phosphate buffer and 0.2 M NaCl, pH 7.4 [phosphate-buffered saline (PBS)] with proteinase inhibitors in concentrations as above. The homogenate was centrifuged at 30,000 *g* for 30 min at 4°C (70.1 Ti rotor, Beckman Instruments). The supernatant was stored at −30°C for later mucin extraction and purification as described below.

Gel permeation chromatography of bile and purification of mucin. All bile samples were processed immediately after collection. Fractionation of biliary proteins and mucin extraction was performed according to the method of Pearson et al. (29), modified by Harvey et al. (13). All steps described below were performed under 4°C. Briefly, to remove bile acids, bile samples (5.0–7.0 ml) were dialyzed for 72 h against distilled water. containing 0.02% NaN_3. The dialyzed bile was centrifuged at 30,000 *g* for 30 min, and the lipid pellet was discarded. The supernatant was chromatographed on a Sepharose 4B-Cl column (80 × 2.6 cm) with PBS containing 0.02% NaN_3 as eluant, at a flow rate of 10 ml/h. Fractions of 5 ml were collected. The column was calibrated with standard molecular mass markers (range from 17 to 670 kDa; Sigma). The excluded fractions (F-I) containing the mucin-type glycoproteins were pooled and concentrated by reverse dialysis with 60% solution of polyethylene glycol to ~5 ml. Further purification of gallbladder mucin was performed by sequential (2×) CsCl-density gradient centrifugation (dgc; starting density = 1.42 g/ml). Centrifugation was carried out in 6-ml polyallomer tubes at 4°C at 300,000 *g* for 6 h in a TV-865 Sorvall vertical rotor (Du Pont Instruments, Bad Homburg, Germany). Six fractions (0.8–1 ml) were collected by tube puncturing from the bottom (Gradient fractionator; Hoefer Scientific Instruments, San Francisco, CA). Analysis of proteins and glycoproteins was performed as described below. Densities were determined by weighing the samples. No qualitative or quantitative differences were observed comparing this mode of CsCl-dgc with CsCl-dgc at 300,000 *g* for 24 h in a 70.1 Ti fixed angle rotor (Beckman Instruments) as described by Smith and LaMont (37) (data not shown). After dgc, the fractions containing mucin were pooled, extensively dialyzed against distilled water, lyophilized, and stored at −30°C for further analysis.

The included fractions from gel chromatography were pooled in two separate fractions according to the apparent molecular mass [F-II < void volume (V_o) > 200 kDa; F-III < 200 kDa], dialyzed for 48 h against distilled water, concentrated, lyophilized, and stored as described above.

Electrophoresis and protein blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 7% or 5–12% gradient separating gels with 3% stacking gel according to the method of Laemmli (20). Samples were run at a constant voltage of 50 mA in a Mighty Small II7CM vertical slab unit (Hoefer Scientific Instruments). Lyophilized pure gallbladder mucin was resuspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5–2% SDS with or without 0.7 M 2-β-mercaptoethanol (Laemmli's sample buffer for reduced and nonreduced conditions, respectively) and heated at 100°C for 5 min. Some mucin samples were reduced under more stringent conditions, i.e., by incubation with Laemmli's sample buffer for 40 h at 60°C as described by Pearson et al. (29). Aliquots of lyophilized samples from FII and FIII and native bile samples were resuspended in 1 ml distilled water. Proteins were precipitated with 7% (vol/vol) trichloroacetic acid (TCA) and delipidated with cooled diethyl ether/ethanol 3:1 (vol/vol) (18). For analytical SDS-PAGE, the gels were stained for proteins with silver (15) and for glycoproteins with the periodic acid-Schiff (PAS) method (23). Electrophoretic transfer of the proteins to nitrocellulose sheets was carried out in a semidry-blotting system at 170 mA for 90 min. To optimize the protein transfer, methanol content in all buffers was reduced to 10% and 0.05% SDS (wt/vol) included in the cathodic buffer. Satisfactory transfer was confirmed by silver and PAS staining of the blotted gels. The sheets were blocked by incubation for 2 h at 37°C with 50 mM Tris·HCl at pH 7.5 containing 0.1% Tween 20 and 0.2 M NaCl (TBS-Tween 0.1%) (1). For fibronectin detection, the membranes were incubated for 2 h with rabbit polyclonal anti-human fibronectin (Calbiochem) or monoclonal anti-human heparin binding fibronectin fragment (clone 3E1; Boehringer Mannheim) antibodies in TBS-Tween 0.05% at a dilution of 1:750. To detect gallbladder mucin, monoclonal anti-human gallbladder mucin antibodies were used (GBM 59) at a dilution of 1:3,000. The antibodies attached to the sheet were detected by incubation with alkaline phosphatase conjugated sheep anti-rabbit (Calbiochem) or anti-mouse (Boehringer Mannheim) immunoglobulins, respectively, and the chromogenic substrates for alkaline phosphatase (5-bromo-4 chloro-3-indolyl phosphate and nitroblue tetrazolium).

Fibronectin quantification. The concentrations of fibronectin in native bile samples were measured in the laboratory of Drs. Gressner and Kropf (Marburg, Germany) using a recently described highly sensitive and specific immunofluorometric assay (19). Briefly, aliquots of native bile stored at −80°C (for 1–4 mo) were quickly thawed by gentle shaking at 37°C and diluted 1:10 and 1:100 (vol/vol) in assay buffer (50 mM Tris·HCl, pH 7.5, 0.3 M KCl, 1 g/dl bovine serum albumin, and 0.05% NaN_3). Fibronectin quantification was carried out with the double antibody-based assay exactly as described before (19). Monoclonal anti-human fibronectin and polyclonal rabbit anti-human fibronectin were used as the coated and

detecting antibodies, respectively. Human-plasma fibronectin (Boehringer Mannheim, Germany) was used for standard curves.

