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Inhibition of Cholesterol Crystal Formation by Apolipoproteins in Supersaturated Model Bile

Abstract. *Apolipoproteins A-1 and A-2 were purified from human plasma. At concentrations present in human bile these proteins prolonged the nucleation time of cholesterol monohydrate crystals when added to model systems of supersaturated bile. In contrast, apolipoprotein C-3 and other serum proteins did not have this effect. Also, when human gallbladder bile was fractionated by gel filtration chromatography, apolipoproteins A-1 and A-2 were among the proteins present in a fraction of bile enriched in potent inhibitors of cholesterol crystal nucleation. These findings suggest that apolipoproteins A-1 and A-2 in supersaturated human gallbladder bile could inhibit the rate of formation of solid cholesterol crystals and thus help to prevent spontaneous cholesterol gallstone formation in humans.*

A critical step in the formation of cholesterol gallstones is the nucleation of excess cholesterol from supersaturated human gallbladder bile. Supersaturated bile, however, does not necessarily lead to the formation of cholesterol crystals or gallstones; for example, more than 50 percent of subjects without gallstones have bile supersaturated with cholesterol (*1, 2*). Earlier we showed that supersaturated bile from gallstone patients differs from that of patients without stones. Nucleation time (the time required for earliest detection of cholesterol monohydrate crystals in an isotropic solution) of supersaturated bile from patients without gallstones was consistently prolonged when compared with bile from patients with cholesterol gallstones (*3, 4*).

Studies conducted in our laboratory (*5*) have provided evidence that biliary proteins contain components capable of inhibiting cholesterol crystal nucleation in human gallbladder bile. Cholesterol crystals were found to form (i) more slowly in supersaturated gallbladder bile from subjects without gallstones than in artificial bile solutions of identical lipid compositon; (ii) more rapidly in supersaturated gallbladder bile from subjects without gallstones after removal of proteins than in the same bile before removal of proteins; and (iii) more slowly in artificial bile solutions after addition of a delipidated, crude protein fraction isolated from human gallbladder bile, an effect that is abolished by Pronase treatment. Further, two protein fractions of gallbladder biles were obtained by molecular sieve chromatography. The first protein fraction eluted before the biliary lipids, and the second protein fraction eluted along with the biliary lipids. This second protein fraction had a more potent effect on cholesterol crystal nucleation inhibition than the first fraction. These data suggest that one or more proteins are present primarily in the second protein fraction of human gallbladder bile, which has been shown to inhibit cholesterol crystal formation. The identity of these putative inhibitors of nucleation remains unknown.

We have also described the presence and amount of apolipoproteins (apo) A-1, A-2, C-2, C-3, and B in human gallbladder and hepatic bile and have presented data indicating that apo A-1 and apo A-2 are present in bile as intact polypeptides (*6*). We suggested that biliary apolipoproteins may be involved in the solubilization of biliary cholesterol and phospholipid in a manner analogous to their functions in plasma, possibly as a supplementary system to bile salts and lecithin (*6, 7*). These data suggested a possible role for apo A-1 and apo A-2, the major protein components of high-density lipoproteins, in gallstone pathogenesis.

In view of these observations, we proposed that the protein components in supersaturated bile responsible for inhibition of cholesterol crystal nucleation include one or another of the apolipoproteins. Moreover, if this hypothesis were correct, these apolipoproteins would be expected to occur primarily in the second fraction.

To examine whether apolipoproteins can inhibit nucleation, we studied the effects of purified serum proteins, including apo A-1 and apo A-2, on the nucleation time of cholesterol crystals in model systems of supersaturated bile (cholesterol saturation index, 1.07 to 1.19; total lipid, 200 g/liter; ratio of phospholipid to bile salt, 0.25) (*8*). Some

