MUC1 cell surface mucin is a critical element of the mucosal barrier to infection

Julie L. McAuley,1 Sara K. Linden,1 Chin Wen Png,1 Rebecca M. King,2 Helen L. Pennington,1 Sandra J. Gendler,3 Timothy H. Florin,1 Geoff R. Hill,4 Victoria Korolik,2 and Michael A. McGuckin1

1Mucosal Diseases Program, Mater Medical Research Institute and The University of Queensland, South Brisbane, Queensland, Australia.
2Institute for Glycomics, Griffith University Institute for Glycomics, Gold Coast, Queensland, Australia.
3Department of Biochemistry and Molecular Biology and Tumor Biology Program, Mayo Clinic College of Medicine, Scottsdale, Arizona, USA.
4Division of Infectious Diseases and Immunology, Queensland Institute of Medical Research, Herston, Queensland, Australia.

Cell surface mucin glycoproteins are highly expressed by all mucosal tissues, yet their physiological role is currently unknown. We hypothesized that cell surface mucins protect mucosal cells from infection. A rapid progressive increase in gastrointestinal expression of mucin 1 (Muc1) cell surface mucin followed infection of mice with the bacterial pathogen Campylobacter jejuni. In the first week following oral infection, C. jejuni was detected in the systemic organs of the vast majority of Muc1+/– mice but never in Muc1+/- mice. Although C. jejuni entered gastrointestinal epithelial cells of both Muc1+/– and Muc1+/- mice, small intestinal damage as manifested by increased apoptosis and enucleated and shed villous epithelium was more common in Muc1+/- mice. Using radiation chimeras, we determined that prevention of systemic infection in wild-type mice was due exclusively to epithelial Muc1 rather than Muc1 on hematopoietic cells. Expression of MUC1-enhanced resistance to C. jejuni cytolytic distending toxin (CDT) in vitro and CDT null C. jejuni showed lower gastric colonization in Muc1+/- mice in vivo. We believe this is the first in vivo experimental study to demonstrate that cell surface mucins are a critical component of mucosal defence and that the study provides the foundation for exploration of their contribution to epithelial infectious and inflammatory diseases.

Introduction
Penetration of mucosal barriers by microbial pathogens, subsequent damage to epithelial cells, and ensuing inflammatory responses cause both acute and chronic diseases with substantial impact on human health. Although mucins have long been regarded as involved in mucosal barrier function, there are no empirical studies, to our knowledge, clearly demonstrating a role for either cell surface or secreted mucins in host defense against infection.

Cell surface mucins are transmembrane glycoproteins expressed at the apical surface of all mucosal epithelial cells. Ten cell surface mucin genes have been identified, and multiple genes are expressed in tissues at greatest risk of infection (1). The extracellular domain of these mucins forms an extremely large thread-like structure covered by a dense array of complex O-linked oligosaccharides and can be shed from the cell surface. Most mucosal pathogens have evolved adhesins for carbohydrates present in the glyocalyx, and many adhesins bind mucin oligosaccharides (2). The cytoplasmic domains of cell surface mucins are highly conserved across species, undergo both serine and tyrosine phosphorylation, and interact with kinases and adaptor molecules (3–7), consistent with a role in signal transduction. However, the primary function of these mucins is not understood.

Mucin 1 (MUC1) is a cell surface mucin broadly expressed in mucosal tissues (8). Consistent with a role in host defense, mucosal epithelial cells transfected with MUC1 are less susceptible to viral invasion in vitro (9, 10), and Muc1+/– mice in conventional housing develop bacterial conjunctivitis and chronic infection of the reproductive tract (11, 12). The respiratory pathogen Pseudomonas aeruginosa binds MUC1, inducing phosphorylation of the cytoplasmic domain (4), demonstrating that MUC1 signals in response to bacteria. MUC1 interacts with growth factor receptors (13) and inhibits the intrinsic pathway of apoptosis (14–16), which suggests that MUC1 modulates a critical balance between growth to maintain barrier function and apoptosis to dispose of infected cells in mucosal epithelia.

