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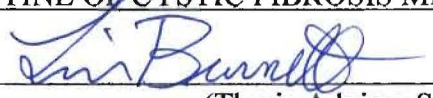
Abstract approved: 

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Abnormal mucus secretions characterize cystic fibrosis (CF). In a mouse model of CF, the pathology manifested in the small intestine is typified by a dehydrated, more acidic environment of the lumen. It is likely that this creates a more favorable environment for bacteria to colonize and proliferate within the small intestine. In this study, the types of cultivable aerobic bacteria of the small intestine were determined by analyzing colony morphology on a nutrient media. Bacterial colony morphologies with differences in abundance between CF and WT mice were isolated and used for proliferation, adherence and antimicrobial activity experiments. A difference was observed in proliferation in only one of five bacterial species in small intestinal supernatant. The adherence experiments only showed a difference between WT and CF small intestinal supernatants in *Escherichia coli*, and only one of four isolates showed any detectable adherence. In addition the antimicrobial activity of soluble compounds was determined by a radial diffusion assay. This assay revealed no difference between the CF mice and the WT mice in all but one of the isolates suggesting that the antimicrobial activity within the small intestine lumen is similar. Also the amount of IgA in the lumen was determined through an ELISA, and a nearly three fold increase was found in the CF mice. These data

suggest proliferation, and soluble antimicrobial compounds might not be critical for controlling abundance of the bacteria that were isolated in the small intestine of CF and WT mice.

MECHANISMS ASSOCIATED WITH THE BACTERIAL POPULATIONS IN THE SMALL INTESTINE OF CYSTIC FIBROSIS MICE

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INTRODUCTION

CYSTIC FIBROSIS

Human Pathology

Cystic fibrosis (CF) is a genetic disease that greatly affects mucus secretion. Mutation results in the misfolding and loss of membrane targeting of a cAMP gated chloride channel, called the cystic fibrosis transmembrane regulator (CFTR). In the mucosal epithelium the absence of CFTR affects the chemical composition of the mucus secreted: sodium ion secretion is increased, chloride ion and water secretion is decreased. (Ismailov *et al.*, 1996; Takacs-Jarret *et al.*, 2001).

In human CF patients the mucus secretion is typically thick, dehydrated, and slightly acidic resulting in significant pathology of the respiratory system. The thick mucus is difficult to clear from respiratory airways, causing an accumulation of fluid in the lung and respiratory tract. Difficulties that arise from the abnormal mucus frequently are exacerbated by secondary pulmonary bacterial infections (Takacs-Jarret *et al.*, 2001; Yu *et al.*, 2000). The typical causative agent of these pulmonary infections is *Pseudomonas aeruginosa*, a Gram negative bacterium that normally is innocuous (Yu *et al.*, 2000).

While the pathologies of the respiratory epithelium are a prime concern, CF pathology is not strictly limited to the pulmonary system. A second pathology in the gastrointestinal system also is of great concern. Thicker mucus and maldigestion in the small intestine might contribute to intestinal obstruction, a characteristic of CF that plagues patients of all ages (Grubb and Boucher, 1999; Yu *et al.*, 2000; Hinojosa-

Kurtzberg *et al.*, 2003). Thicker mucus is an indirect result of the mutation associated with CF. When this chloride channel is affected the transport of water also is decreased (Kunzelmann, 2001). The lack of water being secreted into the lumen causes the mucus becomes dehydrated and increases viscosity. In the small intestine, this increase in viscosity results in a greater chance for particles to become trapped. In addition, the secretion and function of enzymes into the small intestine are affected. Pancreatic enzymes in a normal individual are able to effectively break down food within the lumen of the small intestine. In CF humans the pancreatic duct becomes filled with the thickened mucus, promoting an accumulation of pancreatic enzymes that eventually can lead to the formation of fibrotic tissue in the pancreas (Eggermont, 1996; Grubb and Boucher, 1999). The blockage of the pancreatic duct explains the presentation of symptoms of maldigestion, a characteristic gastrointestinal pathology of CF (Grubb and Gabriel, 1997).

Mouse Pathology

The murine model of CF provides a good experimental system for studying the gastrointestinal pathology of this disease. Unlike humans, the primary pathology of CF in the mouse is located in the gastrointestinal tract and the pulmonary problems are not as pronounced (Grubb and Gabriel, 1997; Grubb and Boucher, 1999). However, the underlying patterns of gastrointestinal pathology are similar. For example, the mouse model displays the same characteristics of maldigestion, likely caused by a blockage of the pancreatic duct and mucus accumulation in the small intestine causing intestinal blockage (Grubb and Gabriel, 1997; Grubb and Boucher, 1999). These two aspects of the CF mouse pathology result in the mortality of the majority of these mice from the

gastrointestinal pathology shortly after weaning (Grubb and Gabriel, 1997; Grubb and Boucher, 1999). Fortunately, a successful colony can be maintained by breeding heterozygotes (*cftr*$^{+/-}$) and the fatal effects of the mutation can be avoided by carefully controlling the diet, making the mouse model a very good disease model for the GI pathology associated with CF.

BACTERIA AND HOST INTERACTIONS IN THE GI TRACT

The GI tract represents a microbial community structured by a delicate balance between the prevention of colonization by unwanted, potentially pathogenic, microbial species yet allowing for the colonization of innocuous or potentially beneficial species. Many of the symbiotic relationships with gut bacteria are considered mutualistic because the host provides a habitat and nutrients for bacterial survival while benefiting from bacterial competition (normal bacteria flora out-competing potentially pathogenic bacteria for space and/or food) and nutrient metabolism that the host is otherwise incapable of performing (Deplancke *et al.*, 2000; Heilig *et al.*, 2002; Wilson and Blitchington, 1996). However, when the immune system is weakened or when physiological abnormalities exist, the relationship changes. For example, normal flora bacteria act as pathogens and cause disease as is the case with *Escherichia coli* in the gastrointestinal tract (Rath *et al.*, 2001). The ability for this relationship to change suggests that the mutualistic association has tenuous boundaries that depend on host and/or environmental factors that promote or prevent bacterial colonization.

Factors Promoting Bacterial Colonization

The environment of the small intestine is conducive for the growth of a variety of different bacterial species. Sterile before birth, it is colonized almost immediately

following birth and as the infant begins to ingest food. The progression of bacterial associations in the gastrointestinal tract has been observed through a progressive stool analysis of human infants throughout the first few months. These types of analysis have shown the first bacterial species to appear in feces after birth are *Enterobacter sp.* and *Streptococci sp.* (Mackie *et al.*, 1999). As the infant ages and begins to ingest different types of food, the community changes. An infant who is consuming breast milk supplemented with formula has a dramatic increase in the number of bacterial species present compared a baby that just is consuming breast milk (Favier *et al.*, 2002). After weaning the bacterial composition in the stool samples from the babies on different initial diets became similar suggesting that diet strongly impacts composition of the bacterial community in the gut (Favier *et al.*, 2002).

Current interests in probiotic therapies demonstrate the importance of diet on bacterial colonization events in the human intestine. These therapies are those in which the human subject purposefully ingests "beneficial" bacteria. Often, the bacterial species ingested can out-compete potentially harmful bacterial species in the intestine when the bacterial flora has been eliminated by the treatment of other illnesses. The lactic acid producing members of the genus *Lactobacilli*, found in "active culture" yogurt, are commonly used as probiotics. *Lactobacilli* have the ability to survive in the gastrointestinal tract and adhere to epithelial cells (Snelling, 2005). Probiotics also provide a way to study factors associated with colonization by the bacterial species in the gut. Colonization by probiotic bacterial species appears to be dependent on hydrophobic membrane molecules. Wadström *et al.*, (1987) demonstrated that *Lactobacilli* that possessed an increase in hydrophobic membrane molecules bound better to epithelial

cells than did those bacteria with more hydrophilic membrane molecules. These *Lactobacilli* use free polyunsaturated fatty acids as a means to facilitate binding to the intestinal mucosa (Kankaapaa *et al.*, 2004). These polyunsaturated fatty acids result in changes associated with the hydrophobicity of the membrane of the organism. Kankaapaa *et al.*, (2004) speculated that environmental changes can induce an alteration of membrane fluidity of some bacterial species to regulate colonization.

