MICROSTRUCTURE OF VESICULAR AGGLOMERATES OF LITHOGENIC BILE

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Views on the mechanisms of formation of cholesterol biliary calculi from lithogenic bile have been revised in recent years [9, 13] because of proof that nonmicellar forms of cholesterol transport exist in bile [20]. Investigations whose results are not in agreement with the postulates of the micellar theory of solubilization of the cholesterol of the bile also have been published [8]. It is affirmed that lithogenic bile is an important, but by no means the only, factor in the formation of biliary calculi [11]. The main agent responsible for targeted transport of cholesterol in the hepatobiliary system, as is now becoming clear, is the vesicular particles, organized on the monolamellar liposome principles, that have been found by various methods [13, 14] in normal bile.

Although model experiments have suggested that cholesterol phospholipid vesicles of the monolamellar (bilayer) liposome type are the immediate precursors of cholesterol calculi [9, 13], nevertheless there has been no detailed characterization of the microstructure of the nonmicellar fraction of lithogenic bile, and the investigation described below was undertaken to remedy this deficiency.

EXPERIMENTAL METHOD

Normal bile from a control group of 10 persons, obtained by means of a catheter [3], and gall bladder bile obtained from 10 patients with cholelithiasis and found to have calculi, were used in the investigation. Samples of normal and lithogenic bile were incubated at 37°C with the addition of indicator amounts of radioactively labeled compounds (cholesterol-^{3}H, lecithine dipalmitate-^{14}C, deoxycholate-^{14}C). After preliminary centrifugation of the bile at 3000 rpm, partition gel chromatography was carried out in Sephactil S-1000. Bile fractions were eluted at the rate of 2 ml/min. The dead space of the column was determined relative to blue dextran. The isolated fractions were detected with a double control, relative both to labeled compounds and to concentrations of cholesterol, phospholipids, and total cholates in the eluates [5, 22]. The structure of the vesicular particles in the fractions of lithogenic bile was studied by scanning and transmission electron microscopy [2, 10]. For scanning microscopy the eluates of the particles were placed on a supporting microfilter, washed with deionized water, and the preparations were then examined in the Nanolab-7 scanning electron microscope ("Opton," West Germany). For transmission electron microscopy eluates of particles were placed on a grid with Parlodon support, sprayed with gold, after which the preparations were stained with phosphotungstic acid (pH 7.4) and examined in the JEM-1200 EX transmission electron microscope ("JEOL," Japan).

EXPERIMENTAL RESULTS

Gel-chromatographic fractionation of normal bile followed by distribution of the fractions on the supporting filter led to the isolation of a fraction of vesicular particles with a hydrodynamic radius of 25-85 nm (Fig. 1), confirmed by the results of scanning electron microscopy (Fig. 2a). This photomicrograph shows part of a microfilter with small vesicular particles, located in the space between the pores with a standard diameter of about 500 nm. The vesicles are spherical, oval, or slightly elongated in shape. Most vesicles are arranged singly, and only a few show a tendency toward adhesion or fusion. In all cases the vesicles consisted of cholesterol and phospholipids with a molar ratio of 1:1 or 1:2. The particles varied in diameter from 30 to 100 nm, on average 80-90 nm. These parameters agree with the results obtained by other investigators, who measured the particles both by electron microscopy and by the laser scatter technique.

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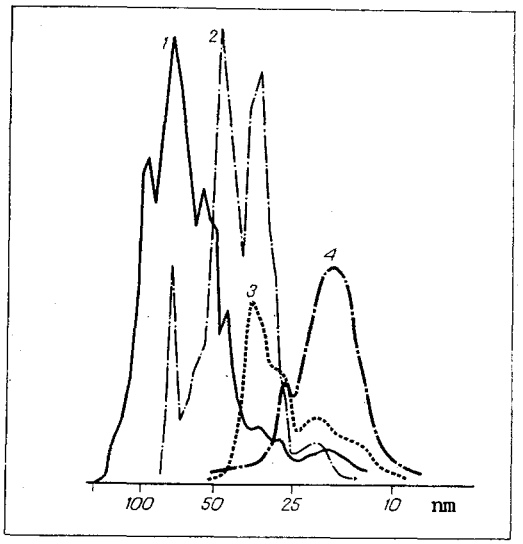

Fig. 1. Elution profile of phospholipids, cholesterol, and cholates during partition gel-chromatography of normal and lithogenic bile. 1) Phospholipids and cholesterol (lithogenic bile); 2) phospholipids and cholesterol (normal bile); cholates (normal bile); 4) cholates (lithogenic bile).

