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Understanding and Modulating Mammalian-Microbial Communication for Improved Human Health

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Abstract

The fact that the bacteria in the human gastrointestinal (GI) tract play a symbiotic role was noted as early as 1885, well before we began to manage microbial infections using antibiotics. However, even with the first antimicrobial compounds used in humans, the sulfa drugs, microbes were recognized to be critically involved in the biotransformation of these therapeutics. Thus, the roles played by the microbiota in physiology and in the management of human health have long been appreciated. Detailed examinations of GI symbiotic bacteria that started in the early 2000s and the first phases of the Human Microbiome Project that were completed in 2012 have ushered in an exciting period of granularity with respect to the ecology, genetics, and chemistry of the mammalian-microbial axes of communication. Here we review aspects of the biochemical pathways at play between commensal GI bacteria and several mammalian systems, including both local-epithelia and nonlocal responses including inflammation, immunology, metabolism, and neurobiology. Finally, we discuss how the microbial biotransformation of therapeutic compounds, such as anticancer or nonsteroidal anti-inflammatory drugs, can be modulated to reduce toxicity and potentially improve therapeutic efficacy.

Keywords

microbiome; drug discovery; β -glucuronidase; irinotecan; NSAIDs

INTESTINAL MICROBIOTA AND HUMAN HEALTH

The interactions between human intestinal cells and symbiotic microbial cells are likely the most diverse that occur in humans because of the sheer number of bacterial cells (10^{14}) and their variability in terms of location, time, environment, and genetics in the gastrointestinal (GI) tract. Furthermore, microbial structural components such as lipopolysaccharide (LPS) in gram-negative bacteria and the products of the microbiome—including peptides, proteins, small molecules, and xenobiotics—vary in time and location in the GI tract (Figure 1).

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Additionally, the overall interactions among bacteria, viruses, and fungi effect changes in the microbial species, in the metabolome, in the cell wall products, and in host signaling at and beyond the epithelial barrier.

In the late nineteenth century, the fact that the human GI tract is filled with bacteria that are in a symbiotic relationship with mammalian physiology was already becoming appreciated (1). A 1937 article published in *The Lancet* described the role bacteria play in creating the sulfanilamide metabolite of sulfa drugs, indicating that in response to therapeutic intervention, human tissues do not act alone (2). By the 1940s, sulfa drugs were being combined with other compounds to treat inflammatory conditions thought to be caused by the intestinal microbiota (3, 4). In the early 1970s, it was suggested that the commensal GI bacteria collectively be considered an organ, as they exhibit metabolic power akin to the human liver (5). Thus, there has long been a growing appreciation for the participation and potential harnessing of what we now call the microbiome in the treatment of human disease. This understanding was greatly advanced upon detailed examinations of GI symbiotic bacteria that started in the early 2000s and upon the first phases of the Human Microbiome Project that were completed in 2012 (6–8). However, as with any large new data set, placing this information in context will be a challenge that must be met over the next several years. Here we review aspects of our current and quickly evolving understanding of the microbiota and the microbiome encoded by it, how the microbiota and microbiome interact with the host GI epithelium, and how they impact human systemic physiology in key ways.

MICROBIOTA-PRODUCED FACTORS THAT INFLUENCE GASTROINTESTINAL HEALTH

Proteins and Peptides

Numerous proteins are secreted by commensal GI bacteria into the intestinal lumen, and some undergo extensive modification either in vivo or ex vivo. Before delving into specific proteins that contribute to GI health, we lay out the foundations for protein-secretion systems in bacterial-host associations (9). Bacteria contain at least seven well-defined secretion systems, termed types I to VII (Figure 2). Gram-negative bacteria contain both inner and outer membranes that enclose the periplasmic space; gram-positive bacteria, in contrast, replace the outer membrane with a thick peptidoglycan layer that encloses the periplasm between it and the inner (and only) membrane. In gram-negative bacteria, the commonly used single-step secretion pathways include the type I, III, IV, and VI systems that move macromolecules across both membranes and outside the cell. Other proteins are exported into the periplasmic space between the outer and inner bacterial membranes and secreted via the universal Sec or two-arginine (Tat) pathways. (A pathway involves transiently interacting proteins, whereas a system typically involves more stable complexes of factors.) They then are translocated through the outer membrane via the type II or type V system, or, less commonly, the type I or type IV system. In gram-positive bacteria, the secretion of proteins across a single membrane is commonly performed via the Sec or Tat pathway. In gram-positive bacteria with an impermeable cell wall (mycomembrane), a specialized type VII secretion system translocates proteins across membranes. A better understanding of these pathways will be required to understand interactions and GI homeostasis.

The beneficial effects of intestinal commensals are many: They prevent intestinal enteropathogens from invading the intestinal wall, create nutrients (10), regulate intestinal angiogenesis (11), lead to maturation and development of gut-associated lymphoid tissues (12), promote oral tolerance (13), enhance mucosal immunity (14), and assist in diversifying preimmune antibody repertoire (15). The immunogenicity of certain bacteria in the gut may

not be as important as the products secreted by specific microbes. For example, stress responses in *Bacteroides fragilis* controlled by the transcription factor Spo0A, as well as sporulation and secretion of the protein YqxM, are required for the development of gut-associated lymphoid tissues in rabbits (16). S-layer proteins, which are surface-layer self-associating proteins that enclose a cell in a two-dimensional array, from *Lactobacillus crispatus* are able to directly interact with collagen molecules on the surfaces of epithelial cells (17). This interaction could be one mechanism at play in preventing enteropathogens (e.g., *Escherichia coli* O157:H7) from seeding and invading the GI lumen.

