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THE ROLE OF BACTERIA IN GALLSTONE PATHOGENESIS

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1. ABSTRACT

Bacteria are often found in high concentrations in brown pigment and less so in cholesterol gallstones. Although it is intriguing to hypothesize that cholesterol stone formation is non-bacterial in nature and principally different from the pathogenesis of “infectious” brown pigment gallstones, it is more likely that significant overlap exists between the two processes. Most gallstones are composite in nature. Using molecular-genetic methods, bacteria can be found in most pure cholesterol gallstones (i.e. those whose structure consists of more than 90% cholesterol). The natural history of the gallstones development is unknown. It is likely that brown pigment stones can evolve in their chemical composition after the termination of the infectious process that initiate their formation, and may further develop into either mixed or nearly pure cholesterol stones. In a similar fashion, cholesterol-poor or black pigment gallstones may act as foreign bodies to enhance the propensity of bacterial colonization in the presence of pre-existing gallstones or

cholangitis, thereby activating pathways of bacterial lithogenesis and resulting in the encasement of cholesterol nuclei with pigment shells and/or in the internal remodeling of extant stones. It is often difficult, if not impossible, to ascertain whether bacterial infection of bile arose before stone formation or *vice-versa*. The development of gallstones (nucleation, assembly of microcalculi, growth, remodeling) includes the interaction of both bacterial and non-bacterial mechanisms, working discontinuously over years and decades and shaping the structural individuality of each stone. At cholecystectomy, the gallstone removed from the patient represents the end product of a long pathologic process. Although our understanding of the exact temporal contribution of bacteria in lithogenesis is incomplete, it is important for the clinician to realize that most gallstones are colonized by a bacterial biofilm, even though the bile may be culture-negative.

2. INTRODUCTION

Gallstones may manifest themselves with dramatic clinical features of obstructive jaundice and acute cholecystitis, or they can stay silent or be minimally symptomatic and be discovered only as incidental findings. They may be located in any part of the biliary tract, but are primarily found in gallbladder and less often in the common bile duct or intrahepatic ductal system. Once gallstones are discovered, they may grow, shrink, or remain the same size for years. Additional stones may form, and existing stones may dissolve or be passed. Gas can appear within gallstones on radiography and disappear later. The radiographic lucency changes with time. All these phenomena, known to those who follow their patients over years and decades, indicate that the gallstone structure and composition can fluctuate and that the gallstones do not appear and remain as a final product, but rather are in a continuous process of growth, dissolution and remodeling.

Despite these well documented observations of dynamic structural changes in the development of gallstones, gallstones are traditionally arbitrarily divided, at the time of cholecystectomy, according to their chemical composition, into three main groups: cholesterol (more than 70% cholesterol), brown pigment gallstones (less than 30% cholesterol) and black pigment gallstones (nearly no cholesterol). Other categories of gallstones (to which most stones belong) are described as composite, combined, complex, or mixed. The existing pathogenic considerations are linked to on-cholecystectomy classification, leading to the oversimplified view that considers cholesterol and black pigment gallstones to be exclusively the result of bile supersaturation and brown pigment gallstones as a product of bacterial infection. Actually, we can only speculate about most events in the long process of gallstone formation, and we understand the development of the inorganic earth minerals much better than that of human gallstones.

3. OCCURRENCE OF BACTERIA IN BILIARY TREE

3.1. Occurrence of bacteria in bile of healthy persons

Studies using small numbers of gallbladder bile samples for culture obtained at laparotomy from patients without biliary disease suggested that the biliary tract is sterile under normal conditions (1, 2). Bile culture studies from elective operations and preoperatively conditioned patients, however, can only detect planktonic bacteria. Bile cultures cannot detect sessile bacterial populations associated with the mucosa, and they are unable to determine the true frequency of bacterobilia. In a forensic autopsy study, bacteria were found in 8% of bile and 23% of the gallbladder wall specimens (3). The isolation of bacteria in the fluid always coincided with the presence of bacteria in the wall, but bacteria were found more often in the gallbladder wall than in the bile (4). Many animal experiments on pathogenesis of gallstones are made on mice and rat models. The bile of these animals is normally sterile (5). However, in dogs, beta-glucuronidase active bacteria could be isolated from gallbladder wall (but not from the corresponding bile) in 50% of all animals (6). In sheep, 50 to 75% of slaughtered animals had positive bile cultures (mostly for Escherichia coli) (7). These investigations, using bile specimens at laparotomy, thus were incomplete, and the findings cannot be accepted as definitive.