We hypothesize that cell surface mucins act as releasable decoy molecules, displaying an array of targets for microbial adhesion, thereby limiting binding of pathogens to other molecules in the glyocalyx. Furthermore, we contend that following release of the extracellular subunit, the cytoplasmic subunit is involved in ensuring appropriate activation, proliferation, or apoptotic responses to microbes that have penetrated overlying mucus. Although Campylobacter jejuni infection is a major cause of gastroenteritis, its mechanisms of pathogenicity are poorly understood. C. jejuni is highly motile within secreted mucus, is chemotactraded toward mucins, and binds to mucin oligosaccharide structures (17, 18), making it an ideal model pathogen for exploration of cell surface mucin function in vivo. In this study, using C. jejuni infection and Muc1+/- mice, we clearly demonstrate in vivo the critical importance of Muc1 in limiting mucosal infection and show that MUC1-expressing intestinal cells have increased resistance to the C. jejuni genotoxin, cytolytic distending toxin (CDT).

Results
Gastrointestinal expression of Muc1 alters rapidly in response to infection. Goblet cell hyperplasia and increased release of secreted mucins are well-characterized responses to mucosal infection. In comparison, little is known of regulation of cell surface mucins in response
Gastrointestinal expression of Muc1 is rapidly upregulated in response to infection with C. jejuni. Muc1 expression was determined by immunohistochemistry with the CT2 cytoplasmic domain antibody in the stomach, small intestine, and large intestine of uninfected Muc1+/+ mice (control) and mice infected with 10⁶ C. jejuni for 2, 6, and 24 hours. Scoring of the proportion of cells staining and the staining intensity for deep glands or the surface epithelium (villus epithelium in the small intestine) was performed blind to treatment. Examples of staining at each time point are provided in Supplemental Figure 1. No staining was seen in uninfected or infected Muc1−/− mice.
The experiment shown in Figure 3B (4 days, 10^6 to 10^7 bacteria) demonstrates that C. jejuni crosses the gastrointestinal barrier in Muc1−/− mice at inoculation doses as low as 10^5 bacteria. Even at this very low dose, all Muc1−/− mice developed systemic infection compared with no detectable systemic organ infection in any Muc1+/+ mice. Colonization levels of gastrointestinal tissues were higher in Muc1−/− mice, regardless of inoculation dose.

Sampling of mice over the first 24 hours of infection revealed rapid transit of C. jejuni through the gastrointestinal tract, reaching the large intestine within 2 hours of inoculation in some mice (see Figure 3C). In Muc1−/− mice, C. jejuni reached the liver of 2 of 4 animals after just 2 hours and 3 of 4 animals by 24 hours, demonstrating that C. jejuni penetrates the gastrointestinal barrier rapidly in Muc1−/− mice. Despite the development of systemic infection, Muc1−/− mice did not develop diarrhea or overt signs of illness, with the exception of a reduced gain in body weight 1–3 days after infection compared with infected Muc1+/− mice and infected and uninfected Muc1−/− mice (not shown). Systemic infection of Muc1−/− mice by C. jejuni was not restricted to the 81116 strain; other strains equally colonized systemic organs (see Supplemental Figure 2).

Long-term infection experiments demonstrated that C. jejuni eventually crossed the gastrointestinal barrier and colonized systemic organs of Muc1−/− mice (see Figure 3D). Fourteen days following infection, colonization levels in gastrointestinal tissues did not differ significantly between Muc1+/+ and Muc1−/− mice, and C. jejuni were cultured from systemic organs of all Muc1+/+ mice. Four weeks following inoculation, C. jejuni continued to colonize the gastrointestinal tract of all Muc1+/+ and Muc1−/− mice, albeit at significantly decreased levels, consistent with partial immunological control (see Figure 3D).

More severe damage to the intestinal epithelium occurs in C. jejuni–infected Muc1+/+ mice. Small foci of epithelial damage in the small intestines were observed in Muc1+/− mice within 24 hours of inoculation with C. jejuni (see Figure 4A). In experiments from 1 to 6 days after inoculation, foci of damage were more frequently seen in Muc1+/− mice. Damage took the form of clusters of enucleated and shed epithelial cells on villi, consistent with necrotic cell death following infection or the action of a bacterial toxin (see Figure 4A and Supplemental Figure 3). These areas of damage had the same morphological appearance as the areas where Muc1 expression was upregulated in wild-type mice (see Supplemental Figure 1). Clusters of apoptotic nuclei were occasionally seen within the small intestinal lumen (Figure 4A). Immunohistochemistry demonstrated localized activation of caspase-3 in damaged small intestinal villous epithelium (Figure 4B). Two hours after infection, multiple foci of villous epithelial cells expressing activated caspase-3 were seen in 4 of 4 Muc1−/− mice and only 1 of 4 wild-type mice, indicating that the infection rapidly triggered foci of epithelial apoptosis, particularly in Muc1−/− mice (Figure 4B).

