Effects of laxative and N-acetylcysteine on mucus accumulation, bacterial load, transit, and inflammation in the cystic fibrosis mouse small intestine

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Submitted 2 May 2007; accepted in final form 29 June 2007

De Lisle RC, Roach E, Jansson K. Effects of laxative and N-acetylcysteine on mucus accumulation, bacterial load, transit, and inflammation in the cystic fibrosis mouse small intestine. Am J Physiol Gastrointest Liver Physiol 293: G577–G584, 2007. First published July 5, 2007; doi:10.1152/ajpgi.00195.2007.—The accumulation of mucus in affected organs is characteristic of cystic fibrosis (CF). The CF mouse small intestine has dramatic mucus accumulation and exhibits slower interdigestive intestinal transit. These factors are proposed to play cooperative roles that foster small intestinal bacterial overgrowth (SIBO) and contribute to the innate immune response of the CF intestine. It was hypothesized that decreasing the mucus accumulation would reduce SIBO and might improve other aspects of the CF intestinal phenotype. To test this, solid chow-fed CF mice were treated with an osmotic laxative to improve gut hydration or liquid-fed mice were treated orally with N-acetylcysteine (NAC) to break mucin disulfide bonds. Treatment with laxative or NAC reduced mucus accumulation by 43% and 50%, respectively, as measured histologically as dilation of the intestinal crypts. Laxative and NAC also reduced bacterial overgrowth in the CF intestine by 92% and 63%, respectively. Treatment with laxative normalized small intestinal transit in CF mice, whereas NAC did not. The expression of innate immune response-related genes was significantly reduced in laxative-treated CF mice, whereas there was no significant effect in NAC-treated CF mice. In summary, laxative and NAC treatments of CF mice reduced mucus accumulation to a similar extent, but laxative was more effective than NAC at reducing bacterial load. Eradication of bacterial overgrowth by laxative treatment was associated with normalized intestinal transit and a reduction in the innate immune response. These results suggest that both mucus accumulation and slowed interdigestive small intestinal transit contribute to SIBO in the CF intestine.

MICE that lack the expression of the cystic fibrosis (CF) transmembrane conductance regulator (Cftr) gene have a severe intestinal phenotype with many of the problems that occur in human CF patients. These include the accumulation of mucus, intestinal obstruction, slowed small intestinal transit, small intestinal bacterial overgrowth (SIBO), inflammation, and failure to thrive (4–6, 30). Of these changes, bacterial overgrowth is of interest because it can be responsible for the majority of the other aspects of the intestinal phenotype (22, 34). Secreted gel-forming mucins, mainly Muc2 in the intestine, bind bacteria and help carry them aborally for efficient clearance from the small intestine. When mucus clearance is impaired, bacteria can proliferate in the mucus and may use it as an energy source (36). Mucus accumulation in the CF intestine is in part due to the dehydrated, acidic environment as well as the altered glycosylation of mucins (38, 40), which may increase the viscosity of mucus. Our laboratory (32) has demonstrated that bacteria that overgrow the CF mouse small intestine colonize the mucus that accumulates in the intestinal lumen. Even though the major intestinal mucin genes (Muc2 and Muc3) do not have increased levels of expression (15, 21), mucin glycoprotein levels are dramatically increased in the CF mouse small intestine (29). Thus, mucus clearance is reduced rather than there is an increase in synthesis in the CF intestine. Interestingly, reduction of bacterial load with antibiotics decreases the accumulation of mucus in the CF intestine without a major effect on mucin gene expression (15). This suggests that secretion from goblet cells is stimulated by bacterial overgrowth. These results indicate that bacteria not only take advantage of mucus to colonize the small intestine but actively participate in stimulating mucus secretion by the intestinal epithelium.

Another aspect of the CF intestinal phenotype related to bacterial overgrowth is that intestinal transit is impaired (1, 13, 19, 27). The interdigestive intestinal motility (migrating motor complex) has a housekeeping function that “sweeps” the lumen clean and thereby removes mucus and embedded bacteria. Dysmotility is a major cause of SIBO (22). We (14) recently showed that interdigestive transit through the CF mouse small intestine is dramatically slower than in wild-type (WT) mice. Thus, a combination of viscous, dehydrated mucus and slower interdigestive motility likely combine to allow SIBO in the CF intestine.

