Abnormal Paneth cell granule dissolution and compromised resistance to bacterial colonization in the intestine of CF mice

Lane L. Clarke,1,2 Lara R. Gawenis,1,2 Emily M. Bradford,1 Louise M. Judd,3 Kathryn T. Boyle,1 Janet E. Simpson,1,4 Gary E. Shull,2 Hiroki Tanabe,5 Andre J. Ouellette,5,6 Craig L. Franklin,3 and Nancy M. Walker1

1Dalton Cardiovascular Research Center and the Departments of 2Biomedical Sciences and 4Veterinary Pathobiology, University of Missouri, Columbia, Missouri 65211; 3Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, Ohio 45267; and Departments of 5Pathology and 6Microbiology and Molecular Genetics, University of California, Irvine, California 92697-4800

Submitted 8 September 2003; accepted in final form 1 January 2004

CYSTIC FIBROSIS (CF) is a lethal genetic disease with an autosomal recessive inheritance pattern (12). The underlying cause of CF is mutations in the CF transmembrane conductance regulator (cfrt) gene, which results in defective function and/or processing of the mutant protein CFTR (33). Normally, CFTR is expressed in the epithelia of various organs and glands in which it functions as a cyclic nucleotide-activated anion channel and exerts regulatory function over other epithelial transport proteins. The major intestinal manifestations of CF are believed to result largely from deficient anion secretion and abnormal Na+ absorption across the epithelium, which lead to insufficient intraluminal hydration and intestinal impaction states such as meconium ileus (5, 27). Histopathologically, the CF intestine is characterized by goblet cell hyperplasia and crypt obstruction with inspissated mucus (2). The obstructive syndrome and histopathological appearance are accurately reproduced in CF mouse models, in particular, CFTR knockout mice, such as the S489X mutant mouse (42). Repeated intestinal infections are not a recognized manifestation of CF, although chronic inflammation of the intestinal wall often persists at subclinical levels in both CF patients and CF mice (15, 36, 41, 47, and L. L. Clarke, unpublished observations). However, our understanding of intestinal microbiology in CF patients is complicated by a number of disease-related factors, including deficient anion secretory responses to bacterial enterotoxins and aggressive patient treatment with antibiotics and digestive enzyme supplements (47).

Paneth cells are granulated epithelial cells found in the small intestine of most vertebrates (25). Unlike other epithelial lineages that derive from stem cells located in the crypts of Lieberkühn, Paneth cells migrate distally to the bottom of the crypt in which they are adjacent to undifferentiated anion secreting cells expressing CFTR (6, 8). In this location, the Paneth cells synthesize and secrete granules containing a variety of peptides and proteins with known host defense functions. The most abundant peptides of Paneth cell granules are a diverse population of 3- to 4-kDa cysteine-rich polypeptides known as enteric α-defensins (or cryptdins), which have a broad range of antimicrobial activity (30). Cryptdins are homologs of myeloid defensins, antimicrobial peptide components of phagocytic leukocytes (39). Human Paneth cells express two α-defensin genes, human α-defensin (HD)-5 and HD-6, whereas the mouse α-defensin family is larger, coding for at least 17 different cryptdin isoforms (29). In addition to the cryptdins, Paneth cell granules contain a number of other proteins that are important in innate immunity of the intestine, including lysozyme, phospholipase A2, TNF-α, matrilysin, and α1-antitrypsin (28). Paneth cell granules are released constitutively into the crypt lumens; however, granule release can be stimulated by cholinergic agonists or during exposure of the...
epithelium to bacterial cell products (4, 37). After Paneth cell granules are secreted, they typically undergo rapid dissolution by a process that apparently requires physiological concentrations of divalent cations in the extracellular milieu (3).

Palliative dietary manipulations, such as provision of a liquid diet or osmotic laxative therapy, are necessary to ensure the growth and survival of CF mice (9, 16). In preliminary studies to evaluate the histopathological changes that develop in the CF mouse intestine after withdrawal of osmotic laxative therapy, we observed the presence of Paneth cell granules entrapped within mucous casts of the intestinal crypts (11). In the mouse, Paneth cell granules are readily identified, because the granules stain eosinophilic and attain a diameter of ~1.5 μm (31). A deficiency in postsecretory dissolution of Paneth cell granules conceivably decreases luminal antimicrobial peptides and compromises innate immune mechanisms of the intestine. This observation is relevant to CF disease in that one explanation for the predispension of CF patients to repeated pulmonary infections is a failure of the endogenous antimicrobial peptide activity in secretions of the airway epithelium (7). In the airways, postsecretory activity of antimicrobial peptides, such as the β-defensins, may be impaired due to abnormal electrolyte composition of airway surface liquid (ASL) in CF patients (7). In vitro studies have shown sensitivity of antimicrobial peptide activity to changes in divalent cation concentration, NaCl concentration, and pH (for a review, see Ref. 38). However, due to difficulties in measuring the composition of the ASL, it remains to be determined whether these constituents are altered in the extracellular milieu of the ASL in CF patients.

