Inflammation of the cystic fibrosis mouse small intestine

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Norkina, Oxana, Simran Kaur, Donna Ziemer, and Robert C. De Lisle. Inflammation of the cystic fibrosis mouse small intestine. Am J Physiol Gastrointest Liver Physiol 286: G1032–G1041, 2004.—The CFTR null mouse [cystic fibrosis (CF) mouse] has a severe intestinal phenotype that serves as a model for CF-related growth deficiency, meconium ileus, and distal intestinal obstructive syndrome. DNA microarray analysis was used to investigate gene expression in the CF mouse small intestine. Sixty-one genes exhibited a statistically significant twofold or greater increase in expression, and 98 genes were downregulated twofold or greater. Of the upregulated genes, most were associated with inflammation and included markers for cells of the innate immune system (mast cells and neutrophils) and for acute-phase genes (serum amyloid A and complement factors). The downregulated genes include 10 cytochrome P-450 genes; several are involved in lipid metabolism, and several are involved in various transport processes. Confirmation by quantitative RT-PCR showed gene expression was significantly increased for mast cell protease 2 (2.9-fold), suppressor of cytokine signaling 3 (2.0-fold), leucine-rich α₂-glycoprotein (21-fold), resistin-like molecule-β (49-fold), and Muclin (2.5-fold) and was significantly decreased for cytochrome P-450 4a10 (28-fold) and cubilin (114-fold). Immune cell infiltration was confirmed histologically by staining for mast cells and neutrophils. These data demonstrate that the CF intestine exhibits an inflammatory state with upregulation of components of the innate immune system.

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the CFTR gene (39). On the basis of their effects on CFTR Cl− channel function, mutations can be classified as severe or mild (40). With severe mutations in both alleles, the earliest manifestations of CF are in the gastrointestinal system, including destruction of the exocrine pancreas beginning in utero (29) and frequent intestinal obstruction in neonates called meconium ileus (30). Gastrointestinal complications persist in CF patients throughout their lives, and distal intestinal obstructive syndrome is common (30). Despite the fact that CF is caused by mutations in a single gene, manifestations of the disease are complex and poorly understood, and there are multiple hypotheses regarding disease pathogenesis (49).

The CF mouse provides a useful model to study the gastrointestinal aspects of this disease. These mice exhibit accumulations of mucus in their intestinal crypts and, if untreated, suffer lethal blockage of the distal small intestines (13). Similar to the human disease, the pathogenesis of these CF-like conditions in this mouse model is not clear. One of the major functions of CFTR in the intestines is fluid secretion, which is accomplished by a combination of Cl− transport by CFTR and regulation of Na+ channels by CFTR (2). The other major function of CFTR in the intestines is pH regulation of the intestinal lumen. Cl− secreted through CFTR can be exchanged by the epithelium for bicarbonate ion, and bicarbonate ion can be secreted directly through the CFTR channel (5). CFTR-dependent bicarbonate-rich secretions are elaborated by the pancreatic and biliary ductal systems, duodenal submucosal glands, and intestinal crypts. Bicarbonate-rich fluid secretion is necessary for neutralization of acid entering the duodenum from the stomach. It was shown previously that the duodenal pH in the CF mouse is abnormally acidic due to lack of CFTR (9), which is similar to human CF patients (1).

Combined loss of fluid volume and abnormal acidity is expected to make exocrine secretions of mucins and glycoproteins into the intestinal lumen poorly soluble and prone to obstructive aggregation (40). However, altered electrolyte transport is likely not the whole explanation of CF pathogenesis. A hallmark of CF is inflammation, and in the CF airways, there is evidence that inflammation may be a consequence of loss of CFTR function even in the absence of pathological microbial infection (4, 47). It has recently been documented that the intestines of CF patients exhibit inflammation (36, 42). One study of CF intestines reported increased plasma proteins in gut lavage fluid, indicative of inflammation (42). In another study, it was reported that several markers of inflammation were upregulated in the small intestine of CF patients despite the fact that there was no appearance of inflammation by conventional histological examination (36). Inflammation in the intestines may not be apparent, because this organ normally has large amounts of commensal microbial colonization shortly after birth, and consequently, the healthy gut contains a large immune component. These studies suggest that there is inflammation of the CF small intestines in the absence of obvious morphological correlates.

