Mucin Dynamics in Intestinal Bacterial Infection

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Abstract

Background: Bacterial gastroenteritis causes morbidity and mortality in humans worldwide. Murine Citrobacter rodentium infection is a model for gastroenteritis caused by the human pathogens enteropathogenic Escherichia coli and enterohaemorrhagic E. coli. Mucin glycoproteins are the main component of the first barrier that bacteria encounter in the intestinal tract.

Methodology/Principal Findings: Using Immunohistochemistry, we investigated intestinal expression of mucins (Alcian blue/PAS, Muc1, Muc2, Muc4, Muc5AC, Muc13 and Muc3/17) in healthy and C. rodentium infected mice. The majority of the C. rodentium infected mice developed systemic infection and colitis in the mid and distal colon by day 12. C. rodentium bound to the major secreted mucin, Muc2, in vitro, and high numbers of bacteria were found in secreted MUC2 in infected animals in vivo, indicating that mucins may limit bacterial access to the epithelial surface. In the small intestine, caecum and proximal colon, the mucin expression was similar in infected and non-infected animals. In the distal colonic epithelium, all secreted and cell surface mucins decreased with the exception of the Muc1 cell surface mucin which increased after infection (p < 0.05). Similarly, during human infection Salmonella St Paul, Campylobacter jejuni and Clostridium difficile induced MUC1 in the colon.

Conclusion: Major changes in both the cell-surface and secreted mucins occur in response to intestinal infection.

Microbial products and inflammatory cytokines stimulate increased production of mucins by mucosal epithelial cells, which can effect a massive discharge of mucin in response to stimuli [10,11,12,13,14,15,16,17,18,19,20,21]. Stimulated mucin release occurs rapidly and is accompanied by a hundredfold expansion of the secretory granules upon hydration. Several pathogens have been shown to interact with mucins [22,23,24,25,26], including EPEC and EHEC which bind to bovine mucins [27]. Upregulation of MUC3 expression in colonic cells has been correlated with decreased binding of EPEC [28,29]. The ability of human milk to limit gastrointestinal bacterial and viral infections has been attributed in part to the presence of large amounts of cell surface mucins, chiefly MUC1 and MUC15 [30,31,32]. Consistent with an important role for Muc1 upregulation in the intestine limiting infection, Muc1−/− mice have a higher rate of systemic infection in a murine Campylobacter jejuni model of gastroenteritis [6]. Many pathogens have evolved mechanisms to subvert the mucin barrier, for example the StcE zinc metalloprotease secreted by EHEC is a mucinase [33].

Goblet cell depletion in infection has been reported previously [34], however, no comprehensive study of expression of all mucins in an animal infection model has been performed. We map the localization along the intestinal tract of all murine mucins for which antibodies are available: Muc1, Muc2, Muc4, Muc5AC, Muc13 and Muc3 (orthologue of human MUC17, therefore hereafter referred to as Muc17. There is currently no Muc3 orthologue annotated in the mouse genome. However, a peptide
identical to a peptide from the human MUC3 mucin has been identified using proteomics on mouse mucins, suggesting the presence of the mouse orthologue to this mucin [35]. In a murine C. rodentium infection model, we demonstrate substantial changes in the amount of virtually all intestinal mucins after 12 days of infection. We further showed mucin binding to C. rodentium, demonstrating that the release of mucins has the capacity to remove bacteria from the epithelial surface.

Materials and Methods

Animals

8–16 weeks old male 129/SvJxC57BL/6 mice were housed under clean conventional conditions, and allowed free access to sterilized food and water. All experiments were approved by the University of Queensland Animal Experimentation Ethics Committee (approval no 059/07). Colonies routinely tested negative for murine viral and bacterial pathogens.

Infection of mice

Citrobactum rodentium strain ICC169 was grown on Lauria-Bertani agar for 20 h at 37°C. Bacteria harvested from plate cultures were suspended in warmed Lauria-Bertani broth. 8 mice were orally inoculated with 10^9 colony forming units (CFU) and sacrificed after 12 days by cervical dislocation. Duplicate samples of duodenum, caecum, small intestine and proximal, mid and distal colon were dissected and collected in either broth or 10% formalin. To assess the number of CFU/g tissue, tissue and fecal samples (collected as defecated samples) were homogenized in broth, serially diluted, plated onto McConkey’s selective agar, and grown for 20 h at 37°C. CFU were enumerated by counting C. rodentium ~1 mm diameter fuschia-coloured colonies. The mice were weighed and diarrhea was scored every second day.

Histological assessment

For analysis of colitis, formalin fixed tissue sections of the small intestine, caecum, proximal and distal colon stained with hematoxylin/eosin were coded to blind the analysis, and the entire section was systematically scored: aberrant crypt architecture (0–5), increased crypt length (0–3), goblet cell depletion (0–3), general leukocyte infiltration (0–3), lamina propria neutrophil counts (0–3), crypt abscesses (0–3), and epithelial damage and ulceration (0–3) were scored (full details in Table S1).