Another colonization factor associated with many bacterial species is membrane-associated lipopolysaccharide (LPS), an outer-membrane glycolipid found especially in Gram negative bacteria. Free LPS can block the binding of *Escherichia coli* to mouse intestinal mucosa (Licht *et al.*, 1996). This study suggests that free LPS can act as a competitive inhibitor occupying receptors so that cell bound LPS cannot bind. Licht *et al.*, (1996) also demonstrated that colonization is influenced greatly by the presence of LPS. LPS deficient strains of *Salmonella typhimurium* were compared to wild-type strains of *Salmonella* in both growth and proliferation in the gastrointestinal tract (Licht *et al.*, 1996). LPS deficient and wild-type strains showed the same ability for growth in gastrointestinal tract. Licht *et al.*, (1996) inferred the LPS macromolecule is not directly needed for growth of this organism. However, when mice were challenged with LPS deficient and wild-type strains, the LPS deficient strain showed a decrease in the ability to colonize the gastrointestinal tract compared to the wild-type strain. Licht concluded that LPS is used by many bacterial species to aid in the colonization of bacteria in the gastrointestinal tract.

Events Preventing Bacterial Colonization

Innate and adaptive immunity can lead to bacterial extinction that results in either the loss of bacterial species or the deterrence of colonization within the small intestine. Innate immunity refers to the cells and compounds, such as Paneth cells, mast cells, macrophages, the complement compounds, and the defensin compounds, which produce a fast, nonspecific response particularly important in controlling bacterial numbers immediately after inoculation. The adaptive immune response is a specific immune response that requires recognition of specific microbial markers. These two components work best in concert with each other. For example, antibody molecules derived from adaptive immune cells can mark a pathogen allowing cells of the innate immune system to locate and kill the pathogen more efficiently.

Secreted antimicrobial compounds mediate much of the innate immunity of the gut mucosa. Many of these antimicrobial compounds are secreted from the Paneth cells at the base of the crypts in the small intestine including a group of small peptide molecules known as the α–defensins (Ouellette *et al.*, 1994; Ouellette *et al.*, 2000; Ayabe *et al.*, 2002). Through the creation of a cDNA library representing transcripts of the murine crypt, Ouellette *et al.*, (1994) discovered that six α-defensin-like compounds (called cryptdins) exist in the mouse small intestinal lumen. Cryptdin four and five possessed the greatest antimicrobial capacity as measured via a radial diffusion assay (Ouellette *et al.*, 1994).

The inflammatory response also is a key component of the immune response of the small intestine. Inflammation is a local response to trauma that helps to stimulate the migration of immune cells to the affected area. This response is composed of many

effector molecules that might have antimicrobial activity. Inflammation acts to prevent bacterial population spikes by eliciting a response to where an outbreak might occur. Inflammation also might also act to keep immune cells activated in the small intestine so there is always a baseline immune response to help regulate bacterial populations.

Components of the adaptive immune system can also act to prevent bacterial colonization. IgA is a class of antibody that can be secreted through the mucosal epithelium and is the most abundant antibody found in mammalian secretions (Macpherson *et al.*, 2001). IgA secretion into the blood is regulated in a very different manner than the IgA that is secreted across the intestinal mucosa. IgA in the serum is activated through a T-cell dependent pathway. However studies with T cell deficient mice have found that intestinal IgA is unaffected (Macpherson *et al.*, 2000). These studies demonstrate that the antibodies found in the small intestine are there because of different B cell stimulation than those found in the serum (Gardby *et al.*, 1998). This suggests that the adaptive immune system associated with the small intestine functions in a specific manner to regulate nonsymbiotic colonization, similar to the innate immune system.

The innate and adaptive immune system can, and often do, work together to provide a more efficient immune response against potential recolonizations. Defensins act both as a way to kill microbes, and as chemoattractants for adaptive immune cells (Lin *et al.*, 2004). The immune cells that have been signaled by the defensins react to certain features of a foreign substance in an attempt to clear this substance. Defensins enhance the mitogenic capability of Concanavalin A *in vitro*, meaning defensins increase

cell division for these immune cells (Tani *et al.*, 2000). Defensins also have adjuvant properties, which could increase antibody production (Tani *et al.*, 2000).

Bacterial Colonization in the CF Small Intestine

A recent investigation demonstrated an increase in the total number of bacteria found in the small intestine of CF mice compared to control mice (Norkina *et al.*, 2004a). This investigation used real time PCR to quantify changes in the abundance of the 16S rDNA region of the bacterial chromosome. Norkina *et al.*, (2004a) showed that there was a nearly forty-fold increase in the copy number of the 16S ribosomal RNA gene in the CF mouse indicating an increase in the abundance of bacteria in the small intestine of CF mice.

Investigations to determine the bacterial community profile of the CF small intestine have been done, but with conflicting results in terms of differences in diversity (Norkina *et al.*, 2004a, Clarke *et al.*, 2004). These two studies have found few similarities in the microbial populations between the two phenotypes, and they disagree in the predominant species of the CF intestine (Norkina *et al.*, 2004a; Clarke *et al.*, 2004). Clarke *et al.*, (2004) isolated *Pseudomonas sp.* in the small intestine, which are of primary concern in the respiratory pathology of CF, and Norkina *et al.*, (2004a) did not. Instead, Clarke *et al.*, (2004) demonstrated that *Enterococcus* sp. and *Proteus* sp. were found exclusively in the CF mouse, but were not the bacterial species of highest abundance. These differences between the two studies are most likely a result of the techniques that were employed. Norkina *et al.*, (2004a) used a shotgun cloning procedure, which identifies bacterial species on the basis of their 16S rDNA sequence, and showed that the small intestine of CF mice had essentially a monoculture of

Escherichia coli. Clarke *et al.*, (2004) identified bacteria by spreading a loop of intestinal contents on a bacterial media.

To date, few studies have been published concerning mucosal immunity in the CF mouse small intestine. Clarke *et al.*, (2004) demonstrated decreased mRNA concentrations for cryptdins and lysozyme in the Paneth cells of CF mice and speculated that this might be responsible for the observed increased bacterial abundance in the small intestine. Norkina *et al.*, (2004b) demonstrated through micro array analysis an increase in the CF mouse in gene expression of many inflammatory markers. Many of these up-regulated inflammatory markers act as signals for antimicrobial compounds. Based on these two studies a relationship might exist among the bacterial community and the regulation of immunological genes associated with the CF small intestine. However, neither study provided evidence for differences in antimicrobial activity in the compounds found in the small intestine.

CF patients have with a propensity for bacterial infections in the pulmonary system, and previous studies using the mouse model show differences in the bacterial community between CF and their WT counterparts that could be related to the gastrointestinal pathology. The differences in the bacterial community most likely exist due to differences in colonization events, extinction events, or competitive interactions. The mucus that lines the WT small intestine is thin, relatively pH neutral, and constantly is being overturned. In the CF mouse small intestine this mucus is thick, dehydrated, acidic, and tends to accumulate. This could lead to an increase in overall bacterial colonization or colonization by specific bacterial species as potentially more colonization

factors are present. This hypothesis will be tested by traditional microbiological methods for community analysis, proliferation and adhesion assays, as described in Chapter 2.