Determination of nucleation-influencing activity. Model bile was prepared according to Groen et al. (11). The final lipid concentrations were (in mM) 130 taurocholate, 13 cholesterol, and 30 phosphatidylcholine. The cholesterol saturation index was 1.2, and total lipid concentration (TLC) was 9 g/dl. The NT of the control varied from 6 to 9 days with different batches of model bile. Within one batch of model bile, the SD of NT was 1 day (n = 40). A nucleation-influencing effect was considered significant when the difference in the nucleation time of the sample (NTs) with respect to the control (NTc) was two times the SD (2 days) (11). Human-plasma fibronectin (Calbiochem) checked for purity with gradient SDS-PAGE (5–12%) under reducing conditions was used for the additional experiments. Aliquots of fibronectin diluted in prefiltered PBS (0.2-μm sterile Minisart filters) were added in duplicate to 500-μl prefiltered (0.2-μm filters) model bile in 1.5-ml screw cup sterile Eppendorf tubes to yield final concentrations of 0.5, 1.2, 10, 20, and 40 μg/ml (n = 4 for each concentration). These concentrations cover the range of fibronectin concentrations found by us and others (7, 41) in gallbladder bile. Equal aliquots of PBS but without fibronectin were added to model bile as controls. NT was determined as described by Holan et al. (16). To validate the model bile for the nucleation-influencing activity assay, the effect of well characterized nucleation-promoting proteins was tested. Biliary pronucleating proteins were isolated from 500 μl lithogenic gallbladder bile by ConA Sepharose chromatography exactly as described before (11). The ConA-binding glycoprotein fraction dissolved in PBS and free of lipids was added to 500 μl of model bile to yield a final protein concentration of 200 μg/ml (n = 2). Equals volumes of PBS without protein were added to model bile as control.

Chemical analysis. Biliary lipids in native and model bile were determined by standard techniques as previously described (18). The cholesterol saturation index was determined using Carey's critical tables (6). Biliary proteins were measured after TCA precipitation and delipidation by the Lowry method modified by Jüngst et al. (18), using human serum albumin as standard. Protein in the eluted chromatographic and dgc fractions was estimated by the Bradford assay (2). Glycoprotein was estimated by the modified PAS method (23) with pig gastric mucin as standard (Sigma).

Statistical analysis. Data are given as means ± SE. Associations between variables were searched with scatter plots and confirmed by linear regression analysis.

RESULTS

Fibronectin levels in bile. Gallbladder bile was obtained from 10 cholesterol gallstone patients as described in MATERIALS AND METHODS. Table 1 shows the bile composition and cholesterol NT for the samples studied. With the use of the double antibody immunofluorometric assay with a detection limit <5 ng/ml (19), fibronectin was detected in bile of all the study patients. Concentrations were found to be 2.57 ± 1.16 μg/ml (mean ± SE; range 0.01–11 μg/ml). No significant correlation was observed between the levels of fibronectin in gallbladder bile and the NT of the samples (r = −0.29, P = 0.4) or any other bile component (TLC r = −0.18; mucin r = −0.25; total protein r = 0.22).

Nucleation influencing activity. Since gallbladder fibronectin was found to be intact antigenically and similar to plasma fibronectin (see Fig. 2), we decided to use plasma fibronectin to study the effect of fibronectin on the nucleation time of supersaturated model bile. All the previous studies on the pronucleating activity of fibronectin have also been performed with plasma fibronectin (4, 7). Fibronectin used showed a single protein band with a molecular mass of 440 kDa on a gradient SDS-PAGE (5–12%) under nonreducing conditions. As shown in Table 2, fibronectin had no effect on the nucleation time of supersaturated model bile in the range of concentrations found in gallbladder bile from gallstone patients (Table 1). In contrast, ConA-binding glycoprotein fraction (200 μg/ml) isolated from lithogenic bile showed a significant nucleation-promoting activity with an NTs/NTc ratio of 0.47 ± 0.02 at a concentration found in native bile.

Evaluation of structure and potential mucin association of biliary fibronectin. Bile obtained from the gallbladders of 10 patients with cholesterol gallstones was fractionated on a Sepharose 4B-Cl column. Figure 1*A* shows a representative chromatographic elution profile of a bile sample. A strongly PAS-positive glycoprotein fraction was excluded from the Sepharose 4B column together with lipids in vesicular form (as assessed by the cloudy appearance of the tubes). This excluded fraction was termed FI. The included fractions were pooled in FII (< V_o > 200 kDa) and FIII (<200 kDa).

Table 1. *Characteristics of gallbladder bile samples*

Patient	Protein, g/l	Mucin, mg/ml	FN, μg/ml	TLC, g/dl	CSI, %	NT, days
1	13.4	0.29	6.03	10.2	149	2
2	31.3	0.14	1.55	20.4	95	11
3	5.4	0.17	0.01	3.7	201	3
4	19.7	0.14	11.0	10.9	110	2
5	8.8	2.20	0.10	17.6	71	>14
6	12.0	0.15	0.28	8.9	85	5
7	33.6	0.92	5.31	12.1	92	ND
8	26.0	0.41	0.62	21.4	101	1
9	9.6	0.25	0.39	12.8	102	2
10	28.3	0.40	0.47	14.2	125	1
Means ± SE	18.7 ± 9.7	0.52 ± 0.63	2.57 ± 3.5	13.2 ± 5.1	113 ± 36	4.6 ± 4.7

Concentrations of total protein, mucin, fibronectin (FN), total lipids (TLC), cholesterol saturation index (CSI), and nucleation time (NT) of gallbladder samples collected from 10 patients with cholesterol gallstones.