In both Muc1+/+ and Muc1−/− mice, C. jejuni was detected immunohistochemically within gastric epithelial cells and intestinal goblet cell thecae 2 hours after inoculation, consistent with chemotaxis toward mucus or another component of mucus and an ability to invade cells (see Figure 4C). C. jejuni appeared within mucosal luminal secretions, and bacteria could be seen being extruded from infected goblet cells undergoing compound mucin exocytosis (see Figure 4C). C. jejuni was also detected within or adjacent to sites of small intestinal epithelial damage in both Muc1+/− and Muc1−/− mice. Examples of C. jejuni within gastric and small and large intestinal submucosal blood vessels were seen in both Muc1+/− and Muc1−/− mice within the first 24 hours of infection. In Muc1−/− mice with
CFU-detected liver infection, C. jejuni was localized within the parenchyma and sinusoids of the liver (Figure 4C). After long-term infection (14 and 28 days), Muc1−/− mice had multiple foci of intestinal epithelial damage at frequencies similar to those observed in the first week of infection in Muc1+/+ mice (see Supplemental Figure 3). Small and large intestinal-associated lymphoid tissue increased progressively in both Muc1−/− and Muc1+/+ mice, and mild inflammation (lymphoid accumulation with aberrant crypts) was observed in a subset of mice of both strains 2 and 4 weeks after inoculation (see Supplemental Figure 3). Mice of both strains developed cystic lesions in the stomach (see Supplemental Figure 3).

Muc1-deficient mice show no evidence of increased susceptibility to infection with Salmonella typhimurium. Salmonella typhimurium circumvents the mucus barrier to infection by selectively targeting M cells (20). The dome epithelium in which M cells reside lacks an overlay of mucus and has an altered glycocalyx (21); however, it is not known whether M cells express Muc1. We compared infection by mice using bone marrow transplantation to evaluate the significance of hematopoietic Muc1 deficiency.

Depletion of phagocytes in Muc1−/− mice only slightly enhanced susceptibility to infection with C. jejuni, with 2 of 5 macrophage- and 1 of 6 neutrophil-depleted mice developing systemic infection at similar levels within 2 days of inoculation with 10^2 S. typhimurium strain 8216915 (see Supplemental Figure 4).

Rapid systemic infection with C. jejuni in Muc1−/− mice is not due to a hematopoietic deficiency in Muc1. In addition to expression by epithelial cells, MUC1 is expressed by hematopoietic cells including erythroid progenitor cells, monocytes, plasma cells, and activated T cells and dendritic cells (22–25). The role of MUC1 on hematopoietic cells is unclear although there is evidence that it associates with kinases and may regulate T cell activation (3, 26, 27). Penetration of the gastrointestinal barrier in Muc1−/− mice infected with C. jejuni substantially predates deployment of the effector arms of adaptive immunity. Nevertheless, it could be argued that a deficiency in Muc1 in hematopoietic cells involved in rapidly engaged innate immunity contributes to the epithelial damage and development of systemic infection we describe. Therefore, we repeated infection experiments after systemic depletion of neutrophils and macrophages and generated chimeric S. typhimurium in Muc1−/− and Muc1+/+ mice as a model for a pathogen known to bypass the mucus barrier. Muc1−/− and Muc1+/+ mice developed systemic infection at similar levels within 2 days of inoculation with 10^3 S. typhimurium strain 8216915 (see Supplemental Figure 4).

Bone marrow transplantation following lethal irradiation clearly demonstrated that increased susceptibility to early systemic infection in Muc1−/− mice is not due to a Muc1 deficiency in hematopoietic cells (see Figure 5C). All Muc1−/− mice transplanted with Muc1−/− (6 of 6) and Muc1+/+ (5 of 5) bone marrow developed systemic infection within 2 days of inoculation with C. jejuni whereas systemic infection was not seen in Muc1+/+ mice transplanted with...
either Muc1+/− (0 of 6) or Muc1−/− (0 of 5) bone marrow. Despite a postulated role for Muc1 in T cell activation, the number of circulating CD3+ T cells and the proportion of activated CD3+CD69+ T cells did not differ among groups of mice in the transplantation experiment (see Supplemental Figure 5).