The mucus associated with the CF small intestine also might affect the mechanisms preventing bacterial colonization. The mucus might act to plug crypts making it difficult for the antimicrobial peptides secreted by the Paneth cells to get into the lumen. Bacterial stimulation of immunological process also could be suppressed as the CF mucus might provide enough of a buffer so that immune cells are not activated, although inflammation is increased (Norkina *et al.*, 2004b). This hypothesis is investigated in Chapter 3

CHAPTER TWO:

ANALYSIS OF THE BACTERIAL COMMUNITY IN THE SMALL INTESTINE OF CF MICE

Introduction

Abnormal mucus secretions characterize cystic fibrosis (CF) (Ismailov *et al.*, 1996; Takacs-Jarret *et al.*, 2001). These secretions are typically dehydrated and slightly acidic, which results in a more viscous mucus. Humans show an inability to clear thick mucus resulting in an increase of fluid in the lung and respiratory tract. Symptoms frequently are exacerbated by secondary pulmonary bacterial infections (Takacs-Jarret *et al.*, 2001; Yu *et al.*, 2000), typically with *Pseudomonas aeruginosa.*

While the pathologies of the respiratory epithelium are a prime concern, CF pathology is not strictly limited to the pulmonary system. A second pathology in the gastrointestinal system also is of great concern. The lack of water being secreted into the lumen causes the mucus becomes dehydrated and increases viscosity (Kunzelmann, 2001). In CF humans the pancreatic duct becomes filled with the thickened mucus, promoting an accumulation of pancreatic enzymes that eventually can lead to the formation of fibrotic tissue in the pancreas (Eggermont, 1996; Grubb and Boucher, 1999). In mouse models of CF, the pathology is primarily manifested in the small intestine and also displays dehydrated, slightly acidic, and viscous mucus as seen humans (Grubb and Gabriel, 1997). It is likely that the physiological abnormalities associated with CF create a more favorable environment for bacteria to colonize and proliferate within the small intestine.

An increase in the total number of bacteria present in the small intestine of CF mice has been demonstrated (Norkina *et al.*, 2004a). Studies to determine the bacterial community profile of the CF small intestine also have been done, but estimation of the bacterial diversity in these profiles was variable (Norkina *et al.*, 2004a; Clarke *et al.*, 2004), most likely a result of the techniques used. Norkina *et al.* (2004a) used a shotgun cloning procedure, which identifies bacterial species on the basis of their 16S rDNA sequence. The small intestine of CF mice had essentially a monoculture of *Escherichia coli* compared to a much more diverse community in wild type (WT) animals (Norkina *et al.*, 2004a). Clarke *et al.*, (2004) identified bacteria by spreading a loop of intestinal contents onto solid media. Their results indicated very little difference in diversity between CF and WT mice, showing that the small intestine of both contained equal numbers of bacteria belonging to the Enterobacteriaceae family and that other types of bacteria, such as *Enterococcus spp.*, *Staphylococcus spp.*, and *Pseudomonas spp.*, were the predominate species in CF mice.

The unique intestinal environment of CF mice results in an altered bacterial community and it is possible that these different communities might contribute to the pathology observed in the small intestine. In this study we specifically looked for evidence of altered bacterial communities by assessing the number and the types of bacteria present. Furthermore, we assessed whether the different small intestine environments of CF and WT mice impact the ability of different species of bacteria to adhere or grow.

Materials and Methods

Animals. CF mice (*cftr*^{tm1UNC}) congenic to the C57Bl/6J background were maintained in the animal facility at the University of Kansas Medical Center by crossing heterozygotes (De Lisle *et al.*, 2001). Genotypes of mice were determined through PCR anlaysis of tail snip samples for the *cftr* gene. Male and female homozygous cage mates between 6 to 9 weeks of age were used for experimentation. To prevent lethal intestinal obstruction of the CF mice and to control for dietary influences, all mice (CF and WT) were provided a liquid diet (Peptamen, Nestlé, Deerfield, Ill.) after weaning.

Sample collection. Mice were killed by CO_2 asphyxiation, and the small intestine was ligated on either end and removed aseptically. The excised small intestine of 18 mice (11 WT/ 7 CF) were flushed with 30 ml of ice cold phosphate-buffered saline (PBS) and six mice (5 WT/ 5 CF) were flushed with 30 ml of sterile ice cold dithiothreitol (DTT, 10 mM). Intestinal flushings were centrifuged for 30 min at 30,000 x g at 4° C. Pellet and supernatant were stored at –20° C until use.

Spread Plating. Intestinal pellets from the DTT flushed mice were thawed to 4° C and ten-fold serial dilutions were performed. A 100 µl sample of each dilution was spread plated onto 100mm Petri plates containing Tryptic Soy Agar (TSA) or MacConkey agar (MAC) and incubated aerobically at 37° C for 48 hours. Each bacterial isolate from the TSA plates also was transferred to MAC and incubated at 37° C for 48 hours, to determine their ability to ferment lactose. Differences in the number of colonies between the two types of media allowed enumeration of lactose fermenting and non-lactose fermenting bacteria. Diversity, based on colony morphology and MAC growth, was assessed using the Shannon-Weiner index of diversity (Krebs, 1985). Four bacterial

isolates were chosen based on differing relative abundance between the two mouse groups and subcultured on TSA to obtain pure cultures.

Proliferation Assay. Protein determinations using BioRad dc (Bio-Rad, Hercules, CA) were performed on the supernatants from WT mice and CF mice and these supernatants normalized to an equivalent protein concentration (0.02 mg/ml) by dilution with PBS. Aliquots of 200 µl were added to wells, in triplicate for each individual mouse (11 WT / 7 CF), on a high binding ELISA/RIA 96-well plate (Corning-Costar Corp., Cambridge, Massachusetts) and incubated overnight at 4°C. Wells were inoculated with 25 µl of a ten fold dilution of overnight TSB culture (*Escherichia coli,* (ATCC 25922) or the small intestinal isolates), and incubated for 270 min at 37°C on a shaker set 250 rpm. Absorbance values at 630 nm were read at 15-minute intervals to measure turbidity during this incubation using a micro plate reader (Biotek, Winooski, VT). Control wells for both WT and CF supernatants were inoculated with ten fold dilution of TSB (no bacteria) and the absorbance of these wells was subtracted from all experimental wells. Except in isolates where growth approximated a linear model, non-linear regression was used to compare changes in absorbance to a 3-parameter sigmoidal model the asymptotic absorbance, and a parameter related to the growth rate were predicted from this model. The 3-parameter model used was:

$$Y = \frac{a}{1+e^{-(X-X0)/b}}$$

where "a" is an estimate of the asymptotic absorbance, "X-X0" is the time delay before detectable growth begins, and "b" is the parameter related to growth rate. These

parameters were compared between WT and CF using 95% confidence limits to determine the statistical significance of the observed differences.

Adherence Assay. Protein determinations using BioRad dc (Bio-Rad, Hercules, CA) were performed on the supernatants from WT mice and CF mice and these supernatants were normalized to an equivalent protein concentration (0.02 mg/ml). Aliquots of 200 µl were added to wells, in triplicate, for each individual mouse (11 WT / 7 CF), on a high binding ELISA/RIA 96-well plate (Corning-Costar Corp., Cambridge, Massachusetts) and incubated overnight at 4°C. After overnight incubation supernatants were removed and approximately (10^6) of *E. coli* or the isolates of the small intestine, in PBS, were added to each well, and incubated for one hour at 37°C on a shaker set 250 rpm. CF or WT supernatant with PBS and no added bacteria were incubated simultaneously to serve as negative controls. Wells were washed four times with PBS to remove non-adherent bacteria. Remaining bacteria were provided 100µl tryptic soy broth and incubated at 37°C on a shaker at 250 rpm. Metabolic activity was determined after 2 hours by the addition of 15 µl of 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO). After 5 minutes, insoluble reduced MTT-formazan was solubilized with acidified isopronanol and absorbance at 630 nm was determined for each well using a micro plate reader. Absorbance values were subtracted from the negative controls and absorbance values of WT and CF coated wells were compared between CF and WT using Student's t-test.

