

A Systematic Evaluation of the role of Infection, Immunity and Inflammation in  
Cholesterol Gallstone Pathogenesis

By

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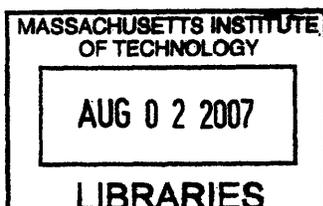
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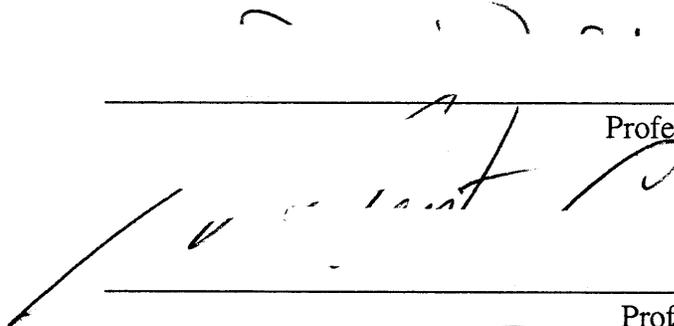
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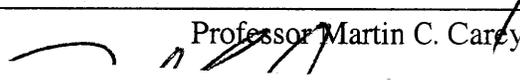
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# A Systematic Evaluation of the role of Infection, Immunity and Inflammation in Cholesterol Gallstone Pathogenesis

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Kirk J. Maurer

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## Abstract

Cholesterol gallstones are exceptionally common and cost nearly 10 billion U.S. dollars annually. Despite a half-century of basic and clinical research questions still remain about cholesterol gallstone pathogenesis. The purpose of the study presented herein is to analyze the roles of infection, and immunity in cholelithogenesis. The first two aims of this work were to analyze the role of enterohepatic *Helicobacter* spp. and the human gastric pathogen *H. pylori* in cholesterol gallstone formation. To test this, we prospectively infected C57L/J mice with a variety of *Helicobacter* spp. and fed infected and uninfected mice a lithogenic diet for eight weeks and analyzed biliary phenotype. Mice infected with *H. bilis* or coinfecting with *H. hepaticus* and *H. rodentium* and fed a lithogenic diet developed cholesterol gallstones at 80% prevalence compared with approximately 10% in uninfected controls ( $P < 0.05$ ). Mono-infections with *H. hepaticus*, *H. cinaedi*, *H. rodentium*, and *H. pylori* gave a cholesterol gallstone prevalence of 40% ( $P < 0.05$ ), 30%, 20% and 20%, respectively; with the exception of *H. hepaticus*, cholesterol gallstone formation in these groups did not differ significantly from uninfected animals. These findings suggest that some *Helicobacter* spp. play a role in the cholesterol gallstone formation in mice and perhaps humans. We further hypothesized that inflammation and immunity were important in cholesterol gallstone formation and that cholelithogenic bacteria were promoting gallstones through immune stimulation. To test this we utilized BALB/c and isogenic *Rag2*<sup>-/-</sup> mice. When fed a lithogenic diet for eight-weeks, wild-type mice developed cholesterol gallstones (27-80% prevalence) significantly more than *Rag2*<sup>-/-</sup> mice (~5%,  $P < 0.05$ ). Transfer of functional splenocytes, or T-lymphocytes to *Rag2*<sup>-/-</sup> mice markedly increased cholesterol gallstone formation (26% and 40% respectively,  $P < 0.05$ ) whereas transfer of B-cells did not (13%). The presence of T-cells and solid cholesterol monohydrate crystals induced proinflammatory cytokine expression in the gallbladder. These studies indicate that T-cells are critical in murine cholelithogenesis and function by promoting gallbladder inflammation. In summary, these results illustrate that microbial pathogens can influence cholesterol gallstone formation; this most likely occurs by modulating the immune response with T-cells being a critical component in this immunomodulation.

Thesis Advisor: James G. Fox  
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## Chapter 1

### **Introduction: The potential role of inflammation, immunity and infection in cholesterol gallstone formation**

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## **Introduction**

Bile is a complex aqueous colloidal system essential for a wide range of physiological functions including the excretion of lipids from the organism and intestinal fat absorption (23, 24). Bile is formed in hepatic canaliculi, small (1-2 $\mu$ m) spaces formed between the tight junctions of hepatocytes. It is composed principally of water and a variety of lipid solutes in micellar and vesicular solution including bile salts, phospholipids (mostly phosphatidylcholine or lecithin), and cholesterol, as well as proteins and bilirubin conjugates (126). Phospholipids and bile salts are essential for the removal from the organism of otherwise insoluble cholesterol molecules in an aqueous environment by solubilizing the sterol to form mixed micelles and unilamellar vesicles (23, 24, 126). Bile is transported from the canaliculi along tubules of increasingly greater diameter until it egresses into the gut at the mid-duodenum. In animals with gallbladders, about half the secreted bile is stored and concentrated in the gallbladder during the interdigestive interval. The gallbladder is connected to the biliary tree via the cystic duct which functions simultaneously as a gallbladder filling and emptying conduit.(126).

Alterations in the relative or absolute proportions of cholesterol, phospholipids, and bile salts can lead to an inability to maintain cholesterol in solution in bile. Most frequently these changes occur due to excess secretion of cholesterol from the liver (120). As absolute cholesterol concentration increases it can no longer be incorporated into mixed micelles and the excess may phase separate forming unilamellar vesicles with biliary phospholipids. Under suitable physico-chemical conditions these can aggregate to form multilamellar vesicles (lamellar liquid crystals) and eventually cholesterol monohydrate

crystals may separate and grow in the gallbladder (23, 24). These crystals may progress to form cholesterol gallstones by agglomeration within a mucin gel. Cholesterol gallstone formation invariably occurs adjacent to the gallbladder wall when cholesterol monohydrate crystals nucleate in the gelled mucin glycoprotein scaffolding (23, 24). The majority of cholesterol gallstones form in the gallbladder; however, in rare situations usually from genetic deficiency of biliary phospholipids, they may form anywhere along the biliary tree even intrahepatically (126). Although a relative excess of cholesterol is necessary for cholesterol gallstone formation it is not sufficient. Indeed, “biliary sludge” composed of agglomerated cholesterol crystals of an ultrasonically-detectable size (~50µm) suspended in mucin gel can be visualized frequently, it may or may not progress to cholesterol gallstones (25, 96, 97). In fact, Lee and colleagues (97) studied patients ultrasonographically for a mean period of over three years and found that only 8% of patients with “biliary sludge” developed cholesterol gallstones whereas in 17.7% of patients, biliary sludge disappeared and in 60.4% biliary sludge disappeared and reappeared.

The study of cholesterol gallstones relies upon multiple methodologies including physical chemical studies of model bile and *ex vivo* bile systems, biliary lipid secretory studies, animal models with particular focus on cholesterol gallstone (*Lith*) genes and epidemiological studies analyzing susceptible and resistant human populations (24, 86, 130, 166, 174). These studies provide the basis for the current knowledge underlying our understanding of the epidemiology and pathogenesis of cholesterol gallstones. Using these methodologies it is now appreciated that cholesterol gallstone formation is

primarily a polygenic trait with significant contributions from the environment (74, 126). Although these studies provided a strong basis for our understanding of the pathogenesis of cholesterol gallstones, many issues remain unresolved. For example, why do so few individuals with supersaturated bile (as evidenced by biliary sludge) proceed to gallstones (97)? Additionally, why do genetically identical murine models challenged with the same lithogenic diet conditions but housed at different research institutions display such marked differences in their proclivity to form cholesterol gallstones (105)?

We hypothesized that the differences in gallstone prevalence in genetically identical mice may be due to differences in their colonization status with gastrointestinal microbes. Initially, we analyzed the ability of enterohepatic *Helicobacter* spp. to contribute to lithogenicity and identified some enterohepatic helicobacters that were capable of contributing to cholesterol gallstones and some that could not (105, 106). Following these initial studies, we further hypothesized that the host immune system may be the driving force in the ability of these organisms (and perhaps others) to promote cholesterol gallstones as well as mucin gel and gallbladder inflammation. Subsequent studies demonstrated that indeed the response of the immune system was important, and probably essential since Rag mice that lack T and B cells seldom (<10%, which is 'rare' on the murine prevalence scale) develop cholesterol gallstones (Chapter 5). A number of previous studies have analyzed the role of the immune system in cholesterol gallstone formation. These studies invariably demonstrated that gallbladder inflammation occurred concomitantly with cholesterol supersaturation and gallstone formation; none however,

conclusively demonstrated that this process was a primary contributing factor rather than a secondary effect of the lithogenic process (12, 67, 139, 140, 167, 168).

The current review focuses on a new aspect of cholesterol gallstone pathogenesis examining the role of infection, inflammation and the immune system's response in the formation of cholesterol gallstones. We analyzed an extensive literature not customarily viewed as being related to cholesterol gallstones and our own recent work focusing on mouse models of infection, inflammation and immunity. Furthermore, this review describes the critical contributions of the gallbladder epithelium to the immune system which in turn plays a key role in cholesterol gallstone formation.

*Biliary epithelium and immunity:*

One cannot describe the potential role of biliary inflammation and the immune system on cholesterol gallstone formation without first describing the interaction of the immune system and the biliary system in general. Anatomically, the biliary tree is often divided into intrahepatic and extrahepatic portions with respect to biliary tissue and disease. This distinction however appears nonessential when describing the interaction of the biliary epithelium with the immune system, since it appears biliary epithelial cells from either location, respond to immunogenic stimuli in a similar fashion (10, 145).

Biliary epithelial cells participate in both innate and adaptive immunity (55, 56, 141). Briefly, the innate immune system is the portion of immunity that induces no immunological memory for the antigen. This system responds to a variety of

evolutionarily conserved patterns found in foreign antigens. In addition to specific cellular subsets including neutrophils, macrophages, eosinophils, basophils and natural killer cells, a variety of proteins also participate in innate immunity including the complement cascade, cytokines, and the proteins of the acute phase response (5, 50, 73). In contrast, the adaptive immune response induces memory to the foreign antigen and is mediated by both B and T lymphocytes. Following activation, the adaptive immune response may also stimulate the production of a variety of cytokines and the recruitment of inflammatory cells (14, 39, 44, 45). There is overlap in these two pathways and perturbation of either pathway may alter the ability of the other to respond appropriately (45).

Human biliary epithelial cells express all known toll-like receptors (TLR) which are essential for antigen-pattern recognition responses of the innate immune system (27, 54, 56, 142). Additionally, myeloid differentiation protein 88 (MyD88), which is a key downstream effector protein of the TLR pathway is also present in these cells (56). Furthermore, biliary epithelial cells possessing dominant negative MyD88 mutants are more susceptible to infection with the intracellular biliary pathogen *Cryptosporidium parvum* indicating that the TLR/MyD88 system is indeed functional in these cells (142).

The relationship between biliary epithelial cells and the adaptive immune response requires a variety of receptor-ligand interactions between epithelial cells, T-cells and 'professional' antigen presenting cells (i.e. dendritic cells). Primary tissue culture cell-lines of human biliary epithelium demonstrate that these cells constitutively express

human leukocyte antigen class I (HLA-I, MHC class I antigens) and little class II antigens (10, 145). However, after stimulation with proinflammatory cytokines the cells express a variety of HLA class II antigens including HLA-DR, HLA-DP, and HLA-DQ (10). Essentially this expression profile demonstrates that biliary epithelial cells are likely to interact with both CD8<sup>+</sup> T-cells (cytotoxic T-cells) as well as CD4<sup>+</sup> T-cells (helper T-cells). In addition to these primary immune receptors, biliary epithelial cells also possess a variety of costimulatory molecules necessary for functional interaction with these immune cells including CD40, ICAM-1, LFA-1 and FAS (2, 3, 18, 68, 81, 141). These protein expression profiles appear to indicate that the biliary epithelium actively participates in adaptive immunity through interaction with T-cells and local antigen-presenting cells.

The second portion of adaptive immunity involves the B-cell response. This response is mediated through immunoglobulins. Immunoglobulins, including IgA, IgG, and IgM, are commonly found in low concentrations in bile (141). However, biliary epithelial cells participate minimally in this process. In humans, IgA is produced by plasma cells lining the bile ducts and then bound by the polymeric immunoglobulin receptor (pIgR) at the basolateral surface of cholangiocytes (71, 117, 122). IgA is then transported to the apical surface and secreted into bile. Evidence exists that this intracellular transport plays a role in protection from intracellular pathogens by binding these antigens during intracellular transit (143). Like IgA, IgM is transported via the pIgR, but in contrast IgG is transported utilizing the FcRn (143). Immunoglobulins reaching intraluminal bile aid in protecting bile from colonization with gastrointestinal pathogens (20, 107).

Aside from expressing the necessary receptors and downstream activators to participate actively in the immune response, biliary epithelial cells also secrete a variety of cytokines and undergo phenotypic changes in response to these inflammatory mediators. Cultured murine gallbladder epithelial cells express *Tnf- $\alpha$* , *Ccl5* (RANTES), and *Mip-2* (Macrophage inflammatory protein 2) (149). Following treatment with LPS these cells upregulate expression of *Mip-2* and *Tnf- $\alpha$* . Additionally, *Il-1 $\beta$* , *Il-6* and *Mcp1* expression is induced following LPS treatment (149). Interestingly, the Th2 cytokine, *Il-10* is not expressed in resting or LPS stimulated gallbladder epithelium (149). It has been established that the biliary epithelium possess receptors for some of these cytokines and chemokines (including TNF- $\alpha$ ) indicating that these secreted cytokines may act in both an autocrine and paracrine manner (141, 154). The addition of exogenous cytokines to cultured biliary epithelial cells induces phenotypic changes in these cells supporting an autocrine role for some of these cytokines. For example, the addition of TNF- $\alpha$ , LPS, IL-1 $\alpha$ , and prostaglandin E2 to cultured human gallbladder epithelial cells alters the transport of sodium, and chloride and diminishes the absorptive function of the gallbladder epithelium (138). A bile duct ligated guinea pig (*Cavia porcellus*) model supports these *in vitro* findings. In this model, the instillation of IL-1 or LPS induces gallbladder wall inflammation and stimulates the release of myeloperoxidase, water and prostaglandin E2 into the gallbladder lumen (136). Systemic administration of proinflammatory cytokines during chemotherapeutic treatment is also associated with changes in the biliary epithelium. Specifically, IL-2 administration to humans causes acalculous cholecystitis (29, 32, 132). Finally, the administration of TGF- $\beta$  to cultured rabbit (*Oryctolagus cuniculi*) gallbladder epithelium induces a mesenchymal, fibrotic

phenotype (114). This *in vitro* phenomenon appears to have an *in vivo* correlate because *TGF- $\beta$*  expression is associated with fibrosis and inflammation during human cholesterol gallstone formation (80).

Highlighting the key immunogenic role of the biliary epithelium are disease states related to immune-mediated destruction. Specifically, primary biliary cirrhosis, primary sclerosing cholangitis and graft versus host disease are all characterized by immune mediated destruction of biliary epithelium (2, 6, 30, 62, 82). Clearly, the biliary epithelium participates actively in both cellular and humoral immunity through antigen presentation, cytokine and chemokine production, and immunoglobulin transport. Moreover, biliary epithelial cells possess all of the necessary cellular components to actively partake in the innate immune system and perturbations of innate immunity appear to predispose to biliary infection (27, 54, 56, 142).

### **The role of immunity, inflammation and infection in cholesterol gallstone pathogenesis**

#### *Immunoglobulins:*

The putative role of biliary immunoglobulins in promoting cholesterol gallstone formation through cholesterol nucleation has been studied extensively. Studies employing model bile support the tenet that biliary immunoglobulins, and in particular IgM and IgG promote the nucleation of supersaturated bile (60, 61). Interestingly, the source of the immunoglobulins appears to influence greatly the ability of these proteins to nucleate bile. IgM and IgA from commercial sources are incapable of nucleating supersaturated

bile, conversely commercially available IgG is pronucleating (61, 164). The anatomic source of immunoglobulin also appears to influence the ability of these molecules to cause cholesterol crystal phase separation. Specifically, biliary immunoglobulins seem to promote nucleation more than immunoglobulins isolated from blood (164). Of the biliary immunoglobulins, IgM demonstrates the greatest ability to induce nucleation, with IgG displaying slightly less ability, and IgA little activity (60, 61, 164). Differences between commercially available IgM and IgM from the sera of patients with Waldenstrom's macroglobulinemia, implies that specific antibodies rather than IgM antibodies *per se* may nucleate cholesterol in bile (164).

Prospective studies utilizing animal models generally do not support a role of immunoglobulins in cholesterol gallstone pathogenesis. In inbred AKR or C57L mice fed a lithogenic diet there is initially a decrease in the amount of IgM and IgA followed by a temporal increase in these immunoglobulins (167). In both strains, the level of IgG remains relatively unchanged. Van Erpecum and colleagues concluded that immunoglobulins are not likely to participate in cholesterol gallstone formation because both gallstone resistant (AKR) and susceptible (C57L) mice alter their immunoglobulin levels with lithogenic diet feeding. Furthermore, gallstone resistant AKR mice actually demonstrate greater biliary immunoglobulin concentrations than do gallstone susceptible C57L mice (167). Although these changes appear to negate the role immunoglobulins play in cholesterol gallstone formation, the data interpretation are made more complicated by pronounced differences in CSI values between C57L mice (~2.0) and AKR mice (~1.5) (127). Therefore, the variable contributions of biliary lipids most likely

confounded these results. However, human studies also suggest that immunoglobulins do not contribute to cholesterol gallstone formation because *ex vivo* bile from humans with gallstones and without failed to demonstrate a correlation between biliary immunoglobulins and cholesterol detection time (111).

A limitation of all of the above studies is that both *in vitro* and *in vivo* studies have focused primarily on the effect of changing immunoglobulin concentrations on solid cholesterol monohydrate nucleation. Unfortunately, although concentration may be important, it is unlikely to be the only factor relevant to this interaction. Importantly, the antigen specificity of the immunoglobulins may be crucial. Indeed, Fab fragments of antibodies isolated from patients with cholesterol gallstones decreased cholesterol crystal observation time *in vitro* whereas commercially available Fab fragments did not produce this effect (99). These data strongly suggest that immunoglobulins may interact with cholesterol in bile in an antigen specific manner and it is these specific Fab fragments that contribute to cholesterol nucleation *in vitro* (99). Therefore, merely quantifying immunoglobulin concentrations in bile appears to provide very limited functional data.

Recent evidence from our laboratories suggest that in a mouse model of cholesterol gallstone formation, mice possessing only T-cells (and therefore lacking B-cells and immunoglobulins) develop gallstones at a greater frequency than do wild-type mice (Chapter 5). In contrast, mice possessing only B-cells do not recapitulate the prevalence of gallstones observed in wild-type mice. However, these mice do form mucin gel at increased levels compared to Rag mice (lacking T and B-cells). These data indicate that

immunoglobulins contribute to *in vivo* nucleation but are not necessary for cholesterol gallstone pathogenesis (Chapter 5).

Based upon the literature a reasonable hypothesis may be proposed that antigen-specific immunoglobulins contribute to the formation of cholesterol monohydrate crystals in a pronucleating biliary environment. The relative production of these specific antibodies probably relates to specific antigens to which the host has been exposed. However, as demonstrated in B-cell deficient mice, immunoglobulins are not necessary for cholesterol gallstone formation to occur.

*The role of mucin glycoproteins:*

The most thoroughly analyzed biliary proteins putatively involved in cholesterol gallstone pathogenesis are the mucin glycoproteins. Mucin proteins are expressed in a variety of tissues. The ones expressed in human biliary epithelia includes MUC1, MUC2, MUC3, MUC5AC, MUC5B and MUC6 (7). However, this list is likely to increase as more mucin genes are characterized and analyzed. These genes encode proteins which can either be expressed on the surface of epithelial cells or secreted by epithelial cells (7, 172). Furthermore, secreted mucins are often divided into gel-forming mucins and soluble mucins based upon their ability or inability to produce large self-aggregated protein lattices or matrices (7, 172). With regard to cholesterol gallstone pathogenesis these designations appear inconsequential since cell surface expressed mucins are critical in mucin gel accumulation and the synthesis and secretion of so-called soluble mucins are up-regulated during cholelithogenesis implying a role for soluble mucins in cholesterol

gallstone formation (175, 176). In both humans and animal models of cholesterol gallstone formation, mucin gel accumulation appears important in cholesterol gallstone formation (87, 89, 93, 95, 175, 176). Moreover, these proteins promote nucleation *in vitro* (4, 89, 98, 156, 182). Taken together both *in vitro* and *in vivo* data support the notion that mucin gel accumulation precedes and most likely promotes cholesterol gallstone formation by nucleating cholesterol crystals from supersaturated bile.

In cultured biliary cell systems, many of these mucin genes are regulated by inflammatory mediators. Specifically, in two different cell-culture systems, both mucin gel accumulation and mucin gene expression are promoted by exogenous addition of LPS, and TNF- $\alpha$ . In human biliary cell cultures, expression of *MUC2*, *MUC3* and *MUC5AC* are all increased by both LPS and TNF- $\alpha$  (186). In the case of *MUC2* and *MUC5AC* regulation, upregulation appears to be due, at least in part, to overlapping signaling pathways, whereas with *MUC3* this regulation appears to be through two independent pathways (186). Utilizing the same cell-culture system, the authors further demonstrated that TNF- $\alpha$  stimulated protein kinase C (PKC) and PKC appeared to serve as the messenger responsible for *MUC2* and *MUC5AC* production (186).

Choi and colleagues (28) obtained similar results utilizing cultured canine gallbladder epithelial cells. The addition of LPS from any of three bacteria stimulated mucin production in a cultured cholecystocyte system from the dog. There appeared to be differences in the ability of LPS from different bacterial species to induce mucin production. For example, LPS from *Escherichia coli* produces the greatest stimulation

whereas LPS from *Pseudomonas aeruginosa* stimulates mucin production the least. Furthermore, TNF- $\alpha$  added to the culture media promoted mucin production, albeit to a lesser degree than LPS. A diminished response to TNF- $\alpha$  could be due to the use of TNF- $\alpha$  of human origin rather than canine origin. The authors noted that these changes in mucin accumulation were not due to cytotoxicity of either LPS or TNF- $\alpha$  since cell death assays demonstrated survival of the cultured cells (28). The interaction of TLR4 and CD14 receptors present on biliary epithelium was not examined in either of these studies. It is reasonable to assume that these receptors participate in the activity of LPS on mucin expression since both CD14 and TLR4 are expressed on biliary epithelium, and the two receptors interact in sensing LPS (19, 34, 35).

Further evidence of the role of the immune system in the regulation of mucin genes comes from *in vivo* studies with T and B-cell deficient (Rag) mice. In Rag mice *Muc1*, *Muc3*, *Muc4*, and *Muc5ac* are all differentially regulated. These genes are generally down-regulated when compared to immunocompetent mice such as wild-type mice or mice adoptively transferred with splenocytes or T-cells (Chapter 5). Furthermore, infection with enterohepatic *Helicobacter* spp. also alters the mucin gene expression pattern in these mice (Chapter 5).

It is becoming clear from these *in vitro* and *in vivo* studies that the expression of mucin genes and production and secretion of mucin glycoproteins are influenced markedly by inflammatory mediators. TNF- $\alpha$  and LPS are likely to be only the “tip of the iceberg” when it comes to inflammatory mediators stimulating mucin gene production in the

gallbladder. If one can cautiously extrapolate from the well characterized respiratory epithelium system a veritable “laundry-list” of cytokines, bacteria, bacterial products and other irritants are known to alter the production of different mucins by upregulating a variety of respiratory mucin genes (133, 144, 161). Perhaps a more important question in this regard is not whether or not inflammatory mediators alter mucin production, but rather under what physiological situations do these inflammatory mediators reach adequate biliary concentrations to exert their effects?

*Other putative pronucleating biliary proteins:*

Other proteins have been demonstrated to nucleate cholesterol monohydrate crystals *in vitro*; however, like the biliary immunoglobulins, their effects *in vivo* are incompletely (or never) characterized with physiological effector levels. Some of these putative pronucleating agents include haptoglobin, phospholipase C, alpha1-acid glycoprotein, albumin and aminopeptidase N (100, 111, 125). In general, *in vivo* studies that analyzed the role of putative pronucleating proteins were hampered by a number of factors: 1) Only recently has large scale proteomic analysis been undertaken which systematically analyzed the composition of the protein fraction of gallbladder bile. (187) Therefore only a handful of proteins have been identified and analyzed to date, 2) Prior methods used to determine quantitative differences of these proteins in bile and gallbladder tissue are relatively insensitive and may be inadequate to demonstrate significant concentration differences, 3) The concentration of individual constituents may not be crucial but rather a cumulative or cooperative effect of multiple proteins which promote phase-separation of crystals or liquid crystals in bile thereby promoting cholesterol gallstone formation.

Moreover, there is little understanding of the interactions between these proteins. Large scale, sensitive proteomic screens (i.e. mass spectroscopy based protocols) are becoming easier and more widely used in pathophysiological and translational research. The utilization of these techniques to compare the gallbladder bile of patients with and without cholesterol gallstones will likely prove fruitful in the future in determining what role if any, the myriad of secreted proteins may play in cholesterol gallstone formation. Furthermore, these techniques could potentially identify proteins in bile that are secreted from the sera in large concentrations during the active stages of gallstone formation. Although these serological proteins may not participate in cholelithogenesis they could serve as serological markers to identify those individuals at greater risk for developing cholesterol gallstones.

*Gallbladder inflammation:*

In both animal models and humans cholesterol gallstone formation is preceded by histopathological alteration of the gallbladder wall indicative of inflammation, including edema, an increases in gallbladder wall thickness, the presence of inflammatory cells and remodeling with concomitant increased production of TGF- $\beta$  (12, 67, 80, 102, 115, 139). These histological changes are often accompanied by alterations in gallbladder contractility and modifications in the ability of the gallbladder epithelium to transport a variety of substances (24, 67, 94, 129, 168). Furthermore, lithogenic bile may alter mechanisms associated with cytoprotection in the gallbladder muscle as demonstrated by the ability of the gallbladder muscle to respond to reactive oxygen species (181).

In an extensive histopathological study, it was demonstrated that gallstone susceptible C57L mice progressively increase gallbladder wall thickness when fed a lithogenic diet. In contrast, in gallstone resistant AKR mice there is only a mild increase in gallbladder wall thickness. Both strains accumulate subepithelial inflammatory cells and edema; however, C57L mice do so to a significantly greater degree. These changes coincide with decreases in the expression in the gallbladder of two aquaporin genes *Aqp1*, and *Aqp8* (168). These findings suggest that during gallstone formation the gallbladder undergoes progressive changes which ultimately result in decreased motility, increased edema and alterations in transport function. The overall contributions of these changes to cholesterol gallstone formation cannot be elucidated at present because gallstone resistant AKR and susceptible C57L mice also display marked differences in the degree of bile supersaturation (127). In  $\text{FXR}^{-/-}$  susceptible mice the gallbladder becomes thickened, edematous and infiltrated with granulocytes following lithogenic diet feeding for a week (168). In contrast  $\text{FXR}^{+/+}$  mice are protected from these changes (115). It is therefore intriguing to hypothesize that FXR participates in gallbladder inflammation as a consequence of lithogenic diet feeding. Again, this interpretation must be tempered with the understanding that  $\text{FXR}^{+/+}$  mice failed to supersaturate bile in contrast to their  $\text{FXR}^{-/-}$  counterparts (115). Additionally, potentially cytotoxic hydrophobic bile salts were also significantly elevated in  $\text{FXR}^{-/-}$  mice (115).

We believe that it is likely that both cholesterol and hydrophobic bile salts are responsible for the histopathological changes noted in the murine gallbladder during cholesterol gallstone formation. Specifically, analysis of *ex vivo* gallbladders obtained from patients

with cholesterol gallstones revealed that smooth muscle cells contain excess cholesterol in their plasma membranes. Furthermore, the isolated muscle tissues displayed less contractility and decreases in the levels of scavengers of reactive oxygen species (181). These changes appear to be associated with dysfunction in the PGE2 receptor which prevents the cytoprotective downstream effect that occurs when PGE2 binds to its receptor. Furthermore, others have noted a positive correlation of gallbladder muscle dysfunction with elevations in the CSI of bile and the histopathological inflammation score of the gallbladder in humans (165). Additionally, oxysterols, which have been demonstrated by Haigh and Lee (53) to be present in lithogenic bile and cholesterol gallstones from human patients, are capable of inducing apoptosis in cultured gallbladder epithelial cells (53, 152). The mechanism mediating this appears to be through the incorporation of these oxysterols into the mitochondria of epithelial cells subsequently stimulating cytochrome-c mediated apoptosis (152).

All of the above mentioned studies have several limitations. Specifically, the direct role of cholesterol toxicity in gallstone formation cannot be separated from the biological ability of cholesterol to induce inflammation. For example, one cannot examine what occurs when identical CSIs are achieved with and without inflammation. However, one can hypothesize reasonably from the prairie dog model of cholelithogenesis that inflammation *per se* is an essential element in cholesterol gallstone formation (95). Specifically, prairie dogs fed high doses of acetylsalicylic acid (ASA or aspirin) fail to form gallstones whereas non-aspirin fed controls developed gallstones (95). Since both of these groups supersaturate gallbladder bile, it is likely that cholesterol supersaturation is

necessary but not sufficient for cholesterol gallstone formation. That is, the downstream inflammatory effects of cholesterol, and likely other proinflammatory mediators, play an integral role in cholelithogenesis. Further evidence arises in studies utilizing isogenic mice deficient in T and B cells (Rag mice) and comparing them to their wild-type counterparts. These studies indicate that both groups of mice supersaturate bile as evidenced by the phase separation of liquid crystals; however, only mice possessing T-cells develop cholesterol gallstones at a high prevalence (40%). Gallstone formation in this case appears to be due, in part, to a potent Th1 immune response which accompanies the formation of solid cholesterol monohydrate crystals in the murine gallbladder. These data imply that cholesterol promotes inflammation via the adaptive immune system, specifically T-cells (Chapter 5).

Hydrophobic bile acids promote inflammation and apoptosis *in vitro* and likely do so *in vivo* (8, 85, 185). In contrast, treatment with the hydrophilic bile acid, ursodeoxycholic acid decreases mucosal inflammation, and decrease biliary protein concentrations associated with inflammation and gallstones (72). In *Helicobacter* spp. infected C57L mice, the relative concentration of hydrophobic bile salts in hepatic bile is increased and hydrophilic bile salts decreased when compared to uninfected mice (Chapter 4). It would appear that one downstream effect of hydrophobic bile salts is the induction of proinflammatory cytokines (85). Since an increase in hydrophobic bile salts is a consistent finding in cholesterol gallstone patients and animal models, it is likely that bile salt mediated inflammation is important in cholelithogenesis (65, 131)

### *Biliary tract bacterial colonization and gallstones*

Bacteria have been implicated in the pathogenesis of cholesterol gallstones for a very long time; however, a definitive cause and effect relationship remained elusive (110, 137, 151). A major factor contributing to this confusion is the chronic nature of cholecystolithiasis. That is, the formation of cholesterol gallstones requires a rather long time period and when they form, they do not disappear spontaneously except by migration into the duodenum which is invariably followed by reformation. Furthermore, even after cholesterol gallstones form, the gallstones may never lead to symptoms in the patient and, when they do so, it could take years (23, 24, 88). Therefore, identification of bacteria in bile or gallbladder tissue of cholesterol gallstone patients may not indicate causality. Rather, the bile, gallbladder mucosa and biliary motility may be altered to allow for subsequent bacterial colonization. Furthermore, bacteria that might have been present at the time of initial stone formation may be cleared and therefore would not be detected by conventional culturing techniques. Further confusing this issue is the ability of some bacteria to directly alter the composition of bile by beta-glucuronidase, cholylglycyl hydrolase, phospholipase A1 or urease activity thereby promoting calcium bilirubinate formation (16, 22, 170). Despite these deficiencies numerous studies have identified bacteria in bile, cholesterol stones and gallbladder tissue from patients with cholesterol gallstones.

Most studies analyzing the role of bacteria in cholesterol gallstone disease classify stones of approximately 50-95% cholesterol as “mixed” stones and any stone >95% cholesterol as a pure cholesterol stone. The other precipitated material in so-called mixed stones

consist of calcium salts (either calcium bilirubinate or calcium carbonate +/- phosphate) (76). Based upon these delineations it would seem that bacterial DNA is highly prevalent in mixed stones appearing in 80-95% of these stones depending upon the study population (92, 159, 160, 180). The nature of the bacteria in these stones varies depending on the study and gram positive, and gram negative, anaerobic and aerobic bacteria have been identified. Some hypothesize that bacteria, through beta-glucuronidation are responsible for nucleation and calcium salt deposition (169, 170). Although this hypothesis is biologically plausible, no studies to date have conclusively demonstrated that it occurs. In fact, *in vitro* studies utilizing *E. coli* or calcium salts failed to induce cholesterol crystal nucleation (177). So-called pure cholesterol gallstones are less consistently associated with bacterial DNA and depending on the study between 0%-90% of stones have been reported to contain DNA (76, 92, 159, 160, 180). Some authors claim that pure cholesterol gallstones possess only gram positive bacteria while others identified a variety of bacteria (76, 180). In at least one instance, the presence of bacteria in the bile of cholesterol gallstone patients was associated with the conversion of cholesterol into oxysterols suggesting that these organisms contribute to some aspects of cholesterol gallstone formation such as inflammation (184).

Recently, there has been a great deal of interest in a putative role of the gastric pathogen *H. pylori* in relation to cholesterol gallstones (1, 40, 106, 112, 118, 119, 121, 155). Interestingly, growth of this organism is inhibited by bile salts both *in vivo* and *in vitro* and chemotactic assays demonstrate that bile salts repel the organism (103, 106, 179). These factors would appear to argue against the ability of this organism to colonize a

healthy gallbladder. Despite this, a number of groups have claimed to have reliably identified *H. pylori* DNA in biliary tissue and gallstones (40, 75, 112, 155). Several problems exist with the data however. First, some samples were collected at endoscopic retrograde cholangiopancreatography (ERCP) (155). Since *H. pylori* colonizes the gastric mucosa of over half the world's population, the statistical chances for sample contamination by gastric *H. pylori* is high. Additionally, the use of 16SrDNA genus-specific primers is commonly used to identify these organisms. Many of these primer sets amplify other non-*H. pylori* helicobacters (105, 106). Furthermore, sequence evaluation of these primer products is generally inadequate to properly speciate *H. pylori*. This is due to high genetic homology with non-*H. pylori* *Helicobacter* spp (124). For example, Avenaud and colleagues (11) identified *H. pylori*-like organisms based upon sequencing of the 16SrRNA gene in human livers; however, rigorous follow up tests proved that these organisms were an unclassified *Helicobacter* sp. (11). The development of "species-specific" primers to other regions of the genome may alleviate some of these concerns. Nonetheless species specific primers are only as good as the number of species that they are proven not to amplify (i.e. having the potential to amplify organisms that they were not validated against). Finally, like the other organisms discussed above, the possibility exists that these organisms are secondary invaders following alterations in the biliary microenvironment and biliary motility secondary to *in situ* cholesterol gallstones. Evidence for this exists in a recent description which demonstrated that *H. pylori* DNA was present in patients with chronic cholecystitis consistent with bouts of cholestasis, but not in asymptomatic patients (40).

In addition to the gastric pathogen *H. pylori*, a variety of enterohepatic helicobacters exist (46, 157). These reside in the small and large intestines and canalicular space of experimentally and naturally infected animals including humans (46, 157). DNA from these organisms were noted in non-Caucasian patients with chronic cholecystitis, gallstones and malignant biliary tract diseases (47, 104). Our work with experimental animal models indicate that these organisms are capable of promoting cholesterol gallstones *in vivo* (105). In contrast, in this same experimental model *H. pylori* could not promote cholesterol gallstone formation (106). Interestingly, a recent study demonstrated that urease positive *Helicobacter* spp. are capable of precipitating calcium salts *in vitro* (16). The authors proposed that this activity may contribute, at least in part, to the ability of these organisms to promote a calcium salt nidus for cholesterol gallstone formation *in vivo* since to date mono-infection with urease-positive organisms or coinfection with at least one urease positive organism induces cholesterol gallstones (16).

For many of the studies described for both *Helicobacter* spp. and other bacteria, PCR was utilized to identify the bacteria. PCR provides a means to detect relatively small quantities of bacterial DNA which serves as a surrogate marker for colonization with organisms that otherwise may not be identified with standard culture methods. Unfortunately, PCR also makes data interpretation difficult. As mentioned previously, just because bacterial DNA is present at the time of examination does not mean that it was a contributing factor to the formation of the stone. Theoretically, analyzing the “core” of stones may provide more reliable data on the inciting cause; however, due to the sensitive nature of PCR the mere act of trying to isolate the core may contaminate it

with nucleic acid from the external stone surface or from the examiner. Additionally, to date, no studies have analyzed the ability of cholesterol gallstones to imbibe DNA. If stones are capable of imbibing DNA then regardless of the analysis (be it core or external) the DNA present may only represent the most recent DNA encountered. Furthermore, when multiple DNA fragments are present and non-specific primers are used (designed to amplify 16SrDNA genes from multiple bacteria) there is the possibility that the DNA present at the highest concentration may be preferentially amplified (21). Since PCR amplification is non-linear, even a small bias in initial amplification may provide logarithmic differences in final DNA concentration. Unfortunately, the most prevalent bacteria may not be the most important bacteria or may not be important at all. The use of more discriminatory PCR techniques such as those utilized to analyze complex environmental samples may aid in circumventing this problem in the future (31, 78, 158, 188).