Human biopsies

Individuals with acute diarrhoea are not usually subjected to colonoscopic examination and biopsy, but archival biopsy material was available from seven patients with acute gastroenteritis. These patients included four previously healthy individuals and three patients with underlying inflammatory bowel disease, as described in Table 1. For controls, biopsies were examined from the same IBD patients when there was no detectable bacterial infection or from the same distal colonic site from age and sex matched healthy individuals (Table 1). The archived biopsies were collected and treated in accordance with the Australian National Statement on Ethical Conduct in Human Research (2007) [http://www.nhmrc.gov.au/publications/synopses/e72syn.htm] guidelines for archived pathological material. There is no consent process required for this type of use. The biopsies were collected between 1/06 and 1/08 for diagnostic histopathology as requested by the attending doctor. The specimens from Muc1 and Muc13 knockout mice were negative for staining with the respective antibodies. The staining pattern of the hHA1-B-1 antibody (raised against the peptide KYTPGFENTLDTVVKNLETKKKNAT [35] were kind gifts from Prof. S. Gendler, Scottsdale, USA, and Prof. G. Hansson, Gothenburg, Sweden. The polyclonal anti sera against the C-terminal sequence of MUC4 (hHA1-B-1) was a kind gift from Prof. K. Carraway, Miami, USA. The MUC5AC antibody (45M1) was purchased from Sigma [37]. Polyclonal antisera (rM13.C and MM2-2) against the cytoplasmic tail of the human MUC13 and murine Muc2 was raised in rabbit using bovine serum albumin conjugated peptides CMQNPSRHSMPRPDYP and CPEDRPYDDLKKK, respectively [38,39], and then purified on a HiTrap NHS-activated HP column (Amersham Biosciences, Uppsala, Sweden) coupled to the same peptides according to manufacturers instructions. The monospecificity of the CT2 and rM13.C antibodies were demonstrated by that gastric and intestinal specimens from Muc1 and Muc13 knockout mice were negative for staining with the respective antibodies. The staining pattern of the hHA1-B-1 antisera was similar to that previously described for MUC4 in human tissue [40,41], and the pre-immune sera from the hHA1-B-1 rabbit did not stain any tissue. The 45M1 antibody recognizes MUC5AC (and no other molecules in gastric tissue extracts) in human and rhesus monkey as demonstrated by that only fractions from density gradient centrifugation that has a density appropriate for MUC5AC (1.4 g/ml) are recognized with this antibody [37], and the mouse gastric tissue used here as a positive control demonstrated a strong surface epithelial cell staining, similar to that of the human and rhesus monkey gastric tissue[37], whereas the intestinal tissue was negative. Similarly, the MM2-2 antibody recognize material from intestinal tissue extracts in fractions of appropriate density from density gradient centrifugation of MUC2/Muc2, show a distinct goblet cell staining pattern in the intestine of mice similar to that demonstrated in human [39], and did not stain the gastric negative control tissue. The specificity of the Muc3-S2 antibody has been confirmed using mass spectrometry [35].

Immunohistochemistry

Mouse samples: Formalin-fixed, paraffin-embedded tissue sections (4 μm) of archived biopsies from mouse stomach, duodenum, caecum, small intestine and proximal-, mid- and distal colon were dewaxed and rehydrated. Antigen retrieval used

### Table 1. Human biopsies used for investigating MUC1 induction in infectious colitis.

| Age | Sex | IBD status* | Control patient | Infectious agent |
|-----|-----|-------------|-----------------|-----------------|
| 59  | F   | No          | Different patient; matched for age, sex and biopsy site | S. St Paul |
| 71  | F   | No          | C. jejuni       |                 |
| 23  | F   | No          | C. jejuni       |                 |
| 46  | M   | CD          | Same patient and biopsy site>12 months from acute infection | C. difficile |
| 19  | M   | CD          | C. difficile    |                 |
| 47  | F   | CD          | C. difficile    |                 |
| 27  | M   | UC          | S. enteridis    |                 |

*CD = Crohn’s disease, UC = ulcerative colitis.

