Microbes and Microbial Toxins: Paradigms for Microbial-Mucosal Interactions
I. Pathophysiological aspects of enteric infections with the lumen-dwelling protozoan pathogen *Giardia lamblia*

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*Eckmann, Lars, and Frances D. Gillin.* Microbes and Microbial Toxins: Paradigms for Microbial-Mucosal Interactions. I. Pathophysiological aspects of enteric infections with the lumen-dwelling protozoan pathogen *Giardia lamblia.* Am J Physiol Gastrointest Liver Physiol 280: G1–G6, 2001.—*Giardia lamblia* is one of the most important causes of waterborne diarrheal disease worldwide, and giardiasis is the most common protozoan infection of the human small intestine. Symptomatic infection is characterized by diarrhea, abdominal pain, and malabsorption, leading to malnutrition and weight loss, particularly in children. The pathogen resides strictly in the lumen of the small intestine, and infection is typically not accompanied by significant mucosal inflammation. Clinical and experimental studies indicate that B cell-dependent host defenses, particularly IgA, are important for controlling and clearing *Giardia* infection, although B cell-independent mechanisms also contribute to this outcome. In contrast to antigiardial host defenses, much less is known about the pathophysiological mechanisms underlying the clinical symptoms of giardiasis, partly because of the current lack of suitable model systems. In addition to being an important human enteric pathogen, *Giardia* is an interesting model organism for gaining basic insights into genetic innovations that led to evolution of eukaryotic cells, since it belongs to the earliest diverging eukaryotic lineage known. The completion of the giardial genome project will increase understanding of the basic biology of the protozoan and will help us to better understand host-pathogen interactions as a basis for developing new vaccination and therapeutic strategies.

GIARDIASIS IS A MAJOR GLOBAL cause of waterborne diarrheal disease, which contributes greatly to the burden of malnutrition and malabsorption leading to childhood mortality, particularly in developing countries (1). *Giardia lamblia* (also known as *G. intestinalis* or *G. duodenalis*) is also of clinical relevance in the USA, since millions of hikers and children in daycare are at risk of infection. Food-borne transmission can also occur, although it is less common than waterborne transmission. This protozoan has the distinction of first being identified by van Leeuwenhoek in his own diarrheic stool. Despite its long history and medical importance, neither the basic biology nor the pathophysiology of *G. lamblia* infection is well understood. Although trophozoites are not invasive and do not secrete any known toxin or cause a consistent inflammatory response, they are capable of causing severe and protracted diarrhea. Villus blunting may be observed in some cases. On the other hand, about half of infected people are asymptomatic and the infection frequently resolves spontaneously. Thus both the duration and symptoms of giardiasis are highly variable in immunocompetent people, some of whom are presumably infected with the same strain (1, 17). It is likely that both immune and nonimmune host factors influence the outcome of infection (10). Since no giardial virulence factor is known, the parasite's capacity to respond to and survive in diverse, hostile environments may be central to pathophysiology. The ability of both prokaryotic and eukaryotic pathogens to monitor and react to environmental signals is crucial for virulence. *G. lamblia* is one of the few microbes that colonize the normal human small intestine, and it may provide clues to host barrier functions and how the parasite evades them.

This themes article is not intended to be comprehensive; space constraints do not allow a full discussion of controversial topics in the field or acknowledgment of all relevant publications. Instead, this article highlights interesting developments in the field and presents a focused view of some promising directions for further research. For more complete information on the immunology and molecular and cell biology of *Gi-
ardia, the reader is referred to several reviews (1, 10, 12, 29).

