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The role of the Concanavalin A-binding fraction in cholesterol crystallization in native human bile

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Background/Aims: Many Concanavalin A-binding glycoproteins have been proposed to influence cholesterol crystallization in human bile. This has been studied mainly by addition of the Concanavalin A-binding fraction to model bile. The physiological relevance of the proteins in native bile is not yet known. The aim of this study was to establish the role of the Concanavalin A-binding fraction in cholesterol crystallization in native human gallbladder bile.
Methods: To determine the effects of the removal of Concanavalin A-binding fraction, fresh human gallbladder bile was incubated with either Concanavalin A-Sepharose or Sepharose alone. Beads were sedimented and crystallization was studied in the supernatant.
Results: Extraction of Concanavalin A-binding fraction decreased crystallization in fast-nucleating biles (Crystal Detection Time ≤4 days). Slow-nucleating biles were not affected. The effect could not be related to the content of known pronucleating proteins (IgA, IgM, haptoglobin, aminopeptidase N and α1-acid glycoprotein), since the slow-nucleating biles contained similar amounts of these proteins.
Conclusions: Although Concanavalin A-binding fraction always accelerated crystallization when added to model bile, removal of the same fraction from native bile often had no effect. We conclude that slow-nucleating biles in particular contain undetermined factors which regulate the activity of pronucleators.

Key words: Biliary proteins; Cholesterol crystallization; Concanavalin A-binding glycoproteins; Gallstones.

NUMEROUS studies have been performed to identify the factor(s) which can explain why cholesterol gallstones form in only a minority of people with cholesterol-supersaturated bile (1–8). Crystallization of cholesterol is a critical step in the development of these gallstones, and Crystal Detection Time (CDT) proved to be a good discriminator between biles of normal subjects and cholesterol gallstone patients (9–11). In gallbladder bile from patients with cholesterol gallstones, CDT is accelerated compared with equally supersaturated bile from patients with pigment stones or healthy controls (9,10,12). Aiming to identify the factor that accelerates crystallization of cholesterol, many studies have focussed on the various proteins present

in bile, because the proteins isolated from bile of cholesterol gallstone patients have been shown to affect crystal formation in model bile (13). Employing Concanavalin A (ConA)-Sepharose affinity fractionation, it became possible to separate mucin from other glycoproteins in bile (14–16). Several proteins were isolated from the ConA-Binding fraction (CABF) and their effect on crystallization was studied. This effect was determined by comparing the CDT of model bile with and without addition of the different proteins. The results of these studies indicated that added immunoglobulins (6,17), α1-acid glycoprotein (7), aminopeptidase N (8,18) and haptoglobin (19) promote cholesterol crystallization in model biles. In addition to crystallization-promoting proteins, inhibitors have been isolated from CABF (3,4,20,21).

Despite the fact that many glycoproteins that putatively influence cholesterol crystallization have been isolated from CABF, the physiological relevance of these factors has not been established. A more appro-

priate way to assess the importance of a factor in cholesterol crystallization is to remove it selectively from native bile. Gallinger et al. (22) removed mucin by ultrafiltration or ultracentrifugation of native bile from cholesterol gallstone patients and could not detect an effect on CDT, even though mucin added to model bile accelerated CDT. This illustrates why the relative importance of other putative promoters and inhibitors is best evaluated by extraction of these proteins one by one from various native bile samples to determine their effect on crystallization kinetics. So far, this procedure has not been carried out successfully for ConA-binding protein. In practice, such a procedure is difficult to accomplish, because large volumes of bile are required and selective extraction of only one protein is not always possible. In the present study, we therefore adopted a more rigorous approach. By incubating bile with ConA-Sepharose, almost all putative promoting and inhibiting proteins which bind to ConA were removed in one step, and the effect on cholesterol crystallization could be studied.

Materials and Methods

Materials

Concanavalin A-Sepharose and Sepharose 4B were obtained from Pharmacia AB (Uppsala, Sweden). Rabbit anti-human IgA (α-chain and secretory component), rabbit anti-human IgM (μ-chain), rabbit anti-human $\alpha 1$-acid glycoprotein, rabbit anti-human haptoglobin and peroxidase-conjugated rabbit anti-human heavy-chain IgA and IgM were obtained from DAKO (Copenhagen, Denmark). Goat anti-human $\alpha 1$-acid glycoprotein was obtained from Sigma Immuno Chemicals (St. Louis, MO, USA). Rabbit anti-human IgM for sandwich ELISA and standard plasma were purchased from Behring (Marburg, Germany). Cholesterol, egg yolk phosphatidylcholine and taurodeoxycholate were obtained from Sigma (St Louis, MO, USA). Taurocholate was from Fluka BioChemika (Buchs, Switzerland). Spectra/Por dialysis membranes with a cutoff of 12–14 kD were obtained from Spectrum Medical Industries (Houston, Texas, USA). Ultrafilters with a cutoff of 10 kD, (YM 10) were from Amicon (Danvers, MA, USA). Dynagard filters with a cutoff of 0.2 μm were from Microgon (Laguna Hills, CA, USA). Microtiter plates with medium binding capacity were purchased from Greiner (Frickenhausen, Germany). All other reagents used were of analytical grade.