In this paper, a DNA microarray approach was used to look at gene expression in the CF mouse small intestine. Increased expression of several genes was found, many of which are components of the innate immune system. Also found were decreases in numerous metabolic enzymes that may be associated with the growth retardation of these mice. The results demonstrate that the CF mouse intestines exhibit an innate immune response, although routine histological examination does not reveal an obvious inflammation. These new data will help understand the pathogenesis of CF in the gastrointestinal system, and this mouse model will serve to investigate the complex pathophysiology of this disease.

MATERIALS AND METHODS

Animals. Mice heterozygous for targeted disruption of the CFTR gene (CF mouse; cftr−/−) on the C57BL/6 background were bred...
and maintained in the University of Kansas Medical Center animal facility as previously described (9). CFTR heterozygotes were bred to prevent intestinal obstruction that occurs in CF mice on solid chow, mice were maintained from postnatal age of 10 days on Peptamen (Nestle, Deerfield, IL), a complete liquid diet (13). To avoid differences due to diet control, littermate mice were also maintained on Peptamen. Animals were housed in a specific pathogen-free facility in barrier-topped cages with Bed O Cobs bedding (13) and with free access to tap water. The mice were killed for experimental analysis at postnatal age of 6–9 wk. All animal procedures were approved by the University of Kansas Medical Center IACUC.

Microarray and quantitative real-time RT-PCR analysis of gene expression. Entire small intestines were removed from mice, flushed with ice-cold PBS, and trimmed free of mesentery. Total RNA was prepared from three control [CFTR(+/+)] and 3 CF mice [CFTR(−/−)] (2 males and 1 female of each genotype) using TRIzol reagent (Life Technologies, Grand Island, NY). RNA samples were submitted to the University of Kansas School of Medicine microarray core facility for probing of the mouse U74Av2 GeneChip from Affymetrix (Santa Clara, CA). All processing and hybridization steps were done according to the manufacturer’s instructions (https://www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf).

Data analysis was performed by using Affymetrix Microarray Suite 5.0 (MAS 5.0) software to obtain the absolute expression signal using the default settings. The entire expression analysis dataset was exported to QuattroPro spreadsheet software (www.Corel.com), and a t-test was performed to compare the three control and three CF samples. To conclude that a gene had increased expression in CF, three criteria were used: 1) the gene had to be called “present” by MAS 5.0 software in all three CF samples; 2) the t-test P value comparing CF to control had to be <0.05; and 3) the average change had to be greater than or equal to twofold. Similarly, to conclude that a gene had decreased expression in CF, the gene had to be called present in all three control samples and meet the other two criteria above.

Genes were functionally categorized by using the online resources: www.ncbi.nlm.nih.gov; Gene Ontology at www.geneontology.org; Mouse Genome Informatics at www.informatics.jax.org; the National Center for Biotechnology Information (NCBI) databases at www.ncbi.nlm.nih.gov; and the scientific literature.

Selected genes were further analyzed by quantitative real-time RT-PCR (QRT-PCR) using 8 control and 11 CF total RNA samples, and expression levels were normalized to GAPDH mRNA (GAPDH levels were not different between the two genotypes according to the array analysis). Reactions were performed with a LightCycler (Roche Biochemicals; www.roche_applied_science.com) using a one-step RT-PCR kit that uses SYBRgreen I for detection of double-stranded DNA product (Qiagen; www.qiagen.com). The mRNA sequences of genes of interest were obtained from GenBank, and when possible, primers were designed to span exon-splice boundaries to minimize potential amplification from any contaminating genomic DNA. Control PCR reactions for each primer pair were performed in which the RT enzyme was not added, and this resulted in a signal of <0.1% of the complete RT-PCR reaction (data not shown). Sequences of PCR primers are listed in Table 1 and the full-size primers have been described previously (45). RT-PCR products were cloned into the pDRIVE vector (Qiagen), and the clones were sequenced to verify that the products are correct (data not shown). To generate standard curves for the quantitative LightCycler reactions, cloned plasmid DNAs were linearized and a dilution series covering 10 pg to 0.01 fg was amplified in parallel with each run of experimental samples. Quantitative RT-PCR data were analyzed by t-test using Systat software (SPSS Science, Chicago, IL). The software was used to identify statistical outliers, which were omitted from the final analysis; no more than one sample per group was identified as an outlier. P values of <0.05 were considered as statistically significant.

Histology, immunohistochemistry, and in situ hybridization. Small intestines from control and CF mice were flushed with ice-cold PBS, immersion was fixed in 4% paraformaldehyde, and 5-μm paraffin sections were prepared, encompassing the whole length of the small intestine. To visualize mast cells, sections were stained with toluidine blue.

