Epithelial attachment alters the outcome of Helicobacter pylori infection

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ABSTRACT Genetically defined in vivo models are needed to assess the importance of target cell attachment in bacterial pathogenesis. Gastric colonization by Helicobacter pylori in human populations is common and persistent, and has various outcomes including peptic ulcers and cancer. The impact of attachment on the course of infection was examined in transgenic mice expressing a human receptor for H. pylori in their gastric epithelium. Persistent infection by a clinical isolate occurred at comparable microbial densities in transgenic mice and surface mucous cells shared by bacteria and acid-secreting parietal cells. Chronic gastritis, and parietal cell loss. This model should help identify bacterial and host genes that produce attachment-related pathology.

Helicobacter pylori colonizes the stomachs of at least half of all humans, surviving largely within the gastric mucus without attaching to host cells (1). The natural history of colonization involves an initial period of rapid bacterial growth associated with gastritis and reduced acid production. In most hosts, the inflammatory response diminishes within several months, leaving an asymptomatic diffuse gastritis with normal acid production that persists for years (2, 3). A subpopulation of colonized individuals develops peptic ulcers (4). In some hosts, inflammation progresses over 3–4 decades, leading to atrophic gastritis that can evolve to adenocarcinoma. H. pylori is estimated to contribute to 30–90% of gastric carcinomas and is a risk factor for mucosal lymphomas (5, 6).

The microbial and host factors that determine the outcome of colonization have been difficult to define, in part because both H. pylori and humans are genetically diverse. One determining factor may be the ability of the microbe to establish physical contact with the gastric epithelium, thereby affecting its ability to influence mucosal epithelial and immune cell populations (7). A number of molecules have been implicated as receptors for H. pylori adhesins (8). Transgenic mice expressing one such receptor, the human blood group antigen Lewisb (Leb), have been used to define the consequences of attachment of clinical isolates to the epithelium. The mouse model is based on the following. In human stomachs, H. pylori is associated with mucus-producing pit cells and their more mature surface mucous cell descendants (9). In vitro binding assays show that attachment to these cells can be mediated by epithelial receptors containing Leb (10). The FVB/N inbred strain of mice expresses lacto-N-fucopentaose I-like oligosaccharides (Fucα1,2Galβ1,3GlcNAcβ) in their pit cell lineage, but not Leb [Fucα1,2Galβ1,3(Fucα1,4)GlcNAcβ] (11). Lacto-N-fucopentaose I functions as an acceptor molecule for GDP-B-L-fucose in the synthesis of Leb by the human Lewis enzyme, which is an α1,3/4 fucosyltransferase. Production of Leb glycoproteins was successfully engineered in the pit and surface mucous cells of FVB/N transgenic mice using a fatty acid binding protein gene promoter linked to the α1,3/4 fucosyltransferase. Leb-positive pit/surface mucous cells were distributed throughout the glandular epithelium in 1 day to 2 year old transgenic animals. Leb was absent in all other gastric cell lineages (11). As described below, we have used this model to show that interactions between a bacterial adhesin and a host receptor can alter disease outcome in an animal without affecting colonization levels, thereby demonstrating that the mode of colonization can be crucial for pathogenesis.

MATERIALS AND METHODS

Clinical Isolates. Gastric biopsies were obtained from adult Peruvians who had clinical and endoscopic evidence of gastritis. The biopsies were used to confirm that they were Leb-positive (11) and to isolate H. pylori. Macerated tissue samples were streaked on selective medium (Brain Heart Infusion agar containing 10% calf blood/6 μg/ml vancomycin/5 μg/ml trimethoprim/8 μg/ml amphotericin B). Single colonies were picked after a 3–5 day incubation at 37°C in CampyPakPlus envelopes (Becton Dickinson) and recultured. Isolates were identified as H. pylori by their morphology and their production of urease and catalase (12).

Animals. Transgenic mice were generated and genotyped as described (11). They were maintained, together with their normal littermates, in a specified pathogen free state and given Pico Lab Rodent Diet 20 (Purina Mills). Gastric epithelial patterns of Lewis epitope production were defined by using a panel of mAbs and immunohistochemical protocols detailed in earlier publication (11, 13).