Table 2. *Effect of FN on NT of model bile*

FN, μg/ml	NTs/NTc
0.5	0.9 ± 0.1
1.2	1.1 ± 0.3
10.0	1.2 ± 0.2
20.0	1.1 ± 0.1
40.0	1.1 ± 0.1

Nucleation time (NT)-influencing activity of fibronectin (FN). Nucleation-influencing activity is expressed as the ratio of NT of model bile with FN (NTs) over the NT of model bile with phosphate-buffered saline, i.e., control (NTc) (11). NT of the controls ranged from 6 to 9 days with a SD of 1 day. Pronucleating activity was defined as a ratio <0.73 and antinucleating activity as a ratio >1.3 (11). Values are means ± SE of 4 experiments.

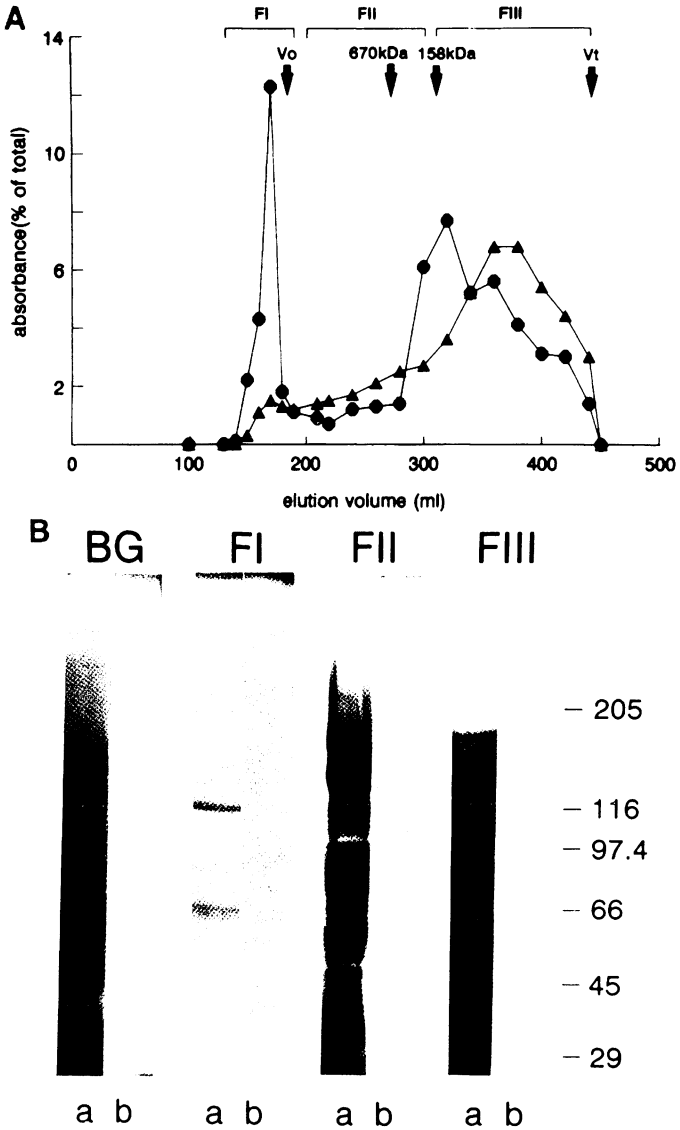

Fig. 1. *A*: Sepharose 4B-Cl chromatography of predialyzed bile sample. Bile (4–7 ml) was applied to the column (80 × 2.6 cm) and eluted at a constant flow rate of 0.3 ml/min at 4°C with PBS (10 mM phosphate buffer, 0.2 M NaCl, 0.02% NaN_3, pH 7.4) as eluant. Fractions of 5 ml of the eluate were collected and screened for glycoprotein (●) by the method of Mantle and Allen (23) and for protein (▲) by the method of Bradford (2). Excluded fractions rich in mucin type-glycoprotein were pooled in FI; the included fractions were separately pooled according to their molecular mass in FII [< void volume (V_0) > 200 kDa] and FIII (<200 kDa). Arrows indicate elution of molecular mass standards. *B*: 5–12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of biliary proteins. Proteins from native bile (BG) and from the pooled eluted fractions after Sepharose 4B-Cl chromatography (*A*; FI,II and III) were precipitated with 7% trichloroacetic acid followed by ether/ethanol (3:1) delipidation. Samples were resuspended in Laemmli's sample buffer under reducing conditions. A constant mass of 50 μg protein or glycoprotein (FI) was applied to each line. The gel was first stained for protein with silver (a) and counterstained thereafter with periodic acid-Schiff reagent (b). Migration of molecular mass markers as indicated (kDa).

Gradient SDS-PAGE with silver stain and counterstaining with PAS (Fig. 1*B*) showed that high molecular mass glycoproteins (mucin) were the main constituents of FI. A high molecular mass polypeptide band is visualized at the top of the gel in native bile and FI; this band stained intensely with PAS. However, other weakly stained low molecular mass proteins coeluted together with mucin in FI. Several polypeptide bands visualized with silver and/or PAS stain were found in FII and FIII. As expected, the most predominant protein in FIII had a molecular mass of 62 kDa and likely corresponded to albumin.