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Antibodies

The MUC1 antibody (CT2[36]) and the Muc17/Muc3-S2 antibody (raised against the peptide KYTPGFENTLTVKNNLETKKNAT [35] were kind gifts from Prof. S. Gendler, Scottsdale, USA, and Prof. G. Hansson, Gothenburg, Sweden. The polyclonal anti sera against the C-terminal sequence of MUC4 (hHA1-B-1) was a kind gift from Prof. K. Carraway, Miami, USA. The MUC5AC antibody (45M1) was purchased from Sigma [37]. Polyclonal antisera (rM13.C and MM2-2) against the cytoplasmic tail of the human MUC13 and murine Muc2 was raised in rabbit using bovine serum albumin conjugated peptides CMQNPSRHSMPRPDYP and CPEDRPYDDLKKK, respectively [38,39], and then purified on a HiTrap NHS-activated HP column (Amersham Biosciences, Uppsala, Sweden) coupled to the same peptides according to manufacturers instructions. The monospecificity of the CT2 and rM13.C antibodies were demonstrated by that gastric and intestinal specimens from Muc1 and Muc13 knockout mice were negative for staining with the respective antibodies. The staining pattern of the hHA1-B-1 antisera was similar to that previously described for MUC4 in human tissue [40,41], and the pre-immune sera from the hHA1-B-1 rabbit did not stain any tissue. The 45M1 antibody recognizes MUC5AC (and no other molecules in gastric tissue extracts) in human and rhesus monkey as demonstrated by that only fractions from density gradient centrifugation that has a density appropriate for MUC5AC (1.4 g/ml) are recognized with this antibody [37], and the mouse gastric tissue used here as a positive control demonstrated a strong surface epithelial cell staining, similar to that of the human and rhesus monkey gastric tissue[37], whereas the intestinal tissue was negative. Similarly, the MM2-2 antibody recognize material from intestinal tissue extracts in fractions of appropriate density from density gradient centrifugation of MUC2/Muc2, show a distinct goblet cell staining pattern in the intestine of mice similar to that demonstrated in human [39], and did not stain the gastric negative control tissue. The specificity of the Muc3-S2 antibody has been confirmed using mass spectrometry [35].
for the detection of the different antibodies was either: 1) 10 mM citric acid, pH 6 at 95°C for 20 min (CT2, Muc17/Muc3-S2); 2) 10 mM citric acid, pH 6 at 95°C for 20 min followed by 10 mM 1,4-dithiothreitol in 0.1 M Tris/HCl buffer, pH 8 at 37°C for 30 min and then 25 mM iodoacetamine in the dark for 30 min (MM2-2); 3) High pH Antigen Retrieval Solution (Dako, RC20); or 4) Rodent decloaker (Biocare Medical, 45M1) at 80°C for 2 h. Sections were then treated with 3% (v/v) hydrogen peroxide for 10 min at room temperature. The sections were washed 3 times between all subsequent steps in phosphate buffered saline, pH 7.4 (PBS) containing 0.05% Tween-20. Non-specific binding was blocked by 10% Milk Diluent/Blocking solution (KPL, Maryland, USA) in PBS (CT2, MM2-2, Muc3-S2, hHAI-B-1 and RC20) or rodent block M (Biocare Medical, 45M1) for 30 min. The sections were incubated with a primary antibody diluted in 10% Milk Diluent/Blocking solution (KPL, Maryland, USA) in PBS containing 0.05% Tween-20 (CT2 1:50, MM2-2 5 µg/ml, Muc17/Muc3-S2 1:2000, hHAI-B-1 1:200, 45M1 1:1000 and rM13.C 30 µg/ml,) for 1 h, then incubated with anti-rabbit antibody conjugated to horseradish peroxidase (HRP), MM2-2,

Figure 1. *C. rodentium* induced colitis and infection load. The number of *C. rodentium* CFU in feces increased over time (A, *p* < 0.05 compared to pre-inoculation, *# p* < 0.05 compared to day 7, Mann Whitney U test, n = 8). At day 12, a higher number of CFU was detected in the large intestine (LI) than in the small intestine (SI) (*p* < 0.05), and low numbers of CFU were found in the internal organs (C). Histological colitis scores were low to moderate, with the distal large intestine being worst affected (D).

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Figure 2. Serum Ig response to *C. rodentium*. Serum concentrations of Ig reactive with *C. rodentium* lysate determined by ELISA in 8 mice prior to and 1, 7 and 12 days following infection.

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Mucins in Infectious Colitis

Small intestine

Caecum

Alcian blue/PAS

Muc1

Muc2

Muc4

Muc13

Muc17

SCL: Apical
CR: Surface

SCL: Apical
CR: Surface

SCL: Apical
CR: Surface

SCL: Apical
CR: Surface

SCL: Apical
CR: Surface

CR: Surface
Base

CR: Surface
Base

CR: Surface
Base

CR: Surface
Base

CR: Surface
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CR: Surface
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CR: Surface
Base