We have proposed that abnormal mucus accumulation in the CF intestine provides a niche for bacterial overgrowth, and we hypothesized that interventions to improve mucus clearance would be beneficial. We used two different oral treatments of CF mice to improve mucus clearance. The first treatment was to maintain mice on solid mouse chow and treat them by replacing their drinking water with an osmotic laxative [polyethylene glycol (PEG)] in a balanced salt solution. Laxative treatment prevents the lethal intestinal obstruction that otherwise occurs in the CF mouse on solid chow and is believed to do so by improving hydration of the gut lumen (10). Laxative-treated CF mice apparently have less mucus accumulation and less dilation of the intestinal crypt lumen compared with liquid-fed mice (9), although this previously has not been quantitatively compared. PEG-based laxative is also used clinically to treat intestinal obstruction in CF patients (11), and its use is associated with a significant decrease in the occurrence of SIBO (18).

The second treatment used in this study was to orally administer N-acetylcysteine (NAC) by addition to the liquid diet. NAC is a mucolytic that acts as a reducing agent to break

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disulfide bonds in mucin molecules and thereby decrease the viscosity of mucus (17, 37). The results of this study show that both laxative and NAC can reduce mucus accumulation and bacterial load but that laxative is much more effective at reducing SIBO and the innate immune response and also is associated with normal interdigestive motility.

MATERIALS AND METHODS

Animals and treatments. Cfr<sup>+/−</sup> mice (cfr<sup>+/−<sub>Unc</sub></sup>) were originally obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were backcrossed on the C57Bl/6 background until congenic (33), and heterozygous mice were backcrossed regularly with the parental strain to prevent genetic drift (as suggested by the Jackson Laboratory, www.jax.org). Cfr<sup>+/−</sup> mice were bred to obtain WT (Cfr<sup>+/+</sup>) and CF (Cfr<sup>−/−</sup>) mice. Occasional Cfr heterozygous mice were used as WT controls, as they do not have any CF phenotype. Mice of both sexes were used between 6 and 10 wk of age for experiments. To increase the power of statistical comparisons (20), groups of WT and CF littersmates were backcrossed on the C57Bl/6 background until congenic (33), and obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were fasted overnight with free access to water or laxative solution as appropriate. In the morning between 8 and 9 AM, mice were gavaged with a 100-μl bolus of 25 mg/ml rhodamine-dextran prepared in 1.5% methylcellulose in saline. At 20 min postgavage, the entire gastrointestinal tract was removed, placed into ice-cold saline, and divided into the stomach and 10 equal segments of the small intestine. In none of the animals did the tracer reach the cecum/large intestine within 20 min postgavage. The luminal contents of the stomach and intestinal segments were rinsed out into 1.75 ml saline. The released material was centrifuged, and the supernatants were used to determine rhodamine fluorescence. Gastric emptying and small intestinal transit were measured as previously described (14). Briefly, mice were fasted overnight with free access to water or laxative solution as appropriate. The results of this study show that both laxative and NAC can reduce mucus accumulation and bacterial load but that laxative is much more effective at reducing SIBO and the innate immune response and also is associated with normal interdigestive motility.

Measurement of bacterial load by quantitative PCR of the bacterial 16S rRNA gene. After the death of the mice, PBS containing 10 mM DTT was flushed through the lumen of the small intestine and flushed material was processed as previously described (32) to extract microbial genomic DNA. The microbial load was measured by quantitative PCR using universal bacterial 16S rRNA-specific primers as previously described (31). The 16S rRNA PCR product from a laboratory strain of Escherichia coli was cloned and used to generate a standard curve for copy number determinations in the real-time quantitative PCR assays as previously described (32).

Measurements of gastric emptying and small intestinal transit. Gastric emptying and small intestinal transit were measured as previously described (14). Briefly, mice were fasted overnight with free access to water or laxative solution as appropriate. The results of this study show that both laxative and NAC can reduce mucus accumulation and bacterial load but that laxative is much more effective at reducing SIBO and the innate immune response and also is associated with normal interdigestive motility.