Results

Bacterial diversity

Bacterial diversity was assessed using the spread plate technique on TSA and MAC. Growth occurred on TSA; however no growth was seen on the MAC agar. Different colony morphologies seen on the TSA plates were assigned a letter designation and the number of different morphologies was assumed to represent the minimum number of species present. Small intestines flushed with DTT showed few potential bacterial species. Also, few differences were seen in diversity between the CF and WT mouse groups and no statistical differences in diversity were seen between the two group's ($P>0.05$, Shannon-Weiner t-test) (Table 1). However, certain colony morphologies were identified to have differing abundances between CF and WT mice.

Four bacterial isolates were subcultured from the TSA spread plates to represent species that were isolated from WT and CF animals. One bacterial isolate (Isolate 1) was unique to the CF mouse phenotype. Two isolates (Isolate 2 and Isolate 3) were found in higher abundance in CF mice. The fourth isolate (Isolate 4) was found in higher abundance in the WT animals.

Proliferation in SI supernatant

The supernatants from CF and WT small intestine flushings were assayed for their ability to sustain the proliferation of *Escherichia coli* and the four bacterial isolates. Proliferation was estimated from either a nonlinear 3-parameter sigmoidal model or linear regression. The 3-parameter model estimates asymptotic absorbance, the time at which the population begins to grow, and a third parameter that is inversely proportional

to the growth rate. The sigmoidal model provided a good fit to the observed curves (r^2 ranging from 0.88 to 0.99).

E. coli incubated in supernatants from WT mice had a growth parameter of 0.015 absorbance units/min with a standard error of 0.185 absorbance units/min. In the CF mouse supernatant, this parameter was 0.019 absorbance units/min with a standard error 0.172 absorbance units/min, which based on 95% confidence limits showed no statistical difference compared to WT supernatants (Figure 1). In the control mice the estimate of asymptotic absorbance was at an absorbance of 0.207 with a standard error 0.016 and in the CF mouse this equilibrium was estimated at an absorbance of 0.160 with a standard error 0.008. However differences in the population equilibrium between CF and control mice were not significant.

Isolate 1 was found only in CF mice. The growth curves in CF and WT supernatant were best fit by linear regression therefore only growth rate could be estimated (Figure 2). Slow growth rates were seen for this isolate, however absorbance values were above the uninoculated mouse supernatants at all time points, and the growth rate was significantly greater than zero (WT, $F=47.14$, $P<0.05$; CF, $F=830$, $P<0.05$) ($r^2=0.87$ for WT mice and 0.99 for CF mice). The growth rate in the control mouse was 1.538×10^{-5} absorbance units/min with a standard error of 2.24×10^{-6} absorbance units/min. In the CF mouse the growth rate was 5.53×10^{-5} absorbance units/min with a standard error of 1.92×10^{-6} absorbance units/min. Based on 95% confidence intervals these differences were statistically significant.

Isolate 2 was a bacterial species isolated from a CF mouse and was found in higher abundance in the CF mouse than in the WT mice. Bacterial growth occurred in

both CF and WT supernatants (WT, F=1461, P<0.05; CF, F=322 P<0.05) (Figure 3). This nonlinear regression produced an r^2 value of 0.99 for WT mice and 0.99 for CF mice. Further analysis showed that the growth parameter for Isolate 2 in WT mice was 0.0120 absorbance units/min with a standard error of 0.148 absorbance units/min. In CF mice this parameter was 0.017 absorbance units/min with a standard error of 0.104 absorbance units/min. . In WT supernatant the asymptotic absorbance was estimated at an absorbance of 0.056 with a standard error 0.022 and in the CF mice supernatants the asymptotic absorbance was identified at an absorbance of 0.046 with a standard error 0.018. No significant differences in the growth parameter and population equilibrium were found when comparing the 95% confidence intervals from estimates given by the 3-parameter sigmoidal model.

The nonlinear regression analysis of Isolate 3 resulted in an r^2 value of 0.99 for WT and 0.98 for CF mice. Growth above the blank was significant (F= 370 for WT (P<0.05) and F=227 for CF (P<0.05)). The growth parameter in WT mice was 0.021 absorbance units/min with a standard error of 0.273 absorbance units/min and 0.017 absorbance units/min with a standard error of 0.176 absorbance units/min in CF mice. When comparing these estimates of growth rate to the 95% confidence intervals no significant differences were seen (Figure 4). When the asymptotic absorbance was compared, control mice showed this asymptote to be estimated at an absorbance of 0.026 with a standard error of 0.0003 and in CF mice the asymptotic absorbance was estimated at an absorbance of 0.028 with a standard error 0.0006 indicating these differences were not significant.

The proliferation and adherence assay was performed with the bacterial species found with higher abundance in WT mice (Isolate 4). Bacterial growth occurred in both CF and WT supernatants (WT, $F=4.49$, $P<0.05$; CF, $F=14.43$, $P<0.05$) (Figure 5). This regression analysis showed an r^2 value of 0.88 for WT mice and 0.99 for CF mice. The growth parameter in WT mice was 0.034 absorbance units/min with a standard error of 0.208 absorbance units/min, and 0.030 absorbance units/min with a standard error of 0.556 absorbance units/min in CF mice. Comparing these values to the 95% confidence intervals demonstrated these differences in the growth parameter were not significant. In the WT, the asymptotic absorbance was estimated at an absorbance of 0.231 with a standard error 0.051 and in the CF mouse the asymptotic absorbance was estimated at an absorbance of 0.269 with a standard error 0.019.

Adherence in SI supernatant

Individual wells of ELISA plates were coated with supernatants from WT or CF mice and inoculated with *E. coli* to evaluate adherence. Metabolic activity, as measured by the MTT assay relative to uninoculated controls, indicated that bacteria adhered to the coated wells and that nearly twice as much activity in wells coated with CF supernatant than in wells coated with WT supernatant ($P<0.05$, Student's t-test)(Figure 6).

Metabolic activity measured via the adherence assay was not detectable for Isolates 1, 2, and 3, but was detectable for isolate 4. This assay for Isolate 4 showed a nearly 1.5 fold increase in binding for WT mice, however these differences were not statistically significant ($P>0.05$, Student's t-test) (Figure7).

Discussion

The abnormal properties of the CF mucus create difficulties in analyzing bacterial communities in the small intestine. The content of the DTT flushing speculatively contains a majority of bacterial species present in the small intestine. This flushing agent acted as a reducing agent that broke down the mucus. By using DTT as a flushing agent more of the mucus could be harvested from the small intestine leading to a more effective release of the bacteria from the mucus. The data presented here show no difference between CF and WT mice in bacterial diversity when the small intestine is flushed with DTT, however there are differences in abundance of certain species. A flushing agent without the ability to loosen the mucus leaves a majority of mucus bound to the intestinal lining. However, since DTT is a reducing agent it might be antimicrobial and could kill certain bacterial species dwarfing any possible changes in diversity. This might be the reason that no growth was seen on MAC agar. MAC agar is selective for gram negative bacteria, and differential for members of the *Enterobacteriaciae* family. Members of this family were present in two previous studies (Norkina *et al.*, 2004; Clarke *et al.*, 2004). Gram negative viability in DTT was not assessed, but the absence of Gram-negative bacteria from our DTT-flushed samples suggests they might be sensitive to DTT.

Different abundance of certain colony morphologies in CF and WT mice might be reflective of differences in the nature of the mucus in CF mice. Various antimicrobial compounds might not have the capacity to penetrate the thicker mucus layer in the CF mouse and therefore bacteria that normally are sensitive to these compounds can survive. While no differences in diversity were seen in this study, there were certain bacteria morphologies in higher abundance in a certain phenotype of mouse. The differences

associated with CF mucus might also have properties that affect certain bacterial species ability to adhere to or colonize the mucus.

Species occurring in only one of the mouse groups might be present because of differences in adherence or more favorable growth conditions. These more favorable conditions might cause changes in the growth rate, and/or a change in the asymptotic absorbance associated within that environment. Similar expectations hold true for bacteria found in differing relative abundances in the two mouse groups.