CR: Surface
Base

CR: Surface
Base
RC) or anti-hamster antibody conjugated to biotin followed by streptavidin-HRP (CT2) for 1 h or with MM HRP-Polymer (Biocare Medical, 45M1) for 20 min. Bound antibody was visualized with diaminobenzidine for 10 min. The sections were counterstained with Harris’s haematoxylin. Gastric sections were used as positive controls for CT2 and 45M1. Human samples: Formalin-fixed, paraffin-embedded tissue sections (4 μm) of archived biopsies were dewaxed and rehydrated. Sections were treated with 1% periodic acid to expose the epitope and then with 3% (v/v) hydrogen peroxide for 30 min at room temperature. The sections were washed 3 times between all subsequent steps in 0.15 M NaCl, 0.1 M Tris/HCl buffer (pH 7.4) containing 0.05% Tween-20. Non-specific binding was blocked by protein block (Dako) for 30 min. The sections were incubated with the BC2 antibody diluted in Antibody Diluent for 1 h, then incubated with Broad Spectrum Poly HRP Conjugate (Zymed Laboratories inc, San Fransisco, USA) for 10 min and then with diaminobenzidine for 10 min. The sections were counterstained with Harris’s haematoxylin.

Staining was classified into one of four categories: high level of staining (approx. 90–100% of cells staining, score = 3), medium level of staining (25–90%, score = 2), low level of staining (1–25%, score = 1) and virtually no staining (<1%, score = 0). The scoring of the stain was performed by an individual blinded to the infection status of the mice. Two of the stains were also analyzed by a second individual blinded to the infection status of the animals, and the results between the two scorers were consistent.

PAS/Alcian blue stain

De-waxed sections were immersed in 100% ethanol for 10 min, rinsed in water for 10 min, immersed in 3% acetic acid for 2 min and stained in 1% Alcian Blue 8GX in 3% acetic acid (pH 2.5) for 2.5 h. Non specific stain was removed with 3% acetic acid and rinsed in water for 10 min. The slides were then oxidized in 1% periodic acid in water at room temperature for 10 min, washed in water for 5 min, immersed in Schiff’s reagent for 10 min, rinsed in water for 5 min and then three times in 0.5% sodium metabisulphite before a final wash in water. To reveal O-acetylated oligosaccharides sections were first treated with 0.1 M KOH for 30 min and then 1 mM periodic acid prior to the Schiff reagent.

Antibody response against C. rodentium

C. rodentium were harvested into PBS, washed once and then sonicated 5 times while on ice with a 3 min interval between sonications. After sonication, the solution was centrifuged at 4000 g for 4 min. The supernatant was diluted to A280 = 0.4 and plated onto Maxisorb plates (Nunc). The microtiter plates were washed 3 times in PBS pH 7.4 containing 0.05% Tween-20 between all ensuing steps. Unbound sites were blocked with 1% bovine serum albumin (BSA) for 1 h. The microtiter plates were incubated with murine sera diluted 1:100 in PBS containing 1% BSA and 0.05% Tween, pH 7.4, for 1 h at room temperature. The microtiter plates were incubated with HRP conjugated anti mouse Ig diluted 1:2000. Bound secondary antibody was visualized using BD OptEIA TMB substrate reagent (BD, San Diego, USA). The reaction was stopped with 1 M sulphuric acid and absorbance at 450 nm was measured.

C. rodentium binding to Muc2

The insoluble complex of Muc2 was extracted from murine intestinal mucosal scrapings as previously described [42]; mucosal scrapings were gently dispersed with a Dounce homogenizer in 6 M guanidinium chloride, 5 mM EDTA, 5 mM N-ethylmaleimide, 10 mM sodium phosphate buffer, pH 7.0, and left stirring at 4°C overnight. After centrifugation (21,000 g, 4°C, 60 min), the soluble material was removed, and the pellets were re-extracted twice, as described above. The final extraction residues (insoluble glycoprotein complex) were solubilized by reduction in 6 M guanidinium chloride, 10 mM dithiothreitol, 5 mM EDTA, 0.1 M Tris-HCl buffer, pH 8.0, at 37°C overnight and then alkylated with 25 mM iodoacetamide in the same buffer and then dialyzed against 4 M guanidinium chloride. After centrifugation, the supernatant was retained. This solubilized “insoluble” MUC2 complex was diluted in 4 M guanidinium chloride and 100 μl/well were coated onto microtiter plates (NUNC P96; Polysorp, Roskilde, Denmark) overnight at 4°C. The microtiter plates were washed 3 times in PBS pH 7.4 containing 0.05% Tween-20 between all ensuing steps. Unbound sites were blocked with 0.5% bovine serum albumin in 1% blocking reagent for ELISA in PBS containing 0.05% Tween-20 for 1 h. The microtiter plates were incubated with biotinylated C. rodentium in 1% in blocking reagent for ELISA containing 0.05% Tween pH 7.4 for 1 h at room temperature. The microtiter plates were incubated with HRP conjugated streptavidin diluted 1:2000. Bound secondary antibody was visualized using 2,2’-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid as a substrate in citric acid- Na2HPO4 buffer, pH 4.3. Absorbance at 405 nm was measured after 40 min. When the 6 M guanidinium HCl-insoluble fraction containing Muc2 was subjected to agarose gel electrophoresis and then blotted onto a membrane, the only PAS positive band present corresponded exactly to the MU2 band identified by Western blotting. However, we have not performed Western blotting for other mucins and very small amounts of other contaminating mucins cannot be excluded.