Immunoblotting of native biliary proteins and eluted protein fractions (FI-III) separated on SDS-PAGE under reducing and nonreducing conditions was carried out with polyclonal and monoclonal anti-human fibronectin antibodies. Under nonreducing conditions anti-human fibronectin antibodies detected a weak single polypeptide band with an apparent molecular mass of 440 kDa in native bile and in FII. This protein was identical to standard plasma fibronectin (data not shown). As expected, under reducing conditions the protein ran as an equimolar doublet with a relative molecular mass of 220 kDa that comigrates with the bands of plasma fibronectin (Fig. 2). Reactivity with anti-human fibronectin antibody was not observed in the mucin-rich excluded fractions (FI) of any of the 10 bile samples.

The excluded fractions (FI) from the Sepharose 4B-Cl chromatography were further purified by CsCl-dgc (2×).

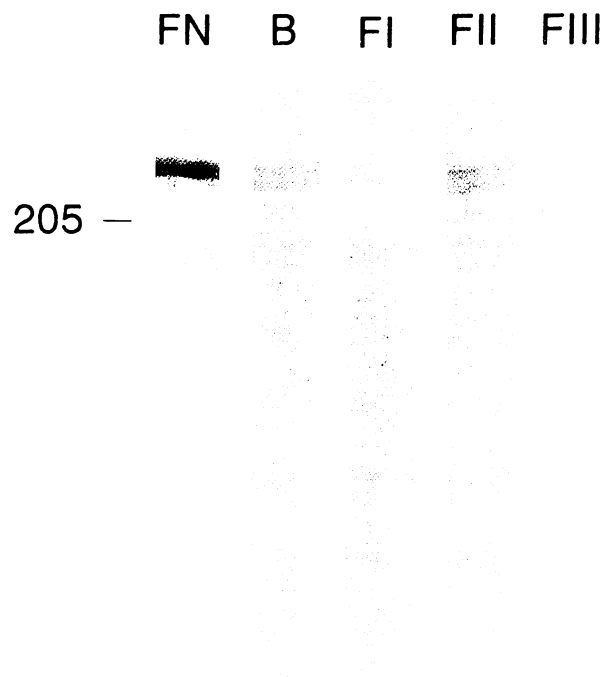

Fig. 2. Detection of fibronectin (FN) in native bile and in biliary protein fractions after Sepharose 4B-Cl chromatography (Fig. 1) using monoclonal anti-human FN antibody. SDS-PAGE was carried out on a 7% gel under reducing conditions. Western blots of standard plasma FN (*lane FN*, 0.1 μg), native biliary proteins (*lane B*, 300 μg), or proteins from the pooled eluted fractions after Sepharose 4B-Cl chromatography (*lanes FI, FII, FIII*; 300 μg protein for each lane) were probed with monoclonal anti-human FN antibody. As shown, standard FN is recognize as a doublet band with a molecular mass of 220 kDa. An identical doublet was shown in native gallbladder bile (*lane B*) and only in FII after gel chromatography. None of the low-molecular-mass proteins that comigrate with mucin in FI (see Fig. 1*B*) react with anti-human FN antibody (*lane FI*).

Because of the particular buoyant properties of mucin, this procedure separates highly glycosylated proteins (high-density *fraction 1*) from noncovalently bound proteins and lipids (lower density *fractions 5–6*; Fig. 3). Mucin recovered after dgc was free of low molecular mass proteins as assessed by gradient SDS-PAGE under nonreducing condition and silver stain. Integrity of the purified mucin was assessed by rechromatography on Sepharose 2B column. One milligram of purified gallbladder mucin was resuspended in PBS and loaded on the column. One-hundred percent of the PAS-positive purified glycoprotein remained in the excluded fractions. No low molecular mass break-down products were found (Fig. 4). Purified gallbladder mucin was then reduced by incubation with 0.7 M mercaptoethanol Laemmli sample buffer for 40 h at 60°C and dialyzed for 24 h against PBS. To assess the degree of reduction achieved, reduced mucin was also fractionated on Sepharose 2B (Fig. 4). Over 85% of the total glycoprotein recovered was included and eluted with a K_{av} of ~0.36, indicating a satisfactory reduction of disulfide bridges (29, 37). SDS-PAGE of pure biliary mucin under reducing conditions (either boiled for 5 min or incubated at 60°C for 40 h as described above) failed to show any mucin-associated low molecular mass protein with silver stain. All the low molecular mass proteins that coeluted with mucin in FI of the Sepharose 4B-Cl chromatography were recovered together with lipids in the low-density fractions (*fractions 5–6*) after CsCl-dgc (Fig. 5). Furthermore, dot blots of purified soluble biliary mucin, mucin extracted from gallbladder epithelial scraping, and proteins recovered from the low-density fractions showed no immunoreactivity with anti-human fibronectin antibodies. In contrast, a strongly positive immunoreactivity of both types of samples was observed with specific anti-human gallbladder mucin antibody (Fig. 6).