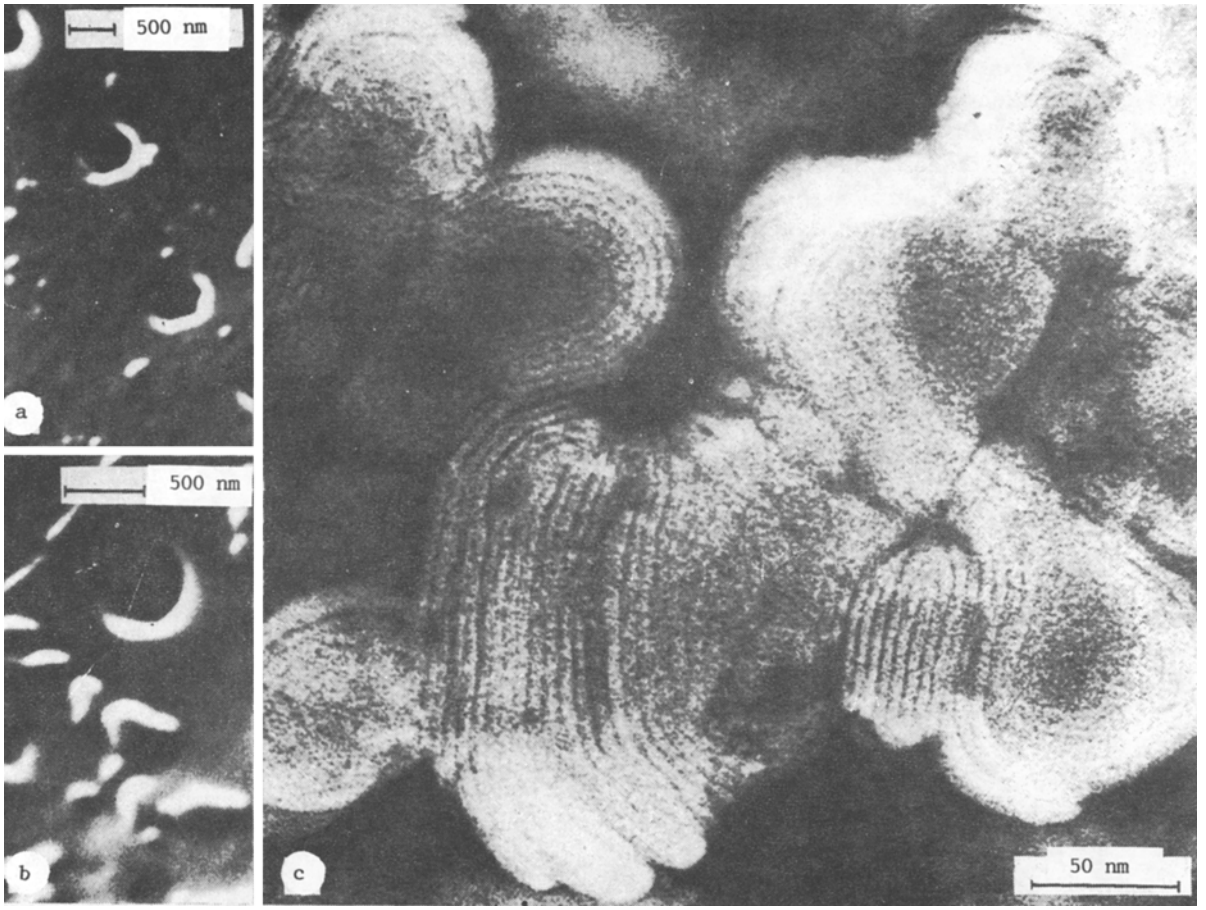

Fig. 2. Microstructure of cholesterol-phospholipid vesicles from normal and lithogenic bile. a) Normal bile (scanning electron microscopy, 15,000×); b) lithogenic bile (scanning electron microscopy, 25,000×); c) lithogenic bile (transmission electron microscopy, 300,000×).