3.2. Occurrence of bacteria in bile of patients with gallstone disease

Data obtained regarding the occurrence of bacteria in bile from patients suffering with gallstone disease are more substantive. However, since symptomatic patients will be more readily operatively treated than asymptomatic carriers, the statistics to occurrence of bacteria are obviously under selective pressure.

Bacteria are regularly found in bile of patients with brown pigment gallstones [53% (8) - 91% (9) - 100% (10)], especially in cases of choledocholithiasis and hepatolithiasis (11,12). Bacteria are less often found in the bile of patients with cholesterol gallbladder gallstones [24% (8,9)] and black pigment gallstones [9% (9) - 19% (13)].

The presence of bacteria in bile varies according to the severity of the biliary tract disease and obstruction, reaching nearly 100% in cases with acute cholangitis (2, 10). In cases of cholangitis, bacteria are found more often in the gallbladder wall and within gallstones than in the bile (4,14).

Bacteria are also often (81%) in the bile of patients with carcinoma of the gallbladder and gallstones (15) and in all patients with cholangiocarcinoma and hepatolithiasis (12).

3.3. Occurrence of bacteria in gallstones

The presence of bacteria within the gallstones positively correlates with the presence of calcium bilirubinate. Bacteria could be demonstrated within brown pigment stones and in the brown pigment portions of cholesterol gallstones *via* bacterial culture, electron microscopy and molecular-genetic methods.

3.3.1. Scanning electron microscopy

Scanning electron microscopy (SEM) demonstrated bacterial microcolonies throughout the interior of brown

pigment stones within layers of calcium bilirubinate (16,17) and in the pigment portions of fresh composite (mixed cholesterol) stones, whereas no bacteria were usually found in the interiors of pure cholesterol gallstones (16,18-20). Bacteria are found coating the surface and crevices of gallstones as an adherent film known as biofilm.

3.3.2. Transmission electron microscopy

With transmission electron microscopy (TEM), the structure of the biofilm can be better appreciated. Multiple species of bacteria within brown pigment gallstones appear to be surrounded with polyanionic material, presumably the bacterial glycocalyx (19). This biofilm, composed of a polysaccharide and glycoprotein network, acts as a protective cover for bacteria and allows them to multiply within the hostile environment of the biliary system (21). There is recent evidence, in fluorescence confocal microscopic studies, to suggest that there is a completely different, unique, micro-ecological system within this biofilm environment. The same species of bacteria, in their sessile form, are phenotypically different from the free-floating planktonic ones, and they induce a low-grade inflammatory response in the surrounding mucosa. They are highly resistant to antibiotics, and it is almost impossible to sterilize the bacterial biofilm. When the stone obstructs a bile duct or the cystic duct, cytokines from the biliary epithelial cells can presumably activate these sessile forms of bacteria. Dramatic intracellular genetic and enzymatic, as well as phenotypic, transformation can occur, leading to an aggressive inflammatory outcome with suppuration and sepsis. When cholecystitis, cholangitis or biliary pancreatitits occurs as a complication of gallstones, the bacteria found in bile, pancreatic secretions and blood are almost always identical to those found in the biofilm (22, 23).

3.3.3. Quantitative PCR

Using quantitative PCR with primer universal for bacteria based on the sequence of 16S ribosomal RNA genes, bacteria at concentrations corresponding to 10^3 -10^6 cfu/gr could be demonstrated in nearly all cholesterol gallstones, even though they were culture negative. Notable exceptions to these observations were found in stones composed of more than 90% cholesterol, in which such concentrations of bacteria could not be demonstrated. No bacterial DNA was found in corresponding sterile bile. Although there was a positive correlation between occurrence of bacterial DNA and "brown pigment content" (24), there was no correlation between concentrations of bacterial DNA and concentrations of calcium bilirubinate (25). The concentrations of bacterial DNA correlated positively with the histological and clinical signs of cholangitis, possibly indicating that the bacterial DNA found in the gallstones does not represent the ancient gallstone pathology, but rather is evidence of ongoing or recently terminated infection(s). The investigations of the stability of the bacterial DNA showed that the concentrations of bacteria detected *via* PCR falls within months. After six month of storage at –20°C, no bacterial DNA could be detected in stones with initial DNA concentrations corresponding to 10^3 cfu/gr. The DNA could still be found in gallstones with initial concentrations corresponding to 10^4 – 10^6 cfu/gr. However, the bacterial concentrations after storage were significantly lower, on the order of 10^3 – 10^4 cfu/gr. A similar approach applying PCR based on sequences of the E. coli beta-glucuronidase gene has also been used (24).