Bile

Gallbladder bile was collected from 23 patients (patient characteristics: Table 1) at laparoscopic cholecystectomy for cholelithiasis. All patients were confirmed

TABLE 1
Patient characteristics ($n=23$)

Male/female	7♂/16♀
Mean age	47.7±2.8
Mean Quetelet Index	26.4±1.2
Blood type	A:7, B:4, AB:1, O:11

to have gallstones by ultrasound examination. Before ligating the cystic duct, bile was collected from the gallbladder by a direct puncture using a sterile 19-G needle and syringe. Care was taken to aspirate the gallbladder constituents completely. The bile samples were transported to the laboratory and processed immediately. Ten microliters of the fresh bile was examined under polarizing light microscopy at 100× magnification to determine the quantity of cholesterol monohydrate crystals, defined as colorless, transparent thin crystals with parallel edges.

Gallstone content

Before the gallbladders were taken to the pathology department, the gallstones were removed, measured and counted; three patients had a solitary stone, seven patients had 2–3 stones and 13 patients had 4 or more stones. Gallstones from all patients were air-dried, crushed and assayed for cholesterol content. One milligram of stone-powder was dissolved in 1 ml iso-propanol and sonicated for 1 h. Cholesterol content was determined using a standard enzymatic procedure. Cholesterol stones were defined to be greater than 70% cholesterol by weight. Only biles from patients with cholesterol stones ($n=23$) are considered in this article. The mean cholesterol content of the stones was 92% (range 76–100).

Extraction of CABF from bile

Fresh human gallbladder bile was centrifuged (10 min, 2000 rpm) and two portions of 3 ml were taken. The remaining bile was stored at −20°C for determination of lipid and protein composition. From one of the 3-ml portions, CABF was removed using ConA-Sepharose-4B. The ConA-Sepharose beads were prewashed three times with ConA-buffer containing 0.2 M NaCl, 10 mM Tris (pH 7.4), 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and 0.02% NaN_3. The beads were pelleted (5 min, 2000 rpm) and the supernatant was carefully removed completely. Three milliliters of bile were added to 1.5 ml packed beads, mixed thoroughly and incubated for 2 h on a roller bank at 20°C. As a control, the other portion of bile was added to 1.5 ml washed packed Sepharose 4B beads and subjected to the same procedure.

After 2 h of incubation on a roller bank at room temperature, the beads with the bile samples were centrifuged for 15 min at 1200 rpm. Supernatant from both ConA and control Sepharose beads was passed through an 0.22 μm Millipore (Millex-GS) filter into a sterile container. Each filtrate was incubated at 37°C to determine CDT. To be able to discriminate between the effects on nucleation and crystal growth, we also examined the result of CABF extraction in biles from which crystals were not removed (unfiltered bile) prior to the crystal detection assay. In 11 separate bile samples the effect of CABF extraction was studied not only in filtered but also in unfiltered bile. The rest of the supernatant was stored at -20°C. The beads were washed three times with 10 ml ConA-buffer and subsequently stacked in a 1×5 cm column, washed again with 15 ml ConA-buffer containing 0.1 M α-D-methylmannopyranoside. The eluate was concentrated on an Amicon-filter (YM10) until approximately 1 ml was left. To remove 0.1 M α-D-methylmannopyranoside, the concentrate was diluted again with 15 ml HEPES buffer (50 mM, pH 7.4), followed by concentration. This procedure was repeated three times. After the final cycle, the concentrate was reconstituted to the volume of the initial sample; i.e. 3 ml.

Crystal detection time and crystal growth determination

In many samples there was insufficient bile for a control incubation of untreated native bile. We therefore compared the effect of incubation with Sepharose in parallel experiments. The effect of incubation with Sepharose beads was studied by comparison in 5 paired bile samples of the CDT in ultrafiltered biles incubated with Sepharose beads with the CDT of untreated ultrafiltered biles.

The bead-treated samples were incubated without shaking at 37°C for a maximum of 21 days. A sample of 10 μl was examined daily. The samples were observed under a light microscope with polarized light at a $100\times$ magnification. The presence of cholesterol monohydrate crystals was determined and if they were present, the number of crystals was counted using a hemocytometer. The crystal detection time (CDT) and the maximum rate of crystal formation (C_{max}) were determined. CDT was defined as the day on which the first cholesterol crystal was observed in a bile sample. C_{max} was defined as the maximal slope of the curve showing the relation between crystal number and time.