In Vitro Bacterial Binding Assays. Formalin-fixed normal human stomachs were obtained from the surgical pathology files of Barnes Hospital (St. Louis, MO). Stomachs from transgenic mice and their normal littermates were fixed in 10% formalin/PBS or frozen and fixed in methanol. Bacteria were cultured in selective medium at 37°C, harvested 1, 3, and 6 days later, labeled with fluorescein isothiocyanate, and applied to tissue sections according to ref 11. Parallel experiments used unlabeled bacteria that were detected, after incubation with the sections, using rabbit antibodies to H. pylori surface proteins (Dako). Blocking controls were performed by pre-
incubating labeled or unlabeled bacteria with purified Lewis-human serum albumin (HSA) neoglycoconjugates (11, 13). Neoglycoconjugates were obtained from IsoSep (Tullinge, Sweden) and typically contained 20–30 oligosaccharides covalently linked to each molecule of HSA.

**Infections.** Clinical isolates were plated on selective medium and incubated for 3 days at 37°C. 10⁵ colony-forming units (cfu) (A₆₀₀ = 0.1) of each isolate (n = 8) were mixed together in 200 μl tryptic soy broth. Organisms were introduced into stomachs by gavage after mice had been placed in metabolic cages for 4 h to allow emptying of their gastric contents. Four hours to 16 weeks later, animals were placed in metabolic cages for 4 h and killed. One half of each stomach was used for histochemical analysis. The other half was homogenized in 0.5 ml tryptic soy broth and serial dilutions of the suspension were plated on selective medium supplemented with polymyxin B (3.3 μg/ml), bacitracin (200 μg/ml), and nalidixic acid (10 μg/ml). Following a 3-day incubation, cfu were defined. Survival rates were the same between transgenic and normal mice after 8 and 16 weeks of infection (88–92%).

**PCR-Based DNA Fingerprinting.** Fingerprinting was performed (14) by using genomic DNA prepared from three individual colonies, and from a full plate of colonies (≈10⁵ cfu), cultured from each stomach. Results were compared with the PCR fingerprints of each input isolate.

**ELISA Assays.** Protocols described in ref. 15 were used to define lipopolysaccharide-associated Lewis epitopes in *H. pylori* isolates and to measure circulating antibodies to Lewis epitopes in mouse sera.

**Analysis of Host Immune Responses.** Stomachs were sectioned parallel to their cephalocaudal axis (≥100 serial 5 μm sections per stomach). Hematoxylin and eosin stained sections were scored for inflammatory changes by three observers in a single blinded fashion. Frozen gastric sections were stained with rat or hamster monoclonal antibodies to mouse CD4, CD8, αβT cell receptor, γδT cell receptor, and CD45R/B220 (PharMingen). Antigen-antibody complexes were detected with tyramide signal amplification (16).

Immune cells were recovered from gastric mucosa pooled from nine transgenic and six normal mice and analyzed by fluorescence-activated cell sorter. Spleens were removed from the infected mice, pooled from each group, and single cell suspensions prepared. Bacteria were cultured in selective medium for 3 days, heat-inactivated, and 10⁶–10⁷ organisms were incubated with 5 × 10⁶ splenocytes/ml in a T cell proliferation assay (17). To detect parietal cell autoantibodies, sera from infected normal and transgenic mice were obtained at the time of death, diluted 1:50 in PBS/blocking buffer (11) and applied to formalin-fixed sections of normal uninfected FVB/N stomachs. Bound antibodies were detected with Cy3-conjugated sheep anti-mouse Ig. Control experiments established that the secondary antibody did not react with any gastric epithelial lineage. Blocking controls were performed by incubating an aliquot of each serum (100 μl of a 1:50 dilution in PBS/blocking buffer) with Le-HSA neoglycoconjugates (10 μg), or with 10⁷ Hp1 cells, overnight at 4°C prior to application to sections.

**Statistical Analysis.** The statistical significance of observed differences between transgenic and nontransgenic mice were analyzed by Student’s t test.

**RESULTS**

**Le³-Dependent Binding of Clinical Isolates of *H. pylori* to Human Pit and Surface Mucous Cells in Vitro Is Reproduced in Transgenic Mice.** Isolates of *H. pylori* were obtained from patients with gastritis living in Lima, Peru. *H. pylori* is endemic in this region and the Le³ phenotype is predominant in Peruvians (18). Therefore, these patients should harbor bacteria that have undergone in vivo selection (15) for their ability to bind Le³. *H. pylori* isolates were recovered from gastric biopsies obtained from 11 patients who expressed Le³ in their pit and surface mucous cells. Vacuolating cytotoxin (VacA) induces acidic vacuoles in epithelial cells and is a virulence factor (19). Cytotoxin-associated protein (CagA) is encoded by a gene located at the end of a pathogenic island (cag) and is considered to be a marker of virulence although its own role is unknown (19, 20). PCR studies revealed that all 11 isolates were vacA⁺ (toxinogenic type MI allele) and cagA⁺.