3.4. Occurrence of bacteria in occluded biliary stents

Bacteria are always present in outer and luminal surfaces of occluded biliary stents (26). These bacteria again exist in a biofilm, which pose a great challenge to the endoscopist. The blockage of stents by bacterial biofilm, and the accompanying sludge, is the major source of morbidity in patients with stent drainage. Although antibiotics may slow the formation of the biofilm and adherent sludge, as mentioned before, it is almost impossible to eradicate the bacteria, and repeated stent replacement has become the only option.

4. BACTERIAL COUNTS

When bacteria are present in otherwise uncomplicated gallstone disease, their numbers are usually sparse. Bacterial counts greater than 10^5 viable organisms per milliliter of bile are found mainly in gallstones disease complicated by cholangitis (27). A recommendation to consider only concentrations higher than 10^5cfu/ml bile as significant is incorrect, since the bile is normally sterile and bile probes are taken under aseptic operative conditions. Similar to microbiologic findings in blood, the significance of bacterobilia cannot be simply correlated with absolute bacterial numbers (28). In cases complicated with acute bacterial cholangitis, bacterial concentrations often reach counts of 10^6 –10^9 cfu/ml bile, leading to demonstrable effects of bacterial metabolism on bile composition. However, acute cholangitis is only a short episode in the history of gallstone disease, whose significance to gallstone pathogenesis is currently unknown (29, 30).

5. MICROBIAL SPECIES IDENTIFIED IN BILE, BILIARY TRACT AND GALLSTONE

The bacterial species found in the bile of patients with biliary disease (both gallstones and cholangitis) indicate that the intestinal flora is a main source of bacterobilia. Escherichia coli (E.coli), Streptococci or Enterococci, Enterobacter, Klebsiella, Pseudomonas, and Proteus are the species most frequently isolated from aerobic cultures. Clostridium and Bacteroides species are often found in anaerobic isolates. In about half or more of the isolates from bile, a mixed infection is found (9, 10, 31-33). Propionibacterium species were isolated from bile in low frequency and only in single studies (32, 34, 35).

A divergent picture is obtained if bacteria are cultured from the gallbladder, from liver biopsies or from gallstones. Two studies from different regions of the United States (Philadelphia, Pennsylvania (27) and Chicago, Illinois (36)), which looked for bacteria in bile, in the gallbladder wall and in liver parenchyma, found Propionibacterium acnes to be the most common anaerobic species. Since Propionibacterium acnes is a typical

representative of a resident skin and mouth flora, the flora adherent to gallbladder mucosa and to gallstones may differ from bacteria found exclusively in bile. Actinomycetes species have been cultured from the center of human gallstones (37), but the description of the isolated bacteria presented is typical for Propionibacteria, which are both closely related and genetically similar to Actinomycetes (38). Using PCR with subsequent cloning and sequencing of bacterial ribosomal RNA genes isolated from "sterile" cholesterol gallstones, Propionibacterium was found to represent the major fraction of the anaerobic sequences in Germany (39) and China (40). Thus, the intestinal bacteria may not be the only source of bacteria in bile.

6. ROUTES OF BACTERIAL INFECTION

6.1. Ascending infection

Under normal conditions, the sphincter of Oddi forms an effective barrier to ascending bacterial migration (41). Free-flowing bile also inhibits colonization (42). In cases of bile stasis, in the presence of intestinal bacterial overgrowth, and in cases of dysfunction of the papilla of Vater, as is typical for patients with duodenal diverticula, an ascending infection is much more likely (43). A high incidence of gallstones in patients with juxtapapillary diverticula is well documented (44-48). Sphincterotomy or biliary-enterostomies allow the free reflux of duodenal contents into the biliary tree and is associated with an increase in brown pigment stone formation and bile duct strictures.

6.2. Portal source of infection

Infusion of high concentrations of E.coli into the systemic circulation, the peri-duodenal lymphatics and the portal circulation of guinea pigs indicated that portal venous blood was the most likely source of infection (49). Similar results with different concentrations of E.coli were obtained in cats (50) and rats (51).