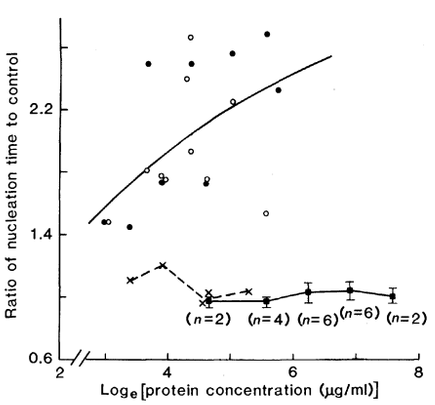

Fig. 1. Effect of apo A-1 (20 to 320 μg/ml), apo A-2 (20 to 250 μg/ml), apo C-3 (30 to 200 μg/ml), and human albumin (100 to 2000 μg/ml) on nucleation time in supersaturated model biles. Data for apo A-1 and apo A-2 were analyzed by a nonlinear regression equation for a rectangular hyperbola: $y = x(1.288 + 0.195)^{-1}$ (standard deviation of regression = 0.373), where y is the ratio of nucleation time to control and x is $\log_e$ (protein concentration). n is the number of observations with albumin; each point for apo C-3 represents a single observation. (●) apo A-1; (○) apo A-2; (x) apo C-3; (■) human albumin.

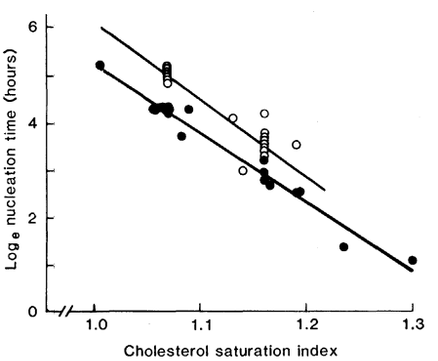

Fig. 2. $\log_e$ (nucleation time) as a function of cholesterol saturation index for a series of model biles without added protein (controls) and for the model biles recombined with apo A-1 and apo A-2. The linear regression equation ($y = ax + b$) for controls is $y = (-14.70 \pm 0.64)x + (19.98 \pm 0.72)$ ($n = 19$, $P < 0.001$; t-test for slope) and for recombinants is $y = (-16.8 \pm 2.29)\ x + (23.05 \pm 2.56)$ ($n = 19$, $P < 0.001$; t-test for slope). For Δa (Δ slope), $P = 0.46$ (not significant); for Δb (Δy-intercepts), $P < 0.001$. At various cholesterol saturation indices, the recombinants nevertheless have consistently longer nucleation times when compared to controls. (○) Recombination with apo A-1 and apo A-2; (●) control.

model solutions were recombined with the purified serum proteins, and other model biles of identical composition were used as controls (*9*). Samples were examined for the appearance of typical cholesterol monohydrate solid crystals and liquid crystals containing lecithin and cholesterol by polarizing light microscopy (*10*).

At concentrations (20 to 320 μg/ml) found in human gallbladder bile, apo A-1 and apo A-2 prolonged the nucleation time of cholesterol monohydrate crystals by a factor of 1.4 to 2.7 times that for control. This effect was concentration-dependent (Fig. 1). In contrast, apo C-3 (30 to 200 μg/ml) and albumin (100 to 2000 μg/ml) had no effect on nucleation time (Fig. 1). The model biles containing apo A-1 and apo A-2 had consistently longer nucleation times, as indicated by their significantly different ordinate intercepts (difference = 3.07, $P < 0.001$ by analysis of covariance) compared to controls at the same cholesterol saturation index (Fig. 2). In addition, haptoglobin (50 to 100 μg/ml), a_1-acid glycoprotein (50 to 200 μg/ml), chromatographically purified porcine gastric mucin (100 to 200 μg/ml), and immunoglobulin A (IgA) (100 to 300 μg/ml) had no effect on nucleation time. None of the proteins tested, including apo A-1 and apo A-2, had any effect on the elapsed time for earliest detection of liquid crystals containing lecithin and cholesterol. Invariably, liquid crystals were initially detectable only 2 to 3 hours before the appearance of solid cholesterol monohydrate crystals in controls, whereas liquid crystals in model biles containing the A apolipoproteins were stable for long periods (10 to 40 hours) prior to the onset of microscopically detectable solid crystals.

