Immunoglobulins as Nucleating Proteins in the Gallbladder Bile of Patients with Cholesterol Gallstones*

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The gallbladder bile of patients with cholesterol gallstones contains pronucleating proteins which accelerate precipitation of cholesterol crystals from bile. In this study we have improved the purification procedure developed earlier for these nucleating proteins and have now identified the nature of these proteins. Gallbladder bile from patients with cholesterol gallstones was applied to concanavalin A affinity columns. The ConA-binding glycoprotein fractions containing the nucleating proteins were then separated by FPLC (fast protein liquid chromatography) using a Superose 12 gel filtration column. Nucleating activity was detected in the high molecular weight (FPLC-1) as well as in the low molecular weight fractions (FPLC-3). Investigation of the high molecular weight fraction by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electroelution and amino acid sequencing suggested that these proteins were immunoglobulins. Immunostaining of Western blots with specific monoclonal antibodies identified the presence of immunoglobulin (Ig) M and IgA in the FPLC-1 fraction. These immunoglobulins were further purified by affinity chromatography employing an antibody exchange (ABx) column which specifically binds immunoglobulins. There was no reduction in the cholesterol nucleating activity in the ABx-bound fraction compared to FPLC-1. Additional studies showed that the FPLC-1 fraction was significantly more potent than the ConA glycoproteins from either rapid and slow nucleating biles. Also the number of crystals formed was significantly greater in the FPLC-1 fraction isolated from cholesterol gallstone biles than from the FPLC-1 fraction from control patient biles. Commercially obtained IgM and IgA had no effect on nucleation, but IgM isolated from the serum of patients with Waldenstrom's macroglobulinemia did accelerate the nucleation of cholesterol. We conclude that the IgM and possibly IgA are pronucleating proteins and may be important in the pathogenesis of cholesterol gallstones in man.

Both antinucleating and pronucleating proteins present in bile have been implicated in the pathogenesis of cholesterol gallstone disease. The antinucleating proteins slow the nucleation of cholesterol from bile (1, 2), while pronucleating proteins accelerate the nucleation of cholesterol crystals (3–8). It is likely that pronucleating and antinucleating proteins coexist in the same bile samples and that the propensity of gallbladder bile to nucleate cholesterol depends on the balance between these opposing influences.

Two pronucleating proteins have been identified, mucous glycoprotein (3, 4) and a glycoprotein that binds to concanavalin A (6–8). During our investigations on the role of mucous glycoproteins in the formation of cholesterol gallstones, we obtained evidence suggesting that a smaller pronucleating protein existed in the bile of patients with cholesterol gallstones (5, 9). This protein appears to bind to concanavalin A and acts in a dose-response manner (10). In this study we have characterized some of the ConA-binding proteins and assessed their ability to accelerate the nucleation of cholesterol from model biles. Using ConA affinity chromatography followed by fast protein liquid chromatography with a gel filtration column, a high degree of purification of the nucleating glycoprotein was attained. Characterization of these proteins indicated that they are rich in IgM and IgA. Dose-response studies were done to determine the lowest consistently effective nucleating dose of the proteins and the nucleating potency of bile proteins from cholesterol gallstone and control patients, serum immunoglobulins from commercial sources, and serum immunoglobulins from patients with Waldenstrom's macroglobulinemia.

EXPERIMENTAL PROCEDURES

Bile Collection

Gallbladder bile was collected from patients during cholecystectomy for cholelithiasis and from patients without gallstones having surgery for Crohn's disease, ulcerative colitis, or intestinal tumors, as previously described (11) using procedures approved by the Human Ethics Committee of the University of Toronto. Written informed consent was obtained prior to bile collection. All control patients were confirmed to be free of stones by ultrasound examination in the perioperative period. Cholesterol gallstones were confirmed to be cholesterol by chemical analysis (12) (i.e. greater than 70% cholesterol by weight). Pigmented stones contained less than 5% cholesterol by weight. Bile was collected only from patients who had normal liver function tests (serum alkaline phosphatase, glutamic oxaloacetic transaminase, total bilirubin) and a functioning gallbladder, as determined by total lipid concentration greater than 5.0 g/dl (13).