In the present study, we provide evidence with light and transmission electron microscopy that postsecretory Paneth cell granules accumulate in the intestinal crypts of CF mice independent of osmotic laxative therapy. The consequences of this phenomenon are investigated with regard to experimental bacterial infection and the mRNA expression of the major antimicrobial peptides that comprise the Paneth cell granules in the mouse intestine.

MATERIALS AND METHODS

Animals. The experiments in this study used gene-targeted weanling mice (4–6 wk old) that were homozygous for the S489X mutation (cftr<sup>−/−</sup>low) (42) and maintained on a C57BL/6J background (>6 generations). The mutant mice were identified by using a PCR-based analysis of tail snap DNA, as previously described (9). All studies involved the comparison of gender-matched cftr<sup>+/−</sup> mice (CF) with their cftr<sup>−/+</sup> littermates, designated as wild-type (WT). The mice were maintained on standard laboratory chow (FormulaLab 5008 Rodent Chow; Ralston Purina, St. Louis, MO) and drinking water containing an osmotic laxative (Colyte; Schwarz Pharma, Seymour, IN) with the following composition (in g/l): 60.00 polyethylene glycol 3350 (PEG), 1.46 NaCl, 0.75 KCl, 1.68 NaHCO<sub>3</sub>, and 5.68 Na<sub>2</sub>SO<sub>4</sub>. The animals were housed in the Animal Care Unit (Association for Assessment and Accreditation of Laboratory Animal Care-accredited), Dalton Cardiovascular Research Center, University of Missouri-Columbia. Ambient temperature in the animal rooms was maintained at 75 ± 4°C and a 12:12-h light-dark cycle was provided. All experiments involving the animals were approved by the University of Missouri-Columbia Institutional Animal Care and Use Committee.

Histology. Small intestine sections (~1-cm length) were excised and immediately submerged in an aqueous fixative containing 2.5% glutaraldehyde, 2.0% paraformaldehyde, 70 mM NaCl, 30 mM NaH<sub>2</sub>PES, and 2 mM CaCl<sub>2</sub>. For light microscopy studies, fixed tissues were embedded in paraffin, sectioned (5-μm thickness), and stained with hemotoxylin and eosin. Sections were examined by using an Olympus BX50WI upright scope and photographed by using a Sen-sicam CCD camera (Cooke, Auburn Hills, MI) fitted with a micro-color filter (Cambridge Research and Instrumentation, Boston, MA). To estimate the number of Paneth cells in WT and CF intestine, the nuclei associated with intracellular Paneth cell granules were counted, because cell borders of the Paneth cells were indistinct in hemotoxylin and eosin sections. Postsecretory dissolution counts were only performed on cryostats with full-length cross sections (2–5 per section) and the results were averaged for each mouse. For electron microscopy, 2-mm sections were additionally fixed in 1% osmium tetroxide and 1% uranyl acetate, dehydrated with ethanol, and infiltrated with epoxy resin. Thin sections (80 nm) were cut and transferred to 200-mesh copper grids before staining with uranyl acetate and lead citrate. Sections were viewed with a JEOL 1200EX transmission electron microscope at 80 kV accelerating voltage.

Microarray analysis. Total RNA was extracted from pooled small intestines of three WT and three CF mice using Tri-Reagent (Molecular Research Center, Cincinnati, OH), and poly(A) RNA was purified by using the MicroPoly(A) mRNA purification kit (Ambion, Austin, TX). Total RNA from WT and CF poly(A) RNA samples were sent to Invitrogen (San Diego, CA) where they were labeled with cyanine 3 (Cy3) and Cy5, respectively, and hybridized with the UniGEM1.31 array representing 9,570 known genes and expressed sequence tags. Microarray data were analyzed by using Microsoft Excel, Incyte Genomics tools, and GeneSpring 2.5 software. The Cy3 and Cy5 signals for each experiment were normalized by using a balancing coefficient determined from the total signal intensity of each dye on the microarray and by analysis of mRNA standards included in the reverse transcription reactions. cDNA elements with a fluorescence signal-to-background ratio of <2.5, <40% coverage of the arrayed gene, or a Cy3 or Cy5 signal intensity of <100 fluorescence units were excluded from analysis. Differential mRNA expression levels were calculated as the ratio of CF to WT fluorescence measurements and are referred to as fold changes, with negative changes indicating downregulation in the CF relative to the WT samples. Only the genes that were downregulated by twofold or greater were included in Table 2. Data were deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under the accession no. GSM9222; a complete list of GEM1.31 array names and their locations on the array is available at the same web address under the accession no. GPL444.