### Table 1. Primers for quantitative RT-PCR and product sizes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cell protease 2</td>
<td>ATG CAG GCC CTA CTA TCC CT</td>
<td>ACA CCT CTC GT</td>
<td>150</td>
</tr>
<tr>
<td>Hematopoietic cell transcript</td>
<td>GGT GGA TAC CTC TAT GAA CA</td>
<td>GGT CTG ATA TCC ATG TCA CA</td>
<td>171</td>
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<tr>
<td>Serum amyloid A3</td>
<td>CAG GAT GAA GCC TTC CAT TG</td>
<td>CCA GCT TCT TCC ATG AAC TG</td>
<td>90</td>
</tr>
<tr>
<td>Leucine-rich α2 glycoprotein</td>
<td>GGA GCA GCT ATG TGC TGT TG</td>
<td>AGT ATC AGG CAT TCC TGG AG</td>
<td>125</td>
</tr>
<tr>
<td>Suppressor of cytokine signaling 3</td>
<td>ATG GTC ACC CAG AGG AAG TT</td>
<td>ATT GTC GGC ATT TCC TTC TT</td>
<td>150</td>
</tr>
<tr>
<td>Transferin receptor</td>
<td>ATG ATG GAT GAA GCA AGG AG</td>
<td>ATT GTC GGC ATT TCC TTC TT</td>
<td>173</td>
</tr>
<tr>
<td>Resistin-like molecule β/FIZZ2</td>
<td>AGG TCC AGG CTG ACT TT</td>
<td>TTA CGG GAT GTC TTA AGG TC</td>
<td>397</td>
</tr>
<tr>
<td>Cytochrome P-450 4A10</td>
<td>GCA AGA GGA GAG TGG TGG AA</td>
<td>GCA AGA GGA GAG TGG TGG AA</td>
<td>483</td>
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### Table 2. Genes with increased expression in the CF intestine associated with inflammation by microarray analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Increase</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innate immune system-associated genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mast cell protease 1 (Mcpt1)</td>
<td>45</td>
<td>X68803</td>
</tr>
<tr>
<td>Mast cell protease 2 (Mcp2)</td>
<td>30</td>
<td>J05177</td>
</tr>
<tr>
<td>Complement factor 1 (C1i)</td>
<td>19</td>
<td>U74810</td>
</tr>
<tr>
<td>Leucine-rich α2 glycoprotein (Lgr)</td>
<td>14</td>
<td>AW230891</td>
</tr>
<tr>
<td>Serum amyloid A3 (Saa3)</td>
<td>13</td>
<td>X03505</td>
</tr>
<tr>
<td>Mast cell carboxypeptidase A3 (Cpa3)</td>
<td>11</td>
<td>J05118</td>
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<tr>
<td>Mast cell chymase 2 (Cma2)</td>
<td>4.9</td>
<td>M68899</td>
</tr>
<tr>
<td>Serum amyloid A2 (Saa2)</td>
<td>3.6</td>
<td>U69438</td>
</tr>
<tr>
<td>Mast cell protease-like (Mcptl)</td>
<td>3.6</td>
<td>M57401</td>
</tr>
<tr>
<td>Complement factor H (C1h)</td>
<td>2.8</td>
<td>M12660</td>
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<tr>
<td>Muclin (crp-dultan, dmb1)</td>
<td>2.4</td>
<td>U37438</td>
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<tr>
<td>Peptidoglycan recognition protein (Pglypr)</td>
<td>2.2</td>
<td>AF076482</td>
</tr>
<tr>
<td>Molecule possessing ankryin-repeats induced by lipopolysaccharide (Mail)</td>
<td>2.2</td>
<td>AA614971</td>
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<tr>
<td>Other inflammation-associated genes</td>
<td></td>
<td></td>
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<tr>
<td>Resistin-like molecule β/Found in inflammatory zone 2 (Retnlb)</td>
<td>256</td>
<td>AA611740</td>
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<td>Hematopoietic cell transcript 1 (HemT1)</td>
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<td>A2242830</td>
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<tr>
<td>Immunoglobulin κ variable chain V5</td>
<td>3.4</td>
<td>U60442</td>
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<tr>
<td>Suppressor of cytokine signaling 3 (SOCS3)</td>
<td>3.3</td>
<td>U88328</td>
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<tr>
<td>Immunoglobulin κ variable chain V8</td>
<td>3.0</td>
<td>U55585</td>
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<tr>
<td>B cell linker (Blnk)</td>
<td>2.8</td>
<td>AF068182</td>
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<tr>
<td>Immunoglobulin κ variable chain V28</td>
<td>2.6</td>
<td>U55576</td>
</tr>
<tr>
<td>Regenerating islet-derived 3y (Reg 3y)</td>
<td>2.5</td>
<td>AV049722</td>
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Table 3. Other genes with increased expression in the CF intestine by microarray analysis