Morphology and morphometric analysis. Mice were fasted overnight with free access to water or laxative solution as appropriate. The entire intestinal tract was resected and placed in ice-cold saline. The mesentery was cut enough to allow extension of the small intestine to its full length. The middle 6 cm of the small intestine was cut into four segments of the small intestine. In none of the animals did the tracer reach the cecum/large intestine within 20 min postgavage. The luminal contents of the stomach and intestinal segments were rinsed out into 1.75 ml saline. The released material was centrifuged, and the supernatants were used to determine rhodamine fluorescence. Gastric emptying and small intestinal transit were measured as previously described (14). Briefly, mice were fasted overnight with free access to water or laxative solution as appropriate. The results of this study show that both laxative and NAC can reduce mucus accumulation and bacterial load but that laxative is much more effective at reducing SIBO and the innate immune response and also is associated with normal interdigestive motility.

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Table 1. Gene-specific primers for quantitative RT-PCR

<table>
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<th>Gene</th>
<th>Description</th>
<th>GenBank Accession No.</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product Size, bp</th>
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<td>Mctp2</td>
<td>Mast cell protease 2</td>
<td>NM_008571</td>
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<td>5′-ACACCTTCTCCTTGAACGGT-3′</td>
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<tr>
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<td>Leucine-rich α-2-glycoprotein</td>
<td>NM_029796</td>
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<tr>
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<td>NM_010416</td>
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<td>Suppressor of cytokine signaling 3</td>
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<td>Remn</td>
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<td>Rpl26</td>
<td>Ribosomal protein L26</td>
<td>BC070397</td>
<td>5′-AATGAGCAACGACGAG-3′</td>
<td>5′-TTTGCGCTGCTTGTG-3′</td>
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RESULTS

Two approaches were used to investigate the consequences of mucus accumulation in the CF small intestine. CF mice were treated with an oral laxative, which improves the hydration status of the intestinal lumen and protects mice from lethal intestinal obstruction (10). Second, the chemical NAC, which has reducing activity and can decrease the viscosity of mucus by breaking disulfide bonds between mucin molecules (17, 37), was used. The dose of NAC we used was similar to that employed to reduce *Helicobacter pylori* infection in mice (24).

Mucus accumulation was estimated morphometrically from the width of intestinal crypts as previously described (15) after tissue had been stained with PAS-AB. Luminal mucus is not always retained in fixed tissues, even when using Carnoy’s solution (unpublished observations). Therefore, we used crypt measurements because mucus in this cul-de-sac is better preserved and more accurately measured than mucus in the intestinal tract lumen proper. Because of this, we may have underestimated the effects of the interventions on the reduction of mucus accumulation.

In untreated CF mice raised on the liquid diet, intestinal crypts were greatly dilated and filled with PAS-AB-reactive mucus that extended up along the villus surface into the intestinal lumen (Fig. 1A). In contrast, WT mice on the liquid diet had narrow crypt lumens with minimal PAS-AB staining in either the crypt lumen or intestinal lumen proper, but there was staining of the luminal surfaces of the epithelium (Fig. 1A). In CF mice treated with laxative, the width and PAS-AB reactivity of the crypts were noticeably less than in liquid-fed CF mice, whereas tissue from WT mice treated with laxative was not noticeably different than that from untreated liquid-fed WT mice (Fig. 1A). The decrease in crypt lumen width in CF mice treated with laxative was confirmed by morphometry. Untreated liquid-fed CF mice had an average crypt width more than sevenfold greater than WT mice (Fig. 1B). With laxative, the CF crypt width was significantly less, such that it was now only about threefold wider on average compared with WT mice (Fig. 1B). Comparable with the effect of laxative, treatment of mice with NAC also reduced crypt lumen width of liquid-fed CF mice (Fig. 1) to about threefold that of WT mice (Fig. 1B).