One of the classical bacterial pathogens of the biliary tree in humans is *Salmonella enterica* ser Typhi, the causative agent of typhoid fever (33, 84). This organism crosses the intestinal epithelial barrier and invades macrophages and spreads systemically (135). After colonizing the liver, the organism can be shed into the gallbladder and either cause acute cholecystitis or chronically colonize the gallbladder (3-5% of those infected) (33, 84). It is well known that chronic colonization with *S. typhi* is associated with gallstones (33, 84). It is less clear whether this organism contributes to stones or alternatively if the presence of gallstones promotes chronic colonization. Recent evidence indicates that *Salmonella* forms bacterial biofilms on the surface of cholesterol gallstones (135). This

biofilm formation would allow for chronic colonization and protect the organism during antibiotic treatment. Biofilm formation is dependent on the presence of bile and organisms cultured without bile, do not readily form biofilms (135). This indicates that this organism is exquisitely adapted to survival in a host gallbladder harboring cholesterol gallstones. Furthermore, biofilm formation is altered when pebbles or glass beads were utilized instead of cholesterol gallstones. This indicates that both bile and cholesterol gallstones are essential for pathogenesis of biofilm formation. Moreover, colonization in the presence of cholesterol gallstones requires expression of several genetic components (134, 135). These data seem to argue that cholesterol gallstones promote chronic colonization with *S. typhi* rather than *S. typhi* promoting gallstones. Regardless of whether gallstones promote colonization or vice-versa, the concomitant presence of gallstones and *S. typhi* markedly promote gallbladder cancer making the interaction between *S. typhi* and cholesterol gallstones of extremely important interest (33, 135).

In summary, a causative role of bacteria in cholesterol gallstone formation in humans is still unclear. It is likely that some bacteria identified in humans with cholesterol gallstones are at least partially responsible for those gallstones, due to their ability to promote inflammation and precipitate calcium salts. Unfortunately, most of the aforementioned studies are primarily descriptive and fail to thoroughly analyze the organisms identified. For example, it would provide considerable insight if it could be determined whether any of the organisms identified possess virulence factors that would allow for their invasion or colonization of the non-diseased biliary tree. Additional information could be garnered by culturing these organisms and utilizing them to

prospectively infect mouse models useful in studying cholesterol gallstone pathogenesis. The authors of the current review have described such a system utilizing the C57L/J mouse model (105, 106). When this mouse is acquired directly from The Jackson laboratory (Bar Harbor, ME) and housed in microisolater cages under SPF conditions and free of enzootic *Helicobacter* spp. they rarely (10%) develop cholesterol gallstones. Purposeful infection with some enterohepatic strains of *Helicobacter* spp. significantly increases gallstone prevalence (to a high of 80%) (105, 106). It will prove valuable to test other putative lithogenic organisms utilizing this model system approach, bearing in mind the caveat that for the assay to be effective the organisms must be capable of colonizing the mouse strain under consideration.

*Chronic inflammatory conditions and gallstones:*

Chronic hepatitis C virus (HCV) infection is associated with gallstone formation (17, 26, 36, 153). The mechanism responsible for this effect may be due to chronic hepatic damage leading to a loss of liver function and cirrhosis (36, 153). However, recently studies demonstrated that HCV infection, independent of liver cirrhosis is associated with increased gallstone prevalence (17, 26). HCV is known to replicate in the gallbladder epithelium (101, 123) and damage of the biliary tree is noted in 22-91% of biopsy specimens from patients with HCV (26). It is therefore possible that the association between non-cirrhotic HCV patients and gallstones is dependent on HCV replication in biliary epithelium leading to local inflammation, alterations in gallbladder contraction and inflammatory-mediated alterations in mucin gene production. Interestingly, one such study notes that the correlation of HCV and gallstones peaks at two-points. The first peak

occurs in patients at 31-40 years old (7% stone prevalence) and the second such peak occurs much later at 61-70 years of age (16% stone prevalence) (26). Perhaps the first prevalence peak represents the effect of HCV infection *per se* whereas the second peak represents the effect of liver dysfunction and cirrhosis. Like most of the bacteria studied, the role of HCV in cholesterol gallstone disease relied on studies examining the presence or absence of gallstones when HCV infection is present. Future studies utilizing modern biochemical and molecular techniques which focus on changes in the gallbladder itself may provide greater insight into the mechanistic relationship of HCV infection and cholesterol gallstones. Further, transgenic mouse models are currently available which express the HCV genome (52, 79, 173) suggesting that these mouse models could prove useful in a cause and effect study of HCV and cholesterol lithogenicity.

Patients with Crohn's disease (CD) are at an increased risk for the development of gallstones (15, 48, 83, 178). Results vary regarding which factors correlate with cholesterol gallstone formation. Factors mentioned include age, female sex, site of disease, surgical resection, and duration of disease (independent of age) (48, 69, 83). Some investigators noted that CD patients with ileal disease or colonic resection supersaturate their bile, likely due to alterations in cholesterol and bile acid absorption from the intestinal tract (49, 128). However, several studies describe a decrease in the cholesterol saturation in the bile of patients with CD especially when the ileum was involved (90, 91). Furthermore, bile acid composition is altered in CD patients and these alterations are often characterized by a decrease in deoxycholic acid and an increase in ursodeoxycholic acid (90, 91, 128). These changes seem paradoxical as ursodeoxycholic

acid enrichment would seem to favor cholesterol gallstone prevention and dissolution (66, 70, 146). Unlike CD patients, patients with UC are not at an increased risk for cholesterol gallstones (15, 83). A hypothesis for the differences in the abilities of UC and CD to influence gallstone formation is that these diseases cause different inflammatory changes (13, 63, 64). Specifically, although not absolute, CD is most commonly associated with a Th1 proinflammatory response whereas UC is more often associated with a Th2 response (13, 63, 64). Currently, no studies are available which describe the gallbladder inflammatory profile in patients with either UC or CD. These studies would provide insight into the relationship of CD, Th1 inflammation and gallstones.

*Inflammation, cholesterol metabolism and Gallstones:*

Inflammation, and more specifically the acute phase response, a key component of innate immunity, produce marked alterations in the metabolism of a variety of proteins and lipids (77). These changes are extensive and beyond the scope of the current paper; however, a leading review was recently published (77). Most of the changes associated with the acute phase response focus on the role of infection on concentrations of cholesterol, and other lipid constituents of plasma. These changes are important due to their potential role in atherogenesis. The section will focus only on those changes which alter cholesterol and bile acid metabolism of the liver as these changes may directly alter the composition of bile. A caveat with respect to the studies that will be described is that most were conducted employing exogenous, acute administration of cytokines or LPS (42, 57, 58, 77, 109). These changes cause rapid (minutes to hours), relatively short-lived (24 hours or less) alterations in hepatic gene transcription and translation. Although these

changes are significant, it remains to be determined if they exist for prolonged periods (days to weeks) (77). For example, it is unclear if with chronic cytokine stimulation, gene transcription levels are down-regulated by compensatory mechanisms. In the case of cholesterol gallstone formation this could prove to be very important as short-lived (hours-days) alterations in bile composition are certainly less likely to promote disease compared to prolonged and sustained alterations.

With this proviso in mind, it is clear from these studies that acute inflammation does alter the hepatic metabolism of either cholesterol or bile salts. In rodents, administration of LPS or proinflammatory cytokines (IL-1, TNF) raises serum cholesterol levels and increases the production of HMG-CoA reductase at both the transcription and translational levels (41, 42, 58). This change increases *de novo* cholesterol synthesis. However, the increase in sterol production is rather modest because other enzymes in cholesterol biosynthesis including squalene synthase, and enzymes downstream of HMG-CoA reductase in the mevalonate pathway are down-regulated (41, 42, 109, 183). Some investigators hypothesize that these changes maintain adequate cholesterol synthesis while redirecting mevalonate metabolites into non-sterol pathways (77). This hypothesis is supported by increases in dolichol phosphate and glycosylated plasma proteins during the acute phase response (113, 148). In contrast to rodents, primates decrease serum cholesterol levels in response to a variety of proinflammatory mediators (9, 37, 147). Unfortunately, the mechanism underlying this change is less well understood but may be due to either alterations in cholesterol secretion or ApoB synthesis (38, 150).

Alterations in bile salt production also occur under these conditions. Specifically, LPS downregulates both the classic and alternative pathway of bile salt synthesis by decreasing production of CYP7A1 (classical), and CYP7B1, CYP27A1 (alternative) (43, 108). Additionally, exogenous administration of LPS decreases hepatocellular uptake and secretion of bile salts by downregulating basolateral and apical bile salt transporters (51, 59, 116, 162, 171). Not surprisingly, because bile salts stimulate the biliary secretion of both phospholipids and cholesterol, LPS administration also decreases expression of phospholipid (MDR2) and cholesterol transporters (ABCG5/ABCG8) on the canalicular membranes of the liver (59, 163, 171).

These changes suggest that acute infections would decrease cholesterol secretion into bile due to intrahepatic cholestasis. Because cholesterol gallstone formation requires only a relative excess of cholesterol, these changes may or may not be lithogenic. Additionally, as discussed previously, these studies are of short duration and may not reflect what occurs with chronic immune and inflammatory stimulation. In fact, during chronic (10 weeks) infection with enterohepatic *Helicobacter* spp. both hepatic bile salt secretion rate and bile salt independent bile flows are increased (Chapter 4). Interestingly, infection also alters the bile salt pool making it more hydrophobic (consistent with a proinflammatory response). These findings highlight the differences between acute studies involving exogenous intravenous administration of cytokines and LPS and those studies involving chronic infection. Further, these data highlight the need to conduct rigorous experiments utilizing pathogens which cause both chronic and acute infections which may provide

entirely different data sets compared to studies utilizing exogenous cytokine and LPS administration.

*Lith genes:*

Large genetic screens have identified a variety of “*Lith*” alleles in mice which promote cholelithogenesis. In the vast majority of cases, these alleles span large chromosomal regions and the genes responsible for gallstone promotion have not been identified. A thorough review of these *Lith* alleles and the potential for them to encode inflammatory mediators was recently published (102). Unfortunately, the *Lith* loci discovered to date occur on virtually every mouse chromosome and span relatively large regions encompassing numerous genes (174). It is therefore highly probable that inflammatory genes are located in many if not most of these *Lith* loci. Unfortunately, the same could be said for genes involved in a variety of other pathways. Therefore, it is our opinion that a more plausible and productive approach to study the role of inflammation and gallstones would be to utilize currently available transgenic and knockout mouse lines with modulations in genes important in inflammation and immune responses and compare these to their wild-type counterparts. Despite the current limitations with *Lith* genetic loci, with continued inbreeding and creation of well characterized congenic mouse strains, these genetic loci will become more refined and some may eventually prove viable in analyzing the influence of inflammation and immunity on cholesterol gallstone formation.

## **Conclusions:**

The gallbladder despite its overtly simple functionality is a complex organ. Like most organs as it becomes inflamed and damaged, it loses its ability to function properly including its concentrating, pH regulation and contractile functions. Unlike most other organs in the body, the gallbladder may be exposed to rather large concentrations of free (unesterified) cholesterol and potentially cytotoxic bile salts. These molecules appear to induce potent inflammatory responses under the appropriate circumstances (8, 85, 168, 185). It would appear that this inflammatory damage, through a variety of undetermined mediators, induces gallbladder hypomotility and promotes the production of pronucleating agents (28, 168, 186). Furthermore, chemotherapeutic intervention can prevent the downstream effects of cholesterol (95). These downstream effects appear to be due, at least in part, to T-cells and a Th1 immune response (Chapter 5). These findings raise the possibility that serological markers of cholesterol gallstone formation may be inflammatory mediators or cellular subsets thereof. If identified, these markers may prove valuable for diagnosis and interventional therapy. Specifically, prevention of prolonged Th1 stimulation may prove useful in the prevention or treatment of cholesterol gallstones either alone or in conjunction with other lipid composition altering therapies. It is important to note that many of the studies described within this review have relied principally upon inbred mice. These mice are genetically homozygous with >99% of their genomes being identical. In contrast, even the most consanguineous of human populations is relatively heterogeneous. The role of inflammation in human cholesterol gallstone disease is therefore likely to be subtle and additive with a variety of contributing and overlapping factors. Additionally, inflammation and immunity may

contribute to cholesterol gallstone formation both environmentally (exposure to pathogens and proinflammatory conditions) and genetically (polymorphisms in genes which promote a greater response to immune stimuli). Clearly, much remains to be learned in this regard and further *in vitro*, animal models and human studies are likely to clarify many of these exciting possibilities.

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**Chapter 2**  
**Identification of cholelithogenic enterohepatic *Helicobacter* species and their role in murine cholesterol gallstone formation.**

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## Abstract

*Helicobacter* spp. are common inhabitants of the hepatobiliary and gastrointestinal tracts of humans and animals and cause a variety of well described diseases. Recent epidemiological results suggest a possible association between enterohepatic *Helicobacter* spp. and cholesterol cholelithiasis, chronic cholecystitis and gallbladder cancer. To test this, we prospectively investigated the effects of *Helicobacter* spp. infection in cholesterol gallstone pathogenesis in the highly susceptible C57L/J mouse model. *Helicobacter* spp.-free adult male C57L mice were infected with several different enterohepatic *Helicobacter* spp. or left uninfected and fed either a lithogenic diet or standard mouse chow for 8 and 18 weeks. At the conclusion of the study, bile was examined microscopically and diagnostic culture and PCR were performed. Mice infected with *H. bilis* or coinfecting with *H. hepaticus* and *H. rodentium* and fed a lithogenic diet developed cholesterol gallstones at 80% prevalence by 8 weeks compared with approximately 10% in uninfected controls. Mono-infections with *H. hepaticus*, *H. cinaedi* and *H. rodentium* gave a cholesterol gallstone prevalence of 40%, 30% and 20%, respectively; the latter two groups did not differ significantly from uninfected animals. Animals fed a chow diet did not develop cholesterol gallstones. Colonization of the gastrointestinal tract with cholelithogenic *Helicobacter* spp. promotes cholesterol gallstone formation; however, this ability is not correlated with the presence of *Helicobacter* spp. in the liver. These findings, along with prior epidemiological studies, suggest that *Helicobacter* spp. play a role in the pathophysiology of cholesterol gallstone formation in mice and perhaps humans.

## Introduction

Cholesterol gallstones, composed predominantly of cholesterol monohydrate crystals within a mucin glycoprotein scaffolding, form in the gallbladder following nucleation and phase separation of cholesterol monohydrate crystals from cholesterol supersaturated bile (3, 28, 36). A variety of risk factors are documented, including ethnicity, gender, obesity, weight loss, dietary intake and concurrent medical conditions and treatments (36).

Currently, the only definitive treatment for gallstones is surgical removal of the gallbladder (cholecystectomy) and the total annual cost of treatment in the United States alone approaches 10 billion dollars (36). Moreover, an ominous complication of gallstone disease is gallbladder cancer (4, 31).

Studies elucidating the pathogenesis of cholesterol gallstones rely predominantly on inbred mouse models (36). In particular, quantitative trait locus (QTL) analysis utilizing gallstone susceptible and gallstone resistant strains illustrates that cholesterol gallstone disease is principally a polygenic trait involving multiple susceptibility loci (*Lith* alleles) on many chromosomes (25, 36, 37, 54). A strain of particular interest is the C57L/J (C57L) mouse because of its high susceptibility to cholesterol gallstones, which develop in 80-100% of males fed a lithogenic diet for eight weeks (23, 48). Additionally, two congenic strains carrying the *Lith1* or *Lith2* loci from the C57L strain in an AKR/J (AKR) background recapitulate the phenotype of the C57L parent (36).

Recent evidence from our laboratories suggests an infectious contribution to the gallstone phenotype in C57L mice. When congenic AKR mice carrying a susceptibility locus

(*Lith2*) from C57L mice (AK.L-*Lith2*<sup>s</sup>) were re-derived and housed under specific pathogen free (SPF) conditions, they failed to develop gallstones. This was established in two independent but identical pilot studies under feeding conditions with a lithogenic diet containing 1% cholesterol, 0.5% cholic acid and 15% dairy fat, lasting 8 weeks (G. Bouchard, B. Paigen M.C. Carey, unpublished observations, 2003). Because of the ubiquitous nature of *Helicobacter* spp. in conventional mouse colonies (41) and the ability of these organisms to cause hepatobiliary disease (11, 41) we considered them likely candidates to be involved in cholesterol cholelithogenesis.

Since the discovery of *H. pylori*, over 25 *Helicobacter* spp. have been isolated from the stomach, intestinal tract, and liver of humans, other mammals and birds (9, 11, 13, 41). Many of these organisms cause extragastric disease and several are able to grow in bile including *H. hepaticus*, *H. bilis*, and *H. pullorum* (9, 13). These non-gastric (enterohepatic) *Helicobacter* spp. generally colonize the distal small intestine, cecum and large intestine, and subsequently the liver where they have been implicated in, or suggested to cause, hepatitis, hepatocellular carcinoma, cholecystitis, typhlocolitis, and colonic adenocarcinoma (9, 11, 13, 41). We demonstrated previously that there is an association between molecular (DNA) evidence of enterohepatic *Helicobacter* spp. colonization and cholecystitis in Chilean women (13). Interestingly, this population is notable for its high prevalence of gallbladder cancer invariably associated with cholesterol gallstone disease (13, 36). Additionally, there is a growing body of literature identifying helicobacter DNA in the gallbladders, bile and stones of patients with gallstones (2, 6, 33).

Due to the high prevalence of enterohepatic *Helicobacter* spp. in mice (9, 11, 16, 41) and the burgeoning evidence in humans (6, 31, 33, 34), we believed it reasonable to hypothesize that *Helicobacter* spp. contribute to cholesterol gallstone formation. To test this hypothesis, prospective infections were performed in the well-characterized, highly susceptible, diet-induced, C57L mouse model of gallstone disease (23, 25, 36, 37, 48) initially utilizing two enterohepatic *Helicobacter* species, *H. hepaticus* and *H. rodentium* (9, 11, 41). We chose these two species because they were present concurrently in the colonies at Brigham and Women's Hospital (BWH, Boston, MA) where much of the original gallstone research was done. Following these coinfection studies we performed monoinfection studies with these enterohepatic *Helicobacter* spp. as well as *H. bilis* and *H. cinaedi* which have been identified in humans with gastrointestinal and hepatobiliary disease (1, 10, 14, 18, 31, 35, 45).

## **Methods**

### *Animals and diet*

*Helicobacter* spp.-free male C57L mice were acquired from The Jackson Laboratory (Bar Harbor, Maine, USA), and housed under SPF conditions in an Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC) accredited facility. These SPF conditions included specific absence of *Helicobacter* spp., *Salmonella* spp., *Citrobacter rodentium*, and known murine viral pathogens. All animal protocols met the approval of the institutions' animal care and use committees. Mice were fed either standard rodent chow, or beginning at 8 weeks of age, a lithogenic diet containing 15% dairy triglycerides, 1.0% cholesterol, and 0.5% cholic acid. This was

continued for eight weeks (37) for both coinfection and monoinfection studies and for eighteen weeks for chronic lithogenic studies.

### *Infection protocols*

*H. hepaticus* strain 3B1 (ATCC 51499), *H. rodentium* (ATCC 700285), *H. bilis* (MU strain), and *H. cinaedi* (CCUG 18818) were grown on blood agar plates under microaerobic conditions at 37°C. For coinfection studies, four-week-old mice were either infected orally three times over five days with approximately  $1.0 \times 10^8$ - $2.0 \times 10^8$  each of *H. hepaticus* and *H. rodentium* suspended in Brucella broth (Becton, Dickinson and Company, Sparks, MD, USA) or sham-dosed with Brucella broth. For monoinfection studies, mice were infected three times over five days with approximately  $1.5 \times 10^8$ - $3.0 \times 10^8$  of the individual *Helicobacter* spp. tested or sham dosed with Brucella broth. For coinfection studies, mice were subsequently re-dosed with a comparable dose of *H. hepaticus* or sham dosed with broth every month thereafter and for monoinfection studies mice were re-dosed with a comparable dose of the appropriate organism every month thereafter for two months.

### *Direct Light and Polarizing microscopy of gallbladder bile and gallstone analysis*

At 16 weeks of age (or 26 weeks of age for prolonged lithogenic studies), mice were euthanized by CO<sub>2</sub> overdose and cholecystectomies were performed immediately. Gallbladders were weighed, opened, and microscopists (M.C.C. and K.J.M.) blinded as to sample identity examined gallbladder bile by both direct and polarized light microscopy for the presence of liquid crystals, solid crystals, sandy stones, and cholesterol gallstones

as defined elsewhere (48). Bile was scored microscopically for mucin gel content on an 0-5 arbitrary scale (53). Gallstones were analyzed for cholesterol content by HPLC as previously described (23).

#### *Histopathological examinations*

Sections of each liver lobe as well as gallbladder and ileocecolic junctions were harvested at necropsy, fixed overnight in 10% neutral-buffered formalin, processed routinely, cut into 4  $\mu\text{m}$  thicknesses, and stained with hematoxylin and eosin. Liver sections were graded for lobular and portal inflammation according to previously defined criteria (39) by a comparative pathologist (A.B.R.) blinded as to sample source.

Gallbladders were assessed morphologically for inflammation, epithelial disorganization, hyperplasia, dysplasia, and hyalinosis. Special stains included a pH 2.5 Alcian blue/periodic acid-Schiff stain for acidic and neutral mucins.

#### *Helicobacter spp. screening tests*

DNA was prepared from post-mortem tissue using a “High pure PCR template preparation kit” (Roche Applied Science, Penberg, Germany) and was either singly amplified from the cecum with genus-specific (for monoinfection studies) or species-specific primers (for coinfection studies) utilizing previously described conditions (16, 17, 40). DNA was amplified from the liver, gallbladder, or cholesterol gallstones by nested amplification with an initial genus-specific primer set utilizing previously described conditions (17). This was followed by a subsequent amplification of 2.5  $\mu\text{l}$  of this product with internal species-specific primers to *H. hepaticus*, *H. rodentium*, or *H.*

*bilis* or a second genus specific primer set for *H. cinaedi* utilizing previously described reaction conditions (16, 40).

#### *Culture of Helicobacter spp.*

Gallbladder and liver were ground in sterile glass tissue grinders with 0.5-1 ml of brucella broth. Ground tissue was streaked onto blood agar plates and grown under microaerobic conditions at 37°C for three weeks. At that time plates were scrutinized for bacterial growth.

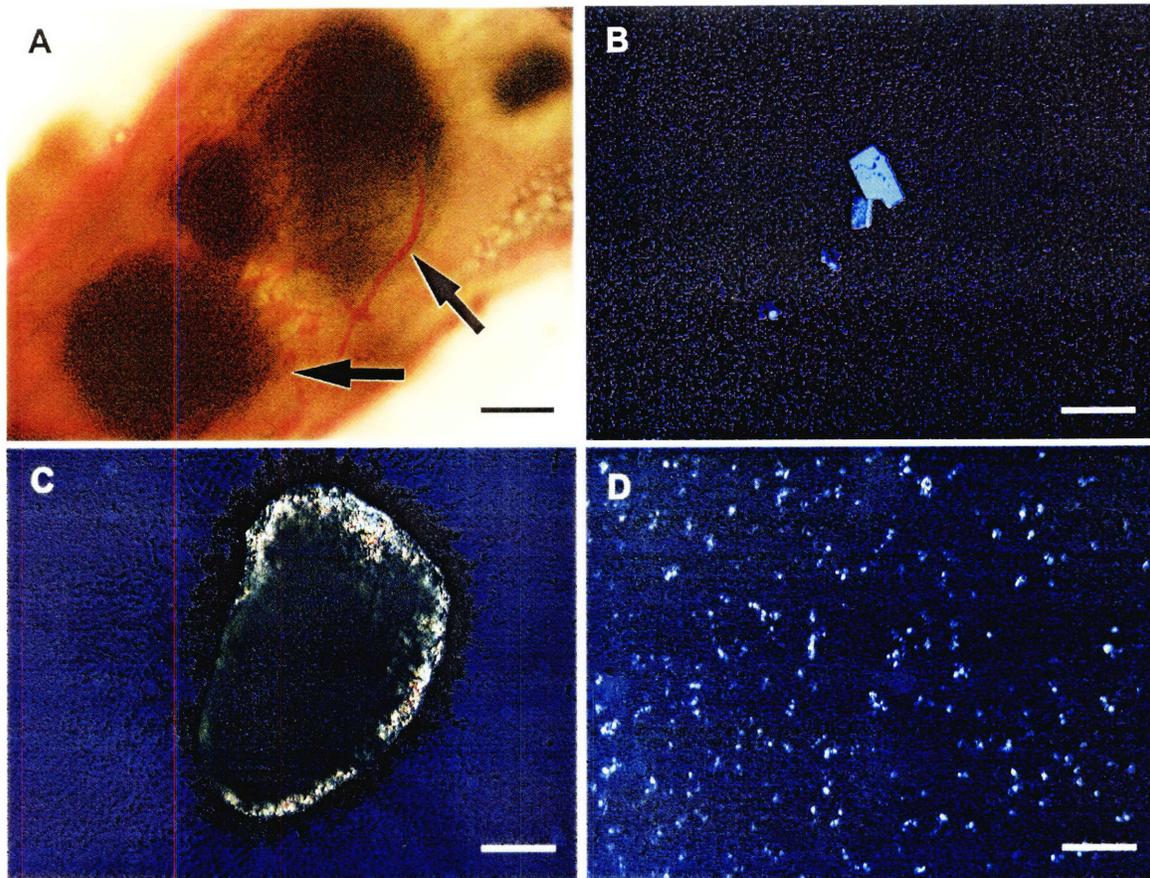
#### *Statistical analysis*

Statistical analyses of mucin score and gallbladder weight were performed by one way analysis of variance (ANOVA), with the Tukey-Kramer post test using InStat 3.0 software (GraphPad, Inc., San Diego, CA, USA). Cholesterol monohydrate crystal as well as sandy and true gallstone formation were analyzed by Fisher's exact test utilizing the same software.

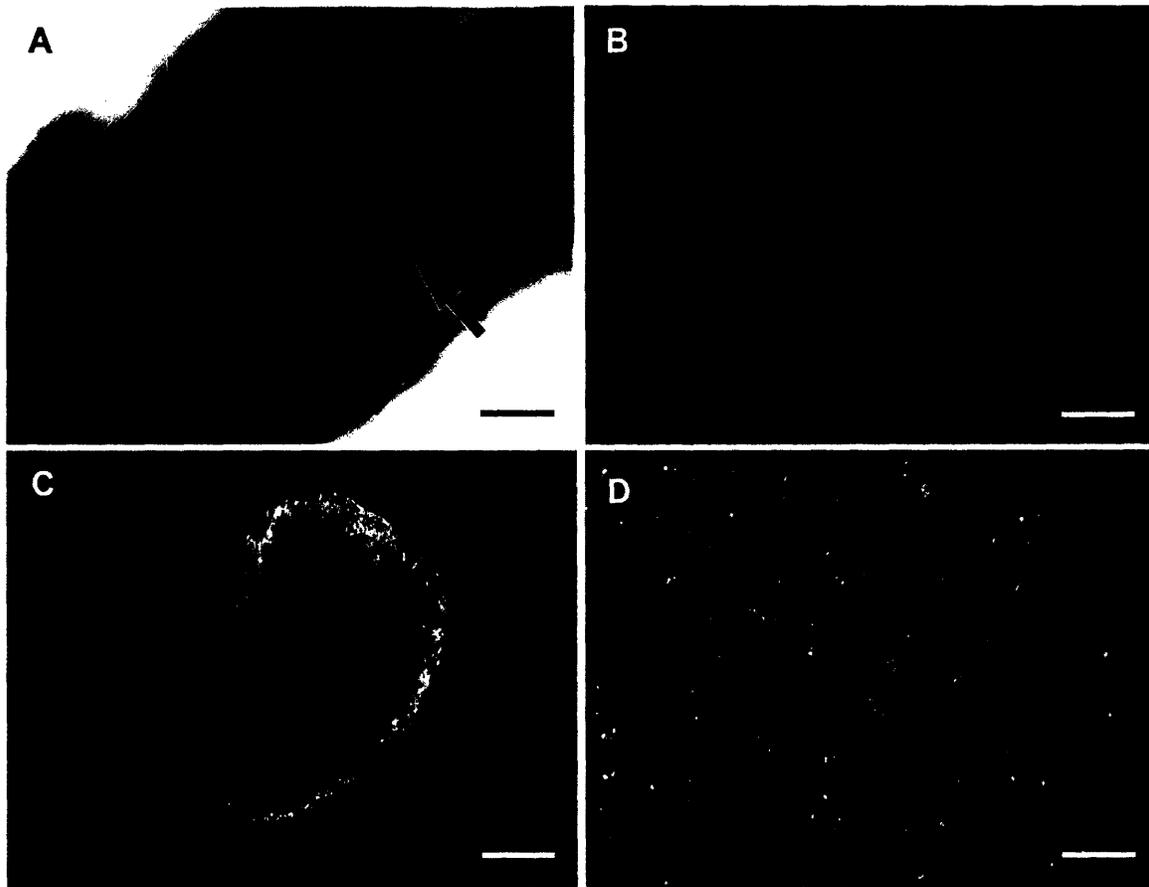
## **Results**

#### *Phenotypic analysis of gallbladder and bile from coinfecting animals*

*Helicobacter* spp. infected mice (all n values = 5-10, see Table 2-1) fed the lithogenic diet developed cholesterol gallstones (48) at a prevalence of 78% and sandy stones at a prevalence of 56%, and all but a single animal progressed beyond the liquid crystalline phase (Table 2-1 and Fig. 2-1). In contrast, uninfected mice and mice that ingested a standard chow diet failed to develop gallstones (Table 2-1 and Fig. 2-1). Interestingly, all



**Figure 2-1:** Morphologic characterization by direct and polarizing light microscopy (48) of gallbladder bile and stones of (A) *Helicobacter* spp. infected C57L mice fed a lithogenic diet for 8 weeks. Macroscopically visible stones (arrows) up to 2 mm diameter within a mouse gallbladder. Progressive stages in development of cholecystolithiasis found in *Helicobacter* spp. infected C57L/J mice fed a lithogenic diet include (B) cholesterol monohydrate crystals (48), sandy stones (48) (not shown) and (C) cholesterol gallstones (48) (these stones have smooth contoured birefringent edges and dark centers due to light scattering and absorption). Birefringent aggregated liquid crystals (48) (D) represent the minimal extent of lithogenesis in uninfected C57L mice fed a lithogenic diet. bar ( $\mu\text{m}$ )= 500 (A); 100 (B); 170 (C); 50 (D).



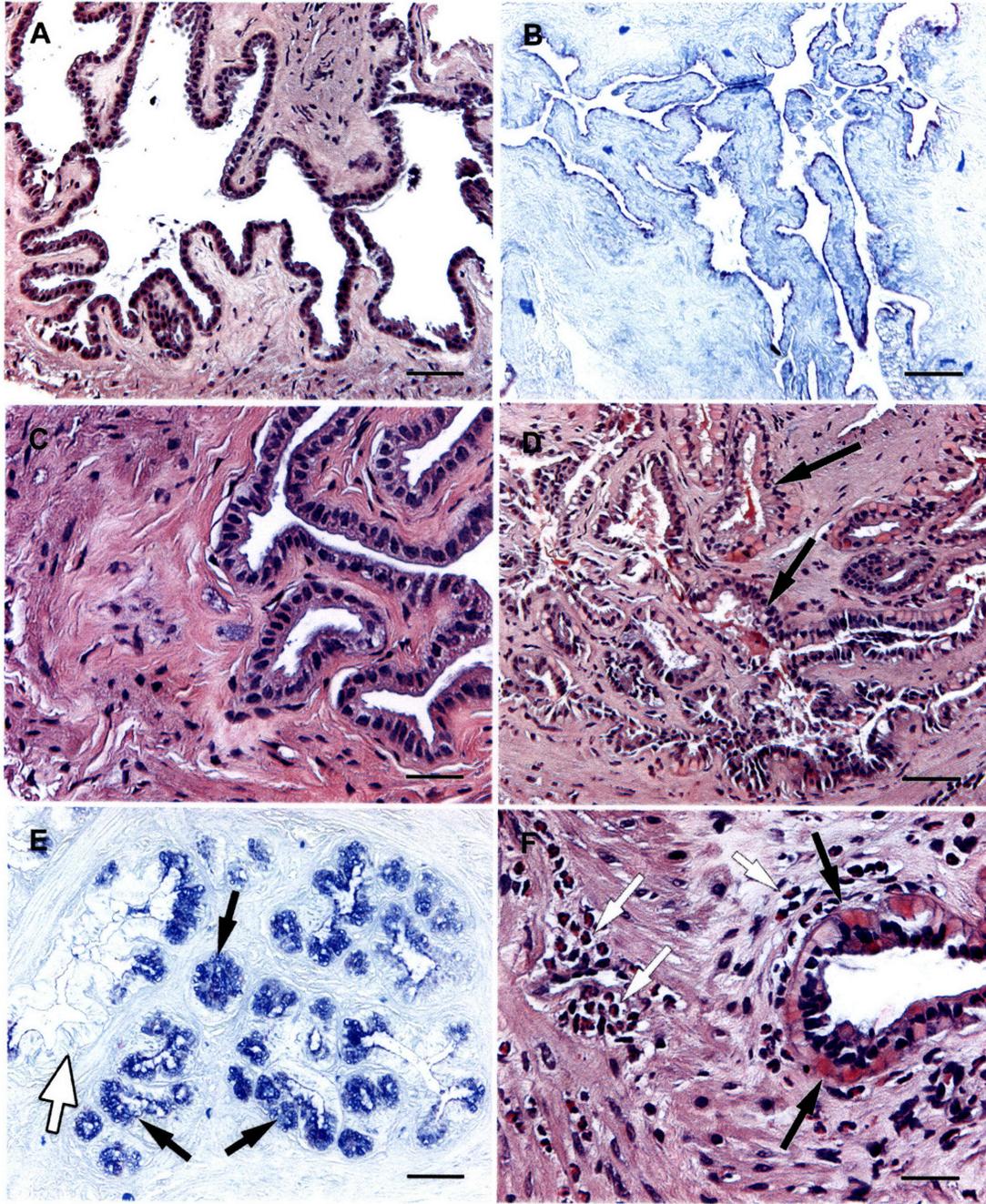
**Figure 2-1:** Morphologic characterization by direct and polarizing light microscopy (48) of gallbladder bile and stones of (A) *Helicobacter* spp. infected C57L mice fed a lithogenic diet for 8 weeks. Macroscopically visible stones (arrows) up to 2 mm diameter within a mouse gallbladder. Progressive stages in development of cholecystolithiasis found in *Helicobacter* spp. infected C57L/J mice fed a lithogenic diet include (B) cholesterol monohydrate crystals (48), sandy stones (48) (not shown) and (C) cholesterol gallstones (48) (these stones have smooth contoured birefringent edges and dark centers due to light scattering and absorption). Birefringent aggregated liquid crystals (48) (D) represent the minimal extent of lithogenesis in uninfected C57L mice fed a lithogenic diet. bar ( $\mu\text{m}$ )= 500 (A); 100 (B); 170 (C); 50 (D).

### *Histopathological analysis of hepatobiliary tissues*

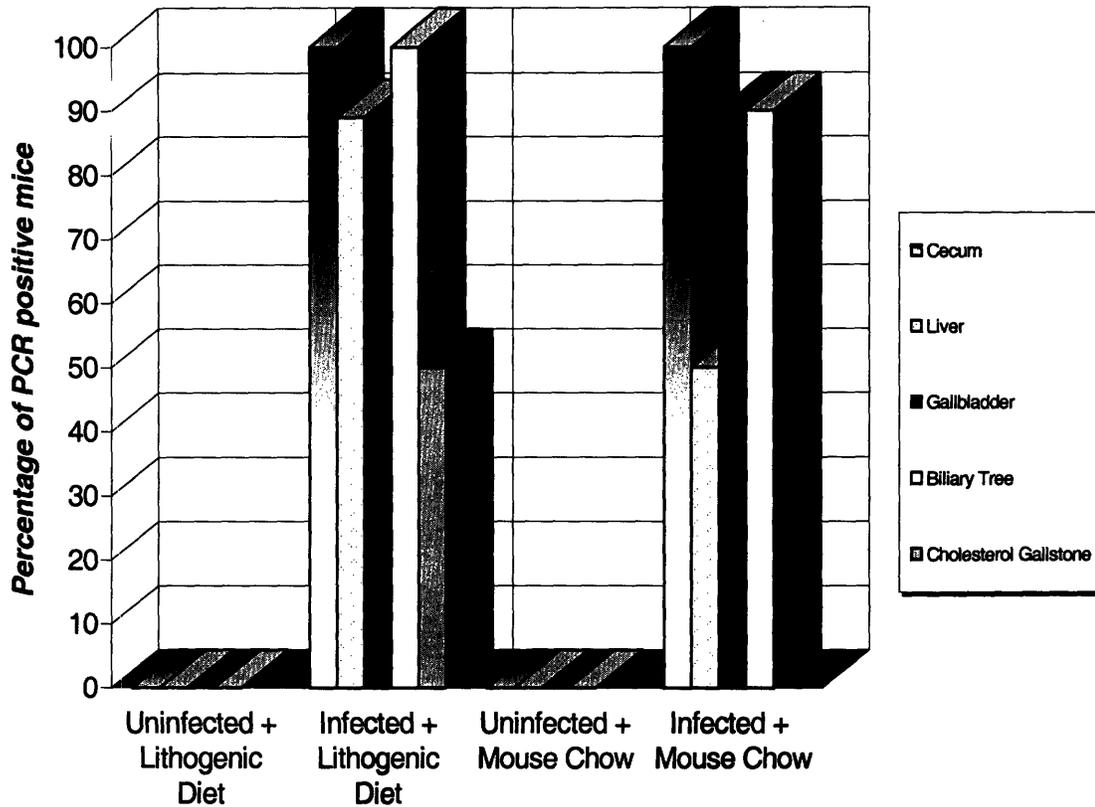
Histological analysis of livers revealed microsteatosis and mild chronic portal inflammation associated with diet but not with *Helicobacter* spp. infection status. A subset of infected and uninfected mice on the lithogenic diet exhibited portal and random lymphohistiocytic infiltrates attributed to hepatobiliary tissue damage caused by steatosis. However, there was no association between hepatic inflammation and risk of gallstones ( $P=1$ ). In contrast, lesions of the gallbladder including hyalinosis, intestinal metaplasia, eosinophilic inflammation, hyperplasia, and dysplastic change were limited to *Helicobacter* spp. infected mice on the lithogenic diet (Fig. 2-2). Interestingly, many of these lesions are seen in humans with gallstones and several of them including dysplasia, hyperplasia, and intestinal metaplasia are considered to be pre-neoplastic (46).