The abbreviations used are: ConA, concanavalin A; FPLC, fast protein liquid chromatography; ABx, antibody exchanger; MES, 2-(N-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Protein Purification

Concanavalin A Affinity Chromatography—Since we have found that proteases are present in gallbladder bile (14), a mixture of proteolytic inhibitors was added to gallbladder bile samples to give a final concentration of phenylmethylsulfonyl fluoride 100 μM, pepstatin 1 μM, antipain 1 μM, and iodoacetamide 1 mM. Gallbladder bile (5-10 ml) was then applied directly to a concanavalin A-Sepharose column (0.7 x 13 cm; 5.0 ml of ConA-Sepharose, Pharmacia, Uppsala, Sweden), and the unbound proteins were eluted with Tris-HCl buffer (100 mM, pH 7.4) containing 0.5 M NaCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM MnCl₂, and 0.02% NaN₃. The column was washed until the eluent was free of lipids and biliary pigments. The bound ConA glycoproteins were then eluted with the above buffer after adding 0.2 M α-D-methylmannopyranoside.

Fast Protein Liquid Chromatography (FPLC)—The ConA-bound glycoproteins from the Sepharose column were separated according to molecular weight by FPLC (Pharmacia, Uppsala, Sweden) employing a Superose-12 HR 10/30 prepacked column (Pharmacia). Briefly, the dialyzed (at 4 °C against distilled water) lyophilized ConA glycoprotein preparations were dissolved in an eluting buffer of 50 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl and 0.02% NaN₃. Aliquots of 200 μl containing 500 μg of protein were injected into the system, and 0.5-ml fractions were collected at a flow rate of 0.4 ml/min. Protein peaks (Fig. 1) were pooled into three fractions: FPLC-1 (>500 kDa), FPLC-2 (110 to ~500 kDa), and FPLC-3 (<130 kDa). For the experiments involving amino acid sequencing, the protein bands followed by rapid destaining (Coomassie Blue for 5.0 min, destaining in acetic acid/methanol/water, 50:165:785 (v/v/v) for 2-3 h). The visualized bands were then cut out and subjected to amino acid sequencing employing an Applied Biosystems gas-phase amino acid sequencer provided by the Protein Analysis Service, Department of Biochemistry at the University of Toronto.

Immunostaining of Western Blots

Immunostaining by the enzyme-linked immunosorbent assay was performed according to the method of Tsang et al. (27). Following transfer, the Immobilon was blocked with 5% Carnation milk in Tris-buffered saline (20 mM Tris, pH 7.4, 150 mM NaCl, 0.02% NaN₃) for 1.0 h at room temperature. The antibody was a gift from Dr. G. Sigurdsson (Enzymobead Reagents-glucose oxidase/lactoperoxidase, Bio-Rad). To increase the sensitivity of the immunoassay, the secondary enzyme-linked antibody was radiolabeled. These 125I-labeled antibodies specific for the heavy chains of their respective immunoglobulins were used to immunostain IgM and IgA under reducing conditions.
Comparison of ConA Glycoproteins and FPLC-1 Protein Fractions Isolated from Slow and Rapid Nucleating Bile

ConA glycoproteins isolated from patients with cholesterol gallstones (n = 7, crystal observation time less than or equal to 2 days) and slow nucleating biles (n = 6, crystal observation time greater than 10 days from control patients or patients with pigment stones) were dialyzed against the Tris-HCl buffer that was used for FPLC (50 mM Tris-HCl, 0.2 M NaCl, 0.02% NaN3, pH 7.4). Following dialysis (three changes, 2 liters each) ConA glycoproteins were concentrated to approximately 0.5–1.0 ml by Amicon filtration using ultrafiltration membranes YM-10 followed by use of Centricon-10 concentrators (Amicon Canada Ltd., Oakville, Ontario). FPLC-1 fractions from these ConA glycoprotein preparations were obtained as previously described (see “Past Protein Liquid Chromatography”). Eluted FPLC-1 fractions were then concentrated using Centricon-10 concentrators to 200–500 μl. Aliquots of the concentrated ConA and FPLC-1 fraction preparations were taken for protein determination by the Lowry assay (21). Protein masses of 25 and 50 μg were aliquoted and brought to a volume of 100 μl with the FPLC buffer and used for cholesterol nucleation assays. Proteins were added to 300 μl of model bile (cholesterol saturation index = 1.2, total lipid concentration = 8.0 g/dl in 10 mM Tris-HCl, 0.12 M NaCl, 0.02% NaN3, pH 7.4) to give a final concentration of 62.5 and 125 μg/ml, respectively. Controls consisted of 100 μl of the FPLC buffer added to 300 μl of model bile. Crystal observation time as well as the number of crystals in a 10-μl aliquot/day were recorded.