Statistics

CFU data were log transformed. Individual data points are presented and the non-parametric Mann Whitney U test was applied to ascertain differences. All statistics were determined using Systat 12 (Systat Software, Inc., San Jose, USA).

Results

C. rodentium infection and associated pathology is greatest in the large intestine

Before inoculation, C. rodentium was not detected in the murine feces (Figure 1A). On day 7, C. rodentium was detected in feces of 5/8 mice and the mean C. rodentium content in feces was 10^5 CFU/g. By day 12, C. rodentium was detected in feces of 6/8 mice and the mean C. rodentium content in feces had increased to 10^6 CFU/g.

Mucins in Infectious Colitis

Figure 3. Tissue localization of mucins in small intestine and caecum. Histologically, the small intestinal villi and caecal crypts are covered by a simple columnar epithelium consisting of enterocytes with interspersed goblet cells. Using immunohistochemistry, we demonstrated that the apical surface of small intestine and caecal epithelia are lined by the cell surface mucins Muc13 and Muc17 as well as a small amount of Muc4 (brown). The goblet cells contain alcian blue positive material (blue), which mainly is Muc2 (brown), but also some Muc4. The photographs were taken using a 20 × magnification. The quantification scores for any of these mucins were not significantly different between non-infected mice (C) and mice infected with C. rodentium for 12 days (C) in these areas of the intestine. (Mann Whitney U test, n = 7). SCL = subcellular localization, RC = region of crypt. The mucin stains are shown in serial sections on the same tissue area, whereas the mucin stain scores represent the average stain scores for the entire specimen for each individual mouse.

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Proximal large intestine

Mucins in Infectious Colitis

Not infected | Infected
---|---
Alician blue/PAS
Muc1
Muc2
Muc4
Muc13
Muc17
of the villous goblet cells expressed Muc4 with weaker staining in the bottom of the crypts. All goblet cells expressed Muc2 and most of the remaining cells of the basal goblet cell lineage there was an increase in mild PAS staining without prior saponification, however, in the remaining cells of the basal goblet cell lineage there were no changes in mucin expression in response to infection.

**Proximal large intestine.** In the proximal large intestine the two distinct crypt and surface lineages of goblet cells were both strongly AB positive and contained Muc2, whereas Muc4 was present in the goblet cells on the surface, but to a lesser degree in the crypts (Figure 4). The enterocytes and their apical surfaces were both weakly positive for Muc4 and more strongly positive for Muc13, whereas Muc17 was restricted to the apical surface of the surface epithelium, and Muc1 was not expressed (Figure 4). Although no statistically significant differences in mucin expression were detected between non-infected and C. rodentium infected proximal colon, the Muc2/AB filled goblet cell thecae had a higher variability in size in the infected animals with groups of crypts being either hypertrophic and bulging, whilst other groups of crypts were partially depleted of mucin (Figure 4). This suggests that both Muc2 production and secretion is increased in the proximal large intestine of infected mice. Saponification prior to mild PAS staining to reveal O-acetylated mucin oligosaccharides revealed O-acetylation in a proportion of the goblet cell lineage found at the base of the crypts of the proximal and mid-colon, as previously reported [44]. Post-infection the loss of goblet cells was reflected in reduced overall mild PAS staining, however, in the remaining cells of the basal goblet cell lineage there was an increase in mild PAS staining without prior saponification, indicating a reduction of O-acetylation in those cells following infection (Figure S1).

**Mid and distal large intestine.** In the mid and distal large intestine, the upper half of the crypts contain the majority of the goblet cells, whereas the lower half of the crypts contain cells of secretory type but with a more vacuolated morphology [45]. AB/PAS staining of the goblet cells revealed medium charged carbohydrates from the middle of the crypt to the top, whereas the bottom of the crypts had a small amount of highly charged carbohydrate chains (Figures 5 and 6). As for the other regions of the intestine these goblet cells expressed Muc2 and to a lesser extent Muc4. In the mid- and distal colon, the apical surface of the lower half of the crypt was weakly positive for Muc1 (Figures 5–6 and Figure S2, intensity score 0–0.5). Muc13 and Muc17 were highly expressed in the cytoplasm of the cells with secretory vacuolated morphology in lower half of the crypt and also expressed on the apical surface of cells in the crypts and the surface epithelium (Figure 6). Muc17 was also present cytoplasmically in the bottom half of the crypt (Figures 5–6). Thus, the tissue localization of Muc17 is similar to that of Muc13 in the mid and distal large intestine, whereas differences occur in the more proximal parts of the intestine.