6.3. Systemic infection

In patients with acute cholangitis, similar bacterial strains were isolated from bile and blood samples, documenting the bacterial migration from bile to blood (52). It is also possible that bacterobilia can result from bacteraemia documented in cases of typhoid fever. Transient bacteraemia with intestinal bacteria is a frequent event during endoscopic investigations. Blood cultures obtained 5 minutes after choledochofiberoscopy were positive for bacteria in 15% of patients, with maximal bacteria counts demonstrable in cultures taken 15 minutes after the investigations. Escherichia coli, Enterococci and Klebsiella species were most frequently recovered. However, the origin of the bacterobilia is possibly intestinal and not biliary.

7. FACTORS CONTROLLING BILIARY INFECTION

7.1. Obstruction

The healthy hepatobiliary tree has a remarkable ability to remove portal bacteraemia. The organisms are quickly eliminated into by the reticulo-endothelial system and may also be discharged down the bile duct into the gut (51). Experiments on cats with and without biliary obstruction using different concentrations of E.coli bacteria (54) showed that, in the absence of biliary obstruction, the feline liver was capable of trapping 10^2 to 10^5 E.coli delivered in 1 hr in reticulo-endothelial system. As more bacteria entered the portal circulation, the clearance mechanism was saturated and bacteria began to appear in biliary tract. Thus, in unobstructed animals, bacteria could be found in bile only if high concentrations of bacteria were applied. In chronic obstruction, the biliary tract is vulnerable to bacterial infection with bacteria excreted at an earlier time and in larger quantities (54, 55). Investigations into bacterial translocation in humans (56) with and without obstructive jaundice demonstrated bacterial translocation in 24% and bacterobilia in 20% of patients with obstructive jaundice, as compared to 6.5% and 3.5%, respectively, in controls with chronic cholecystitis.

7.2. Foreign bodies

Besides stasis, foreign bodies in the biliary tract were shown to facilitate bacterobilia. Implants of rubber and silicon pieces in the temporarily occluded rat biliary tract (55) led to bacterial colonization and biofilm formation on the surface of implanted material and on the mucosal surface of the biliary tract, but not in the biliary tract mucosa of rats without implants. Bile cultures and SEM examination of implanted human cholesterol gallstones, polyethylene tubes, and the corresponding gallbladder mucosa in cats were negative in the first two weeks after the implantation. At six weeks, bacterial colonization and proliferation led to formation of bacterial biofilm (detected with SEM) on the polyethylene tubes and the gallstones. With further proliferation of bacteria, the microorganisms started to disperse into the bile and resulted in suppurative cholangitis and positive bile cultures. The bile ducts were normal in appearance until the initiation of cholangitis, and, when examined after 12 weeks, the common bile ducts were plugged with biliary sludge. Adherent bacterial biofilms were then demonstrated on the mucosal surface. This experiment showed that transient bacterobilia exists in the feline biliary tract. The foreign body implants permitted the adhesion and retention of transient biliary bacteria on their surfaces and initiated the formation of adherent biofilms by these bacteria, which persisted until the system was sampled (54).

7.3. Host immunity

Little is known about host immunity in gallstone disease. Older age has a significant effect on rate of positive bile cultures among patients with symptomatic gallstones (57, 58). Bile secretory immunglobulin-A concentrations of patients with biliary infections were remarkably lower than either those of patients without biliary infection or those of normal controls (59). On the other hand, bacterial infection of the biliary tree may stimulate bile duct cells to increase bile volume to inhibit hepatocytic transport activity of bile acids and bilirubin, contributing to mucosal clearance of bacteria (60).

7.4. Bacterial pathogenicity

The analysis of 20 species of bacteria recovered from pigment and mixed cholesterol gallstones showed that

all had P1 fimbriae and alpha-galactosyl residues and non-mannose specific fimbriae (20). Unfortunately, these observations were not followed with further studies. To date, there have been no formal and focused studies addressing the molecular-genetic or phenotypic pathogenicity-related properties of biliary E.coli (the main species found in bile with cholangitis) or of other biliary bacterial species.

8. MECHANISMS OF BACTERIA-INDUCED LITHOGENESIS

Gallstones are created when the constituents of the bile precipitate out of solution. One of the possibilities to trace the role of bacteria in pathogenesis of gallstones is to investigate the potential contribution of bacterial metabolism to the precipitation of the different substances that comprise gallstones.