Each of the 11 isolates was cultured for 1, 3, and 6 days, and then applied to fresh or formalin-fixed sections of transgenic and normal mouse stomachs (n = 10 animals/group) and formalin-fixed sections of 10 normal Le³-positive human stomachs. All isolates bound to both human and transgenic mouse stomachs. Binding was limited to Le³-positive pit and surface mucous cells and was blocked by pre-treatment of the isolates with Le³-HSA neoglycoconjugate but not by Le³-HSA, Le³-HSA, Le³-HSA, or HSA alone (Fig. 1 A–C). Binding was not affected by bacterial growth phase, or by the presence or absence of flagella. None of the isolates bound to nontransgenic Le³-negative stomachs.

**A Comparable High-Density Infection Is Established in Normal and Transgenic Mice but with Sustainable Differences in Bacterial Distribution.** Eight of the isolates were pooled (10⁷ cfu per isolate) and administered in a single gavage to 3–4-month-old transgenic and normal mice. Animals were killed 4 h, 1 day, and 1, 4, 8, or 16 weeks later (9–11 mice/group/time point/experiment; 2–3 experiments/time point). Gastric contents were cultured on selective medium. Four hours after gavage, the number of viable organisms was 7 orders of magnitude less than in the inoculum. There was a marked increase in the number of cfu recovered from the stomachs of mice killed over the course of the next 4 weeks. There was no statistically significant difference in the percentage of transgenic and normal animals infected 8 or 16 weeks after inoculation (89 vs. 80%, respectively, at 8 weeks; 75 vs. 73% at 16 weeks), nor was there a significant difference in the total number of viable organisms in their stomachs (Fig. 1D).

In contrast, there was a distinct difference in the distribution of the organisms: after 8 and 16 weeks, bacteria were only seen in the mucus layer in normal animals while in transgenic mice, bacteria were associated with both the mucus layer and pit/surface mucous cells (Fig. 1 E–H). PCR fingerprinting of *H. pylori* recovered after 8 and 16 weeks of infection revealed that only one of the eight input isolates (Hp1) was present in transgenic mice but not in normal mice (Fig. 1D).

**Bacterial Attachment Affects the Cellular and Humoral Immune Responses of the Host.** After 8 weeks of infection, a patchy chronic active gastritis was evident throughout the glandular epithelium of normal and transgenic mice (n = 28 animals/group). Gastritis was not present in the stomachs of controls that had been gavaged with medium alone (n = 20/group). Surveys of hematoxylin and eosin-stained serial sections revealed that the severity of the inflammation was worse in transgenic animals (Fig. 2A, B), and that the gastritis was distributed over a greater area of the corpus of the stomach. Immunohistochemical and fluorescence-activated cell sorter analyses disclosed that ~50% of the lymphocytic infiltrate in both groups was composed of αβ T cell receptor-positive T cells and 10% of B cells. However, there was a 2–3-fold greater fractional representation of macrophages and NK1.1-positive natural killer cells in infected transgenic hosts. In vitro stimulation of splenic T cells by Hp1 produced a significantly greater proliferative response when cells were obtained from infected transgenic vs. normal animals (Fig. 2C).

Parietal cell autoantibodies have been correlated with the occurrence of atrophic gastritis in *H. pylori* infected humans (21, 22). To screen for autoantibodies, sera from infected transgenic and normal mice were incubated with sections prepared...
from uninfected normal mouse stomachs. After 4 weeks of infection, neither group had detectable autoantibodies to any gastric epithelial lineage. After 8 weeks, 21 of 28 transgenic animals (75%) had autoantibodies that reacted with parietal and zymogenic cells in both groups of mice, whether or not they are infected. After 16 weeks, autoantibodies were detectable in 87% of transgenic and 26% of nontransgenic animals (P < 0.05; n = 25/group). Uninfected normal and transgenic mice gavaged 8 or 16 weeks earlier with any time during persistent infection. Mean values ± 1 SD are plotted. (E and F) Frozen sections of an unperfused transgenic mouse stomach following an 8-week infection. (E) Double exposure after staining with Leb mouse mAb, fluorescein isothiocyanate-conjugated donkey anti-mouse Ig, rabbit antisera against H. pylori surface proteins, and Cy3-conjugated sheep anti-rabbit Ig. (F) Triple exposure of another section, incubated with the reagents in E plus the nuclear stain bis-benzimide (blue). Bacteria (yellow-orange) are attached to Leb-positive pit and surface mucous cells (green; e.g., solid arrows in E). (G and H) Frozen sections from an 8-week infected nontransgenic mouse, processed as in E and F, respectively. In the Leb-negative stomach, bacteria (red) are only evident in the luminal mucus (open arrow in G) and not associated with the epithelium (H). (Bars = 25 μm.)

