Time Dependent Changes in the Concentration and Type of Bacterial Sequences Found in Cholesterol Gallstones

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The role of bacteria in gallstone formation could not be conclusively evaluated until bacterial presence or absence in a stone was consistently shown. Cultural bacteriologic investigations at the time of cholecystectomy, however, led to the assumption that cholesterol gallstones were free of bacteria. In this study, we used a culture independent, molecular genetic approach to detect, quantify, and identify bacteria in cholesterol gallstones from 100 patients at the time of cholecystectomy and 6 months following. Bacterial growth was recorded in the culture in 9 of 100 gallstones; bacterial DNA, however, was detected in 82 of 91 sterile gallstones. High concentrations corresponding to between 10^6 to 10^7 bacteria/g were detected in 11 stones and low concentrations of 10^5 bacteria/g were detected in 71 sterile stones. The infection in stones with a positive bacterial culture was characterized by the predominance of single bacterial sequence(s) of the bacteria cultured. A similar predominance, indicating a recent infection, was found in sterile gallstones with low DNA concentrations. A high diversity of non-repeating bacterial sequences, possibly arising from previous overlapping infections, was found in sterile gallstones with high concentrations of bacterial DNA. After 6 months concentrations of bacterial DNA fell significantly in all groups of gallstones. As bacterial DNA is quickly destroyed upon storage, but is nevertheless readily found in most gallstones at the time of cholecystectomy, there must be a mechanism by which it is replenished. One such mechanism is the frequently reoccurring, possibly self-terminating infection and another one is the permanent colonization of the gallstone with bacteria at low concentrations. Both can promote cholecystolithiasis. (HEPATOLOGY 1998;27:662-665.)

Our knowledge about the cholesterol gallstone comes primarily from the investigation of its composition at the time of cholecystectomy and from brief model experiments with saturated bile. Gallstone formation, however, takes years, and the vast majority of events that occur in the time between cholesterol crystal nucleation and their consolidation into a gallstone is not accessible to a direct investigation. Gallstone formation is, therefore, incompletely understood. Because cholesterol gallstones are more than 70% cholesterol and because bacteria are not regularly cultured at the time of cholecystectomy, the role of bacteria in the pathogenesis of cholesterol gallstones has been questioned.[1] Bacteria, however, are a good explanation for the initiation of nucleation.[2] Bacterial polymers are ideal substances for both binding loose cholesterol crystals to a massive gallstone and for building a matrix in which cholesterol later crystallizes.[3] Unfortunately, there is no convincing model to prove either of these hypotheses and the data about the occurrence of bacteria in bile,[4,6-7] the billiary tree,[5,8] and in the gallstones[8] are inconsistent.

Bacteriologic findings depend on bacterial vitality and culturability. As bacteria growing in the gallstone environment may need special culture requirements, methods other than culture must be used to verify whether gallstones are really bacteria free. We previously adopted a molecular genetic approach to unravel the role of bacteria in cholesterol gallstone formation.[9] With nested polymerase chain reaction (PCR) and a primer set universal for bacteria, followed by cloning and sequencing of the PCR products, we detected and identified traces of bacterial DNA in almost all cholesterol gallstones with a negative bacterial culture. The relevance of these findings was uncertain, because nested PCR can detect even the slightest, possibly insignificant, quantities of DNA and also because detected DNA can come from both living bacteria and dead microorganisms which were incorporated and conserved for many years.

The aim of the present study is to quantify bacterial DNA in gallstones molecular genetically and also to understand whether different concentrations of bacteria are associated with particular bacterial sequences, cholesterol concentrations, gallstone numbers, and patients′ clinical data. To determine whether bacterial DNA in gallstones represent an ongoing infection or past events, we looked for changes in bacterial concentrations and sequences in sterile gallstones, and in gallstones with positive bacterial culture 6 months after cholecystectomy; we then compared the molecular genetic with the culture findings.

PATIENTS AND METHODS

Patients

This study was performed with previous approval of the institutional review board and with the patients′ informed consent. The gallstones were obtained from the clinic of surgery, Friedrichshain

Abbreviations: PCR, polymerase chain reaction; cfu, colony forming unit.