8.1. Biochemical properties of bacteria contributing to lithogenesis

8.1.1. Beta-glucuronidase

The early observation that brown pigment gallstones with a characteristically high content of calcium bilirubinate are often associated with Escherichia coli infection led to an assumption that bacterial beta-glucuronidase may be involved in bacterial lithogenesis by facilitating the deconjugation of bilirubin diglucuronide to free bilirubin in bile, which may then precipitate as calcium bilirubinate and act as a nidus for brown pigment gallstone formation. Subsequent experiments in which E.coli was incubated with human bile showed that sediment consisting chiefly of calcium bilirubinate was produced (61). Beta-glucuronidase is today the most accepted and affirmed mechanism of bacterial involvement in the gallstones pathogenesis (62-67). In dogs, where spontaneous bacterobilia is common, brown pigment stones could be produced simply by narrowing the cystic duct with ligatures. In all cases of thus newly formed stones, beta-glucuronidase-producing Streptococci and Staphylococci were found in the bile, gallbladder and liver. Alternatively, gallstones have been produced in animal models by injection of E. coli into the spleen (6).

In selected cases, bile infection was documented as an initial event in the pathogenesis of brown pigment stones in humans (68). In Connecticut, two cases have been reported of infants less than 3 months of age, who presented with obstructive jaundice and cholelithiases. Neither infant had any congenital anatomic abnormality of the biliary tract leading to stasis, and yet both had cultures of gallbladder bile that grew abundant bacteria. In both cases, unconjugated bilirubin accounted for a large percentage of the total bile biliary pigments measured. Bacterial beta-glucuronidase activity was high in both cases (69). Similar experience has been reported from France (70).

8.1.2. Phospholipase

Both calcium palmitate, which is detected in considerable amounts (about 15% by dry weight) in brown pigment gallstones, and fatty acids (10-20% of brown pigment stone content) have been linked to bacterial phospholipase activity (71). Phospholipase releases palmitic acid and fatty acids from phosphatidylcholine, which then may combine with ionized calcium. Since fatty acids are both saturated and unsaturated (72). Since saturated fatty acids, even when ionized, have lower aqueous solubility than unsaturated fatty acids a preferential precipitation of calcium palmitate results (73). The phospholipase activity was found to be elevated in common duct bile of patients with recurrent common duct stones (71). The mean phospholipase activity in the infected bile was significantly higher than in the absence of bacteria (73).

8.1.3. Bile acids and bacterial hydrolases

Bile acids play an important role in solubilizing cholesterol, and they participate in the solubilization of bilirubin. A decreased concentration of bile salts in the bile diminishes micellar solubilization of bilirubin as well as cholesterol, favoring the formation of gallstones. Bile acids present in normal bile are almost exclusively in the conjugated form. Bacterial hydrolases can deconjugate bile acids side chains, and thus facilitate both active and passive absorption of free bile acids. Bile acid levels were significantly reduced in bile of patients with brown pigment gallstones and bacterobilia, and bile acids have been found in large amounts within corresponding brown pigment gallstones. Significantly elevated concentrations of free bile acids were also found in brown pigment stones with sterile bile, but not in cholesterol gallstones, leading to the conclusion that elevated concentrations of bile acids can be used as a surrogate to document previous bacterial metabolism (74).

8.3. Mucin

Mucin is an important constituent of gallstones. It could be shown in experimental and human gallstone disease that mucin secreted by the gallbladder and biliary duct epithelium can function as a pro-nucleating agent, providing a framework upon which precipitates or crystals can grow and work later as a bridging and coagulation factor to solidify amorphous material into shaped gallstones (75-81). Gallbladders with brown pigment stones and combinations stones with a brown periphery contained more copious mucin in the mucosal tissue than did gallbladders with cholesterol gallstones (82). The secretion and properties of mucin can be profoundly influenced by biliary infection *via* prostaglandins, lipopolysaccharides and other bacterial toxins, the secondary inflammatory response, and toxic metabolites.

8.4. Prostaglandin

Prostaglandins are known to stimulate mucin release in the gastrointestinal tract. In vivo, high concentrations of prostaglandins and hexosamine were found to be significantly higher in a severe cholecystitis group than in the mild group and higher in infected bile than in non-infected bile (83). In vitro, prostaglandins stimulate mucin secretion from gallbladder epithelial cell cultures (84).