These observations indicate that apo A-1 and apo A-2 inhibit the rate of formation of cholesterol monohydrate crystals, possibly by stabilizing liquid crystals and retarding their phase transition to solid crystals in metastably supersaturated model bile systems moving toward equilibrium. To explore the related issue of whether apo A-1 and apo A-2 are present primarily in the second protein fraction containing potent inhibitors of cholesterol crystal formation (*5*), we fractionated native gallbladder biles freshly collected from subjects without gallstones ($n = 10$) into 15-ml portions by gel filtration chromatography (Fig. 3). Protein, cholesterol, phospholipid, and bile salts for each fraction were measured as described (*11*), and apo A-1 and apo A-2 were measured by radioimmunoassays (*6*).

All of the phospholipid and cholesterol in bile eluted together in a single peak (Fig. 3). A bile salt peak also eluted with the phospholipid and cholesterol. The simultaneous elution of these three components suggests that micelles containing a mixture of bile salt, phospholipid, and cholesterol were isolated. Total proteins in bile were fractionated into two major peaks, the second peak eluting along with the mixed micelles, as we had observed earlier (*5*). The major peaks of apo A-1 and apo A-2 in bile also eluted in the region of the mixed micelles, being present primarily in fractions 15 to 20 (Fig. 3). Electrophoretic studies of fractions indicated that the proteins of the initial protein peak are accounted for mainly by IgA or IgG heavy and light chains (or both), IgA secretory component, and a_2-macroglobulin. In addition to apo A-1 and apo A-2, the proteins of the second peak, as assessed by electrophoretic techniques, are accounted for mainly by albumin, transferrin, orosomucoid (a_1-acid glycoprotein), and haptoglobin (*12*).

These observations indicate that apo A-1 and apo A-2 can specifically inhibit the rate of formation of cholesterol crystals in vitro and are among the biliary proteins isolated by molecular sieve chromatography that contain potent inhibitors of cholesterol nucleation. These results are consistent with the hypothesis (but do not prove) that apo A-1 and apo A-2 can be responsible, at least in part, for preventing gallstone formation in human bile supersaturated with cholesterol. The possibility that apo A-1 and apo A-2 in bile could inhibit an apparent nucleation process by retardation of crystal growth from nuclei (*9*) or by directly altering the solubilization of cholesterol in a manner analogous to their role in plasma (*7*) cannot be excluded. No apparent quantitative difference was detectable in bile apo A-1 and apo A-2 concentrations between normal and gallstone patients (*6*). However, additional