The isolates from this study were harvested from either a WT mouse or a CF mouse based on unique occurrences, or large differences in relative abundance. Bacterial Isolate 1was unique to the CF mouse, and therefore would be expected to have a more favorable growth rate, asymptotic absorbance, and/or adherence ability in this environment. The population growth expectations were met with this particular species, however no adherence was seen nor was the ability to test asymptotic absorbance as the growth curve did not follow a sigmoidal model. However this species was able to grow at a significantly faster rate in CF mice compared to WT. The two bacterial isolates (Isolates 2 and 3) that showed a higher abundance in the CF animals might be expected to have higher growth rates, asymptotic absorbance, and adherence in the CF supernatant, however they showed no proliferation differences between the two groups, and no detection of adherence was seen. This implies that these bacterial species are suited for both environments and preference between the CF and WT environment is minimal. A higher abundance of a bacterial isolate (Isolate 4) in the WT mice suggest higher growth rates, asymptotic absorbance, and adherence in the WT supernatant, however they showed no proliferation differences between the two groups, and no detection in

adherence was seen. This could imply that this bacterium is equally suited for both environments and preference between the CF and WT environment is minimal.

The adherence assay used for this study demonstrated that only two of the five bacterial species used could be detected. This assay was performed at the same time as the proliferation assay and growth rates had not yet been determined. Therefore the stop point was based on a middle point of the growth curve for *E. coli*. This point might not have been optimum for the isolates contributing to our inability to detect adherence, and assay optimization for each isolate could have been done to have detectable results. Furthermore, there were fewer bacteria at the addition of growth media in the adherence assay which could have been accounted for in determining when to add MTT.

Proliferation and adherence could be used to explain abundance differences of certain bacterial between mouse groups. Differences were seen in the abundance of certain bacterial species in CF or WT mice; however the only difference seen in the proliferation assay was for Isolate 1. The only species to show differences in adherence was a lab strain of *E. coli*. Other mechanisms must be in place to explain these abundance differences. One mechanism could be related to the immune system causing bacterial extinction. Lack of and/or non functional antimicrobial compounds might lead to an increase in abundance of certain species in the CF mouse small intestine. The adherence assay showed no detection with the methods used in this study. However differences cannot be ruled out because this was not fully optimized to show if differences in adherence exist in the bacterial isolates.

TABLE 1. Small Intestinal Bacterial Community of CF and WT Mice Flushed with DTT

	Colony Morphology	Abundance (CFU/ml x 10^4)
CF	a (Isolate 2)	8.94
N=5	b	27.2
	c	7.17
	d* (Isolate 1)	0.27
WT	a	3.71
N=5	b (Isolate 3)	3.06
	c	3.62
	e† (Isolate 4)	0.05
	f**	0.1

* found only in three CF mice.

† found only in two WT mice.

** found only in one WT mouse.

Time (min)

Abs (630 nm)
0.25
0.2
0.15
0.1
0.05
0
0
100
200
300
400
Time (min)
CF Model
WT Model

Figure 1. Rate of *E. coli* growth in WT and CF small intestinal supernatant. Results are expressed as the average absorbance of the uninoculated wells per min subtracted from the average absorbance of the mouse (11 WT / 7 CF) wells per min. Error bars represent standard error of the mean. No differences were seen between the measures of growth rate or populate density equilibrium.

Abs (630nm)
0
0.005
0.01
0.015
0.02
0
100
200
300
Time (min)
CF
WT

Figure 2. Rate of Isolate 1 growth in WT and CF small intestinal supernatant. Results are expressed as the average absorbance (A630) of the uninoculated wells per min subtracted from the average absorbance of the mouse (11 WT / 7 CF) wells. Error bars represent standard error of the mean. Differences were seen between the measures of growth rate or populate density equilibrium ($P<0.05$ Linear Regression, ANOVA).

Abs (630 nm)
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
100
200
300
400
Time (min)
CF Model
WT Model

Figure 3. Rate of Isolate 2 growth in WT and CF small intestinal supernatant. Results are expressed as the average absorbance of the uninoculated wells per min subtracted from the average absorbance of the mouse (11 WT / 7 CF) wells. Error bars represent standard error of the mean. No differences were seen between the measures of growth rate or populate density equilibrium.

Abs (630 nm)
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
100
200
300
400
Time (min)
CF Model
WT Model

Figure 4. Rate of Isolate 3 growth in WT and CF small intestinal supernatant. Results are expressed as the average absorbance of the uninoculated wells per min subtracted from the average absorbance of the mouse (11 WT / 7 CF) wells. Error bars represent standard error of the mean. No differences were seen between the measures of growth rate or populate density equilibrium.

Abs (630 nm)
0
0.05
0.1
0.15
0.2
0.25
0.3
Time (min)
0
100
200
300
400
CF Model
WT Model

Figure 5. Rate of Isolate 4 growth in WT and CF small intestinal supernatant. Results are expressed as the average absorbance of the uninoculated wells per min subtracted from the average absorbance of the mouse (11 WT / 7 CF) wells. Error bars represent standard error of the mean. No differences were seen between the measures of growth rate or populate density equilibrium.

Abs (630nm)
0
0.01
0.02
0.03
0.04
0.05
0.06
WT
CF

Figure 6. Adhesion of *E. coli* in WT and CF small intestinal supernatant. Results are expressed as the mean absorbance (630 nm) for each mouse group (11WT / 7 CF). Error bars represent standard error of the mean. A nearly two fold difference was seen between the adhesion in WT compared to CF ($P<0.05$, Student's T-test).

Abs (630nm)
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
WT
CF

Figure 7. Adhesion of Isolate 4 in WT and CF small intestinal supernatant. Results are expressed as the mean absorbance (630 nm) for each mouse group (11WT / 7 CF). Error bars represent standard error of the mean. No difference was seen between the adhesion in WT compared to CF ($P>0.05$, Student's T-test).

CHAPTER THREE:

ANALYSIS OF THE INNATE IMMUNITY IN THE SMALL INTESTINE OF CF MICE

Introduction

Abnormal mucus secretions characterize cystic fibrosis (CF) (Ismailov *et al.*, 1996; Takacs-Jarret *et al.*, 2001). Human patients typically have a pulmonary pathology related to this thick mucus. Symptoms frequently are exacerbated by secondary pulmonary bacterial infections (Takacs-Jarret *et al.*, 2001; Yu *et al.*, 2000), typically with *Pseudomonas aeruginosa* that the body normally is normally able to control.

The CF pathology is not strictly limited to the pulmonary system. A second pathology in the gastrointestinal system also is of great concern. Lack of water being secreted into the lumen causes the mucus to become dehydrated and increases viscosity (Kunzelmann, 2001). In CF humans the pancreatic duct becomes filled with the thickened mucus, promoting an accumulation of pancreatic enzymes that eventually can lead to the formation of fibrotic tissue in the pancreas (Eggermont, 1996; Grubb and Boucher, 1999). In mouse models of CF, the pathology is primarily manifested in the small intestine and also displays dehydrated, slightly acidic, and viscous mucus as seen humans (Grubb and Gabriel, 1997). It is likely that these conditions of the disease impair natural immune defenses of the gut epithelium, which might lead to the differences in bacterial abundance demonstrated by Norkina *et al.*, 2004a, and the compositional differences not described in Chapter 2

To date, one study has been published concerning mucosal immunity in the CF mouse small intestine. Clarke *et al.*, (2004) demonstrated decreased mRNA

concentrations for cryptdins and lysozyme in Paneth cells of CF mice. These data suggest a decrease in abundance of these antimicrobial compounds in the lumen of the CF small intestine and might explain the increased bacterial abundance in the CF mouse small intestine. Also, chapter 2 demonstrated bacterial species with differing abundances between the two mouse groups, with no difference in proliferation or adherence.