Determination of the extraction efficacy of the Concanavalin A-Sepharose beads

The efficacy of protein extraction with ConA-Sepharose has been tested previously with gel electro-

phoresis and Western blotting. No protein could be demonstrated in the eluate of bile that had been loaded on a ConA-Sepharose column (14). In the present study we additionally tested the efficacy of Concanavalin A-Sepharose fractionation by determining the extraction of two important Concanavalin A-binding proteins: IgA and haptoglobin. Using an ELISA (see below), IgA and haptoglobin were measured in the supernatant of both ConA-Sepharose-treated- and Sepharose-treated bile. The amount of IgA and haptoglobin found in the ConA-treated bile divided by the amount found in Sepharose-treated bile was considered a measure for the extraction-efficacy of CABF.

Determination of the effect of the different extracted proteins on crystal appearance time in model bile

For the preparation of supersaturated model bile with a cholesterol saturation index (CSI) of 1.6, taurocholate and taurodeoxycholate were dissolved in methanol/water (85:15) and mixed with lecithin in chloroform. After addition of cholesterol in chloroform, the mixture was dried under nitrogen at 50°C to evaporate the organic solvents and then subsequently lyophilized. The dry lipid film was solubilized in HEPES buffer (50 mmol/l, pH 7.4) containing 100 mmol/l NaCl and 0.02% NaN_3 and equilibrated for 2 h at 56°C. Each eluate from the ConA-Sepharose-treated bile samples was mixed with an equal volume of model bile. The mixtures were filtered over a 0.2 μm ME Dynagard filter and incubated at 37 °C. At different time points the cholesterol crystal mass in the samples was measured at 840 nm, using a (Behring BN 100) nephelometer. Light scattering was calculated as total crystal mass in μg/ml according to a linear standard curve. Crystallization curves were fitted with the computer program Fig-P (Biosoft, Ferguson, MO) by using a sigmoidal model (Hill type equation).

ELISA

The amount of four glycoproteins (IgA, IgM, haptoglobin and α1 acid glycoprotein), was measured separately in the different bile fractions. Microtiter plates were coated with rabbit antihuman IgA (1:5000) or rabbit antihuman IgM (1:1000), goat antihuman haptoglobin or goat anti-human α1 acid glycoprotein respectively, for 16 h at 4°C. The plates were washed twice with PBS/Tween (0.05%, v/v). Blocking was obtained with 1% (w/v) BSA in PBS/Tween 0.05% for 1 h at 37°C. After washing twice with PBS/Tween 0.05%, the wells were incubated with serially-diluted samples (in PBS/Tween with 0.1% BSA) or standard plasma for 2 h at 37°C. For haptoglobin and α1 acid glycoprotein, this procedure was followed by incubation with rabbit

TABLE 2

Lipid composition of bile samples before and after addition of Sepharose- or Concanavalin A-Sepharose beads

	Untreated bile	ConA-Sepharose-treated bile	Sepharose-treated bile
Cholesterol (mM)	14.4±2.1	12.3±1.6	12.3±1.4
Phospholipids (mM)	29.7±6.3	22.3±4.8	22.0±4.4
Bile salts (mM)	105.6±8.3	61.4±7.2	66.6±10.9
CSI	1.58±0.19	2.08±0.33	2.04±0.32

anti-human haptoglobin (1:3000) or anti-α1 acid glycoprotein (1:2000). After washing four times in PBS/Tween, incubation followed by peroxidase-conjugated rabbit antihuman, IgA (1:3000) or IgM (1:1000), or peroxidase-conjugated goat anti-rabbit IgG (1:3000) diluted in PBS/Tween buffer containing 0.1% BSA for 1 h at 37°C. Color development was performed with 0.1 mg/ml 3,3',5,5'- tetramethylbenzidine, 0.0036% H_2O_2 and 0.1 mol/l sodium acetate, pH 5.5.

The intra-assay coefficient of variance and inter-assay coefficient of variance in all ELISA procedures described were less than 8 and 10%, respectively.

Aminopeptidase N

Levels of aminopeptidase N were determined by measuring its enzymatic activity for hydrolysis of L-alanyl-ß-naphthylamide. The β-naphthylamide product was determined fluorometrically (excitation 340 nm, emission 410 nm) (23).

Protein and lipid composition

Protein in gallbladder bile was determined by the method of Bradford (24) after delipidation according to Wessel & Flügge (25). Protein in the Concanavalin A-binding fraction was determined directly by the method of Bradford (24). Cholesterol, phospholipid and bile-salt concentrations were determined using standard enzymatic procedures (26–28). Cholesterol saturation indices were calculated according to Carey's critical tables (29).

Statistical analysis

Statistical evaluations were performed with the Mann-Whitney U-test between groups and the Wilcoxon Signed Rank Test within groups. Data are given as mean±SE.