GIARDIAL LIFE CYCLE

Giardia undergoes two pathogenetically important types of differentiation: encystation is required for survival outside the host, and excystation is required for infection. Giardia takes advantage of host conditions throughout its descent through the human gastrointestinal tract (12). The dormant, quadrinucleate cyst persists for months in cold fresh water, and ingestion of as few as 10 cysts can initiate infection. Exposure of ingested cysts to gastric acid during passage through the host stomach triggers excystation. After entry into the small intestine and stimulation by intestinal pH, bicarbonate, and protease, the parasite emerges and divides into two equivalent binucleate flagellated trophozoites. The trophozoites colonize the human small intestine below the entrance of the common bile duct, where they attach to enterocytes and mucus or swim in the intestinal fluid. Giardia synthesizes few of its cellular building blocks and must gain most nutrients from the host (1, 2, 29). Because of the maturation and sloughing of enterocytes and the downward flow of intestinal contents, trophozoites must remain motile to stay in the small intestine. If they are carried downstream, they must encyst to survive outside the host. Exposure to signals from the small intestine (slightly elevated pH, bile) leads to encystation (12). To understand giardial survival in the host and environment, it is important to define the cellular mechanisms that enable this parasite to survive in the lumen of the host small intestine and secrete the cyst wall that protects it from the external environment and from gastric acid upon infection of a new host. Other intestinal parasites also have a cyst or oocyst form that survives outside the host and causes infection, but only the giardial life cycle has been completed in vitro. This makes it a valuable model for differentiation of other enteric pathogens, such as Entamoeba and Cryptosporidium.

BIOLGICAL IMPLICATIONS OF THE GIARDIAL GENOME ANALYSIS

Genome analyses of a range of bacterial pathogens have been highly informative because they provide a blueprint from which to analyze the basic biological functions of pathogens as well as their ability to interact with the host under various physiological conditions. In addition to its medical importance, G. lamblia provides an interesting and important model organism for genome analysis because of its relatively small genome (~12 million base pairs on 5 chromosomes coding for an estimated 5,000 genes) and because it belongs to one of the earliest diverging eukaryotic lineages. The evolutionary distance between Giardia and yeast is at least as great as that separating yeast and humans. Giardia lacks mitochondria and peroxisomes and has some prokaryotic characteristics, particularly in pathways important for energy generation (7, 29). Therefore, it is a valuable model for gaining basic insights into genetic innovations that led to evolution of eukaryotic cells. Random sequencing has led to more than threefold coverage of the genome as of June 2000 (16). Sequence assembly and gene mapping and annotation are in progress. Updated genome information can be accessed on the website of the Giardia genome project database at www.mbl.edu and through the National Center for Biotechnology Information at www.ncbi.nlm.nih.gov.

Results of Giardia genome analysis will be helpful in addressing important questions related to human health. For example, identification of key differences in the metabolic pathways of Giardia and the host may lead to novel targets for therapy. The success of the 5-nitroimidazoles (Flagyl or metronidazole) in treating giardial infections is due to the unusual anaerobic energy metabolism of the parasite, involving pyruvate and the enzyme pyruvate-ferredoxin oxidoreductase (PFOR), a bacterial type of metabolic pathway not found in the human host (7, 29). However, metronidazole can have significant side effects, and the drug is only ~85–95% effective. Although giardial drug resistance is a minor clinical problem at this time, the emergence of 5-nitroimidazole-resistant trichomonads and of multiple drug resistance in other human pathogens (e.g., Mycobacterium tuberculosis) once thought to be safely controllable with antimicrobial drugs serves as a warning that the current state of affairs with Giardia may not remain stable indefinitely. Therefore, identification of novel pharmacological targets in Giardia through a combination of genomic and biochemical analysis will be important for enlarging the repertoire of future treatment options.

