Brief Definitive Report

Gene Structure of the *Helicobacter pylori* Cytotoxin and Evidence of Its Key Role in Gastric Disease

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Summary

The gram negative, microaerophilic bacterium *Helicobacter pylori* colonizes the human gastric mucosa and establishes a chronic infection that is tightly associated with atrophic gastritis, peptic ulcer, and gastric carcinoma. Cloning of the *H. pylori* cytotoxin gene shows that the protein is synthesized as a 140-kD precursor that is processed to a 94-kD fully active toxin. Oral administration to mice of the purified 94-kD protein caused ulceration and gastric lesions that bear some similarities to the pathology observed in humans. The cloning of the cytotoxin gene and the development of a mouse model of human gastric disease will provide the basis for the understanding of *H. pylori* pathogenesis and the development of therapeutics and vaccines.

The recently discovered, gram negative, microaerophilic bacterium *Helicobacter pylori* colonizes the human gastric mucosa and establishes a chronic infection that is tightly associated with atrophic gastritis, peptic ulcer, and gastric carcinoma (1–5). *H. pylori* infection is a worldwide problem, since in developing countries it affects over 80% of the population older than 20. Also in developed countries the infection is present in 20% of the population by the age of 30 rising to over 50% by the age of 60. Clinical isolates of *H. pylori* can be classified into two groups based on the presence or absence of the vacuolating cytotoxin (6, 7) whose expression is linked to a surface exposed immunodominant antigen (CagA) (8, 9). Since high titers of serum antibodies to the CagA protein are detected in all patients with duodenal ulcer (8) and most of those with gastric carcinoma (10, 11), it has been proposed that disease development requires infection with cytotoxin-producing strains.

The cytotoxin causes massive vacuolation in several mammalian cell lines (6), and similar vacuoles have also been observed in the gastric epithelia of patients with active chronic gastritis associated with *H. pylori* infection (12), indicating that the cytotoxin can contribute significantly to the pathogenesis of gastritis. Cell vacuolation in vitro can be blocked and reversed by inhibitors of V-type ATPases and potentiated by inhibitors of the Na^+-K^+ ATPase (13, 14), suggesting that the mechanism of action of the toxin is due to aberrant cation transport within the target cells. The purified toxin has been described as a protein of ~87 kD that is found in the bacterial culture supernatants, and the sequence of the NH_2-terminal 23 amino acids has been determined (7).

Despite the epidemiological correlation between infection with cytotoxic strains and disease (8) and the in vitro evidence for the presence of a cytotoxin, the in vivo roles of infection and cytotoxin have not been established due to the lack of a suitable animal model. *H. pylori* does not colonize the gastric mucosa of mice or other small laboratory animals. To overcome this limitation, we administered *H. pylori* extracts and purified cytotoxin orally to mice. Using this model, extracts from cytotoxic *H. pylori* strains and purified cytotoxin induced a gastric pathology with some similarities to that observed in *H. pylori*–associated human disease.

In addition, we have cloned the gene coding for the cytotoxin responsible for the gastric lesions and determined the nucleotide sequence. Antisera against recombinant fragments of the toxin were used to study the synthesis and processing of the protein.

Materials and Methods

Purification of the Cytotoxin. Toxic activity was concentrated from *H. pylori* culture supernatant by precipitation with 50% ammonium sulphate, recovered in 20 mM sodium phosphate buffer, pH 7.0, and applied to a CM-Sepharose CL-6B column. The toxin was eluted with a gradient of 0–0.5 M NaCl. SDS-PAGE of frac-

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tions eluted at ~150 mM NaCl indicated purification to homogeneity of a 94-kD protein that was recognized by antisera raised against recombinant fusion proteins. The purified material contained no detectable urease either by immunoblot using urease-specific antibodies or by enzymatic activity (15).

Treatment of Mice. 6-wk-old BALB/c mice were deprived of food, but allowed free access to water. After 24 h the mice received an administration of saline alone (0.5 ml) or containing 5 μg of purified cytotoxin or 100 μg of *H. pylori* sonic extract. The samples were introduced via catheter inserted into the eosophagus to ensure delivery. The administration was repeated after 48 h. After a further 48 h, the mice were killed and portions of the gastric mucosa were fixed in 4% formalin and embedded in paraffin. 7 μm sections were cut, rehydrated, and stained in hematoxilin and eosin.

Gene Cloning and Sequencing. *H. pylori* DNA was prepared and plasmid libraries were prepared as described previously (8). Two oligonucleotide mixtures were prepared: one containing all possible combinations of the codons coding for the amino acid sequence AFFTTV and one complementary to all combinations of the sequence GTAVGT. These mixes were used at a concentration of 4 μM with 100 ng of total *H. pylori* DNA in a polymerase chain reaction with Taq polymerase. The reaction was cycled through 1 min at 94°C, 2 min at 48°C, and 2 min at 72°C 30 times. The product of the degenerate PCR was labeled by random priming and used to screen a library of HindIII restriction fragments cloned in the bluescript SK(+) plasmid. An ~3 kbp fragment was isolated that contained the first 273 bp of the coding sequence. A 120-bp fragment was derived from the 3-kb fragment by digestion with EcoRI and was used to screen a library of EcoRI fragments. Clones containing an ~7-kb overlapping fragment were isolated. The nucleotide sequence of the coding region and flanking regions of the gene were determined using the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH). Sequence analysis programs used were Wordsearch, FastA, and Peptidestructure as implemented in Version 7 of the Genetics Computer Group sequence analysis package.