Results

Efficacy of the Concanavalin A-extraction method

To investigate the capacity of ConA-Sepharose in the experimental set-up used, the extraction of two import-

ant ConA-binding proteins, IgA and haptoglobin, was determined. The extraction efficacy was 96±1.9% for IgA and 98±1.2% for haptoglobin. Although bile samples were added to packed beads, some dilution was inevitable due to the fluid in and adhering to the beads. We therefore measured the lipid content of the samples before and after addition of both Sepharose and ConA-Sepharose. Treatment, whether ConA or plain Sepharose, induced comparable reductions in the contents of bile salts (39%), in phospholipids (25%) and in cholesterol (15%), resulting in an increase of the CSI of about 30% (Table 2).

Incubation of bile with Sepharose beads did not result in a significant change in CDT compared to untreated ultrafiltered bile (4.2±1.7 days and 4.8±1.3 days, respectively).

Crystal detection time and the maximum rate of crystal formation

Removal of CABF from the bile samples caused prolongation or no change in CDT. The size of the crystals as observed under the microscope was comparable between paired Sepharose and ConA-treated bile samples. Significant acceleration of CDT by removal of CABF was never observed, indicating that none of the biles contained a surplus of ConA-binding inhibiting factor.

The result of a typical experiment is shown in Fig. 1. Removal of the CABF increased CDT from 1 to 4 days and dramatically reduced the number of crystals formed. Fig. 2 summarizes the results for all 23 patients. CABF-extraction did not significantly alter
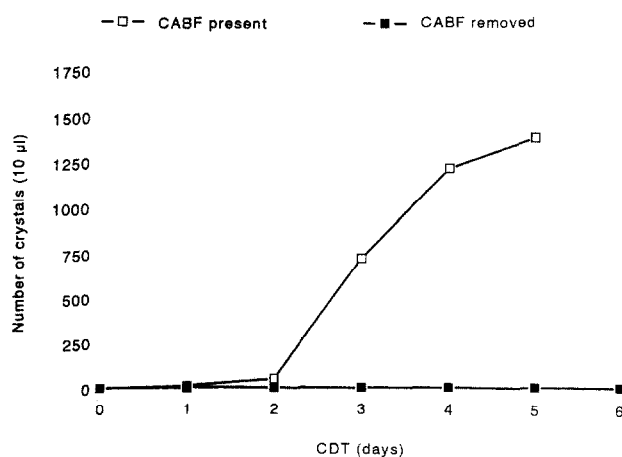

Fig. 1. Effect of Concanavalin A-binding Fraction (CABF) extraction on the number of crystals formed in a fast-nucleating bile sample. □=native bile with CABF, ■=native bile without CABF.
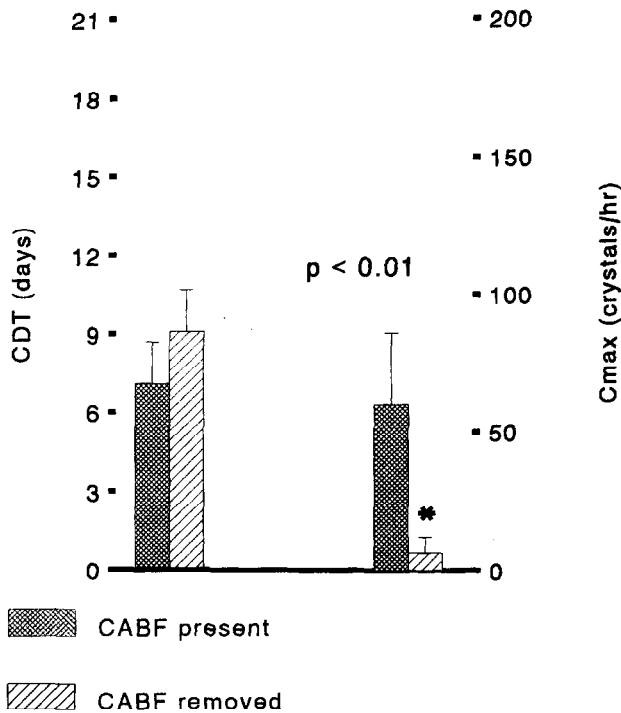
Fig. 2. Effect of Concanavalin A-binding Fraction (CABF) extraction on crystal detection time (CDT) and maximal rate of crystallization (C_{max}) as described in the methods section. Data are presented as mean±SE.

Fig. 3. Crystal Detection Time (CDT) and maximum rate of crystal formation (C_{max}) in slow-nucleating bile samples in the absence and presence of the Concanavalin A-binding Fraction (CABF). Data are derived from the group presented in Fig. 2. Patients with a CDT>4 days were selected. Data are given as mean±SE.

CDT, but dramatically decreased maximal crystallization.