**C. rodentium caused focal damage and elongation of crypts in the mid and distal colon, with patches of normal epithelium**
interspersed with the more inflamed elongated crypts. In infected animals, the distal colonic crypts were depleted of AB, Muc2 and Muc4 positive material (p<0.05 for AB/PAS, Figure 6). In some areas the goblet cell thecae were depleted from the base of crypts but some goblet cell thecae remained at the top of crypts, and there was a massive amount of secreted mucus and shed goblet cells (see arrow in Figure 5) in the lumen. The mid large intestine also tended to be depleted of stored Alcian blue positive material at the bottom of the crypt and partially of the purple goblets from mid length to top (Figure 6), although the variability between animals was larger in this location. The more elongated crypts tended to be more depleted of mucus linking goblet cell depletion with localized infection and/or inflammation.

In the C. rodentium infected animals the apical Muc1 expression in the lower half of the crypt was increased in the mid and distal colon, and de novo expression occurred apically on the surface in the lower half of the crypt was increased in the mid and distal large intestine with infection and the apical expression in the crypt. The mucin stains are shown in serial sections on the same tissue area, whereas the mucin stain scores represent the average stain scores for the entire specimen for each individual mouse.

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Discussion

This work demonstrates that six mucins have distinctly different expression patterns throughout the murine intestinal tract, indicating that they are differentially regulated and are likely to make different functional contributions to the mucosal barrier. The cellular content of most mucins changes in response to infection, with Muc1 being the only mucin to be induced whereas all other mucins were depleted, albeit to differing degrees. As mucins are strategically positioned between the vulnerable mucosa and the bacterial contents of the intestinal lumen [46], the changes in mucin concentrations and the consequent changes in mucin binding to bacteria are likely to affect both host-commensal and host-pathogen interactions.

Muc1/MUC1 appears to play a significant role in host defense in acute infection. In the murine infection model of C. jejuni, Muc1 is upregulated acutely in the intestine in response to infection [6]. We now show that C. rodentium infection also induces Muc1 in mouse colon, and similarly that S. St Paul, C. jejuni and C. difficile induced MUC1 in human colon during infection. Thus, Muc1/ MUC1 is induced in response to infection by a range of different pathogens in both mice and humans. As Muc1−/− mice have a higher rate of systemic C. jejuni infection [6], Muc1/MUC1 induction appears to be an effective host defense used by both human and mouse innate immunity. MUC1 expression in biopsies from inflammatory bowel disease patients with chronic inflammation was also higher during acute infection compared to the inflammatory bowel disease control biopsies. This indicates that MUC1 is upregulated in response to acute infection and that chronic inflammation by itself is not sufficient to substantially upregulate MUC1.

Previous studies have shown that mucins bind to several pathogens [6,22,23] and we show here that mucins bind to C. rodentium. Increased secretion of mucin in response to infection has been demonstrated using in vitro airway epithelial cells co-cultured with Burkholderia cenocepacia and Listeria monocytogenes inoculated rat ileal loops [47,48]. The depletion in the amount of Alcian blue positive mucous material as well as Muc2, Muc4, Muc13 and Muc17 we detected here is also likely to occur as a consequence of increased secretion and therefore be part of the host defence. In a recently published study, Muc2 depletion in C. rodentium infected mice was shown to be dependent on T- and B-cells [49]. However, for such a host defence to be effective the mucus must be replenished. In our infected specimens we saw large amounts of shed whole cells, including goblet cells (Figure 5). The factors causing premature death of the goblet cells with infection are unknown, although it appears that C. rodentium directly interacts with and infect murine goblet cells during infection [49] and may therefore result in goblet cell death. Shielding of these cells would delay replenishment of the mucus layer providing an advantage to the mucosal pathogen. In addition, several pathogens have been shown to specifically inhibit mucin synthesis [50,51]. A further possibility is that the goblet cells experience endoplasmic reticulum stress due to the enormous amount of protein synthesis that they effect during infection and that the consequent unfolded protein
response shuts down mucin production and if more prolonged or severe causes apoptosis [39,52,53]. It seems likely that the host attempts to remove pathogens by increasing secretion of mucins to trap and remove pathogens from the epithelial surface, whereas the pathogen tries to eliminate this host defence by inhibiting the mucin synthesis and causing shedding of the mucus producing epithelial cells. Interestingly, goblet cell depletion during infection does not appear to occur in the respiratory tract where goblet cell hyperplasia and mucus hypersecretion can continue for extended periods [54]. Furthermore, changes in mucin secretion is likely to affect the resident microflora due to the large change in glycosylated compounds available for bacterial degradation [55,56].