Diminution of gastrointestinal flora with antibiotics increases susceptibility of Muc1−/− mice to systemic infection with C. jejuni. Previous studies show increased gastrointestinal levels of C. jejuni after infection of germ-free (28) and antibiotic-treated mice (29). Pretreatment of Muc1−/− and Muc1+/− mice with broad-spectrum antibiotics to reduce normal microflora significantly increased the levels of gastrointestinal tract colonization, caused several histological foci of intestinal damage, and induced early (within 2 days) systemic infection in all Muc1+/− mice, abolishing the differences in susceptibility between Muc1−/− and Muc1+/− mice (see Figure 6A). This introduced the possibility that the enhanced susceptibility to infection of Muc1−/− mice was due to an alteration in their intestinal bacterial flora. However, quantitative assessment of the intestinal bacterial flora of Muc1−/− and Muc1+/− mice demonstrated equivalent numbers of bacteria and a virtually identical relative abundance of the major phyla and subphyla (see Figure 6B).

Expression of MUC1 in intestinal cells increases resistance to C. jejuni CDT in vitro. The type of intestinal damage we observed in mice infected with C. jejuni was consistent with the action of a toxin. C. jejuni produces a genotoxin (CDT) with nuclease activity that triggers cell-cycle arrest and increased apoptosis in vitro (30). MUC1 has been shown to modulate apoptosis and cell-cycle progression in malignant epithelial cells in response to genotoxic stress, in part by interacting with the regulatory domain of the p53 transcription factor (14–16). HeLa cervical cancer cells express MUC1 and have been shown to be susceptible to CDT (31). Treatment of HeLa cells with strain 81116–derived CDT resulted in almost complete redistribution of the MUC1 extracellular domain from the cell surface to the nucleus within 24 hours (see Figure 7A). CDT induced both cell-cycle arrest and apoptosis in HeLa cells in a dose-dependent manner. Stable partial knockdown of MUC1 with short-hairpin RNA (shRNA) did not enhance CDT-induced cell-cycle arrest or apoptosis over that seen with a control shRNA (representative experiment at 48 hours shown in Figure 7B, similar results were obtained at 72 and 96 hours). Because HeLa cells have very low functional p53 due to the presence of the HPV E6 protein (32), we transfected MUC1 in the p53-expressing HCT116 intestinal cell line in which transfection of MUC1 had previously been shown to increase resistance to chemotherapeutic genotoxins (16). In this cell line, CDT caused the MUC1 extracellular domain to accumulate in cytoplasmic vesicles, but it was not seen within the nucleus (see Figure 7C). In HCT116 cells, CDT caused dose-dependent cellular distension and cell-cycle arrest but did not induce apoptosis at doses resulting in complete arrest. HCT116 cells expressing MUC1 proliferated more in the presence of CDT, showing that MUC1 protects intestinal cells from the activity of C. jejuni CDT (results from 2 independent experiments are shown in Figure 7D). The MUC1 cytoplasmic domain translocated to the nucleus in a small proportion of CDT-affected cells within 24 hours of exposure to CDT with very strong nuclear staining. This pattern is consistent with transient relocation to the nucleus or relocation only in the most severely affected cells, and a role for MUC1 in modulation of transcription (Figure 7E). p53 showed diffuse colocalization with the MUC1 cytoplasmic domain in the cytoplasm of untreated cells and strong colocalization in cytoplasmic vesicles in CDT-treated cells (see Figure 7E), p53 translocated to the nucleus in the vast majority of CDT-treated MUC1-expressing HCT116 cells. Taken together, these data support a p53-mediated mechanism of MUC1 resistance to CDT.