Bacterial flagellins recognize Toll-like receptor 5 (TLR5) and ICE protease-activating factor (18, 19), each of which promotes intestinal innate immunity under normal conditions (i.e., non-disease states). Glycolipids from bacteria recognize receptors on intestinal cells (e.g., TLR2) and are deemed important in regulating barrier function and integrity (20, 21). C-type lectin receptors on dendritic cells and macrophages recognize bacterially secreted extracellular glycoproteins, and these interactions are thought to be important for tolerance (22). The serine protease inhibitor (serpin) from *Bifidobacterium longum* subsp. *longum* NCC2705 directly interacts with host factors and inhibits pancreatic and neutrophil elastases; thus, serpins have anti-inflammatory effects in vivo (23). *Bifidobacterium animalis* subsp. *lactis* BB-12 produces a pentapeptide in a stationary phase of growth. This pentapeptide regulates both interleukin-6 (IL-6) and c-myc in human promyelocytic leukemia cells (HL-60 cells) and binds to and activates the orphan nuclear receptor ROR- γ (24). Thus, its role in intestinal physiology (inflammation and cancer pathways) is salient to the chemical interaction between bacterial and mammalian cells but remains to be more clearly defined. Numerous secreted proteins from probiotic strains known to be natural human commensals have been characterized; a large majority of them are unidentified, but their interactions with the host are becoming well defined (reviewed in 25). In spite of this important progress, further characterizing the bacterial secretome is an unmet need.

Chemical Metabolites from the Microbiota

The chemical repertoire in the human gut is expansive, and, given that variations in diet are accompanied by colony count within both time and space, investigating the effects of bacterial metabolites on the host is compelling. Chemical factors produced by the microbiota can be divided broadly into intermicrobial (those that act between different types of microbes) and intramicrobial (those that act between similar microbes) factors, as well as those necessary for host-microbe interactions (26). Factors involved in iron exchange with the host and with iron metabolism include siderophores such as enterobactin (produced by all strains), aerobactin (gut commensals), yersiniabactin (uropathogenic *Yersinia*), and salmochelin (uropathogenic strains) (27). The exact role of these siderophores in invasion and pathogenicity has not been fully characterized. Similarly, there have been reports of siderophore-peptide associations; however, their biological role remains elusive (28).

Microbial aging is a well-recognized phenomenon in mammals that directly relates to microbial metabolic diversity (29, 30). The types of metabolites seen include short-chain fatty acids (e.g., butyrate, associated with the *Firmicutes*) known to alter the energy status of colonic epithelial cells; bile acids (e.g., taurocholates, *Lactobacillus*) known to facilitate enteric absorption of nutrients; choline metabolites (e.g., methylamine, *Bifidobacterium*) known to modulate lipid and glucose metabolism; phenolic and phenyl derivatives (e.g., hippuric acid, *Clostridia*) that aid in xenobiotic metabolism; indoles (e.g., indole 3-propionate, *Clostridia*) known to protect against enteric stress; and less well-defined metabolites that appear to have significant effects on host metabolism (reviewed in 30). Metabolic exchange factors, including those involved in quorum sensing (a bacterial process used to control cell population density), are also important in maintaining microbial

communities and host homeostasis. Acyl homoserine lactones mediate interand intraspecies bacterial interactions and are necessary for colonization of the cattle rumen by enterohemorrhagic *Escherichia coli* (31). Autoinducer 3 is a bacterial chemical exchange factor that activates virulence genes in the microbiome by an uncharacterized mechanism (32).

Xenobiotics

That the gut microbiota metabolizes xenobiotics is clear (e.g., irinotecan) (33), but appreciating how xenobiotics shape the physiome of the gut microbiota itself is perhaps more significant (34). Specifically, particularly important xenobiotics such as antibiotics damage cell wall components and lead to significant changes in the composition of commensal bacteria. Furthermore, xenobiotics serve as substrates for metabolic enzymes involved in the biotransformation of a parent drug to its (either active or inactive) metabolites (34). In addition, the gut microbiota likely alters host drug metabolism either through metabolite expression or through the direct action of microbial products on hepatic drug-metabolizing enzymes (35–38). For example, germ-free mice exhibit significant upregulation of host cytochrome P450 (Cyp) enzymes such as Cyp3a11 and Cyp2c29, as well as the host xenobiotic sensors PXR (pregnane X receptor) and CAR (constitutive androstane receptor); taken together, these data support a core role for gut commensals in host xenobiotic detoxification pathways (29).

An example of the importance of the enteric microbiome in the host drug response is seen with the cholesterol-lowering drug simvastatin (39). Secondary bile acids and the microbial metabolite of cholesterol, coprostanol, are predictors of the efficacy of simvastatin, suggesting that microbial metabolism can play an important role in determining the efficacy of the drug in the host (40). Similarly, microbial enzymes such as sugar-scavenging β -glucuronidases directly impact the toxicity of chemotherapy drugs such as irinotecan, as discussed below (33). Cysteine-conjugated drugs are heavily influenced by the presence of commensal bacteria that produce a conjugating β -lyase (41, 42). The bioavailability of phytoestrogens, plant components resembling 17β -estradiol that are implicated in cancer growth and/or prevention, is largely dependent on gut microbial metabolism. For example, equol and 8-PN are bacterial metabolites of daidzein (soy) and isoxanthohumol (hop), respectively. These metabolites have higher biological activity than do their precursors, but they display large interindividual variability (43, 44).