8.5. Cellular inflammatory response

There is a direct correlation between the number of colonies present per ml of choledochal bile and the severity of biliary disease. Patients with acute cholangitis had significantly more pyocites present in choledochal bile, as compared to patients with gallstones or to patients with CBD stones without cholangitis (85).

8.6. Lipopolysaccharides

The investigation of the interrelationship between bile lipopolysaccharides (LPS) concentrations and beta-glucuronidase activity from samples obtained during endoscopic retrograde cholangiography (ERC) demonstrated very high concentrations of LPS to be a significant predictor of common duct stones independent of beta-glucuronidase levels or the presence of juxtapapillary diverticula. These observations led to the conclusion that, in choledocholithiasis, gram-negative bacteria can produce pathogenetic factors other than beta-glucuronidase activity (86). LPS of E.coli, Klebsiella pneumoniae and Pseudomonas aeruginosa stimulates the secretion of mucin in cultured dog gallbladder epithelial cells (87). It is likely that other inflammatory cytokines, activated by the presence of bacterial products such as LPS, may have an effect on biliary epithelial cell secretion.

8.7. Oxycholesterols

Recently, oxidized species of cholesterol have been found in human bile and gallstones. These are likely to be a product of leucocytic peroxidation of biliary cholesterol, as a consequence of chronic bacterial infection, as when a biofilm is present. Interestingly, most of the sterols in brown pigment gallstones, hitherto thought to be cholesterol, are in fact found to be oxysterols. The amount of oxysterols in pigment gallstones correlated with the quantity of bacterial DNA present in these stones, suggesting that there is a causal relationship (88). This finding raises interesting and provocative speculations concerning the long-term sequelae of stones with bacteria. A number of oxysterols have been shown to be cytotoxic, pro-inflammatory, and carcinogenic. Whether these compounds are etiologically related to cellular inflammation, fibrosis and cancer formation has not been studied.

8.9. Bacterial biofilm

Endoprostheses blockage, recurrent jaundice and cholangitis are often complications of biliary stents. Macroscopically, the material in blocked stents resembles biliary sludge and is composed of bacteria and amorphous material with embedded crystals of calcium bilirubinate, calcium palmitate, and cholesterol. At high magnification, the material embedding bacteria can be seen to be arranged radially around the microorganisms, thus differing from the randomly distributed mucus or cell debris, leading to the conclusion that the formation of a bacterial biofilm and the trapping of additional bacteria, bile, and intestinal components is the primary pathogenic factor (89-91). E.coli, Proteus mirabilis and Enterococci were often isolated from blocked biliary stents (26, 32).

The pathogenicity of bacterial beta-glucuronidase has been proposed in the process of biliary stent clogging, on the basis of the observation that one-third of the isolates were beta-glucuronidase producing bacteria (26). However, this hypothesis was not supported by the findings in an *in vitro* model (92), in which cholesterol or brown pigment stones could be found within biliary stents (93). Endoprostheses perfused with artificially contaminated bile contained significantly more sludge than those perfused with sterile bile. The amount of sludge varied with the bacterial species used. Endoprostheses perfused with bacteria producing beta-glucuronidase activity were not associated with a particularly large amount of sludge (92).

8.10. Intestinal Bacteria

Some studies have suggested that there is an excess of deoxycholic acid (DCA) in the bile acid pool with cholesterol supersaturation of bile being prevalent in patients with cholesterol gallstones (94, 95). Others have not found this to be so (96). One possible explanation for this increase is a conversion of cholic acid (CA) to DCA by intestinal bacteria and enrichment of bile acid pool with more DCA than CA. The search for potential causes of DCA excess revealed that the fecal anaerobic microflora of these patients contained a high level of CA-7alpha-dehydroxylating activity and 1000-fold increased counts of CA-7alpha-dehydroxylating bacteria. The suppression of this microflora by oral intake of ampicillin reverted CA-DCA metabolism nearly to normal (97). The analysis of fecal flora from patients with gallstone disease and controls showed that all isolated 7alpha-dehydroxylating bacteria belong to the genus Clostridium in gallstones patients. No 7alpha-dehydroxylating bacteria could be isolated from a small number of controls using the same isolation technique. Thus, the intestinal microflora may be an important factor for cholesterol gallstone formation in a subgroup of patients (98). Experiments based on comparison of germfree and conventional or mono-contaminated mice demonstrated that the intestinal bacteria could also enhance the secretion of bile acids and inhibit gallstone formation (99).