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![Fig. 1. Effects of laxative and N-acetylcysteine (NAC) on mucus accumulation and tissue morphometry in cystic fibrosis (CF) mice. A: representative images of the middle portion of the small intestine from wild-type (WT) and CF mice. After fixation in Carnoy’s solution, tissues were processed for periodic acid-Schiff (PAS)-Alcian blue (AB) staining. The following samples are shown: control (untreated with the liquid diet), laxative (laxative treatment with solid chow), and NAC (NAC added to the liquid diet) from WT and CF mice. B: quantitation of the effects of laxative and NAC on mucus accumulation in CF mice. Mucus accumulation was estimated from the widths of intestinal crypts. Random images of PAS-AB-stained crypts were collected using a ×10 objective. Crypt lumen widths were measured as described in MATERIALS AND METHODS. Data are means ± SE from 8 liquid-fed WT mice (1,794 crypts), 9 laxative-treated WT mice (3,965 crypts), 6 NAC-treated WT mice (1,983 crypts), 6 liquid-fed CF mice (1,983 crypts), 3 laxative-treated CF mice (1,612 crypts), and 6 NAC-treated CF mice (933 crypts). Lax, chow-fed mice treated with laxative in drinking water; Liq, liquid-fed untreated mice; NAC, liquid-fed mice treated with NAC. C and D: morphometric analysis of the effects of laxative and NAC on intestinal tissues in CF mice. Sections from each group of mice were analyzed as described in MATERIALS AND METHODS. Only sections showing the entire crypt-villus axis were measured. C: villus height; D: crypt depth. Data are means ± SE from n = 3 WT and 3 CF mice for each treatment group (crypt-villus axes from 93 laxative-treated WT mice, 116 liquid-fed WT mice, 105 NAC-treated WT mice, 54 laxative-treated CF mice, 70 liquid-fed CF mice, and 103 NAC-treated CF mice). NS, not statistically significant.

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bacterial load, we used quantitative PCR for the bacterial 16S rRNA gene as previously described (32). As previously shown, untreated liquid-fed CF mice have SIBO compared with WT mice on the liquid diet (Fig. 2). Treatment of CF mice with laxative reduced bacterial overgrowth by 92%, and the 16S levels in laxative-treated CF mice were not statistically significant compared with WT mice on laxative (Fig. 2). In contrast, treatment with NAC was less effective than laxative, and bacterial load in NAC-treated CF mice was reduced by 63% (Fig. 2).

The above results show that laxative has measurable benefits for the CF intestinal phenotype, including better body weight gain, reduced mucus accumulation, and almost total eradication of bacterial overgrowth. Although NAC also decreased mucus accumulation, the bacterial load was less effectively reduced compared with laxative treatment. These data indicate that mucus alone is not responsible for bacterial overgrowth in the CF small intestine. Another factor that is likely involved in bacterial overgrowth in the CF intestine is slower transit during the interdigestive period. We (14) recently reported that interdigestive small intestinal transit is dramatically slower in liquid-fed CF mice compared with WT mice on the same diet. To compare interdigestive intestinal transit in treated CF mice, we used a rhodamine-dextran gavage after an overnight fast and measured fluorescence in the stomach and along the small intestine 20 min later. Under none of the conditions tested was there any fluorescence in the cecum or large intestine at 20 min postgavage (data not shown).

There was a small reduction in gastric emptying in untreated CF mice raised on the liquid diet compared with WT mice on the same diet, but due to high variability the difference was not statistically significant (Fig. 3). There was a trend to greater gastric emptying in laxative-treated WT mice compared with WT mice on chow with plain drinking water, but the difference was not significant (Fig. 3). When CF mice were treated with...
laxative, gastric emptying was significantly greater than in liquid-diet raised CF mice, and the degree of gastric emptying was not different compared with laxative-treated WT mice (Fig. 3). Treatment of liquid-fed CF mice with NAC also tended to increase gastric emptying, but the difference was not statistically significant compared with untreated liquid-fed CF mice (Fig. 3).

By measuring the distributions of rhodamine-dextran along the small intestine, the degree of interdigestive intestinal transit was determined. As shown in Fig. 4, transit in CF mice on the liquid diet was dramatically less compared with WT mice on the same diet. The raw data were transformed to calculate the GCF in the small intestine, which allows statistical comparisons (Fig. 4). The GCF of CF mice on the liquid diet was significantly different than WT mice on the liquid diet (Fig. 4). When treated with laxative, transit in CF mice was similar to that of WT mice treated in the same manner (Fig. 4), and there were no significant differences in GCF values when WT and CF laxative-treated animals were compared (Fig. 5). For comparison, WT mice raised on chow without laxative treatment were also studied. The fluorescence did not extend quite as far distally in WT mice with laxative treatment compared with untreated chow-fed WT mice (Fig. 4), but differences were not statistically significant based on GCF values (Fig. 5). There was greater transit in WT mice raised on the liquid diet compared with those on chow (Fig. 4), and GCF values were significantly different (Fig. 5). In contrast to laxative, treatment of mice with NAC did not alter transit in either WT or CF mice compared with those on the liquid diet alone (Figs. 4 and 5).