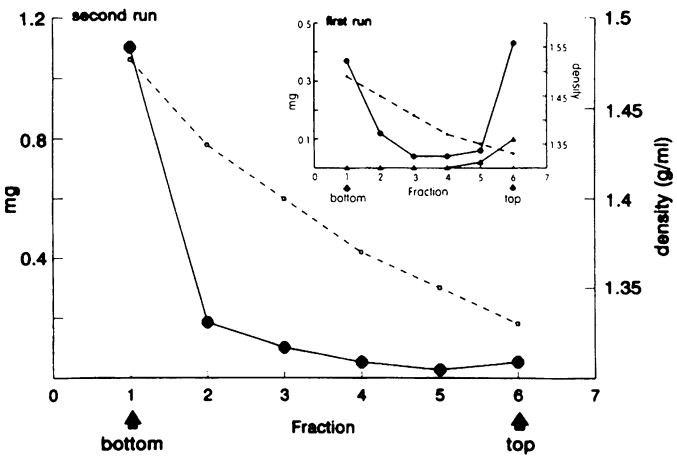

Fig. 3. Sequential CsCl-density gradient centrifugation of mucin-rich void volume fractions. FI of Sepharose 4B chromatography (Fig. 1A) was centrifuged in a vertical rotor at 300,000 *g* for 6 h. Starting density was 1.42 g/ml (dotted line). After centrifugation, the tubes were fractionated into 6 equal fractions (0.8–1 ml) by puncturing the bottom and were analyzed for glycoprotein (●) by the method of Mantle and Allen (23) and protein (▲) by the method of Bradford (2). *Inset*, initial CsCl-density gradient centrifugation (dgc) step. Duplicate samples of *fractions 1* and *2* from the initial dgc were diluted, brought to a density of 1.42 g/ml, and centrifuged once more under identical conditions (main graph).

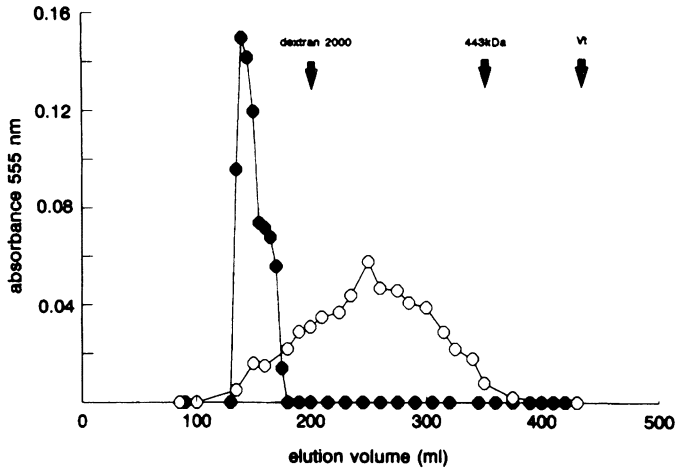

Fig. 4. Sepharose 2B chromatography of 1 mg of purified human gallbladder mucin before (●) and after reduction by incubation at 60°C for 40 h with 0.7 M mercaptoethanol (○). The column (85 × 2.6 cm) was eluted at a constant flow rate of 10 ml/h, and fractions of 5 ml were collected. The eluant was 0.2 M NaCl, 0.02% NaN_3, pH 7.4. Glycoprotein was estimated by the method of Mantle and Allen (23). Arrows indicate elution of molecular mass standards.

DISCUSSION

Fibronectin, a well-characterized multifunctional extracellular matrix and plasma protein (33), has been implicated to be a pronucleating agent of cholesterol in bile (7). In addition, it has recently been postulated that fibronectin closely interacts with mucin (35). In this context, the aim of the present study was to further define the possible role of fibronectin in the pathogenesis of cholesterol gallstone disease.

The concentrations of fibronectin in human gallbladder bile from cholesterol gallstone patients measured with an immunofluorometric assay (19) were in the same range as the concentrations previously described for gallstone patients using other methods (7, 41). In addition, we have found with immunoblot analysis using specific polyclonal and monoclonal antibodies against plasma fibronectin that bile fibronectin was antigenically intact and showed a similar molecular mass to plasma fibronectin (i.e., a doublet with 220 kDa under reducing conditions). No lower molecular mass proteolytic fragments of fibronectin were found.

The source of fibronectin in bile is unknown. Since hepatocytes appear to synthesize and secrete plasma-type fibronectin (39), it has been postulated that biliary fibronectin is of hepatic origin (7). However, in hepatocytes sorting and secretion of fibronectin into bile have not been demonstrated. Fibronectin is one of many plasma glycoproteins present in bile (27). Its concentration in bile is < 1% of the normal plasma concentration (300 μg/ml) (40). This is in the range of bile/plasma ratio measured for other plasma proteins (albumin, α_2-macroglobulin, and IgG) that use a hepatic paracellular pathway to reach the bile (27). Therefore, in addition to active secretion plasma fibronectin may passively diffuse through hepatic paracellular shunts into bile. However, fibronectin may also reach gallbladder bile by diffusion across the gallbladder epithelium. Fibronectin