<table>
<thead>
<tr>
<th>Metabolic enzymes</th>
<th>Increase</th>
<th>Accession</th>
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<tbody>
<tr>
<td>3-oxoacyl CoA transferase (Oxct)</td>
<td>3.9</td>
<td>AY843222</td>
</tr>
<tr>
<td>Phospholipase A2, group V (Pla2g5)</td>
<td>3.8</td>
<td>U66873</td>
</tr>
<tr>
<td>Squalene epoxidase (Sple)</td>
<td>3.6</td>
<td>D42048</td>
</tr>
<tr>
<td>Carbonic anhydrase 4 (Car4)</td>
<td>3.2</td>
<td>U37091</td>
</tr>
<tr>
<td>Glutathione peroxidase 2 (Gpx2)</td>
<td>2.9</td>
<td>X91864</td>
</tr>
<tr>
<td>Glutathione reductase 1 (Gsr)</td>
<td>2.5</td>
<td>AI851983</td>
</tr>
<tr>
<td>Farnesyl diphosphate synthetase (Fdp)</td>
<td>2.5</td>
<td>AY846851</td>
</tr>
<tr>
<td>Stearoyl CoA desaturase 2 (Scd2)</td>
<td>2.4</td>
<td>M26270</td>
</tr>
<tr>
<td>Serine protease inhibitor, Kazal type 4 (Spink4)</td>
<td>2.4</td>
<td>Y11505</td>
</tr>
<tr>
<td>Isopentyl-diphosphate Δ isomerase (Idi)</td>
<td>2.4</td>
<td>AA716963</td>
</tr>
<tr>
<td>Squalene synthase/farnesyl diphosphate farnesyl transferase 1 (Fdhf1)</td>
<td>2.1</td>
<td>D29016</td>
</tr>
<tr>
<td>Asparagine synthetase 1 (Asns)</td>
<td>2.0</td>
<td>U38940</td>
</tr>
</tbody>
</table>

Table 2 and 3 are partial lists of upregulated genes. The remainder did not fit apparent classification. All upregulated genes are included in the GEO datasets (see MATERIALS AND METHODS).

blue. To visualize neutrophils, sections were immunolabeled with a rat anti-mouse neutrophil antibody (Sorotec MCA771GA; www.sorotec.com) followed by a mouse preadsorbed rabbit anti-rat-biotin secondary antibody (Vector Labs BA-4001; www.vectorlabs.com). This was followed by use of the Vectastain Elite ABC kit, development with the VIP peroxidase substrate, and counterstaining with methyl green (Vector Labs). To visualize endogenous immunoglobulins, tissue sections were immunolabeled omitting the primary antibody step and using a goat anti-mouse Ig-biotin (Vector Labs) as the secondary antibody in the Vectastain Elite ABC procedure. As a negative control, goat anti-rabbit Ig-biotin was used in place of the anti-mouse-biotin.

For in situ hybridization, digoxigenin-labeled anti-sense and sense RNA probes were prepared by in vitro transcription using T7 and SP6 RNA polymerases and a digoxigenin RNA labeling mix (Roche). RNA probes were prepared by in vitro transcription using T7 and SP6 RNA polymerases and a digoxigenin RNA labeling mix (Roche). This array has probes sets for 6,000 expressed sequence tags (ESTs). Total small intestinal RNA from three control and three CF littermates was used to probe microarrays. The complete datasets are deposited in the Genome Expression Omnibus (GEO) website (accession nos. GSM11902, GSM11903, GSM11904, GSM11905, GSM11906, GSM11910; www.ncbi.nlm.nih.gov/geo/). There were 61 genes significantly upregulated 2- to 250-fold in the CF small intestine. Of these genes with known or predicted function, the category with the most upregulated genes is the immune system, and most of these are associated with innate immunity (Table 2). The innate response genes include several mast cell markers: the neutrophil marker leucine-rich α2-glycoprotein (Lrg) (33); the acute-phase proteins, serum amyloids and complement factors; two genes whose expression is induced by bacterial products (molecule possessing ankyrin-repeats induced by lipopolysaccha-
Other upregulated genes associated with inflammation but not specifically with innate defenses, are listed in Table 2. The highly upregulated gene RELMβ/FIZZ2 is associated with inflammation (23), but it also has been suggested to be a growth factor (43) and has recently been shown to have resistin activity and induces hepatic insulin resistance in vivo in rats (37). Also upregulated are the cell marker hematopoietic cell transcript 1 (HemT1) (51); the negative regulator of cytokine signaling, suppressor of cytokine signaling (SOCS3) (46); the stress-related Reg3γ (32); the B cell linker (Blnk) signaling adaptor protein (14); and three probe sets for the variable chain.