### *Helicobacter* spp. infection status of co-infected mice

Co-infected mice fed either chow or lithogenic diet demonstrated consistent cecal colonization with both *Helicobacter* species when screened by PCR (Fig. 2-3) (16, 40). Molecular (DNA) evidence of enterohepatic *Helicobacter* spp. was present in a region of the hepatobiliary tree (either gallbladder or liver) in most infected animals (Fig. 2-3) (16, 17, 40). No uninfected animals demonstrated molecular evidence of colonization in either the cecum or hepatobiliary tree (Fig. 2-3). PCR analysis of four gallstones from *H. hepaticus* plus *H. rodentium* co-infected mice revealed that helicobacter DNA was present in 50% of stones (Fig. 2-3). None of the gallbladders of *Helicobacter* spp. co-infected animals fed the lithogenic diet (n=5) were culture positive even after prolonged (3 weeks) incubation.



**Figure 2-2:** Panels (A-F): Compared with sections from uninfected mice on a lithogenic diet (A, B, C), histopathologic examination of gallbladder tissue from *Helicobacter* spp. infected mice demonstrate (D; arrows) epithelial disorganization with eosinophilic luminal secretory products, hyperplasia and mucous intestinal-type metaplasia (black arrows; note absence of mucous staining in hyalinized epithelium, white arrow; E), and epithelial hyalinosis (glassy pink-red material, black arrows) and patchy eosinophil-predominant inflammation (white arrows; F). Histologic stains: hematoxylin and eosin (A, C, D, F) and Alcian blue/periodic acid-Schiff pH 2.5 (B, E); bar ( $\mu\text{m}$ )= 170 (A, B, D, E); or 85 (C, F).



**Figure 2-3:** Prevalence of helicobacter DNA in various tissues from coinfecting experimental groups. DNA was prepared from tissue using the “high pure PCR template preparation kit” (Roche Applied Science, Penberg, Germany) and was either singly amplified from the cecum with species specific primers utilizing previously described conditions (17) or amplified from the liver, gallbladder, or gallstones by nested amplification with an initial genus specific primer set utilizing previously described conditions (17). This was followed by a subsequent amplification of 2.5  $\mu$ l of this product with species specific primers to *H. hepaticus* and *H. rodentium* with reaction conditions (16, 40) as previously described. Biliary tree refers to a positive result from either intrahepatic bile ducts or gallbladder.

### *Phenotypic analysis of gallbladder and bile in monoinfected mice*

In mice fed a lithogenic diet for eight weeks, the biliary phenotype varied greatly depending upon the organism the mice were infected with. Specifically, when compared to uninfected mice, monoinfection with *H. rodentium* or *H. cinaedi* did not increase normalized gallbladder weight, mucin score, cholesterol monohydrate crystal formation, sandy stone formation, or cholesterol gallstone formation significantly (Table 2-2). In contrast, monoinfection with *H. bilis* and *H. hepaticus* significantly increased cholesterol monohydrate crystal formation, sandy stone formation and cholesterol gallstone formation when compared with uninfected mice (Table 2-2). Additionally, *H. bilis* infection increased mucin score significantly when compared to uninfected mice (Table 2-2). A sampling of stones (n=2 from infected, and uninfected) was analyzed for cholesterol content and both infected and uninfected were composed primarily of cholesterol (monohydrate) confirming that they were indeed cholesterol stones. This was further corroborated by their creamy white to tan appearance to the unaided eye as well as the agglomeration of cholesterol monohydrate crystals in sandy and true stones by polarizing and direct light microscopy.

Histopathological changes in the gallbladder mucosa observed in coinfecting animals were also noted in some monoinfected animals and were rarely detected in uninfected animals. Almost invariably, these histopathological changes were found concomitantly with sandy stones or true cholesterol gallstones in both infected and uninfected animals.

### *Prolonged lithogenic diet feeding*

To determine if *Helicobacter* spp. were merely altering the rates of cholesterol cholelithogenesis, we fed uninfected mice and *H. hepaticus* infected mice a lithogenic diet for 18 weeks. Interestingly, prolonged exposure to the lithogenic diet did not significantly alter the prevalence of cholesterol gallstone formation in either group when compared to their counterparts fed the lithogenic diet for eight weeks (Table 2-2). When compared to uninfected mice, *H. hepaticus* infected mice displayed a significantly higher mucin score, with a higher prevalence of both cholesterol monohydrate crystals and sandy stones (Table 2-2).

### *Infection status of hepatobiliary and gastrointestinal tissues*

All C57L mice infected with *Helicobacter* spp. were confirmed to be infected by cecal PCR (except for *H. bilis* infected animals that were confirmed to be positive by cecal culture); all uninfected animals tested negative for enterohepatic *Helicobacter* spp. by cecal PCR (Table 2-2). The presence of *Helicobacter* spp. DNA in liver of infected animals was variable and the presence or absence of DNA in liver did not correlate with gallstone formation. None of the *H. hepaticus* (n=10) or *H. rodentium* (n=10) infected mice were culture positive for *Helicobacter* spp. in the liver or gallbladder.

Table 2-2: Biliary phenotype and colonization status of monoinfected and uninfected male C57L mice on a lithogenic diet

Organism	Lith Diet (weeks)	Normalized Gallbladder Weight (mg/g) <sup>a</sup>	Mucin Score	Liquid Crystals	Solid Crystals	Sandy Stones	Cholesterol Gallstones	Cecal PCR	Liver PCR	Hepatobiliary Culture
<i>H. hepaticus</i> (n=20)	8	0.85 (± 0.13)	1.20 (± 0.12)	95%	65% <sup>c</sup>	35% <sup>b</sup>	40% <sup>b</sup>	100%	60%	0% (n=10)
<i>H. rodentium</i> (n=10)	8	0.69 (± 0.15)	1.55 (± 0.31)	100%	40%	30%	30%	100%	80%	0% (n=10)
<i>H. bilis</i> (n=5)	8	0.73 (± 0.35)	2.00 <sup>b</sup> (± 0.35)	80%	100% <sup>c</sup>	100% <sup>c</sup>	80% <sup>c</sup>	100%	60%	ND <sup>d</sup>
<i>H. cinaedi</i> (n=5)	8	0.72 (± 0.08)	1.40 (± 0.19)	100%	20%	20%	20%	100%	80%	ND
Uninfected (n=30)	8	0.59 (± 0.065)	0.93 (± 0.11)	100%	20%	7%	10%	0%	ND	ND
<i>H. hepaticus</i> (n=10)	18	0.90 (± 0.18)	2.45 <sup>b</sup> (± 0.32)	100%	100% <sup>c</sup>	100% <sup>c</sup>	50%	100%	67%	ND
Uninfected (n=10)	18	0.57 (± 0.09)	1.55 (± 0.19)	100%	20%	20%	20%	0%	ND	ND

<sup>a</sup> Numerical data are presented as mean +/- standard error of the mean.

<sup>b</sup>  $P < 0.05$ , and <sup>c</sup>  $P < 0.005$  when compared to controls fed the lithogenic diet for the same time-period.

<sup>d</sup> N.D. = Not determined

## Discussion

This study presents systematic information detailing what appears to be a paradigm shift in our understanding of cholesterol gallstone pathogenesis, at least in this inbred mouse model and perhaps in humans. These data demonstrate rigorously that enterohepatic *Helicobacter* spp., play a notable role in the development of cholesterol gallstones in the murine gallbladder. Indeed, without this infection the C57L mouse, the most frequently used polygenic model of cholesterol gallstone disease, does not acquire gallstones with any appreciable frequency.

The ability to promote cholesterol gallstones in C57L mice is *Helicobacter* species specific. Of the organisms tested, co-infection with *H. hepaticus* and *H. rodentium* and mono-infection with *H. bilis* displayed the greatest impact in promoting cholesterol

gallstone formation. *H. hepaticus* was intermediate in its ability, and *H. rodentium* and *H. cinaedi* did not influence cholesterol cholelithogenesis significantly compared with uninfected controls. We propose the appellation “cholelithogenic” *Helicobacter* spp. to differentiate those species that are capable of promoting cholesterol cholelithogenesis from those that do not. We suspect that with further testing other *Helicobacter* spp. and perhaps other related and unrelated microorganisms may prove capable of promoting cholesterol gallstone formation.

We tested mice at the facilities where most of the original studies were done (BWH) by anaerobic and aerobic fecal culture. Those results were compared with results of cultures from mice in the current study to determine if other pathogens may have contributed to the historical prevalence of gallstones at the prior facility. We found no differences in pathogen status between the two (BWH and MIT) facilities (data not shown). Moreover, the only other mouse pathogen present at both MIT and BWH was *Klebsiella oxytoca*, an organism rarely associated with disease and then only with utero-ovarian infection in mice (7). The possible pathogenic roles that the normal microbiota of the gut plays in the complex process of cholesterol gallstone disease is intriguing but cannot be answered without prospective studies in gnotobiotic and germ-free animals.

In our studies the ability of some but not all *Helicobacter* spp. to promote cholesterol cholelithogenesis implies that promotion of cholesterol gallstone formation is mediated by species-specific bacterial products or a reaction of the host to these products, possibly

through cytokines and other pro-inflammatory mediators. If promotion of cholelithogenesis were due to a non-specific host response to colonization then all *Helicobacter* organisms tested should have promoted gallstone formation equally. In particular, an interesting aspect of this phenomenon was that the bacteria tested did not colonize the biliary tree (at least in the culturable state when tested at sacrifice) and yet cholelithogenesis was promoted. This is evidenced by the lack of correlation between bacterial DNA in the liver and stone formation as well as, our inability to culture organisms even after prolonged incubation and the necessity for nested PCR to identify DNA in the liver and gallbladder. This raises several questions concerning the mechanism behind the relationship between these bacteria and cholesterol gallstone pathogenesis and the manner in which past studies have been conducted in an attempt to detect a possible infectious contribution to pathogenesis of cholesterol gallstone disease. Many of these studies relied upon culture and PCR of the hepatobiliary tree to determine a possible role for *Helicobacter* spp. and other bacteria in cholesterol gallstone formation (6, 13, 19, 29, 31, 32). A number of investigators found no correlation between the presence of *Helicobacter* spp. DNA in these tissues and the presence of cholesterol gallstones (6, 32). However, based upon the results of the present study, it is clear that these organisms do not have to be present in high numbers in the hepatobiliary tree to promote cholelithogenesis. In fact, our study demonstrates that the most appropriate anatomic site to determine whether *Helicobacter* spp. are indeed an epidemiological risk factor in mouse and human cholelithogenesis would be the distal small intestinal tract, cecum and proximal large intestine. Furthermore, because our studies suggest a *Helicobacter* species-specific effect, future investigations should focus on identifying the

organisms at the species level by acceptable methods and not rely merely on the presence or absence of helicobacters as a genus.

Several potential mechanisms or combinations thereof could be suggested to explain the contribution of *Helicobacter* spp. to cholesterol cholelithogenesis. First, the organisms may produce a soluble antigen or antigens that modulate key hepatobiliary genes in the lithogenic pathway. Based upon the present data, an attractive population of genes are the *Muc* alleles of the gallbladder epithelium involved in the production of mucin (28, 36). Alternatively, because enterohepatic *Helicobacter* spp. colonize, among other sites, the distal ileum they may modulate enterohepatic cycling of conjugated bile acids either directly or through genetic regulation of absorption at the enterocyte level or by modulating gastrointestinal transit time. An intestinal role is further suggested by our finding in this murine model of a lack of correlation between colonization of the hepatobiliary tree by *Helicobacter* spp. and cholesterol gallstone formation. In addition to other possible mechanisms, an antigenic product of *Helicobacter* spp. or a host response (e.g. cytokines or other inflammatory mediators) could change the phase equilibria of bile or accelerate the nucleation kinetics of solid cholesterol monohydrate crystals from liquid crystals.

Interestingly, mice infected with both *H. rodentium* and *H. hepaticus* developed cholesterol gallstones at a consistently higher rate (78%) than infection with either of these organisms alone which produced gallstones only at a moderate prevalence (*H.*

*hepaticus*) or rarely (*H. rodentium*). This implies microbiotic synergism in that individually, *H. rodentium* and *H. hepaticus* may not possess all of the necessary cholelithogenic factors to promote gallstone formation but together can do so effectively. Alternatively, colonization by one of these organisms may modulate a host response towards the other organism. In this regard, studies conducted on gastric *Helicobacter* spp. (*H. felis*) infected animals demonstrate that the presence of other pathogens may promote or inhibit gastric disease. (12, 42)

The possibility that enterohepatic *Helicobacter* spp., influence human cholesterol gallstone formation is a provocative concept for several reasons. First and historically, the disease was presumed to be solely a physical-chemical phenomenon occurring at the level of the gallbladder; there then followed the realization that a genetic (polygenic component) was crucial in altering the secretory rates of biliary lipids from the liver and now it appears that a “random event” is required. This supports the concept that a necessary component may also be chronic intestinal infection with an enterohepatic *Helicobacter* spp. It is worth noting that at the beginning of the 20<sup>th</sup> century Lord Berkeley Moynihan, a notable British surgeon avouched that “a gallstone is a tombstone erected to the memory of the organism within it” (38). Although this statement exhibits magnificent prescience by a thoughtful surgeon, it does not hold entirely true with respect to our findings since not all cholesterol gallstones in the current study contained helicobacter DNA. Another salient aspect of this concept relates to the fact that the prevalence of gallbladder cancer is intimately associated with prevalence of gallstone disease (4, 13, 31). In experimental animals both excess biliary cholesterol and *H.*

*hepaticus* produce hepatobiliary cancer (24) and hepatocellular carcinoma (15, 50) respectively, either by themselves or in the presence of other cocarcinogenic or promoting cofactors. It is therefore tempting to speculate that *Helicobacter* spp., some of which are known carcinogens (43), and cholesterol (or an oxidized product thereof) may act synergistically to promote tumors of the biliary tree in individuals with lithogenic bile and cholesterol gallstones. Further evidence supporting this hypothesis derives from studies involving Japanese and Thai patients with gallstones and biliary tract malignancies (31) and Chilean women with chronic cholecystitis (13). These populations have high prevalence rates of gallstones and biliary cancers (13, 31) and both of these studies found an association between molecular (i.e. DNA) evidence of enterohepatic *Helicobacter* spp. infection and neoplastic biliary disease.

In the recent past, a putative role of *Helicobacter* spp. in human cholesterol gallstone disease has been proposed and questioned critically because helicobacter DNA has been amplified in hepatobiliary samples from unaffected individuals (6) and sometimes helicobacter DNA is not identified in hepatobiliary tissues of cholesterol gallstone patients (8). However, it is clear from our data that *Helicobacter* spp. infection per se plays a role in cholelithogenesis, but is not sufficient in or of itself, to produce cholesterol gallstones. Moreover, only certain strains of *Helicobacter* spp. promote cholesterol cholelithogenesis in the setting of lithogenic bile in vivo. Clearly, genetic background and non-infectious environmental factors (i.e. the lithogenic diet) are essential in the mouse model in initiating the physical chemical conditions, i.e. lithogenic bile predisposing to the disease. Mouse strain is clearly important since cholesterol gallstone

resistant mice from the original facility (37) were almost certainly colonized with these *Helicobacter* spp but their biles are obstinate in developing supersaturation. Genetics also play a role in how a host responds to infection with *Helicobacter* spp. For example, A/JCr mice can develop severe hepatitis and hepatocellular carcinoma when infected with *H. hepaticus*; however, some other mice strains display little hepatic disease when infected (22, 49, 50). Finally, this study utilized the C57L mouse which typically has a cholesterol saturation index (CSI) of approximately 1.5 at the eight week time-point (48) of lithogenic diet feeding. This value roughly correlates with CSI values observed in normal weight individuals with cholesterol gallstones (5); however, the value is lower than the median found in very obese individuals with gallstones (5). Further work is needed to determine whether *Helicobacter* spp. play a similar role at high CSI values (>1.60) such as those seen in morbidly obese patients (5). On the basis of the present studies we propose a hypothetical biological model demonstrating that three pathophysiological factors, environment, genetics, and enterohepatic *Helicobacter* spp. infection, contribute to cholesterol gallstone formation in C57L mice, as well as metaplasia, dysplasia and potentially the progression to gallbladder cancer.

Of interest is that in the past a number of investigators have identified the presence of bacteria (non-*Helicobacter* spp.) in the extrahepatic biliary tree, gallbladder and gallstones of humans and hypothesized on their putative role in so-called “mixed” (i.e. plus calcium bilirubinate) cholesterol gallstone formation (27, 30, 44). In particular Lee and colleagues tentatively identified *E. coli* and *Pseudomonas* spp. principally in “mixed” cholesterol gallstones containing 65-94% cholesterol and brown pigment gallstones with

16 and 25% cholesterol harvested from human gallbladders without current cholecystitis. These authors hypothesized that bacteria may indeed play a role in “mixed” cholesterol - bilirubinate gallstone formation (27). However, to date none of the organisms identified in these studies have been analyzed prospectively and because the bacteria identified are among the normal microbiota inhabiting the human distal small intestine and colon, their presence in the biliary tree may be secondary to recurrent biliary stasis and subclinical cholecystitis from cholesterol gallstones leading to the “mixed” bilirubinate-cholesterol gallstones (27, 30, 44). Development of this mouse model should allow for a prospective analysis of the primary versus secondary roles of these and other components of the gut microbiota in relation to gallstone pathogenesis.

Based on our data (Table 2-1), we believe we can now propose a realistic hypothesis for the role of *Helicobacter* spp. (and perhaps other bacteria) in cholesterol gallstone pathogenesis. We suggest that certain helicobacters change the thermodynamics of cholesterol crystal nucleation from the liquid crystalline state (i.e. the initial phase separation) in gallbladder bile (Table 2-1). Cholesterol supersaturation of bile is a necessary but not sufficient event for cholesterol gallstone formation, and both gallbladder hypomotility and accumulation of mucin gel (as a nucleation matrix) are also required. Theoretically, nucleation of solid cholesterol monohydrate crystals can occur homogeneously, but this is thermodynamically unfavorable and might require a enormous degree of cholesterol supersaturation (26). Alternatively, a far more favorable event thermodynamically is heterogeneous nucleation which involves a nucleating agent(26) that provide a hydrophobic surface for molecular deposition and these agents

can nucleate bile with only modest degrees of supersaturation. Multiple studies have suggested the presence of nucleating agents in humans with cholesterol gallstones (26). Likely nucleating agents identified previously include mucins, immunoglobulins and other biliary proteins, some of which have been functionally and chemically characterized (20, 26, 28, 55). We have demonstrated in the current study that particular *Helicobacter* spp. significantly increase mucin production in C57L mice fed a lithogenic diet (Table 2-1). Additionally, we showed earlier that *H. hepaticus* infection causes a three-fold increase in biliary IgA production, and it is logical to presume that *H. bilis* would have a similar effect (52). Furthermore, it is provocative to suggest that *Helicobacter* spp. may produce other pronucleating agonists in response to biliary exposure or, alternatively, that these organisms may induce production in the host of nucleating agents either directly or through chronic immune stimulation. Most importantly, it has been demonstrated that different *Helicobacter* spp. exhibit very divergent protein expression profiles in response to their exposure to bile (21). Perhaps this difference in part explains why some, but not all, of these related organisms promoted cholesterol gallstone formation in our studies. Additionally, preliminary data (KJ Maurer, MC Carey JG Fox unpublished observations, 2004) from our laboratories suggest that global changes occur in hepatic gene expression from enterohepatic helicobacter infection even without inclusion of a lithogenic diet, including genes thought to play a role in cholesterol homeostasis. It is theoretically possible that one or more of these upregulated genes may play a role in altering biliary lipid composition either qualitatively or quantitatively.

In conclusion, our work represents a provocative new dimension in our understanding of cholesterol gallstone pathogenesis in C57L mice, and that these findings are likely to be applicable to humans. Dissecting the mechanisms responsible for these observations, including physical-chemical, petrological, genomic/proteomic and infectious will require further critical hypothesis and experimentation in several mouse models with different enterohepatic as well as gastric *Helicobacter* spp. and related organisms. Provided the new concept holds true for human gallstone disease, as some earlier epidemiological evidence would imply, then a paradigm shift may be in the offing that may lead to major modifications in the prevention and treatment of cholesterol gallstone disease, and possibly prevention and management of gallbladder and other hepatobiliary diseases including hepatobiliary and perhaps pancreatic cancers.

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**Chapter 3**  
***Helicobacter pylori* and cholesterol gallstone formation in C57L/J mice: a prospective study**

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## Abstract

Recently we demonstrated that cholesterol gallstone prone C57L/J mice rarely develop gallstones unless they are infected with certain cholelithogenic enterohepatic *Helicobacter* species. Since the common gastric pathogen *H. pylori* has been identified in the hepatobiliary tree of cholesterol gallstone patients, we wished to ascertain if *H. pylori* is cholelithogenic, by prospectively studying C57L infected mice fed a lithogenic diet. Weanling, *Helicobacter* spp.-free male C57L mice were either infected with *H. pylori* SS1 or sham dosed. Mice were then fed a lithogenic diet (15% dairy triglycerides, 1.0% cholesterol and 0.5% cholic acid) for eight weeks. At 16-weeks of age, mice were euthanatized, the biliary phenotype was analyzed microscopically, and tissues were analyzed histopathologically. *H. pylori* infection did not promote cholesterol monohydrate crystal formation (20% vs. 10%), sandy stone formation (0% for both) nor gallstone formation (20%) when compared to uninfected mice fed the lithogenic diet (10%). Additionally, *H. pylori* failed to stimulate mucin gel accumulation in the gallbladder or alter gallbladder size when compared with uninfected animals. *H. pylori* infected C57L mice developed moderate to severe gastritis by 12 weeks and the lithogenic diet itself produced forestomach lesions which were exacerbated by the infection. We conclude that *H. pylori* infection does not play any role in murine cholesterol gallstone formation. Nonetheless, the C57L mouse develops severe lesions of both the glandular and non-glandular stomach in response to *H. pylori* infection and the lithogenic diet respectively.

## **Introduction**

Gallstones are an exceptionally common cause of morbidity worldwide and cholelithiasis with or without cholecystitis is the most common gastrointestinal disease requiring inpatient treatment in the United States (45). Despite five decades of intense basic and clinical research (6, 11, 27, 41, 42) there is currently no effective non-surgical treatment for definitive management of gallstones, hence the disease is a serious surgical and economic burden with the median per patient cost exceeding 10,000 U.S. dollars (45).

The term gallstones or cholelithiasis is a generic description encompassing both pigment and cholesterol gallstones (41). Cholesterol gallstones are the most common gallstones encountered in the Western world (41). These stones result from cholesterol supersaturation of bile and phase separation of cholesterol-rich liquid crystals and solid crystals with subsequent crystal agglomeration and stone growth in the gallbladder within a mucoglycoprotein gel scaffold (8, 9, 41). Cholesterol gallstone formation is a polyfactorial disease with heterogeneous contributions from both genetics and environment (41). Specifically, genetic susceptibility to cholesterol gallstones is, with very rare exceptions inherited as a polygenic trait (6, 10, 11, 27, 41, 42, 54). Cholesterol gallstone susceptibility genes require environmental triggers including diet, obesity, estrogenic drugs and other complex factors to express the cholesterol gallstone phenotype (41).

The C57L/J mouse is utilized extensively to investigate cholesterol gallstone genetics and pathogenesis (25, 42, 51, 52). When fed a lithogenic diet containing 1.0% cholesterol

and 0.5% cholic acid for eight weeks, this animal model historically develops cholesterol gallstones with an 80% prevalence rate (25, 51). Our initial studies with this model were conducted at facilities where the mice were enzootically infected with enterohepatic *Helicobacter* spp (33). Recently, we demonstrated that in the absence of infection with specific enterohepatic *Helicobacter* spp., prevalence of cholesterol gallstones was much less than we reported previously, approximating 10% (33).

We and others, found serological and molecular evidence that enterohepatic *Helicobacter* spp. may be associated with several chronic hepatobiliary diseases of humans (3, 15, 30, 32, 38, 50). Others have reported the identification of *H. pylori* in hepatobiliary tissue from patients with benign and malignant hepatobiliary disease (7, 39, 47). In some of these studies, bile procurement and sample processing had the potential to bias the results towards identification of *H. pylori*. For example, endoscopic retrograde cholangiopancreatography (ERCP), was utilized to collect bile samples from patients with known gastric *H. pylori* colonization, leading to potential contamination of bile samples with *H. pylori* from the gastric mucosa; particularly so when sensitive detection methods such as PCR are utilized (7). Moreover, in some cases, *H. pylori* was identified on the basis of sequencing segments of its 16SrRNA gene (47). However, this method is known to be unsatisfactory for properly speciating helicobacters due to high 16SrRNA sequence homology among organisms in this large genus (40). Moreover, *in vitro* studies have revealed that *H. pylori* is sensitive to bile and is chemotactically repelled by pure solutions of both conjugated and unconjugated bile salts (22, 55). Because of these equivocal findings in humans and our recent identification of a number of enterohepatic

*Helicobacter* spp. that promote murine cholelithogenesis, we wished to ascertain whether *H. pylori* infection exhibited the ability to induce cholesterol gallstones in the C57L mouse model of “infectious” cholesterol cholelithogenesis (33) .

## **Methods**

### *Animal sources and husbandry*

All animal protocols were reviewed and approved by the MIT Committee on Animal Care. Three to four-week old *Helicobacter* spp. free C57L/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were divided into three groups for study. In the first group mice remained uninfected and were fed a standard lithogenic diet containing 15% dairy fat, 1.0% cholesterol and 0.5% cholic acid (n=10) (25). The second group was infected with *H. pylori* SS1 (28) and fed a rodent chow diet (n=5), and the third group was infected with *H. pylori* SS1 and fed a standard lithogenic diet (n=10). Group numbers were chosen based upon a power of 90-95% to statistically detect similar changes in gallstone prevalence compared to changes noted in our initial studies (33). Mice were housed in polycarbonate microisolator cages under specific pathogen free (SPF) conditions (free of *Helicobacter* spp., *Citrobacter rodentium*, *Salmonella* spp., endoparasites, ectoparasites and known murine viral pathogens) in an Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility. Mouse rooms were kept at constant temperature and humidity on a 12-hour regular light to dark cycle and mice received food and water *ad libitum*. Animals were fasted for 12 hours prior to CO<sub>2</sub> induced euthanasia. Mice were fed a standard rodent chow (Purina Mills, St. Louis MO) containing less than 0.05% cholesterol until

eight weeks of age. At this time point mice were either continued on a standard rodent chow or converted to the lithogenic diet.

#### *H. pylori infection*

*H. pylori* strain SS1 (28) was grown for 24-48 hours in Brucella broth (Becton Dickinson, Co., Franklin Lakes NJ) containing 5% heat inactivated fetal calf serum. Broth cultures were centrifuged at 8000 rpm for 20 minutes at 4°C and the bacterial pellet was resuspended in Brucella broth at a turbidometric (O.D.= 660 nm) reading of between 0.6-1.2. Four to five wk old mice were infected with 0.2mL of resuspended bacteria (n=15) or sham dosed with 0.2 mL Brucella broth (n=10) by oral gavage three times over a five-day period. At monthly intervals thereafter mice were given 2 more doses of *H. pylori* SS1 by gavage utilizing the same protocol.

#### *Bile Analysis and tissue processing*

Mice were euthanized with CO<sub>2</sub> in the fasting state at 16 weeks of age. A ventral midline incision was made and the gallbladder was removed intact. Full gallbladders were weighed and their contents were examined under direct and polarized light microscopy by a microscopist (KJM) blinded as to sample identity. Bile was scored for mucin content, presence of liquid crystals, solid cholesterol monohydrate crystals, sandy stones, and true cholesterol gallstones as described (27, 52). Statistical analyses of mucin score and gallbladder weight were performed by one way analysis of variance (ANOVA), with the Tukey-Kramer post test using InStat 3.0 software (GraphPad, Inc., San Diego, CA, USA). Cholesterol monohydrate crystals as well as sandy and true gallstone

formation were analyzed by Fisher's exact test utilizing the same software. Stomachs were removed aseptically, and opened longitudinally along the greater curvature. Approximately 30 mg of glandular stomach was flash frozen in liquid N<sub>2</sub> and stored at -80°C for subsequent quantitative PCR. Three, 10 mg segments of the liver from different liver lobes were collected aseptically and frozen at -20°C for subsequent PCR.

### *Histopathology*

At necropsy, liver, gallbladder, stomach, duodenum, pancreas, and ileocecolic junction were collected, trimmed and fixed in 10% neutral-buffered formalin. Tissues were processed routinely, paraffin embedded, cut at 4 µm thickness, and stained with hematoxylin and eosin (H&E). Additional stomach and gallbladder sections were stained with Alcian blue/periodic-acid Schiff (pH 2.5) for acidic and neutral mucins (18). Tissues were evaluated by a comparative pathologist blinded as to sample identity (ABR). Gastritis was scored on an ascending 0—4 scale using previously defined criteria (17). Inflammation of the squamous forestomach was scored using criteria similar to those for the glandular compartment. Squamous epithelial hypertrophy/hyperkeratosis was scored as 0: normal thickness, 1: 2X normal thickness, 2: 3X normal thickness, 3: 4X normal thickness, or 4: >4X normal thickness. The presence or absence of intraepithelial microabscesses was also recorded. Mean histologic grades were compared between multiple groups by Kruskal-Wallis one-way analysis of variance followed by Dunn's post-test using Prism 3.0c for Macintosh (GraphPad, Inc., San Diego, CA). Direct comparisons were made with Mann Whitney U test. Prevalence of microabscesses was

evaluated using Fisher's exact test.  $P$  values  $\leq 0.05$  were considered statistically significant.

### *Real Time Quantitative PCR*

Chromosomal DNA from broth-grown *H. pylori* SS1 and total DNA from mouse stomachs were prepared using the "High Pure Template Preparation Kit" according to the supplier's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). To quantify colonization levels of *H. pylori* strain SS1 within gastric mucosa, a real-time Quantitative PCR assay (Q-PCR) was developed based upon the nucleotide sequence of *H. pylori ureB* gene in the ABI Prism TaqMan 7700 sequence detection system (A/B Applied Biosystem, Foster City, CA). Two primers (Forward: 5'-CAAATCGCTGGCATTGGT-3'; Reverse: 5'-CTTCACCGGCTAAGGCTTCA-3') and an internal probe (5'-AACAAAGACATGCAAGATGGCGTTAAAAACA-3') were designed to hybridize within the 100-bp region (nucleotides 273 to 373) of the single copy *ureB* gene (AF508016) of *H. pylori* SS1 using Primer Express software (Applied Biosystem, Foster City, CA) (40). Q-PCR conditions were described previously (20). The specificity of these oligonucleotides for *H. pylori* was tested using DNA isolated from *H. felis* (ATC49179), *H. mustelae* (ATCC43772), and '*H. heilmannii*'. Serial 10-fold dilutions (from  $5 \times 10^5$  to 5) of the *H. pylori* SS1 genome copies, estimated from an average mass value (1.66 Mb) obtained from the two published *H. pylori* genomes, were used to generate a standard curve (2, 49). Copy numbers of gastric mucosal *H. pylori* SS1 DNA in mice were then calculated and normalized to  $\mu\text{g}$  of murine chromosomal

DNA determined by Q-PCR, using the mammalian 18S rRNA gene-based primers and probe mixture (Applied Biosystems, Foster City, CA) described elsewhere (53).

### *Liver PCR*

Livers were harvested and DNA was extracted using the Roche DNA “High Pure Template Preparation Kit” (Roche Molecular Biochemicals, Indianapolis, IN, USA) per the manufacturer’s recommendations. Two rounds of PCR amplification were performed: The first round of amplification utilized the genus specific primer set C97 and C05 which amplifies an approximately 1200 bp amplicon (16, 19). PCR reactions were performed utilizing 5µL template DNA and “PuRe Taq Ready To-Go PCR beads” (Amersham Biosciences, Uppsala, Sweden) utilizing previously described reaction conditions (16, 19). Following this initial reaction, a second nested amplification was performed utilizing the genus specific C97 and C98 primer sets which amplify an approximately 400 bp amplicon (16, 19). This reaction utilized 1 µL of template DNA from the first reaction and followed conditions described previously (16, 19). Known *H. pylori* SS1 colonized murine gastric tissue was included as positive control and known uninfected mouse tissue was used as negative control.

## **Results**

### *Biliary phenotype*

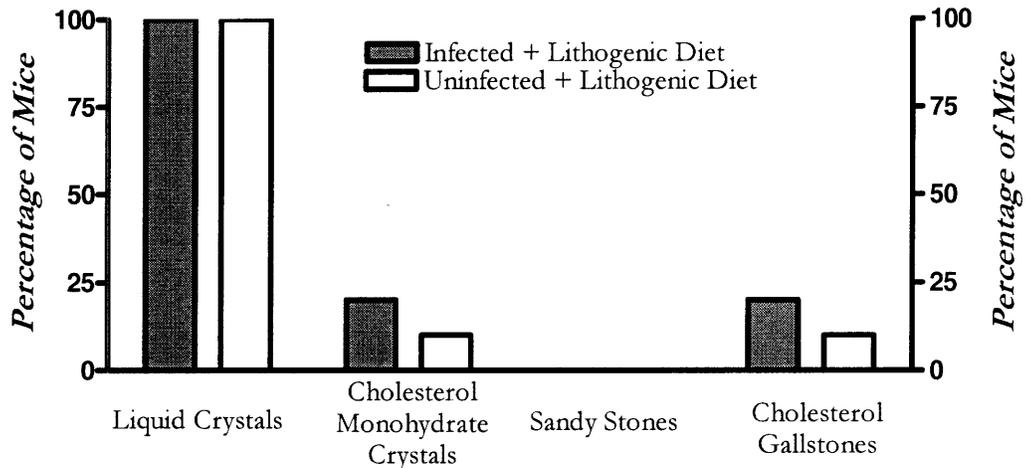
Regardless of infection status, the gallbladder bile of all mice fed the lithogenic diet, displayed cholesterol-phospholipid liquid crystals indicating supersaturated bile (Figure 1). Mice fed a chow diet did not develop liquid crystals in bile, confirming the well

known requirement for a modified diet to supersaturate gallbladder bile and induce solid crystal phase-separation in this mouse strain (33, 51). *H. pylori* infected mice fed the lithogenic diet developed slightly more cholesterol monohydrate crystals (20%) and true cholesterol gallstones (20%) when compared to control animals (10% for each), but these changes were not statistically significant ( $P=1.0$ , Fig. 3-1). Moreover, no differences in sandy stone formation were noted (0% for both groups, Fig. 3-1). Mucin gel scores and normalized gallbladder weight for animals fed the lithogenic diet were statistically greater than those fed a standard chow diet ( $P\leq 0.05$ ), however, neither differed among animals fed the lithogenic diet regardless of infection status ( $P>0.05$ ) (Fig. 3-2). *H. pylori* infected animals fed a chow diet did not develop mucin gel formation (score of 0, Fig. 3-2) and had a similar normalized gallbladder weight compared to uninfected mice fed a chow diet as described in our previous studies (Fig. 3-2, (33)).

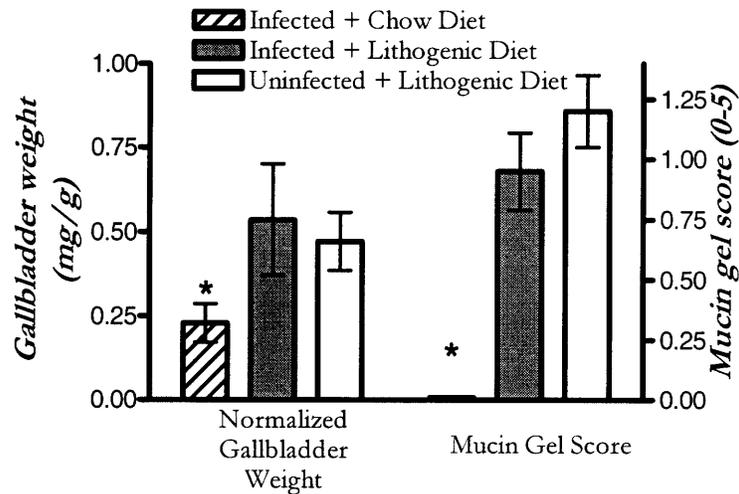
#### *Real-time quantitative PCR*

To validate the specificity and sensitivity of the Q-PCR assay for *H. pylori*, *H. pylori* SS1 DNA in parallel with the DNA templates from three gastric helicobacters, *H. felis*, *H. mustelae*, and '*H. heilmanii*', was detected using the primers and probes designed for the *ureB* gene. The quantitative PCR assay detected a minimum of 5 copies of the *H. pylori* SS1 genome (Lane 6, Fig. 3-3), whereas there was no amplification from 10 ng (approximately equal to  $5 \times 10^5$  genome copies based on the size of the *H. pylori* genome) of DNA from *H. felis*, *H. mustelae*, or '*H. heilmanii*' ((Lane 7-9, Fig. 3-3). The mean number of *H. pylori* organisms per  $\mu\text{g}$  of host DNA in the gastric corpus of chow fed infected animals was  $2.46 \times 10^5$  whereas in lithogenic fed infected animals those

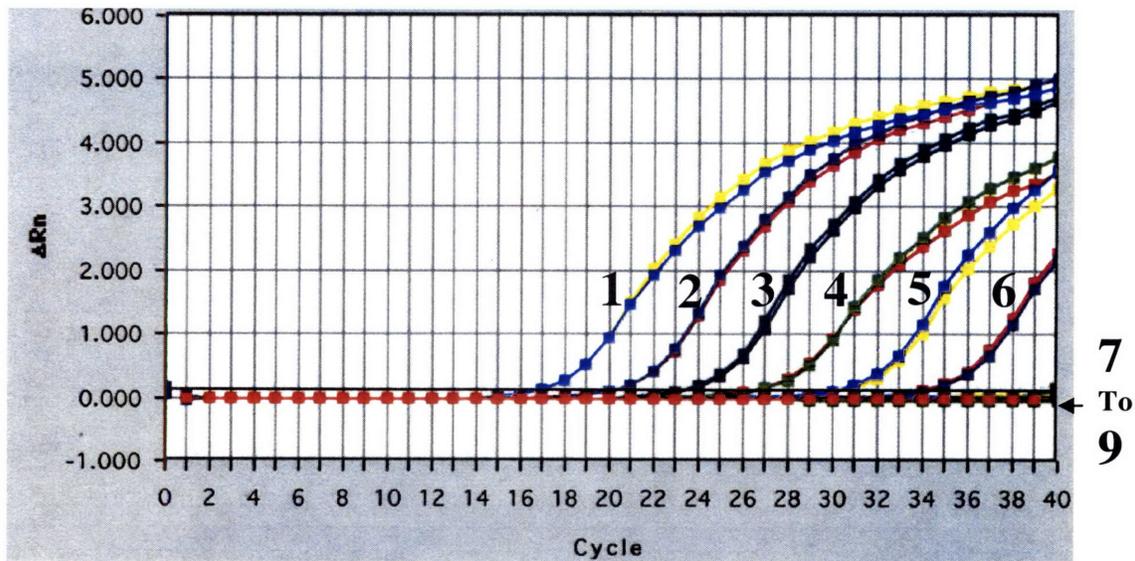
numbers were reduced significantly by approximately 1 log unit ( $P < 0.05$ ) with only  $6.01 \times 10^4$  organisms per  $\mu\text{g}$  of host DNA (Fig. 3-4).