Our working hypothesis is that the intestinal pathology of CF mice is due, in part, to an increased bacterial load within the small intestine. Norkina *et al.*, (2004a) demonstrated that the small intestine of CF mice indeed have a higher concentration of bacteria. In this study, we investigated whether the abnormalities associated with CF has an impact on mucosal immunity. The antimicrobial activity of CF mouse supernatants were compared to these of WT mouse supernatants to bacterial species associated with small intestine. We particularly paid attention to the antimicrobial activity against those bacterial species found in differing abundance in CF or WT mice. These assays demonstrated few differences in antimicrobial activity between the two groups. The adaptive immune system was also investigated as luminal IgA concentrations were compared between the CF and WT mice.

Materials and Methods

Animals. All animals were housed and maintained at the University of Kansas Medical Center. The CF mouse model was derived from *cftr*^{tm1UNC} mice (Jackson Laboratories, Bar Harbor, ME), by backcrossing onto a C57BL/6 background strain and maintained by breeding heterozygotes (De Lisle *et al.*, 2001). Genotypes of mice were determined through PCR of tail snip samples. Homozygotes of both sexes were used

between 6 to 9 weeks of age. To prevent lethal intestinal obstruction, all mice were provided a liquid diet (Peptamen, Nestlé, Deerfield, Ill.) after weaning.

Sample collection. Ten mice (five CF, five WT) were killed by CO_2 asphyxiation. The small intestine was ligated where the stomach meets the small intestine and where the small intestine meets the cecum and removed aseptically. The excised small intestine was massaged and flushed with 30 ml of ice-cold, sterile 10 mM dithiothreitol (DTT) (Sigma, Saint Louis, MO). Intestinal flushings then were centrifuged for 30 min at 30,000 x g at 4° C. Supernatants were stored at –20° C until all mice were processed (about three months). Supernatants were thawed on ice to 4° C and then evaporated to dryness using a vacuum concentrator. Each lyophylate was resuspended in 150 µl phosphate buffered saline (1 mM PBS) and centrifuged for three minutes at 16,000 x g to pellet insoluble material. Resuspended lyophylates were stored overnight at 4°C.

Bacteria used in the experiments. As described in chapter 2, four bacterial isolates were obtained from spread plates to represent species that were differentially isolated in WT and CF animals. Isolate 1, Isolate 2 and Isolate 3 are either unique to or found at higher abundance in CF animals, while Isolate 4 is found at higher abundance in WT animals. *Listeria monocytogenes* (Lab stock, Emporia State University) also was used since it is known to be sensitive to intestinal cryptdins of mice (Eisenhauer *et al.*, 1992).

Radial Diffusion Assay. The radial diffusion assay was performed as described by Eisenhauer *et al.*, 1992. A ten-fold dilution of log phase bacteria (*L. monocytogenes* or the SI isolates) in tryptic soy broth (TSB) was performed in 7.2 ml of melted radial diffusion agar (1% w/v agarose, 10 mM PBS, 0.02% Tween 20) cooled to 50 °C. The inoculated agar was poured into a sterile petri plate and allowed to solidify. Wells were

punched into the solid agar material using a sterile metal tube (3mm outside diameter). Five µl of each mouse supernatant were loaded into the wells. A dilution series (range 200 µg to 12.5 µg) of human neutrophil peptide-1 (HNP-1) (Sigma, Saint Louis, MO), a protein of similar structure and function to the antimicrobial compounds of the small intestine, was added to different wells. Control wells were loaded with PBS, Penicillin/Streptomycin (1000units penicillin/1 mg Streptomycin) (Sigma, Saint Louis, MO), or 10 mM DTT lyophilized using the vacuum concentrator and resuspended in PBS and one-tenth TSB to 150 µl. The plates were incubated at 40° C for five hours to allow the diffusion of the loaded material into the agar. The diffusion agar was then overlaid with five ml of 2x TSA and incubated overnight at 37° C. The diameters of the zones of inhibition (ZOI) excluding the well diameter were measured to the nearest millimeter. The mean ZOI of CF and WT supernatants were compared for each bacterial species using Student's t-test.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was used to determine the abundance of IgA in the lumen of CF and WT mice. Eighteen mice (seven CF, eleven WT) were killed by CO_2 asphyxiation. The small intestine was ligated where the stomach meets the small intestine and where small intestine meets the cecum and removed aseptically. The excised small intestine was massaged and flushed with 30 ml of ice cold sterile PBS. Intestinal flushings were then centrifuged for 30 min at 30,000 x g at 4° C. Supernatants were stored at –20° C until all mice were processed (about three months). Supernatants were thawed to 4° C and processed using a mouse IgA ELISA kit (Bethyl Labs, Montgomery, TX) following the protocol furnished by the manufacturer. The substrate for this enzyme reaction was 3, 3', 5, 5'-tetramethylbenzidine (TMB). A

standard curve was prepared from IgA dilutions. The mean abundance of CF and WT IgA antibodies were compared using Student's t-test.

Results

The relative antimicrobial capacity of CF and WT supernatants was assessed using the common gut pathogen *Listeria monocytogenes*. A dose-dependent ZOI was observed around the wells loaded with varying amounts of HNP-1. The antibiotic loaded well had a large ZOI (20 mm) (data not shown) while PBS and DTT/TSB lyophylate did not inhibit growth (Figure 1A). The SI supernatant samples showed varying zones of inhibition with an average of 7.6 mm with a standard deviation of 1.6 mm for the WT and 6.8 mm with a standard deviation of 1.5 mm for the CF mice (Figure 1B). There was no statistical difference between the two groups ($P>0.05$, Student's t-test).

The antimicrobial capacity of supernatants of WT and CF mice was also assessed using the three bacteria isolates found in higher abundance in CF mice (Isolates 1, 2, 3). A dose-dependent ZOI was observed around the wells loaded with varying amounts of HNP-1, while PBS and DTT/TSB lyophylate did not inhibit growth (data not shown). This assay yielded an average ZOI of 8.2 mm with a standard deviation of 5.8 mm in the CF mouse and 12.4 mm average with a standard deviation of 2.4 mm in the WT mouse for Isolate 1(Figure 2). Isolate 2 demonstrated a mean ZOI of 2.4 mm with a standard deviation of 2.1 mm in the CF mouse and 2.3 mm average with a standard deviation of 2.2 in the WT mouse (Figure 3). Isolate 3 had a mean ZOI of 6.8 mm with a standard deviation of 3.8 mm in the CF mouse and 0 mm in the WT mouse (Figure 4). The SI supernatant from control mice did not inhibit the growth of this organism as no zones of

inhibition were observed in any of the wells loaded with control supernatant. Isolate 3 was the only organism to have a statistical difference ($P<0.05$, Student's t-test).

The bacterial species that was higher abundance in WT mice (Isolate 4) showed sensitivity only to high concentrations of HNP-1. This isolate was not sensitive to either WT or CF supernatant.

Supernatants of both WT and CF mice were used to assess the concentration of the IgA antibody using the ELISA technique. A standard curve, using mouse IgA indicated that the assay was sensitive to15 ng/ml. The WT mice had a concentration of 19800 ng/ml of IgA with a standard deviation of 6160 ng/ml of IgA. The CF mice had a concentration of 39900 ng/ml of IgA with a standard deviation of 15400 ng/ml of IgA (Figure 5). There was a nearly three fold difference between the two mouse groups ($P<0.05$, Student's t-test).

Discussion

We hypothesized that the intestinal pathology of CF mice is due, in part, to the increased bacterial load within the small intestine. An immunocompromise might provide an explanation for why an increase in bacterial load exists in the CF model. We investigated both innate and adaptive mucosal immunity.