Genomic analysis is also likely to aid in understanding the pathophysiological consequences of infection, particularly diarrhea. Through homology searches, it may be possible to identify potential toxins that cause increased fluid secretion by the intestinal epithelium. For example, cloning and sequencing of a variable surface protein (VSP) gene, CRP136, which is expressed in a laboratory-selected strain of metronidazole-resistant G. lamblia, has revealed a region that shares ~57% homology with a group of snake toxins, sarafotoxins, that cause intestinal symptoms similar to those observed after Giardia infection (28). Although no functional studies have been published on this gene product, these data nonetheless indicate the potential of finding candidate toxins through systematic genomic analysis. Biochemical and physiological approaches using the most relevant model systems currently available in vitro (e.g., Caco-2 epithelial monolayers) and in vivo (e.g., ligated intestinal loops) will allow evaluation of the potential relevance of candidate toxin genes. Such studies would be complemented, ultimately, by determining the biological consequences of interfering with the expression of the candidate gene from Giardia. In this regard, techniques to interfere with gene expression have become available recently for Giardia. For instance, inhibition of PFOR in G. lamblia by a virus-mediated hammerhead ribozyme has shown that
this enzyme enhances anaerobic growth of *Giardia* but also promotes susceptibility toward metronidazole (7).

Since *Giardia* undergoes antigenic variation, genomic analysis will elucidate the number and organization of VSP genes and aid in understanding their regulation and function. Trophozoites are covered with a dense layer of VSPs consisting of a single VSP species (11). Although VSPs are highly variable, certain sequence features are conserved, suggesting as yet unknown structural and functional importance. All VSPs are type I integral membrane proteins that are variable, except for a highly conserved COOH terminal membrane-spanning region and five-amino-acid cysteine-rich region (18). VSPs are unusually cysteine rich (>11%) throughout the protein. Most of the cysteine residues are in a CXXC tetrapeptide motif (11). X can be any amino acid, although there is a bias toward hydrophilic residues, which may affect the reactivity of disulfide bonds. The prevalence of CXXC motifs is absolutely characteristic of these proteins; however, their function remains unknown (3). VSPs switch spontaneously and at high frequency (10^3 to 10^4 per generation) in vitro (18). The molecular mechanism of switching is not known but does not appear to require DNA rearrangements (26). In addition, since *Giardia* is binucleate and probably polyploid (2), the parasites must have a sophisticated mechanism to ensure that only a single VSP gene is expressed. Expression of different VSPs can be selected by physiological conditions because they differ in sensitivity to proteases. Exposure to antibodies in vitro or in vivo also selects against the corresponding VSP and allows trophozoites expressing different VSPs to take over a giardial population (17). In addition, encystation and excystation lead to randomization of VSPs. After completion of the life cycle of a giardial culture expressing a major VSP, the excysting cells express a variety of VSPs. This may be a novel form of immune evasion that can help to explain the high rate of reinfection in endemic areas (26). The giardial genome is estimated to contain at least 150 VSP genes (2, 18). In addition, the VSP repertoire of genetically distant isolates may differ greatly (18). Thus, although VSPs are immunogenic in human and animal infections, they are not likely to be good vaccine candidates. Genomic and biological analyses have also facilitated the identification of several invariant surface proteins. These are more plausible vaccine candidates than VSPs, although they are less abundant on the cell surface than VSPs.

**PATHOPHYSIOLOGICAL ASPECTS OF GIARDIA INFECTION**

Two of the most important clinical symptoms of giardiasis are diarrhea and malabsorption (1). The mechanisms underlying these host responses are only poorly understood, but a number of abnormalities have been reported after *Giardia* infection, including changes in sodium uptake and digestive enzyme activities. One of the best-established and most studied pathological changes in experimental giardiasis is inhibition of the activities of several digestive enzymes, including sucrase and maltase, during the acute phase of the infection. A correlation exists between the infectious load and the extent of enzyme inhibition, which suggests that the effects might be caused by factors released by live parasites. However, similar changes in digestive enzyme activities can be evoked in immunized mice that are challenged with giardial antigens, even in the absence of live trophozoites, implicating a function of the host immune system in these events. A recent study provides support for the latter interpretation (21). Immunocompetent mice infected with *Giardia* showed a diffuse loss of brush border microvillus surface area, which correlated with a significant reduction in maltase and sucrase activities at the peak of the infection, whereas T cell-deficient nude mice had no change in microvillus surface area despite a similar parasitic load (21). The host factors and mechanisms mediating the T cell effects on microvillus structure in giardiasis are not known at present but are likely to involve one or more cytokines other than interleukin (IL)-6 (21).