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Hospital, Berlin, Germany. Molecular genetic analysis of gallstones was done at the IV, Medical Clinic, Charité Hospital, Humboldt University, Berlin, Germany. The microbiologic diagnosis was performed in the microbiology laboratory of Friedrichshain Hospital.

Symptomatic patients who underwent elective, laparoscopic cholecystectomy for cholecystolithiasis were considered. Patients with acute symptoms at the time of the operation or with pigment gallstones were excluded. One-hundred consecutive patients, operated between January and June of 1995, met these criteria. All patients had received 1 gr of cefotaxim and 500 mg of metronidazole iv before the operation. The stones and a sample of corresponding bile were transported to the molecular genetic laboratory within two hours after cholecystectomy. The biggest stone was analyzed from each patient. Bacterial culture of the same stone was made in parallel.

After the initial investigation the gallstones were frozen in their corresponding bile and stored at -20°C for 6 months.

Methods

DNA was extracted from 100 mg of stone (native weight) according to our previous protocol[9] and resuspended in 20 µL of high performance liquid chromatography water (J.T. Baker, Deventer, Holland). The same procedure was used to extract DNA from 500 µL of corresponding bile. The 5′ half of the 16S ribosomal RNA gene (600 base pairs) was amplified with PCR using a primer set universal for bacteria, as follows: 5′CTGGTTCCGGCGAAGAGTTTGAT(c/t)(c/a)TGGCTCAG-3′, 5′-CTCGCTCCGGCGAACCGC(g/t)(a/g)CTGCTGGCAC-3′. The thirteen additional nucleotides (underlined amino acids) were added at the 5′ ends for cloning (PharMingen, San Diego, CA). To prevent false positive PCR results, pre- and post-PCR steps were carried out in different laboratories and control experiments omitting template DNA were performed in parallel.

Two microliters (10 mg of stone/50 µL of bile) were taken for PCR. The concentration of bacteria in this sample was measured by electrophoretic comparison after 25 and 30 thermocycles of a sample's PCR to the PCR of standard bacterial dilutions of 10^5,10^4,10^3,10^2, and 10 colony forming units (cfus) per µL. Each amplification was repeated at least four times to check for consistency of results (twice after 25 cycles and twice after 30). The PCR–negative DNA samples were additionally amplified together with bacterial DNA corresponding to 10^3 cfu/µL to exclude PCR suppression and false negative results.

The standard bacterial dilutions were made from XL1 Blue MRF′ *Escherichia coli* cells (Stratagene, La Jolla, CA). For visualization, the agarose gel was stained with ethydium bromide. Dilutions of 10 to 10^2 cfus/10 mg gave no PCR signal. Weakly positive PCR products corresponding to the 10^3 dilution signal could not be cloned after straight PCR. The DNA of the first 20 gallstones with low bacterial concentrations (10^3 cfu/10mg) was, therefore, amplified again with nested PCR and then cloned and sequenced according to our previous protocol.[9] After 6 months of storage a nested PCR of all stones with previous concentrations of 10^3 cfu/10 mg was done.

To determine the bacterial composition of PCR products with a positive signal corresponding to concentrations of 10^4 to 10^5 cfu/10 mg, amplified 16S ribosomal RNA sequences were cloned into a pCMV-LIC vector according to a Ligation Independent Cloning Protocol (PharMingen). Fifty clones were sequenced for each stone sample on the LI-COR Model 4000 Sequencer (LI-COR, Lincoln, NE). Bacterial sequences were analyzed and compared with 16S ribosomal RNA primary structures retrieved from public databases.[10-11]

Bacteriologic analysis of gallstones was performed in the routine microbiologic laboratory of Friedrichshain Hospital. Ten milligrams of stone was diluted in 500 µL of physiologic saline and plated on columbia agar with sheep blood (5%), chocolate, and MacConkey agar The plates were incubated at 37°C aerobe and microaerophil (blood agar) and examined after 48 hours. To isolate anaerobes, specimens were plated on prereduced schaedler agar and inoculated into enriched thioglycolate broth. The plated media were incubated in GasPak jars and examined after 120 hours. The thioglycolate broth was incubated for 7 days. Anaerobes were identified using a commercial identification kit API ID 32A (bio Merieux, Neurtingen, France).