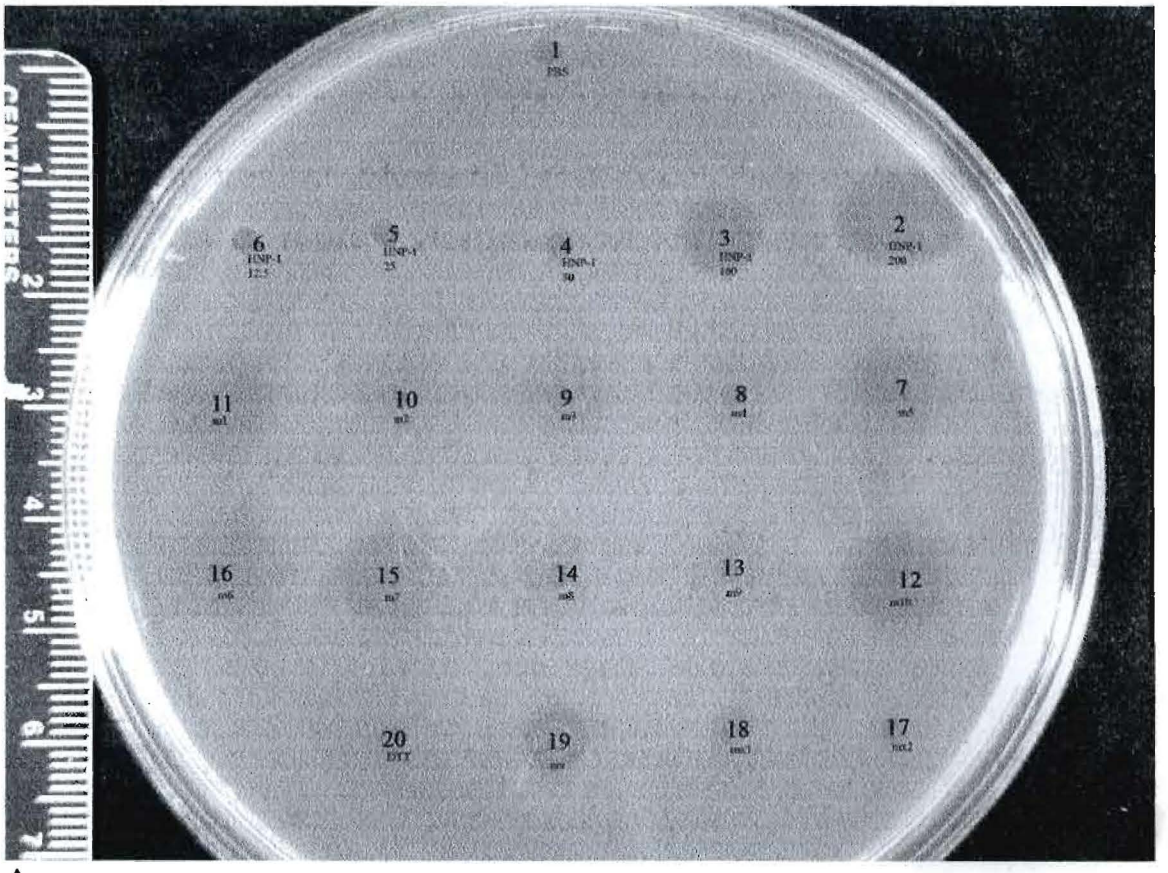
Listeria monocytogenes was used as a test organism to determine the relative antimicrobial activity of small intestine supernatants from CF mice and WT mice. Because of the increased bacterial abundance in CF small intestine (Norkina *et al.*, 2004a) we predicted that the antimicrobial activity of the CF mice supernatants would be less. This experiment indicated that *L. monocytogenes* was equally sensitive to supernatants derived from CF and WT mice.

Lack of and/or non functional antimicrobial compounds in the CF small intestine would expectantly lead to an increase in an increase in bacterial abundance. This might also explain why certain bacterial species were found in higher abundance in the CF animal. Bacterial Isolates 1, 2, and 3 were all found in higher abundance in the CF small intestine. However no difference was observed in the specific antimicrobial activity between the mouse groups for Isolate 1 and 2. This suggests that the antimicrobial compounds in the small intestine are functional and can help regulate the abundance of these two species. Isolate 3 was more sensitive to the antimicrobial activity of CF supernatant than to WT supernatant. Because Isolate 3 was found with much higher abundance in CF animals this result was surprising. DTT was used as a flushing agent to loosen and harvest as much of the mucus as possible. However if antimicrobial compounds were blocked by mucus from getting into the lumen the DTT could release these trapped compounds, resulting in larger ZOI in CF supernatants.

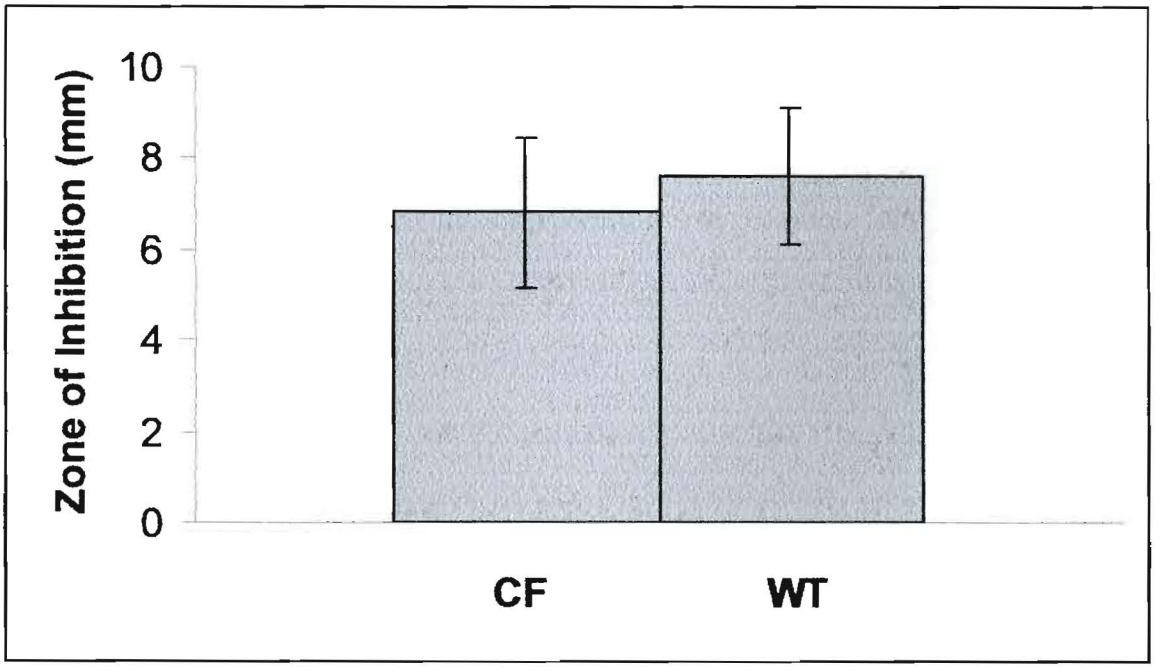
The bacterial species (Isolate 4) that demonstrated a higher abundance in the small intestine of the WT mouse showed no sensitivity to any of the mouse samples. This result, along with the observation that there was relatively small ZOI with even large concentrations of HNP-1 suggests that this bacterial species is relatively insensitive to cryptdin –like compounds.

The ELISA indicated a difference in the IgA concentration between CF and WT mice. The thick mucus associated with CF animals might have positive or negative effects on the antibody concentration. This thick mucus could lead to a lack of antigen presentation to B-cells causing a decrease in the antibody concentration, or the increase in bacterial abundance could lead to an increase in the antibody titer. Also, the ineffective

pancreatic enzymes associated with CF (Grubb and Boucher, 1999) might result in poor degradation of these proteins which might cause a biased comparison between the two groups. The increase in IgA found in CF mice suggests that antibodies are able to get to the lumen and possibly attach to bacteria.


CENTIMETERS
1
PBS
6
5
4
3
2
11
10
9
8
7
16
15
14
13
12
20
19
18
17

A.