Giardiasis can be accompanied by severe diarrhea, yet relatively little is known about the underlying mechanisms. In *G. lamblia*-infected gerbils, glucose-stimulated absorption of sodium and water is decreased compared with uninfected controls, whereas basal absorption is not different between infected and control animals (5). *G. lamblia* infection of gerbils also accelerates gastrointestinal transit and smooth muscle contractility, which could play a role in the pathogenesis of giardial diarrhea (8). In contrast to gerbils, infection of mice with *G. lamblia* has been reported to cause net secretion of sodium and chloride in the basal state, whereas control animals showed net absorption of these ions under the same conditions (13). The reasons for these different results are not clear but may relate to differences in giardial strains or species-specific host responses. Gerbils, for example, develop diarrheal symptoms after giardial infection, but mice do not.

**HOST DEFENSES AGAINST GIARDIA**

Clinical and experimental data show that the infected host develops adaptive immune responses against *Giardia*, since most patients infected with *Giardia* clear the infection spontaneously, even in the absence of treatment (17). Furthermore, persons living in areas where giardiasis is endemic have fewer episodes of *Giardia* infections and reduced severity of infections relative to people coming into endemic areas from places with little giardiasis (10). Experimental studies also show that effective immune defenses exist against *Giardia*, since mice and cats infected with *Giardia* spontaneously clear the infection and are resistant to secondary challenge with the pathogen (10). Acquired immunity against *Giardia* is not complete, and reinfections are common, although it is not clear whether this is due to incomplete immune defenses or
variations in the infecting pathogen. In any case, in the absence of adequate host defenses, *Giardia* infections are more common and severe. For example, children under 6 years of age, who have an immature immune system, are more susceptible to *Giardia* infection but not to infection with another enteric pathogen, Entamoeba histolytica, which has a similar transmission route. Adults with primary immunodeficiencies, particularly common variable immunodeficiency (CVID) and, to a lesser degree, selective IgA deficiency, also have an increased incidence of *Giardia* infections (1, 10, 15).

A full understanding of the crucial host defenses against *Giardia* is important for developing vaccination strategies to prevent infection and for improved therapeutic approaches in case of treatment failure with current therapies. In addition, *Giardia* serves as an instructive model system for characterizing host strategies important for controlling luminal microorganisms in the intestine. This may have implications for understanding the pathophysiology of infections with other luminal pathogens (e.g., nematodes) as well as the role of luminal commensals in human health and disease. A consideration of antigiardial host defenses needs to take into account two key features of the infection. First, *Giardia* reside strictly in the lumen of the intestine and do not invade the mucosa. This requires that an effective host defense must be active in the intestinal lumen. Second, *Giardia* infection is typically characterized by little or no mucosal inflammation (19). A massive influx of effector cells into the mucosa and/or intestinal lumen is probably not important for clearing infection, which suggests that most of the cells required for an effective defense against *Giardia* are likely to be present in the normal mucosa. Furthermore, host defenses that are usually induced during an inflammatory response, e.g., those induced by proinflammatory cytokines such as tumor necrosis factor-α or IL-1, are less likely to be important for clearing *Giardia* infection relative to infections normally accompanied by inflammation (e.g., *Salmonella* infection).