Addition of Commercial Immunoglobulins and Isolated Serum Immunoglobulins

Human secretory IgA (pooled colostrum) and human IgM (pooled serum) were purchased from Cappel (Organon Teknika Corp., West Chester, PA). Serum immunoglobulins purified from individual patients were kindly provided by Dr. M. Schiff of the Department of Immunology, University of Toronto. Serum IgA was purified from multiple myeloma patients and IgM from patients with Waldenstrom’s macroglobulinemia. SDS-PAGE showed that proteins from all sources (commercial and serum isolates) were free of contaminants. Immunoglobulins from these two sources were added to both model and heated abnormal biles at a final concentration of 250 μg/ml. A few studies were also performed at lower concentrations.

Other Chemical Methods

Biliary lipids, namely cholesterol, phospholipid, and bile salts contained in model biles and heated abnormal biles were determined by standard techniques (12, 28, 29). The cholesterol saturation index was determined using Carey’s critical tables (30).

Statistics

Data were evaluated by random block design with one way layout of treatments, and Tukey’s studentized range test using SAS (31). The number of crystals formed over the observation period with the addition of ConA glycoproteins and FPLC-1 fractions were analyzed using the Generalized Linear Interactive Modeling System (Numerical Algorithms Group Inc., Downer’s Grove, IL).

RESULTS

Separation of ConA Glycoproteins by FPLC—Since the results of previous studies indicated that pronucleating proteins are present in the glycoproteins binding to ConA, our first experiments were directed at further purification of these glycoproteins. ConA glycoproteins from gallbladder bile of patients with cholesterol gallstones were separated according to molecular weight by FPLC on a Superose 12 gel filtration column. Using this method a characteristic pattern for ConA-bound glycoproteins isolated from the biles was observed (Fig. 1). There were two large peaks at the high and low molecular weight ranges. These were designated FPLC-1 and FPLC-3, respectively. The smaller series of peaks between FPLC-1 and FPLC-3 was considered a single fraction, FPLC-2. The quantity of protein contained in the void volume (FPLC-1) varied from patient to patient.

Addition of FPLC Fractions to Model Bile and Heated Abnormal Bile—Having isolated three molecular weight ConA-binding glycoprotein fractions, we then sought to determine their activity with respect to the nucleation of cholesterol. Addition experiments were first performed using a model bile system. Results of adding equal masses of unseparated ConA glycoproteins and the three FPLC fractions (FPLC-1 > 500 kDa, FPLC-2 110–500 kDa; FPLC-3 < 110 kDa) from 12 patients with cholesterol gallstones to model bile are shown in Fig. 2A. Although there was some heterogeneity from patient to patient, ConA glycoproteins FPLC-1 and FPLC-3 significantly reduced the crystal observation time compared to the control model bile. Similar results were obtained by adding the fractions from 10 patients with cholesterol gallstones to another test system, heated abnormal biles (simulated control bile) (Fig. 2B). In three cases there was enough protein isolated from the sample to permit additions to both test systems. The results were generally the same, with reduction of the crystal observation time occurring after addition of FPLC fractions 1 and 3 and the ConA fraction.

Polyacrylamide Gel Electrophoresis and Amino Acid Sequencing of FPLC Fractions—In order to further characterize the active FPLC fractions, polyacrylamide gel electrophoresis was performed. Under nonreducing conditions, FPLC-1 contained high molecular weight proteins consistent with the gel filtration results (see below). Having identified strong nucleating activity in the FPLC-1 fraction, it was of interest to further characterize these polypeptides. As a first step in this direction we decided to determine the amino acid sequence of two FPLC-1 protein subunits. The proteins contained in FPLC-1 were electrophoresed under reducing conditions and a 75- and a 60-kDa subunit were identified. These bands were electroeluted onto Immobilon for amino acid sequencing. Fifteen cycles were completed for the electroeluted 75- and 60-kDa proteins. For the 75-kDa protein, 9 out of 15 amino acids could be matched to the amino acid sequence of a standard immunoglobulin heavy chain (Sequences of Proteins of Immunological Interest; U.S. Department of Health and Human
additions to heated abnormal bile

FPLC saturation index

both under nonreducing and reducing conditions (Fig. 3, A

FIG. 2. Results of additions of ConA glycoproteins and FPLC fractions. Panel A, addition to model bile (n = 12, cholesterol saturation index = 1.2, total lipid concentration = 8 g/dl). Panel B, additions to heated abnormal bile (n = 10, simulated control bile). *p < 0.05 when compared to control value.