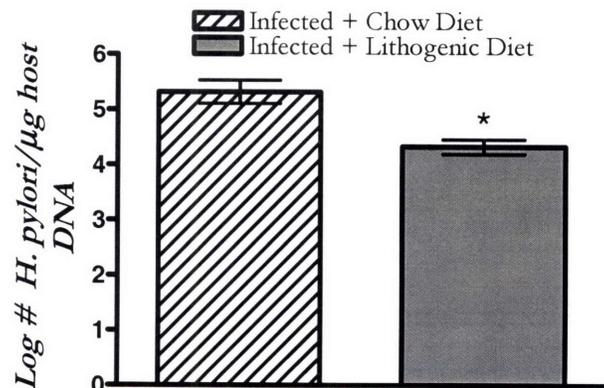
**Figure 3-1:** Biliary phenotype determined by polarized light microscopy of gallbladder bile. Mice infected with *H. pylori* and fed a lithogenic diet do not differ in biliary phenotype when compared to uninfected animals fed the same diet. Data for *H. pylori* infected animals fed a chow diet are not displayed because chow fed C57L mice fail to supersaturate their bile with cholesterol and therefore do not demonstrate any of the ascribed biliary phenotypes.



**Figure 3-2** (preceding page): Mucus gel score and normalized gallbladder weight (mg of gallbladder/g of mouse) of all groups of mice. Lithogenic diet feeding, independent of *H. pylori* status increases mucin accumulation and normalized gallbladder weight ( $*P \leq 0.05$ ) when compared to animals fed a standard chow diet. *H. pylori* infection does not significantly increase mucin content or normalized gallbladder weight when compared to uninfected animals fed the same diet ( $P > 0.05$ ). All data are presented as the mean  $\pm$  SEM.



**Figure 3-3:** A standard curve detailing the sensitivity and specificity of the quantitative PCR assay developed. Plots 1 to 6:  $5 \times 10^5$ ,  $5 \times 10^4$ ,  $5 \times 10^3$ ,  $5 \times 10^2$ , 50 and 5 copies of the *H. pylori* SS1 genome were used as the initial template in these reactions; plots 7 to 9: 10 ng of genomic DNA from *H. felis*, '*H. heilmannii*', and *H. mustelae* were the initial template. All of these failed to amplify and are illustrated by the red dots following the y-intercept. The  $r^2$  from the linear regression is  $>0.99$ .



**Figure 3-4:** Data are presented as log numbers of *Helicobacter pylori* organisms per  $\mu\text{g}$  of host DNA  $\pm$  SEM. Lithogenic diet fed animals show significantly less organism numbers present in the gastric mucosa than rodent chow fed animals ( $*P \leq 0.05$ ).

### *Liver PCR*

Livers of infected animals were uniformly negative on initial PCR screening..

Subsequent nested amplification also failed to amplify any *H. pylori* DNA from either the infected group fed chow or the infected group fed the lithogenic diet (data not shown).

This contrast markedly with the positive PCR screens from the livers of enterohepatic *Helicobacter* infected C57L mice in our earlier study (33)

### *Histopathology : Liver and Gallbladder*

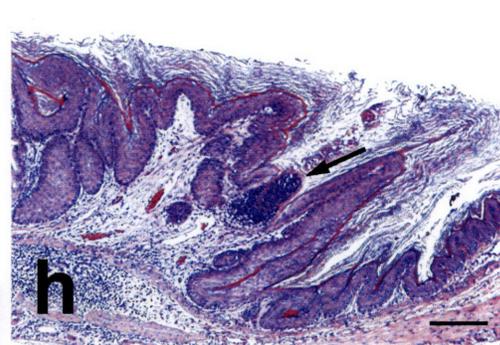
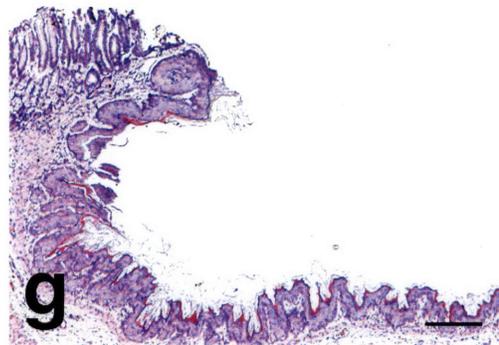
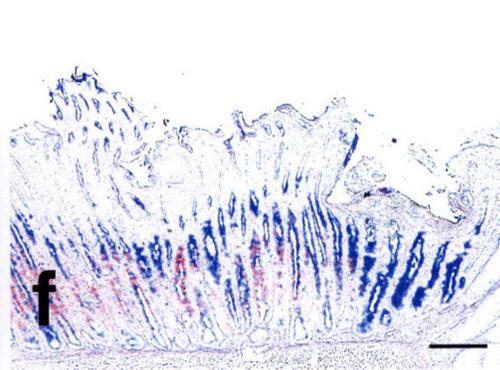
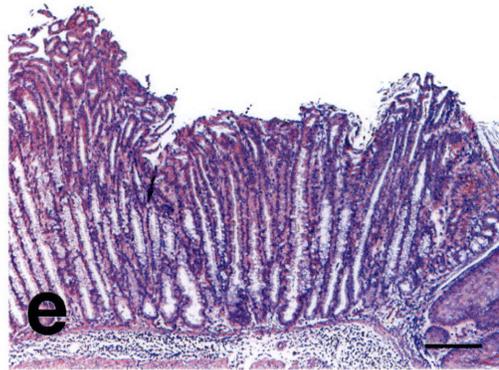
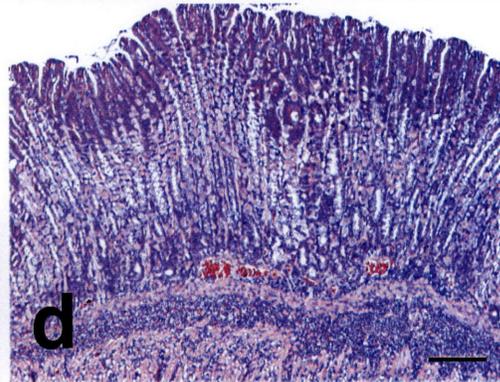
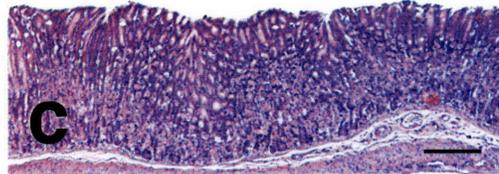
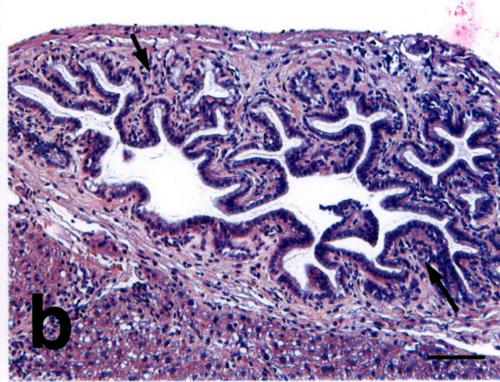
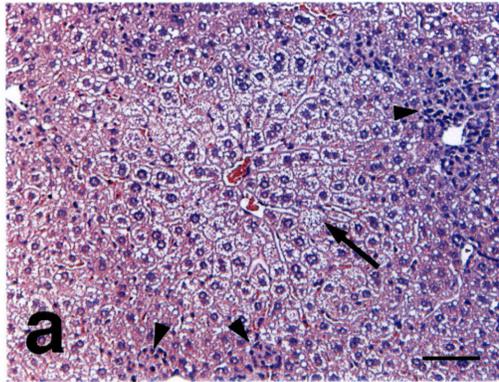
The livers of C57L mice fed the lithogenic diet demonstrated a lobular pattern of hepatocellular microsteatosis concentrated in acinar zones 2 and 3 (mid-zonal and centrilobular, respectively; Fig. 3-5a). In contrast, macrosteatosis characterized by medium to large round clear cytoplasmic lipid vacuoles was mild, patchy, and usually most prominent in the periportal regions. Mild-to-moderate portal mononuclear inflammation was noted in a subset of mice on the lithogenic diet, but its occurrence was recorded equally in *H. pylori* infected and uninfected animals (Fig.5a). Small lipogranulomata comprised of macrophages with phagocytosed lipofuscin-like material were sometimes seen (Fig. 3-5a). Mild and inconsistent gallbladder lesions were evident in some mice fed the lithogenic diet. Microscopic findings included eosinophilic and lymphocytic cholecystitis (Fig. 3-5b), small islands of mucous metaplasia, and scattered epithelial cell hyalinosis with rare intraluminal crystals (data not displayed). Unlike the severe gallbladder lesions in mice fed the lithogenic diet infected with specific enterohepatic *Helicobacter* spp. (33), gallbladder lesions in the present study were inconsistent, mild, and unassociated with *H. pylori* infection status. Moreover, there were

no hepatobiliary lesions in *H. pylori* infected mice on the chow diet. Duodenum, pancreas and ileocecolic junction were within normal limits in all mice regardless of diet or infection status.

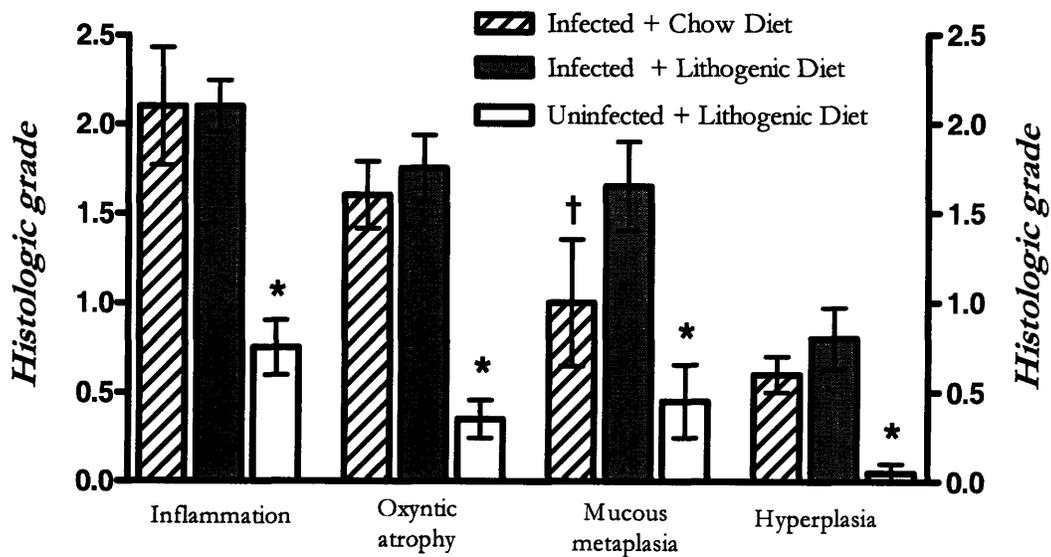
#### *Histopathology: Stomach*

Two distinct histological patterns of disease were produced in the stomach, one associated with *H. pylori* infection and the other with the lithogenic diet. Compared with uninfected mice on the lithogenic diet (Fig. 3-5c), *H. pylori* infected mice on either chow or lithogenic diet developed moderate mixed mononuclear and granulocytic proliferative gastritis of the cardia and corpus with oxyntic gland atrophy and mucous metaplasia (Fig. 3-5d). No significant intestinal metaplasia or dysplasia was evident. Using H&E staining, mucous metaplasia of the oxyntic mucosa was characterized by foamy change in the cytoplasm of parietal cells (Fig. 3-5e). Mucous metaplasia was confirmed by Alcian blue/PAS staining at pH 2.5, demonstrating transformation of surface mucins from the neutral gastric-type (red) to the acidic intestinal-type (blue). Additionally, there was heavy production of mixed mucins in the parietal cell zone, with intestinal-type mucins concentrated at the upper and lower boundaries of the cellular columns and gastric-type mucins in the middle (Fig. 5f). Compared to uninfected controls, scores of the lesions in the glandular stomach incorporating all criteria were significantly increased in *H. pylori* infected mice, regardless of diet ( $P \leq 0.05$ ; Fig. 3-6). However, there were no differences in mean scores between infected groups on different diets except for an additive effect of the lithogenic diet on mucous metaplasia ( $P \leq 0.05$ ; Fig. 3-6).

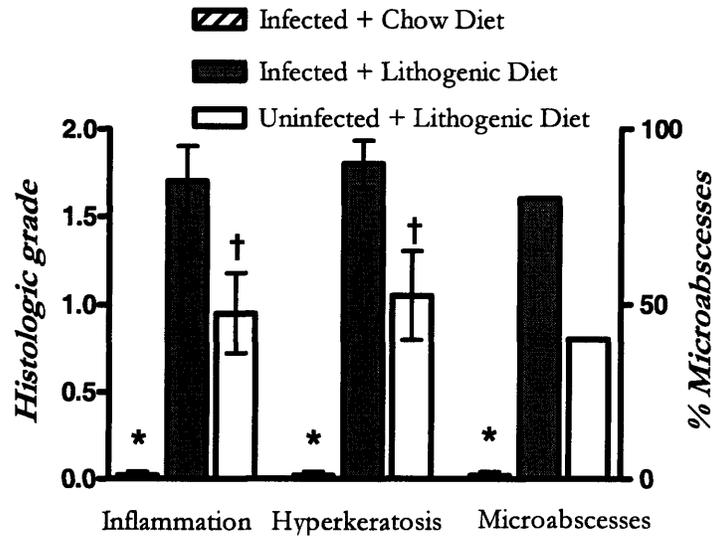
The second pattern of gastritis, associated with the lithogenic diet, affected the anterior squamous compartment (forestomach). Histologic changes consisted of moderate mixed inflammation and edema of the lamina propria and submucosa, and hypertrophy of the squamous epithelium with orthokeratotic hyperkeratosis and frequent intraepithelial abscesses (Fig. 3-5h). Microabscesses, generally 1-2 mm, were composed of degenerate neutrophils and epithelial cells, often with a central core of keratotic debris (Fig. 3-5h). In some instances, microabscesses contained embedded hair shafts or vegetative material from the diet, although it was not clear whether the foreign bodies induced the inflammation or became entrapped within pre-existing lesions. In contrast, no squamous defects developed in *H. pylori* infected mice on the chow diet (Fig. 3-5g). However, in mice ingesting the lithogenic diet, there was a statistically significant additive effect of *H. pylori* infection on the degree of squamous hyperkeratosis ( $P \leq 0.05$ ; Fig. 3-7) and inflammation ( $P \leq 0.05$ ). *H. pylori* infected mice on the lithogenic diet were twice as likely to develop microabscesses of the squamous stomach as uninfected mice (80% vs. 40%, respectively), although this difference did not reach statistical significance ( $P=0.17$ ).



**Figure 3-5** (preceding page): Histopathology of liver, gallbladder and stomach from mice on lithogenic diet and/or infected with *H. pylori* and euthanatized at 16 weeks of age. (a) Hepatic lobule in liver of *H. pylori* infected mouse on lithogenic diet with centrilobular and midzonal microsteatosis (arrow), patchy macrosteatosis (larger clear vacuoles), mild portal mononuclear inflammation (arrowhead, right) and lipogranulomas (arrowheads, lower left). Identical lesions were present in uninfected mice on the lithogenic diet (not shown). (b) Gallbladder from (a) with mild eosinophilic and lymphocytic inflammation in the lamina propria (arrows). (c) Histologically normal gastric corpus from uninfected mouse on lithogenic diet. (d) Moderate-to-severe inflammation and hyperplasia in *H. pylori* infected C57L mouse on chow diet. (e) Marked mucous metaplasia in *H. pylori* infected mouse on lithogenic diet. Note cytoplasmic foamy change in parietal cell zone. (f) pH 2.5 Alcian blue/PAS stain of (e). Abnormal intestinal-type acidic mucins (blue) on the gastric surface, and cytoplasmic accumulation of both acidic and neutral mucins in the parietal cell zone. (g) Histologically normal squamous stomach in *H. pylori* infected mouse on chow diet. (h) Hypertrophic pleating and marked hyperkeratosis associated with mucosal and submucosal inflammation and edema of squamous stomach from *H. pylori* infected mouse on lithogenic diet. Note intraepithelial microabscess comprised of degenerate epithelial and polymorphonuclear inflammatory cells (arrow).



**Figure 3-6:** Comparison of lesion grades in the glandular stomach of C57L mice. Mean grade of all lesion criteria was increased by *H. pylori* infection compared with sham inoculation ( $*P \leq 0.05$ ). An additive effect of lithogenic diet with *H. pylori* infection was observed for mucous metaplasia ( $\dagger P \leq 0.03$ ).



**Figure 3-7:** Comparison of the lesion grades in the squamous stomach of C57L mice. The lithogenic diet induced squamous hypertrophy and hyperkeratosis, inflammation and intraepithelial abscesses. *H. pylori* infection increased severity of lesions in mice on the lithogenic diet ( $\dagger P \leq 0.05$ ). In contrast, *H. pylori*-infected mice on the chow diet did not develop lesions in the squamous forestomach.

## Discussion

The literature ascribing a role of *H. pylori* in causing human hepatobiliary disease has been both confusing and inconclusive (4, 7, 13, 39, 47). In this study, we demonstrated that *H. pylori*, unlike some enterohepatic *Helicobacter* spp. (33) does not promote cholesterol cholelithogenesis in the C57L mouse model. *H. pylori* does not increase cholesterol gallstone formation nor does it promote any of the preliminary stages (cholesterol monohydrate crystals, sandy stones) in cholelithogenesis. One might argue that a doubling of the prevalence of cholesterol gallstone formation in infected mice would be important when dealing with a large population (such as humans with *H. pylori*); however, since *H. pylori* does not increase gallbladder mass (a surrogate marker of gallbladder motility), nor mucin gel accumulation (in fact infected animals had less mucin gel accumulation than uninfected animals) which are markers of cholelithogenesis

it is likely that this increase is merely due to population variation (as our statistical analyses indicate). In addition, our PCR data indicate that *H. pylori* does not colonize the hepatobiliary tree. This finding is not surprising since to date there is not one single report of successful bacterial isolation of these organisms from the liver or biliary tree of experimentally infected animals and only a single letter reporting the isolation of *H. pylori* from a human liver with Wilson's disease (44).

In the past, authors claimed to have identified *H. pylori* like organisms in hepatobiliary tissue by 16SrRNA amplification and sequencing. (47). Unfortunately, speciating *Helicobacter* based on sequencing a small (400-1000 base pair) segment of the 16SrRNA gene is fraught with error since this region is highly conserved among the *Helicobacter* spp. (40). Highlighting this concern, Avenaud and colleagues (4) identified organisms that appeared to be *H. pylori* based upon sequencing of the 16SrRNA gene from the liver of humans. Based upon stringent follow up tests, these investigators discovered that these organisms were a previously unidentified (and still unclassified) *Helicobacter* sp. that is phylogenetically related to, but not, *H. pylori* (4).

We hypothesize that the presence of *H. pylori* in the biliary tree and liver of human patients with cholesterol gallstones is either a secondary colonizer (due to biliary changes from gallstones, cholestasis, or chronic cholecystitis), or the DNA denotes one or more related *Helicobacter* spp. Perhaps, in the presence of disease induced by other *Helicobacter* spp. or other factors, the biliary microenvironment changes to allow for secondary invasion by *H. pylori*. For example, we demonstrated previously (33) that

some cholelithogenic *Helicobacter* spp. increase gallbladder mucin gel accumulation significantly as well as contributing to mucinous metaplasia of the gallbladder in C57L mice fed the lithogenic diet. An increase in mucin gel accumulation, or a change in mucin species may promote attachment of *H. pylori* to the biliary epithelium.

Interestingly, others have noted that the type of mucin produced in the stomach is pivotal for *H. pylori* colonization and in fact some normal gastric mucins are bactericidal (24).

Consistent with a possible alteration of the biliary microenvironment leading to secondary invasion by *H. pylori*, Myung and colleagues (39) found that patients that are PCR positive for *H. pylori* exhibit a significantly lower bile pH than those that were PCR negative. Further evidence for secondary invasion by *H. pylori* was recently demonstrated by multiplex PCR in an Iranian population (14). *H. pylori*-like DNA could be amplified from bile of gallstone patients with pathologic evidence of chronic cholecystitis but not from the bile of asymptomatic gallstone patients (14). These authors did not attempt to culture for bacteria other than *Helicobacter* spp. However, others (21, 37, 46) have cultured the biliary tree and bile from humans and experimental animal models with cholecystitis and demonstrated numerous bacterial species which are considered part of the normal distal gastrointestinal tract, and nasopharyngeal microbiota (21, 37, 46). These organisms often colonize the biliary tree secondarily, especially following obstructive cholestasis or sphincter of Oddi ablation. It is likely that *H. pylori* behaves in a similar manner and may colonize the biliary tree due to impaired biliary drainage, changes in mucus content or character and other biochemical alterations affecting the nature and concentration of biliary lipids. To validate or disprove these

hypotheses a thorough chemical and physical-chemical analysis of bile in the presence and absence *Helicobacter pylori* DNA in the hepatobiliary tree will be necessary.

In planning the present experiments, we were concerned that the lithogenic diet would inhibit *H. pylori* colonization, because *in vitro* studies have demonstrated bactericidal effects of bile acids on *H. pylori* (22). Indeed, in the present work, there was a significant log unit decrease in the number of *H. pylori* organisms in the stomachs of lithogenic diet fed infected mice when compared to the chow fed group (Fig 4). Interestingly, there was no significant change in the extent or severity of glandular gastritis between the two infected groups. In fact, in both lithogenic diet- fed and chow- fed mice a robust gastritis was noted. Historically, the *H. pylori* SS1 strain causes minimal lesions in susceptible mouse strains at 3 months (12 weeks) post-infection and C57BL/6 mice which are characterized as a susceptible strain, often require six months of *H. pylori* infection to demonstrate moderate gastric inflammation (28). The C57L mouse appears to be exquisitely susceptible to *H. pylori* induced gastritis (regardless of diet fed) and displays similar lesion scores to INS-GAS hypergastrinemic mice at this early time-point (17). This observation merits further investigation in view of the established fact that INS-GAS mice progress to adenocarcinoma after 6 months of colonization with *H. pylori* (17).

In addition to infectious gastritis in both chow fed and lithogenic fed animals, non-infected mice that received the lithogenic diet developed lesions of the squamous forestomach. These lesions included hypertrophy, hyperkeratosis, and inflammation with microabscess formation of the epithelium. Furthermore, some of these diet- induced

lesions were exacerbated significantly by infection with *H. pylori*. Moreover, the squamous epithelial lesions are reminiscent of the lesions caused in part by diet and stress in the *pars esophagea* of swine which consist of hyperkeratosis and ulceration (12). Swine stomachs are commonly colonized by *Helicobacter suis*; however, the contribution of these helicobacters to gastric disease in swine is not completely understood (26, 43). We propose that in domestic swine, like the inbred mice in this study, dietary composition induces lesions in the *pars esophagea* and that *H. suis* exacerbates these lesions. The lesions in the squamous stomach demonstrate that under modified dietary conditions, *H. pylori* can exacerbate disease in areas in close anatomic proximity to where these organisms colonize typically, i.e. the glandular stomach. The squamous forestomach of the mouse is a non-glandular area separated anatomically from the glandular gastric compartment by the limiting ridge (29). Histologically, this region is lined by stratified squamous epithelium (29). Due to its anatomic separation and histological appearance, it is essentially an extension of the mouse esophagus and is similar to the esophagus of humans. This raises the possibility that this mouse model could be used to study the influence of diet and *H. pylori* to contribute to chronic esophageal diseases such as esophagitis and esophageal cancer.

Diet and *H. pylori* status in C57L mice also appeared to exhibit synergism in causing lesions of the glandular stomach, most notably metaplasia. The cholic acid, triglyceride, and cholesterol components of the diet may either singly or together be contributing to gastric lesions. For example, cholic acid promotes azoxymethane induced aberrant crypt foci in rats (5, 48). However, analysis of the forestomach of bile acid-fed rats did not

demonstrate any notable lesions (56). Interestingly, diets high in fat and cholesterol (so called “Western diets”) have been correlated with risk for esophageal adenocarcinoma, gastric cardia adenocarcinoma, esophageal squamous cell carcinoma, and common gastric adenocarcinoma (31, 34, 35). Additionally, cholesterol free radicals are known to promote a variety of diseases including atherogenesis (1, 23, 36). It is therefore reasonable to hypothesize that inflammation from *H. pylori* induced gastritis may promote oxidation of cholesterol or fatty acids in humans consuming high cholesterol or high animal triglyceride diets. These oxidized products could then further contribute to gastric and potentially esophageal cancers, possibly due in part to free radical damage.

In summary, *H. pylori* infection does not contribute to cholesterol gallstone formation in the C57L mouse model. We believe that the purported suggestions in the literature that *H. pylori* causes cholesterol gallstones in humans are suspect and like many other bacteria, *H. pylori* is likely to be a secondary colonizer of the hepatobiliary tree following complicated gallstone disease and/or iatrogenic interventions. In addition, the C57L mouse is highly susceptible to *H. pylori* induced gastritis and dietary induced nonglandular lesions of the squamous forestomach. This mouse strain may provide an important new model to study the effects of diet and chronic *H. pylori* inflammation on the development of gastric and esophageal cancers.

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## Chapter 4

### Alterations in hepatic lipid secretion do not account for *Helicobacter* spp. cholesterol gallstone promotion in C57L/J mice

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## Abstract

Infection with *Helicobacter hepaticus* and *H. rodentium* significantly promotes cholesterol gallstones in C57L/J mice by as much as 70%. One possible mechanism to explain this finding is that these organisms alter the concentration of biliary lipids to promote a more favorable lithogenic environment. To test this hypothesis we infected 4-5 week-old C57L/J mice with both *H. hepaticus* and *H. rodentium* and compared them to uninfected mice. Both groups were fed the lithogenic diet for 6 weeks. After 6 weeks of lithogenic feeding hepatic bile was collected in anesthetized mice for 20 minutes. Bile flow, the concentrations of bile salts, phospholipids and cholesterol were calculated. Infected mice did not differ from uninfected mice with regard to the concentration of bile salts, phospholipids, and cholesterol. However, infected mice displayed a significant increase in ( $P < 0.05$ ; 88.5%) the percentage of hydrophobic bile when compared to uninfected mice (86%). Furthermore, infected mice secreted bile and bile salts significantly more ( $P < 0.05$ ; 0.6313  $\mu\text{mol/hr/100g}$ , 25.07 mL/hr/100g) when compared to uninfected mice (0.5145  $\mu\text{mol/hr/100g BW}$  and 16.50 mL/hr/100g). Biliary secretion is increased in a bile salt independent manner based upon a bile salt independent flow rate of 283.0  $\mu\text{L/hr/100g}$  in uninfected mice and 379.1  $\mu\text{L/hr/100g}$ . These data indicate that although *Helicobacter* spp. infection do physiologically alter hepatic bile and bile secretion these changes are unlikely to explain the ability of these organisms to promote cholesterol gallstone formation.

## **Introduction**

Some *Helicobacter* spp. are capable of promoting cholesterol gallstones in mouse models (15, 16). One possible mechanism to explain this finding is that these organisms alter the concentration of biliary lipids. Several findings support this hypothesis. First, preliminary microarray data generated from livers of *H. hepaticus* and *H. rodentium* infected and uninfected mice indicates that chow fed C57L/J mice alter a variety of enzymes regulating lipid metabolism (14). Furthermore, rodent models of inflammation demonstrate that proinflammatory cytokines alter the production of hepatic cholesterol (5-7, 10, 11). It is currently unclear whether these changes in gene expression correspond to alterations in biliary phenotype. Furthermore, unlike these models of acute infection enterohepatic *Helicobacter* spp. are chronic Th1 inducing pathogens and it is unclear if lipid metabolism and transport are altered similarly in chronic infection (8, 27). Therefore, this study tests not only how *Helicobacter* spp. may contribute to cholesterol gallstones but also furthers the understanding of how biliary transport is affected by chronic infection.

## **Methods**

### *Animal Housing and Husbandry*

C57L/J mice were acquired from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in an Association for the Assessment and Accreditation of Laboratory Care (AAALAC) facility and received food and water *ad libitum*.

### *Infection protocol and lithogenic diet feeding*

Mice were infected with both *H. hepaticus* and *H. rodentium* at 4-5 weeks-old as described previously and boosted with *H. hepaticus* at monthly intervals thereafter (15, 16). Four weeks following initial infection mice were fed a lithogenic diet containing 1% cholesterol 0.5% cholic acid and 15% dairy triglyceride. Mice were fed the lithogenic diet for 6 weeks.

### *Bile collection*

Mice were anesthetized with Avertin (2-2-2 Tribromoethanol, 0.4-0.75 mg/g) intraperitoneally. Following anesthetic administration mice were clipped and aseptically prepared with alternating iodine scrub and 70% isopropyl alcohol. Mice were placed supine underneath a dissecting microscope. The skin and abdominal wall were longitudinally incised along the midline. The common bile duct was identified and isolated. The cystic duct was ligated and the common bile duct was ligated at its egress. A small hole was made in the common bile duct proximal to the ligature and the bile duct was cannulated. Hepatic bile was collected for 20 minutes. At the completion of surgery mice were euthanized and collected bile was stored at -20°C for future analysis.

### *Analysis of cholesterol, bile salts and phospholipids*

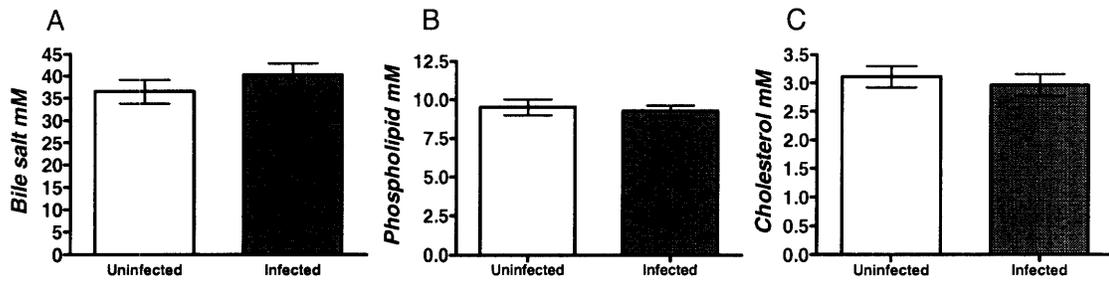
Cholesterol was isolated as described previously (21) and quantified by high-performance liquid chromatography (HPLC) (25). Total bile salts were calculated by the 3 alpha-hydroxysteroid dehydrogenase assay and bile salt species were calculated by HPLC (18,

22). Phospholipids were calculated via Bartlett's assay (2). Bile cholesterol saturation index (CSI) was calculated using the critical tables (3).

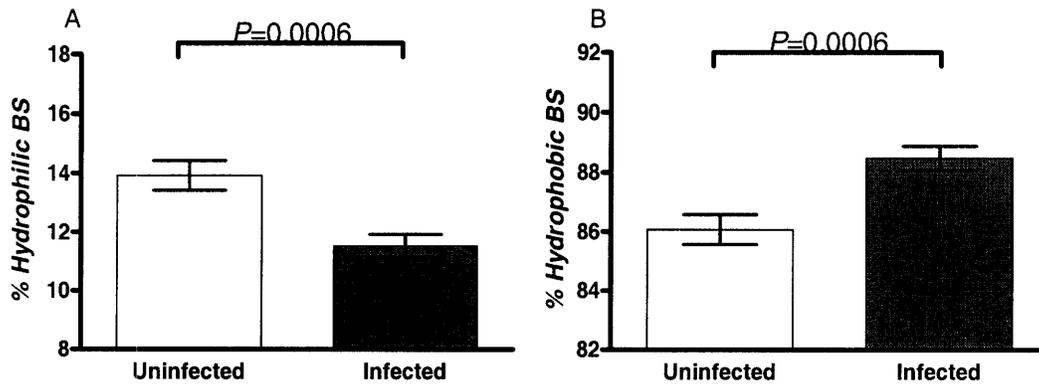
## **Results**

### *Bile salt, cholesterol and phospholipids concentration*

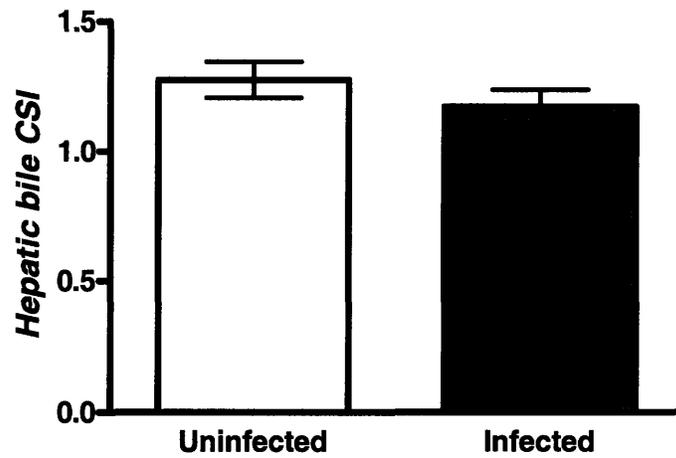
The concentrations of bile salts, cholesterol and phospholipids did not vary between infected or uninfected groups (Fig. 4-1). This was verified in two independent studies analyzing the concentration of these constituents in hepatic bile. Although the total concentration of bile salts did not differ, infected mice displayed a significant decrease in hydrophilic bile salts (Fig. 4-2A,  $P < 0.05$ ;  $\alpha$  and  $\beta$  muricholate and ursodeoxycholate) and a significant increase in hydrophobic bile salts (Fig. 4-2B,  $P < 0.05$ , taurocholate, taurochenodeoxycholate, and taurodeoxycholate). Both infected and uninfected mice developed supersaturated hepatic bile and the groups did not demonstrate significant differences in CSI (Fig. 4-3). Both groups plot within the two-phase zone of the phase diagram (Fig. 4-4) indicating phase separation into micelles and liquid crystals.



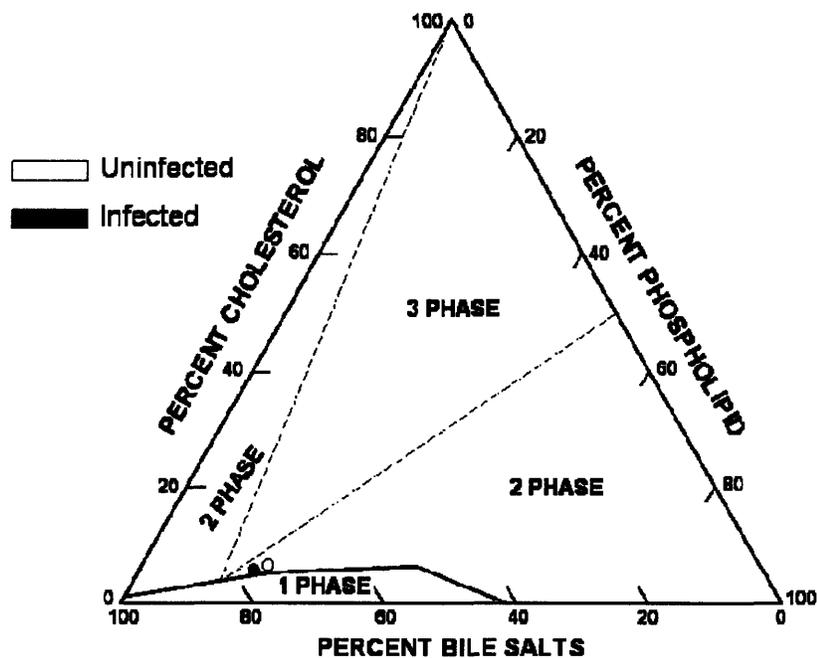
**Figure 4-1:** Concentration of bile salts (A), phospholipids (B) and cholesterol (C) from hepatic bile of *H. hepaticus* and *H. rodentium* infected and uninfected mice. No significant differences were noted between uninfected and infected mice.



**Figure 4-2:** Percent of hydrophilic (A, tauro  $\alpha$  muricholate, tauro  $\beta$  muricholate, tauroursodeoxycholate) and hydrophobic (B, taurocholate, taurochenodeoxycholate, taurodeoxycholate) bile salts present in hepatic bile of *H. hepaticus* and *H. rodentium* infected and uninfected mice. Infected mice display significant decreases in percent of hydrophilic and significant increases in percent hydrophobic bile salts.



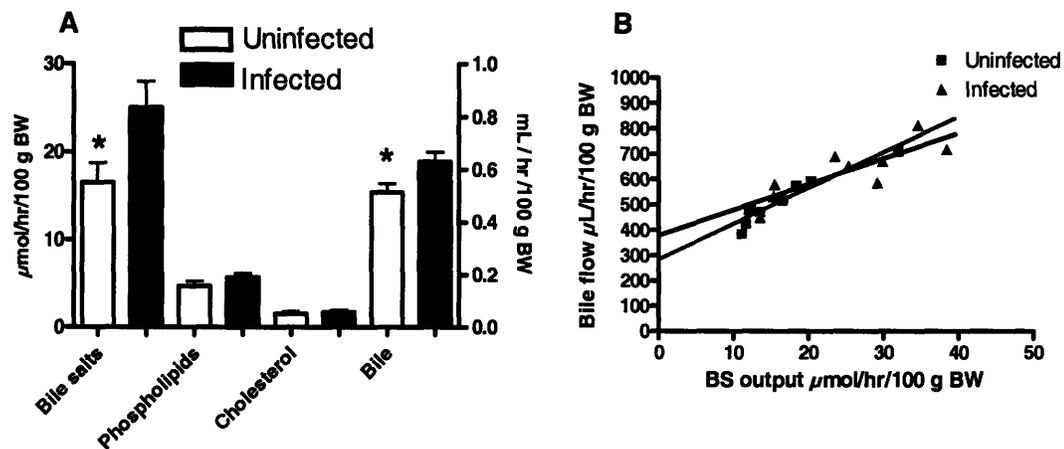
**Figure 4-3:** Hepatic bile cholesterol saturation index (CSI) in *H. hepaticus* and *H. rodentium* infected and uninfected mice. Infection does not significantly alter the CSI of hepatic bile.