Real-time quantitative PCR. Epithelial cells from the small intestine were isolated by an EDTA chelation method, as previously described (23). Total RNA was extracted by using Tri-Reagent (Molecular Research Center), and 4 μg were reverse transcribed by using first-strand cDNA synthesis (Invitrogen). Samples were eliminated if they were contaminated with genomic DNA as identified by the PCR amplipicn pattern from the β-actin primers. cDNA samples were evaluated by using primers designed to simultaneously amplify all 17 cryptdins (i.e., termed “cryptdins” in Refs. 13, 14) and β-actin. The primers used to amplify cryptdins were Defcf<sub>n380</sub> R 5′-GGTGAT-GCATCAGACCCCCGACACTGAT-3′ and Defcf<sub>p130</sub> F 5′-AA-GAGCAGTTAATCTGGGAGGACGC-3′ and for β-actin R 5′-TCT-CAGCCTGGTGTTGGGAA-3′ and β-actin F 5′-TGTTACCCAATT-GGGACGACA-3′. The relative mRNA concentration in the samples was estimated by using a LightCycler (Roche, Indianapolis, IN) and FastStart DNA Master SYBRgreen according to the manufacturer’s directions. Products were amplified by using the following conditions: β-actin-1 cycle at 95°C for 10 min and 35 cycles at 95°C for 0 s, 56°C for 5 s, 72°C for 25 s, and fluorescence acquisition on attaining 86°C; cryptdin-1 cycle at 95°C for 10 min and 45 cycles at 95°C for 0 s, 58°C for 5 s, 72°C for 10 s, and fluorescence acquisition on attaining 80°C. A melting curve analysis was performed after each PCR to ensure specificity of quantification. The relative concentration of
cDNA in each sample was determined by using a standard curve, as previously described (20), and normalized to the concentration of β-actin in each sample to control for differences in cell number and the quality of the RNA preparation.

Northern blot analysis. Total RNA was extracted from whole small intestines using the RNeasy Mini Kit and DNAase I treatment (Qiagen). Total RNA (8 μg/sample) was mixed with gyloxal sample buffer (BioWhittaker Molecular Applications, Rockland, ME), separated by 1% agarose gel electrophoresis in MOPS buffer, and transferred to Hybond-N + nylon membrane (Amersham Biosciences, Piscataway, NJ). Hybridization was performed as described previously (43) using [32P]dDNA probes for cDNA, lysozyme, and the mouse L32 ribosomal protein (as a loading control).

Acid-urea-PAGE immunoblots. Whole small intestines from gender-matched WT and CF littermate pairs were excised after death and the lumens were flushed gently with 50 ml of ice-cold water. The intestines were submerged in ice-cold 30% acetic acid, homogenized, and extracted by rotary shaking overnight at 4°C. Extracts were centrifuged at 100,000 g for 2 h at 4°C and clarified supernatants were lyophilized after extensive dialysis, as previously described (30). Lyophilized samples were dissolved in 5% acetic acid, protein content was measured by the Bradford assay, and 250 μg of protein/samples were analyzed by acid-urea-PAGE (AU-PAGE) in 12.5% gels at 250 V for 3 h (3, 4). After AU-PAGE separation, resolved proteins were transferred to 0.22-μm nitrocellulose membranes and blocked with 5% skim milk; the membrane was stained for 1 h with Coomassie blue R-250 and washed. Band densitometry of a digital image of the gel was performed by using Image Pro Plus 4.1 (Silver Spring, MD).

Aerobic bacterial flora. Under aseptic conditions, small intestines from gender-matched WT and CF pairs were exposed and opened longitudinally in the midjejunum for introduction of a sterile inoculation loop. The loop was used to inoculate plates for aerobic culture under the following conditions: 5% sheep blood agar incubated at 35°C-7% CO2 for 24 h, cetramide agar (selective for Pseudomonas) incubated at 42°C for 24–48 h, and MacConkey agar (selective for gram-negative species) incubated at 35°C for 24 h. Identification of bacterial colonies for genus and species was performed by the University of Missouri Research Animal Diagnostic Laboratory. Bacterial identification was based on colony morphology, gram staining, bacterial morphology, and secondary function tests, e.g., lactose fermentation, catalase activity.