A. Non-Reduced SDS-PAGE

1 2 3 4 5 6 7

200

116

92.5

67

45

31

21.5

14.4

B. Reduced SDS-PAGE

1 2 3 4 5 6 7

200

116

92.5

67

45

31

21.5

14.4

FIG. 3. SDS-PAGE analysis. A constant mass of protein (50 μg) as determined by the Lowry procedure was applied to each lane. Proteins were stained with Coomassie Brilliant Blue. (gels 16 × 14 cm, 1.5 mm thick). Panel A, nonreduced SDS-PAGE of ConA eluate and FPLC fractions. Lane 1, molecular weight markers. Lane 2, IgM standard. Lane 3, sIgA standard. Lane 4, ConA-bound fraction. Lane 5, FPLC-1. Lane 6, FPLC-2. Lane 7, FPLC-3. Panel B, reduced SDS-PAGE of ConA eluate and FPLC fractions. Lane 1, molecular weight markers. Lane 2, IgM standard. Lane 3, sIgA standard. Lane 4, ConA-bound fraction. Lane 5, FPLC-1. Lane 6, FPLC-2. Lane 7, FPLC-3.

Services, National Institutes of Health). The 60-kDa protein sequence matched with seven amino acids of the standard. These results suggested that the 75- and 60-kDa bands might consist of the heavy chains of immunoglobulins.

In order to further examine this possibility, the ConA and FPLC fractions were run against standards of IgA and IgM both under nonreducing and reducing conditions (Fig. 3, A and B). In the nonreduced gel (A) the IgM standard (lane 2) ran as a single very high molecular weight band and the IgA standard (lane 3) ran as two somewhat lower molecular weight bands. The latter bands are probably the dimer and the tetramer of IgA. In FPLC-1 (lane 5) bands were observed at both the IgM and IgA levels. It was of interest that the dimer was also seen in FPLC-2 (lane 6), as the protein band of highest molecular weight in that fraction.

The ConA fraction (lane 4) contained protein bands distributed over the entire molecular weight range of the gel. With few exceptions the molecular weights of the protein bands in the FPLC fractions corresponded to the expected molecular weight ranges, with FPLC-1 (lane 5) containing the highest molecular weight bands, FPLC-3 (lane 7) the bands of lowest molecular weight, and FPLC-2, bands of intermediate weight. There was a small overlap between fractions. One exception to the expected molecular weight distribution was a faint band at 35 kDa in the ConA and all three FPLC fractions. This band is ConA itself, a small amount of which eluted from the column with the glycoproteins, ran on FPLC with the respective molecular weight class, but then separated from the glycoproteins under the conditions of SDS-PAGE electrophoresis. This has been determined using a ConA standard. Addition of ConA to model bile does not affect the crystal observation time (data not shown). A very small molecular mass protein in the 14 kDa range also appeared in FPLC-2 (lane 6). A band was present in the same position in the IgA standard; this band is in the position expected for the J chain of IgA. Possibly J chain separates from the IgA dimer in FPLC-2 during SDS-PAGE and then runs at this anomalous molecular weight. Note that an intermediate molecular weight band can also be seen in the IgA standard (lane 3). This band has the approximate molecular weight of secretory component. In evaluating the molecular weights of bands in Fig. 3A, it should be remembered that under nonreduced conditions proteins are often in a more globular form, than after reduction. This affects their movement through the gel, and under nonreduced conditions they may have a spuriously low molecular weight.