Anthranoids are obtained from the dried leaflets and pods of plants such as senna plants, *Cascara sagrada*, *Frangulae cortex* and rhubarb. They are present mostly as sugar derivatives, and owing to this β -glycosidic linkage, they are carried unabsorbed into the large intestine where microbial metabolism starts and the active aglycone anthrone is released (45). Anthranoids have potent laxative-like effects in the intestine, and excess use causes melanosis coli, which is controversially associated with the potential for colon carcinogenesis (46). A more dramatic cautionary tale related to drug discovery and the microbiota is the metabolic interaction between the new antiviral drug orivudine and the established chemotherapeutic 5-fluorouracil (5-FU), which resulted in the death of 18 patients in the 1990s (47). The microbial selective metabolic conversion of orivudine to (*E*)-5-(2-bromovinyl)uracil interfered with the metabolic clearance of 5-FU.

In 1937, the sulfa-type antibiotics prontosil and neoprontosil were shown to be converted to sulfanilamide (2), their active metabolite, and by 1941, the reductions performed by the microbiota were being harnessed to develop new compounds that joined antibacterials with the anti-inflammatory salicylic acid (3, 4). A greater appreciation of the chemical role of the intestinal bacteria began in 1972 with the demonstration that the azo bond in sulfasalazine, a drug used in ulcerative colitis, is cleaved by microbial enzymes (48). Since then, it has been

clearly shown that bond reductions are catalyzed by intestinal bacteria for therapeutic drugs including nitrazepam, clonazepam, misonidazole, omeprazole, sulfinpyrazone, sulindac, digoxin, and zonisamide (49). Other chemical transformations (and some of the drugs impacted) subsequently carried out by microbial symbionts include hydrolysis (sorivudine), dehydroxylation (levodopa), acetylation (5-aminosalicylic acid), deacetylation (phenacetin), N-oxide bond cleavage (ranitidine), proteolysis (insulin), denitration (glyceryl trinitrate), amine formation (chloramphenicol), and deconjugation of glucuronides and sulfates (estrogens, indomethacin) (49). As early as 1967, it was suggested that the most serious clinical complication of chloramphenicol, bone marrow aplasia, was caused by a metabolite of this drug that was produced by the intestinal bacterial symbionts (50).

Specific enzymes and pathways in gut commensals are responsible for the metabolic biotransformation of many drugs, including omeprazole, digoxin, lactulose, and sorivudine, as well as carcinogens such as 2-amino-3-methylimidazo(4,5-f)quinolone (49). The role of commensals in controlling antibiotic resistance is also emerging as a significant area for therapeutic intervention (40). There is increasing understanding that microbial products can shape both intestinal and liver carcinogenesis as well as pathogenic infections (51–53). A recent example is the demonstration that the gut commensal *Bacteroides thetaiotaomicron* which expresses fucosidase enzymes, contributes to enteropathogenic *Escherichia coli* (EPEC) virulence by cleaving fucose from mucin, thereby activating the *FusKR* signaling cascade to modulate virulence gene expression in enterohemorrhagic *Escherichia coli* (54).

MAMMALIAN MECHANISMS FOR RECOGNITION OF MICROBIOTA-PRODUCED FACTORS

Given the intimate relationship between the microbiota and the tissues of the mammalian GI tract, it is not surprising that several mechanisms potentiate communication between bacterial and mammalian cells. Lipoteichoic acid (LTA), a cell wall component in gram-positive bacteria, binds to extracellular gelsolin proteins from mammalian cells, mediating host immune recognition of bacterial LTA (55). LPS from gram-negative bacteria efficiently binds TLR4, which aids in mediating host innate immune recognition (56–59).

Polysaccharide A produced by *Bacteroides fragilis* promotes immune tolerance via TLR2 signaling in Foxp3⁺ Treg cells (60, 61). More recently it has been demonstrated that flagellin, the primary protein component of bacterial flagella, directly interacts with its receptor, TLR5; this interaction may lead to allergic asthma. The knockdown of TLR5 can promote gut inflammation through the inability to manage proteobacteria signaling (62, 63).

The enteric bacterial protein Tir (translocated intimin receptor) shares sequence similarity with the host cellular immunoreceptor tyrosine-based inhibition motifs (ITIMs). Tir from EPEC interacts with the host cellular tyrosine phosphatase SHP-1 (Src homology region 2 domain-containing phosphatase-1) in an ITIM-phosphorylation-dependent manner. The association of Tir with SHP-1 facilitates the recruitment of SHP-1 to the adaptor TRAF6 [tumor necrosis factor (TNF) receptor associated factor 6] and inhibits the ubiquitination of TRAF6. ITIMs of Tir suppress EPEC-stimulated expression of proinflammatory cytokines and inhibit intestinal immunity to infection with *Citrobacter rodentium*. These findings demonstrate a new pathway for bacterial ITIM-containing proteins to inhibit innate immune responses (64).