Salmonella challenge. Inocula of the cryptdin-sensitive Salmonella typhimurium PhoP− mutant strain were grown in tryptcase soy broth at 37°C to midlog phase from single colonies (4). Bacteria were centrifuged (5 min), resuspended in PBS, and quantified by plate counts of serial dilutions on tryptcase soy agar. Pairs of gender-matched WT and CF littermates in wire-bottomed cages were fasted 6–8 h before inoculation using a small gastric gavage needle (1–5 × 106 cfu of bacteria in 100 μl PBS). Mice were returned to cages with food and bedding. Mice were killed after 24 h, and 2 cm of terminal ileum were removed. The ileum was opened longitudinally and was briefly washed twice in 1-ml volumes of PBS with gentle vortexing. The washes were combined (termed “rinses”) and homogenized using a polypropylene pestle driven by a Pellet Pester Motor (Kimble/Kontes, Vineland, NJ). The rinsed ileum was homogenized in 0.5 ml of sterile PBS. Rinses and ileal homogenates were serially diluted, plated on Salmonella-selective brilliant green agar, and incubated overnight at 35°C. Bacterial counts of ileal homogenates were normalized to 1-cm length of ileum. All colonies that grew on brilliant green agar had the same morphology. Selected colonies were confirmed to be Salmonella species by standard microbiological biochemical analyses using the string test (3% KOH), oxidase test, incubation on TLT media series (1), API 20E commercial test kit (BioMerieux, Hazelwood, MO), and positive response to Group O Salmonella antiserum poly A-I and Vi (Becton-Dickinson, Franklin Lakes, NJ).

Statistics. A Student’s t-test was used to compare two treatment groups. A one-way ANOVA with a post hoc Tukey’s t-test was used to compare multiple treatment groups. A P value < 0.05 was considered statistically significant. All data are expressed as means ± SE.

RESULTS

Paneth cell granules accumulate in the intestinal crypts of CF mice. The predominant disease phenotype of CF mice is severe intestinal impaction, a condition that carries a high mortality rate in the postweaning period. Obstructing intestinal impactions in the CF mice can be prevented by oral administration of an osmotic laxative, Coleyte (active ingredient, 3350 PEG), in the drinking water (9). As shown in Fig. 1A, almost all CF mice survive when provided with PEG-treated drinking water during the postweaning period. However, if PEG treatment is withdrawn from the drinking water (Fig. 1B), most CF mice succumb within a 10-day period. Necropsy examination of these mice revealed obstructing impactions, primarily located in the distal small intestine at the jejunoileal junction. In contrast to the effect on CF mice, survival of WT littermates is unaffected by removal of PEG from the drinking water after a period of treatment (9).

As shown in Fig. 2A, histopathology of the CF intestine near an obstruction revealed formation of inspissated mucus casts in the intestinal crypts, which in many cases extended beyond the villi and fused with luminal content (11). In contrast, overt mucus accumulation was not apparent in the intestine of PEG-treated CF or WT mice killed at the same age (see Fig. 2, B and C). Closer examination of the crypt lumens in the untreated CF intestine revealed the presence of eosinophilic granules, consistent in appearance and size with Paneth cell granules, within the cores of all mucus casts that were observed (Fig. 2D). Most surprisingly, Paneth cell granules also accu-

---

**Fig. 1.** Polyethylene glycol (PEG) laxative treatment of cystic fibrosis (CF) mice. A: survival of postweaning CF mice treated with PEG laxative in drinking water (n = 77). B: survival of postweaning CF mice (35 days old) after withdrawal of PEG treatment (n = 14).
mulated within the crypt lumens of the PEG-treated CF mice, despite the absence of overt mucus accretion within the crypts (Fig. 2E). The accumulation of Paneth cell granules within the crypt lumens of the CF mouse intestine contrasts sharply with the histopathological appearance of the intestinal crypts in WT littermates. As shown by the representative image in Fig. 2F, granules are observed within the Paneth cells but are only rarely observed within the WT crypt lumens. The numbers of Paneth cells/crypt did not differ significantly between CF and WT intestine (CF mean, 5.8 ± 0.8 vs. WT mean, 4.7 ± 0.5 Paneth cells/crypt, n = 5–4, not significant). However, the number of Paneth cells/crypt in the CF intestine may be underestimated, because the accumulations of Paneth cell granules occasionally obscured visualization of the cell nucleus.