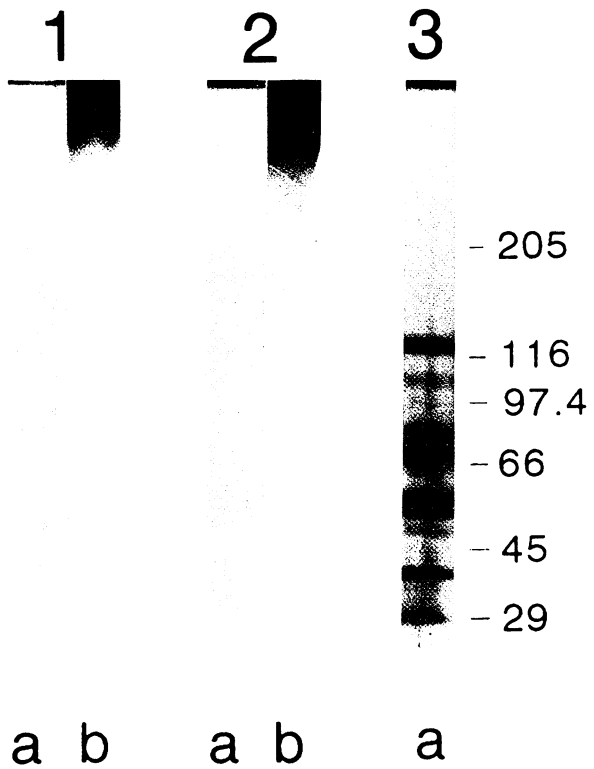

Fig. 5. Five to 12% gradient SDS-PAGE of purified gallbladder mucin. Biliary mucin was isolated by Sepharose 4B-Cl chromatography and purified by sequential CsCl-dgc (Figs. 1*A* and 3). The high-density *fractions 1* and *2* from the 2nd run were pooled, dialyzed, and lyophilized. Mucin was resuspended in Laemmli's sample buffer. Reduction was carried out by incubation with 0.7 M mercaptoethanol at 60°C for 40 h. *Lane 1*, 100 μg nonreduced mucin; *lane 2*, 100 μg reduced mucin; *lane 3*, low-density *fractions 5–6* from the 1st run of the CsCl-dgc. The gel was stained with silver (a) and counterstained with periodic acid-Schiff reagent (b). After reduction, both the polypeptide band at the top of the gel (a) and the single periodic acid-Schiff band (b) became broader. Reduction did not release any mucin "link protein." The position of the molecular mass standards is shown at right (kDa).

can be produced by macrophages, fibroblasts, polymorphonuclear leukocytes, and epithelial cells. Gallbladder inflammation is a frequent finding in gallstone patients. Biliary fibronectin appears to be higher in acute cholecystitis than in chronic cholecystitis and stone-free patients (7, 41). Increased levels of fibronectin have been measured in body fluids other than plasma as a consequence of epithelial damage and/or inflammation (19, 31, 34). We have observed that biliary concentrations of fibronectin did not correlate to the total lipid or bile salts content, i.e., biliary concentrations were independent of the degree of concentration of hepatic bile by the gallbladder. The absence of such a correlation points toward the gallbladder as the source of fibronectin in gallbladder bile. Conceivably therefore, fibronectin may leak through a damaged gallbladder epithelium into bile. Further studies are needed to better define the origin of fibronectin in bile.

Fibronectin failed to accelerate cholesterol nucleation in model bile in the range of concentrations measured in gallbladder bile of cholesterol gallstone patients (Table 2). This finding is supported by a recent study of Bruijn et al. (4) in which plasma fibronectin used in concentrations similar to in our experiments had no effect on cholesterol crystal growth in model bile. There are, however, major differences between both the latter study and our data and the study of Chijiiwa et al. (7) in which a marked pronucleating activity of plasma fibronectin was found. The reasons for this discrepancy are unclear. Methological differences in the preparation of the model bile may at least in part explain the different findings. We used a well-defined supersaturated model bile that has previously been shown to be a good in vitro model for cholesterol nucleation assays (11, 14, 25). Positive control assays using ConA-binding biliary proteins exerted the expected pronucleating effect in this model bile. Chijiiwa et al. (7) used a relatively fast nucleating bile (4 days) with a comparatively low sensitivity for pronucleating activity.

Studies on the cholesterol pronucleating activity of fibronectin in bile have all been performed using plasma

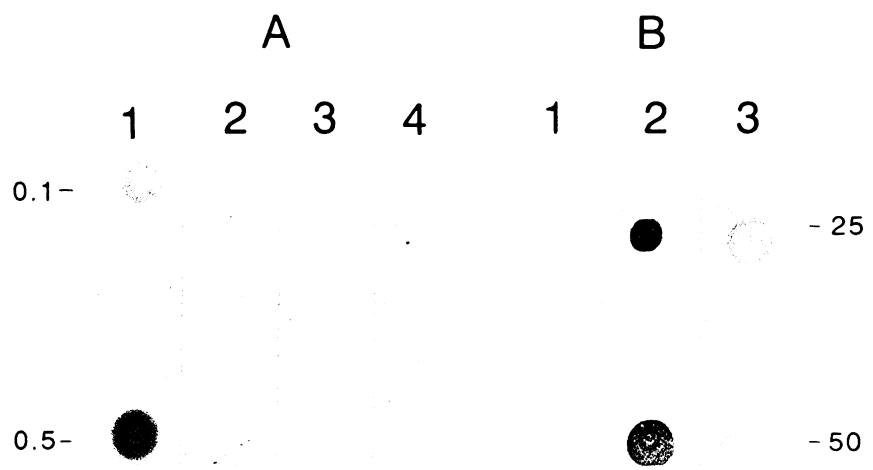

Fig. 6. Dot blot analysis of purified biliary mucin and mucin extracted from gallbladder epithelial scraping. Soluble biliary mucin and mucin from gallbladder epithelial scraping were extracted and purified as described in MATERIAL AND METHODS. Standard plasma fibronectin (*lane 1*), purified soluble biliary mucin (*lane 2*), and mucin from epithelial scrapings (*lane 3*) were probed with either monoclonal anti-human fibronectin (*A*) or monoclonal anti-human gallbladder mucin (*B*) antibodies. *Lane 4*, low-density fraction from the 1st run of the CsCl-dgc step. Amount of protein (μg) applied to each row of dots is show at right for mucin and low-density fractions proteins and at left for fibronectin.

fibronectin. Chijiiwa et al. (7), Bruijn et al. (4), and we have used plasma fibronectin because it appears that fibronectin in bile is identical to plasma fibronectin. To test this view, we have performed gel chromatography, SDS-PAGE, and Western blots on native bile. These experiments show identical immunoreactivity and identical molecular mass of bile and plasma fibronectin (Fig. 2).