In the small intestine and proximal large intestine, *C. rodentium* was present, albeit in lower amounts than in the distal large intestine, and yet there were few signs of mucosal damage, inflammation or changes in mucins. Furthermore the highest concentrations of bacteria were detected in the colons of animals with the highest amount of colonic damage, mucin depeletion, and systemic infection. Even within animals, damage and inflammation in the distal colon was often patchy suggesting that focal bacterial penetration of the barrier leads to localised mucin release and inflammatory responses. Why these bacteria penetrate the barrier in the distal colon remains to be elucidated but could be due to an increased bacterial pathogen load in the distal colonic niche, or an ability to degrade the mucin glycoforms in this region, and/or involve induction of pathogenicity-related genes in the bacteria due to local environmental cues. We have demonstrated that MUC2 is one such cue for *C. jejuni* which switches on pathogenicity genes in response to mucin exposure [57]. An IgG response has been shown to be integral to clearance of *C. rodentium* infection [58] and it appears pertinent for our understanding of the onset of humoral immunity that the *C. rodentium* specific Ig production at this stage of infection was restricted to those mice in which the bacteria had penetrated the mucosal barrier.

*C. rodentium* infection causes colonic inflammation, mucosal hyperplasia, epithelial dysfunction in association with increased permeability to luminal bacteria and a vigorous Th1 inflammatory response [59]. Thus *C. rodentium* infection elicits mucosal inflammation with similarities to inflammatory bowel disease, and has therefore been used to investigate the relationship between inflammation and anti-bacterial immunity in the gut [4]. MUC5AC, which is a product of normal gastric mucosa, is absent from normal colon but frequently present in colorectal adenomas and colon cancers [60,61], and expressed in patients with

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**Figure 6. Tissue localization of mucins in the distal large intestine.** The apical surface of the distal large intestinal epithelium and crypts are lined by the cell surface mucins Muc13 and Muc17 as well as a small amount of Muc4 (brown). The goblet cells contain Alcian blue positive material (blue = highly charged carbohydrate structures, purple = medium charged carbohydrate structures), which mainly is Muc2 (brown), although the goblet cells of the surface also contain Muc4. The photographs were taken using a 20× magnification. The quantification scores for Alcian blue, Muc1, Muc13 and Muc17 were significantly different between non-infected mice (○) and mice infected with *C. rodentium* for 12 days (●) (**p<0.05, Mann Whitney U test, n = 7). SCL = subcellular localization, RC = region of crypt. The mucin stains are shown in serial sections on the same tissue area, whereas the mucin stain scores represent the average stain scores for the entire specimen for each individual mouse.

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**Figure 7. *C. rodentium* binding to Muc2.** ELISA plates were coated with the “insoluble” Muc2 complex or with BSA and incubated with biotinylated *C. rodentium*. Statistics: Mean±SD. ***p<0.001 (t-test, n = 6) (A). Large amounts of bacteria can be found entangled in the secreted Muc2 in *C. rodentium* infected animals (B–D). By brightfield observation using a 20× lens (B) the Muc2 is visualized as a brown stain, and the presence of large amounts of bacteria within the secreted Muc2 can be seen by reflected light Nomarski differential interference observation (C; 40× lens and D; 100× lens).

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ulcerative colitis [61]. MUC5AC was not detected in the infected mice in our study, suggesting that induction of MUC5AC may arise from more long term effects of chronic inflammation. However, similar to our findings in murine C. rodentium infection, a reduction in MUC2 expression occurs in UC adjacent to ulceration and in active colitis [62,63], and MUC1 expression was upregulated in severe UC at the site of rupture of crypt abscesses [63]. Our non-infected human biopsies from IBD patients only had a very low amount of MUC1, but they did not contain any crypt abscesses, which may induce MUC1 in a similar manner to acute infection.

In conclusion, in response to intestinal infection mucin secretion is increased and most mucins are depleted from the epithelium whilst MUC1 is upregulated. As mucus bind pathogens, increasing the secretion of mucus is likely to aid the host by trapping and removing pathogens from the epithelial surface. Pathogens may subvert this host defence by inhibiting mucin synthesis and secretion or causing premature shedding of the mucus producing epithelial cells. Further exploration of the dynamic host mucin – bacterial interactions that occur in the mucin covered mucosal interface will increase our understanding of human susceptibility to infection.

Supporting Information

Table S1  Histological scoring of murine colitis.
Found at: doi:10.1371/journal.pone.0003952.s001 (0.03 MB DOC)

Figure S1  Mild PAS staining with and without prior saponification on a non-infected and C. rodentium infected mouse. Found at: doi:10.1371/journal.pone.0003952.s002 (7.16 MB TIF)

Figure S2  Expression of Muc1 in the distal large intestine is upregulated in response to infection with C. rodentium. Representative examples of Muc1 expression determined by immunohistochemistry in the distal colon of mice infected with C. rodentium for 12 days and non-infected mice. The photographs were taken using 20× magnification. Found at: doi:10.1371/journal.pone.0003952.s003 (26.10 MB TIF)
References

1. Tobe T, Sasakawa C (2002) Species-specific cell adhesion of enteropathogenic Escherichia coli is mediated by type IV bundle-forming pili. Cell Microbiol 4: 29–42.