**Figure 4-4:** Triangular coordinates and phase diagram in *H. hepaticus* and *H. rodentium* infected and uninfected mice. Both infected and uninfected mice plot within the 2 phase region of the diagram indicating that both micelles and liquid crystals are present.

### *Bile salt, cholesterol, and phospholipids secretion rates*

Significant differences in bile salt excretion were noted between infected and uninfected mice with infected mice increasing bile salt excretion (\*;  $P < 0.05$ , Fig. 4-5A). No significant changes were noted in either cholesterol or phospholipids excretion between the groups. Bile secretion was significantly increased in infected mice when compared to uninfected mice (\*;  $P < 0.05$ , Fig. 4-5A). It appears from linear regression that the increase in bile flow is bile salt independent in infected animals (Fig. 4-5B). Furthermore, the slope of the line, which is inversely proportional to micellar size is greater in uninfected mice indicating that infected mice possess larger micelles. This data is consistent with the greater hydrophobicity of the bile in these mice.



**Figure 4-5:** A: Bile salt, phospholipids, cholesterol secretion (left Y-axis) and total bile secretion (right Y-axis) rate in hepatic bile from *H. hepaticus* and *H. rodentium* infected and uninfected mice. Infection significantly increases the secretion of bile salts and overall bile secretion rate (\*;  $P < 0.05$ ). B: A plot of bile salt output vs. overall bile flow of infected and uninfected mice. Linear regression was used to extrapolate the bile flow at the Y-intercept which indicates bile salt independent flow. Note infected mice demonstrate greater bile salt independent flow and a lowered slope of the extrapolated line indicating larger micelles in bile.

## Discussion

The overall concentration of biliary lipids was not significantly altered between infected and uninfected mice. Therefore, it would seem unlikely that *Helicobacter* spp. promote cholelithogenesis by altering the concentration of lipids in hepatic bile. However, based upon the current data it cannot be discounted that further alterations of lipids occur in the gallbladder and thus promote cholesterol gallstone formation. Aside from these findings two interesting changes did occur with infection. Specifically, infection increases the amount of hydrophobic bile salts in hepatic bile and infection increases bile salt independent bile flow and secretion of bile salts. An increase in hydrophobic bile salts is consistent with promotion of a proinflammatory biliary environment and with previous descriptions of cholesterol gallstone formation (13, 23, 24). Also, it appears that chronic infection induces an increase in bile salt excretion rather than the decrease that is frequently noted in acute models of infection (9, 12, 17, 20, 26). Unlike models of acute infection chronic infection does not appear to induce intrahepatic cholestasis (4, 17). Increasing secretion of bile could be due to either an increase in glutathione or bicarbonate in bile creating an osmotic gradient (1). It is intriguing that *H. hepaticus* possesses a urease enzyme and reside at the bile canaliculus and thus may alter the concentration of bicarbonate through conversion of urea and hydroxyurea to CO<sub>2</sub> which would be converted to bicarbonate at bile pH.

Recent evidence indicates that a Th1 inflammatory response of the gallbladder is important in cholesterol gallstone formation (Chapter 5). These findings were conducted in *Rag2*<sup>-/-</sup> and wild-type BALB/c mice. *Helicobacter* spp. are not nearly as essential in

BALB/c mice since they do not alter production of cholesterol monohydrate or sandy stone formation and only modestly increase cholesterol gallstone formation. However, based upon the Rag mouse model and the current study it appears likely that *Helicobacter* spp. promote cholesterol gallstone formation by promoting a Th1 mediated immune response. This seems likely due to the known ability of these organisms to promote Th1 immunity, and the new knowledge that these Th1 immune responses are vital in cholesterol gallstone formation (8, 19, 27). Perhaps the C57L/J mouse requires *Helicobacter* spp. infection to promote this immune response whereas BALB/c mice do not. This data is supported by histopathological evidence of inflammation appearing only in the gallbladders of infected C57L/J mice whereas inflammation occurs in both infected and uninfected BALB/c gallbladders (15). These data point out that inflammation may participate in cholelithogenesis at both the genetic and environmental level. Specifically, some strains of mice (BALB/c) appear to be highly susceptible to biliary inflammation without infection. This could be due to genetic polymorphisms of inflammatory genes. In contrast, C57L/J mice appear resistant to gallbladder inflammation in the *Helicobacter* spp. uninfected state and therefore require environmental pathogens to promote cholesterol gallstone formation.

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**Chapter 5**  
**T-cell function is critical in cholesterol gallstone formation: the instrumental role of**  
**adaptive immunity in murine cholelithogenesis**

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## **Abstract**

The formation of cholesterol gallstones is complex involving contributions from genes and environmental factors. Although gallbladder inflammation is believed common during cholelithogenesis, the role of immunological factors in cholesterol gallstone formation is unknown. Here we analyzed the role of adaptive immunity in cholesterol cholelithogenesis utilizing immunocompetent BALB/c and isogenic *Rag2*<sup>-/-</sup> mice. When fed a lithogenic diet for eight-weeks, wild-type mice developed cholesterol gallstones (27-80% prevalence) significantly more so than *Rag2*<sup>-/-</sup> mice (~5%,  $P < 0.05$ ). Transfer of functional splenocytes, or T-lymphocytes to *Rag2*<sup>-/-</sup> mice markedly increased cholesterol gallstone formation (26% and 40% respectively,  $P < 0.05$ ) whereas transfer of B-cells was not cholelithogenic (13%). The adaptive immune response increased the expression of *Muc* genes and accumulation of mucin gel. In addition, the presence of T-cells and solid cholesterol monohydrate crystals induced proinflammatory cytokine expression in the gallbladder. These studies show that T-cells are critical in murine cholelithogenesis and act through modulation of gallbladder inflammation.

## **Introduction**

Cholesterol gallstones are composed predominantly of cholesterol monohydrate crystals within a mucin glycoprotein scaffolding (30). They form following nucleation and phase separation of cholesterol monohydrate crystals from supersaturated bile (30). Despite a half-century of basic and clinical research there is currently no satisfactory, non-surgical treatment for definitive management of gallstone patients; as a consequence, the disease continues to be a serious economic burden with an annual cost in the United States approaching 10 billion dollars (30).

Most commonly, gallstones result from the interaction of multiple genes and environmental factors (30). A large twin study proposed that in symptomatic gallstones, genes contribute 25% to the phenotype, shared environmental factors 13%, and unique environmental factors 62% (20). Regardless of the predisposing causes, cholesterol gallstones form when excess hepatically-secreted cholesterol phase-separates in bile as unilamellar vesicles which eventually fuse into multilamellar vesicles (liquid crystals)(4, 30). Cholesterol gallstone formation proceeds when these liquid crystals nucleate cholesterol monohydrate crystals in an inflamed and hypomotile gallbladder (3, 4, 18, 30). Biliary proteins, in particular mucin glycoproteins, promote cholesterol nucleation and precipitation (24, 45). In addition, a variety of other proteins including biliary immunoglobulins may be pronucleating (14-16).

*In vivo* studies analyzing the pathogenesis of cholesterol gallstones rely predominantly on inbred mouse models (27, 30). These models allow investigators to analyze genetic

differences in cholelithogenesis under well-defined conditions. However, despite these controlled environments, different cholesterol gallstone susceptibility prevalence rates are often noted in inbred mice under seemingly identical conditions (27). Recently we demonstrated that several murine enterohepatic bacterial pathogens, specifically *Helicobacter hepaticus*, *H. bilis*, and coinfection with *H. hepaticus* and *H. rodentium* increase cholesterol gallstone formation by as much as 70% in C57L/J mice. These organisms likely contributed to the susceptibility differences noted historically (27). Enterohepatic *Helicobacter* spp. are enzootic in mouse colonies worldwide and colonize the intestine and bile canaliculi (10). Interestingly, the human gastric pathogen *H. pylori*, and two urease negative enterohepatic helicobacters are not able to promote cholesterol gallstone formation in this model (1, 27, 28). Aside from the gallstone phenotype, the most notable findings in mice infected with cholelithogenic *Helicobacter* spp. is an increase in gallbladder mass (a surrogate marker for gallbladder stasis), mucin gel, inflammation, and hyperplasia of the gallbladder epithelium (27). However, *Helicobacter* spp. infection does not alter the absolute or relative compositions of biliary lipids (26).

*Helicobacter* spp. generally cause disease by inducing a Type 1 (Th1) mediated pro-inflammatory immune response (11, 46). In many cases, disease is mediated by the adaptive immune system. Most notably, without an adaptive immune response, *H. pylori* fails to produce gastritis (9). When adaptive immunity is restored by splenocyte transfer, gastritis ensues. In the case of *H. pylori* this effect is mediated by T-cells (8, 9).

Based upon these data we hypothesized that the promotion of cholesterol gallstones by *Helicobacter* spp. occurs via adaptive immunity. Further, we hypothesized that mechanistically, such promotion could occur in one of two ways: Either by increased production of pronucleating immunoglobulins; or T-cell mediated production of pro-inflammatory cytokines altering mucin production (or other unidentified pronucleating proteins) and promoting gallbladder inflammation and hypomotility.

### **Methods:**

#### *Animal maintenance and husbandry*

Mice were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility. Animals were provided with rodent chow (Purina Mills, St. Louis MO) and water *ad libitum* and housed in micro-isolator cages under SPF conditions (free of known bacterial, including *Helicobacter* spp., viral and parasitic mouse pathogens). Mice were fasted for approximately 12 hours prior to being euthanized via CO<sub>2</sub> overdose followed by necropsy.

#### *Helicobacter spp. infection studies*

*Helicobacter hepaticus* 3B1 and *H. rodentium* ATCC 700285 were grown on blood agar plates (Remel; Lenexa, KS) under microaerobic conditions at 37°C. Bacteria were removed from plates and suspended in Brucella broth (Becton, Dickinson and Company, Franklin Lakes, NJ) to a final optical density of 0.6-2.0 at 660nm. Mice were infected as previously described at 4-6 weeks of age via gavage and redosed at 4-week intervals with *H. hepaticus* (27, 28).

### *Bile analysis, tissue harvest, and histopathological analysis*

Gallbladders were removed intact and weighed. Bile was removed from full gallbladders and analyzed for mucin gel accumulation and biliary lipid phenotype by direct and polarized light microscopy. Mucin gel was scored visually by microscopy on a scale of 0-5 (0=0% mucin , 1=20% of the gallbladder filled with mucin, 2= 40% of the gallbladder filled with mucin , 3=60% of the gallbladder filled with mucin, 4=80% of the gallbladder filled with mucin and 5=100% of the gallbladder filled with mucin) (27, 28, 44). Empty gallbladders were immediately flash-frozen in liquid N<sub>2</sub> and stored at -80°C for transcription analysis or they were fixed in 10% neutral-buffered formalin. Formalin fixed gallbladders were routinely processed and stained with hematoxylin and eosin. Gallbladder sections were analyzed by a comparative pathologist blinded to sample identity (ABR).

### *BALB/cJ and Rag mouse studies*

BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME; referred to as Jackson) and C.129S6(B6)-*Rag2*<sup>tm1Fwa</sup> N12 (Rag mice) were bred at MIT following acquisition from Taconic Farms, Inc. (Germantown, NY; referred to as Taconic). Each mouse strain was divided into two groups, one infected with both *Helicobacter hepaticus* and *H. rodentium* and the other group uninfected, thereby providing four groups (n=15-18 mice per group). Mice were fed a standard rodent chow diet until 8-10 weeks of age, at which time they were converted to the lithogenic diet (containing 0.5% cholic acid, 1% cholesterol and 15% dairy derived triglyceride). Mice were fed the lithogenic diet for 8

weeks and were then euthanized with CO<sub>2</sub> and analyzed for biliary phenotype as described (27, 28).

#### *BALB/cAnNTac, Rag, Nude, and Adoptive Transfer Studies*

BALB/cAnNTac, and C.Cg/AnNTac-*Foxn1*<sup>nu</sup> N9 (nude mice) were acquired from Taconic. Rag mice were either acquired directly from Taconic or were bred at MIT from mice originally obtained from Taconic. All mice for these studies were infected with both *H. hepaticus* and *H. rodentium*. Adoptive transfer of splenocytes, T-cells or B-cells occurred when Rag mice were approximately 8-9 weeks of age (approximately one-week prior to initiation of feeding lithogenic diet). Seven mouse groups in total were included in these studies: Rag mice (n=36), Rag mice receiving splenocytes (n=27), nude mice (n=12), Rag mice receiving T-cells (n=15), Rag mice receiving B-cells (n=15), and Wild-type mice (n=15). Mice were fed a standard rodent diet (Purina Mills, St. Louis MO) until 9-10 weeks of age at which time they were converted to the lithogenic diet. Mice were fed the lithogenic diet for 8 weeks and were then humanely euthanized and the biliary phenotype analyzed (27, 28, 44).

#### *Splenocyte transfer*

Splenocytes prepared from naïve BALB/cAnNTac donors were adoptively transferred into Rag recipients. Spleens were aseptically removed from euthanized donor mice and single cell suspensions from the pooled spleens were prepared in RPMI-1640 medium supplemented with 10% fetal bovine serum. After osmotic lysis of red blood cells, splenocytes were washed and examined for viability with Trypan blue exclusion. Cell

number was quantified by an automated cell-counter and validated by counting with a hemocytometer. Finally, the cell concentration was adjusted such that each mouse received  $2 \times 10^6$  viable splenocytes intraperitoneally in 0.2 ml serum-free Hanks balanced salt solution (HBSS pH 7.2).

### *T-cell transfer*

For T-cell transfer studies, splenocytes were prepared first as described above. Initial enrichment utilized Dynal® Mouse T Cell Negative Isolation Kit (Invitrogen Corporation; Carlsbad, CA) per the manufacturer's recommendations. Briefly, the splenocytes were incubated with a cocktail of magnetized-beads coupled to antibodies that bind non-T-cell populations. The bead-bound non-T cell fraction was then separated from the T-cell enriched fraction by placing the sample on a magnetic column. This initial purification provides a highly purified T-cell population. Following this initial negative selection strategy, a second flow cytometry-based approach was used to further eliminate contaminating B-cells. The T-cell enriched fraction was incubated with Cy-labeled anti-B220 antibody (Pharmingen, BD Biosciences, San Diego, CA) and subjected to cell-sorting to "gate out" B cells stained with the antibody. An aliquot of the collected cells was stained with FITC labeled pan-T-cell specific  $\alpha\beta^+$  TCR-specific antibody (Cedarlane, Burlington, NC) confirming a T cell population of >95% purity. Finally, these highly purified T cells were injected retroorbitally with  $2 \times 10^6/0.2$  ml HBSS into recipient mice anesthetized with isoflurane.

### *B-cell transfer*

For B-cell transfer, splenocytes were first prepared as described. Initial enrichment utilized Dynal® Mouse B Cell Negative Isolation Kit (Invitrogen Corporation; Carlsbad, CA) per the manufacturer's instructions. Briefly, the splenocytes were incubated with a cocktail of magnetized-bead coupled antibodies that bind non-B-cell populations and the bead-bound non-B cell fraction was retained on magnetic column as described. Further, to remove any residual T cells, the B-cell enriched fraction was incubated with FITC-labeled pan-T-cell specific  $\alpha\beta^+$  TCR-specific antibody (Cedarlane, Burlington, NC). During sorting, the FITC-labeled T cells were eliminated and B cells were isolated. An aliquot of the sorted cells was labeled with anti-B220 antibody (Pharmingen, BD Biosciences, San Diego, CA) and shown to contain >95% pure B-cell population on re-analysis. Finally, these highly purified B cells with  $2 \times 10^6/0.2$  ml HBSS were injected retroorbitally into recipient mice anesthetized with isoflurane.

### *mRNA preparation and cDNA preparation*

The mouse gallbladder is small and as a result contains minute concentrations of RNA. To maximize RNA yield, the RNeasy Micro Kit (Qiagen Corporation; Valencia, CA), a kit designed to maximize RNA yield from small tissue samples was utilized per the manufacturer's recommendations. Following mRNA isolation, cDNA was prepared utilizing SuperScript™ III First-Strand Synthesis System (Invitrogen Corporation; Carlsbad, CA) according to the manufacturer's recommendation for *Muc* gene analysis or ReactionReady™ First Strand cDNA Synthesis Kit (Superarray Bioscience; Frederick, MD) for cytokine gene expression analysis.

### *Muc gene expression analysis*

To determine mucin (*Muc*) gene expression, four murine mucin gene (*Muc 1*, *Muc 3*, *Muc 4*, and *Muc 5ac*) Fam-labeled primer-probe sets were acquired (Applied Biosystems; Foster City, CA). Quantitative PCR (QPCR) was performed utilizing the ABI-Prism 7700, and *Muc* gene expression levels were normalized to *Gapdh* expression; differences between groups (n=  $\geq 10$  per group) were analyzed via  $\Delta\Delta C_T$  method utilizing either uninfected Rag mice or infected Rag mice as the comparative delta value.

### *Inflammatory gene expression analysis*

Large scale transcriptional screening of genes involved in immunity and inflammation was performed utilizing the RT<sup>2</sup> Profiler™ PCR Array Mouse Inflammatory Cytokines & Receptors (Superarray Bioscience; Frederick, MD). This kit is a SYBR green 96 well based quantitative PCR assay which includes 84 immune and inflammatory based genes, 5 housekeeping genes (*Gusb*, *Hprt1*, *Hspcb*, *Gapdh*, *Actb*) and appropriate controls. Expression of each gene of interest is normalized to the housekeeping genes. Groups (n=5-6 per group) are compared by the  $\Delta\Delta C_T$  method for each individual gene utilizing Rag mice that developed cholesterol monohydrate crystals as the comparative delta. Additionally, relative expression of three common, well characterized Th1 cytokine genes (*Il-1 $\beta$* , *Ifn- $\gamma$* , and *Tnf- $\alpha$* ) and three common, well characterized Th2 cytokine genes (*Il-4*, *Il-10*, and *Il-13*) were analyzed to determine the relative Th1/Th2 balance of each group.

### *Statistical analyses*

All statistical analyses were performed utilizing GraphPad Prism (GraphPad, Inc; San Diego, CA). Cholesterol monohydrate, sandy stone and cholesterol gallstone formation were compared by Fisher's exact test. Non-parametric data were analyzed by the Mann-Whitney Test. Gene expression analysis was performed utilizing one-way analysis of variance and individual data pairs were analyzed by the unpaired t-test. Data were considered statistically significant if the *P* value was < 0.05.

## **Results**

### *Biliary phenotype of BALB/cJ mice compared to Rag mice*

To determine the role of adaptive immunity in cholesterol gallstone formation, BALB/cJ (The Jackson Laboratories, Bar Harbor, ME; Jax) mice were compared to T and B-cell deficient Rag mice (C.129S6(B6)-*Rag2*<sup>tm1Fwa</sup>N12) on a BALB/c background. Regardless of infection status, BALB/cJ mice display significant increases in mucin gel accumulation, and increased prevalence of cholesterol monohydrate crystals, sandy stones and true cholesterol gallstones when compared to Rag mice (*P*<0.05; Fig. 5-1 A-F). Infected BALB/cJ mice significantly increase cholesterol gallstone formation compared to their uninfected counterparts (*P*<0.05; Figure 5-1 A); however, infection does not significantly alter any of the other phenotypic parameters analyzed. Gallbladders from BALB/cJ mice were infiltrated with modest numbers of infiltrating eosinophils and lymphocytes, and developed mild mucosal hyperplasia compared to Rag mice that displayed little or no inflammation and lacked hyperplasia (Fig. 5-1 G-H). These data strongly suggest that adaptive immunity is important in cholesterol gallstone formation

and in BALB/cJ mice *Helicobacter* spp. infection plays a minimal role since changes were noted only in the terminal stage of cholelithogenesis.

#### *Biliary phenotype of Rag mice following splenocyte transfer*

To determine whether transfer of wild-type lymphocytes could restore cholesterol gallstone susceptibility, splenocytes were harvested from BALB/cAnNTac (WT) mice and transferred to their Rag counterparts. Since we wished to utilize a genetically homogeneous group to eliminate any genetic effects on phenotype, mice from Taconic Farms Inc. (Germantown, NY; Taconic) were chosen because we utilized Rag mice of Taconic genetic origin. Additionally, nude mice (C.Cg/AnNTac-*Foxn1nu*N9) on a BALB/c background were analyzed to determine if B-cells, in the absence of T-cells, are relevant in cholelithogenesis.

Both WT and Rag mice receiving splenocytes (ST) exhibit a statistically higher prevalence of gallstones when compared to Rag mice ( $P < 0.05$ , Fig. 5-2A). The ST group also significantly increase cholesterol monohydrate crystal formation compared to Rag mice ( $P < 0.05$ , Fig. 5-2A). WT, nude and ST mice all display significant increases in mucin-gel accumulation when compared to Rag mice ( $P < 0.05$ , Fig. 5-2B). Interestingly, infected WT (BALB/cAnNTac) mice express a lower prevalence of cholesterol monohydrate crystals, sandy stones and true cholesterol gallstones when compared to BALB/cJ mice (Fig. 5-1A and 5-2A). These data indicate that restoration of the cholesterol gallstone phenotype occurs with transfer of functional lymphocytes.

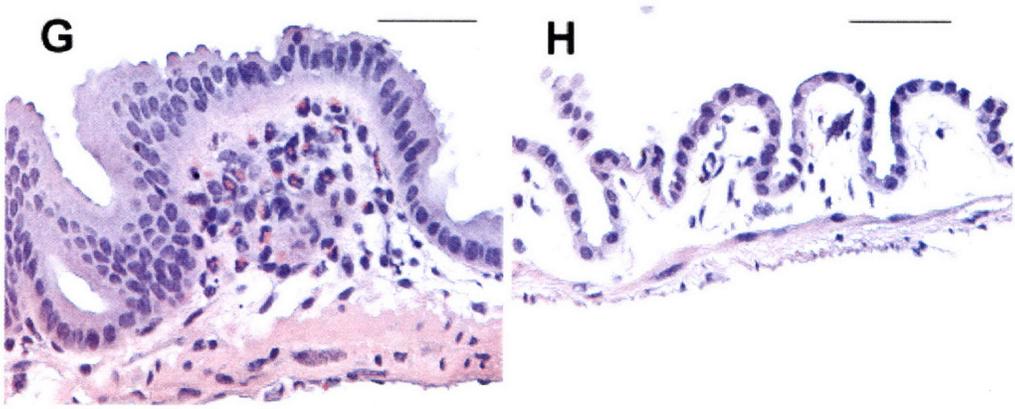
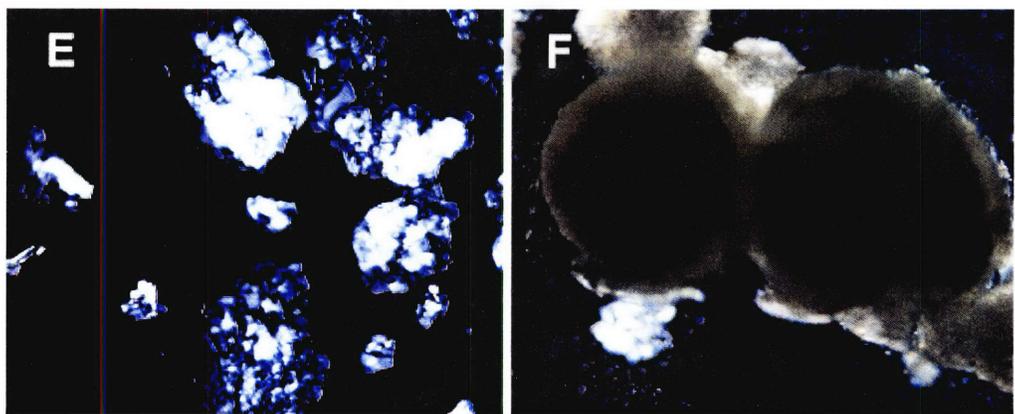
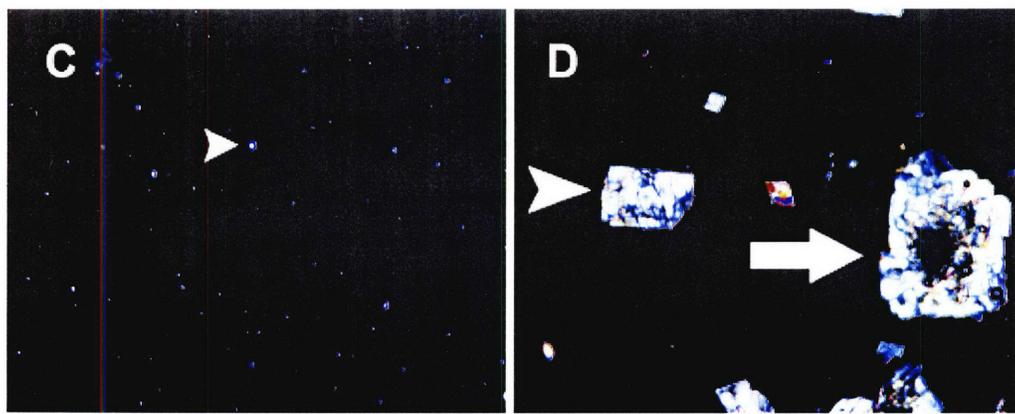
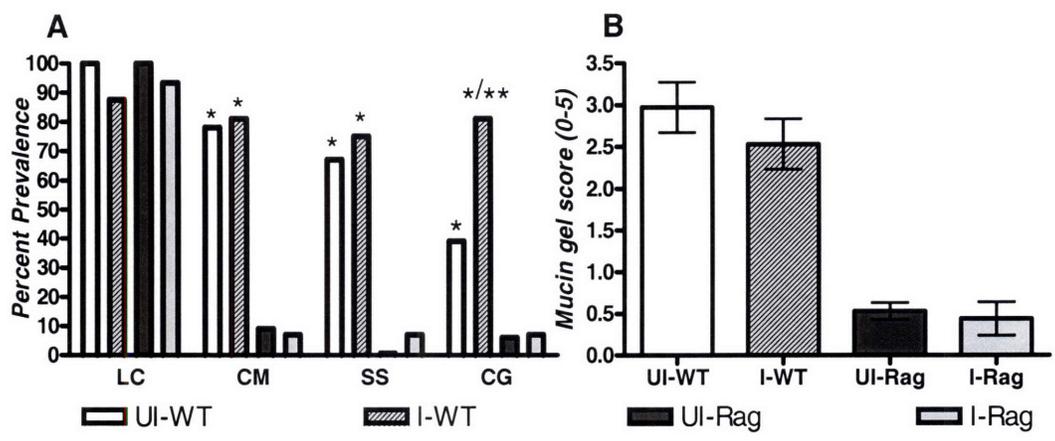
Furthermore, B-cells alone are not capable of inducing the gallstone phenotype, although

they do promote mucin gel accumulation. Finally, cholelithogenesis is substrain dependent in the BALB/c mouse.

#### *Mucin gene expression analysis*

Uninfected BALB/cJ mice upregulate *Muc1* when compared to either uninfected or infected Rag mice ( $P<0.05$ , Fig. 5-3A) and upregulate *Muc4* compared to all other groups ( $P<0.05$ , Fig. 5-3C). In contrast, infected BALB/cJ mice increase *Muc3* expression compared to uninfected Rag mice ( $P<0.05$ , Fig. 5-3B).

Mucin gene expression patterns differ in WT mice and ST mice ( $P<0.05$ , Fig. 5-3E-H). ST mice upregulate *Muc3* when compared to all other groups ( $P<0.05$ , Figure 5-3F), *Muc4* when compared to WT mice ( $P<0.05$ , Fig. 5-3G), and *Muc5ac* when compared to either nude or Rag mice ( $P<0.05$ , Fig. 5-3H). Nude mice increase expression of *Muc1* compared to ST mice (Fig. 5-3E). Finally, WT mice display an increased expression of *Muc5ac* compared to Rag mice ( $P=0.06$ , Fig. 5-3H). These data demonstrate that expression of mucin genes is dependent upon the commercial source of the inbred mice, immune status and status of *Helicobacter* spp. infection.

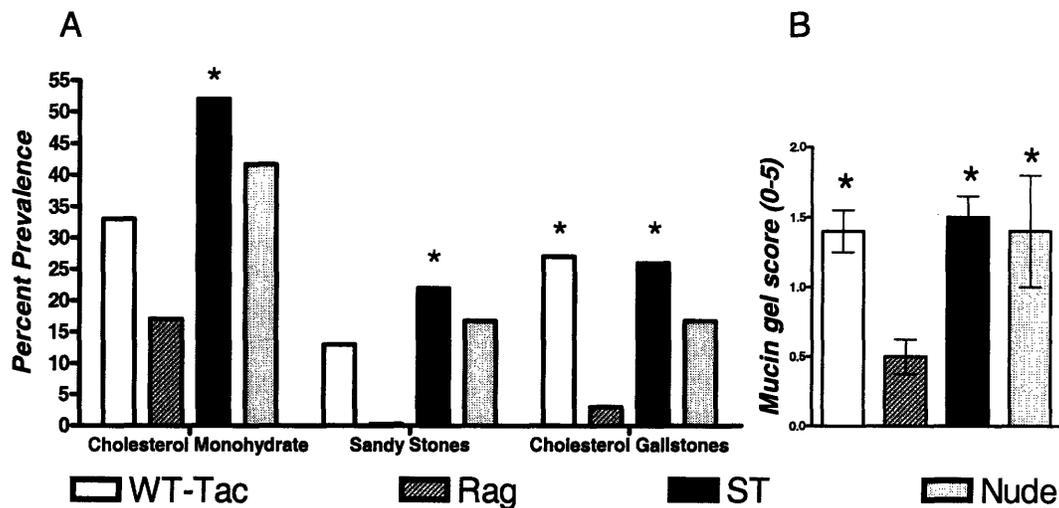


**Figure 5-1** (following page): Data represent percent prevalence of liquid crystals (LC), cholesterol monohydrate crystals (CM), sandy stones (SS) and cholesterol gallstones (CG) (A) and mucin gel score (B) (0-5). Virtually all mice formed liquid crystals (A, C white arrowhead) indicating that bile is supersaturated with cholesterol and phase separated. Those mice without liquid crystals developed cholesterol monohydrate crystals (D white arrowhead, E) or beyond indicating that they possessed lithogenic bile; however; they rapidly progressed beyond the liquid crystalline stage. Either uninfected (UI-WT) or infected BALB/cJ (I-WT) mice formed cholesterol monohydrate (A, D), sandy stones (A, D white arrow, E), and cholesterol gallstones (A, F) significantly more frequently than uninfected (UI-Rag) or infected Rag mice (I-Rag) and accumulated significantly more mucin gel (B) than Rag mice. Infected BALB/cJ mice also had an increased prevalence of cholesterol gallstones when compared to uninfected BALB/cJ mice. Gallbladder tissue of infected mice (G) was infiltrated by a mixed eosinophilic/lymphocytic tissue population. Additionally, these tissues were typically thickened and mild mucosal hyperplasia was noted. In contrast, Rag mice (H) rarely possessed inflammatory infiltrates in the gallbladder and mucosa of Rag mice generally consisted of cuboidal epithelium that was rarely hyperplastic.

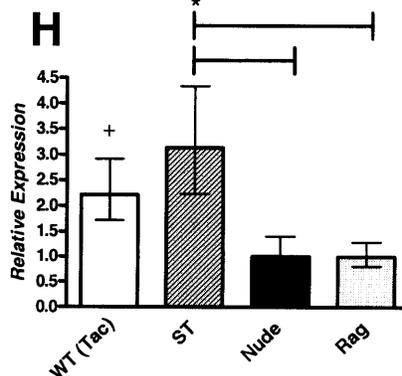
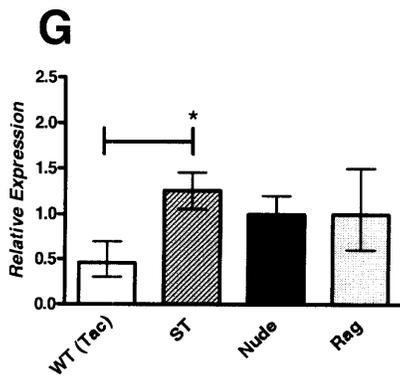
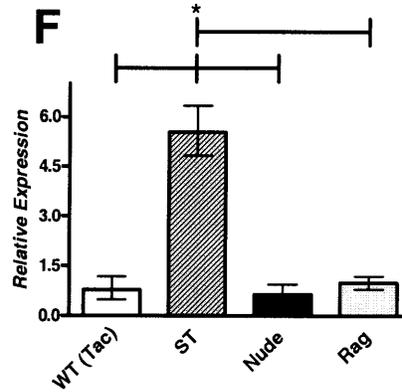
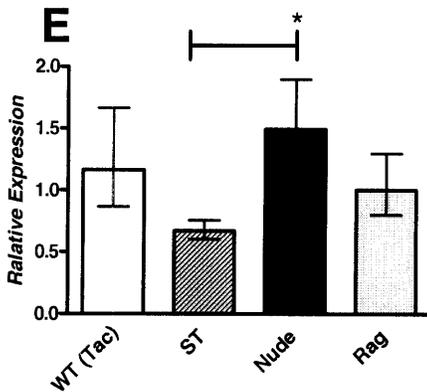
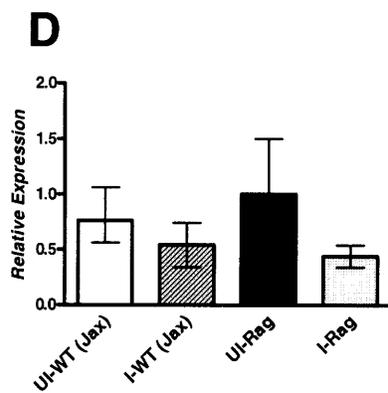
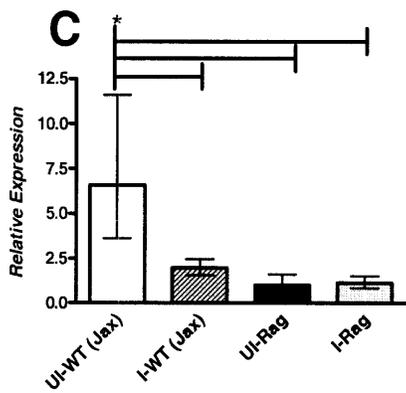
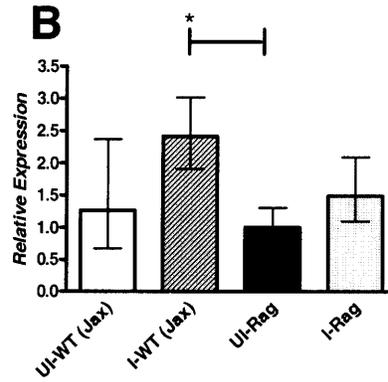
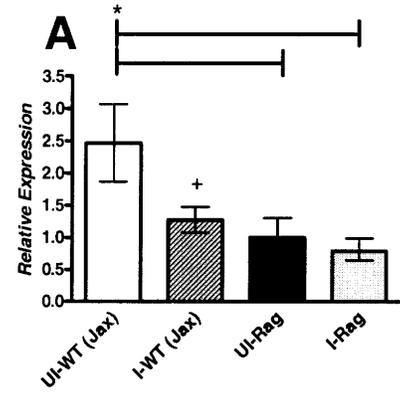
G,H: Bars= 60  $\mu$ M

\*:  $P < 0.05$  compared to Rag mice

\*\*: $P < 0.05$  compared to uninfected BALB/cJ mice



**Figure 5-2:** Data represent percent prevalence of cholesterol monohydrate crystals, sandy stones and cholesterol gallstones (A) and mucin gel score (B) in BALB/cAnNTac (WT), Rag mice, Rag mice receiving splenocytes (ST), and nude mice. All mice developed liquid crystals (not shown). Mice receiving splenocytes formed cholesterol monohydrate crystals, sandy stones, and cholesterol gallstones significantly more frequently than Rag mice. Further, WT mice displayed an increased prevalence of cholesterol gallstones when compared to Rag mice. WT, ST, and nude mice all significantly increased mucin gel accumulation when compared to Rag mice as indicated by the mucin gel score (B). BALB/cAnNTac mice exhibited a lower prevalence of cholesterol monohydrate crystals, sandy stones and cholesterol gallstones when compared to BALB/cJ mice (Fig 1A and Fig 2A). \*:  $P < 0.05$  when compared to Rag mice



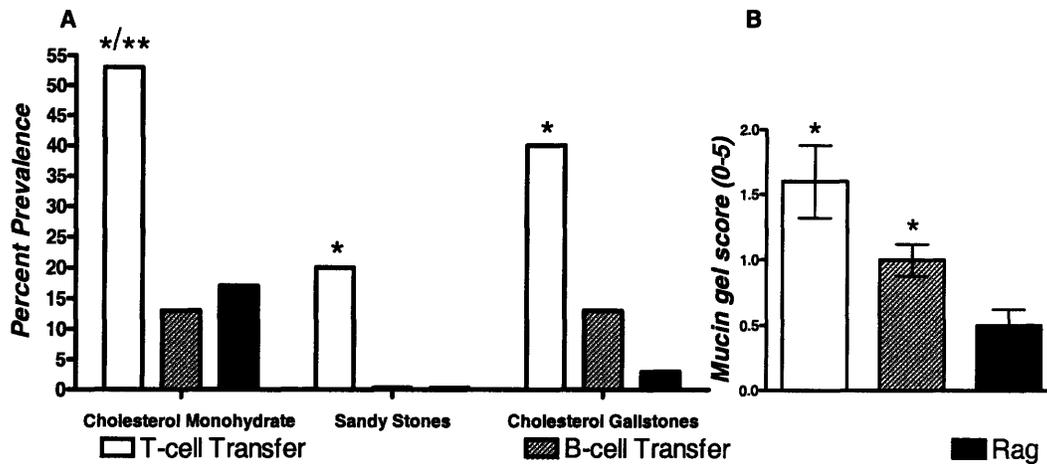
**Figure 5-3** (preceding page): Data represent gallbladder expression of *Muc1* (A), *Muc3* (B), *Muc4* (C), and *Muc5ac* (D) from the BALB/cJ study or *Muc1* (E), *Muc3* (F), *Muc4* (G), and *Muc5ac* (H) from the second BALB/cAnNTac and splenocyte transfer study. Gene expression is normalized to *Gapdh* expression and groups are compared by the  $\Delta\Delta C_T$  method utilizing uninfected Rag (UI-Rag) mice (A, B, C, and D) or infected Rag mice (I-Rag) (E, F, G, and H) as the baseline (i.e. 1) expression level. Uninfected BALB/cJ mice (UI-WT) significantly upregulated *Muc1* (A) and *Muc4* (C) when compared to Rag mice of either infection status. Additionally, uninfected BALB/cJ mice significantly increased expression of *Muc4* (C) when compared to infected BALB/cJ (I-WT) mice. Infected BALB/cJ mice significantly increased *Muc3* expression when compared to uninfected Rag mice (B). Infected BALB/cJ mice also upregulated *Muc1* expression to a nearly significant level compared to infected Rag mice (A). ST mice demonstrated significant increases in *Muc3* (F) compared to all other groups, *Muc4* compared to wild-type mice, (G) and *Muc5ac* compared to either nude or Rag mice (H). Wild-type mice significantly increased expression of *Muc5ac* compared to Rag mice (H). Nude mice demonstrated a significant increase in *Muc1* compared to ST mice (E).