To investigate further the phenomenon of Paneth cell granule accumulation in CF mouse intestine, histological sections were evaluated at the ultrastructural level using transmission electron microscopy. As shown in Fig. 3A, presecretory granules within Paneth cells in the intestinal crypts are electron-dense structures that are surrounded by an electron-lucent “halo,” known to consist largely of O-linked GalNAc glycoconjugates (24). Figure 3B shows a mucous cast within the crypt lumen of an untreated CF mouse. Note the presence of electron-dense Paneth cell granules encased within the cast. A few of the postsecretory Paneth cell granules appear to possess remnants of the glycoconjugate halo (see arrow in Fig. 3B). In PEG-treated CF mouse intestine (see Fig. 3C), the crypt lumens do not contain a mucoid cast, but Paneth cell granules have accumulated within the crypt and appear to be undergoing a limited dissolution process. Note that the granules are less electron dense and have feathery edges compared with granules within the Paneth cell or those entrapped in mucus casts.

Tissue-associated cryptdin peptides in the CF intestine. Cryptdins (α-defensins) constitute a major component of microbiocidal peptide activity in Paneth cell secretions of the mouse intestine (48). Procryptdins formed within the Paneth cells are extensively processed intracellularly to an active form by matrilysin-dependent enzymatic cleavage (3, 40). Therefore, the accumulation of Paneth cell granules within the crypts of CF intestine should yield an increase in processed cryptdins.

that are associated with the intestinal tissue. To evaluate this possibility, entire small intestines were removed from PEG-treated WT and CF gender-matched littermates, flushed free of luminal content and homogenized for AU-PAGE analysis. As shown in Fig. 4, A and B, the processed cryptdin peptide content of tissue homogenates from the CF intestine was greater than in the paired WT intestine.

Endogenous aerobic bacterial flora in CF mice. Although the CF intestine has increased cryptdin peptide content, abnormal dissolution of granule peptides would be expected to diminish the antimicrobial activity in the luminal environment of the CF intestine. Because the antimicrobial activity of the Paneth cell secretions is believed to provide a biochemical barrier that limits bacterial colonization of the epithelium (28), we first investigated whether the endogenous aerobic bacterial flora in the distal small intestine was altered in the CF intestine compared with their WT littermates. Table 1 shows the aerobic bacterial species that were isolated from cultures of the mucosal surface of the distal jejunum. Primary bacterial pathogens were not identified in isolates from either the CF or WT intestine. Several aerobic bacterial species were common between CF and WT jejunum. In this sampling, a greater number of bacterial species were isolated from the CF intestine. Some bacterial isolates from the CF samples were not present in the WT intestine, e.g., Enterococcus, but these species were not found in the majority of CF mice. Two species from the Pseudomonas genus (P. flourescens and P. stutzeri) were isolated from both WT and CF intestine (5 isolates in WT; 11 isolates in CF).

Salmonella challenge of the CF mouse intestine. Primary pathogens were not detected in aerobic cultures of the CF intestine; therefore, we sought to determine whether the CF mouse intestine has reduced protection against a murine enteric pathogen. CF mice and gender-matched WT littermates were orally challenged with 1–5 \times 10^8 cfu of a cryptdin-sensitive mutant (PhoP^-) strain of S. typhimurium (26). After 24 h, the amount of recoverable S. typhimurium in the terminal ileum was determined by serial dilutions plated on Salmonella-selective brilliant green agar. As shown in Fig. 5, both rinses of the ileum and the ileal homogenates from the CF mice had significantly greater recovery of S. typhimurium (0.5–1.0 log) than that from the WT littermates. All bacterial colonies that grew on brilliant green agar plates exhibited morphology consistent with S. typhimurium and random checks of colonies via biochemical analyses confirmed isolation of S. typhimurium.

Downregulation of cryptdin and lysozyme mRNA expression in the CF mouse intestine. The accumulation of Paneth cell granules within the intestinal crypts of CF mice raised the question of whether increased expression of antimicrobial peptides was present in the CF intestine. Initial studies employed a limited microarray analysis of small intestinal RNA to screen for alterations in gene expression in the intestine from three pairs of CF mice and their gender-matched littermates. Surprisingly, the IncyteGenomics UniGem1.31 microarray revealed that 8 of 15 downregulated genes were components of Paneth cell granules. As shown in Table 2, lysozyme and seven defensin-related cryptdin genes were downregulated by approximately twofold. To validate the cryptdin and lysozyme findings of the microarray, Northern blot analysis of RNA from small intestine was used to compare cryptdin expression between five pairs of CF mice and their WT littermates. Cryptdin expression was assessed by using a probe corresponding to nucleotides 80–352 present in all 17 of the known murine cryptdin cDNA sequences (13, 14). As shown by the blot in Fig. 6A, mRNA expression of cryptdins was slightly but consistently reduced in CF intestines compared with their paired WT intestines. When the amounts of mRNA for cryptdins and lysozyme were normalized to the mRNA expression of the ribosomal protein L32 (Fig. 6B), cryptdins and lysozyme were reduced in the paired analysis by −28 ± 12 and −32 ± 9%, respectively. However, a wide range of reduction (−11 to −69%) was noted for mRNA expression of cryptdins. Therefore, quantitative real-time PCR using RNA from isolated small intestinal epithelial cells was employed as a third test of cryptdin mRNA expression. As previously described (13, 14), consensus primers designed to amplify products corresponding to nucleotides 80–352 in all murine cryptdin cDNA sequences were used to probe for cryptdins. With the use of this method,
mRNA expression of cryptdins, when normalized to β-actin mRNA expression, was significantly reduced in the CF intestinal epithelium relative to that of the WT littermates (CF, 7.8 ± 4.3 vs. WT, 25.8 ± 8.7 cryptdins/β-actin mRNA; n = 5; P < 0.05).