CDT-null C. jejuni show lower gastric colonization but equivalent penetration of the intestinal barrier in Muc1+/− mice in vivo. Chloramphenicol-resistant C. jejuni strain 81-176 with the CDT-B gene deleted (ACDT-B) has been shown to cause less inflammation in a 4- and 8-week chronic infection model in NF-KB p50−/− p65−/− mice (33). Wild-type 81-176 and an isogenic ACDT-B mutant were inoculated into Muc1+/− and Muc1−/− mice and colonization of gastrointestinal and systemic tissues analyzed after 48 hours (Figure 8A).
Wild-type 81-176 showed greater colonization of the gastrointestinal and systemic tissues of Muc1−/− compared with Muc1+/+ mice, verifying the susceptibility of Muc1−/− mice to C. jejuni infection. In Muc1−/− mice, significantly lower levels of colonization by the ΔCDT-B mutant occurred in the stomach but not in the intestine. This finding suggests that in the stomach, where Muc1 expression is highest, MUC1-mediated resistance to the action of CDT is a component of the consistently lower levels of C. jejuni colonization in wild-type mice. However, the ΔCDT-B mutant crossed the gastrointestinal barrier in Muc1−/− mice, indicating that the action of CDT is not the primary determinant of systemic infection in mice lacking Muc1. Quantitative assessment of concentrations of apoptosis-associated activated caspases 3 and 7 in a segment of the terminal ileum of mice infected in the experiment shown in Figure 8A revealed increased apoptosis in infected mice, with trends toward greater apoptosis in Muc1−/− than Muc1+/+ mice infected with wild-type C. jejuni, and lower apoptosis in mice infected with the ΔCDT-B mutant (Figure 8B). The variation among animals in these samples is consistent with the focal nature of activated caspase-3 staining observed by immunohistochemistry.

**Discussion**

This study provides what we believe is the first empirical in vivo evidence that mucins are a critical element of gastrointestinal mucosal defense against pathogens. Alterations in expression of Muc1 in the gastrointestinal tract following bacterial infection, together with evidence that C. jejuni more heavily colonizes the gastrointestinal tract, induces more epithelial damage, and readily crosses the gastrointestinal barrier in Muc1−/− mice, demonstrate that this mucin is part of a dynamic epithelial response to the presence of bacterial pathogens. Bone marrow transplantation provided conclusive evidence that Muc1 expression by epithelia, and not the contribution to innate or adaptive immunity of Muc1 expressed by cells of hematopoietic lineage, protected mice from systemic infection by C. jejuni. Furthermore, phagocyte depletion experiments demonstrated Muc1 was a more significant element of protection from systemic C. jejuni infection than phagocytes. By manipulating MUC1 expression in epithelial cells in vitro we showed that MUC1 protects cells from a C. jejuni genotoxin. We propose that this mucin, rather than being part of a static barrier, is part of a dynamic response to mucosal infection that interacts with and is regulated by elements of both innate and adaptive immunity. We propose that the cell surface mucins have evolved as a critical defense against potential pathogens that penetrate the physical and chemical barrier provided by secreted mucus.

Our data provide clear experimental evidence confirming previous uncontrolled observational studies that suggested Muc1−/− mice housed under conventional conditions were more susceptible to reproductive tract and ocular infection by environmental bacteria (11, 12). Our observations of increased susceptibility to...
infection by mucin-binding bacteria in Muc1+/− mice are consistent with molecular epidemiological studies linking polymorphisms in the length of the MUC1 extracellular domain with development of gastritis and gastric cancer following infection with the mucin-binding gastric pathogen H. pylori (34–37). While Muc1+/− mice showed dramatically increased susceptibility to systemic infection by C. jejuni, no differences were demonstrated with an intestinal pathogen, S. typhimurium, that targets M cells, thereby circumventing the cell surface mucin barrier.

Based on our data and the biochemical properties of cell surface mucins, we propose that cell surface mucins act at 2 levels in host defence against infection. Initially, the large extracellular domain (for Muc1 estimated to be 200–500 nm in length; ref. 38) expressing diverse and complex oligosaccharides acts as a releasable decoy ligand for bacterial adhesins, thereby limiting attachment of pathogens to other cell surface molecules and subsequent invasion. Cell surface mucins are cleaved at a SEA module during synthesis, facilitating subsequent release of the extracellular domain (39). The MUC1 extracellular domain can also be released by proteolytic cleavage by the enzymes TACE (ADAM17) (40) and MT1-MMP (41). Proteases may cleave the mucin following bacterial adherence or highly motile C. jejuni infection, facilitating subsequent release of the extracellular domain. In particular, due to the absence of human milk reduces the in vivo environment. In particular, due to the absence of human milk reduces the colonization of human mucin-lysosome, stimulating the phosphorylation of Akt and Bad, and upregulating mitochondrial Bcl-2, which targets M cells, thereby circumventing the cell surface mucin barrier.