From the microarrays, 98 genes were found to be significantly downregulated twofold or greater in the CF samples. Ten cytochrome P-450 enzymes were downregulated 2- to 28-fold in the CF small intestine (Table 4). Another 16 genes associated with lipid metabolism and lipid transport were moderately downregulated two- to sixfold (Table 4). Several other transport protein genes were also downregulated (Table 4). Other downregulated genes do not fall into obvious functional groups and are not listed in Table 4 (available in the complete datasets: GEO accession nos. GSM11902, GSM11903, GSM11904, GSM11905, GSM11906, GSM11910).

Confirmation of altered gene expression for selected genes using quantitative real-time RT-PCR. From the genes with altered expression in the CF small intestine by microarray analysis, some were chosen for verification of expression changes by quantitative real-time RT-PCR. The upregulated genes analyzed were mast cell protease 2 (Mcpt2), HemT1, the acute-phase protein Saa3 (serum amyloid A3), the neutrophil marker Lrg, SOCS3, the secreted signaling protein RELMβ/FIZZ2, and a putative innate defense glycoprotein, Muclin. Quantitative RT-PCR (QRT-PCR) was also performed for the transferrin receptor, although it was not identified on the arrays. Expression of the transferrin receptor has been reported to be increased in the human CF duodenum (36), and it has been demonstrated that iron homeostasis is impaired in CF patients (38). The downregulated genes measured by QRT-PCR were the cytochrome enzyme P-450 4a10 (Cyp4a10) and the multiligand endocytic receptor protein cubilin (Cubn).

All of the genes examined by QRT-PCR were significantly altered in the CF intestine (Figs. 1 and 2). The upregulated genes had the following increases: McptP2, 27-fold; HemT1, 17-fold; SAA3, 2.9-fold; Lrg, 21-fold; SOCS3, 2-fold; trans-

![Fig. 1. Quantitative RT-PCR measurement of genes overexpressed in cystic fibrosis (CF) small intestine. Total RNA was analyzed by real-time quantitative RT-PCR. Data are presented as means ± SE and were analyzed for statistical significance between control and CF samples. A: mast cell protease 2, n = 8 control, 10 CF, P < 0.02. B: hematopoietic cell transcript 1 (HemT1), n = 8 control, 11 CF, P < 0.001. C: serum amyloid A3, n = 7 control, 10 CF, P < 0.02. D: leucine-rich α2-glycoprotein, n = 8 control, 11 CF, P < 0.02. E: suppressor of cytokine signaling 3, n = 8 control, 10 CF, P < 0.005. F: transferrin receptor, n = 8 control, 11 CF, P < 0.02. G: resistin-like molecule β, n = 3 control, 10 CF, P < 0.001. H: Muclin, n = 8 control, 10 CF, P < 0.005. Note, for resistin-like molecule-β (RELMβ), all but 3 of the control samples were below detection levels of the QRT-PCR reaction.](https://www.ajpgi.org/10.1152/ajpgi.00314.2003)
ferrin receptor, 1.3-fold; RELMβ, 49-fold; and Muclin, 2.5-fold (Fig. 1). The fold increase for RELMβ/FIZZ2 by QRT-PCR is a lower estimate because expression was below the sensitivity of the assay (2 copies per nanogram of total RNA) in five of the eight control samples, which were omitted from the calculation. The downregulated genes decreased 28-fold (Cyp4a10) and 114-fold (Cubn) (Fig. 2).

In situ hybridization of Muclin, serum amyloid A3, and resistin-like molecule β. Muclin is a high molecular weight sulfated, O-glycosylated protein expressed on the apical surface of many epithelia including the crypts of the small intestine (8). By in situ hybridization, Muclin in the control mouse small intestine is expressed mostly in the crypts as previously reported (3), and there is a gradient of expression with the highest levels in the proximal small intestine (Fig. 3, A–C). In the CF intestine, the Muclin in situ hybridization signal was increased, and there was labeling throughout the small intestine (Fig. 3, D–F). Also, the signal was observed further up on the villous epithelial cells, especially in the proximal intestine (Fig. 3D). The sense probe control was negative throughout the small intestine (Fig. 3, H–I).