DISCUSSION

The unique staining and size characteristics of Paneth cell granules in the murine intestine enabled the observation that postsecretory events of Paneth cell granules are altered in the CF mouse intestine. Paneth cell granules stain bright red with hemotoxylin and eosin and attain a diameter of ~1.5 μm in the mouse intestine, which yields a granule-to-crypt diameter ratio of ~1:17 (31). From this visual record, light microscopy studies revealed that Paneth cell granules are retained and accumulate within the crypts after release from the Paneth cells in the CF murine intestine. In the untreated CF mouse intestine, the postsecretory granules in the crypt lumens were encased in mucus casts. In CF mice provided an osmotic laxative on a continuous basis, the granules were found to accumulate within the intestinal crypts in the absence of overt mucus accretion. The latter observation suggests that abnormal dissolution of the granules may not simply be due to the mucus coating on the granules. Paneth cell granule accumulation in the intestinal crypts of CF patients has not been reported, but it is possible that abnormal dissolution has gone undetected. In humans, Paneth cell granule diameter is ~1 μm (34), yielding an estimated granule-to-crypt diameter ratio of ~1:60 that might limit recognition of the granules within the mucus and debris of the CF intestine. Studies at the ultrastructural level may be necessary to determine whether abnormal dissolution of postsecretory antimicrobial peptide granules contributes to disease in the intestine and other affected organs of CF patients.

Table 1. Endogenous aerobic bacterial flora isolated from the jejunum of gender-matched CF and WT littermate pairs

<table>
<thead>
<tr>
<th>Species</th>
<th>WT</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>1/8</td>
<td>1/8</td>
</tr>
<tr>
<td>Alcaligenes xylosoxidans</td>
<td>0/8</td>
<td>2/8</td>
</tr>
<tr>
<td>Brevundimonas vesicularis</td>
<td>0/8</td>
<td>1/8</td>
</tr>
<tr>
<td>Enterococcus sp.</td>
<td>0/8</td>
<td>3/8</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1/8</td>
<td>1/8</td>
</tr>
<tr>
<td>Gram + rods</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td>Pasteurella pneumotropica</td>
<td>4/8</td>
<td>3/8</td>
</tr>
<tr>
<td>Proteus sp.</td>
<td>0/8</td>
<td>1/8</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>1/8</td>
<td>3/8</td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>4/8</td>
<td>8/8</td>
</tr>
<tr>
<td>Staphylococcus sp. (not S. aureus)</td>
<td>5/8</td>
<td>8/8</td>
</tr>
<tr>
<td>Streptococcus sp. alpha hemolytic</td>
<td>8/8</td>
<td>7/8</td>
</tr>
<tr>
<td>Total isolates</td>
<td>32</td>
<td>46</td>
</tr>
</tbody>
</table>

n = 8 Littermate pairs. CF, cystic fibrosis; WT, wild type.

Fig. 4. Activated cryptdins in intestinal tissue homogenates from WT and CF mice. A: samples (250 g) of protein extracts from small intestinal homogenates were resolved by acid-urea PAGE and gels were stained with Coomassie blue. Comparisons were made between gender-matched littermate pairs of WT and CF mice. The boxed region denotes that positions at which murine cryptdin peptides migrate in the acid/urea gel system (3). Purified cryptdin 4 (Crp4) is shown for reference. B: densities of all bands/lane within boxed region were summed after background subtraction for each mouse. Graph depicting average of summed densities for WT and CF mice (n = 3). *Significantly different from WT by paired t-test.