Several ligand-regulated transcription factors, including peroxisome proliferator-activated receptor γ (PPAR γ), aryl hydrocarbon receptor (AhR), vitamin D receptor (VDR), liver X receptor (LXR), and farnesoid X receptor (FXR), have been implicated as either the direct or indirect targets of microbial sensing (65–70). The clinical relevance of these findings is

demonstrated by the fact that dietary constituents of cruciferous vegetables, for example, appear to regulate innate immunity. The mechanism involves the activation of AhR by metabolites that are extracted and metabolized by the microbiota or provided to the host by digestion (71). Furthermore, the production of methyl or acetyl groups from bacteria could affect the host one-carbon pathway, and these small chemical groups appear to be directly involved in chromatin modifications (72). Whereas these mechanisms are at play with the host epithelial cell layers, complementary mechanisms of immune recognition also involve antigen sampling by mucosal immune cells. These include dendritic cell (DC) extension to the luminal surface, B cell-mediated immunoglobulin A production, and epithelial regulation of DC function and tolerance in the lamina propria. DCs that express CD103⁺, an α E β 7 integrin, have recently been shown to play a key role in regulating oral tolerance through the induction of regulatory Foxp3⁺ T cells that express gut-homing receptors in the mesenteric lymph node (73).

HARNESSING MICROBIOTA FOR GASTROINTESTINAL HEALTH

In recent years, an example of the successful intestinal targeting of microbiota for the purposes of therapy involved fecal replacement (displacement) of *Clostridium difficile* infection using fecal admixtures from uninfected individuals and rodents (74, 75). The proposed mechanisms involve competition and the establishment of a bacterially diverse community that displaces *C. difficile* along with an increase in both *Bacteroidetes* and *Clostridium* clusters IV and XIVa and a decrease in certain *Proteobacteria* species. This idea has now been explored or proposed for other conditions, such as inflammatory bowel disease (76). *Faecalibacterium prausnitzii* a member of the normal human microbiota, has been associated with extension of the period of remission in patients with Crohn's disease (77). Similarly, the microbiota of the spore-forming clostridial clusters IV and XIVa have been associated with the capacity to induce colonic Foxp3⁺ T cells in mice (78–80). In a few cases, the effector molecules of specific microbiota members have been identified, the most prominent example being polysaccharide A, which is produced by human commensal *Bacteroides fragilis* and induces IL-10 expression from regulatory Foxp3⁺ T cells (81).

Together, these data support the possible use of probiotics as cocktail therapies for a variety of conditions. Probiotic use has shown clinical efficacy in the prevention of antibiotic-associated diarrhea, the prevention of severe necrotizing enterocolitis in preterm infants, symptom alleviation in irritable bowel syndrome, and a reduction in respiratory tract infections (82). Several modes of action by which probiotics could contribute to human health have been suggested: (a) antagonism of pathogens by competition; (b) stimulation of the production of bacteriocins and other antimicrobial factors, thereby limiting numbers of mucosa-associated bacteria and preventing bacterial penetration of host tissues; (c) modulation of epithelial barrier integrity, often via strengthening tight junctions; (d) epithelial signaling induced by interaction of probiotics or probiotic effector molecules that modulate immune cell functions via production of cytokines and other stromal factors; (e) generation of tolerizing DCs and regulatory CD4⁺Foxp3⁺ T cells; and (f) induction of IL-10-producing Tr1 (Type 1 regulatory T) cells (83, 84).

No single probiotic supplement drives all of the aforementioned effects. Hence, there is need for the rational selection of specific probiotic strains, each matched with a precise clinical indication. On the positive side, safety concerns with probiotics are likely minimal, although there must be caution regarding certain microbial metabolites (e.g., indoles) that undergo host metabolism (sulfation, in this case) that could be detrimental for those with poor kidney function; such patients might experience increased levels of circulating toxic indoxyl sulfate (35). Clinical evidence, however, suggests that probiotic cocktails may reduce the circulation of some uremic toxins (85).

Genetically engineered bacteria constitute another approach for therapeutic targeting of the GI microbiota in disease (86). For example, lactic acid bacteria (LAB) engineered to overexpress elafin, a natural protease inhibitor expressed in healthy intestinal mucosa that has pleiotropic anti-inflammatory properties in vitro and in animal models, substantially reduce colitis in mice (87). Similar approaches have been used to engineer LAB that lack cell wall components (e.g., LTA) that can aggravate inflammation (88) and polyposis (89). A second example is the use of low-virulence strains of *Helicobacter pylori* that lack the Pdx cluster and thus reduce induction of intestinal inflammation (90). Similarly, LAB strains engineered to express high levels of B-group vitamins could be employed to overcome certain types of vitamin deficiency (91). Although these approaches rely on known probiotics with a history of safety, potential issues still remain. Indeed, some LAB could produce amines that are harmful to humans (92), and some probiotics have the potential to worsen protein malnutrition (93, 94). It would appear essential that the genetic and chemical factors associated with potential probiotic therapies are carefully characterized during their development toward clinical applications. Still, there is great potential application of cell-based therapies for a range of human conditions.

ALLEVIATING IRINOTECAN-INDUCED ENTEROPATHY

Because the toxicity of irinotecan (also called CPT-11)—delayed and dose-limiting diarrhea—is reasonably well understood, we examine it in less detail than we examine the GI toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs) that only recently are being appreciated for producing severe lower GI enteropathy (see below). Chemotherapy-induced diarrhea (CID) is a common and serious side effect that leads to dehydration, electrolyte imbalance, renal insufficiency, immune dysfunction, and sometimes death (95–97). Some chemotherapeutic regimens are associated with 50–80% incidence of diarrhea, with more than 30% involving interference in activities of daily living, hospitalization, or cardiovascular compromise (96–98). The cost of hospitalization following CID was > \$10,000 per patient in 2004, which is 50–70% higher than the cost of hospitalization for other common chemotherapy-induced toxicities, including febrile neutropenia, cardiac toxicity, and stomatitis (99, 100). Several cancer therapies are known to cause GI toxicity (96, 97, 101, 102), although the mechanisms underlying CID are complex, often incompletely understood, and typically agent specific (102, 103).