Expression of the acute-phase gene Saa3 was found to be essentially absent in control small intestinal tissue, at least using the signal development time of 4.5 h (Fig. 4, A–B). By contrast, the CF tissue exhibited isolated clusters of high expression in villous epithelial cells (Fig. 4, C–D). The label was predominantly in the proximal region of the small intestine and became undetectable beyond the middle portion of the intestine (Fig. 4D). Also shown is the sense probe control, which was negative throughout the small intestine (Fig. 4, E–F).

RELMβ/FIZZ2 is of unknown function, but it has been associated with growing crypt cells in the normal colon and with small intestinal tumor growth in the min mouse, which has a mutation in the adenomatous polyposis gene (43) as well as with inflammation in lungs (44). In the control small intestine, RELMβ/FIZZ2 was virtually absent (Fig. 5, A–C). In contrast, in the CF small intestine, most crypts had one or more RELMβ/FIZZ2 positive cells (Fig. 5, D–E). The sense probe gave no signal (Fig. 5, F–H).

Histological analysis of immune cells in the small intestines of CF mice. Control and CF intestines were stained for mast cells using toluidine blue and for neutrophils using a rat

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**Fig. 2.** Quantitative RT-PCR measurement of genes downregulated in CF small intestine. Total RNA was analyzed by real-time quantitative RT-PCR. Data are presented as means ± SE and were analyzed for statistical significance between control and CF samples. A: cytochrome P-450 4a10, n = 7 control, 9 CF, P < 0.01. B: cubilin, n = 7 control, 9 CF, P < 0.001.

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**Fig. 3.** In situ hybridization for Muclin mRNA in control and CF small intestine. Tissue sections were probed with antisense and sense digoxigenin-labeled single-stranded RNAs and all sections were developed for 1 h. A–C: control small intestine shows a gradient of Muclin mRNA from proximal to distal. The label is observed mostly in the crypt epithelium with weaker labeling of the lower villous epithelial cells. D–F: CF small intestine also shows a gradient for Muclin mRNA from proximal to distal, but the signal is stronger than in control, and the signal extends further into the villous epithelium, especially in the proximal region (D). G–H: sense-strand probe shows no signal on CF small intestine sections.
monoclonal antibody. Consistent with the gene expression data, the small intestine of the CF mouse exhibits an increase in mast cells (Fig. 6). Mast cells are very rare in control mouse small intestines (Fig. 6, A and C). Over the length of the entire small intestine in 5-μm sections, fewer than five mast cells were observed per mouse. In contrast, examination of two CF mice revealed 448 and 517 mast cells in 5-μm longitudinal sections through the entire length of the small intestine. Mast cells in the CF tissue were found in the connective tissue surrounding the submucosal glands in the duodenum (Fig. 6 B) and, more abundantly, near lymphatic nodules in the muscularis externa and along the serosal mesothelium, with a few cells in the submucosa (Fig. 6 D). Mast cells were very rare in the lamina propria of the CF intestine and were not observed in the control tissue. Shown in Fig. 6 E is a higher magnification image of an individual mast cell found at the serosal mesothelium in the CF intestine to illustrate the characteristic metachromatic staining of the cell’s granules by toluidine blue.

Immunocytochemistry confirmed that upregulation of Lrg is associated with an increased number of neutrophils in the CF intestine. Control mouse intestines have some neutrophils, especially in the submucosa near lymph nodules (Fig. 7A). Neutrophils are rare in the submucosa away from lymph nodules (Fig. 7C). By contrast, there are many more neutrophils in the CFTR null intestine, especially in all the tissue layers around lymph nodules (Fig. 7B) as well as in the submucosa and lamina propria underlying the surface epithelium (Fig. 7D).

Because some immunoglobulin genes were upregulated on the microarray, immunostaining for endogenous immunoglobulins was performed by using only the secondary biotinylated anti-mouse Ig reagent (see materials and methods). The control mouse intestine had a moderate signal in the lamina propria and no signal in the lumen of the intestine (Fig. 8A). The CF intestine had an increase in lamina propria immunoreactivity, and the aggregated material in the intestinal lumen was strongly positive (Fig. 8B). The negative control samples for control and CF tissue, using an anti-rabbit Ig-biotin conjugate, had virtually no immunoreactivity (data not shown).