Fig. 5. Challenge of gender-matched littermate pairs of CF and WT mice with avirulent Salmonella typhimurium (oral dose of 1–5 × 10^8 cfu). Shown are bacterial counts of the PhoP^− mutant of S. typhimurium in PBS rinses and intestinal homogenates of the terminal ileum collected 24 h postinoculation. Bacterial counts for intestinal homogenates are normalized to 1-cm length of the terminal ileum. Bars represent means ± SE for n = 6 mouse pairs. *Significantly different from WT.
Abnormal dissolution of Paneth cell granules in the intestine of CF mice apparently compromises innate host defense against enteric colonization with pathogenic bacteria. Cryptdins provide the major microbiocidal activity in Paneth cell granules. Inhibition of cryptdins was observed in the CF small intestine, despite evidence that processed cryptdins were abundant in the CF intestine (48). In the present study, cryptdin activation predisposes these animals to intestinal infection with pathogenic bacteria. Cryptdins provide the major microbiocidal activity in Paneth cell granules. Inhibition of cryptdins was observed in the CF small intestine, despite evidence that processed cryptdins were abundant in the CF intestine (48). In the present study, cryptdin activation predisposes these animals to intestinal infection with pathogenic bacteria.

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Gene Name</th>
<th>Balanced Differential Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA871663*</td>
<td>defensin related cryptdin, related sequence 7</td>
<td>-3.6</td>
</tr>
<tr>
<td>AA669146*</td>
<td>defensin related cryptdin, related sequence 10</td>
<td>-3.0</td>
</tr>
<tr>
<td>AA667003</td>
<td>glutathione S-transferase, mu 1</td>
<td>-2.9</td>
</tr>
<tr>
<td>AA47336*</td>
<td>cytochrome P-450, steroid inducible 3a11</td>
<td>-2.7</td>
</tr>
<tr>
<td>AA871641*</td>
<td>defensin related cryptdin, related sequence 2</td>
<td>-2.6</td>
</tr>
<tr>
<td></td>
<td>Public domain EST</td>
<td>-2.4</td>
</tr>
<tr>
<td>A036879*</td>
<td>lysozyme</td>
<td>-2.3</td>
</tr>
<tr>
<td>AA669181*</td>
<td>defensin related cryptdin 3</td>
<td>-2.2</td>
</tr>
<tr>
<td>AA69173*</td>
<td>defensin related cryptdin peptide</td>
<td>-2.2</td>
</tr>
<tr>
<td>AA871410*</td>
<td>defensin related cryptdin 5</td>
<td>-2.1</td>
</tr>
<tr>
<td>AA623172*</td>
<td>defensin related cryptdin peptide</td>
<td>-2.1</td>
</tr>
<tr>
<td>AA881013</td>
<td>cytochrome P-450, 2c37</td>
<td>-2.1</td>
</tr>
<tr>
<td>W34862</td>
<td>glutathione S-transferase, mu 6</td>
<td>-2</td>
</tr>
<tr>
<td>AA27986</td>
<td>cytochrome P-450, 3a25</td>
<td>-2</td>
</tr>
<tr>
<td>AA22002</td>
<td>cytochrome P-450, 2c40</td>
<td>-2</td>
</tr>
</tbody>
</table>

*Genes whose peptide products are constituents of murine Paneth cell granules.

Given that the Paneth cell numbers were similar in the CF and WT intestine, it is possible that the accumulation of Paneth cell granules in the CF crypts may result from increased expression of the antimicrobial peptides. However, the consensus of microarray analysis, quantitative real-time PCR, and Northern blot analysis indicated that mRNA expression of cryptdins and lysozyme was similar or modestly decreased in the CF compared with WT intestine. Although the decrease in cryptdin and lysozyme mRNA expression was relatively minor, downregulated expression of granule constituents during postsecretory granule accumulation has been previously reported for pepsinogen in chief cells of the gastric epithelium from gastric H^+-K^-ATPase knockout mice (44). In the absence of acid secretion, pepsinogen granules accumulate within the gastric pit lumens and mRNA expression studies show downregulation of the pepsinogen gene. One explanation for this phenomenon is that an abnormal concentration of peptides within the crypt lumen (either too low due to lack of dissolution or too high due to toxicity), would compromise host defense against enteric pathogens in the CF murine intestine.