Fig. 3B demonstrates the results obtained under reducing conditions in the same fractions. The following component polypeptide chains of the immunoglobulin standards were observed. In lane 2 the heavy and light chains of IgM were identified, while in lane 3 the light and heavy chains of IgA (at about 60 and 30 kDa), secretory component (75 kDa), and J chain (14 kDa) may be seen. FPLC-1 (lane 5) contained three major protein bands with apparent molecular masses of 75, 60, and 30 kDa. These bands corresponded to the heavy chains of IgM (75 kDa) and IgA (60 kDa) and their light chains (30 kDa). Note that secretory component and IgM heavy chain run at the same molecular mass so that by this technique it is not possible to determine whether the 75-kDa band in FPLC-1 is either or both. FPLC-2 (lane 6) contained several bands, two of which ran in the positions of IgA heavy and light chain, in keeping with the presence of IgA dimer in this fraction in the nonreduced gel. There was little 75-kDa band in this fraction. FPLC-3 (lane 7) contained a number of proteins. One ran at 75 kDa, leading to the suggestion that the nucleating potency of FPLC-3 might be due to a detached immunoglobulin subunit such as secretory component or IgM heavy chain. We decided to concentrate our efforts at trying to identify the proteins contained in the FPLC-1 peak.

Western Blotting of FPLC-1 Proteins—To further examine the nature of the FPLC-1 peak proteins, the proteins were analyzed by Western blotting as shown in Fig. 4. Again the analysis was performed under both nonreduced and reduced
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Further Purification of the FPLC-1 Fraction—As only immunoglobulins were detectable in FPLC-1, it was highly likely that these immunoglobulins were the pronucleating elements in this fraction. However, it seemed remotely possible that FPLC-1 might contain a non-immunoglobulin, with potent pronucleating activity, but which was present in such small quantities that it was undetectable on the gels. To rule out this possibility, we used affinity chromatography to bind the immunoglobulins in FPLC-1, and we then examined the nucleating potency of the bound fraction to determine if it retained the potency of FPLC-1. To further purify FPLC-1, affinity chromatography was performed employing Bakerbond ABx, a media with specific affinity for immunoglobulins. Approximately 90% of the proteins of FPLC-1 bound to the Bakerbond ABx column (Fig. 5). Immunostaining of Western blots of the bound ABx fraction confirmed the presence of IgM and IgA (Fig. 6). Silver staining of PAGE of the ABx bound proteins showed the absence of other contaminating protein bands (Fig. 7). Rechromatography of the bound ABx fraction onto a Superose 6 column resulted in the elution of a single protein peak in the included volume at a molecular mass range of 650–950 kDa (Fig. 5, inset). In six studies the bound ABx fraction proteins accelerated the crystal observation time of heated abnormal bile from 5.0 ± 1.7 to 1.5 ± 0.5 S.D. days (day 1 in three studies and day 2 in three studies, p < 0.002). These crystal observation times are slightly more rapid than those obtained when the same mass of FPLC-1 was added to the same biles. Thus, one may conclude that nucleating potency is maintained after elimination of proteins not adhering to an immunoglobulin affinity column. These studies sustain the contention that the pronucleating potency is due to immunoglobulins and not to a trace substance not visible on Coomassie Blue-stained gels.

The ABx-unbound protein fraction was also shown to contain traces of IgA and IgM as determined again by immunostaining. The failure to obtain complete binding of the immunoglobulins to Bakerbond ABx could possibly be due to denaturation of the immunoglobulins during the isolation procedures. Failure of complete binding of immunoglobulins to the column was also observed when standard IgA or IgM was applied (results not shown), and in fact the elution profile was virtually identical to that in Fig. 5, i.e. 10–15% of the standard immunoglobulins failed to bind. The crystal observation time was used as the end point for nucleation in this study.

Comparison of ConA Glycoprotein and FPLC-1 Protein Fractions Isolated from Slow and Rapid Nucleating Biles—In
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**Fig. 6.** Western blots of the bound ABx fraction run under reducing conditions and immunostained with monoclonal antibodies. *Lane 1, ABx-bound proteins stained with Coomassie Blue. Lanes 2–4 as indicated. Positive reactions are for IgM heavy chain, IgA heavy chain, and secretory component, in lanes 2–4, respectively (original gels 8.2 × 6.2 cm, 1.0 mm thick).*

**Fig. 7.** Silver-stained SDS-PAGE of reduced ConA eluate, FPLC-1 proteins, and ABx-bound proteins. *Lane 1, IgM standard. Lane 2, IgA standard. Lane 3, ConA-bound fraction. Lane 4, FPLC-1. Lane 5, bound ABx proteins.*