The C. jejuni CDT has nuclease activity and triggers cell-cycle arrest in vitro (30). C. jejuni mutants lacking CDT show equal gastrointestinal tract colonization but impaired invasiveness into blood, spleen, and liver in SCID mice (31) and diminished chronic enteritis in NF-kB-deficient mice (33), demonstrating the importance of this toxin in vivo, at least in immunocompromised animals. Our demonstration of greater resistance to CDT by MUC1-expressing human intestinal cells in vitro and the absence of increased gastric colonization in Muc1−/− mice by C. jejuni lacking CDT is consistent with a role for MUC1 in suppressing the activity of genotoxins in the gastrointestinal tract. MUC1 is phosphorylated in response to binding bacteria (4), interacts with growth factor receptors involved in epithelial repair (13), and inhibits the intrinsic pathway of apoptosis in malignant epithelial cells in response to genotoxic stress by (a) localizing to mitochondria, stimulating the phosphorylation of Akt and Bad, and upregulating mitochondrial Bel-X (14, 16) and (b) binding to the regulatory domain of p53 and moving in a complex to the nucleus, attenuating activation of Bax transcription (15). MUC1 binding to p53 also promotes transcription of p21, facilitating cell-cycle arrest at the G1 checkpoint (15). The rapid upregulation and enhanced cytoplasmic localization of the MUC1 cytoplasmic domain in gastrointestinal epithelial cells exposed to C. jejuni in vivo are therefore consistent with an intracellular role for this mucin. We demonstrate that, in vitro, the MUC1 extracellular domain is internalized and the cytoplasmic domain translocates to the nucleus in CDT-affected cells. We et al. estimated that,
in MUC1-transfected HCT116 cells, about 4% of the cytosolic pool of the MUC1 cytoplasmic domain is bound to p53 (15). We show colocalization of the MUC1 cytoplasmic domain and p53 and translocation of both proteins to the nucleus in CDT-treated intestinal cells. The lack of a protective effect of MUC1 in HeLa cells is likely to be due to their lack of functional p53 to partner MUC1 (31), with the lack of p53 perhaps also explaining the high level of apoptosis induced by CDT in this cell line. Translocation of the MUC1 extracellular domain to the nucleus in CDT-treated HeLa cells was unexpected and introduces a possible role for p53 in MUC1 stabilization and recycling during stress. Although the ability of MUC1 to protect from genotoxic agents, targets goblet cell thecae in vivo, and shows enhanced pathogenicity in the absence of just 1 cell surface mucin. We show that MUC1 protects cells from the actions of a bacterial genotoxin, suggesting that cell surface mucins are not simple blocking molecules on the cell surface but rather have evolved wider roles in protecting epithelial cells from xenobiotic toxins. Polymorphisms in cell surface mucin genes themselves, genes encoding and regulating the glycosyltransferases that assemble their complex carbohydrates, and genes that regulate appropriate mucin production and secretion could all conceivably predispose individuals to both acute infection and chronic inflammatory disease. The results of this study should underpin further exploration of the normal function of members of the cell surface mucin family and their contribution to epithelial diseases.

Methods

Animals. Mac1+/– mice on a 129/SvJ background (43) were maintained as a breeding colony, and wild-type 129/SvJ Mac1+/– mice were purchased from the Walter and Eliza Hall Institute (Melbourne, Victoria, Australia). Mice were sex and age matched within experiments (6–12 weeks of age unless otherwise specified), 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 nos. 734/05, 586/04, 389/03, and 200/02). Colonies routinely tested negative for murine viral and bacterial pathogens.

Bacterial culture and preparation of CDT. C. jejuni strains 81116, 331, and 81-126 were grown on solid selective agar (2% Columbia agar, 1% bacteriological agar, 5% defibrinated horse blood, Skirrow Selective Supplement; Oxoid) under microaerophilic conditions (5% O2, 15% CO2, 80% N2; BOC Gases) at 42°C for 48 hours. CDT was prepared from cultured C. jejuni 81116 as described (31) with 1 AU corresponding to the toxin prepared from murine intestinal mucus by repetitive extraction of the insoluble mucin in 0.1 M guanidinium hydrochloride (Sigma-Aldrich).
samples collected in either broth or 10% formalin. To assess the number of CFUs/g tissue, samples in broth were homogenized, serially diluted, plated onto selective agar, and grown as above.