In conclusion, the present study provides evidence that abnormal dissolution of Paneth cell granules in the intestine of CF mice is complicated by the inability of the CF intestine to generate fluid secretion in response to bacterial enterotoxins and inflammatory mediators (5, 10). Additional confounding factors include vigorous antibiotic therapy and digestive enzyme supplementation in CF patients and housing in specific, pathogen-free environments for CF mice. Nonetheless, there are isolated reports suggesting that the microbial ecology of the CF intestine is altered in CF patients. Several reports indicate that the carriage rates of Clostridium difficile in CF patients may attain 50%, which is approximately twice that in non-CF patients undergoing antibiotic therapy (32, 46). Unlike the non-CF patients colonized with C. difficile, the majority of CF patients did not show symptoms of gastrointestinal illness. Furthermore, in one report, increased aerobic bacterial isolates were present in stool specimens from CF patients compared with the other patient group and included significantly increased rates of colonization by Pseudomonas aeruginosa, Enterococcus, Staphylococcus, and Lactobacillus (46). Together with the present findings, these data suggest that differences exist in microfloral colonization in the CF small intestine. An important consideration in this regard is that both CF patients and CF mice also exhibit low-grade inflammation in the intestinal wall (15, 36, 41, 47). The etiology of the inflammation, like the more intensively studied inflammatory process in CF lungs (33), remains unknown. In the CF mouse intestine, perhaps spontaneous inflammation reflects intensified activity by other components of the innate or acquired immune systems that are necessary to compensate for a deficiency in antimicrobial peptide defense.
tion or too high due to limited dissolution of many granules) may exert feedback control of peptide expression at nearby epithelial cells. Although the mRNA expression of cryptdins decreased modestly, CF intestinal tissue content of processed cryptdin peptides increased, as would be predicted from the accumulation of postsecretory Paneth cell granules. However, it is possible that the amount of processed cryptdin peptide that is dispersed into the intestinal lumen is decreased in the CF compared with WT intestine. Thus the total amount of processed cryptdin peptides in the intact CF intestine may actually be unchanged or decreased relative to WT intestine, which would reconcile the peptide measurements with the mRNA expression studies.

The results of the present study raise the central question of why Paneth cell granules undergo limited dissolution in CF intestinal crypt lumens. Several factors warrant consideration. First, fluid secretion resulting from the osmotic force of the PEG laxative was apparently inadequate to yield rapid dissolution of the granules. Failure of osmotically induced secretion to hydrate the crypt contents may indicate either insufficient osmotic force by laxative concentrations of PEG or the possibility that the osmotic force of PEG is applied mainly at the villus rather than the crypt epithelium. Second, even if hydration of the crypt lumens were adequate, the composition of intracryptal fluid may be altered in CF. Given CFTR’s well-known role in bicarbonate transport, it is possible that the pH of the crypt lumen is inappropriate for rapid dissolution of the peptides. Suboptimal pH may alter peptide conformation or configuration within the postsecretory granule. Although we are unaware of any measurements of crypt luminal pH in the CF intestine, recent studies (22) of submucosal gland secretion in isolated airway mucosa from CF patients suggest that the pH is not altered. However, these findings are countered by other reports (19, 35, 45) of acidic pH in the secretions of other glands affected in CF disease. Another environmental variable may be the available divalent cation concentration within the lumen of CF intestinal crypts. Chelation of divalent cations is typically required during the isolation process to prevent dissolution of intact Paneth cell granules (3). Previous studies (17, 18, 21) of CF intestine and salivary glands have shown increased binding of Ca$^{2+}$ in mucus secretions that is apparently due to the acidic and sulfated nature of CF mucus. These characteristics of CF mucus are recapitulated in the intestine of the CF mouse model (Ref. 11 and L. R. Gawenis and L. L. Clarke, unpublished observations). Third, it is unknown whether the composition and packaging of the granules during formation within the Paneth cells are abnormal in the CF intestine. On the basis of these considerations, further studies of Paneth cell biology and the composition of the crypt lumen environment will be necessary to understand abnormal dissolution of Paneth cell granules in the CF murine intestine.

In summary, studies of a murine model of CF show that postsecretory Paneth cell granules accumulate within the intestinal crypts. This anomaly was associated with decreased resistance to intestinal colonization by an enteric pathogen (*S. typhimurium*) and modest downregulation of cryptdins/lysozyme mRNA expression. Because the size of Paneth cell granules relative to crypt diameter is considerably less in humans than mice, it remains to be determined whether abnormal granule dissolution is also a feature of intestinal disease in CF patients. Interestingly, some reports indicate that CF patients may also have higher rates of intestinal colonization with bacteria, although these studies are confounded by various treatment modalities. If antimicrobial peptide defense is compromised, then the low-grade inflammation that is observed in the intestinal wall of both CF patients and CF mice may represent compensation by other components of the innate and acquired immune systems to prevent bacterial colonization in the small intestine.

ACKNOWLEDGMENTS

The authors acknowledge the expert technical assistance of the University of Missouri Research Animal Histopathology Laboratory, Cheryl Jensen at the University of Missouri Electron Microscopy Core, and Cecelia Wylde at the University of Missouri Research Animal Diagnostic Laboratory.

GRANTS

This study was supported by Cystic Fibrosis Foundation Grant CLARKE03G0 (to L. L. Clarke) and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-48816 (to L. L. Clarke), DK-50594 (to G. E. Shull), and DK-44632 (to A. J. Ouellette).

REFERENCES