Irinotecan is relatively unique among anticancer drugs because its GI toxicity has been linked to a specific mechanism of action. Irinotecan is associated with the highest rates of diarrhea (~80%) and severe diarrhea (~15%) among chemotherapeutics (100, 102, 104, 105). Because single-agent irinotecan is so toxic, the drug is typically employed in combination therapies to treat a range of solid tumors. Still, diarrhea is its primary dose-limiting side effect; as such, irinotecan is frequently prescribed below its recommended dose to prevent CID, and therefore its therapeutic benefit is lower than its potential (96, 102). Irinotecan-induced GI toxicity is caused by bacterial β -glucuronidase enzymes expressed in commensal microflora in the lower GI tract (106–108).

Typical CID remedies include chemotherapy dose reductions, delays in treatment, and discontinuation of treatment, all of which can compromise chemotherapy outcomes (96, 103, 109). Reduced chemotherapeutic dose intensity has been associated with decreased overall and disease-free survival (109). Approximately 65% of patients with CID require a lower dose (i.e., fewer mg/kg) at some point in their chemotherapy courses, 45% require fewer doses (i.e., less often), and 71% experience a temporary or permanent cessation of treatment (96). Pharmacologic treatments for CID include loperamide as well as some off-label use of octreotide and tincture of opium, all of which offer limited benefit in mild diarrhea and minimal benefit in severe CID (101, 110–114). More complicated cases require

vigilant medical monitoring, aggressive pharmacologic treatment, intravenous fluids in outpatient settings, and even hospitalization (101). The mean hospital stay to treat severe CID is eight days (99, 100). Antibiotic-based approaches have been proposed and evaluated because of the role that commensal bacteria play in irinotecan-induced diarrhea (111, 115–118). The success of these nonspecific strategies is limited as they may promote pathogenic bacterial infection and impede gut immune homeostasis. Furthermore, the literature contains conflicting reports on the impact that antibiotics have on irinotecan toxicity; most show some alleviation (111, 116–118), whereas one study did not find a statistical difference (115). Alternative strategies to develop analogs or reformulations of SN-38, the active metabolite of irinotecan, have been in clinical development for nearly a decade, but their efficacy in comparison with irinotecan is unproven (119–123).

The mechanism of action for irinotecan-induced GI toxicity is well characterized. Intravenous irinotecan is converted by carboxylesterases into the active, cytotoxic form, SN-38, which kills tumor cells (124, 125). SN-38 undergoes extensive phase II glucuronidation and is inactivated to SN-38G by liver UDP-glucuronosyltransferases (107, 126, 127). SN-38G is excreted via the biliary ducts into the GI lumen and serves as a substrate for the microbiotic bacterial β -glucuronidase enzyme that removes the glucuronide group to reactivate SN-38, which is toxic to the GI tract. Bacterial β -glucuronidase inhibitors were hypothesized to prevent reactivation of SN-38 and reduce or eliminate severe diarrhea (108, 125). This hypothesis was tested through the identification of numerous potent bacterial β -glucuronidase inhibitors, one of which, when administered orally, protected mice from irinotecan-induced GI toxicity (33) (Figure 3). This was the first study that demonstrated the ability to selectively and nonlethally inhibit a component of the microbiome to effect a particular potential clinical outcome. As explained in more detail below, this approach has proven successful for a second class of therapeutics, NSAIDs, and related methods might be applied to drugs or endogenous compounds involved in glucuronide-based metabolic recycling.

PATHOGENESIS AND CLINICAL SIGNIFICANCE OF NONSTEROIDAL ANTI-INFLAMMATORY DRUG ENTEROPATHY

NSAIDs are among the most extensively used drugs worldwide, and some have been employed for decades. It has long been recognized that GI injury is a major adverse effect of NSAID therapy. Whereas most of the focus to date has been on gastropathy, which is easier to monitor, injury to the small intestine (enteropathy) may also occur frequently, as revealed by novel diagnostic techniques including video capsule endoscopy or balloon enteroscopy. The injury presents as erosions and bleeding of the mucosa, ulceration, and, in more severe cases, even perforations. The clinical impact of NSAID enteropathy has been recently reviewed (128, 129). Surprisingly, several analyses have demonstrated that approximately 70% of all chronic recipients of NSAIDs develop injury to the distal small intestine (130, 131). Despite this apparently high incidence, there is currently no effective and safe treatment that protects the small bowel from NSAID-associated enteropathy. One of the major reasons for the lack of specific therapies is our limited understanding of the mechanisms underlying the damage.