Cholesterol content analysis was performed at the Institute of Biochemistry, Charité Hospital, Humboldt University, Berlin, Germany, according to our previous protocol.[9]

Statistics

Where appropriate, non-parametric data were expressed by median value (range), normally distributed data were expressed as mean, and differences between or across groups were evaluated by ANOVA. Statistical analysis was done using χ^2 and t tests. $P < .05$ was considered significant.

RESULTS

Bacterial growth was recorded in the culture of 9 of 100 gallstones. Seven stones were infected by only one bacterium (2x *Escherichia coli*, *Streptococcus sp.*, *Enterococcus sp.*, *pseudomonas aeruginosa*, *Staphylococcus sp.*, and *Ervinia herbicola*); and 2 stones had a mixed infection (*Kluyvera sp.* + *Streptococcus sp.* and *Streptococcus* + *Staphylococcus sp.*). Quantitative PCR showed all stones with positive bacterial culture to have concentrations of bacteria between 10^4 and 10^5 cfu/10 mg. Eleven of 91 sterile gallstones had concentrations of bacteria comparable with those of culture positive stones. Seventy-one sterile gallstones had bacterial concentrations of 10^3 cfu/10 mg and no bacterial DNA was detected in only 9 stones. Quantitative PCR of corresponding bile was positive in only 5 patients with positive stone culture (both patients with mixed infection, 2x *Escherichia coli*, *Enterococcus sp*). Bacterial DNA concentrations of 10^5 cfu/mL were observed in bile.

Based on the concentration of bacterial DNA and the culture findings, the analyzed gallstones fell into four following groups: gallstones with positive bacterial culture and high bacterial DNA concentrations, sterile gallstones with high concentrations of bacterial DNA, sterile gallstones with low concentrations of bacterial DNA, and gallstones with no detectable bacteria. The patients′ mean age, female/male ratio, mean stone cholesterol content, and the percent of patients with solitary versus multiple gallstones in these four groups are summarized in Table 1. Except for the cholesterol

TABLE 1. Patients' Mean Age, Female/Male Ratio, Stone Cholesterol Content, and the Number of Patients With Solitary Gallstones

	Gallstones With Positive Culture	Sterile Gallstones With High Concentrations of Bacterial DNA	Sterile Gallstones With Low Concentrations of Bacterial DNA	Sterile Gallstones Without Bacterial DNA
Total number of patients/gallstones	9	11	71	9
Number of patients with a solitary stone	1	2	12	1
Mean age	48	49	51	58
	ns	ns	ns	ns
Female/Male	7/2	6/5	54/17	3/6
Mean percent cholesterol ± SD	83 ± 5.11	82.5 ± 8.2	81.7 ± 6.4	93.9 ± 2.8 $P < .001$

TABLE 2. Concentrations of Bacterial DNA Detected in Cholesterol Gallstones Initially and After 6 Months of Storage at −20°C

Number of Gallstones With cfu/10 mg	Gallstones With Positive Culture		Sterile Gallstones With High Concentrations of Bacterial DNA		Sterile Gallstones Low Concentrations of Bacterial DNA		Sterile Gallstones Without Bacterial
	Initially	After 6 Months	Initially	After 6 Months	Initially	After 6 Months	Initially
0-10^2		1				71	9
10^3		5*		3*	71*		
10^4	6	3	6	4			
10^5	3		5	4			
Mean number of different bacterial sequences ± SD	1.2 ± 0.4	7.3 ± 2.4 $P < .001$	23.5 ± 6 $P < .001$	3.6 ± 1.3	3.9 ± 2.2		
Patients with a single sequence dominating	9/9	8/8	0/11	8/11	16/20		

*Cloning and sequencing was possible only after nested PCR.

concentration, there were no statistically significant differences between groups with different concentrations of bacterial DNA.