This analysis both the time of appearance as well as the number of crystals formed over the observation period were recorded. Results are shown in Fig. 8. In both patients with and without cholesterol gallstones, the FPLC-1 protein fraction was significantly more effective than the ConA glycoproteins both in terms of the observation time as well as the number of crystals formed. While the crystal observation time was more rapid after the addition of protein fractions from patients with cholesterol gallstones the more impressive difference between patient groups was in the number of crystals formed particularly in the first few days of observation (Fig. 8). Addition of proteins in lower doses than those shown was also attempted. Although acceleration of the crystal observation time was sometimes seen, consistent results were not obtained. When buffer only was added to the model biles nucleation was never seen before day 10.

**Addition of Commercial Immunoglobulins and Isolated Serum Immunoglobulins—**Neither IgM or IgA obtained from commercial sources accelerated nucleation. IgM isolated from four individuals with Waldenstrom’s macroglobulinemia consistently accelerated nucleation in two model systems. In one study addition of IgM from the four patients to a heated abnormal bile accelerated the crystal observation time to 4–6 days compared to a control time of greater than 10 days. Nucleation was also accelerated in model biles. The four protein extracts were added to model biles on three occasions (12 experiments). Mean crystal observation time was 4.0 ± 3.0 days in the additions versus 18.1 ± 2.5 days for controls. Serum IgA isolated from patients with multiple myeloma had no effect on the nucleation of cholesterol.

**DISCUSSION**

Considerable research has been directed toward the identification of pronucleating and antinucleating agents in bile. Holzbach and co-workers (2, 32) identified two anti-nucleating apolipoproteins and recently an antinucleating glycoprotein binding to Helix pomatia. Mucous glycoprotein is pronucleating in model bile systems (3) and native gallbladder bile (4). Our investigations showed that other pronucleating proteins are present in bile since biliary proteins, separated from mucous glycoproteins, decreased the crystal observation time of native control bile (5). Furthermore, proteins isolated from
the bile of patients with cholesterol gallstones were more effective than proteins from control patients. The pronucleating protein was then identified by Groen et al. (6-8) to be a glycoprotein binding to concanavalin A, and we have shown that it acts in a dose-response manner (10).

The major finding of this study is that IgM and possibly biliary IgA are pronucleating proteins. The evidence is summarized as follows. A high molecular weight fraction, FPLC-1 accelerates crystal observation time in two separate test systems, model bile and heated abnormal bile. FPLC-1 proteins examined by SDS-PAGE under nonreducing conditions have the same molecular weight as IgM and IgA standards, and immunoblotting confirms the identity of these bands as IgM and IgA. Reduction of the proteins in FPLC-1 results in the appearance of protein bands at molecular weights of the component chains of the immunoglobulins and again immunoblotting confirms them to be the component chains of IgM and IgA. Further purification of FPLC-1 on an immunoglobulin affinity column results in no loss of nucleating potency, suggesting that the nucleating effect of FPLC-1 is not due to a trace substance not detected on the gels. Silver-stained PAGE of the bound ABx proteins confirms the purity of the immunoglobulins. Finally the addition of purified IgM from patients with Waldenström's macroglobulinemia accelerated the nucleation of cholesterol in both model and heated normal gallbladder bile.

The chromatographic material ABx is a silica-based mixed-mode ion-exchange matrix. Chromatography employing ABx has been shown to be superior to other chromatographic procedures for purification of immunoglobulins (anion-exchange, cation-exchange, and gel filtration) (15-17). The procedure is simple, rapid and the immunoglobulins are eluted under mild conditions which prevent denaturing and maintain their immunoreactivity. Furthermore, all classes of immunoglobulins bind to the column (17) and not just IgG as in the case with protein A. It has been reported that immunoglobulins of purity greater than 99% are obtained from ascites or cell culture fluid in a single step using an ABx liquid chromatography column (18, 19). Thus, we believe that these methods are sufficiently specific to rule out the possibility that the pronucleating protein is a trace undetected substance.