DISCUSSION

In this work, global gene expression changes were investigated in the small intestine of a mouse model of CF. The
largest functional group of genes upregulated in the CF intestine are components of the immune system, and most of them are associated with innate responses. A major phenotype of the CF mouse is low body weight that is ~30% less than controls at 4–8 wk of age for mice maintained on the liquid Peptamen diet (9). Low body weight is also a condition of human CF patients (16), but the cause of this failure to thrive is not fully understood. It partially reflects inadequate digestion and nutrient absorption, but may also be contributed to by the abnormal energy expenditure consumed by inflammation in affected organs. Inflammation in CF is of major concern in the airways (52), and there is recent evidence of inflammation in the human CF small intestine (36, 42). Inflammation occurs in the intestine and is expected to contribute to the energy imbalance in this disease (35).

Genes increased in the CF mouse small intestine include factors produced by epithelial cells with roles in innate immunity: serum amyloids, complement factors H and I, and Mucin [also known as deleted in mammalian brain tumor 1 (dmbt1)]. Serum amyloids are expressed in normal human small intestine and they are upregulated as part of the acute phase (48). Their function is not yet clear, but recent evidence suggests serum amyloids act as chemoattractants for mast cells (34).

Mucin was also upregulated in the CF intestine. Mucin was discovered and subsequently cloned in our lab (8, 10) and it was cloned independently by others and called CRP-ductin (3) and dmbt1 (31). Mucin is a sulfated, mucinlike glycoprotein expressed on the apical plasma membrane of many epithelial cells, and its expression is upregulated in response to increased microbial load in the mouse intestines (24, 45). The function of Mucin is not proved, but its upregulation in inflammation, location on the apical surface, and mucinlike biochemical composition (8) are consistent with it having a protective role as part of the innate defenses of the epithelium (26, 45).

Neutrophils and mast cells were increased in the CF intestine. Neutrophils are an important component of the innate defenses and they secrete bactericidal agents and kill microbes by phagocytosis (17). Mast cells are rare in the healthy mouse intestine (32, 33), unlike humans in whom intestinal mast cells are common. Mast cells in the intestine are associated with nematode infections (50) and IgE-mediated responses (53). Through released factors (histamine, proteases, prostaglandins, leukotrienes, and cytokines), mast cells increase permeability and motility to flush parasites out of the gut (7). The terminal ileum and proximal colon of neonatal CF mice have increased tissue permeability as indicated by a 30–40% decrease in

Fig. 6. Histochemical staining for mast cells with toluidine blue. A: control duodenum is mostly devoid of mast cells in all areas. B: CF duodenum has several mast cells (arrowheads) in the connective tissue around the submucosal glands (SMG). The crypts, villi, and lamina propria did not contain mast cells. C: control intestine is devoid of mast cells near lymph nodules. D: CF intestine has large numbers of mast cells (arrowheads) near lymph nodules, mostly in the muscularis externa (ME) layer and along the serosal mesothelium, and a few in the submu cosa (SM) layer. E: high-magnification image of a mast cell along the mesothelium from the CF intestine showing the characteristic metachromatic staining of mast cell granules by toluidine blue.
tissue resistance measured in a Ussing chamber (19). Increased intestinal permeability is a characteristic of human CF (22) but the cause of this has not been determined, and it may be that mast cell products are involved.

Interestingly, the microarray analysis did not show an increase in the CF intestinal RNA for T cell markers [6 probe sets for T cell markers; 1 probe set for a T cell-restricted myosin heavy chain (MHC) protein, and probe sets for CD4 and CD8; MHC genes (22 probe sets); or macrophage markers (14 probe sets)]. With the exception of immunoglobulin-k genes and the Blnk gene, no genes associated with the adaptive immune system were found to be upregulated in the CF intestine. An increase in immunoglobulin content in the CF mouse intestine was demonstrated by immunostaining, and human CF patients have increased immunoglobulins in the intestinal lumen (42).

Inflammation of the CF intestine is limited, suggesting the influence of anti-inflammatory factors. Possible anti-inflammatory mechanisms are the increased expression of the suppressor of cytokine signaling 3 gene (SOCS3) and the negative regulators of the complement system, factors I and H. The SOCS genes are antagonistic to cytokine signaling by blocking the JAK/STAT pathway or otherwise blocking specific gene expression by inhibiting transcription factors (18). It has been suggested that SOCS3 specifically plays a negative regulatory function in intestinal inflammation by interfering with the STAT3 pathway (46). Complement factor I cleaves the active C3b component of the complement system thereby inactivating it and acting as a negative regulator of complement-mediated inflammation (17). The activity of factor I is enhanced by complement factor H, which increases the affinity of factor I for its C3b target (12).