2. Mundy R, Schuller S, Girard F, Fairbrother JM, Phillips AD, et al. (2007) Host Pathogen Binding and Adhesive Properties of H6 and HT Flagella of Attaching and Effacing Escherichia coli. J Bacteriol 199: 7426–7435.

3. Mack DR, Ahare S, Hyde I, Lei S, Hollingsworth MA (2003) Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. Gut 52: 827–833.

4. Penniston JA, Patton S, Hanshaw M (1996) Glycoproteins of the human milk fat globule in the protection of the breast-fed infant against infections. Biology Of The Neonate 74: 143–162.

5. Yolken RH, Peterson JA, Vonderfecht SL, Fouts ET, Midthun K, et al. (1992) Human milk mucin inhibits rotavirus replication and prevents experimental diarrheal infection. Journal of Clinical Investigation 90: 1884–1891.

6. Smirnova MG, Birchall JP, Pearson JP (2000) TNF-alpha in the regulation of intestinal goblet cells. Cell Immunol 214: 239–248.

7. Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, et al. (2008) Aberrant MUC15 expression as a soluble, secretable form in lactating mammary gland and blood. Biochim Biophys Acta 1783: 293–303.

8. Schroeder JA, Thompson MC, Gardiner MM, Gengl SJ (2001) Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation in the mouse mammary gland. J Biol Chem 276: 13057–13064.

9. Komatsu M, Arango ME, Carraway KL (2002) Synthesis and secretion of MUC15. Biochim Biophys Acta 158: 74–81.

10. Komatsu M, Arango ME, Carraway KL (2002) Synthesis and secretion of MUC15. Biochim Biophys Acta 158: 74–81.
Porphyromonas gingivalis interference with salivary mucin synthesis. IUBMB Life 56: 153–159.

51. Slomiany BL, Slomiany A (2006) Cytosolic phospholipase A2 activation in Helicobacter pylori lipopolysaccharide-induced interference with gastric mucin synthesis. IUBMB Life 58: 217–223.

52. Marciniak SJ, Ron D (2006) Endoplasmic reticulum stress signaling in disease. Physiol Rev 86: 1133–1149.

53. Kaser A, Lee AH, Franke A, Glickman JN, Zeissig S, et al. (2008) XBP1 Links ER Stress to Intestinal Inflammation and Confers Genetic Risk for Human Inflammatory Bowel Disease. Cell 134: 743–756.

54. Thornton D, Rousseau K, McGuckin M (2008) Structure and Function of the Polymeric Mucins in Airways Mucus. Annu Rev Physiol. pp 459–486.

55. Hooper LV, Gordon JI (2001) Glycans as legislators of host-microbial interactions: spanning the spectrum from symbiosis to pathogenicity. Glycobiology 11: 1R–10R.

56. Byrd JC, Bresalier RS (2004) Mucins and mucin binding proteins in colorectal cancer. Cancer & Metastasis Reviews 23: 77–99.

57. Tu Q, McGuckin M, Mendz G (2008) Campylobacter jejuni response to human mucin MUC2: modulation of cell morphology and virulence determinants. J Med Microbiol; in press.

58. Masuda A, Yoshiida M, Shiomi H, Iezawa S, Takagawa T, et al. (2008) Fc(γ) Receptor Regulation of Citrobacter rodentium Infection. Infect Immun Epub ahead of print.

59. Skinn AC, Vergnaud N, Zammer SR, Wallace JL, Cellars L, et al. (2006) Citrobacter rodentium infection causes iNOS-independent intestinal epithelial dysfunction in mice. Canadian Journal of Physiology & Pharmacology 84: 1301–1312.

60. Byrd JC, Bresalier RS (2004) Mucins and mucin binding proteins in colorectal cancer. Cancer & Metastasis Reviews 23: 77–99.

61. Forgue-Lafitte ME, Fabiani B, Levy PP, Maurin N, Flejou JF, et al. (2007) Abnormal expression of M1/MUC5AC mucin in distal colon of patients with diverticulitis, ulcerative colitis and cancer. Int J Cancer 121: 1543–1549.

62. Hinoda Y, Akashi H, Suwa T, Isoh F, Adachi M, et al. (1998) Immunohistochemical detection of MUC2 mucin core protein in ulcerative colitis. J Clin Lab Anal 12: 150–153.

63. Longman RJ, Poullom R, Corfield AP, Warren BF, Wright NA, et al. (2006) Alterations in the composition of the supramucosal defense barrier in relation to disease severity of ulcerative colitis. J Histochem Cytochem 54: 1335–1340.
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