Bile is a rich source of secretory IgA and free secretory component (33-35), particularly in rodents. Immunoglobulins are probably secreted in bile to protect against bacteria and perhaps to remove harmful antigens from the circulation in the form of immunocomplexes (36). A secretory transmembrane glycoprotein with affinity for polymeric immunoglobulins selectively transports both polymeric IgA (37-40) and polymeric IgM (41) from blood-to-bile in the rat. Transepithelial transport is less efficient in man, due to lack of secretory component (42, 43). In man, polymeric immunoglobulins appear to enter by endocytosis through intrahepatic and extrahepatic biliary epithelium (42, 44) and are derived not only from plasma (45) but from local synthesis (46). Numerous plasma cells containing IgA are found around accessory glands of major bile ducts and beneath the epithelium (46). Recently, it has been suggested that the gallbladder is an important source of biliary IgA and IgM in man (47). Compared to the hepatic bile, the gallbladder bile is enriched in IgA and IgM, and immunoglobulin secreting cells are present in normal human gallbladder (48).

The pronucleating activity might be due to an immunoglobulin complex rather than to the immunoglobulin itself. However, this seems unlikely as the molecular weight of the immunoglobulins in FPLC-1 corresponded to that of standards. If a complex were present one would expect FPLC-1 bands to run at a somewhat higher molecular weight; this does not rule out the presence of an immune complex with an antigen of low molecular weight.

It remains to be determined which immunoglobulins are active, in what form they are active, as well as how immunoglobulins actually alter the process of crystallization. This study suggests that the pronucleating immunoglobulin will be of tetrameric size or greater. The active FPLC-1 fraction contains tetrameric IgA and pentameric IgM, while the IgA in FPLC-2 is dimeric. Pentameric serum IgM from patients with Waldenström's macroglobulinemia was also active, but serum IgA whether from multiple myoloma patients or commercial sources was not. It is well known that in man most biliary IgA immunoglobulins are polymeric (45) and associated with secretory component, while most serum IgA immunoglobulins are monomeric (36). Why the commercially obtained IgM was ineffective is not clear, as one would expect it to be pentameric. Possibly, the processes used to purify the IgM have altered its capacity to induce nucleation. To examine this issue further, it will be necessary to study the components of FPLC-1, particularly to determine if pure tetrameric IgA is pronucleating, since all the pronucleating activity might reside in polymeric IgM.

Throughout we have referred to the crystal observation time rather than the nucleation time since it is now widely recognized that this useful test cannot differentiate between the various steps that lead to the production of crystals large enough to be seen with a light microscope (49). These steps include movement of lipid between lipid carriers, aggregation of vesicles, the actual nucleation of unit crystals and crystal growth. In this study proteins obtained from patients with cholesterol gallstones produced a much greater effect on the number of crystals in model bile than did proteins from control patients. This difference was more striking than the difference in the actual time of appearance of crystals which usually was only a few days. Such an effect on crystal number would be most likely attributable to an action on crystal growth, as opposed to earlier steps in crystal formation. However, there are other possible actions of immunoglobulins on the crystallization process. Immunoglobulins bind phospholipids (50) and interact with lipid membranes causing release of anions and glucose from vesicles (51). Nucleation of cholesterol crystals requires the aggregation and perhaps fusion of vesicles that have a high cholesterol-to-phospholipid ratio (52-56). Phospholipid-binding immunoglobulins could promote nucleation by aggregating vesicles or, by acting as a phospholipid transfer protein, participate in the enrichment of vesicular cholesterol that is known to occur in the biliary tree as a result of preferential transfer of phospholipid from vesicles to micelles (54). The immunoglobulin might also be an autoantibody directed against an antinucleating substance such as an apolipoprotein. More specific isolation of the pronucleator is required to determine the mechanism action of the protein.

This study has not dealt directly with quantitative aspects of immunoglobulins in bile. We have developed an enzyme-linked immunoassay for the measurement of immunoglobulins (IgA, IgG, and IgM) in the bile. The concentration of proteins added to the model bile systems in this study was well within the range of immunoglobulins in gallbladder bile. Initial studies do show differences in immunoglobulin concentrations in different patients groups, and in particular these differences may be related between IgA concentration and rapidity of nucleation. However, there is considerable variation in concentrations as would be expected in this system in which solute concentration is so substantially affected by
water movement out of bile in the gallbladder. In order to analyze the quantitative aspects, a large number of samples will have to be measured and the results analyzed by a multivariate approach. This is in progress.

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