To investigate whether the reduction of mucus accumulation and bacterial load with laxative and NAC also affected the innate immune response that occurs in CF mice on the liquid diet (33), the expression of selected genes (Table 1) was measured by quantitative RT-PCR. We (33) have previously demonstrated that the CF mouse small intestine has significant increases in expression levels of genes associated with innate defenses, and this is accompanied by an influx of mast cells and neutrophils. It was also found that eradication of bacterial overgrowth in CF mice with broad-spectrum antibiotics reduced this innate immune response (32). Consistent with previous work, a marker of differentiated mast cells, mast cell protease 2 (Mcpt2), was dramatically upregulated in the CF small intestine in liquid-fed mice (Fig. 6). When CF mice were treated with laxative, there was significantly less Mcpt2 expression compared with CF mice on the liquid diet (Fig. 6). In contrast, treatment with NAC did not affect Mcpt2 expression levels in CF mice (Fig. 6). Similarly, the acute-phase gene leucine-rich α2-glycoprotein (a neutrophil marker and expressed by high endothelial venules during inflammation), hematopoietic cell transcript 1 (unknown function), and resistin-like molecule β (an innate defense gene) were all dramatically upregulated in the untreated CF intestine, and all were significantly downregulated in laxative-treated CF mice but were not significantly affected in NAC-treated CF mice (Fig. 6). The anti-inflammatory gene suppressor of cytokine signaling 3 (Socs3), while significantly upregulated in untreated CF mice, was not affected by either laxative or NAC treatments (Fig. 6). Unexpectedly, Socs3 was upregulated in WT mice treated with laxative or NAC compared with untreated liquid-fed WT mice (Fig. 6), but the importance of this was not apparent.
DISCUSSION

Mucus accumulation in the CF intestine is a significant problem that contributes to intestinal obstruction and probably to bacterial overgrowth. To better understand the relationship of mucus to bacterial overgrowth and intestinal transit, we compared untreated CF mice with those treated with an osmotic laxative or treated with the mucolytic NAC. Both laxative and NAC treatments reduced mucus accumulation in the CF mouse small intestine by 40–50% compared with untreated CF mice. However, while laxative treatment reduced bacterial overgrowth by ~90% in CF mice, NAC was much less effective and bacterial overgrowth was reduced by only ~60% in treated CF mice. Similarly, laxative treatment resulted in a strong downregulation of inflammatory marker genes, whereas NAC had little effect.

The mechanisms by which laxative and NAC can reduce mucus accumulation are distinct. Laxative delivers a nonabsorbable osmolyte into the gut lumen that causes the retention of ingested fluid as well as an influx of water from the body into the lumen. This increases the hydration state of the luminal contents. Better hydration decreases mucus viscosity and allows easier clearance of it from the intestinal lumen. On the other hand, NAC reduces the viscosity of mucus by breaking disulfide bonds between and within mucin molecules.

Besides reducing the viscosity of mucus, NAC has other actions that potentially might contribute to improving the luminal environment in CF. NAC can serve as a precursor for the cellular antioxidant glutathione, and there is strong evidence that oxidative stress is important in tissue damage in CF (8). A benefit of orally administered NAC in CF was demonstrated in a recent phase 1 clinical trial that showed a decrease in airway neutrophil levels (39). This was believed to be due to the antioxidant activities of NAC. Our mouse study did not find a significant effect on expression of the inflammatory marker genes we measured, so there does not seem to be a benefit from the antioxidant properties of NAC on the CF intestinal phenotype. NAC has also been reported to inhibit sodium absorption by nasal epithelial cells (35), and, if this occurs in the intestine, it would tend to improve the hydration state of the intestinal lumen and may have contributed to the reduction in mucus accumulation in NAC-treated CF mice. The efficacy of NAC on hydration would not appear to be very great as the overall result of NAC administration was limited compared with laxative treatment.
In addition to reducing mucus accumulation and SIBO, a major effect of laxative treatment was a normalization of interdigestive small intestinal transit in CF mice. Improved transit is expected to contribute to the eradication of SIBO. The mechanism by which laxative normalized transit in the CF intestine is not known at present. One possibility could be due to the fact that osmotic laxatives cause distension of the intestinal wall and thereby stimulate motility (12, 25). Laxative-treated mice were given access to the laxative solution during the overnight fast prior to transit measurements, so it is possible that there was enough laxative in the gut to have an effect on transit of the tracer. However, the fluorescent tracer used for all these experiments was prepared in a similar osmotically active compound (methylcellulose), which makes an acute effect of PEG seem unlikely. It is also possible that the gut had become adapted to the laxative treatment, which lasted for at least 5 wk before experiments. Chronic exposure of the gut to the osmotic effects of the laxative could cause adaptation and modification of the interdigestive motility behavior.