\*:  $P < 0.05$  when compared to bracketed group

+:  $P < 0.06$  when compared to infected Rag mice

#### *Biliary phenotype of Rag mice adoptively transferred with T or B-cells*

To determine whether T or B-cells alone are critical effectors in cholesterol gallstone formation, individual cell types were harvested, purified and transferred to Rag mice. Rag mice receiving T-cells (T-cell) form cholesterol monohydrate crystals significantly more frequently compared to Rag mice or mice receiving B-cells (B-cell) ( $P < 0.05$ , Fig. 5-4A). Moreover, they demonstrate a significant increase in cholesterol gallstone prevalence compared to Rag mice ( $P < 0.05$ , Fig. 5-4A). Both B and T-cell reconstituted mice increase accumulation of mucin gel compared to Rag mice ( $P < 0.05$ , Fig. 5-4B). These data indicate that although both B and T-cells induce mucin gel accumulation, only T-cells increase cholesterol gallstone formation.



**Figure 5-4:** Data represent percent prevalence of cholesterol monohydrate crystals, sandy stones and cholesterol gallstones (A) and mucin gel score (B) in Rag mice receiving T-cells only (T-cell), Rag mice receiving B-cells only (B-cell), or Rag mice receiving no cells. All mice developed liquid crystals (not displayed). Mice receiving T-cells only demonstrated an increased prevalence of cholesterol monohydrate crystals, sandy stones, and cholesterol gallstones when compared to Rag mice. Further, these mice had an increased prevalence of cholesterol monohydrate crystals when compared to B-cell mice (A). Both T-cell and B-cell mice significantly accumulated mucin gel when compared to Rag mice (B).

\*:  $P < 0.05$  when compared to Rag mice

\*\* :  $P < 0.05$  when compared to B-cell mice

#### *Cytokine and inflammatory gene product expression analysis*

Others have described the ability of cholesterol monohydrate crystals to induce inflammation in otherwise healthy gallbladders (35). To determine if T-cell mediated cholesterol gallstone formation could be due, at least in part, to their ability to promote local cytokine production following solid cholesterol phase separation as cholesterol monohydrate crystals, sandy stones or cholesterol gallstones, cytokine expression was analyzed in gallbladder tissue. Mice were divided into four groups based upon lymphocyte status (T-cell positive or Rag) and biliary phenotype (formation of liquid crystals or progressing beyond the liquid crystalline stage). Therefore, the following four

groups were analyzed: 1. Rag mice developing cholesterol monohydrate crystals whether free or agglomerated into stones (Rag CM); 2. Rag mice developing only liquid crystals (Rag LC); 3. T-cell mice developing cholesterol monohydrate crystals (T-cell CM); 4. T-cell mice developing only liquid crystals (T-cell LC).

T-cell CM mice consistently increase expression of genes indicative of a Th1 inflammatory response (Fig. 5-5). In contrast, T-cell LC mice dampen their Th1 response (Fig. 5-5). Furthermore, in the absence of T-cells (Rag mice), solid cholesterol crystals do not promote a Th1 inflammatory response (Fig. 5-5). Essentially, Rag CM mice respond to solid cholesterol crystals in an opposing manner to mice with T-cells (Fig. 5-5). These global changes are easily demonstrated by comparing the mean expression of three well-described Th1 cytokines (*Il1 $\beta$* , *Ifn- $\gamma$* , *Tnf- $\alpha$* ) with three well described Th2 cytokines (*Il4*, *Il10*, *Il13*). Th1 cytokines are highly expressed in T-cell CM mice compared to all other groups ( $P < 0.05$ , Fig. 5-5N). In contrast, common Th2 cytokines are not expressed differentially nor are they expressed to any large degree in any group (Fig. 5-5O). Additionally, *Tgf- $\beta$* , which was shown previously to be expressed during cholesterol gallstone induced gallbladder damage and fibrosis (22) is highly expressed in T-cell CM mice (Table 5-1).



**Figure 5-5** (preceding page): Data represent gallbladder expression of genes generally involved in a Th1 immune response. Expression values are normalized to five control genes included in the assay and compared to Rag CM mice by the  $\Delta\Delta C_T$  method and logarithmically converted and expressed in relation to Rag CM mice (A-M). Common Th1 cytokines (*Il1- $\beta$* , *Ifn- $\gamma$* , and *Tnf- $\alpha$* ) (N) and Th2 cytokines (*Il-4*, *Il-10*, *Il-13*) (O) are analyzed by taking the mean  $C_T$  value of each gene group relative to the five control genes, logarithmically converting this value and multiplying by  $10^4$  for ease of display. All graphs depict mean expression +/- standard error of the mean (both calculated prior to logarithmic conversion). T-cell mice which developed cholesterol monohydrate crystals significantly increase Th1 cytokines compared to all other groups analyzed. Additionally, for all groups analyzed Th1 cytokines are expressed 10 fold or greater compared to Th2 cytokines (N-O).

\*: $P < 0.05$  when compared to bracketed group

#: $P < 0.05$  when compared to all Rag mice (Rag CM + Rag LC)

$\Delta$ : $P < 0.05$  when compared to all other groups

Table 5-1: Fold changes of gallbladder inflammatory gene expression relative to Rag CM mice

Gene Name <sup>a</sup>	Rag LC	T-cell LC	T-cell CM
<b>Th1 genes</b>			
<i>Casp1</i>	6.3 (3.9-9.5) <sup>b</sup>	2.3 (1.4-3.5)	9.9 (6.7-13.8)*
<i>Ccl1</i>	1.3 (0.4-4.1)	0.9 (0.4-1.9)	32.9 (7.3-147.9)†
<i>Ccl5</i>	23.3 (13.8-38.9)‡	8.0 (4.4-14.4)	26.4 (16.8-40.9)‡
<i>Ccr5</i>	3.8 (2.5-5.7)‡	1.1 (0.5-2.2)	5.2 (3.8-7.1)‡
<i>Cxcl 5</i>	0.6 (0.4-0.8)	1.0 (0.5-1.8)	19.0 (5.5-65.7)†
<i>Cxcl9</i>	27.6 (8.8-86.6)	4.4 (1.1-18.0)	40.3 (22.3-72.8)‡
<i>Cxcl10</i>	6.2 (3.5-11.1)	1.0 (0.4-2.6)	14.9 (7.8-28.2)‡,*
<i>Cxcl11</i>	29.8 (15.8-56.2)‡	15.4 (7.3-32.6)	133.9 (63.9-280.7)‡
<i>Cxcr 3</i>	9.6 (6.0-15.6)‡	2.7 (0.9-8.3)	24.2 (8.4-69.2)‡
<i>Ifn-γ</i>	30.7 (15.4-57.9)‡	3.3 (0.8-13.5)	125.4 (55.8-266.1)‡,*
<i>Il1β</i>	3.1 (2.3-4.0)‡	1.1 (0.7-1.7)	6.7 (4-11.3) ‡,*
<i>IL8 rb</i>	2.7 (0.6-12.8)	1.5 (0.4-5.1)	43.0 (20.7-89.4)‡,*†
<i>Tnf-α</i>	6.7 (3.2-13.8)	2.0 (1.1-3.7)	31.0 (14.4-66.8) ‡,*†
<b>Th2 genes</b>			
<i>Ccl22</i>	4.3 (3.4-5.5)‡	0.5 (0.2-1.4)	11.7 (4.3-31.6)
<i>Ccr3</i>	8.3 (5.7-12.1)‡	2.0 (1.1-3.7)	7.2 (4.7-11.1)‡
<i>Il10ra</i>	5.2 (3.8-6.9)‡	2.8 (2.1-3.8)	6.8 (4.5-10.3)‡
<b>Mixed function Genes</b>			
<i>Ccl2</i>	19.5 (12.1-31.4)‡	1.9 (0.6-5.4)	16.0 (8.6-29.7)‡
<i>Ccl8</i>	25.2 (16-40)	10.2 (4.7-22.0)	43.0 (22.1-83.7)‡
<i>Ccl9</i>	7.9 (4.5-14.1)‡	1.3 (0.7-2.6)	3.5 (1.8-6.9)
<i>Ccr1</i>	2.1 (1.5-2.8)*	0.6 (0.4-0.8)	2.2 (1.4-3.4)
<i>Ccr2</i>	11.8 (7.8-17.7)‡	2.5 (1.3-4.9)	12.8 (9.2-17.9)‡,*
<i>Ccr4</i>	7.7 (2.0-29.0)	15.9 (7.7-32.8)	226.8 (85.2-603.4)‡,†
<i>Cxcl 4</i>	0.9 (0.7-1.0)	0.45 (0.3-0.8)	2.3 (1.5-3.6)*
<i>Il2rg</i>	3.7 (2.4-5.7)‡	1.1 (0.6-2.1)	5.5 (3.6-8.4)‡
<i>Il 15</i>	7.9 (5.4-11.5)‡	3.3 (2.5-4.3)	10.9 (4.3-27.9)
<i>IL16</i>	8.7 (6.7-11.1)‡	5.1 (3.8-6.9)	8.7 (5.2-14.4)‡
<i>Ltb</i>	5.8 (4.2-8.0)‡	2.0 (1.2-3.2)	5.2 (3.5-7.7)‡
<i>Tgfb1</i>	1.6 (1.3-2.0)	0.7 (0.5-1.0)	2.1 (1.7-2.5)*
<b>Innate immune genes</b>			
<i>Crp</i>	2.5 (2.3-2.8)‡,*,**,¥	1.5 (1.3-1.7)	0.7 (0.5-1.1)
<b>Undefined genes</b>			
<i>Il1f6</i>	2.0 (0.8-5.0)	4.1 (2.9-5.8)	73.4 (28.2-191.2)‡,+,†
<i>Il1r2</i>	4.7 (2.6-8.4)	0.7 (0.2-1.9)	22.1 (7.8-62.9)‡,*†
<i>IL17b</i>	32.4 (19.2-54.9) ‡,*	1.3 (0.4-4.0)	44.0 (14.6-132.3)‡
<b>T-cell expressed genes</b>			
<i>Tnfsf5</i> (CD40L)	0.52 (0.2-1.3)	21.4 (8.3-55.0)‡,+,†	40.8 (16.1-103.5) ‡,+,†

a: Only genes demonstrating significant changes are displayed: *Abcf1*, *Bcl6*, *Blr1*, *C3*, *Ccl3*, *Ccl4*, *Ccl6*, *Ccl7*, *Ccl12*, *Ccl17*, *Ccl19*, *Ccl20*, *Ccl22*, *Ccl24*, *Ccl25*, *Ccr6*, *Ccr7*, *Ccr8*, *Ccr9*, *Cxcl1*, *Cxcl12*, *Cxcl13*, *Cxcl15*, *Gpr2*, *Il1a*, *Il1f8*, *Il1r1*, *Il2rb*, *Il3*, *Il4*, *Il10*, *Il10ra*, *Il10rb*, *Il11*, *Il2rb*, *Il13*, *IL13ra1*, *Il5ra*, *Il6ra*, *Il6st*, *Il18*, *Il20*, *Itgam*, *Itgb2*, *Lta*,

*Mif*, *Scye1*, *Spp1*, *Tnfrsf1a*, *Tnfrsf1b*, *Tollip*, and *Xcr1* did not demonstrate any significant changes between any of the groups and are not displayed.

b: Data represent fold changes and ranges compared to Rag mice developing cholesterol monohydrate crystals (expression of 1 for each gene). Ranges are based on plus or minus standard error of the mean prior to logarithmic conversion.

\*:  $P < 0.05$  vs. T-cell LC mice

†:  $P < 0.05$  vs. Rag mice (combined)

‡:  $P < 0.05$  vs. Rag CM mice

\*\* :  $P < 0.05$  vs. T-cell CM mice

+:  $P < 0.05$  vs. Rag LC mice

¥:  $P < 0.05$  vs. T-cell mice (combined)

## Discussion

This study provides the first detailed description linking adaptive immunity to cholesterol gallstone pathogenesis. It is clear from the data that T-cells promote cholesterol gallstone formation markedly. Additionally, we demonstrate that T-cells and solid cholesterol crystal phase separation induce a potent, Th1-mediated, local inflammatory response.

However, in the absence of T-cells, solid cholesterol crystals down-regulate inflammatory genes. This downregulation likely prevents subsequent cytokine-mediated damage and ensuing gallbladder motility defects.

The potential influence of inflammation and the immune system on cholesterol gallstone formation was first evidenced by Lee and coworkers in a diet-induced prairie dog model of cholesterol gallstones (25). When treated with high-dose acetylsalicylic acid (ASA), these rodents maintained supersaturated bile but did not nucleate solid cholesterol monohydrate crystals or form cholesterol gallstones (25). However, ASA has a wide range of *in vivo* activities and the exact mechanism whereby it prevented cholesterol gallstone formation was never elucidated (13). Additional publications attempting to link the immune system to cholesterol gallstone formation have suggested the importance of

immunoglobulins in promoting nucleation (14-16, 29, 41). However, the *in vivo* role of immunoglobulins in cholesterol gallstone pathogenesis has never been demonstrated. Moreover, additional *in vitro* studies indicated that inflammatory products influence mucin production in cultured gallbladder epithelial cells (21, 37). The influence of inflammatory mediators on mucin gene expression is also well established in studies on the respiratory tract epithelium (38). Moreover, in several previous investigations, attempts were made to examine the *in vivo* influence of inflammation on cholesterol gallstone pathogenesis (42, 43). These investigations invariably concluded that gallbladder inflammation and immunoglobulin production occurred when mice were fed a lithogenic diet (42, 43). By their design, these investigations could not definitively determine a role for inflammation and immunity; Both gallstone susceptible (C57L) and resistant (AKR) strains were studied (42, 43) and these strains of mice display marked differences in their ability to supersaturate bile. Therefore, the influence of genes responsible for bile supersaturation could not be eliminated from consideration in these models (42, 43). The current murine study eliminates this variable by utilizing isogenic strains of mice differing only in their ability to mount an adaptive immune response.

In the present study, B-cells and therefore immunoglobulins were not needed to achieve a high prevalence of cholesterol gallstone formation in our models. Although B-cells do promote mucin gel formation it appears, that the *in vivo* role of biliary immunoglobulins in promoting cholesterol gallstone formation is minor.

Gallbladder mucin gel formation differs in mice depending on the commercial source of the mouse as well as the immune status. Although four *Muc* genes were examined in the current study, no single gene appeared to play a central role in cholesterol gallstone pathogenesis. However, our expression data indicated a general trend of increased *Muc* gene expression in mice with a functioning adaptive immune system. Also, there are more than four *Muc* genes expressed in the murine gallbladder (45). However, the complete *Muc* and *Muc*-like genes are extensive and their complete expression profile in the murine gallbladder remains to be elucidated (6).

It is unclear from the present results whether a Th1 response promotes cholesterol monohydrate crystal formation or is the result of cholesterol monohydrate crystal formation on the gallbladder mucosa (i.e. an immune response to a noxious stimulus). It is possible that the Th1 response may be both promoted by and in turn promote cholesterol monohydrate crystal formation thus creating a repetitive injurious cycle. Promotion of cholesterol monohydrate crystals could occur through modulation of *Muc* genes and accumulation of mucin gel. This event, although likely important, does not appear sufficient in cholelithogenesis because mice possessing only B-cells accumulate mucin gel, albeit to a lesser degree than mice with only T-cells. A plausible mechanism for these observations based upon our data is that mucin gel accumulation promotes cholesterol monohydrate crystal formation; but it is the subsequent cytokine response to accumulated cholesterol monohydrate crystals that determines progression of the disease. Our data from Rag mice that developed cholesterol monohydrate crystals illustrates that these mice down-regulate Th1 cytokines, chemokines and receptors and as a result do not

progress to cholesterol gallstone formation. Interestingly, mice adoptively transferred with T-cells and developing only liquid crystals displayed less pronounced cytokine expression than Rag mice that developed liquid crystals. These mice may represent a population with a greater percentage of regulatory T-cells, thereby dampening the immune response. Regardless of the inciting cause, it is clear that cholesterol gallstone pathogenesis in this model is strongly associated with the stimulation of a robust Th1-mediated immune response.

The data in the current study parallel several important findings in the well-established paradigm underlying pathogenesis of *Helicobacter pylori*. In the absence of T-cells, *H. pylori*-mediated gastritis is markedly attenuated (9). However, when T-cells are transferred into the host, gastritis ensues and occurs via a Th1-mediated immune response (8, 9). Additionally infected Severe Combined Immunodeficiency (SCID) mice which, like Rag mice, lack functional T and B cells, develop more severe disease than infected wild-type mice transplanted with splenocytes (8, 9). Indeed mice receiving T-cells demonstrated a higher prevalence of cholesterol gallstones than their wild-type counterparts. This result may represent transfer of a subset of T-cells that promote inflammation (T-effector cells) or clonal expansion of a reactive subset of cells in the host. Demonstrated previously is the effect of such T-effector populations in mouse models of inflammatory bowel disease and cancer (33, 34).

BALB/cJ (Jackson) and BALB/cAnNTac (Taconic) mice demonstrate markedly different cholesterol gallstone prevalence rates. These differences could be due to either genetic or

environmental influences. Genetic differences may derive from either contamination prior to the separation of these two substrains, or due to genetic drift (the cumulative effect of spontaneous mutations over time), after the lines separated. Although genetic drift cannot be discounted, a lack of genetic contamination of BALB/c mice was demonstrated previously and therefore seems unlikely (17). Alternatively, environmental factors could be important. Specifically, husbandry practices differ between Taconic and Jax. Taconic rederives their mice in a germ-free state and then colonizes them purposefully with altered Schaedler flora (ASF) containing eight defined gastrointestinal bacteria (7, 40). These mice are then maintained within a specific pathogen free (SPF) barrier, where they may become colonized with other non-pathogenic microbes. In contrast, Jax does not rederive their mice into a germ-free state nor do they colonize the mice with ASF. Instead, they are maintained in a SPF environment and possess an undefined gut microflora (40). Indeed microbial differences were confirmed in a recent analysis of Jax mice, which demonstrated that they possessed a diverse population of intestinal microbes (23). Interestingly, Jax mice were found to be free of lactobacilli which account for 2 of the 8 bacteria in ASF (39). Lactobacilli protect against Th1-mediated disease and diminish proinflammatory cytokine production (31, 32). It is intriguing to hypothesize that these organisms afford mice of Taconic origin protection against cholesterol gallstone formation. In support of this hypothesis, is the observation that a variety of inbred strains of Taconic mice were resistant to infection with *Giardia lamblia* whereas isogenic Jax mice were susceptible. When these mice were co-housed *Giardia* spp. resistance was transferred to the Jax mice indicating that resistance was transmissible (40). Further evidence for the role of indigenous microbes was

demonstrated in studies using BALB/cAnNCr (from the National Cancer Institute, Bethesda, MD) mice which are genetically most closely related to BALB/cAnNTac mice (both are of the BALB/cAnN lineage). However, BALB/cAnNCr (of unknown *Helicobacter* spp. status), unlike BALB/cAnNTac mice (27% gallstone prevalence when infected with *Helicobacter* spp.), develop 100% prevalence of cholesterol gallstones when fed a diet containing 1% cholesterol and 0.5% cholic acid (35). Therefore these mice behave phenotypically more like the genetically less related BALB/cJ strain (80% gallstone prevalence when infected with *Helicobacter* spp).

In summary, T-cell function appears critical in the pathogenesis of murine cholesterol gallstones. We propose a biological model whereby adaptive immunity alters the production of mucin gel and expression of mucin genes. The presence of T-cells and cholesterol monohydrate crystals leads to gallbladder inflammation that promotes tissue damage and subsequent gallbladder motility defects as well as further production of pronucleating agents. Furthermore, we hypothesize that infectious agents and other Th1-inducing conditions are likely to contribute to cholesterol gallstone formation in this model as well as humans. For example in humans, hepatitis C virus infection and Crohn's disease, both chronic Th1-inducing diseases, are correlated positively with increased prevalence of cholesterol gallstones (2, 5, 12, 19). To date no detailed population studies have focused on analyzing the inflammatory environment of the human gallbladder in the presence or absence of cholesterol crystals and/or gallstones. The current study argues poignantly for the importance of conducting such studies. Our systematic research describes a hitherto unappreciated new dimension in cholesterol gallstone pathogenesis

which may prove crucial in human patients. Understanding the importance of the adaptive immune system in cholesterol gallstone formation may change not only the way we study cholesterol gallstone pathogenesis and disease, but could alter the paradigms whereby the disease is treated and possibly prevented. The findings in the present study are reminiscent of other diseases in both mouse models and humans (i.e. gastric ulcers, diabetes mellitus, atherosclerosis) which were once thought to represent merely disturbances in biochemistry, biophysical-chemistry and metabolism but can now be clearly identified as polyfactorial conditions with strong immunologic components (36, 47).

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## Chapter 6

### Conclusion

It is clear from the studies described that cholesterol gallstone formation is a complex polyfactorial disease. Based upon the literature and the data described in this manuscript it appears that at a minimum supersaturated bile and an intact adaptive immune system are required for cholesterol gallstone formation. Furthermore, it seems likely that the type of immune response generated is critical. Specifically, a polarized Th1 response is generated in response to cholesterol monohydrate crystals when the host possesses functional T-cells. Like cholesterol supersaturation, which occurs because of both genetic and environmental factors, the immune system also appears to be influenced by both of these factors. In BALB/cJ mice environmental pathogens are not necessary to develop a high prevalence of cholesterol gallstones (~40%) whereas without infection with cholelithogenic *Helicobacter* spp. C57L/J mice rarely develop stone (~10%).

The studies described in this thesis analyzed only a narrow spectrum of murine pathogens and further analyzed only the adaptive arm of the immune system. Three critical areas not addressed in the current study are the role of other pathogens, the role of the indigenous microbiota, and the role of the innate immune response in cholesterol gallstone formation. Based upon differences in cholesterol gallstone susceptibility between substrains of BALB/c mice it appears likely that the indigenous microbiota of the host is important in susceptibility and resistance to cholesterol gallstones. Furthermore, since the biliary epithelium possesses all the components of adaptive immunity it seems likely that this system may too be important in cholelithogenesis.

Utilizing the data acquired in these murine studies it is possible to extrapolate and hypothesize about the role of infection and inflammation in human cholesterol gallstone formation. It is likely that genetic polymorphisms in genes involved in the immune system influence cholesterol gallstone formation. For example, a person who is genetically more predisposed to develop a Th1 immune response may be more susceptible to cholesterol gallstone formation. Environmental exposure to Th1 inducing agents will further contribute. A caution to these scenarios is that without supersaturated bile inflammation cannot contribute to cholesterol gallstone formation.

In conclusion I propose the following biological model to describe the relationship between bile supersaturation, the immune system and cholesterol gallstones (Fig. 6-1). Genes and environmental factors (diet, rapid weight loss, others) promote supersaturation of bile. As bile becomes supersaturated cholesterol monohydrate crystals form. In a Th1 favorable environment the formation of these crystals promotes inflammation which causes gallbladder wall damage, an increase in mucin gel production and the production of other pronucleating substances. These conditions in turn increase cholesterol monohydrate crystal formation by promoting a favorable nucleating environment. As inflammation progresses the gallbladder wall becomes increasingly damaged and nonfunctional leading to an inability to clear mucin gel and cholesterol monohydrate crystals and eventual progression to cholesterol gallstones. In contrast, when the gallbladder is not polarized in a Th1 manner the gallbladder remains functional and there is no or little increase in mucin gel. Cholesterol monohydrate crystals can then be

expelled or chronically harbored in the gallbladder and cholesterol gallstones do not form.

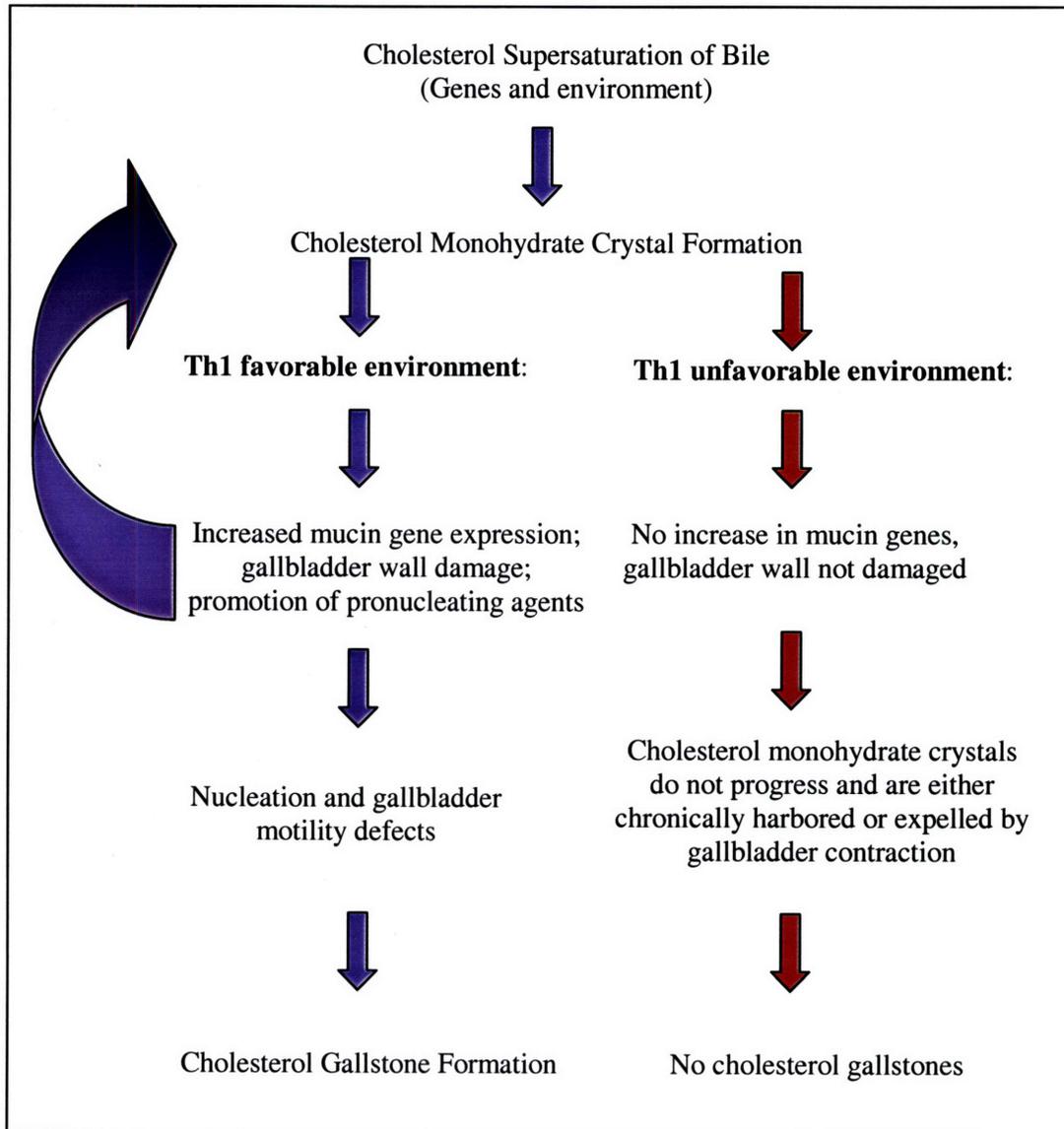


Figure 6-1:  
A schema depicting a proposed biological model of the formation of cholesterol gallstones under Th1 favorable and unfavorable conditions in the gallbladder

## **Appendix One**

### **Identification of cholelithogenic enterohepatic helicobacter species and their role in murine cholesterol gallstone formation**

## BASIC-LIVER, PANCREAS, AND BILIARY TRACT

# Identification of Cholelithogenic Enterohepatic *Helicobacter* Species and Their Role in Murine Cholesterol Gallstone Formation

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See editorial on page 1126.

**Background & Aims:** *Helicobacter* spp are common inhabitants of the hepatobiliary and gastrointestinal tracts of humans and animals and cause a variety of well-described diseases. Recent epidemiologic results suggest a possible association between enterohepatic *Helicobacter* spp and cholesterol cholelithiasis, chronic cholecystitis, and gallbladder cancer. To test this, we prospectively investigated the effects of *Helicobacter* spp infection in cholesterol gallstone pathogenesis in the highly susceptible C57L/J mouse model. **Methods:** *Helicobacter* spp-free adult male C57L mice were infected with several different enterohepatic *Helicobacter* spp or left uninfected and fed either a lithogenic diet or standard mouse chow for 8 and 18 weeks. At the conclusion of the study, bile was examined microscopically and diagnostic culture and polymerase chain reaction were performed. **Results:** Mice infected with *Helicobacter bilis* or coinfecting with *Helicobacter hepaticus* and *Helicobacter rodentium* and fed a lithogenic diet developed cholesterol gallstones at 80% prevalence by 8 weeks compared with approximately 10% in uninfected controls. Mono-infections with *H hepaticus*, *Helicobacter cinaedi*, and *H rodentium* gave a cholesterol gallstone prevalence of 40%, 30%, and 20%, respectively; the latter 2 groups did not differ significantly from uninfected animals. Neither infected nor uninfected mice fed a chow diet developed cholesterol gallstones. **Conclusions:** These findings, along with prior epidemiologic studies, suggest that *Helicobacter* spp play a major role in the pathophysiology of cholesterol gallstone formation in mice and perhaps humans.

ing nucleation and phase separation of cholesterol monohydrate crystals from cholesterol supersaturated bile.<sup>1-3</sup> A variety of risk factors are documented, including ethnicity, sex, obesity, weight loss, dietary intake, and concurrent medical conditions and treatments.<sup>2</sup> Currently, the only definitive treatment for gallstones is surgical removal of the gallbladder (cholecystectomy) and the total annual cost of treatment in the United States alone approaches \$10 billion.<sup>2</sup> Moreover, an ominous complication of gallstone disease is gallbladder cancer.<sup>4,5</sup>

Studies elucidating the pathogenesis of cholesterol gallstones rely predominantly on inbred mouse models.<sup>2</sup> In particular, quantitative trait locus analysis using gallstone-susceptible and gallstone-resistant strains shows that cholesterol gallstone disease is principally a polygenic trait involving multiple susceptibility loci (*Lith* alleles) on many chromosomes.<sup>2,6-8</sup> A strain of particular interest is the C57L/J (C57L) mouse because of its high susceptibility to cholesterol gallstones, which develop in 80%-100% of male mice fed a lithogenic diet for 8 weeks.<sup>9,10</sup> Additionally, 2 congenic strains carrying the *Lith1* or *Lith2* loci from the C57L strain in an AKR/J (AKR) background recapitulate the phenotype of the C57L parent.<sup>2</sup>

Recent evidence from our laboratories suggests an infectious contribution to the gallstone phenotype in C57L mice. When congenic AKR mice carrying a susceptibility locus (*Lith2*) from C57L mice (AKR-*Lith2*) were re-derived and housed under specific pathogen-free

Cholesterol gallstones, composed predominantly of cholesterol monohydrate crystals within a mucin glycoprotein scaffolding, form in the gallbladder follow-

Abbreviations used in this paper: PCR, polymerase chain reaction.

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conditions, they failed to develop gallstones. This was established in 2 independent but identical pilot studies lasting 8 weeks under feeding conditions with a lithogenic diet containing 1% cholesterol, 0.5% cholic acid, and 15% dairy fat (G. Bouchard, B. Paigen, M. C. Carey, unpublished observations, 2001-2002). Because of the ubiquitous nature of *Helicobacter* spp in conventional mouse colonies<sup>11</sup> and the ability of these organisms to cause hepatobiliary disease,<sup>11,12</sup> we considered them likely candidates to be involved in cholesterol cholelithogenesis.

Since the discovery of *Helicobacter pylori*, more than 25 *Helicobacter* spp have been isolated from the stomach, intestinal tract, and liver of humans, other mammals, and birds.<sup>11-14</sup> Many of these organisms cause extragastric disease and several are able to grow in bile, including *Helicobacter hepaticus*, *Helicobacter bilis*, and *Helicobacter pullorum*.<sup>13,14</sup> These nongastric (enterohepatic) *Helicobacter* spp generally colonize the distal small intestine, cecum, and large intestine and subsequently the liver, where they have been implicated in, or suggested to cause, hepatitis, hepatocellular carcinoma, cholecystitis, typhlocolitis, and colonic adenocarcinoma.<sup>11-14</sup> We showed previously that there is an association between molecular (DNA) evidence of enterohepatic *Helicobacter* spp colonization and cholecystitis in Chilean women.<sup>13</sup> Interestingly, this population is notable for its high prevalence of gallbladder cancer invariably associated with cholesterol gallstone disease.<sup>2,13</sup> Additionally, there is a growing body of literature identifying *Helicobacter* DNA in the gallbladders, bile, and stones of patients with gallstones.<sup>15-17</sup>

Due to the high prevalence of enterohepatic *Helicobacter* spp in mice<sup>11,12,14,18</sup> and the burgeoning evidence in humans,<sup>5,15,17,19</sup> we believed it reasonable to hypothesize that *Helicobacter* spp contribute to cholesterol gallstone formation. To test this hypothesis, prospective infections were performed in the well-characterized, highly susceptible, diet-induced, C57L mouse model of gallstone disease<sup>2,7-10</sup> initially using 2 enterohepatic *Helicobacter* spp: *H hepaticus* and *Helicobacter rodentium*.<sup>11,12,14</sup> We chose these 2 species because they were present concurrently in the colonies at Brigham and Women's Hospital (Boston, MA), where much of the original gallstone research was performed.<sup>2,4</sup> Following these coinfection studies, we performed monoinfection studies with these enterohepatic *Helicobacter* spp as well as *H bilis* and *Helicobacter cinaedi*, which have been identified in humans with gastrointestinal and hepatobiliary disease.<sup>5,20-25</sup>

## Materials and Methods

### Animals and Diet

*Helicobacter* spp-free male C57L mice were acquired from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility. These specific pathogen-free conditions included specific absence of *Helicobacter* spp, *Salmonella* spp, *Citrobacter rodentium*, and known murine viral pathogens. All animal protocols met the approval of the institutions' animal care and use committees. Mice were fed either standard rodent chow or, beginning at 8 weeks of age, a lithogenic diet containing 15% dairy triglycerides, 1.0% cholesterol, and 0.5% cholic acid. This was continued for 8 weeks<sup>8</sup> for both coinfection and monoinfection studies and for 18 weeks for chronic lithogenic studies.

### Infection Protocols

*H hepaticus* strain 3B1 (ATCC 51499), *H rodentium* (ATCC 700285), *H bilis* (MU strain), and *H cinaedi* (CCUG 18818) were grown on blood agar plates under microaerobic conditions at 37°C. For coinfection studies, 4-week-old mice were either infected orally by gavage 3 times over 5 days with approximately  $1.0 \times 10^8$  to  $2.0 \times 10^8$  each of *H hepaticus* and *H rodentium* suspended in Brucella broth (Becton Dickinson and Co, Sparks, MD) or sham dosed with Brucella broth. For monoinfection studies, mice were infected 3 times over 5 days with approximately  $1.5 \times 10^8$  to  $3.0 \times 10^8$  of the individual *Helicobacter* spp tested, or sham dosed with Brucella broth. For coinfection studies, mice were subsequently redosed with a comparable number of *H hepaticus* or sham dosed with broth every month thereafter; for monoinfection studies, mice were redosed with a comparable number of the appropriate organism every month thereafter for 2 months.

### Direct Light and Polarizing Microscopy of Gallbladder Bile and Gallstone Analysis

At 16 weeks of age (or 26 weeks of age for prolonged lithogenic studies), mice were killed by CO<sub>2</sub> overdose and cholecystectomies were performed immediately. Gallbladders were weighed and opened, and microscopists (M.C.C. and K.J.M.) who were blinded as to sample identity examined gallbladder bile by both direct and polarized light microscopy for the presence of liquid crystals, solid crystals, sandy stones, and cholesterol gallstones as defined elsewhere.<sup>10</sup> Bile was scored microscopically for mucin gel content on a 0-5 arbitrary scale.<sup>26</sup> Gallstones were analyzed for cholesterol content by high-performance liquid chromatography as previously described.<sup>9</sup>

### Histopathologic Examinations

Sections of each liver lobe as well as gallbladder and ileocecolic junctions were harvested at necropsy, fixed overnight in 10% neutral-buffered formalin, processed routinely,

**Table 1.** Phenotypic Characterization of Murine Gallbladders and Their Contents

	Gallbladder weight (mg)/ mouse weight (g) (mean $\pm$ SEM)	Mucin (mean $\pm$ SEM)	Liquid crystals (%)	Solid crystals (%)	Sandy stones (%)	Cholesterol gallstones (%)
Infected + lithogenic diet (n = 9)	0.93 $\pm$ 0.14 <sup>a</sup>	1.94 $\pm$ 0.33 <sup>b</sup>	7/9 (78)	8/9 (89) <sup>a</sup>	5/9 (56) <sup>b</sup>	7/9 (78) <sup>a</sup>
Uninfected + lithogenic diet (n = 9)	0.58 $\pm$ 0.05	1.11 $\pm$ 0.07	9/9 (100)	1/9 (11)	0/9 (0)	0/9 (0)
Infected + mouse chow (n = 10)	0.54 $\pm$ 0.05	0.15 $\pm$ 0.15	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
Uninfected + mouse chow (n = 5)	0.42 $\pm$ 0.03	0 $\pm$ 0	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

NOTE. Male C57L mice uninfected and coinfecting with *H hepaticus* and *H rodentium* were studied.