A tight association between the "sticky" fibronectin and phospholipid vesicles has been reported (32). This observation appeared to support the view that fibronectin may exert pronucleating activity and may be the mechanism of its pronucleating effect in bile (7). However, as we have recently shown (25), a tight interaction of proteins with biliary vesicles does not necessarily implicate pronucleating activity of proteins. Indeed, in this previous study only three of the six proteins bound to vesicles isolated from hepatic bile of cholesterol gallstone patients exerted pronucleating activity. For the present study, it is of interest that fibronectin was not found to be bound to purified native biliary vesicles.

Gallbladder mucin is known to be a pronucleator in model bile. In addition, since it is the major determinant of bile viscosity it may profoundly influence gallbladder emptying and cholesterol crystal growth (21). The integrity of the macromolecular structure of mucin is essential for these mucus functions. For years, it has been postulated that mucin contains so-called integral link proteins that hold monomeric basic units together by covalent bonds to form the macrostructure of the large functionally active mucin (windmill model) (38). In intestinal mucin, the antigenically and chemical properties of a 118-kDa link protein appeared to be identical to a fibronectin fragment (35). A similar mucin-link protein has been previously postulated for gallbladder mucin (29). However, our gel chromatographic fractionation studies in combination with SDS-PAGE (Fig. 1*B*) and immunoblotting analysis (Figs. 2, 5, and 6) clearly show that fibronectin does not interact with soluble biliary mucin or mucin purified from gallbladder epithelial scrapings. Furthermore, under effective reducing conditions, purified gallbladder mucin did not release any other low molecular mass link proteins as assessed by gradient SDS-PAGE with silver stain. This observation is supported by elaborate studies of Smith and LaMont (37), which also failed to detect a mucin-link protein in bovine gallbladder mucin. Low-molecular-mass proteins (120–30 kDa) that coeluted with mucin in the V_0 fraction of gel chromatography (Fig. 1) were completely separated from mucin-type glycoproteins after a first or second run of CsCl-dgc (Figs. 3 and 5), showing that these proteins were not covalently bound to mucin. It is therefore, quite conceivable that most of these low- molecular-mass proteins coelute in the V_0 fraction because of a close binding to biliary vesicles and not to mucin (25).

In conclusion, fibronectin is present in gallbladder bile of cholesterol gallstone patients in concentrations similar to body fluids other than plasma. Bile fibronectin is antigenically intact and has a molecular mass similar to plasma fibronectin. In a wide range of concentrations, fibronectin has no effect on the nucleation time of supersaturated model bile. Purified human gallbladder mucin does not interact with biliary fibronectin nor does it require an integral link protein to maintain its macromolecular structure. Taken together, our data do not support a role of fibronectin in cholesterol gallstone disease.

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REFERENCES

1. **Batteiger, B., W. J. Newhall, and R. B. Jones.** The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. *J. Immunol. Methods* 55: 297–307, 1982.
2. **Bradford, M. M.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254, 1976.
3. **Bruijn, M. A. C., K. Mok, N. Domingo, H. Lafont, G. N. J. Tytgat, and A. K. Groen.** Further characterization of the cholesterol crystallization promoting lipoprotein in biliary Con A-binding protein (Abstract). *Gastroenterology* 102: A310, 1992.
4. **Bruijn, M., K. Mok, T. Out, B. Goldhoorn, G. N. J. Tytgat, and A. K. Groen.** A novel lipoprotein fraction is the major cholesterol crystallization promoting activity in bile (Abstract). *Falk Symposium* 68: 2, 1992.
5. **Busch, N., N. Matiuck, S. Sahlin, S. P. Pillay, and R. T. Holzbach.** Inhibition and promotion of cholesterol crystallization by protein fractions from normal human gallbladder bile. *J. Lipid Res.* 32: 695–702, 1991.
6. **Carey, M. C.** Critical tables for calculating the cholesterol saturation of native bile. *J. Lipid Res.* 19: 945–955, 1978.
7. **Chijiiwa, K., A. Koga, T. Yamasaki, K. Shimada, H. Noshiro, and F. Nakayama.** Fibronectin: a possible factor promoting cholesterol monohydrate crystallization in bile. *Biochim. Biophys. Acta* 1086: 44–48, 1991.
8. **Fahim, R. E. F., R. D. Specian, G. G. Forstner, and J. F. Forstner.** Characterization and localization of the putative "link" component in rat small-intestinal mucin. *Biochem. J.* 243: 631–640, 1987.
9. **Gollish, S. H., M. J. Burnstein, R. G. Ilson, C. N. Petrunka, and S. M. Strasberg.** Nucleation of cholesterol monohydrate crystals from hepatic and gallbladder bile of patients with cholesterol gallstones. *Gut* 24: 836–844, 1983.
10. **Groen, A. K.** Non-mucus glycoproteins as pronucleating agents. *Hepatology* 11: 189S–194S, 1990.
11. **Groen, A. K., C. Noordam, J. A. G. Drapers, P. Egbers, P. L. M. Jansen, and G. N. J. Tytgat.** Isolation of a potent cholesterol nucleation-promoting activity from human gallbladder bile: role in the pathogenesis of gallstone disease. *Hepatology* 11: 525–533, 1990.
12. **Groen, A. K., R. Ottenhoff, P. L. M. Jansen, J. Van Marle, and G. Tytgat.** Effect of cholesterol nucleation-promoting activity on cholesterol solubilization in model bile. *J. Lipid Res.* 30: 51–58, 1989.
13. **Harvey, P. R. C., C. A. Rupar, S. Gallinger, C. N. Petrunka, and S. M. Strasberg.** Quantitative and qualitative comparison

of gallbladder mucus glycoprotein from patients with and without gall stones. *Gut* 27: 374–381, 1986.