To investigate whether the effect of the procedure was related to CDT, we separated the group into fast (CDT≤4 days) and slow nucleators (CDT≥4 days), as suggested by Jüngst et al. (30). The mean CSI of the slow-nucleating biles (1.43±0.13) was not significantly different from the CSI of fast-nucleating biles (1.74±0.30, p=0.62, Table 3). Removal of the CABF had no significant effect on CDT or C_{max} in slow-nucleating biles (Fig. 3). In contrast, in the fast-nucleating biles a clear effect was observed on both CDT

and C_{max} (Fig. 4). The mean CDT increased from 1.7±0.3 to 4.2±0.9 days (p=0.01), whereas the mean C_{max} decreased from 106±42 to 11±10 crystals per hour (p<0.002). Note that C_{max} was more than 100× faster for the fast-compared to the slow-nucleating biles.

In the procedure described above, to remove cholesterol crystals, the samples are filtered through a 0.22 μm filter after incubation with Concanavalin A-Sepharose or Sepharose. To investigate the influence of this, we omitted the filtration step in 11 bile samples and compared the results with the procedure with filtration. There was no significant difference in the effect of CABF extraction on C_{max} in filtered and unfiltered bile (Fig. 5). Of course, determination of CDT is meaningless in unfiltered samples.

To establish whether the effect on CDT and the effect on C_{max} could be explained by the same factor in the CABF, we determined if there was a correlation between the effect of extraction of the CABF on the CDT and the effect of CABF extraction on C_{max}. The

TABLE 3

Lipid composition of slow (n=13) and fast (n=10) nucleating bile samples

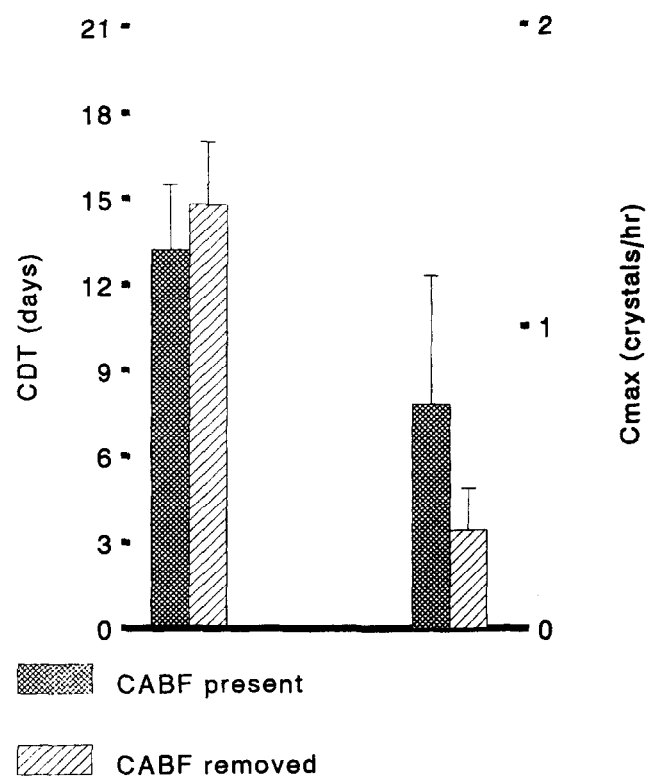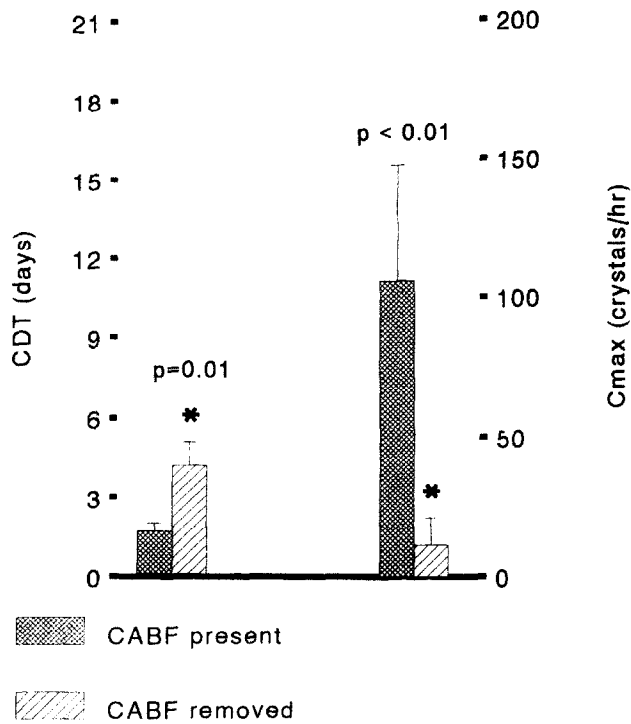|	Total group (n=23)	Fast-nucleating bile (n=10)	Slow-nucleating bile (n=13)
Cholesterol (mM)	16.8±1.7	16.6±2.2	17.1±2.8
Phospholipids (mM)	34.6±3.1	31.5±3.5	38.8±5.5
Bile salts (mM)	114.5±9.4	111.8±14.7	117.9±11.0
Total lipids (g/dl)	8.8±0.6	8.3±0.7	9.4±0.9
CSI	1.60±0.2	1.74±0.30	1.43±0.13