Numbers are significantly greater (<sup>a</sup> $P < .005$  and <sup>b</sup> $P < .05$ ) compared with uninfected mice fed the same diet or mice fed a standard mouse chow.

cut into 4- $\mu$ m thicknesses, and stained with H&E. Liver sections were graded for lobular and portal inflammation according to previously defined criteria<sup>27</sup> by a comparative pathologist (A.B.R.) blinded as to sample source. Gallbladders were assessed morphologically for inflammation, epithelial disorganization, hyperplasia, dysplasia, and hyalinosis. Special stains included a pH 2.5 alcian blue/periodic acid-Schiff stain for acidic and neutral mucins.

### *Helicobacter* Spp Screening Tests

DNA was prepared from postmortem tissue using a high pure polymerase chain reaction (PCR) template preparation kit (Roche Applied Science, Penberg, Germany) and was either singly amplified from the cecum with genus-specific primers (for mono-infection studies) or species-specific primers (for coinfection studies) using previously described conditions.<sup>18,28,29</sup> DNA was amplified from the liver, gallbladder, or cholesterol gallstones by nested amplification with an initial genus-specific primer set using previously described conditions.<sup>28</sup> This was followed by a subsequent amplification of 2.5  $\mu$ L of this product with internal species-specific primers to *H hepaticus*, *H rodentium*, or *H bilis* or a second genus-specific primer set for *H cinaedi* using previously described reaction conditions.<sup>18,29,30</sup>

### Culture of *Helicobacter* Spp

Gallbladders and livers were ground in sterile glass tissue grinders with 0.5–1 mL of Brucella broth. Ground tissue was streaked onto blood agar plates and grown under microaerobic conditions at 37°C for 3 weeks. At that time, plates were scrutinized for bacterial growth.

### Statistical Analysis

Statistical analyses of mucin score and gallbladder weight were performed by one-way analysis of variance with the Tukey–Kramer posttest using Instat 3.0 software (GraphPad, Inc, San Diego, CA). Cholesterol monohydrate crystal formation as well as sandy and true gallstone formation were analyzed by Fisher exact test using the same software.

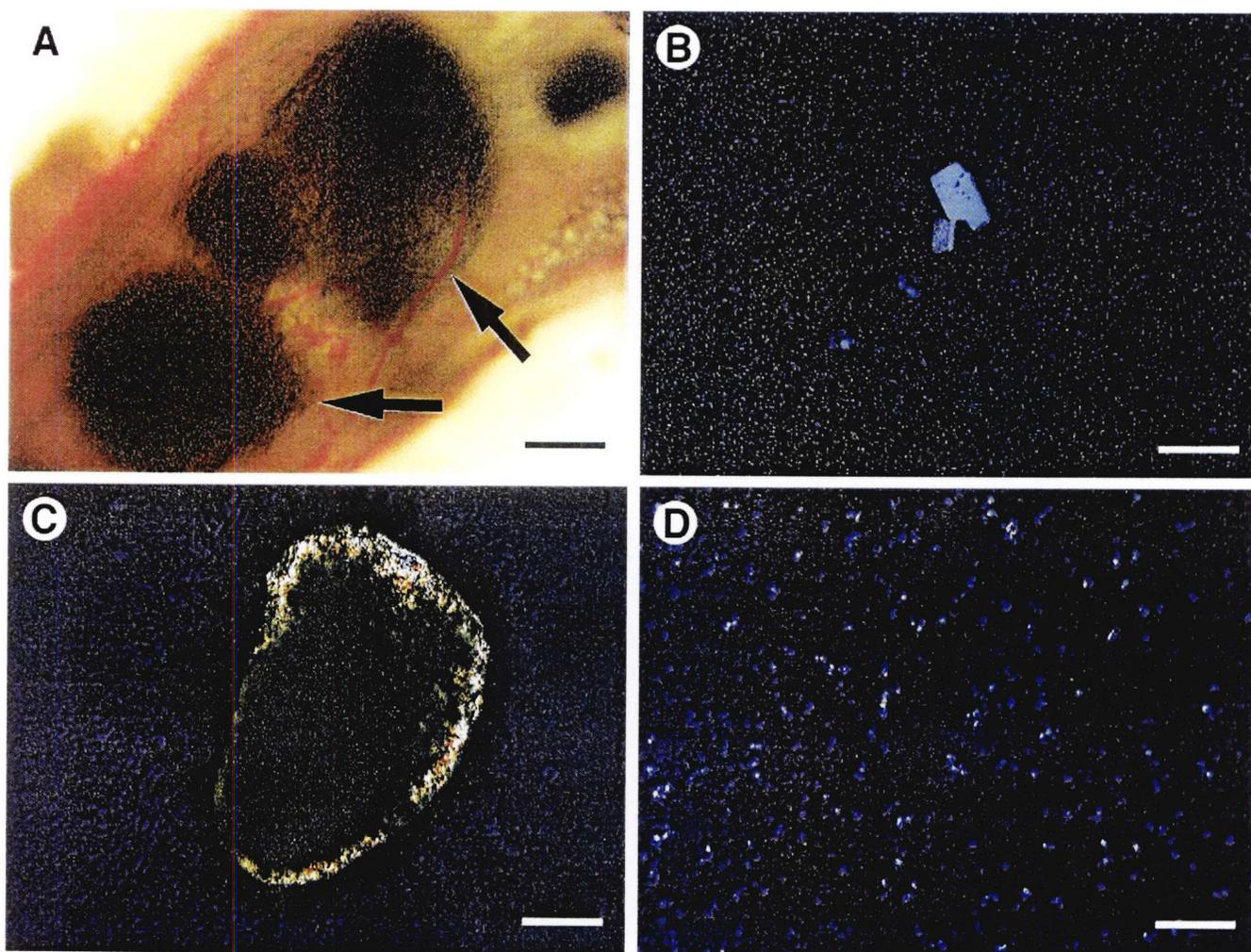
## Results

### Phenotypic Analysis of Gallbladder and Bile From Coinfected Animals

*Helicobacter* spp–infected mice (all n values = 5–10; see Table 1) fed the lithogenic diet developed cholesterol gallstones<sup>10</sup> at a prevalence rate of 78% and sandy stones at a prevalence rate of 56%, and all but a single animal progressed beyond the liquid crystalline phase (Table 1 and Figure 1). In contrast, uninfected mice fed a lithogenic diet and infected or uninfected mice that ingested a standard chow diet failed to develop gallstones (Table 1 and Figure 1). Interestingly, all uninfected mice fed the lithogenic diet progressed to the liquid crystalline phase in the nucleation sequence,<sup>10</sup> but only one mouse progressed beyond that to cholesterol monohydrate crystals (Table 1 and Figure 1). Infected, or uninfected mice that ate standard chow diets, did not develop lithogenic bile, confirming the need for a modified diet in the development of gallstones in C57L mice<sup>31</sup> (Table 1). Infected animals fed a lithogenic diet displayed significantly higher ( $P < .05$ ) mucin scores<sup>26</sup> compared with uninfected animals or those ingesting standard mouse chow, and gallbladders of infected animals consuming the lithogenic diet were significantly larger ( $P < .005$ ) than those of uninfected animals or those fed standard chow diets (Table 1).

### Histopathologic Analysis of Hepatobiliary Tissues

Histologic analysis of livers revealed microsteatosis and mild chronic portal inflammation associated with diet but not with *Helicobacter* spp infection status. A subset of infected and uninfected mice on the lithogenic diet exhibited portal and random lymphohistiocytic infiltrates attributed to hepatobiliary tissue damage caused by steatosis. However, there was no association between hepatic inflam-



**Figure 1.** Morphologic characterization by direct and polarizing light microscopy<sup>10</sup> of gallbladder bile and stones of (A) *Helicobacter* spp–infected C57L mice fed a lithogenic diet for 8 weeks. Macroscopically visible stones (arrows) up to 2 mm in diameter within a mouse gallbladder. Progressive stages in development of cholecystolithiasis found in *Helicobacter* spp–infected C57L/J mice fed a lithogenic diet include (B) cholesterol monohydrate crystals,<sup>10</sup> sandy stones<sup>10</sup> (not shown), and (C) cholesterol gallstones<sup>10</sup> (these stones have smooth contoured birefringent edges and dark centers due to light scattering and absorption). (D) Birefringent aggregated liquid crystals<sup>10</sup> represent the minimal extent of lithogenesis in uninfected C57L mice fed a lithogenic diet. Bars: A, 500  $\mu$ m; B, 100  $\mu$ m; C, 170  $\mu$ m; D, 50  $\mu$ m.

mation and risk of gallstones ( $P = 1$ ). In contrast, lesions of the gallbladder, including hyalinosis, intestinal metaplasia, eosinophilic inflammation, hyperplasia, and dysplastic changes, were limited to *Helicobacter* spp–infected mice on the lithogenic diet (Figure 2). Interestingly, many of these lesions are observed in humans with gallstones; several of them, including dysplasia, hyperplasia, and intestinal metaplasia, are considered to be preneoplastic.<sup>32</sup>

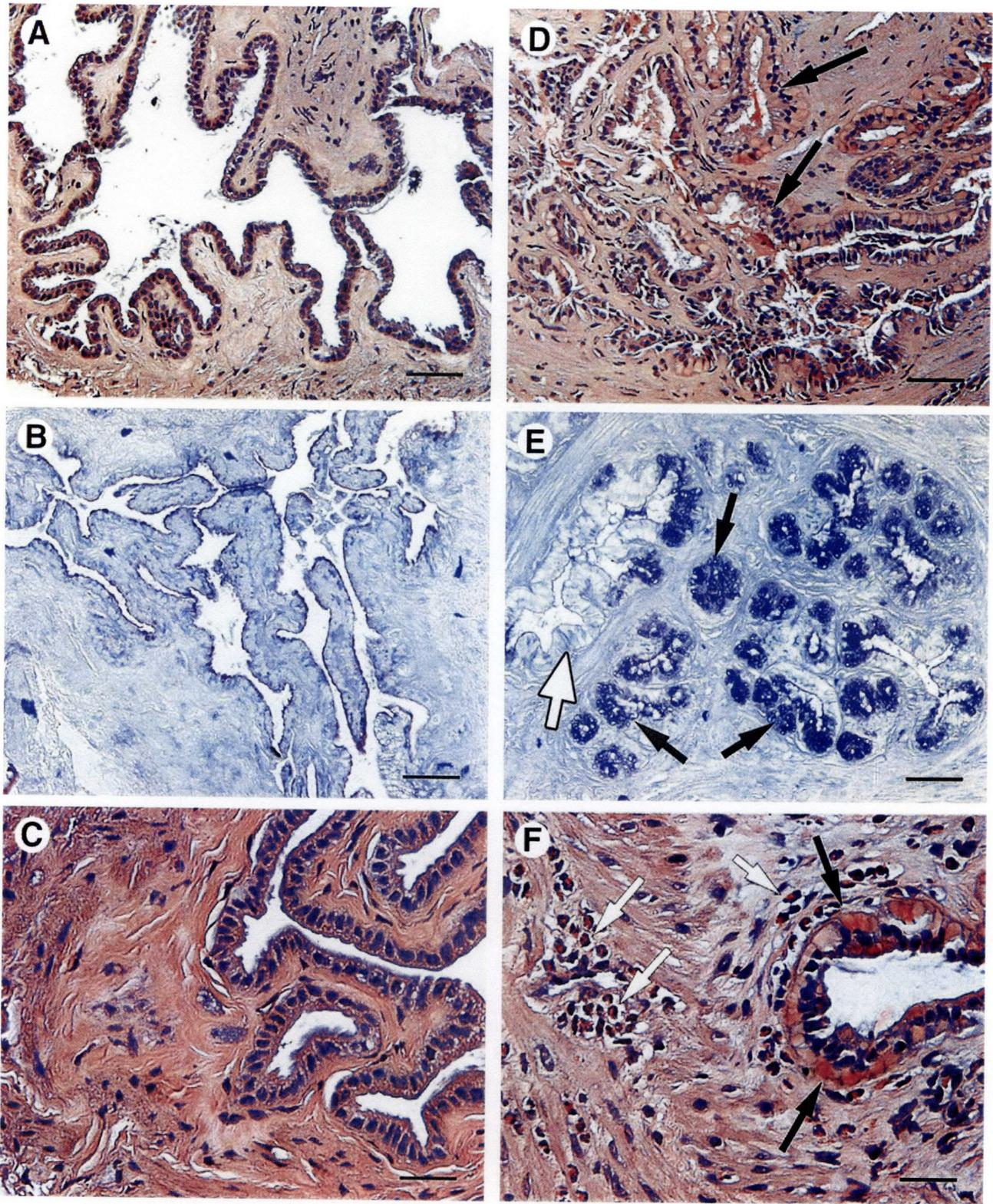
#### ***Helicobacter* Spp Infection Status of Coinfected Mice**

Coinfected mice fed either chow or the lithogenic diet showed consistent cecal colonization with both *Helicobacter* spp when screened by PCR (Figure 3).<sup>18,29</sup> Molecular (DNA) evidence of enterohepatic *Helicobacter* spp was present in a region of the hepatobiliary tree (either

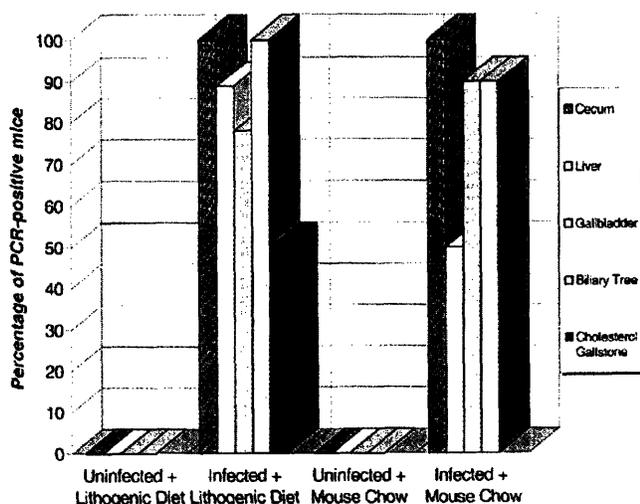
gallbladder or liver) in most infected animals (Figure 3).<sup>18,28,29</sup> No uninfected animals showed molecular evidence of colonization in either the cecum or the hepatobiliary tree (Figure 3). PCR analysis of 4 gallstones from *H hepaticus* plus *H rodentium* coinfecting mice revealed that *Helicobacter* DNA was present in 50% of stones (Figure 3). None of the gallbladders of *Helicobacter* spp coinfecting animals fed the lithogenic diet ( $n = 5$ ) were culture positive even after prolonged (3 weeks) incubation under appropriate microaerobic conditions.

#### **Phenotypic Analysis of Gallbladder and Bile in Monoinfected Mice**

In mice fed a lithogenic diet for 8 weeks, the biliary phenotype varied greatly depending on the organism with which the mice were infected. Specifically,



**Figure 2.** Compared with (A–C) sections from uninfected mice on a lithogenic diet, histopathologic examination of gallbladder tissue from *Helicobacter* spp.-infected mice shows (D; arrows) epithelial disorganization with eosinophilic luminal secretory products, (E) hyperplasia and mucous intestinal-type metaplasia (black arrows; note absence of mucous staining in hyalinized epithelium, white arrow), and (F) epithelial hyalinosis (glassy pink-red material, black arrows) and patchy eosinophil-predominant inflammation (white arrows). Histologic stains: A, C, D, and F, H&E; B and E, alcian blue/periodic acid–Schiff pH 2.5. Bars: A, B, D, and E, 170  $\mu$ m; C and F, 85  $\mu$ m.



**Figure 3.** Prevalence of *Helicobacter* DNA in various tissues from coinfecting experimental groups. DNA was prepared from tissue using the high pure PCR template preparation kit and was either singly amplified from the cecum with species-specific primers using previously described conditions<sup>28</sup> or amplified from the liver, gallbladder, or gallstones by nested amplification with an initial genus-specific primer set using previously described conditions.<sup>28</sup> This was followed by a subsequent amplification of 2.5  $\mu$ L of this product with species-specific primers to *H hepaticus* and *H rodentium* with reaction conditions as previously described.<sup>18,29</sup> Biliary tree refers to a positive result from either intrahepatic bile ducts or gallbladder.

when compared with uninfected mice, monoinfection with *H rodentium* or *H cinaedi* did not significantly increase normalized gallbladder weight, mucin score, cholesterol monohydrate crystal formation, sandy stone formation, or cholesterol gallstone formation (Table 2). In contrast, monoinfection with *H bilis* and *H hepaticus* significantly increased cholesterol monohydrate crystal formation, sandy stone formation, and cholesterol gallstone formation when compared with uninfected mice (Table 2). Additionally, *H bilis* infection increased mucin score significantly when compared with uninfected mice (Table 2). A sampling of stones ( $n = 2$  per group) was analyzed for cholesterol content and stones were composed primarily of cholesterol (monohydrate), confirming that they were indeed cholesterol stones. This was further

corroborated by their creamy white to tan appearance to the unaided eye and the agglomeration of cholesterol monohydrate crystals in sandy and true stones by polarizing and direct light microscopy.

Histopathologic changes in the gallbladder mucosa observed in coinfecting animals were also noted in some monoinfected animals and were rarely detected in uninfected animals. Almost invariably, these histopathologic changes were found concomitantly with sandy stones or true cholesterol gallstones in both infected and uninfected animals.

### Prolonged Lithogenic Diet Feeding

To determine if *Helicobacter* spp were merely altering the rates of cholesterol cholelithogenesis, we fed uninfected mice and mice infected with *H hepaticus* a lithogenic diet for 18 weeks. Interestingly, prolonged exposure to the lithogenic diet did not significantly alter the prevalence of cholesterol gallstone formation in either group when compared with their counterparts fed the lithogenic diet for 8 weeks (Table 2). When compared with uninfected mice, *H hepaticus*-infected mice displayed a significantly higher mucin score, with a higher prevalence of both cholesterol monohydrate crystals and sandy stones (Table 2).

### Infection Status of Hepatobiliary and Gastrointestinal Tissues

At the termination of experiments all mice infected with *Helicobacter* spp were confirmed to be infected by cecal PCR (except for *H bilis*-infected animals, which were confirmed to be positive by cecal culture); all uninfected animals tested negative for enterohepatic *Helicobacter* spp by cecal PCR (Table 2). The presence of *Helicobacter* DNA in the liver of infected animals was variable, and the presence or absence of DNA in the liver did not correlate with gallstone formation. None of the *H hepaticus*- ( $n = 10$ ) or *H rodentium*- ( $n = 10$ ) infected mice were culture positive for *Helicobacter* spp in the liver or the gallbladder.

**Table 2.** Biliary Phenotype and Colonization Status of Monoinfected and Uninfected Male C57L Mice on a Lithogenic Diet

Organism	Lithogenic diet (wk)	Normalized gallbladder weight (mg/g) (mean $\pm$ SEM)	Mucin score (mean $\pm$ SEM)	Liquid crystals (%)	Solid crystals (%)	Sandy stones (%)	Cholesterol gallstones (%)	Cecal PCR (%)	Liver PCR (%)	Hepatobiliary culture (%)
<i>H hepaticus</i> ( $n = 20$ )	8	0.85 $\pm$ 0.13	1.20 $\pm$ 0.12	95	65 <sup>b</sup>	35 <sup>a</sup>	40 <sup>a</sup>	100	60	0 ( $n = 10$ )
<i>H rodentium</i> ( $n = 10$ )	8	0.69 $\pm$ 0.15	1.55 $\pm$ 0.31	100	40	30	30	100	80	0 ( $n = 10$ )
<i>H bilis</i> ( $n = 5$ )	8	0.73 $\pm$ 0.35	2.00 <sup>a</sup> $\pm$ 0.35	80	100 <sup>b</sup>	100 <sup>b</sup>	80 <sup>b</sup>	100	60	ND
<i>H cinaedi</i> ( $n = 5$ )	8	0.72 $\pm$ 0.08	1.40 $\pm$ 0.19	100	20	20	20	100	80	ND
Uninfected ( $n = 30$ )	8	0.59 $\pm$ 0.065	0.93 $\pm$ 0.11	100	20	7	10	0	ND	ND
<i>H hepaticus</i> ( $n = 10$ )	18	0.90 $\pm$ 0.18	2.45 <sup>a</sup> $\pm$ 0.32	100	100 <sup>b</sup>	100 <sup>b</sup>	50	100	67	ND
Uninfected ( $n = 10$ )	18	0.57 $\pm$ 0.09	1.55 $\pm$ 0.19	100	20	20	20	0	ND	ND

ND, not determined.

<sup>a</sup> $P < .05$  and <sup>b</sup> $P < .005$  when compared with controls fed the lithogenic diet for the same time period.

## Discussion

This study presents systematic information detailing what appears to be a paradigm shift in our understanding of cholesterol gallstone pathogenesis, at least in this inbred mouse model and perhaps in humans. These data show rigorously that enterohepatic *Helicobacter* spp play a notable role in the development of cholesterol gallstones in the murine gallbladder. Without this infection, the C57L mouse, the most frequently used polygenic model of cholesterol gallstone disease,<sup>2</sup> does not acquire gallstones with any appreciable frequency.

The ability to promote cholesterol gallstones in C57L mice is *Helicobacter* spp specific. Of the organisms tested, coinfection with *H hepaticus* and *H rodentium* and monoinfection with *H bilis* displayed the greatest impact in promoting cholesterol gallstone formation. *H hepaticus* was intermediate in its ability, and *H rodentium* and *H cinaedi* did not influence cholesterol cholelithogenesis significantly compared with uninfected controls. We propose the appellation "cholelithogenic" *Helicobacter* spp to differentiate those species that are capable of promoting cholesterol cholelithogenesis from those that do not. We suspect that, with further testing, other *Helicobacter* spp and perhaps other related and unrelated microorganisms may prove capable of promoting cholesterol gallstone formation.

We tested mice housed at the facilities (Brigham and Women's Hospital) where most of the original studies<sup>2,4</sup> were performed by anaerobic and aerobic fecal culture. Those results were compared with results of cultures from mice in the current study to determine if other pathogens may have contributed to the historical prevalence of gallstones at the prior facility.<sup>2,4</sup> We found no differences with respect to other pathogens between the 2 facilities (Brigham and Women's Hospital and Massachusetts Institute of Technology) (data not shown). Moreover, the only other mouse pathogen present at both Massachusetts Institute of Technology and Brigham and Women's Hospital was *Klebsiella oxytoca*, an organism rarely associated with disease and then only with uterovarian infection in mice.<sup>33</sup> The possible pathogenic roles that the normal microbiota of the gut play in the complex process of cholesterol gallstone disease is intriguing but cannot be answered without prospective studies in gnotobiotic and germ-free animals.

In our studies, the ability of some but not all *Helicobacter* spp to promote cholesterol cholelithogenesis implies that promotion of cholesterol gallstone formation is mediated by species-specific bacterial products or a reaction of the host to these products, possibly through cytokines and other proinflammatory mediators. If pro-

motion of cholelithogenesis were due to a nonspecific host response to colonization, then all *Helicobacter* organisms tested should have promoted gallstone formation equally. In particular, an interesting aspect of this phenomenon was that the bacteria tested did not colonize the biliary tree (at least in the culturable state when tested at the time the mice were killed) and yet cholelithogenesis was promoted. This is evidenced by the lack of correlation between bacterial DNA in the liver and stone formation and our inability to culture organisms even after prolonged incubation and the necessity for nested PCR to identify DNA in the liver and the gallbladder. This raises several questions concerning the mechanism behind the relationship between these bacteria and cholesterol gallstone pathogenesis and the manner in which past studies have been conducted in an attempt to detect a possible infectious contribution to the pathogenesis of cholesterol gallstone disease. Many of these studies relied on culture and PCR of the hepatobiliary tree to determine a possible role for *Helicobacter* spp and other bacteria in cholesterol gallstone formation.<sup>5,13,15,34,36</sup> A number of investigators found no correlation between the presence of *Helicobacter* DNA in these tissues and the presence of cholesterol gallstones.<sup>15,36</sup> However, based on the results of the present study, it is clear that these organisms do not have to be present in high numbers in the hepatobiliary tree to promote cholelithogenesis. In fact, our study shows that the most appropriate anatomic site to determine whether *Helicobacter* spp are indeed an epidemiologic risk factor in mouse and human cholelithogenesis would be the distal small intestinal tract, cecum, and proximal large intestine. Furthermore, because our studies suggest a *Helicobacter* spp-specific effect, future investigations should focus on identifying the organisms at the species level by acceptable methods and not rely on the presence or absence of *Helicobacter* as a genus.

Several potential mechanisms or combinations thereof could be suggested to explain the contribution of *Helicobacter* spp to cholesterol cholelithogenesis. First, the organisms may produce a soluble antigen or antigens that modulate key hepatobiliary genes in the lithogenic pathway.<sup>2</sup> Based on the present data, an attractive population of genes is the *Muc* alleles of the gallbladder epithelium involved in the production of mucin.<sup>1,2</sup> Alternatively, because enterohepatic *Helicobacter* spp colonize, among other sites, the distal ileum, they may modulate enterohepatic cycling of conjugated bile acids either directly or through genetic regulation of absorption at the enterocyte level or by modulating gastrointestinal transit time. An intestinal role is further suggested by our finding in this murine model of a lack of

correlation between colonization of the hepatobiliary tree by *Helicobacter* spp and cholesterol gallstone formation. In addition to other possible mechanisms, an antigenic product of *Helicobacter* spp or a host response (eg, cytokines or other inflammatory mediators) could change the phase equilibria of bile or accelerate the nucleation kinetics of solid cholesterol monohydrate crystals from liquid crystals (see Table 1).

Interestingly, mice infected with both *H rodentium* and *H hepaticus* developed cholesterol gallstones at a consistently higher rate (78%) than infection with either of these organisms alone, which produced gallstones only at a moderate prevalence (*H hepaticus*) or rarely (*H rodentium*). This implies microbiotic synergism in that, individually, *H rodentium* and *H hepaticus* may not possess all of the necessary cholelithogenic factors to promote gallstone formation but together can do so effectively. Alternatively, colonization by one of these organisms may modulate a host response toward the other organism. In this regard, studies conducted on gastric *Helicobacter* spp (*H felis*)—infected animals show that the presence of other pathogens may promote or inhibit gastric disease.<sup>37,38</sup>

The possibility that enterohepatic *Helicobacter* spp influence human cholesterol gallstone formation is a provocative concept for several reasons. First and historically, the disease was presumed early on to be solely a physical/chemical phenomenon occurring at the level of the gallbladder<sup>1,3</sup>; there then followed the realization that a genetic (polygenic) component was crucial in altering the secretory rates of biliary lipids from the liver,<sup>2</sup> and now it appears that a “random event” is required, supporting the concept that a necessary component may also be chronic intestinal infection with an enterohepatic *Helicobacter* spp. It is worth noting that at the beginning of the 20th century, Lord Berkeley Moynihan, a notable British surgeon, avouched that “a gallstone is a tombstone erected to the memory of the organism within it.”<sup>39</sup> Although this statement exhibits magnificent prescience by a thoughtful surgeon, it does not hold entirely true with respect to our findings because not all cholesterol gallstones in the current study contained *Helicobacter* DNA. Another salient aspect of this current findings relates to the fact that the prevalence of gallbladder cancer is intimately associated with the prevalence of gallstone disease.<sup>4,5,13</sup> In experimental animals, both excess biliary cholesterol and *H hepaticus* produce hepatobiliary cancer<sup>40</sup> and hepatocellular carcinoma,<sup>11,12</sup> respectively, either by themselves or in the presence of other cocarcinogenic or promoting cofactors. It is therefore tempting to speculate that *Helicobacter* spp, some of which are known carcinogens,<sup>43</sup> and cholesterol (or an oxidized product thereof) may act synergistically

to promote tumors of the biliary tree in individuals with lithogenic bile and cholesterol gallstones. Further evidence supporting this hypothesis derives from studies involving Japanese and Thai patients with gallstones and biliary tract malignancies<sup>5</sup> and Chilean women with chronic cholecystitis.<sup>13</sup> These populations have high prevalence rates of gallstones and biliary cancers,<sup>5,13</sup> and both of these studies found an association between molecular (DNA) evidence of enterohepatic *Helicobacter* spp infection and neoplastic biliary disease.

In the recent past, a putative role of *Helicobacter* spp in human cholesterol gallstone disease has been proposed and questioned critically because *Helicobacter* DNA has been amplified in hepatobiliary samples from unaffected individuals,<sup>15</sup> and sometimes *Helicobacter* DNA is not identified in hepatobiliary tissues of patients with cholesterol gallstones.<sup>11</sup> However, it is clear from our data that *Helicobacter* spp infection per se plays a role in cholelithogenesis but is not sufficient in or of itself to produce cholesterol gallstones. Moreover, only certain strains of *Helicobacter* spp promote cholesterol cholelithogenesis in the in vivo setting of lithogenic bile. Clearly, genetic background and noninfectious environmental factors (ie, the lithogenic diet) are essential in the mouse model in initiating the physical/chemical conditions (ie, lithogenic bile) predisposing to the disease. Mouse strain is clearly important because cholesterol gallstone-resistant mice from the original facility<sup>8</sup> were almost certainly colonized with these *Helicobacter* spp but their bile is obstinate in developing supersaturation.<sup>10</sup> Genetics also play a role in how a host responds to infection with *Helicobacter* spp. For example, A/JCr mice can develop severe hepatitis and hepatocellular carcinoma when infected with *H hepaticus*; however, some other mouse strains display little hepatic disease when infected.<sup>41,45,46</sup> Finally, this study used the C57L mouse, which typically has a cholesterol saturation index of approximately 1.5 at the 8-week point<sup>10</sup> of lithogenic diet feeding. This value roughly correlates with cholesterol saturation index values observed in normal-weight individuals with cholesterol gallstones<sup>17</sup>; however, the value is lower than the median found in very obese individuals with gallstones.<sup>17</sup> Further work is needed to determine whether *Helicobacter* spp play a similar role at high cholesterol saturation index values (>1.60), such as those seen in morbidly obese patients.<sup>17</sup> On the basis of the present work, we propose a hypothetical biologic model showing that 3 pathophysiologic factors (environment, genetics, and enterohepatic *Helicobacter* spp infection) all contribute to cholesterol gallstone formation in C57L mice, as well as metaplasia, dysplasia, and potentially the progression to gallbladder cancer.

Of interest is that, in the past, a number of investigators have identified the presence of bacteria (non-*Helicobacter* spp) in the extrahepatic biliary tree, gallbladder, and gallstones of humans and hypothesized on their putative role in so-called "mixed" (ie, plus calcium bilirubinate) cholesterol gallstone formation.<sup>48-50</sup> In particular, Lee et al tentatively identified *Escherichia coli* and *Pseudomonas* spp principally in "mixed" cholesterol gallstones containing 65%–94% cholesterol and brown pigment gallstones with 16% and 25% cholesterol harvested from human gallbladders without current cholecystitis. These investigators hypothesized that bacteria may indeed play a role in "mixed" cholesterol-bilirubinate gallstone formation.<sup>50</sup> However, to date, none of the organisms identified in these studies have been analyzed prospectively and, because the bacteria identified are among the normal microbiota inhabiting the human distal small intestine and colon, and hydrolyze conjugated bilirubin to unconjugated bilirubin, their presence in the biliary tree may be secondary to recurrent biliary stasis and subclinical cholecystitis from cholesterol gallstones leading to the "mixed" bilirubinate-cholesterol gallstones.<sup>48-50</sup> Development of this mouse model should allow for a prospective analysis of the primary versus secondary roles of these and other components of the gut microbiota in relation to gallstone pathogenesis and complications.

Based on our data (Table 1), we believe we can now propose a realistic hypothesis for the role of *Helicobacter* spp (and perhaps other bacteria) in cholesterol gallstone pathogenesis. We suggest that the most likely scenario is that certain *Helicobacters* change the kinetics of cholesterol crystal nucleation from the liquid crystalline state (ie, the initial phase separation) in gallbladder bile (Table 1). Cholesterol supersaturation of bile is a necessary but not sufficient event for cholesterol gallstone formation, and both gallbladder hypomotility and accumulation of mucin gel (as a nucleation matrix) are also required.<sup>2,3,51</sup> Theoretically, nucleation of solid cholesterol monohydrate crystals can occur homogeneously, but this is thermodynamically unfavorable and might require an enormous degree of cholesterol supersaturation.<sup>51</sup> Alternatively, a far more favorable event thermodynamically is heterogeneous nucleation, which involves a nucleating agent<sup>51</sup> that provides a hydrophobic surface for molecular deposition, and these agents can nucleate bile with only modest degrees of supersaturation. Multiple studies have suggested the presence of nucleating agents in humans with cholesterol gallstones.<sup>51</sup> Likely nucleating agents identified previously include mucins, immunoglobulins, and other biliary proteins, some of which have been functionally and chemically characterized.<sup>1,51-53</sup> We have

shown in the current study that particular *Helicobacter* spp significantly increase mucin production in C57L mice fed a lithogenic diet (Table 1). Additionally, we showed earlier that *H hepaticus* infection causes a 3-fold increase in biliary immunoglobulin A production, and it is logical to presume that *H bilis* would have a similar effect.<sup>54</sup> Furthermore, it is provocative to suggest that *Helicobacter* spp may produce other pronucleating agonists in response to biliary exposure or, alternatively, that these organisms may induce production in the host of nucleating agents either directly or through chronic immune stimulation. Most importantly, it has been shown that different *Helicobacter* spp exhibit very divergent protein expression profiles in response to their exposure to bile.<sup>55</sup> Perhaps this difference explains in part why some, but not all, of these related organisms promoted cholesterol gallstone formation in our studies. Additionally, preliminary data (K.J.M., M.C.C., J.G.F., unpublished observations, 2004) from our laboratories suggest that global changes occur in hepatic gene expression from enterohepatic *Helicobacter* infection even without exhibition of a lithogenic diet, including genes believed to play a role in cholesterol homeostasis. It is theoretically possible that one or more of these differentially expressed genes may play a role in altering biliary lipid composition either qualitatively or quantitatively.

In conclusion, our work represents a provocative new dimension in our understanding of cholesterol gallstone pathogenesis in C57L mice and these findings are likely to be applicable to humans. Dissecting the mechanisms responsible for these observations, including physical/chemical, petrological, genomic/proteomic, and infectious, will require further critical hypothesis and experimentation in several mouse models with different enterohepatic and gastric *Helicobacter* spp, as well as related organisms. Provided the new concept holds true for human gallstone disease, as some earlier epidemiologic evidence would imply, then a paradigm shift may be in the offing that may lead to major modifications in the prevention and treatment of cholesterol gallstone disease and possibly prevention and management of gallbladder and other hepatobiliary diseases, including hepatobiliary and perhaps pancreatic cancers.

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## **Appendix Two**

***Helicobacter pylori* and cholesterol gallstone formation in C57L/J mice: a prospective study**



## *Helicobacter pylori* and cholesterol gallstone formation in C57L/J mice: a prospective study

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Divisions of <sup>1</sup>Comparative Medicine and <sup>2</sup>Biological Engineering, Massachusetts Institute of Technology, Cambridge; and <sup>3</sup>Department of Medicine, Harvard Medical School, and Division of Gastroenterology, Brigham and Women's Hospital and Harvard Digestive Diseases Center, Boston, Massachusetts

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Maurer, Kirk J., Arlin B. Rogers, Zhongming Ge, Ashley J. Wiese, Martin C. Carey, and James G. Fox. *Helicobacter pylori* and cholesterol gallstone formation in C57L/J mice: a prospective study. *Am J Physiol Gastrointest Liver Physiol* 290:G175–G182, 2006. First published August 18, 2005; doi:10.1152/ajpgi.00272.2005.—Recently, we demonstrated that cholesterol gallstone-prone C57L/J mice rarely develop gallstones unless they are infected with certain cholelithogenic enterohepatic *Helicobacter* species. Because the common gastric pathogen *H. pylori* has been identified in the hepatobiliary tree of cholesterol gallstone patients, we wanted to ascertain if *H. pylori* is cholelithogenic, by prospectively studying C57L infected mice fed a lithogenic diet. Weanling, *Helicobacter* spp.-free male C57L mice were either infected with *H. pylori* SS1 or sham dosed. Mice were then fed a lithogenic diet (1.0% cholesterol, 0.5% cholic acid, and 15% dairy triglycerides) for 8 wk. At 16 wk of age, mice were euthanized, the biliary phenotype was analyzed microscopically, and tissues were analyzed histopathologically. *H. pylori* infection did not promote cholesterol monohydrate crystal formation (20% vs. 10%), sandy stone formation (0% for both), or true gallstone formation (20%) compared with uninfected mice fed the lithogenic diet (10%). Additionally, *H. pylori* failed to stimulate mucin gel accumulation in the gallbladder or alter gallbladder size compared with uninfected animals. *H. pylori*-infected C57L mice developed moderate to severe gastritis by 12 wk, and the lithogenic diet itself produced lesions in the forestomach, which were exacerbated by the infection. We conclude that *H. pylori* infection does not play any role in murine cholesterol gallstone formation. Nonetheless, the C57L mouse develops severe lesions of both the glandular and nonglandular stomach in response to *H. pylori* infection and the lithogenic diet, respectively.

lithogenic; bile; cholesterol crystals; mucin; gastritis

GALLSTONES are an exceptionally common cause of morbidity worldwide, and cholelithiasis with or without cholecystitis is the most common gastrointestinal disease requiring in-patient treatment in the United States (45). Despite five decades of intense basic, clinical, and epidemiological research (6, 11, 27, 41, 42), there is currently no definitive nonsurgical treatment for the management of gallstones; therefore, the disease is a serious surgical and economic burden with the median per patient cost exceeding 10,000 US dollars (45).