An increased incidence of giardiasis is observed in patients with CVID, an immunodeficiency characterized by T and B cell defects causing impaired production of most immunoglobulin isotypes, and those with selective IgA deficiency (15). CVID patients are more susceptible than IgA-deficient patients to *Giardia* infection. Some studies even have suggested that IgA-deficient patients are not at increased risk for giardiasis (15), although IgA deficiency is a heterogeneous condition that is only rarely characterized by a complete absence of IgA antibodies. In addition to patients with primary immunodeficiencies, an increased incidence of *Giardia* infections has been reported for patients with acquired immunodeficiency due to HIV infection, although infections with other pathogens, especially Cryptosporidium, are often clinically more important in these patients. Together, the clinical data indicate that B cells, particularly IgA, and CD4 T cells are important for controlling *Giardia* infection in humans. Further support for this conclusion comes from experimental *Giardia* infections in mice, in which in vivo ablation of CD4 T cells and B cells leads to chronic giardiasis (14, 24, 25). We have recently found that IgA knockout mice, which have a complete absence of IgA antibodies, cannot clear infection, although they control infection better than B cell knockout mice. These data indicate that antigiardial IgA is important for controlling and eliminating *Giardia* infection, although other immunoglobulin isotypes also play a role in this outcome and may be able to compensate, at least partially, for absent IgA. The mechanism(s) by which antigiardial IgA can control infection are not clear, but antigiardial antibodies of other isotypes have been shown to be cytotoxic to *Giardia* and to interfere with giardial differentiation (10).

Although B cell-dependent defenses are especially important for eradicating chronic *Giardia* infection, B cell-independent mechanisms also play a role in controlling acute infection (22). In addition, nonimmune host factors may influence the duration or severity of giardiasis, since giardial infections are variable even in immunocompetent hosts. Several components of the normal small intestinal milieu have been shown to affect giardial viability or differentiation in vitro. For example, bile salts are potent detergents that inhibit the growth of many microbes in the small intestine. Yet bile salts strongly stimulate giardial growth and encystation. Similarly, the intestinal mucus layer is thought to protect the epithelium, but trophozoites avidly attach to mucus strands. On the other hand, trophozoites are killed by products of lipolysis in normal intestinal fluid, such as unsaturated free fatty acids and lysophospholipids. *Giardia* are also killed by normal human breast milk in vitro but not by cow or goat milk. This is due to release of unsaturated free fatty acids from milk triglycerides by a specific lipase in human milk but not cow or goat milk. However, *Giardia* are protected from killing by milk through association with intestinal mucus and by bile salts above their critical micellar concentration and by the slightly alkaline intestinal pH. Antibacterial cationic peptides that protect mucosal surfaces, such as the NH₂ terminal peptide lactoferricin, from milk lactoferrin and intestinal defensins produced by crypt Paneth cells, also kill trophozoites in vitro (4).

Many intracellular pathogens are known to be killed by nitric oxide (NO), although the role of NO in controlling infections with extracellular pathogens is less well established. Polarized intestinal epithelial cells produce NO and release it, or its products, preferentially on the apical side, suggesting that this mediator might affect nearby trophozoites. In vitro, NO donors inhibit giardial growth but do not kill trophozoites (9). In addition, NO inhibits both encystation and excystation of *Giardia* and thus could interfere with parasite transmission. Interestingly, *Giardia* has evolved a strategy to disarm this potent defense. Trophozoites actively metabolize arginine, depriving enterocytes of the substrate for NO production (9). Enterocyte NO production in the presence of trophozoites can be restored by supplementation with excess arginine. Thus
arginine consumption and NO production define a novel cross-talk between a noninvasive pathogen and the host intestinal epithelium. Together, these in vitro studies suggest that nonimmune host defenses and the parasite’s ability to evade them may help determine the outcome of giardial infection. The in vivo role of these nonimmune defenses in controlling Giardia infection will be an important topic for future studies.