A third possibility is that laxative treatment, in addition to a numerical reduction in bacteria, also resulted in normalization of the composition of microflora in the intestine. A more normal composition of the microflora may have a positive effect on interdigestive transit. This is a complex issue because there is a two-way interaction between intestinal flora and interdigestive transit (2). Normal interdigestive transit is known to be a major factor in controlling bacterial load in the small intestine (22). At the same time, the composition of bacteria in the intestine affects intestinal motility by mechanisms not yet well defined. Work in germ-free rats versus those colonized with conventional flora or specific bacterial strains has shown that the microbial ecology of the gut has significant effects on gut behavior (23). Animals that are germ free or colonized only with E. coli have slower small intestinal transit compared with rats colonized a conventional species mixture.

It is important to note that the predominant species that overgrows the CF mouse small intestine on the liquid diet is E. coli, which is expected to contribute to the decreased interdigestive transit (14). Although laxative treatment reduced bacterial load in the CF intestine to levels near the WT intestine, it is not known what bacteria remain in the laxative-treated CF intestine and how the bacterial composition compares with that of WT mice. A culture-based analysis of aerobic bacteria in laxative-treated CF mice did not show marked differences in the composition of bacterial species in their small intestine compared with WT mice (9), so it may be that laxative-treated CF mice have a normal composition of bacterial species. However, in that study, CF mice treated with laxative did have about fivefold greater survival in the terminal ileum of orally administered Salmonella typhimurium, demonstrating that laxative treatment did not fully correct the CF intestine’s ability to deal with bacteria. We are in the process of determining the composition of bacterial species in the CF mouse treated with laxative using the inclusive and powerful molecular analysis of 16S rRNA genes. This is expected to be informative as to whether the bacterial species composition is more normal and might explain the positive effect of laxative on interdigestive transit in the CF small intestine.

Modification of the bacterial ecology of the gut may be beneficial in CF. It has been reported that there are increased levels of fecal calprotectin and nitric oxide in rectal dialysis fluid from CF patients, indicating inflammation (7). That study also showed that after administration of the probiotic Lactobacillus GG, these inflammatory parameters were significantly decreased. It will be of interest to see if probiotics can improve motility and the other aspects of the CF intestinal phenotype.

Because laxative treatment had such wide-ranging positive effects on the CF intestinal phenotype, it is difficult to know which aspect of its actions is most important. It is interesting to note that the prokinetic drug cisapride (a 5-HT4 agonist) has shown improvements in gastrointestinal symptoms in CF patients with intestinal obstruction (26). In a more recent study (4), cisapride was also shown to improve gastrointestinal motility in CF patients. It is possible that correcting interdigestive transit by itself may prove to be sufficient to reduce mucus accumulation and eradicate SIBO in the CF intestine, and this deserves further investigation.

In summary, both laxative and NAC significantly reduced mucus accumulation in the CF intestine. However, only laxative was very effective at reducing SIBO and decreasing the expression of innate immune response-related genes. In addition, laxative treatment also normalized small intestinal transit in the CF mouse. These data suggest that the amount of mucus as well as interdigestive small intestinal transit are involved in controlling bacterial load but that normal transit may be the more important factor.

ACKNOWLEDGMENTS

We thank Lauren Meldi and Racquel Sewell for managing the mouse colony and Maureen Flynn for help with the morphometric analysis.

GRANTS

This work was supported by Cystic Fibrosis Foundation Grants DELISL05G0 and DELISL06G0.

REFERENCES