A number of genes increased in the CF intestine suggest an increase in bacterial load. Genes that respond to the presence of lipopolysaccharide and peptidoglycan (MaiL and Pglyrp, respectively) were increased. Also, 10 cytochrome P-450 genes had decreased expression on the microarrays, and downregulation was confirmed by QRT-PCR for Cyp4a10. P-450 genes are downregulated during the acute phase and in response to lipopolysaccharide (6). It has been proposed that P-450 downregulation is permissive for elevated levels of leukotrienes and prostaglandins as part of the inflammatory response (6). An increased bacterial load may also account for upregulation of immunoglobulin genes, because microbial antigens could activate B cells to become functional plasma cells. It will require further work to determine whether bacterial overgrowth occurs in the CF intestine and whether it has a role in CF pathogenesis.

The other downregulated gene confirmed by QRT-PCR was Cubn. Cubn is a multiligand endocytic receptor normally expressed in the kidney and intestine, and in the intestine, it is required for endocytosis of intrinsic factor-vitamin B12 complex (15). CF patients have reduced vitamin B12 absorption (20), and a decrease in Cubn could explain this. Despite decreased vitamin B12 absorption, CF patients are not vitamin B12 deficient (20).

The gene most strongly upregulated in the CF small intestine was RELMβ/FIZZ2. This gene is associated with cell growth and with inflammation. Under normal conditions, RELMβ is expressed at high levels in crypts of the colon, and at low levels in the small intestine (23, 43). Spontaneous duodenal tumors develop in the min mouse, which has a mutated APC gene, and these rapidly growing cells express RELMβ at high levels (43).

By in situ hybridization, RELMβ/FIZZ2 is expressed in colonic crypt cells (23, 43), and we found expression in small intestinal crypt cells of the CF mouse. Crypt expression is consistent with a role in cell growth, and we previously reported hypertrophy of the CF mouse small intestine (9). However, RELMβ/FIZZ2 protein was reported by immunocytochemical staining to be in colonic goblet cells (21). The

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Fig. 7. Immunocytochemical staining for neutrophils. A: control intestine has neutrophils (dark staining spots) near lymph nodules. B: CFTR null intestine has an obvious increase in neutrophils in the submucosa near lymph nodules. C: away from lymph nodules, the CFTR null tissue has very few neutrophils in the submucosa (arrows). D: away from lymph nodules, the control tissue has many neutrophils in the submucosa and in the lamina propria.

Fig. 8. Immunocytochemical staining for endogenous immunoglobulins. Sections were incubated without a primary antibody and then incubated with an anti-mouse Ig-biotin secondary antibody and processed by using an avidin-biotin kit. A: control tissue shows moderate immunoreactivity in the lamina propria (LP). B: CF tissue also shows immunoreactivity in the lamina propria as well as very high reactivity in the protein aggregated in the intestinal lumen (arrows).
authors of that work did not comment on how the mRNA can be expressed in crypt cells, whereas the protein is expressed in goblet cells. It is conceivable that the immunohistochemical signal represents RELMβ/FIZZ2 protein secreted from crypt cells that had adhered to goblet cell mucus. This issue will require more investigation to clarify.

The RELMβ gene was also discovered independently and named FIZZ2 (23). FIZZ2 was shown to be upregulated in lung tissue after antigen challenge in control but not STAT6 null mice (44). RELMβ/FIZZ2 expression was upregulated in the mouse colon by introduction of bacteria into germ-free mice and by lipopolysaccharide treatment of intestinal cell lines (21).

Upregulation of RELMβ/FIZZ2 in the CF intestine is consistent with a role for this gene in inflammation. At the same time, upregulation is also consistent with a role in cell growth. Further work will be needed to clarify the role(s) of this interesting gene in CF.

In summary, these data present the novel finding of an innate immune response in the CF mouse small intestine, which may have a role in CF pathogenesis. A consequence of intestinal inflammation may be an increase in circulating immune cells and cytokines. It is tempting to speculate that intestinal inflammation results in elevated systemic inflammation and may predispose the body toward a hyperresponsive state. Because the intestine is a site of early CF-related dysfunction, intestinal inflammation may lead to an increased activity of the immune system early in life. This may prime the body for the excessive response that occurs when the lungs become challenged by microbes in CF. These hypotheses provide a framework to investigate CF pathogenesis and may lead toward better therapeutic approaches for treatment of overactive immune responses that are central to CF disease.

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