Stones with a positive bacterial culture were characterized by the predominance of a single bacterial sequence. Predominance was defined as one sequence constituting more than 50% of all the clones sequenced. Sequence analysis of the PCR product from each of the 7 monoinfected stones and from 3 corresponding PCR–positive bile samples showed only the sequences of the cultured bacterium. In the two stones with a mixed positive culture, sequences of both cultured bacteria were observed in the following ratios: 34/16 and 42/8. In the corresponding biles only the sequence of the most numerous bacterium was found.

The pattern of sequences in the 11 sterile gallstones with high concentrations of bacteria (10^4-10^5 cfu/10 mg) was completely different. In each stone, a thorough mix of bacterial sequences was observed (on average 23.4 different sequences per 50 clone) with no one sequence repeating in more than 10% of all the clones sequenced (Table 2).

A sequence analysis of sterile cholesterol gallstones with low concentrations of bacteria was performed after nested PCR. The nested PCR products of the first twenty stones were cloned and sequenced. Sixty-nine different bacterial sequences were found and the mean number of different bacterial sequences per stone was 3.6. Despite this high diversity, a single sequence was predominant in some stones. However, the predominant sequences found in sterile gallstones with low DNA content and in gallstones with positive bacterial culture were not the same. Propionibacteria-like sequences, coli-like sequences, and sequences previously described as belonging to gram-positive bacteria with high DNA G+C content (guanidine and cytosine content higher than 55%) were predominant in 7.4, and 4 stones, respectively. An alcaligenes-like sequence was predominant in one stone and a non-repeating mix of sequences was observed in four stones.

After six months of storage at −20°C, no bacterial DNA could be detected in the 71 stones with low concentrations of bacteria. In other groups, there was also a considerable reduction in the concentration of bacterial DNA (Table 2). This reduction was accompanied by marked changes in the sequence patterns and bacterial diversity. In stones with a positive culture, sequences of cultured bacteria still dominated; however, new sequences appeared and made up to 20% of all the clones sequenced. The mean number of different bacterial sequences per 50 clones grew from 1.3 to 7.3. An opposite change was observed in sterile gallstones with high concentrations of bacterial DNA. In these stones the diversity of bacteria diminished significantly. The mean number of different sequences found per 50 clones fell from 23.5 to 3.6 and one sequence dominated in some stones appearing in 50% of all the clones sequenced (Table 3).

DISCUSSION

Previously we showed that all cholesterol gallstones, with the exception of pure cholesterol stones (more than 91% of cholesterol), harbor traces of bacterial DNA.[9] This time we had the same trend in results. Stones without bacterial DNA did have significantly higher cholesterol concentrations ($P < .001$) than stones with; nevertheless, among stones with bacterial DNA, we found no positive correlation between the concentration of bacterial DNA in a stone and the percentage of cholesterol in the stone; thus, the particular role of bacteria in cholesterol gallstone pathogenesis remains puzzling. It is certain, however, that the widespread conjecture that cholesterol gallstones arise in a bacteria free bile environment is oversimplified. DNA concentrations corresponding to 10^5 cfu/g are readily found in most sterile cholesterol gallstones. Although bacterial concentrations in gallstones with positive bacterial culture are higher, these high concentrations of 10^6 to 10^7 cfu/g are not restricted to gallstones with positive

TABLE 3. Bacteria Cultured and Predominant Sequences (more than 50% of all clones sequenced identical) Found in Cholesterol Gallstones

Bacteria cultured from gallstones
2 × *Escherichia coli,* 3 × *Streptococcus sp.,* 1 × *Enterococcus sp.,* 1 × *Pseudomonas aeruginosa,* 2 × *Staphylococcus sp.,* 1 × *Ervinia herbicola,* 1 × *Kluyvera sp.*

Predominant sequences
Gallstones with positive culture
Initially: identical to the sequences of bacteria cultured from the same gallstones
After 6 months of storage: 2 × *Escherichia coli,* 1 × *Ervinia herbicola*
Sterile gallstones with high concentrations of bacterial DNA
Initially: no repeating sequences
After 6 months of storage: 2 × *bacillus species,* 2 × *coli-like,* 2 × *Alcaligenes eutrophus-like,* 1 × *Carnobacterium divergens,* 1 × *Burkholderia cepacia*
Sterile gallstones with low concentrations of bacterial DNA
Initially: 4 × coli-like, 7 × *propionibacteria-like,* 4 × unknown, 1 × alcaligenes-like.
After 6 months of storage: no bacteria were detected.

culture. Eleven of 91 sterile gallstones had the same high concentration of bacteria as infected gallstones.