CABF present

CABF removed

Fig. 4. Crystal Detection Time (CDT) and maximum rate of crystal formation (C_{max} in fast-nucleating bile samples in the absence and presence of the Concanavalin A-binding Fraction (CABF). Data are derived from the group presented in Fig. 2. Patients with a CDT ≤ 4 days were selected. Data are given as mean$\pm$SE.
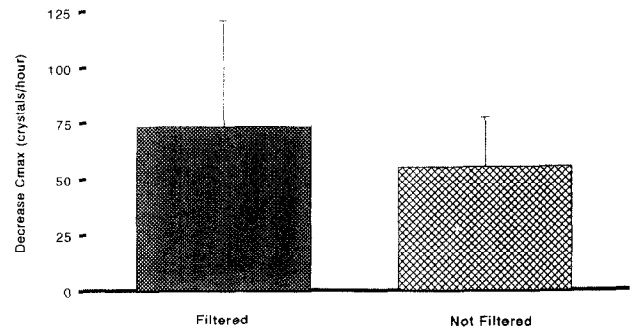

Fig. 5. Effect of Concanavalin A-binding Fraction (CABF) extraction on maximum rate of crystal formation (C_{max}) in 11 bile samples. After incubation with Concanavalin A-Sepharose or Sepharose, bile was either filtered or not filtered through a 0.22 µm filter. The difference in C_{max} between parallel incubations (plus or minus CABF) was calculated and is presented as Δ C_{max}. Data are given as mean$\pm$SE.
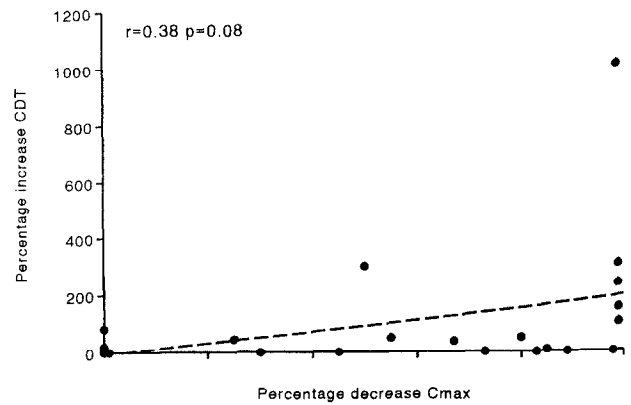
results are plotted in Fig. 6 where the percentage increase in CDT is plotted against the percentage decrease in Cmax. No significant relation ($r=0.38$, $p=0.08$) was observed between CDT and decrease of C_{max}, because in 6 samples removal of CABF produced a large decrease in C_{max} with no effect on CDT, suggesting that more than one factor in CABF is involved in regulating crystallization.

Fig. 6. Relation between Δ CDT and Δ C_{max}. For experimental details, see legend to Fig. 2. The difference for the condition with or without the Concanavalin A-binding Fraction was calculated. This difference is expressed as percentage of the value in Sepharose-treated bile samples.

TABLE 4

Content of total protein and five different Concanavalin A-binding glycoproteins in native bile, determined by the method of Bradford (total protein), ELISA (IgA, IgM, α1-acid glycoprotein, haptoglobin) and enzymatic activity (aminopeptidase N)

	Mean concentration in bile
Total protein	2.9$\pm$0.6 g/l
Haptoglobin	54.7$\pm$15.6 mg/l
IgA	189.2$\pm$22.4 mg/l
IgM	186.0$\pm$32.2 mg/l
A1–acid glycoprotein	69.4$\pm$8.9 mg/l
Amino-peptidase N	1306.6$\pm$206.4 U/l

Correlation between five different Con A-binding glycoproteins and the effect on CDT and C_{max}

To investigate if one of the known ConA-binding promoting proteins was mainly responsible for the effect that extraction of the CABF had on CDT and C_{max}, the contents of IgA, IgM, α1-acid glycoprotein, aminopeptidase N and haptoglobin were measured in the native bile samples (Table 4). The mean total amount of protein in the bile samples was 2.9 g/l (range 0.6–12.2). All individual proteins except IgM ($r=0.3$, $p=0.13$) correlated highly ($r=0.5$–0.8, $p\leq 0.01$) with total protein. Subsequently, the concentrations of the different proteins were plotted against the CDT and against the effect extraction of the CABF had on CDT and C_{max}.

No correlation was found between the amount of IgA, IgM, haptoglobin α1-acid glycoprotein, aminopeptidase N or the total amount of protein and either CDT or the effect of extraction.