INTESTINAL MICROBIOTA AND GIARDIA INFECTION

The intestinal tract is colonized by commensal bacteria (called, in its entirety, the intestinal microbiota), which may play a role in the pathogenesis of Giardia infections. The composition and numbers of commensal bacteria vary greatly from segment to segment in the intestine, with increasing numbers from oral to caudal. The intestinal microbiota has physiological functions in the host (e.g., assistance in carbohydrate metabolism), although germ-free animals are generally healthy and reproduce normally. Perhaps more importantly, the intestinal microbiota plays a role in the pathogenesis of enteric infections and inflammation. This function can be beneficial for the host, as indicated by the observation that germ-free animals are more susceptible to infections with pathogenic bacteria than littermates with conventional microbiota. For example, the oral LD<sub>50</sub> for Salmonella infection is <10<sup>3</sup> in germ-free mice compared with 5 × 10<sup>6</sup> in conventional mice, whereas the LD<sub>50</sub> for intraperitoneal infection is comparable between the two groups (6). This function of the microbiota, often referred to as “colonization resistance,” is likely to be related to fierce microbial competition for space and nutrients in the intestinal lumen, particularly in the colon, although effects on the development and activation status of the mucosal immune system may also play a role. On the other hand, the intestinal microbiota can also have detrimental effects on the host. In the context of immune dysregulation, for example, the microbiota, or specific components thereof, contributes to the development and persistence of intestinal inflammation. This is illustrated by the finding that mice in which the gene for the anti-inflammatory cytokine IL-10 is genetically disrupted develop severe colitis if they harbor a conventional flora but not if they are kept in a germfree state (20). The exact mechanisms underlying this effect are not known, but microbial interactions with the intestinal epithelium leading to production of proinflammatory signals by the epithelium may be involved.

Recent studies suggest that the intestinal microbiota also play a role in determining severity and symptoms of Giardia infections. In one study, mice from one commercial vendor were found to be more resistant to infection with Giardia than isogenic mice from a different vendor, and this resistance could be transferred by housing animals together (23). Treatment of mice with the nonabsorbable antibiotic neomycin made both groups of mice equally susceptible to infection. Together, these data show that the intestinal microbiota is important for determining resistance to Giardia infection and raise the possibility that probiotic therapy may be useful in preventing infection or as an adjunct for treating infection (23). These data may also suggest an explanation for differences in the results of different groups of investigators. In another study, germ-free mice were found to have a reduced influx of mononuclear cells into the mucosa after G. lamblia infection compared with mice with a conventional microbiota, although fecal cyst output was comparable between the groups (27). Although more detailed studies are required to fully characterize the function of the intestinal microbiota in the pathogenesis of Giardia infections, on the basis of these studies one can speculate that the intestinal microbiota has two, in some ways opposing, functions for the host in regard to Giardia infection. It protects against infection, while at the same time, if infection occurs nonetheless, it may be responsible for some of the pathophysiological consequences of the infection for the host.

OUTLOOK

With the approaching completion of the giardial genome project, research in giardial biology will enter the postgenomic era of characterizing the functions of a multitude of giardial genes and their roles in the pathogenesis of the infection. Although this offers a plethora of choices for detailed studies, we highlight here briefly several areas of research we believe to be central to the understanding of giardial biology. First, assessment of gene expression profiles in Giardia during proliferation and differentiation in vitro, and in response to various physiological conditions in vivo, will be important for understanding the ability of Giardia to adapt to distinct niches in the gastrointestinal environment. Such studies need to be paralleled by characterization of mechanisms of gene regulation in Giardia. Second, VSPs are the most abundant surface proteins of Giardia, yet their function remains unknown. It will be important to conduct structure/function studies for this group of proteins and assess the functional consequences of interfering with their expression. Third, although substantial progress has been made in defining the role of specific host immune defenses in controlling and clearing Giardia infections, little is known about the role of nonimmune defenses in determining the outcome of infection. Several potential defense mechanisms (e.g., defensins, lactoferrin, and NO) have been characterized in vitro, and their roles should be tested in mouse models. Fourth, despite the successful use of mouse models for characterizing host immune defenses, few good in vitro or in vivo models are available to date to define the mechanisms underlying Giardia-induced diarrhea and malabsorption. Development of better model systems will promote functional and structural investigations into this important area of Giardia research. Together, these studies are likely to provide important new insights into the pathogenesis of infection with this clinically impor-
tant enteric pathogen and point to new directions for prevention and treatment.

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