Phagocyte depletion. Mice were depleted of macrophages by i.p. injection of 1 mg Carrageenan-λ. (IV) (Sigma-Aldrich) in 200 μl PBS, pH 7.2, 48 hours prior to oral inoculation with bacteria. Mice were depleted of neutrophils by i.p. injection of 150 μg RB68CS antibody in 200 μl PBS daily for 3 days prior to and including the day of challenge with bacteria. Control mice were injected with the same volume of PBS.

Bone marrow transplantation. Mice (6–8 weeks) were irradiated with 2 doses of 500 cGy delivered 3 hours apart in a Gammacell irradiator (Nordion) (demonstrated in preliminary experiments to be lethal in this strain of mice in the absence of bone marrow transplantation and to result in complete chimerism following transplantation). Following a 60-minute recovery period, 5 × 10⁸ bone marrow cells isolated from the femur and tibia of donor mice were injected i.v. in 200 μl sterile L15 medium. Mice were kept for 6 months to ensure full engraftment and maturation of the immune system before experimental challenge with C. jejuni.

Histological assessment and immunohistochemistry. Gastrointestinal tract specimens were fixed in 10% buffered formalin and embedded in paraffin. For analysis of histological damage, tissue sections stained with H&E were coded to blind the analysis, and the entire section was systematically examined and areas of damage recorded. Muc1 was detected in tissue sections with the CT2 cytoplasmic domain antibody (13) following antigen retrieval in citric acid using a standard biotin-streptavidin detection system and 3,3′-diaminobenzidine tetrahydrochloride as chromagen, then counterstaining with hematoxylin. The surface epithelium and glands in the stomach, small intestine, cecum, and large intestine were scored blind to treatment for the presence of Muc1 in the apical membrane and cytoplasm. The proportion of cells staining (0, no/weak staining; 1, 0–25% of cells; 2, 26–50%; 3, 51–75%; 4, 76–100%) and the intensity (0, no/weak staining; 1–3, increasing intensity of staining) were scored. Sections from infected and uninfected Muc1+/− mice were included as controls for staining specificity and scored negatively. C. jejuni was similarly detected using biotinylated NCL-C-JEJUNI anti-flagella antibody (Novocastro Laboratories) and cleaved caspase-3 with a rabbit polyclonal antibody (Calbiochem).

Antibiotic treatment. Reduction of normal flora within the gastrointestinal tract of mice was achieved by oral administration of the broad-spectrum antibiotics vancomycin hydrochloride (250 mg/ml) and imipenem-cilastatin (250 mg/ml) (Merck Sharp & Dohme) in drinking water for 7 days and antibiotic was withdrawn 24 hours prior to inoculation with C. jejuni.

Quantitation of intestinal and fecal bacteria. The entire large intestine, including luminal contents, of Muc1+/− and Muc1+/+ mice was homogenized, fixed with 3% formaldehyde, filtered onto 0.2 μm Anodisc 25-mm filters (Whatman), and stained with SYBR green (Invitrogen); the number of bacteria was counted under fluorescence microscopy using Image-Pro Plus, version 5.0.0.39 (MediaCybernetics) and expressed per gram of intestinal tissue. DNA was extracted from feces of Muc1+/− and Muc1+/+ mice and quantitative PCR used to amplify 165 ribosomal DNA from the major intestinal bacterial groups using the following primers: universal 165 DNA (45); Bacteroides-Prevotella-Porphyromonas, Clostridium coccoides and Lactobacillus groups, and Enterococcus spp (46); Clostridium leptum subgroup (47); and Desulfovibrio group (48). Bifidobacterium genus primers were as follows: forward primer, 5′-TTAGTGTCAGGCGCTACG-3′; reverse primer, 5′-ATTAC- TACGACTCCGCTTCA-3′. Results were expressed as a proportion of universal bacterial 165 ribosomal DNA primers.