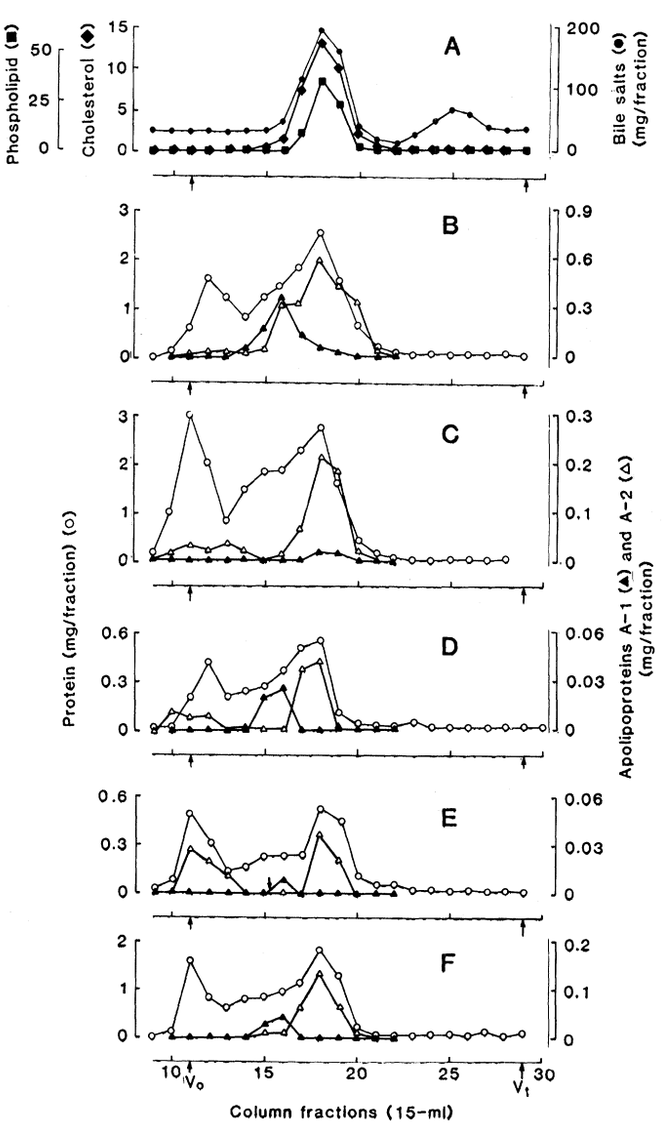

Fig. 3. Gel chromatography of normal human gallblader bile on a Sephacryl S-200 (2.7 by 80 cm) column (Pharmacia). Fresh bile (5 ml) was eluted at 100 ml/hour in a buffer containing 5 m*M* taurodeoxycholate, 0.01*M* Hepes buffer, 0.14*M* NaCl, 0.1 percent EDTA, and 0.02 percent NaN_3 at *p*H 7.5. Fractions (15 ml each) were collected and assayed for bile salts (●), cholesterol (◆), phospholipid (■), protein (○), and apo A-1 (▲) and apo A-2 (△). The totally excluded (V_0) and totally included (V_t) volumes are indicated (arrows). (A) Elution pattern of bile lipids representative of nearly identical sample profiles obtained from five patients. (B to F) Elution patterns of total bile proteins and apo A-1 and apo A-2 from the same five samples.

studies measuring the amounts of apo A-1 and apo A-2 present in bile in subjects without gallstones but whose bile is supersaturated with cholesterol, particularly in relation to apo A-1 and apo A-2 serum concentrations from these patients, are needed. Also, isolation, purification, and characterization of biliary apo A-1 and apo A-2 from these same patients is needed to understand better the roles of the apolipoproteins. Evidence for the presence of nucleating factors in the gallbladder bile of animal models or patients with cholesterol gallstones has been shown (*13*). Thus, formation of cholesterol gallstones may represent a dynamic process reflecting a balance between nucleating and antinucleating factors in the milieu of bile supersaturated in cholesterol. Whenever an isolated protein is recombined with lipid in vitro, it may not be as effective as would be the case in its normal in vivo state. Nevertheless, the magnitude of the prolongation of nucleation time (that is, only a twofold increase) observed in vitro in our study may not be sufficient to prevent gallstone formation in all susceptible patients. Finally, we speculate that a quantitative or qualitative (that is, functional) deficiency in the effector proteins capable of inhibition of nucleation, such as apo A-1 and apo A-2, may constitute a second important risk factor for cholesterol cholelithiasis in addition to supersaturation of bile in cholesterol.