Eluate of isolated CABF on crystallization in model bile

To be able to correlate the crystallization-promoting activity of CABF in native bile with the activity in a model bile system, we eluted the bound fraction from the ConA-Sepharose beads and, after dialysis, concentrated back to the initial volume. The CABF were mixed 1:1 with a supersaturated model bile, and crystalli-

Fig. 7. Crystallization-promoting activity in the Concanavalin A-binding Fraction (CABF) isolated from fast- and slow-nucleating biles. CABF was isolated as described in the Methods section. The fraction was concentrated back to the initial volume of the bile sample and mixed with supersaturated model bile (●). In the control incubation (□) model bile was mixed with HEPES buffer. Activity was determined in the crystal growth assay for the time points indicated in the figure.

zation-promoting activity was determined in the crystal growth assay. Figure 7 shows the activity for 5 samples derived from fast-nucleating biles and 5 samples from slow-nucleating biles.

The addition of CABF accelerated and decreased crystal appearance time in all cases, but the magnitude of the effect varied considerably from patient to patient, and there was no significant difference between slow- and fast-nucleating native bile samples. Eluates from control Sepharose columns were tested as well, but showed no activity. We also incubated model bile with ConA-Sepharose beads. The eluate of this incubation was tested but showed no activity.

Discussion

Crystallization of cholesterol in bile is an extremely complex process involving a multitude of biliary components. In the early eighties a role for proteins in this process was proposed (31). Despite intensive research, conclusive evidence for the importance of proteins in the crystallization process is still lacking. We have attempted but failed to eliminate possible important nucleating proteins one by one from the bile, by using immunoaffinity chromatography, probably because biliary components interfere with the antibody-antigen interaction. In this study we therefore used a more rigorous approach and simply removed all Concanavalin A-binding proteins by using Con A-Sepharose. The results were surprising. Of the 23 bile samples tested, only 10 showed a significant effect of removal of CABF. These bile samples nucleated quickly and showed, in most cases, fast crystal growth. The effect of CABF extraction was more pronounced on crystal growth than on CDT. Interestingly, some samples showed only an effect on CDT and no decrease in crystal growth, indicating the influence of more than one activating factor.

The validity of the method is based on two assumptions. Firstly, extraction of protein should be highly effective; otherwise, when present in great excess, incomplete removal of a promoter will have relatively little effect on its crystallization activity. Secondly, the procedure should not selectively influence biliary lipid composition. Incubation of bile with column material inevitably leads to dilution. We therefore controlled our procedure by incubating a parallel bile sample with Sepharose 4B. It has been reported that Concanavalin A binds hydrophobic compounds. In the present study, we observed no difference between the incubation with Sepharose or ConA-Sepharose. Interestingly, the CSI of the bile increased after incubation with column material; depletion was greatest for bile salts. Apparently, the distribution volume is much larger for simple and
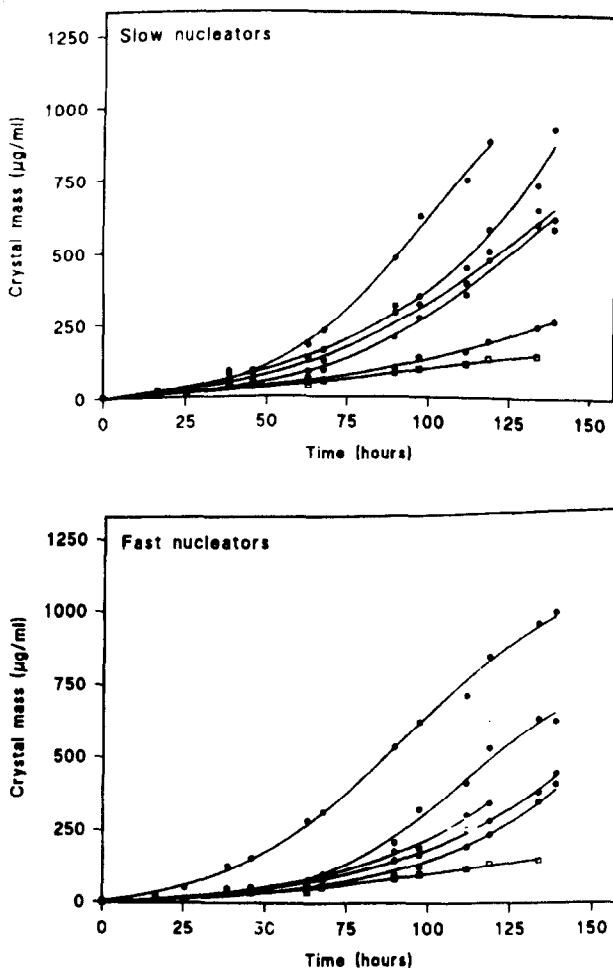
mixed micelles than for vesicles. The pores in the beads are too small for the vesicles.

ConA-Sepharose extraction proved to be highly efficient. The major protein in CABF, IgA, was 95% removed, and haptoglobin was 98%. However, since extraction was not 100%, the influence of CABF on crystallization may have been somewhat underestimated. When one of the promotors serves as a nucleation nidus, the residual presence of about 5% could explain why particularly the number of crystals formed is affected, whereas the influence on CDT was somewhat less pronounced.