14. **Harvey, P. R. C., G. A. Upadhya, and S. M. Strasberg.** Immunoglobulins as nucleating proteins in the gallbladder bile of patients with cholesterol gallstones. *J. Biol. Chem.* 266: 13996–14003, 1991.
15. **Heukeshoven, J., and R. Dernick.** Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* 6: 103–112, 1985.
16. **Holan, K. R., T. Holzbach, R. E. Hermann, A. M. Cooperman, and W. J. Claffey.** Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology* 77: 611–617, 1979.
17. **Hynes, R.** Molecular biology of fibronectin. *Annu. Rev. Cell Biol.* 1: 67–90, 1984.
18. **Jüngst, D., T. Lang, C. Von Ritter, and G. Paumgartner.** Role of high total protein in gallbladder bile in the formation of cholesterol gallstones. *Gastroenterology* 100: 1724–1729, 1991.
19. **Kropf, J., E. Quitte, and A. M. Gressner.** Time-resolved immunofluorometric assays with measurement of a europium chelate in solution: application for sensitive determination of fibronectin. *Anal. Biochem.* 197: 258–265, 1991.
20. **Laemmli, U. K.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature Lond.* 227: 680–685, 1970.
21. **LaMont, J. T., B. F. Smith, and J. R. L. Moore.** Role of gallbladder mucin in the pathophysiology of gallstones. *Hepatology* 4, *Suppl.*: 51S–56S, 1984.
22. **Lee, T. J., and B. F. Smith.** Bovine gallbladder mucin promotes cholesterol crystal nucleation from cholesterol-transporting vesicles in supersaturated model bile. *J. Lipid Res.* 30: 491–498, 1989.
23. **Mantle, M., and A. Allen.** A colorimetric assay for glycoprotein based on the periodic acid/Schiff stain. *Biochem. Soc. Trans.* 6: 607–609, 1978.
24. **Mantle, M., and G. Stewart.** Intestinal mucin from normal subjects and patients with cystic fibrosis. *Biochem. J.* 259: 243–253, 1989.
25. **Miquel, J. F., L. Nunez, A. Rigotti, L. Amigo, E. Brandan, and F. Nervi.** Isolation and partial characterization of potent cholesterol pronucleating glycoproteins bound to native biliary vesicles. *FEBS Lett.* In press.
26. **Mok, K., T. Out, M. De Bruijn, B. Goldhoorn, G. N. J. Tytgat, and A. K. Groen.** An evaluation of the quantitative importance of individual pronucleating proteins in biliary concanavalin A-binding protein fraction (Abstract). *Gastroenterology* 102: A323, 1992.
27. **Mullock, B. M., L. J. Shaw, B. Fitzharris, J. Peppard, M. J. R. Hamilton, M. T. Simpson, T. M. Hunt, and R. H. Hinton.** Source of proteins in human bile. *Gut* 26: 500–509, 1985.
28. **Paumgartner, G., and T. Sauerbruch.** Gallstones: pathogenesis. *Lancet* 338: 1117–1121, 1991.
29. **Pearson, J. P., R. Kaura, W. Taylor, and A. Allen.** The composition and polymeric structure of mucus glycoprotein from human gallbladder bile. *Biochim. Biophys. Acta* 706: 221–228, 1982.
30. **Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti.** Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell* 26: 259–267, 1981.
31. **Rennard, S. I., and R. G. Crystal.** Fibronectin in human bronchopulmonary lavage fluid. *J. Clin. Invest.* 69: 113–122, 1981.
32. **Rossi, J. D., and B. A. Wallace.** Binding of fibronectin to phospholipid vesicles. *J. Biol. Chem.* 258: 3327–3331, 1983.
33. **Ruoslathi, E.** Fibronectin and its receptors. *Annu. Rev. Biochem.* 57: 375–413, 1988.
34. **Salonen, E. M., T. Vartio, V. Miggiano, C. Stähli, B. Tacacs, G. Virgallita, G. De Petro, S. Bartali, and A. Vahri.** A rapid and highly sensitive solid-phase enzyme immunoassay specific for human fibronectin using a characterized monoclonal antibody. *J. Immunol. Methods* 72: 145–156, 1984.
35. **Slomiany, A., K. Okasaki, S. Tamura, and B. L. Slomiany.** Identity of mucin's "118-kDa link protein" with fibronectin fragment. *Arch. Biochem. Biophys.* 286: 383–388, 1991.
36. **Smith, B. F.** Gallbladder mucin as a pronucleating agent for cholesterol monohydrate crystals in bile. *Hepatology* 11: 183–188, 1990.
37. **Smith, B. F., and J. T. LaMont.** Hydrophobic binding properties of bovine gallbladder mucin. *J. Biol. Chem.* 259: 12170–12177, 1984.
38. **Strous, G. J., and J. Dekker.** Mucin-type glycoproteins. *Crit. Rev. Biochem. Mol. Biol.* 27: 57–92, 1992.
39. **Tamkun, J. W., and R. O. Hynes.** Plasma fibronectin is synthesized and secreted by hepatocytes. *J. Biol. Chem.* 258: 4641–4647, 1983.
40. **Webb, K. S., and G. H. Lin.** Urinary fibronectin. Potential as a biomarker in prostatic cancer. *Invest. Urol.* 17: 401–404, 1980.
41. **Wilton, P. B., A. P. Dalmasso, and M. O. Allen.** Complement in local biliary tract defense: dissociation between bile complement and acute phase reactants in cholecystitis. *J. Surg. Res.* 42: 434–439, 1987.