There is no question that the pathogenesis of NSAID enteropathy is distinct from that in the stomach; therefore, different therapeutic approaches are required (132). Although earlier studies on enteropathy mechanisms focused on the role of prostaglandin synthesis inhibition (the mechanism of action of NSAIDs), more recent studies have clearly demonstrated that numerous off-target effects are equally important contributors to the enteropathy. A recently established multiple-hit concept includes a description of the complex mode of action of NSAID enteropathy (133). Briefly, the first determinant is the delivery of NSAID

metabolites to the small intestinal lumen via hepatobiliary excretion. Because most NSAIDs have a carboxylic acid moiety, the parent compound or oxidative metabolite(s) produced in the liver are conjugated to glucuronic acid and excreted into the bile as acyl (ester) glucuronides via the conjugate export pump (Mrp2). In addition, phenol (ether) glucuronides can be formed from ring-hydroxylated NSAID metabolites and excreted through the same mechanisms (134). In the small intestine, these glucuronoconjugates can be enzymatically cleaved, releasing the aglycone(s) that are taken up at the brush-border membranes of the enterocytes.

The parent NSAID, or its metabolites, can be further metabolized by the gut epithelia to potentially reactive intermediates. These intermediates can covalently bind to cellular protein targets, can induce increased oxidant stress, or can damage numerous intracellular organelles, including the endoplasmic reticulum (ER) and mitochondria. ER and mitochondrial stress can damage cells and lead to an energy crisis or even cell death. This initial injury to the tight epithelial junction leads to an increase in mucosal permeability. As a consequence, luminal bacteria can reach deeper layers of the lamina propria and interact with TLRs, which activate proinflammatory cytokines and cause inflammation. The infiltrating neutrophils not only produce an oxidative burst and release proteases but also can further metabolize NSAIDs by, for example, activity of myeloperoxidase [the major oxidizing enzyme in polymorphonuclear neutrophils (PMNs)]. Desmethyldeschloroben-zoylindomethacin, the major metabolite of indomethacin, is further oxidized by myeloperoxidase to a reactive iminoquinone (135). During this multistep pathogenesis of NSAID-induced mucosal injury, bacteria clearly play a distinct role.

BACTERIAL CELLS AND NONSTEROIDAL ANTI-INFLAMMATORY DRUG ENTEROPATHY

General Aspects of the Microbiota and Enteropathy

It has been known for decades that the presence or absence of specific intestinal bacteria can greatly impact the toxic response in the GI tract to NSAIDs. For example, abiotic-treated (no intestinal bacteria), gnotobiotic-treated (defined intestinal bacteria), and antibiotic-treated rats were highly resistant to the ulcerogenic effects of certain NSAIDs including indomethacin (136–138). Reinoculation of mice with specific bacteria restored the sensitivity to the ulcerogenic effects of NSAIDs (137). Both the abundance and the specific type of bacteria can influence responses to NSAIDs. For example, oral administration of *E. coli* to mice and subsequent treatment with indomethacin greatly aggravated the extent of ulceration in the small intestine compared with *E. coli*-deficient vehicle controls receiving indomethacin (M. Bopst & U.A. Boelsterli, unpublished data) (Figure 4). In contrast, NSAIDs were unable to induce ulcers in gnotobiotic rats monoassociated with *Lactobacillus acidophilus* or *Bifidobacterium adolescentis* (138). Taken together, these data indicate that intestinal bacteria are an important determinant in the development of NSAID-induced enteropathy.

Several reasons could explain the causal role that bacteria play in modulating the extent of small intestinal damage (Figure 5). First, bacteria have been implicated in releasing LPS and other cell wall components that could specifically bind to and activate TLRs, entailing an inflammatory response. Second, bacteria are endowed with numerous enzymes that could participate in the metabolic activation or detoxication of NSAIDs. There is evidence that both pathways play a key role. Importantly, as outlined below, administration of NSAIDs can alter the relative composition of commensal microbiota, causing dysbiosis (an imbalance in the microbiota), which can greatly influence the ulcerogenic role of bacteria in the small intestine.

NSAID-Induced Dysbiosis

NSAID-induced enteropathy is not only dose dependent but also greatly influenced by the relative composition of the intestinal microbiome (Figure 5). For example, inoculation with *E. coli* or *Eubacterium* readily induced ulcers in gnotobiotic rats treated with DUP-697 [5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl) thiophene (BFMeT), a candidate nonacidic NSAID in development], whereas inoculation with *Bifidobacterium* or *Lactobacillus* did not, despite identical serum concentrations of the NSAID in all treatment groups (138). These and other findings imply that shifts in the composition of the microbiota may alter the response to NSAIDs and increase or decrease the risk for NSAID-associated ulceration. These modulations in the relative composition and count of bacteria may be brought about by a range of factors (e.g., stress), including the administration of drugs (138, 139). In fact, NSAIDs themselves can alter the composition of the microbiota; for example, diclofenac or BFMeT administration caused an increase in the number of gram-negative bacteria (including *E. coli*) in rats, while at the same time decreasing the abundance of gram-positive bacteria (including *Bifidobacterium adolescentis* and *Lactobacillus acidophilus*) (138, 140). Although the phenomenon of unbalanced overgrowth with specific bacteria after NSAID administration has been recognized for several decades, the exact reasons for this dysbiosis are not known. One possible explanation could be the documented antibacterial activity of certain NSAIDs, such as diclofenac (141); however, whether this activity could be directly responsible for the shift in the composition of the microflora is not known.

Furthermore, drugs given concomitantly with NSAIDs may further alter the composition of the microbiota. For example, the proton pump inhibitors omeprazole or lansoprazole, administered together with naproxen, caused significant dysbiosis compared with an NSAID administered alone. The mechanisms are not fully understood, but in this case the changes in gastric pH over an extended period of time could be a plausible explanation (142).