Parietal Cell Autoantibodies: Shared Lea Immunodeterminants between Bacteria and Host Cells. H. pylori contains two fucosyltransferase genes, one of which is an α1,3 fucosyltransferase that produces Lea (20, 23, 24). Five of the input isolates, including Hp1, expressed Lea epitopes ex vivo. All eight isolates expressed Leb. Production of these bacterial Lewis epitopes persisted after 8 and 16 weeks of infection in transgenic and normal mice (7–11 animals/group/time point). Bacterial Leb and Lea immunodeterminants were not detected before or after infection.

Binding of parietal cell autoantibodies was blocked by preincubation of sera with Lea- and Leb-positive Hp1 cells, or with Lea-HSA, Leb-HSA, Lea-HSA, or HSA alone. At least 50% of parietal cells in uninfected normal and transgenic FVB/N mice have detectable Lea immunodeterminants. Lea is limited to parietal cells in both groups of mice, whether or not they are infected. None of the clinical isolates bound to Lea-positive parietal cells in vitro (Fig. 2F). ELISA assays revealed a significantly higher titer of circulating antibodies to purified H. pylori lipopolysaccharide-associated Lea and to Lea-HSA in transgenic mice with parietal cell autoantibodies, compared with infected transgenic or normal mice without detectable autoantibodies (P < 0.05; 7–8 animals/group at the 8-week time point).

Gastritis, Parietal Cell Loss, and Mucosa-Associated Lymphoid Tissue (MALT) in Transgenic Animals. Mice inoculated with hybridoma cells secreting antibodies to an H. pylori lipopolysaccharide-Lewis epitope that mimics parietal cell Lewis epitopes develop gastritis (25–27). In our model, development of parietal cell autoantibodies was directly correlated with more extensive and pronounced reactive atypia in the gastric epithelium, and more pronounced parietal cell loss. Areas of reactive atypia contain elongated and architecturally distorted gastric units (glands) with a general increase in proliferating epithelial cell populations, moderate to severe nuclear atypia, and altered nuclear to cytoplasmic ratios (Fig. 2B). A panel of antibodies and lectins (28) were used to establish that the foci of reactive atypia were associated with loss of parietal cells and a block in terminal differentiation of pepsinogen-producing zymogenic (chief) cells. These pathologic changes were not seen in uninfected transgenic mice.

The granulocytic component of the gastritis was lost and its lymphocytic component diminished in both groups of mice after 16 weeks of infection. Areas of mild reactive atypia lacking parietal and zymogenic cells were present in 33% of transgenic mice (Fig. 2J), but not in any normal littermates. The normal mouse stomach lacks organized MALT. Persistent H. pylori infection in humans is associated with formation of MALT (29). MALT was not evident after 8 weeks of infection in transgenic or normal mouse stomachs. After 16 weeks, MALT, composed predominantly of B220-positive B cells, was present in 8 of 15 (53%) transgenic animals (Fig. 2J), while smaller and less numerous mucosal lymphoid aggregates were present in only 3 of 15 (20%) normal mice (P < 0.05).
Clinical Isolate Hp1 Is Sufficient to Produce the Spectrum of Host Responses to Infection. To determine whether infection with Hp1 alone could produce gastric pathology, or whether early collaborative interactions between several isolates are necessary, transgenic and normal mice were gavaged with 10^7 cfu of Hp1 and killed 8 and 16 weeks later (10 mice/group/time point/experiment; n = 2 independent experiments). The percentage of mice in each group that were infected, the density and distribution of bacteria within their stomachs, the incidence of autoantibodies that bound to parietal cells in a Lex-dependent manner, and the inflammatory responses were similar to that observed in mice gavaged with the mixture of eight isolates.

We have not yet defined the time course of predominance of Hp1, nor do we know why it becomes the predominant organism. Hp1 was obtained from a patient with active ulcer disease. Some of the other isolates had growth rates in vitro that were similar to Hp1. Nonetheless, Hp1 had one distinguishing phenotypic characteristic: when cultured, it was able to retain a helical (spiral) form to a greater degree than any of the other isolates. When initially isolated from patients, H. pylori is present in the helical form, which is considered to be the actively growing form of the organism (30, 31). Conversion to a coccoid form has been reported to occur ex vivo during the course of nutrient deprivation, or in vivo after treatment with antibiotics or inhibitors of parietal cell H^+K^+ATPase (32–35).