Slow-nucleating bile samples were not significantly influenced by CABF removal. We therefore conclude that in these samples crystallization is not controlled by ConA-binding protein. CABF has been shown to contain inhibitory proteins (3,4), in addition to the pronucleating activity. It can thus not be ruled out completely that the removal of compensating activities explains the lack of effect, but we consider this possibility highly unlikely. Firstly, CDT has been shown to be unaffected by pronase digestion of protein; the inhibitors proposed so far are all sensitive to proteolytic degradation (32). Secondly, it seems highly unlikely that pro- and antinucleating activity would always be exactly in balance. We propose that in the slow-nucleating samples either the lipid components control the nucleating process or inhibitors are present which specifically block the activity of CABF. Interestingly, a number of fast-nucleating, slowly growing samples also show a lack of CABF influence. In these samples either non-Con A-binding proteins are active or cholesterol saturation is the main controlling factor. Indeed, in the samples not responding to removal of CABF a significant correlation between CDT and CSI was observed ($r=-0.55$, $p=0.05$).

In two recent studies (33,34), it has been shown that CABF can contain cholesterol crystals. The origin of these crystals is not clear; presumably they form during elution of the bound glycoproteins from lipoprotein particles. We have shown before that, when testing activity of CABF in model bile, the presence of such crystals does not significantly influence promoting activity in our experimental setup because they are removed by a filtration step (35). In principle, formation of cholesterol crystals during the incubation in native bile with ConA-Sepharose will also not influence the type of study, presented here, because they are removed anyway at the start of the nucleation assay. We have, however, shown previously that ultracentrifugation or ultrafiltration of bile does not rigorously remove all micro-crystals present in bile (35). If these micro-crystals do bind to ConA-

Sepharose and are present only in fast-nucleating biles, this could explain part of the effect in these biles. To investigate this possibility, we tested the effect of ConA-Sepharose on crystallization in bile from which crystals were not removed prior to the procedure. Of course, an effect on CDT cannot be studied under these conditions, but a specific effect of the proteins on crystal growth should still be apparent. As shown in Fig. 5, there was no significant difference in the effect on inhibition of crystal growth, whether or not crystals were present at the onset of the crystal detection assay. To address the question which factor is responsible for the activity seen in CABF, we chose two independent strategies. First the concentration of the previously reported nucleating factors was determined in all bile samples. Secondly, total promoting activity in CABF was determined in a model bile system. There was no correlation between the concentration of the promotors in native bile and the ability to influence crystallization parameters. The data were analyzed using multi-variate analysis. No correlation was found between the content of IgA, IgM, α1-acid glycoprotein, aminopeptidase N or haptoglobin and the degree of influence of removal or addition on either CDT or crystal growth. In an earlier study, we quantified the crystallization-promoting activity by assessing the so-called Nucleation Promoting Activity Titer (36). In the present study, activity was quantified using the much less laborious assay system developed by Busch et al. (37). In accordance with our earlier study, promoting activity was found in all CABFs, although there was variation among patients. Since we had only three single-stone patients in this group, we did not test whether the activity in these patients was lower.

Surprisingly, the samples which showed no effect of CABF-extraction also had considerable promoting activity in the isolated fraction. We can only speculate about possible explanations for this paradoxical finding. The lack of effect of removal of CABF on crystallization kinetics was almost invariably found in slow-nucleating samples. It cannot be excluded that the crystallization path in these samples differs from the fast crystallizing samples. Proteins than do not influence this path. Perhaps a more plausible explanation is the presence of potent non-Concanavalin A-binding inhibitors in slowly-crystallizing bile. In the past decade, the group of Holzbach has proposed several inhibitory candidates, including apolipoprotein A I (38) and a helix pomatia binding 128 kD protein (4), and recently Busch et al. have added IgA and its secretory component to this list (39). Except apolipoprotein A I, these proteins bind to Concanav-

alin A and therefore cannot account for the observed results. We also do not consider a role for apolipoprotein A I very likely. Activity of the protein has been shown to be sensitive to protease treatment. Pattinson & Willis could not find an effect of pronase on crystallization in slow or fast-nucleating samples (40). Activity of a pronase sensitive inhibitor should have led to acceleration of crystallization. We have shown that non-protein compounds such as FFA and BSP are potent inhibitors of promotor-induced crystallization (41,42). Whether organic anions or related compounds present in human bile are also capable of controlling cholesterol crystallization is presently under investigation.

Conclusions

In this study, we have shown that although bile from cholesterol gallstone patients always contains potent crystallization promotors, this activity controls cholesterol precipitation only in fast-nucleating biles. We speculate that this specific activity is inactivated in slowly-crystallizing supersaturated bile by as yet uncharacterized factor(s).

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