BACTERIAL MOLECULES AND NONSTEROIDAL ANTI-INFLAMMATORY DRUG ENTEROPATHY: LIPOPOLYSACCHARIDE

Bacterial LPS (endotoxin), produced by gram-negative bacteria (e.g., *E. coli*), has been implicated in activating a potent inflammatory response in the small intestine, which further aggravates the ulcerative injury. LPS binds to TLR4 in the lamina propria of the mucosa, activating a signaling pathway that leads to proinflammatory cytokine production and massive infiltration of PMNs (143) (Figure 5). A major proinflammatory cytokine that increases via TLR4 signaling following indomethacin treatment is tumor necrosis factor- α (TNF- α) (144). The causal involvement of TNF- α in the development of enteropathy has been convincingly demonstrated with anti-TNF- α antibodies or TNF- α -null mice, as both types of mice are protected against indomethacin-induced injury (145, 146). Similarly, intraperitoneal administration of antineutrophil antibody attenuates indomethacin-induced enteropathy (147), providing evidence for a causal role of PMNs in the pathogenesis of NSAID enteropathy.

The following factors likely explain why the normal presence of gram-negative bacteria in the gut does not spontaneously lead to massive and uncontrolled inflammation. First, TLR4 is expressed neither at the brush borders nor at the microvilli surfaces of enterocytes; instead, it is expressed at the basolateral surfaces. Therefore, direct contact between the ligand and TLR4 is minimized. LPS also downregulates TLR4 because of the overwhelming presence of LPS in the intestinal lumen. Second, RegIII γ (regenerating islet-derived protein 3 gamma) peptide, which is secreted by specialized cells and which has anti-gram-positive bacterial properties, keeps bacteria at a safe distance of approximately 50 μ m; similar mechanisms likely exist for gram-negative bacteria (148). Third, intestinal alkaline

phosphatase can dephosphorylate (and thus inactivate) the lipid A moiety of LPS. Intestinal alkaline phosphatase is expressed on the apical surfaces of enterocytes and secreted into the lumen (149), thus protecting the intestinal barrier.

BACTERIAL ENZYMES AND NONSTEROIDAL ANTI-INFLAMMATORY DRUG ENTEROPATHY: β -GLUCURONIDASE

Bacteria directly influence NSAID-induced enteropathy via microbial enzyme-catalyzed hydrolysis of NSAID glucuronides in the gut. Certain commensal bacteria express the inducible *gus* gene, which encodes for β -glucuronidase (Figure 3). The composition of the microbiome greatly determines the overall β -glucuronidase activity; for example, *E. coli* has much higher (approximately 30-fold) β -glucuronidase activity against the standard substrate, *p*-nitrophenyl- β -D-glucuronide, than do other bacterial operational taxonomic units (OTUs) (150, 151). Furthermore, there are region-selective differences in the activity of β -glucuronidase along the small intestine; the activity is significantly higher in the distal part of the small intestine relative to the proximal part (152, 153). β -Glucuronidase can release the parent NSAID or oxidative metabolites from its/their respective glucuronide conjugates. Consequently, the aglycone(s) are taken up by the enterocytes, leading to increases in the oral systemic bioavailability of the aglycone (154). At the same time, the aglycones can also be further metabolized by intestinal cytochrome P450s, and these metabolites (or their precursors) may induce ER and mitochondrial stress, as outlined above.

TARGETING GASTROINTESTINAL BACTERIA TO ALLEVIATE NONSTEROIDAL ANTI-INFLAMMATORY DRUG ENTEROPATHY

Because there is a clear mechanistic basis for a causal role of bacteria in the pathogenesis of NSAID enteropathy, the specific targeting of one or several mechanisms offers a promising new approach to prevent small intestinal injury in experimental models and ultimately in patients treated with NSAIDs. Three major approaches have been used: first, to target the composition of the microbiota, thus preventing or reversing dysbiosis; second, to antagonize the LPS-TLR axis, thus attenuating the proinflammatory response; and third, to inhibit bacterial β -glucuronidase, thus limiting the exposure of the enterocytes to the free aglycones released from the glucuronides. The first and third approaches are discussed below.

Antibiotics, Probiotics

It has been suggested that antibiotic prophylaxis with poorly absorbable antibiotics should be reevaluated as a pharmacologic intervention to protect against NSAID enteropathy (155). Although certainly effective, this approach will likely not be pursued clinically because intestinal bacteria are needed for normal health and metabolism. However, administration of probiotics can prevent colonization by more pathogenic bacteria and thus protect against NSAID enteropathy (156, 157). More data, especially with regard to specific strains, dose responses, and treatment duration, would help make this approach clinically routine.