Besides the vesicular fraction, normal bile also contained a fraction of particles with a hydrodynamic ratio of under 25 nm (Fig. 1), consisting of salts of bile acids and residual amounts of cholesterol, and also a fraction of particles consisting of macromolecular complexes of cholesterol, phospholipids, and salts of bile acids with peak values of hydrodynamic radius of about 35 nm. Although as regards the composition and size of this last fraction the particles can be classed as mixed micelles, nevertheless neither fraction could be demonstrated electronmicroscopically. Elution of pigments was observed to begin with the appearance of macromolecular complexes with a hydrodynamic radius of less than 10 nm or equal to it and it continued after the disappearance of these complexes from the eluate.

Gel-chromatographic fractionation of cystic bile containing cholesterol calculi yielded three fractions of particles lying within the range of hydrodynamic radius of 10-125 nm (Fig. 1). The main fraction of particles, consisting of cholesterol and phospholipids with a molar ratio of 1:1 or 1:3, had peak values of its elution profile between 25 and 100 nm, and differed considerably in its morphological characteristics from vesicles of native bile. Comparison of the photomicrographs (see Fig. 2: a and b) shows that only individual particles in this bile fraction (Fig. 2b) preserve their morphology, whereas the majority of vesicles are in the aggregated state, in the form of agglomerates of vesicles which may attain a diameter of 200 nm or more. It has to be pointed out that the other two fractions of particles with peak values of their hydrodynamic radius of 10-50 and 23-27 nm (Fig. 1) could not be found electron-microscopically. Particles of the first fraction were composed mainly of salts of bile acids and traces of cholesterol, whereas those of the second fraction contained small amounts of phospholipids in addition to the compounds mentioned above.

A unique feature of the behavior of the long-chain phospholipids when dispersed in aqueous solution such as bile is that they can join together spontaneously into macromolecular complexes, exhibiting as they do so a wide range of phase-ordered liquid-crystalline states, differing in their molecular texture: lamellar, spherical, cylindrical, and hexagonal [7, 15]. However, with the method of scanning electron microscopy which we used it was impossible to identify the phase state of the cholesterol—phospholipid fraction in lithogenic bile. In connection with ideas on the universal role of vesicular agglomerates in the formation and growth of biliary calculi [4], it is important to obtain more precise characteristics of the microstructure of the particles in this particular fraction.

To do this we used a combination of transmission electron microscopy with the method of negative staining of preparations particles. Although this method has been used to investigate similar bile fractions [2], this is nevertheless the first time it has been used to identify the phase state of lipids in bile.

The investigations showed that cholesterol—phospholipid agglomerates (see Fig. 2c) consist of lamellae, grouped either in pockets or in the form of stratified vesicles, with a microstructure reminiscent of multilamellar liposomes. The thickness of the lamellae in all cases was 5-6 nm, which is thicker than the bimolecular layer of phospholipids in liposomes and is close to the thickness of the bilayer of cell membranes. If the illustrative material which we obtained and published previously [1] is compared, it will be seen that the morphology of lamellar agglomerates of lithogenic bile corresponds to the descriptions of the lamellar phase of lipids, formed by dispersion of lecithin and cholesterol in water.

The theory of the crystalline origin of cholesterol biliary calculi [6] rules out any participation of liquid-crystalline phases in the mechanism of cholesterol precipitation. The authors cited start with the assumption that bile is a monophasic (micellar solution) or, at most, a biphasic (micelluar solution plus crystalline cholesterol) system. A stable triphasic system, including a lamellar phase also, is possible, according to the proponents of the micellar theory, only if lecithin concentrations in the bile are abnormally high [16]. Meanwhile several investigators [9, 13, 16] suggest on the basis of experimental data that an important condition for crystallization of cholesterol in lithogenic bile is the presence in it of a lamellar phase, formed by lecithin and cholesterol.

Our factual material confirms this view and leads to the conclusion that a morphological feature of the lithogenic state of bile is that it must contain two alternative lipid textures: stratified vesicles of the multilamellar lysosome type and aggregates of lamellar, grouped in packets. Despite differences in the morphology of the two formations, their structural basis is a bimolecular layer of phospholipids and cholesterol. Since intermediate transitions between the lamellar and hexagonal phase have been found [7] in model lipid—water systems, and since this transformation has been shown to depend on temperature and on the degree of hydration and concentration of bivalent ions, it must be expected that during inflammation of the gall bladder various phase transitions and structural transformations of the biliary lipids are possible. Considering the properties of the lameller phase of incorporating cholesterol and glycoproteins, and the wide spectrum of lipophilic and hydrophilic compounds [1], it must be submitted that the morphological variants of this phase are not only precursors, but also structural components of cholesterol calculi.

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