The high incidence of bacterial DNA in sterile gallstones raises the question of why the bacteria cannot be cultured. Either they are dead at the time of the operation or they arise from infections with unculturable or unusual microorganisms with fastidious culture requirements. It is also possible that there are no intact bacteria and no bile infection in the gallstones; rather, a gallstone works as a filter/sponge holding the traces of bacterial DNA molecules that pass with the bile. In the last case, one would expect significant concentrations of bacterial DNA in sterile bile or a stability of bacterial DNA within a gallstone which is necessary for accumulation. Both suppositions were not confirmed. There was no bacterial DNA in the corresponding bile of sterile gallstones, and the bacterial DNA within the gallstone proved to be unstable. Because bacterial DNA disappears quickly upon storage and, yet, can be found readily in almost all stones immediately after cholecystectomy, it must be refilled either by series of reoccurring and overlapping infections or by a permanent colonization of the gallstone.

An ongoing infection in patients with a positive bacterial culture was characterized by the predominance of a single bacterial sequence constituting nearly all of the clones sequenced and identical with the sequence of the bacteria cultured. Only sterile gallstones with low concentrations of bacterial DNA had a similar predominance of single bacterial sequences. In this group among predominant sequences, propionibacterium-like sequences were especially frequent. Propionibacterium is very sensitive in its growth requirements. Its exclusive presence in sterile gallstones with low bacterial concentrations but not in other groups strengthens the possibility that some slowly growing bacteria is capable of permanently colonizing the gallstone at low concentrations under physiologic conditions but are quickly destroyed upon extracorporation.

In contrast, sterile gallstones with high concentrations of bacterial DNA had a high diversity of unrelated bacterial sequences, an average 23.5 sequences per stone. No sequence was predominant initially or appeared regularly in other stones of the same group. A simultaneous infection with over 20 different pathogens is unlikely. A better explanation of this diversity is the explanation of short, overlapping, consecutive infections of the biliary tree with intestinal bacteria, all of which have been terminated for some time, and none, therefore, predominant at the time of investigation. The high incidence of bacteria cultured from the bile of patients with cholecystolithiasis and common bile duct stones, but not subjects without gallstone disease, is consistent with this proposal.[12]

If clinically unapparent infections of the gallstones occur often and if bacteria repeatedly visit the biliary tree, then the bacterial diversity should not be restricted to a single group of cholesterol gallstones, as was, indeed, the case. Even in gallstones with a positive culture in which a single sequence initially predominated, an increase in bacterial diversity and a decrease in the concentration of bacterial DNA was detected after 6 months. Apparently the exponential decline of the dominant population after cholecystectomy uncovers the remnants of previous infectious agents whose decomposition had already slowed down.

A shrinking diversity of bacterial sequences, some of which predominated after 6 months of storage in the group of sterile gallstones with high bacterial concentrations, is confusing but is easy to explain if the predominating sequences are considered. These were assigned to resistant bacterial rods, such as *Bacillus*, *Alcaligenes*, *Carnobacterium*, and *Burkholderia*, which are typical representatives of pit and earth bacteria, which survive under extreme conditions. No *Propionibacteria* sequences were found here. Because the DNA and bodies of different microorganisms disintegrate at different rates, some microorganism and their DNA can persist longer in the gallstone and be found later than others.

When we started our investigation 5 years ago, we simply wanted to see whether bacteria occur in the cholesterol gallstone more frequently than the culture data indicate. Using a molecular genetic approach we found an unexpectedly rich and extremely complex microflora. This should not be further neglected. However, our hope to use bacterial DNA as a paleontologic marker was not fulfilled. Bacterial DNA disappears too quickly and as the formation of the gallstone takes years, the bacteria we detected do not document the initial pathogenic events but an ongoing process. One part of this process is the reoccurring, and possibly self-terminating infections; another one is the permanent colonization of a gallstone by slowly growing microorganisms. Both components can contribute to gallstone formation.

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