Selective Nonlethal β -Glucuronidase Inhibition and NSAID Enteropathy

A promising approach is the use of bacteria-specific inhibitors to protect against NSAID-induced enteropathy. Such inhibitors must be selective such that they do not impact the mammalian β -glucuronidase, which is essential for proper lysosomal storage. In contrast, microbial β -glucuronidases appear to be nonessential and are present to scavenge for glucuronide sugar carbon sources (33). For example, the selective, nonlethal β -glucuronidase inhibitor termed Inhibitor-1 (Inh-1) was able to alleviate the extent of small intestinal injury caused by an ulcerogenic dose of diclofenac in mice (158). Not only were

the ulcers reduced in size and number, but the biochemical indicators of enteropathy (i.e., serum alkaline phosphatase activity and serum fluorescein isothiocyanate–dextran concentrations after oral administration of the fluorescent permeability marker) were also reduced in the Inh-1-pretreated mice receiving diclofenac. Importantly, the β -glucuronidase inhibitor did not interfere with the hepatobiliary excretion of diclofenac glucuronides, suggesting that the inhibitory effect occurred more distally, likely in the small intestine (K. Saitta & U.A. Boelsterli, unpublished data). We have recently found that the enteric-protective effects of the β -glucuronidase inhibitor Inh-1 were not diclofenac specific; they could also be achieved for other NSAIDs, including indomethacin (Figure 4) or ketoprofen (K. Saitta & U.A. Boelsterli, unpublished data). Each NSAID tested featured a carboxylic acid moiety that would be expected to be glucuronidated, leading to excretion via the hepatobiliary route and enabling the glucuronides to undergo processing by β -glucuronidases in the GI tract and during enterohepatic recirculation. Taken together, these data suggest that NSAID toxicity, and perhaps efficacy, could be improved through the use of compounds such as Inh-1.

CONCLUSION

Considerable recent progress has been made in understanding the interactions that exist among components of the microbiota, and between the microbiota and human tissues in the GI tract. Furthermore, emerging evidence suggests that interkingdom chemistry in the GI tract can be modulated for therapeutic gain (33, 158). “Interkingdom” in this context refers to the processes performed by mammalian eukaryotes in symbiosis with commensal bacteria. Deeper elucidation of the manner in which features of microbial ecology work together to advance the collective survival of the bacteria and to impact mammalian systemic physiology will have great value. Additionally, the ability to intervene in acute or long-term modes of action using diet, pharmacology, and/or designed probiotic species appears to be a potentially rewarding goal now in sight.

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Figure 1.

A general schematic illustrating microbial factors that influence the epithelial barrier in the intestine. Each of these factors varies by bacterial colony counts, spatial distribution, and time of sampling. The mammalian GI epithelial layer is rendered in orange with villi at the bottom, and schematics of the cell surfaces of Gm+ and Gm- bacteria arising from a range of differently shaped forms of microbiota are presented below. The microbiota encode the microbiome, which generate proteins, peptides, metabolites, and altered xenobiotics that impact the types of communication indicated. Abbreviations: GI, gastrointestinal; Gm+, gram-positive bacteria; Gm-, gram-negative bacteria.

Figure 2.

This schematic illustrates the basics of several types of secretion pathways in bacteria, and they are outlined to indicate the level of complexity involved in bacterial-human cell communication considering only the prokaryotic side of the equation. Abbreviations: ABC, ATP-binding cassette; ATP, adenosine triphosphate; Clp B, a chaperone ATPase; GI, gastrointestinal; IM, inner membrane; MFP, membrane fusion protein; MM, mycomembrane; OM, outer membrane; OMP, outer membrane protein; VirB, a DNA transfer protein first identified in *Agrobacterium tumefaciens*; Y-E-G, a complex of transfer proteins associated with type II systems.

Figure 3.

The microbiota plays direct roles in the drug-induced gastrointestinal (GI) toxicities of the anticancer drug irinotecan (33) and nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac (158) and indomethacin.

Figure 4.

Colonization of the gastrointestinal tract with *E. coli* aggravates indomethacin-induced enteropathy in mice. Male C57BL/6 $\times$ 129/SvEv mice that tested negative for *E. coli* before the beginning of the study were inoculated by oral gavage with 10^{11} cells of *E. coli*. Positive colonization was confirmed after 2 weeks. Indomethacin (85 mg/kg/day, sc, for 2 days) was administered 2 weeks after successful colonization in the test (*E. coli* colonized) group, and the small intestines of the test and control groups were analyzed for pathological lesions 24 h post indomethacin discontinuation or control. Data are mean $\pm$ SD ($n = 5$ mice per group). Asterisk indicates $P < 0.05$ (M. Bopst & U.A. Boelsterli, unpublished data).

Figure 5.

Multiple relationships between bacteria and NSAIDs in drug-induced enteropathy. **1** NSAIDs can lead to unregulated overgrowth of the small intestine with bacteria and also change the relative composition of commensal OTUs, thus inducing dysbiosis. **2** Certain OTUs differentially express β -glucuronidase, which can catalyze the hydrolytic cleavage of NSAID glucuronides in the lumen, enabling uptake into enterocytes of the aglycones (parent NSAID and/or oxidative metabolites of NSAID). These compounds can be further metabolized in the epithelial cells to reactive intermediates, and/or they can induce ER or mitochondrial stress, which leads to cell injury and increased permeability of the epithelia. **3** Gram-negative bacteria release LPS; if LPS escapes inactivation by intestinal enzymes including alkaline phosphatase, and if the intestinal permeability is increased by NSAIDs, LPS can reach deeper layers of the mucosa and bind to TLR4 (other bacteria can release other cell wall components that act as ligands for other TLRs). TLR signaling results in

activation of proinflammatory cytokines and recruitment of PMNs. In addition to producing pro-oxidants and proteases, PMNs may also be involved in enzymatic activation of NSAID metabolites to reactive intermediates. Abbreviations: ER, endoplasmic reticulum; GI, gastrointestinal; LPS, lipopolysaccharide; MPO, myeloperoxidase; NSAID, nonsteroidal anti-inflammatory drug; PMN, polymorphonuclear neutrophil; TLR, Toll-like receptor; OTU, operational taxonomic unit.