Zone of Inhibition (mm)
10
8
6
4
2
0
CF
WT

B.

Figure 1. Radial diffusion assay performed with *Listeria monocytogenes*. Figure 1A. demonstrates ZOI's wells 2-6 is HNP-1 standard curve, middle two rows are CF and WT mouse supernatants. Well 20 represents a DTT control, and well 1 represent a PBS control. Wells 17-19 are a dilution series of a previous mouse that also demonstrate a dose dependency on the size of the well. Figure 1B the mean ± standard deviation of ZOI seen in both CF and WT mice.

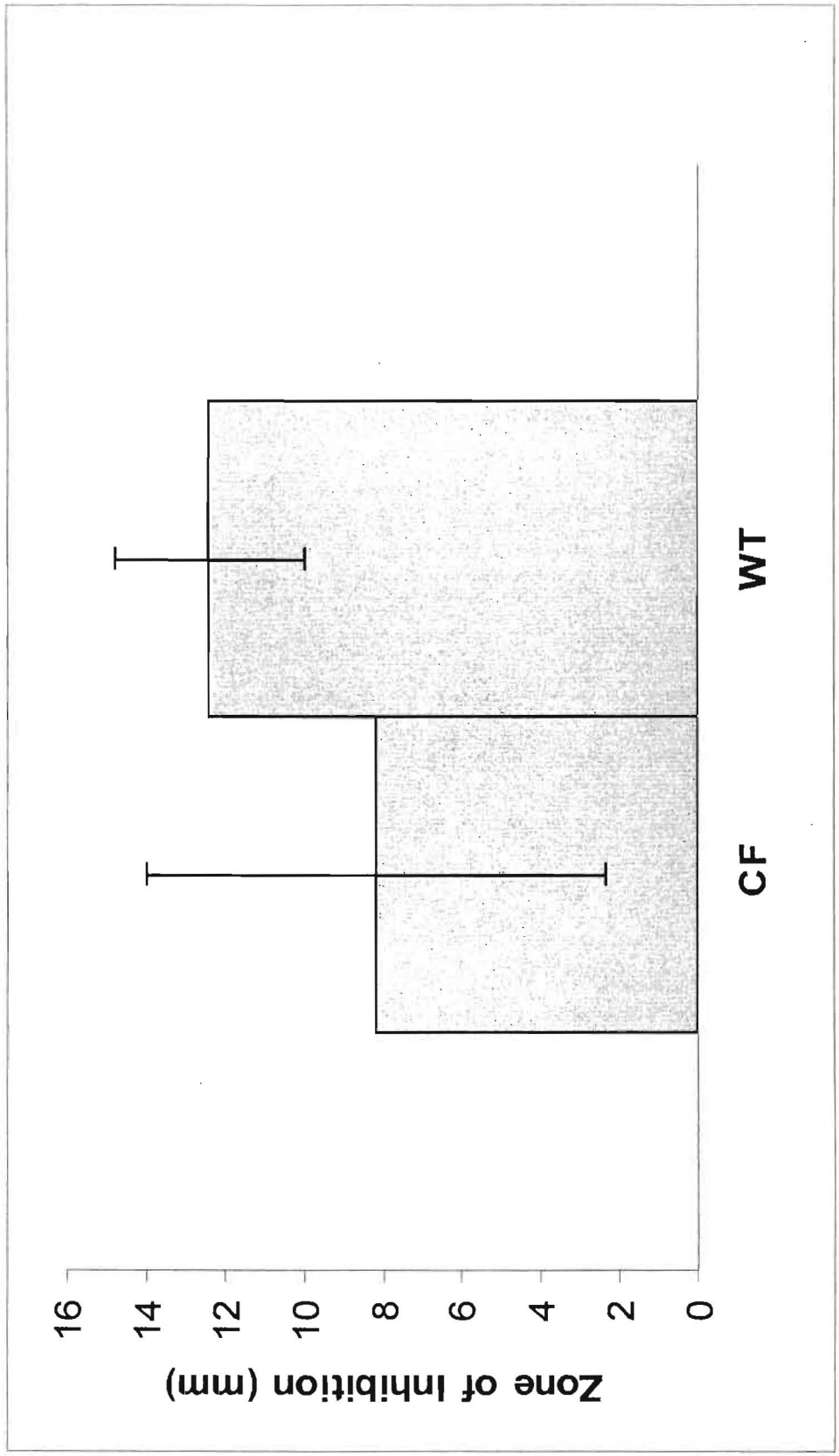

Zone of Inhibition (mm)
0
2
4
6
8
10
12
14
16
CF
WT

Figure 2. Radial Diffusion assay for Isolate 1 showing the mean ± standard deviation of ZOI observed in CF and WT supernatants. No differences seen between the two mouse groups. (P>0.05, Student's t-test)

Zone of Inhibition (mm)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
CF
WT

Figure 3. Radial Diffusion assay for Isolate 2 showing the mean ± standard deviation of ZOI observed in CF and WT supernatants. No differences seen between the two mouse groups. (P>0.05, Student's t-test)

Zone of Inhibition (mm)
0
2
4
6
8
10
12
CF
WT

Figure 4. Radial diffusion assay for Isolate 3. No zones of inhibition seen for the WT mouse, but zones of inhibition were observed in the CF mouse samples. ($P<0.05$, Student's t-test)

IgA concentration ng/ml
0,00
10000,00
20000,00
30000,00
40000,00
50000,00
60000,00
CF
WT

Figure 5. Results from ELISA comparing mean ± standard deviation IgA concentrations observed in CF and WT supernatants. A nearly 3 fold increase was seen in the CF mice supernatants. ($P<0.05$, Student's t-test)

CHAPTER FOUR:

SUMMARY

Bacterial diversity of the small intestine was assessed by colony morphology on TSA plates. The diversity between CF and WT mice showed no difference, however there were differences seen in the abundance of certain bacterial species in each mouse group. These differences in composition could be the result of favorable environmental changes which promote adherence and proliferation, or to unfavorable environmental changes which promote bacterial extinction. These potential mechanisms were investigated using bacterial isolates that differed in relative abundance between CF and WT mice.

The proliferation assay showed no difference in the asymptotic absorbance or the parameter related to growth rate for most isolates and *E. coli*. Only Isolate 1 differed between CF and WT mice. However, this was also the only bacterial species that did not have a sigmoidal curve and very minor changes in absorbance occurred over four hours. It is likely that this isolate never got out of lag phase, and never reached logarithmic growth so comparisons of growth rate and asymptotic absorbance are tenuous. These results suggest that the changes in the small intestine environment due to CF do not affect proliferation or population density equilibrium of these bacteria.

The adherence assays showed that bacteria might be able to specifically adhere to one mouse phenotype better than the other. However, we were only able to detect adherence in Isolate 4 and *E. coli* therefore comparisons between CF and WT could not be made. This assay used metabolic activity and bacterial growth to measure bacterial adherence. This could cause some bias in the assay as some bacterial species grow at

different rates as demonstrated by the proliferation experiments. Therefore more optimization could be done with this assay with the other isolates.

The radial diffusion assay could show if differences in antimicrobial activity between CF and WT by changes in the size of ZOI. This assay demonstrated that no differences in antimicrobial activity between CF and WT mice using *Listeria monocytogenes*, Isolate1, Isolate 2. Isolate 4 showed no sensitivity to CF or WT supernatant suggesting possible other unidentified mechanisms are used in the CF animal to keep the abundance lower than in WT mice. Isolate 3 demonstrated a difference in antimicrobial activity, however this antimicrobial activity of mouse supernatant does not explain the difference in abundance. This bacterial species was found in higher abundance in the CF small intestine, but was more sensitive to CF supernatant.

Finally the IgA concentration analysis showed a higher concentration of IgA in CF mice. This increase in IgA suggests that there is no deficiency in antibody production and secretion. In fact it might be higher because there is a higher abundance of bacteria in the small intestine of CF mice.

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