9. PIGMENT VS: CHOLESTEROL CHOLELITHIASIS FROM THE DEVELOPMENTAL POINT OF VIEW. CONCLUDING REMARKS

Our knowledge of gallstone pathogenesis is based on analysis of gallstones, bile and the biliary tree at the time of cholecystectomy, autopsy data, in vitro tests and experiments with young animals. Gallstone formation in humans, however, occurs preferentially among older patients and takes years or decades. The vast majority of events that occur in the time between cholesterol crystal nucleation and stone extraction is not observable by direct investigations. The developmental history of gallstones is incompletely understood and is reconstructed with highly speculative hypotheses.

The theory about bacterial pathogenesis of gallstones is old. The main publications between 1890 and 1960 are adequately reviewed by AJH Rains (37). However, the marked differences in composition, bacterial findings and location within the biliary tree, contrary to later belief, excluded bacteria from the list of potential mechanisms of cholesterol and black pigment stone formation. How weighty are these differences?

9.1. Appearance

The appearance of cholesterol stones in their cut-surface shows radial or radially laminated structures interrupted with concentric pigment layers alternating with layers of cholesterol and documenting different episodes of

growth and resolution under changing environments. Some of the layers have a composition typical for "infectious brown pigment stones", *i.e.* calcium bilirubinate and palmitate, while others are composed of pure cholesterol. The mucin content changes from layer to layer. The concentration of bacterial DNA differs in different portions of gallstones. Most brown pigment gallstones are composed of amorphous, structurally unformed material. Only small portions of brown pigment stones have a concentric structure and are possibly older. They are then presumably found only in the gallbladder. The high grade of structural organization of cholesterol gallstones and the lack of such in most brown pigment stones indicates that age may be the main difference between them. Abundant literature follows the development of composite stones over years, and it is obvious that their differently composed layers cannot emerge simultaneously. On the other hand, brown pigment stones can emerge within weeks (69).

9.2. Location in the biliary tree

Although the significant proportion of brown pigment stones in East Asia form in the gallbladder, brown pigment gallstones are found preferentially in the common duct regardless of their geographical distribution. Obstruction of the common duct is more likely to cause acute cholangitis and symptoms leading to ERC or surgery. Common duct stones that lead to obstruction have therefore much less time and opportunity for the processes of remodeling which take place in the gallbladder. The long-term fate of a brown pigment stone after the termination of infection remains unexplored.

9.3. Infection

20% to 30% of cholesterol stones and 50% to 100% of brown pigment stones have a positive bile culture (8, 9, 10, 11, 13, 14). Molecular detection methods show that this difference is mainly quantitative and that PCR can detect bacterial sequences in almost all cholesterol gallstones. On the other hand, it is obvious that the frequency of the bacterobilia correlates with the local inflammation. Since acute cholangitis is much more probable in the common duct tract than in the gallbladder, the high frequency of bacteria in common duct gallstones is expected.

9.4. Calcium bilirubinate

Most of the bacterial species involved in cholangitis initially appear to have originated from the intestines. Beta-glucuronidase is a characteristic enzyme of E.coli, but is also present in many other intestinal bacteria. High activity of bacterial beta-glucuronidase, in turn, leads to high concentrations of calcium bilirubinate in obstructing common duct stones, as part of the scenario of an unresolved cholangitis.

9.5. Calcium carbonate

10% of cholesterol stones and the average black pigment stones are opaque on abdominal flat films. The opacity is due to the presence of calcium carbonate or phosphate at levels greater than 4% of the dry weight of the gallstone. Brown pigment stones are nearly always radiolucent, as the concentration of inorganic salts in these stones is low. This difference in concentrations of calcium carbonate is usually used to stress the principal structural difference between "infectious" brown and "non-infectious" black pigment and cholesterol gallstones. However, it is not clear why bacterial infection would be negatively associated with the precipitation of calcium carbonate. Another simple explanation is also possible. Since the opacity of gallbladder stones tends to grow with years, it is imaginable that the differences in concentrations of inorganic salts found in brown pigment and cholesterol or black pigment gallstones represent the differences in the age of the gallstones.

Although it is obvious that cholesterol and pigment gallstones follow divergent etiologic pathways, similar pathogenic mechanisms work simultaneously, and may overlap at different times to shape individual morphological and chemical characteristics. It is also possible that many pathogenetic links are still unknown, and that there are other factors working in concert to produce stones in different parts of the biliary apparatus.

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