The term gallstones or cholelithiasis is a generic description encompassing both pigment and cholesterol gallstones (41). Cholesterol gallstones are the most common gallstones en-

countered in the Western world (41). These stones result from liver-induced cholesterol supersaturation of bile and phase separation of cholesterol-rich liquid crystals and solid crystals with subsequent crystal agglomeration and stone growth within the gallbladder in a mucoglycoprotein gel (8, 9, 41). Cholesterol gallstone formation is a polyfactorial disease with heterogeneous contributions from both genetics and environment (41). Specifically, genetic susceptibility to cholesterol gallstones is, with very rare exceptions, inherited as a polygenic trait (6, 10, 11, 27, 41, 42, 54). Cholesterol gallstone susceptibility genes require environmental triggers including diet, obesity, estrogenic drugs, and other complex and unknown factors to express the cholesterol gallstone phenotype (41).

The C57L/J mouse is studied extensively as a model to investigate cholesterol gallstone genetics and pathogenesis (25, 42, 51, 52, 54). When fed a lithogenic diet containing 1.0% cholesterol and 0.5% cholic acid for 8 wk, this animal historically develops cholesterol gallstones with an 80% prevalence rate (25, 51). Our initial studies with this mouse model were conducted at facilities where the mice were enzootically infected with enterohepatic *Helicobacter* spp. (33). Recently, we (33) demonstrated that, in the absence of infection with specific enterohepatic *Helicobacter* spp., the prevalence of cholesterol gallstones in mice despite them being fed a lithogenic diet for 8 wk was much less than what we reported previously, approximating 10%.

We and others (3, 15, 30, 32, 38, 50) have found serological and molecular evidence in humans that enterohepatic *Helicobacter* spp. may be associated with several chronic hepatobiliary diseases. Others (7, 39, 47) have reported the identification of *H. pylori* in hepatobiliary tissues from patients with benign and malignant hepatobiliary disease. In some of these studies, bile procurement and sample processing had the potential to bias the results toward identification of *H. pylori*. For example, endoscopic retrograde cholangiopancreatography was utilized to collect bile samples from patients with known gastric *H. pylori* colonization, leading to potential contamination with *H. pylori* from the gastric mucosa (7). Moreover, in some cases, *H. pylori* was identified on the basis of sequencing segments of its 16S rRNA gene (47). However, this method is unsatisfactory for speciating *Helicobacters* due to the high 16S rRNA sequence homology among organisms in this genus (40). Moreover, *in vitro* studies have revealed that *H. pylori* is sensitive to bile and is chemotactically repelled by solutions of

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both conjugated and unconjugated bile salts (22, 55). Because of these equivocal findings in humans and our recent identification of a number of enterohepatic *Helicobacter* spp. that promote murine cholelithogenesis, we wanted to ascertain whether *H. pylori* infection exhibited the ability to induce cholesterol gallstones in the C57L mouse model (33).

## MATERIALS AND METHODS

### Animal Sources and Husbandry

All animal protocols were reviewed and approved by the Massachusetts Institute of Technology Committee on Animal Care. Three- to four-week-old male *Helicobacter* spp.-free C57L/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were divided into three groups for study. In the first group, mice remained uninfected and were fed a standard lithogenic diet containing 1.0% cholesterol, 0.5% cholic acid, and 15% dairy fat ( $n = 10$ ) (25). The second group was infected with *H. pylori* SS1 (28) and fed a rodent chow diet ( $n = 5$ ), and the third group was infected with *H. pylori* SS1 and fed a lithogenic diet ( $n = 10$ ). Group numbers were chosen based on a 90–95% power to statistically detect similar changes in the gallstone prevalence rate compared with changes noted in our initial study (33). Mice were housed in polycarbonate microisolator cages under specific pathogen-free conditions (free of *Helicobacter* spp., *Citrobacter rodentium*, *Salmonella* spp., endoparasites, ectoparasites, and known murine viral pathogens) in an Association for the Assessment and Accreditation of Laboratory Animal Care-accredited facility. Mouse rooms were kept at constant temperature and humidity on a 12:12-h regular light-dark cycle, and mice received food and water ad libitum. Animals were fasted for 12 h before CO<sub>2</sub>-induced euthanasia. Mice were fed a standard rodent chow (Purina Mills; St. Louis MO) containing <0.05% cholesterol until 8 wk of age. At this time point, mice were either continued on a standard rodent chow or converted to the lithogenic diet.

### *Helicobacter pylori* Infection

*H. pylori* SS1 (28) was grown for 24–48 h in Brucella broth (Becton Dickinson; Franklin Lakes, NJ) containing 5% heat-inactivated fetal calf serum. Broth cultures were centrifuged at 8,000 rpm for 20 min at 4°C, and the bacterial pellet was resuspended in Brucella broth at a turbidometric (optical density = 660 nm) reading of between 0.6 and 1.2. Four- to five-wk-old mice were infected by oral gavage with 0.2 ml of resuspended bacteria ( $n = 15$ ) or sham dosed with 0.2 ml Brucella broth ( $n = 10$ ) three times over a 5-day period. At monthly intervals thereafter, mice were given two more doses of *H. pylori* SS1 by gavage using the same protocol.

### Bile Analyses and Tissue Processing

Mice were euthanized with CO<sub>2</sub> in the fasted state at 16 wk of age. A ventral midline incision was made, and the gallbladder was removed intact. Full gallbladders were weighed, and their contents were examined under direct and polarized light microscopy by a microscopist (K. J. Maurer) blinded to sample identity. Bile was scored for mucin gel content, presence of liquid crystals, solid cholesterol monohydrate crystals, sandy stones, and true cholesterol gallstones as previously described (27, 52). Statistical analyses of mucin gel score and gallbladder weight were performed by one-way ANOVA with the Tukey-Kramer posttest using Instat 3.0 software (GraphPad; San Diego, CA). The frequency of cholesterol monohydrate crystals as well as sandy and true gallstone formation were analyzed by Fisher's exact test using the same software. Mouse stomachs were removed aseptically and opened longitudinally along the greater curvature. Approximately 30 mg of glandular stomach were flash frozen in liquid N<sub>2</sub> and stored at –80°C for subsequent quantitative PCR. Three 10-mg segments of the liver from different

liver lobes were collected aseptically and frozen at –20°C for subsequent PCR.

### Histopathological Examinations

At necropsy, the liver, gallbladder, stomach, duodenum, pancreas, and ileocecolic junction were collected, trimmed, and fixed in 10% neutral buffered formalin. Tissues were processed routinely, paraffin embedded, cut into 4- $\mu$ m-thick slices, and stained with hematoxylin and eosin. Additional stomach and gallbladder sections were stained with Alcian blue/periodic acid Schiff (PAS; pH 2.5) for acidic and neutral mucins (18). Tissues were evaluated by a comparative pathologist (A. B. Rogers) blinded to sample identity. Gastritis was scored on an ascending 0–4 scale using previously defined criteria (17). Inflammation of the squamous forestomach was scored using criteria similar to those for the glandular compartment. Epithelial hypertrophy/hyperkeratosis of the squamous stomach was scored as follows: 0, normal thickness; 1, two times normal thickness; 2, three times normal thickness; 3, four times normal thickness; or 4, greater than four times normal thickness. The presence or absence of intraepithelial microabscesses was also recorded. Mean histological grades were compared between multiple groups by Kruskal-Wallis one-way ANOVA followed by Dunn's posttest using Prism 3.0c for Macintosh (GraphPad). Direct comparisons were made with the Mann Whitney *U*-test. The prevalence of microabscesses was evaluated using Fisher's exact test. *P* values of  $\leq 0.05$  were considered statistically significant.

### Real-Time Quantitative PCR

Chromosomal DNA from broth-grown *H. pylori* SS1 and total DNA from mouse stomachs was prepared using the High Pure Template Preparation Kit according to the instructions of the supplier (Roche Molecular Biochemicals; Indianapolis, IN). To quantify colonization levels of *H. pylori* strain SS1 within the gastric mucosa, a real-time quantitative PCR assay was developed based on the nucleotide sequence of the *H. pylori* ureB gene in the ABI Prism TaqMan 7700 sequence detection system (A/B Applied Biosystems; Foster City, CA). Two primers (forward: 5'-CAAATCGCTGGCATTGGT-3' and reverse: 5'-CTTCACCGGCTAAGGCTTCA-3') and an internal probe (5'-AACAAAGACATGCAAGATGGCGTAAAAACA-3') were designed to hybridize within the 100-bp region (nucleotides 273–373) of the single-copy ureB gene (AF508016) of *H. pylori* SS1 using Primer Express software (Applied Biosystems) (40). Quantitative PCR conditions were as described previously (20). The specificity of these oligonucleotides for *H. pylori* was tested using DNA isolated from *H. felis* (ATC49179), *H. mustelae* (ATCC43772), and "*H. heilmannii*." To generate a standard curve, serial 10-fold dilutions (from  $5 \times 10^5$  to 5) of *H. pylori* SS1 genome copies, estimated from an average mass value (1.66 Mb) obtained from the two published *H. pylori* genomes, were used (2, 49). Copy numbers of gastric mucosal *H. pylori* SS1 DNA in mice were then calculated and normalized to micrograms of murine chromosomal DNA determined by quantitative PCR using a mammalian 18S rRNA gene-based primers and probe mixture (Applied Biosystems) as described elsewhere (53).

### Liver PCR

Livers were harvested, and DNA was extracted using the Roche DNA High Pure Template Preparation Kit (Roche Molecular Biochemicals) per the manufacturer's instructions. Two rounds of PCR amplification were performed. The first round of amplification used the genus-specific primer set C97 and C05, which amplifies an amplicon of ~1,200 bp (16, 19). PCRs were performed using 5  $\mu$ l template DNA and "PuRe Taq Ready To-Go PCR beads" (Amersham Biosciences; Uppsala, Sweden) with previously described conditions (16, 19). After this, a nested amplification was performed using the genus-specific C97 and C98 primer sets, which amplify an amplicon

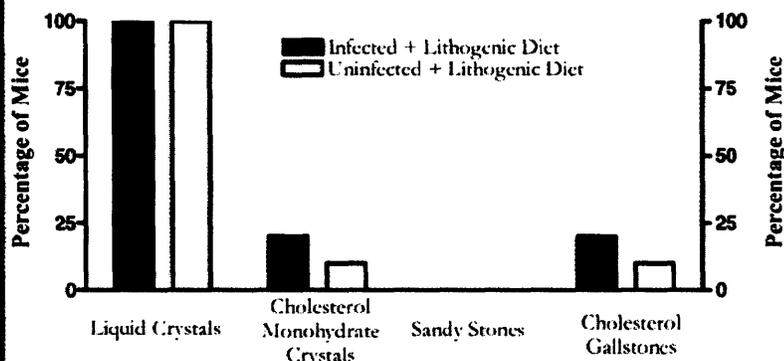


Fig. 1. Biliary phenotype determined by polarized light microscopy of gallbladder bile. Mice infected with *Helicobacter pylori* and fed a lithogenic diet do not differ in biliary phenotype compared with uninfected animals fed the same diet. Data for *H. pylori*-infected animals fed a chow diet are not displayed because chow-fed C57L mice fail to supersaturate their gallbladder bile with cholesterol and therefore do not demonstrate any of the ascribed biliary phenotypes.

of ~400 bp (16, 19). This reaction used 1 µl template DNA from the first reaction and followed the conditions described previously (16, 19). Included as a positive control was known *H. pylori* SS1 colonized murine gastric tissue, and proven uninfected mouse tissue was used as a negative control.

RESULTS

Biliary Phenotype

Regardless of the infection status, the gallbladder bile of all mice fed the lithogenic diet displayed cholesterol-phospholipid liquid crystals, indicating phase-separated supersaturated bile (Fig. 1). Mice fed a chow diet did not develop liquid crystals in bile, confirming the well-known requirement for a modified diet to supersaturate gallbladder bile and induce both liquid and solid crystal phase separation in this mouse model (33, 51). *H. pylori*-infected mice fed the lithogenic diet developed more cholesterol monohydrate crystals (20%) and true cholesterol gallstones (20%) compared with control animals (10% for each), but these changes were not statistically significant ( $P = 1.0$ ; Fig. 1). Moreover, no differences in sandy stone formation were noted (0% for both groups; Fig. 1). Mucin gel scores and normalized gallbladder weight for animals fed the lithogenic diet were significantly greater than those fed a standard chow diet ( $P \leq 0.05$ ); however, neither differed among animals fed the lithogenic diet regardless of infection status ( $P > 0.05$ ; Fig. 2). *H. pylori*-infected animals fed a chow diet did not develop mucin gel formation (score of 0; Fig. 2) and exhibited normalized gallbladder weights comparable with uninfected mice fed a chow diet (Fig. 2) based on our previous study (33).

Real-Time Quantitative PCR

To validate the specificity and sensitivity of the quantitative PCR assay for *H. pylori*, *H. pylori* SS1 DNA in parallel with the DNA templates from three gastric *Helicobacters* (*H. felis*, *H. mustelae*, and *H. "heilmanii"*) was detected using the primers and probes designed for the ureB gene. The quantitative PCR assay detected a minimum of five copies of the *H. pylori* SS1 genome (Fig. 3, lane 6), whereas there was no amplification from 10 ng (approximately equal to  $5 \times 10^5$  genome copies based on the size of the *H. pylori* genome) of DNA from *H. felis*, *H. mustelae*, or *H. "heilmanii"* (Fig. 3, lanes 7–9). The mean number of *H. pylori* organisms per microgram of host DNA in the gastric corpus of chow-fed animals was  $2.5 \times 10^5$ , whereas in lithogenic diet-fed infected animals these values were reduced by ~1 log unit ( $P < 0.05$ ) with  $6.0 \times 10^4$  organisms/µg host DNA (Fig. 4).

Liver PCR

Livers from *H. pylori*-infected animals were uniformly negative on initial PCR screening. Subsequently, nested amplification also failed to amplify any *H. pylori* DNA from either the infected group fed chow or the infected group fed the lithogenic diet (data not shown). This result contrasts markedly with the positive PCR results from the livers of enterohepatic *Helicobacter*-infected C57L mice in our earlier study (33)

Histopathology

*Liver and gallbladder.* Livers of C57L mice fed the lithogenic diet demonstrated a lobular pattern of hepatocellular microsteatosis concentrated in acinar zones 2 and 3 (mid-zonal and centrilobular, respectively; Fig. 5a). In contrast, macrosteatosis characterized by medium to large round clear cytoplasmic lipid vacuoles was mild, patchy, and mostly prominent in the periportal regions. Mild to moderate portal mononuclear cell inflammation was noted in a subset of mice fed the lithogenic diet, but its occurrence was recorded equally in *H. pylori*-infected and uninfected animals (Fig. 5a). Small lipogranulomata comprised of macrophages with phagocytosed lipofuscin-like material were sometimes seen (Fig. 5a). Mild and inconsistent gallbladder lesions were evident in some mice

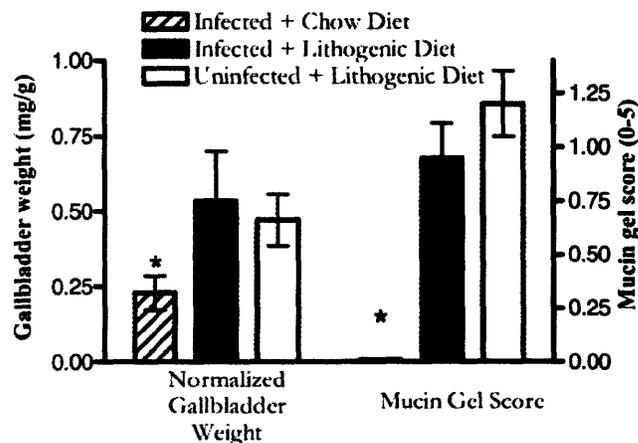
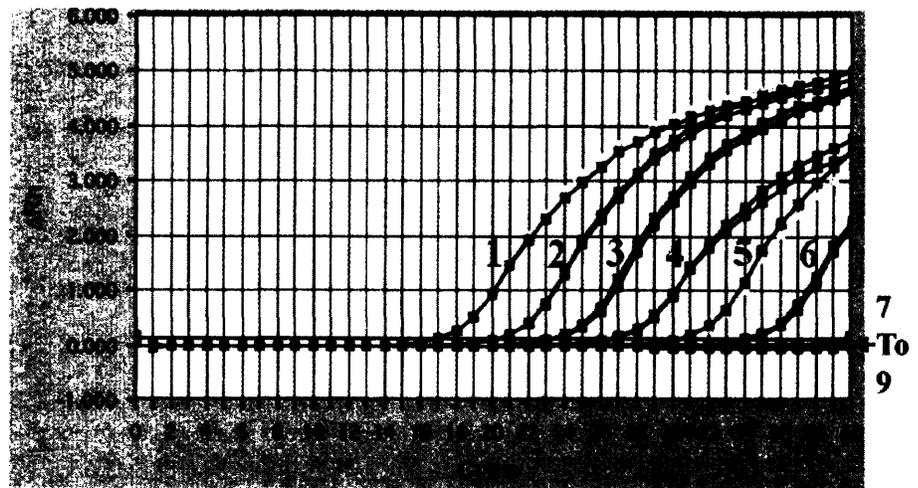


Fig. 2. Mucus gel score and normalized gallbladder weight (mg gallbladder/mouse body wt) for all groups of mice. Lithogenic diet feeding of mice independent of *H. pylori* status increases mucin accumulation and normalized gallbladder weight ( $P \leq 0.05^*$ ) compared with animals fed a standard chow diet. *H. pylori* infection does not significantly increase mucin gel content or normalized gallbladder weight compared with uninfected animals fed the same diet ( $P > 0.05$ ). All data are presented as means  $\pm$  SE.

Fig. 3. Standard curve detailing the sensitivity and specificity of the quantitative PCR assay developed. In plots 1–6,  $5 \times 10^5$ ,  $5 \times 10^4$ ,  $5 \times 10^3$ ,  $5 \times 10^2$ , 50, and 5 copies of the *H. pylori* SS1 genome were used as the initial template in these reactions. In plots 7–9, 10 ng of genomic DNA from *H. felis*, *H. "heilmannii,"* and *H. mustelae* were the initial template. All of these failed to amplify and are illustrated by the red symbols after the y-intercept. The  $r^2$  from the linear regression is  $>0.99$ .



fed the lithogenic diet. Microscopic findings included eosinophilic and lymphocytic cholecystitis (Fig. 5b), small islands of mucous metaplasia, and scattered epithelial cell hyalinosis with rare intraluminal crystals (data not shown). Unlike the severe gallbladder lesions in mice fed the lithogenic diet infected with specific enterohepatic *Helicobacter* spp. (33), gallbladder lesions in the present study were inconsistent, mild, and unassociated with *H. pylori* infection status. Moreover, there were no hepatobiliary lesions in *H. pylori*-infected mice on the chow diet. The duodenum, pancreas, and ileoceocolic junction were within normal limits in all mice regardless of diet or infection status.

**Stomach.** Two distinct histopathological patterns were produced in the stomach: one associated with *H. pylori* infection and the other with the lithogenic diet. Compared with uninfected mice fed the lithogenic diet (Fig. 5c), *H. pylori*-infected mice fed either chow or the lithogenic diet developed moderate mixed mononuclear and granulocytic cell proliferative gastritis of the cardia and corpus with atrophy of oxyntic glands and mucous metaplasia (Fig. 5d). No appreciable intestinal metaplasia or dysplasia were evident. With the use of staining by hematoxylin and eosin, the mucous metaplasia of the oxyntic mucosa was characterized by a foamy change in the cytoplasm of parietal cells (Fig. 5e). Mucous metaplasia was confirmed by

Alcian blue/PAS staining at pH 2.5, demonstrating transformation of surface mucins from the neutral gastric type (red) to the acidic intestinal type (blue). Additionally, there was heavy production of mixed mucins in the parietal cell zone, with intestinal-type mucins concentrated at the upper and lower boundaries of the cellular columns and gastric-type mucins in the middle (Fig. 5f). Compared with uninfected controls, scores of the lesions in the glandular stomach incorporating all criteria were significantly increased in *H. pylori*-infected mice regardless of diet ( $P \leq 0.05$ ; Fig. 6). However, there were no differences in mean scores between infected groups fed different diets except for an additive effect of the lithogenic diet on mucous metaplasia ( $P \leq 0.05$ ; Fig. 6).

The second pattern of gastritis, associated with the lithogenic diet, affected the anterior squamous compartment (forestomach). Histological changes consisted of moderate mixed inflammation and edema of the lamina propria and submucosa and hypertrophy of the squamous epithelium with orthokeratotic hyperkeratosis and frequent intraepithelial microabscesses (Fig. 5h). Microabscesses, generally 1–2 mm, were composed of degenerate neutrophils and epithelial cells, often with a central core of keratotic debris (Fig. 5h). In contrast, no squamous defects developed in *H. pylori*-infected mice fed the chow diet (Fig. 5g). However, in mice fed the lithogenic diet, there was a statistically significant additive effect of *H. pylori* infection on the degree of squamous hyperkeratosis ( $P \leq 0.05$ ; Fig. 7) and inflammation ( $P \leq 0.05$ ). *H. pylori*-infected mice fed the lithogenic diet were twice as likely to develop microabscesses of the squamous stomach as uninfected mice (80% vs. 40%, respectively), although this difference did not reach statistical significance ( $P = 0.17$ ).

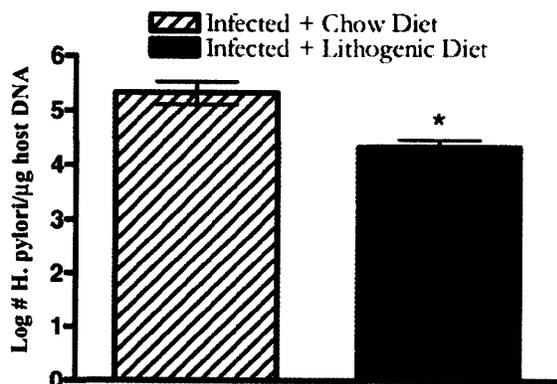


Fig. 4. Data are presented as log numbers of *H. pylori* organisms per microgram of host DNA  $\pm$  SE. Lithogenic diet-fed animals show significantly fewer organisms (by a log unit) present in the gastric mucosa compared with the situation in rodent-chow fed animals ( $*P \leq 0.05$ ).

**DISCUSSION**

The literature ascribing a putative role for *H. pylori* in causing human hepatobiliary disease has been both confusing and inconclusive (4, 7, 13, 39, 47). In this study, we demonstrated that *H. pylori*, unlike some enterohepatic *Helicobacter* spp. (33), does not promote cholesterol cholelithogenesis in the C57L mouse model. Moreover, *H. pylori* does not promote any of the preliminary stages (cholesterol monohydrate crystals, sandy stones) in the physical chemistry of cholelithogenesis. One might argue that a doubling of the prevalence of choles-

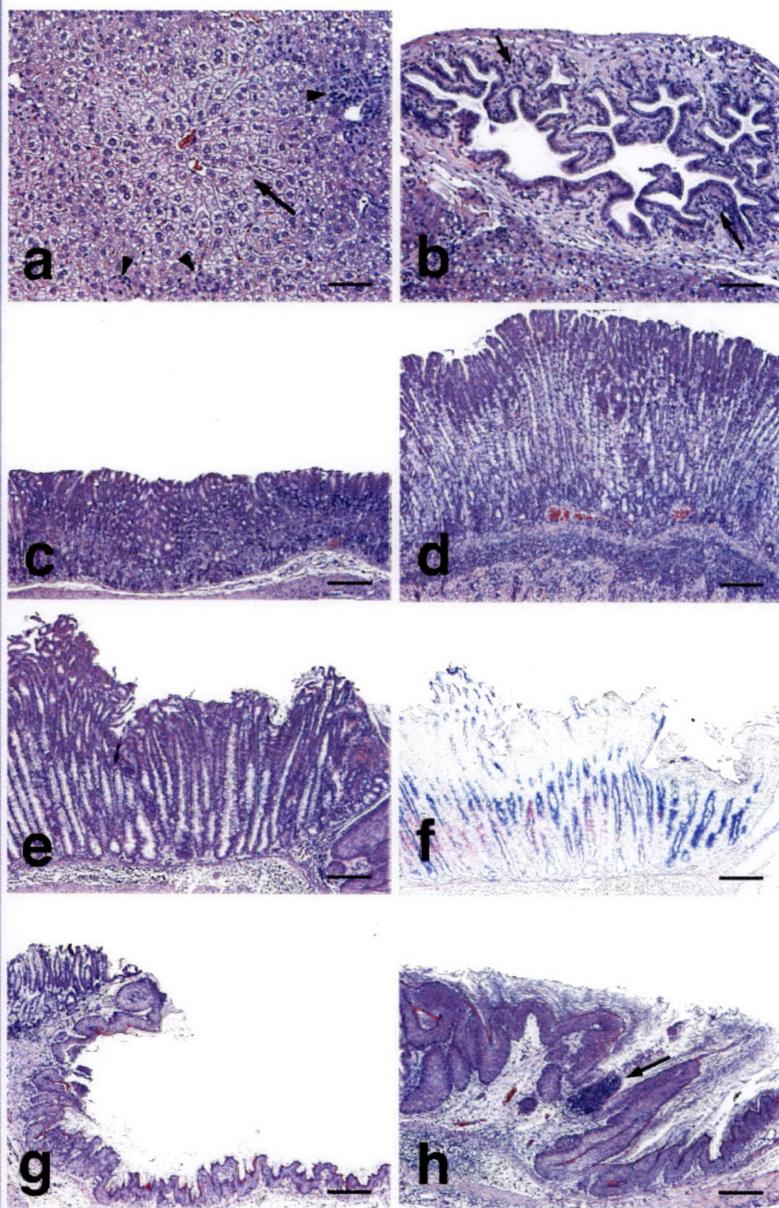


Fig. 5. Histopathology of the liver, gallbladder, and stomach from mice fed lithogenic diet and/or infected with *H. pylori* and euthanized at 16 wk of age. *a*: Hepatic lobule in the liver of a *H. pylori*-infected mouse fed the lithogenic diet with centrilobular and midzonal microsteatosis (arrow), patchy macrosteatosis (larger clear vacuoles), mild portal mononuclear inflammation (right arrowhead), and lipogranulomas (bottom left arrowheads). Identical lesions were present in uninfected mice fed the lithogenic diet (not shown). *b*: Gallbladder from the same mouse shown in *a* with mild eosinophilic and lymphocytic inflammation in the lamina propria (arrows). *c*: Histologically normal gastric corpus from an uninfected mouse fed the lithogenic diet. *d*: Moderate to severe inflammation and hyperplasia in a *H. pylori*-infected C57L mouse fed the chow diet. *e*: Marked mucous metaplasia in a *H. pylori*-infected mouse fed the lithogenic diet. Note the cytoplasmic foamy change in the parietal cell zone. *f*: pH 2.5 Alcian blue/periodic acid Schiff stain of the same mouse shown in *e*. Note the abnormal intestinal-type acidic mucins (blue) on the gastric surface and cytoplasmic accumulation of both acidic and neutral mucins in the parietal cell zone. *g*: Histologically normal squamous stomach in a *H. pylori*-infected mouse fed the chow diet. *h*: Hypertrophic pleating and marked hyperkeratosis associated with mucosal and submucosal inflammation and edema of the squamous stomach from a *H. pylori*-infected mouse fed the lithogenic diet. Note the intraepithelial microabscess composed of degenerate epithelial and polymorphonuclear inflammatory cells (arrow).

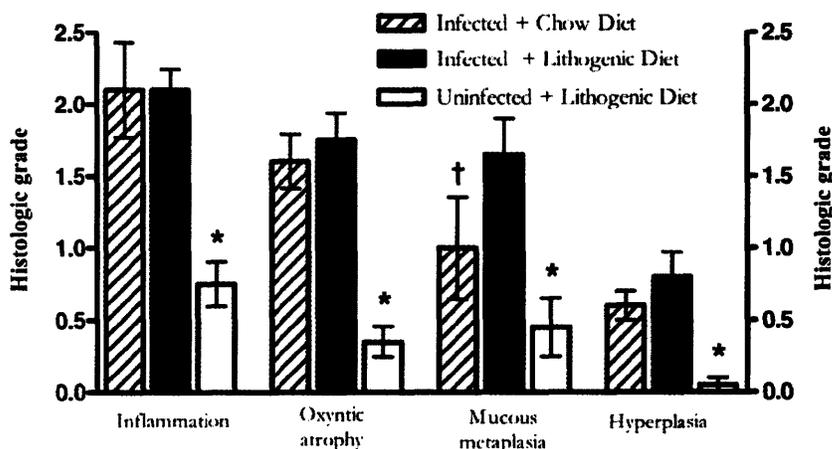
terol gallstone formation in infected mice would be important when dealing with a large population (such as humans with *H. pylori*); however, because *H. pylori* does not increase gallbladder mass (a surrogate marker of gallbladder hypomotility) or mucin gel accumulation (infected animals had, in fact, less mucin gel accumulation than uninfected animals), which are markers of cholelithogenesis, it is likely that this increase is merely due to population variation (as our statistical analyses indicate). In addition, the PCR results indicate that *H. pylori* does not colonize the murine hepatobiliary tree. This finding is not surprising because to date there is not a single report of successful bacterial isolation of these organisms from the liver or biliary tree of experimentally infected animals and only a single communication (44) reporting the isolation of *H. pylori* from a human liver damaged by Wilson's disease.

In the past, authors claimed to have identified *H. pylori*-like organisms in hepatobiliary tissue by 16S rRNA amplification

and sequencing. (47). Unfortunately, speciating *Helicobacters* based on sequencing a small (400–1,000 bp) segment of the 16S rRNA gene is fraught with error, because this region is highly conserved among *Helicobacter* spp. (40). Highlighting this concern, Avenaud and colleagues (4) identified organisms that appeared to be *H. pylori* based on sequencing of the 16S rRNA gene from the liver of humans. On the basis of stringent followup tests, these investigators discovered that these organisms were a previously unidentified (and still unclassified) *Helicobacter* sp. that is phylogenetically related to, but not, *H. pylori* (4).

We hypothesize that the presence of *H. pylori* in the biliary tree and liver of human patients with cholesterol gallstones is either a secondary colonizer (due to biliary changes from gallstones, cholestasis, or chronic cholecystitis) or the DNA denotes one or more related *Helicobacter* spp. Perhaps, in the presence of disease induced by other *Helicobacter* spp. or other

Fig. 6. Comparison of lesion grades in the glandular stomach of C57L mice. Mean grades of all lesion criteria were increased by *H. pylori* infection compared with sham inoculation (\* $P \leq 0.05$ ). An additive effect of the lithogenic diet with *H. pylori* infection was observed in the case of mucous metaplasia ( $\dagger P \leq 0.03$ ).



factors, the biliary microenvironment changes to allow for secondary invasion by *H. pylori*. For example, we (33) demonstrated previously that some cholelithogenic *Helicobacter* spp. increase gallbladder mucin gel accumulation significantly as well as contribute to mucinous metaplasia of the gallbladder in C57L mice fed the lithogenic diet. An increase in mucin gel accumulation or a change in mucin species may promote attachment of *H. pylori* to the biliary epithelium. Interestingly, others have noted that the type of mucin produced in the stomach is pivotal for *H. pylori* colonization, and, in fact, some normal gastric mucins are bactericidal (24). Consistent with a possible alteration of the biliary microenvironment leading to secondary invasion by *H. pylori*, Myung and colleagues (39) found that patients that are PCR positive for *H. pylori* exhibited a significantly lower biliary pH than those that were PCR negative. Further evidence for secondary invasion by *H. pylori* was recently demonstrated by multiplex PCR in an Iranian population (14). In this work, *H. pylori*-like DNA could be amplified from bile of gallstone patients with histological evidence of chronic cholecystitis but not from the bile of asymptomatic gallstone patients (14). These authors did not

attempt to culture for bacteria other than *Helicobacter* spp. However, others (21, 37, 46) have cultured the biliary tree and bile from humans and experimental animal models with cholecystitis and demonstrated numerous bacterial species that are considered part of the normal distal gastrointestinal tract and nasopharyngeal microbiota. These organisms often colonize the biliary tree secondarily to biliary disease, especially after obstructive cholestasis or sphincter of Oddi ablation. It is likely that *H. pylori* behaves in a similar manner and may colonize the biliary tree due to impaired biliary drainage, changes in mucus content or character, and other biochemical alterations affecting the chemistry and concentration of biliary lipids. To validate or disprove these hypotheses, a thorough chemical and physical-chemical analysis of bile in the presence and absence *H. pylori* DNA in the hepatobiliary tree would be necessary.

In planning the present experiments, we were concerned that the lithogenic diet would inhibit *H. pylori* colonization, because *in vitro* studies have demonstrated bactericidal effects of bile acids on *H. pylori* (22). Consistent with this notion, there was a significant log unit decrease in the present work in the number of *H. pylori* organisms in the stomachs of lithogenic diet-fed infected mice compared with the chow-fed group (Fig. 4). Interestingly, there was no significant change in the extent or severity of glandular gastritis between the two infected groups. In fact, in both lithogenic diet-fed and chow-fed mice, a robust gastritis was noted. Historically, the *H. pylori* SS1 strain causes minimal lesions in susceptible mouse strains at 3 mo postinfection, and C57BL/6 mice, which are characterized as a susceptible strain, often require 6 mo of *H. pylori* infection to demonstrate moderate gastric inflammation (28). The C57L mouse appears to be exquisitely susceptible to *H. pylori*-induced gastritis (regardless of the type of diet fed) and displays similar lesions and scores to INS-GAS hypergastrinemic mice at this early time point (17). This observation merits further investigation in view of the known fact that INS-GAS mice progress to adenocarcinoma after 6 mo of colonization with *H. pylori* (17).

In addition to infectious gastritis, noninfected mice that ingested the lithogenic diet developed lesions of the squamous forestomach. These lesions included hypertrophy, hyperkeratosis, and inflammation with microabscess formation of the epithelium. Furthermore, some of these diet-induced lesions were exacerbated significantly by infection with *H. pylori*.

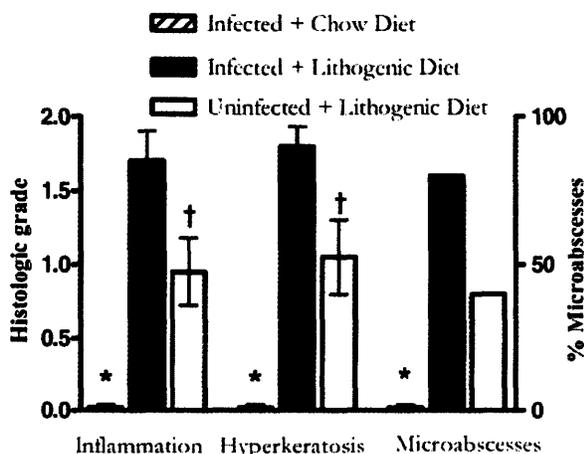


Fig. 7. Comparison of the lesion grades in the squamous stomach of C57L mice. The lithogenic diet induced squamous hypertrophy and hyperkeratosis, inflammation, and intraepithelial abscesses. *H. pylori* infection increased the severity of lesions in mice fed the lithogenic diet ( $\dagger P \leq 0.05$ ). In contrast, *H. pylori*-infected mice fed the chow diet did not develop lesions in the squamous forestomach. \*Significance compared with either infected group ( $P < 0.05$ ).



Moreover, the squamous epithelial lesions are reminiscent of the lesions caused in part by diet and stress in the *pars esophagea* of swine, which consist of hyperkeratosis and ulceration (12). Swine stomachs are commonly colonized by *H. suis*; however, the contribution of these helicobacters to gastric disease is not completely understood (26, 43). Like the inbred mice in this study, we propose that in domestic swine, dietary composition induces lesions in the *pars esophagea* and that *H. suis* exacerbates these lesions. The lesions in the squamous stomach of mice demonstrate that under modified dietary conditions, *H. pylori* can exacerbate disease in areas in close anatomic proximity to where these organisms colonize typically, i.e., the glandular stomach. The squamous forestomach of the mouse is lined by stratified squamous epithelium (29) and hence is a nonglandular zone, which is essentially an extension of the mouse esophagus separated anatomically from the glandular gastric compartment by the "limiting ridge" (29). These concepts raise the possibility that this mouse model could be used to study the contributions of diet and *H. pylori* infection to chronic esophageal diseases such as esophagitis and even esophageal cancer.

Any explanation for the synergism of diet and *H. pylori* infection in causing lesions of the glandular stomach in C57L mice, most notably metaplasia, must be speculative. The cholic acid triglyceride and cholesterol components of the diet may either singly or together be contributing to gastric lesions. For example, cholic acid promotes azoxymethane-induced aberrant crypt foci in rats (5, 48). However, analysis of the forestomach of bile acid-fed rats did not demonstrate any notable lesions (56). Interestingly, in humans, diets high in fat and cholesterol (so-called "Western diets") correlate with the risk for esophageal adenocarcinoma, gastric cardia adenocarcinoma, esophageal squamous cell carcinoma, and common gastric adenocarcinoma (31, 34, 35). Additionally, cholesterol free radicals are believed to promote a variety of diseases in mice and humans including atherogenesis (1, 23, 36). It is reasonable, therefore, to hypothesize that gastric inflammation from *H. pylori* may promote oxidation and free radical derivatives of cholesterol or fatty acids in humans consuming high dietary levels of these lipids and that these oxidized products could further contribute to chronic gastric and potentially esophageal diseases.

In summary, *H. pylori* infection in this prospective study does not contribute to cholesterol gallstone formation in the C57L mouse model. We believe that the purported suggestions in the literature that *H. pylori* causes cholesterol gallstones in humans are suspect and that, in parallel with many other bacteria, *H. pylori* is likely to be a secondary colonizer of the hepatobiliary tree after complicated gallstone disease and/or iatrogenic interventions. In addition, the C57L mouse is highly susceptible to *H. pylori*-induced gastritis and dietary-induced nonglandular lesions of the squamous forestomach, an anatomic region analogous in structure and function to the esophagus. This mouse strain may provide an important new model to study the effects of diet and chronic *H. pylori* infection on the development of chronic gastric and esophageal inflammation and perhaps neoplasia.

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