DISCUSSION

There are several reasons why H. pylori infection provides an excellent opportunity to study the role of attachment in bacterial pathogenesis. Colonization occurs frequently but with varied outcomes. Although bacteria can exist within the gastric mucus without host cell attachment, a variety of potential epithelial receptors for bacterial adhesins have been described. Genetic diversity among hosts and H. pylori could generate various combinations of adhesins and receptors within and between infected populations that could determine...
whether the organism attaches to the epithelium of a colonized individual or remains in the mucus.

Our study illustrates how it is possible to test the biological consequences of attachment. A putative receptor for *H. pylori* adhesins (Leb) was produced in a clinically relevant target cell lineage in a genetically defined transgenic host. Nontransgenic FVB/N mice provided an ideal control: they were colonized with similar efficiency and microbial density for equivalent periods as their isogenic transgenic littermates, indicating that the receptor was not required to establish and maintain an infection. Distinct, sustainable differences in microbial compartmentalization occurred between normal and transgenic mice, establishing that the genetically engineered Leb receptor was biologically active. In addition, both infected and infected normal and transgenic FVB/N mice constitutively express Lewis\(^b\) epitopes in their acid-producing parietal cells. Because these epitopes are often present in the lipopolysaccharide of clinical *H. pylori* isolates, they provide an opportunity to determine if bacterial attachment affects the host response to shared carbohydrate structures. This system differs from other reported models of *H. pylori* infection because microbial attachment to the host can be genetically manipulated while keeping the bacterial strain “constant” (36).

A microbial basis for autoimmunity in chronic inflammatory diseases is widely postulated but remains largely unproven. The effect of *H. pylori* attachment was to promote development of autoantibodies to acid-producing parietal cells, chronic gastritis, and loss of parietal cells. The mechanisms underlying autoantibody production likely include molecular mimicry between constitutively expressed bacterial lipopolysaccharide- and parietal cell-associated Leb epitopes as well as facilitated presentation of the bacterial epitopes to a recruited gastric mucosal immune population. While we cannot rule out subtle attachment-induced alterations in the structure of Hp1 or host Leb epitopes, comparisons of Hp1 recovered from normal and transgenic mice indicated that the presence or absence of Leb in the host did not induce a change in the classes of Lewis immunodeterminants produced by the microbe. Moreover, the presence or absence of Hp1 did not elicit a change in Lewis immunodeterminants produced by transgenic or normal mice.

The results of a diphtheria toxin-induced ablation of parietal cells in another FVB/N transgenic mouse model (28) underscore the potential contributions of parietal cell destruction to *H. pylori* pathogenesis. The parietal cell is the only principal cellular target of the toxin in the gland (37). The lineage ablation experiment disclosed that parietal cells are required to complete differentiation of zymogenic cells. Ablation of parietal cells produced augmented proliferation of stem cells in the absence of inflammation (28). Amplified stem cells and their immediate committed daughters expanded beyond their normal niche. Cells with the morphologic appearance of gastric stem cells and their immediate committed daughters eventually formed lesions with adenomatous features and extended through the muscularis mucosa into the submucosa (Q. Li, A. Syder, R. G. Lorenz, S. M. Karam and J. I. Gordon, unpublished manuscript). These findings, together with the results described in this report, suggest that if a host is colonized by a strain that expresses adhesins that promote attachment to epithelial receptors, and if that strain also expresses surface antigens that mimic host (parietal) cell structures, then the course of infection may be skewed toward autoimmunity, atrophic gastritis with parietal cell loss, and perhaps neoplasia.

Engineering carbohydrate receptors for bacteria in transgenic animals may be a generally useful approach for examining whether host cell attachment simply provides a more effective means for delivering constitutively expressed bacterial gene products, or whether intimate association with cellular targets serves to modify patterns of bacterial or host gene expression. The sequence of the *H. pylori* genome is now known (20). Further definition of the factors that mediate attachment-related *H. pylori* pathology should come from comparisons of gene expression and genome structure in isolate Hp1 recovered before and after infection of the Leb transgenic mice and their normal littermates. Because Hp1 is genetically manipulatable, using these mice to assess the effects of disrupting *H. pylori* genes should also be informative.

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