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8. Nucleation times were determined by polarizing microscopy as described (*3*) with several modifications. Each heated isotropic mixture was filtered through a detergent-free, 0.22-μm filter (Millipore) into a 2-ml capped glass micro-vial; the vial was pressurized with N_2, sealed, and incubated at 37°C. The interval between time zero and the first detection of birefringent cholesterol monohydrate crystals was designated the nucleation time. As is well known, in the microparticulate size range of these experiments a distinction between crystal growth from nuclei versus true nucleation is difficult to determine, and these two processes have often been thought to occur simultaneously. A model bile was constructed by a combination of methods (*1, 2*). The cholesterol saturation index was calculated from the total and relative biliary lipid compositions [M. C. Carey, *J. Lipid Res.* **19**, 945 (1978)].
9. The model bile solutions were incubated at 55°C with shaking until they were isotropic. The solutions were recombined with the protein solutions in Hepes buffer and saline (0.01*M* and 0.14*M*, respectively) at *p*H 7.5 and heated at 37°C; controls were recombined with an identical amount of Hepes buffer–saline only. Both solutions were incubated at 45°C for 5 hours. High-density $d = 1.063$ to 1.21 g/ml) and low-density $d < 1.006$ g/ml) lipoproteins were obtained from normal, pooled human plasma by sequential ultracentrifugation [R. J. Havel, *J. Clin. Invest.* **34**, 1345 (1955)]. Apolipoproteins A-1, A-2, and C-3 were purified as described [S. J. T. Mao *et al.*, *Biochemistry* **14**, 4127 (1975); S. I. Barr *et al.*, *Biochim. Biophys. Acta* **663**, 491 (1981)].
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11. Proteins were measured fluorometrically by a modified fluorescamine assay [J. V. Castell, M. Cervera, R. Marco, *Anal. Biochem.* **99**, 379 (1979)] after trichloroacetic acid precipitation, organic delipidation, and alkaline hydrolysis (*5*). Cholesterol, phospholipid, and bile salts were measured as described (*3*).
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Nucleotide Sequence of a Human *Blym* Transforming Gene Activated in a Burkitt's Lymphoma

Abstract. *The nucleotide sequence of a human* Blym-*1 transforming gene activated in a Burkitt's lymphoma cell line was determined. This sequence predicts a small protein of 58 amino acids that is 33 percent identical to the predicted product of chicken* Blym-*1, the activated transforming gene of chicken B cell lymphomas. Both the human and chicken* Blym-*1 genes exhibit significant identity to an amino-terminal region of transferrins.*

The *Blym*-1 transforming gene was first isolated by transfection of NIH 3T3 cells with DNA of chicken B cell lymphomas (*1, 2*). The nucleotide sequence of chicken *Blym*-1 indicated that it encoded a small protein of 65 amino acids that was partially identical to the amino-terminal region of transferrin family proteins (*2*). The cloned chicken *Blym*-1 gene hybridized to a small family of sequences in DNA's of both chicken and human cells, suggesting that it was a member of a small gene family that was conserved in vertebrate evolution (*2*). Subsequently, a biologically active transforming gene detected by transfection of DNA's of six Burkitt's lymphoma cell lines was isolated with the use of chicken *Blym*-1 as a hybridization probe, indicating that the transforming gene activated in these human B cell neoplasms was a member of the gene family defined by hybridization to chicken *Blym*-1 (*3*). We now present the complete nucleotide sequence of the transforming gene (designated human *Blym*-1) isolated from one of these Burkitt's lymphoma cell lines and describe its relationship to chicken *Blym*-1 and to transferrin.

Earlier we showed that the entire biologically active human *Blym*-1 gene was contained within a 1.0-kilobase (kb) Eco RI fragment (*3*). This fragment was therefore subcloned into the Eco RI site of pBR322 for sequence analysis. The resulting plasmid, pHuBlym-1, induced transformation of NIH 3T3 cells with an efficiency of approximately 4.5×10^3 foci per microgram of DNA. For sequencing by the method of Maxam and Gilbert (*4*) (Fig. 1), pHuBlym-1 was digested with Eco RI, Pst I, or Ava I, labeled at either the 5′ or 3′ end with ^{32}P, digested with an appropriate second restriction enzyme, and purified by gel electrophoresis. For sequencing by the dideoxy chain-termination procedure (*5*) (Fig. 1), the 1.0-kb Eco RI fragment was isolated and digested with Pst I, and the resulting Eco RI–Pst I fragments were inserted in both possible orientations into the M13 bacteriophage vectors mp8 and mp9 (*6*). Most of the sequence was determined from both strands. In addition, all of the sequence except for 33 nucleotides (positions 265 to 297) was determined from two independent clones.

The nucleotide sequence of the 1.0-kb Eco RI fragment containing human *Blym*-1 (Fig. 1) confirms the positions of the Pst I and Ava I restriction enzyme sites, which we had previously mapped within this region. However the nucleotide sequence did not identify a Bam HI site, which was mapped in the initial λ clone of human *Blym*-1 approximately 200 nucleotides from the left-hand