Manipulation of MUC1 gene expression in vitro. HCT116 intestinal cancer cells were transfected with a MUC1 eDNA containing 22 VNTR repeats in the pcDNA3 vector (Invitrogen) or the vector alone. Stable G418-resistant clones were isolated and MUC1 expression determined by

![Figure 8](image)

**Figure 8**

ΔCDT-B C. jejuni show lower gastric colonization and lower intestinal apoptosis but equivalent penetration of the intestinal barrier in Muc1+/− mice in vivo. (A) Concentrations of C. jejuni in gastrointestinal and systemic tissues of Muc1+/− (−) and Muc1+/+ (+) 129/SvJ mice 48 hours after oral inoculation with 10⁶ C. jejuni strain 81176 (CDT +) and ΔCDT-B (CDT −). Mean ± SEM of CFUs/g tissue. Colonization frequency is shown at the base of each histogram. ANOVA, Tukey’s post-hoc test; *P < 0.05; **P < 0.01. (B) Concentrations of activated apoptosis-associated caspases 3 and 7 determined by the Caspase-Glo 3/7 luminescence assay in 2 pooled 5-mm segments of the terminal ileum of mice in the experiment described in A. A linear scale is used because individual samples showed dilution linearity. RFU, relative fluorescence units.
in intestinal extracts using a modification (49) of the Caspase-Glo CDT for 1–7 days and then harvested with trypsin and combined with any arrested cells; therefore cells were harvested and manually counted using a hemocytometer. Activity of activated caspases 3 and 7 was determined in intestinal extracts using a modification (49) of the Caspase-Glo 3/7 luminescence assay (Promega).

In vitro cocultures. HCT116 cells stably transfected with MUC1 or the pcDNA3 vector were grown in 24-well plate wells in 10% FCS in RPMI1640 for 5 days after reaching confluence and transferred to micro-aerophilic gas (5% O2, 15% CO2, 80% N2) for 2 hours at 37°C prior to the introduction of C. jejuni prepared as above. After 1 or 2 hours, monolayers were washed twice with PBS and lysed with 0.02% Triton X-100 in PBS; CFUs were determined as above. To measure invasion, after washing, monolayers were cultured in fresh medium with 400 μg/ml gentamicin for a further 2 hours at 37°C under microaerophilic conditions and then harvested and CFUs determined.

Assessment of cell-cycle arrest and apoptosis. HCT116 and HeLa cells were established as monolayer cultures in 24-well plates at densities of 2 to 5 × 104 cells/well 1–2 days prior to being exposed to a range of concentrations of CDT for 1–7 days and then harvested with trypsin and combined with any nonadherent cells. For cell-cycle analysis, cells were fixed for 24 hours in 70% ethanol, stained with 7-amino-actinomycin D (7-AAD), assessed by flow cytometry, and analyzed using the Cylchred, (version 1.0.2; http://www.cardiff.ac.uk/medicine/haematology/cytonetuk/documents/software.htm). For assessment of apoptosis, harvested cells were stained with annexin V-PE (BD) and 7-AAD and analyzed by flow cytometry for cells in early (annexin V negative, 7-AAD positive) and late (annexin V and 7-AAD positive) apoptosis. Cell-cycle dynamics in CDT-affected HCT116 cells could not be assessed by flow cytometry due to the distension and clumping of arrested cells; therefore cells were harvested and manually counted using a hemocytometer. Activity of activated caspases 3 and 7 was determined in intestinal extracts using a modification (49) of the Caspase-Glo 3/7 luminescence assay (Promega).
etic mice with Campylobacter jejuni: colonization of intestine and spread to lymphoid and reticulo-
29. Field, L.H., Underwood, J.L., and Berry, L.J. 1984. The role of gut flora and animal passage in the col-
30. Lara-Tejero, M., and Galan, J.E. 2000. A bacterial toxin that controls cell cycle progression as a deoxy-
protein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell.
63:1129–1136.
33. Fox, J.G., et al. 2004. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type Campylobacter jejuni but not with C. jejuni lack-
ing cytotoxul distending toxin despite persistent colonization with both strains. Infect. Immun.
72:1116–1125.
Genet. 9:548–552.
37. Silva, F., et al. 2003. MUC1 polymorphism confers increased risk for intestinal metaplasia in a Colombi-
45. Rupf, S., Merte, K., and Eicherh, K. 1999. Quantifi-
tion of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J. Appl. Microbiol.
97:1166–1177.
47. Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T., and Tanaka, R. 2004. Use of 16S rRNA gene-

matory transcription factors is required for mas-
sive liver apoptosis induced by bacterial lipopoly-