Expression of Cytotoxin Fragments. DNA fragments containing the sequences coding for amino acids 34–132 (region A), amino acids 262–428 (region B), amino acids 751–1,000 (region C), amino acids 1,001–1,121 (region D), and amino acids 1096–1220 (region E) were prepared either by PCR or restriction enzyme digestion of the cloned gene and cloned, in frame, in the appropriate version of the expression vector pex34 (16). Fusion proteins were extracted from *Escherichia coli* harboring the plasmids as described (17) and purified by SDS-PAGE before the immunization of rabbits.

Preparation of H. pylori Proteins. Total proteins were extracted from *H. pylori* cells using 6 M guanidinium hydrochloride as described (8). Culture supernatants were concentrated by precipitation with 50% ammonium sulphate. The precipitate was resuspended in phosphate buffered saline and dialyzed against the same buffer.

Protein Sequencing. Protein fragments were electroeluted after PAGE, applied to ProSpin tubes and sequenced in a pulsed liquid protein sequencer (model 477A; both from Applied Biosystems, Inc., Foster City, CA).

PCR Analysis of Cytotoxin Negative Strains. Based on the sequence shown in Fig. 2, oligonucleotides of length 20 were prepared to amplify the following regions of the sequence: Nucleotides 1–780, 332–934, 768–1,464, 1,468–2,692, 2,646–3,693, 3,259–3,925, and 3,861–4,087. The products of PCR using the cloned gene, total DNA from three cytotoxic strains (CCUG 17874, 60190, G39), and three cytotoxin negative strains (TX30, G21, G50) were analyzed by agarose gel electrophoresis.

Results

A Model of H. pylori–induced Ulceration. Oral administration in mice of a sonicate of a cytotoxin producing strain of *H. pylori* caused epithelial vacuolation and infiltration of mononuclear inflammatory cells in the lamina propria (Fig. 1 *b*). The vacuolation of cells seen in the proximity of the more severe epithelial lesions has also been observed in patients suffering from *H. pylori* associated active chronic gastritis (12) and can be induced by the cytotoxin in cells in vitro (6). Occasional ulceration with substitution of the mucosal tissue with early stage granulation tissue and loss of gastric gland structure was observed (Fig. 1 *c*). Administration of saline did not cause any significant mucosal damage (Fig. 1 *a*). Administration of sonic extract from a noncytotoxic strain of *H. pylori* (G21) did not cause epithelial lesions and resulted in gastric histology essentially identical to that shown in Fig. 1 *a*. Thus, in this model, we have produced erosive lesions in the murine gastric mucosa and confirmed the association of these lesions with cytotoxin producing strains.

Administration of vacuolating cytotoxin, purified to homogeneity, caused similar epithelial lesions in the absence of extensive inflammation. Of six mice treated with purified toxin, all revealed localized regions of cell necrosis accompanied by loss of cytoplasm and general loss of gastric gland architecture (Fig. 1 *d*). In two of the six mice, gastric ulceration was also observed (Fig. 1, *e* and *f*). The ulceration was characterized by focal mucosal injury and the presence of inflammatory exudate within the healing tissue. Of 10 control mice and 6 mice administered with 5 μg of purified *H. pylori* urease, all showed normal gastric histology (data not shown). We conclude that the cytotoxin alone is responsible for most *H. pylori*–induced epithelial erosion.

The Cytotoxin Gene. To isolate the gene coding for the cytotoxin, two oligonucleotide mixtures were prepared corresponding to the sequences capable of coding for the first 6 amino acids and complementary to the last 6 amino acids of the 23 known NH_2-terminal amino acids (7). The mixtures were completely degenerate at the third base of every codon. A polymerase chain reaction was carried out with total DNA from strain CCUG 17874 of *H. pylori* and a fragment of the expected size was obtained. The PCR product was used to probe a library of *H. pylori* DNA digested with HindIII and cloned in the bluescript plasmid vector. Clones containing an ~3-kbp insert were isolated. Clones containing an ~7-kbp insert that overlapped with 120 bp of the HindIII clone were subsequently isolated from a library of EcoRI restriction fragments. These two fragments contained the complete gene. The gene contains a single long open reading frame capable of coding for a protein of 1,296 amino acids with a calculated molecular mass of 139.7 kD flanked by consensus promoter sequences, the ribosome binding site, and terminator sequences (18, 19) (Fig. 2). The first 33 amino acids of the putative gene product resemble bacterial signal peptides (20) suggesting that the protein is exported by a *sec*-dependent mechanism. From position 34–56 the sequence is identical to the 23 amino acids determined by NH_2-terminal sequencing of the purified cytotoxin.

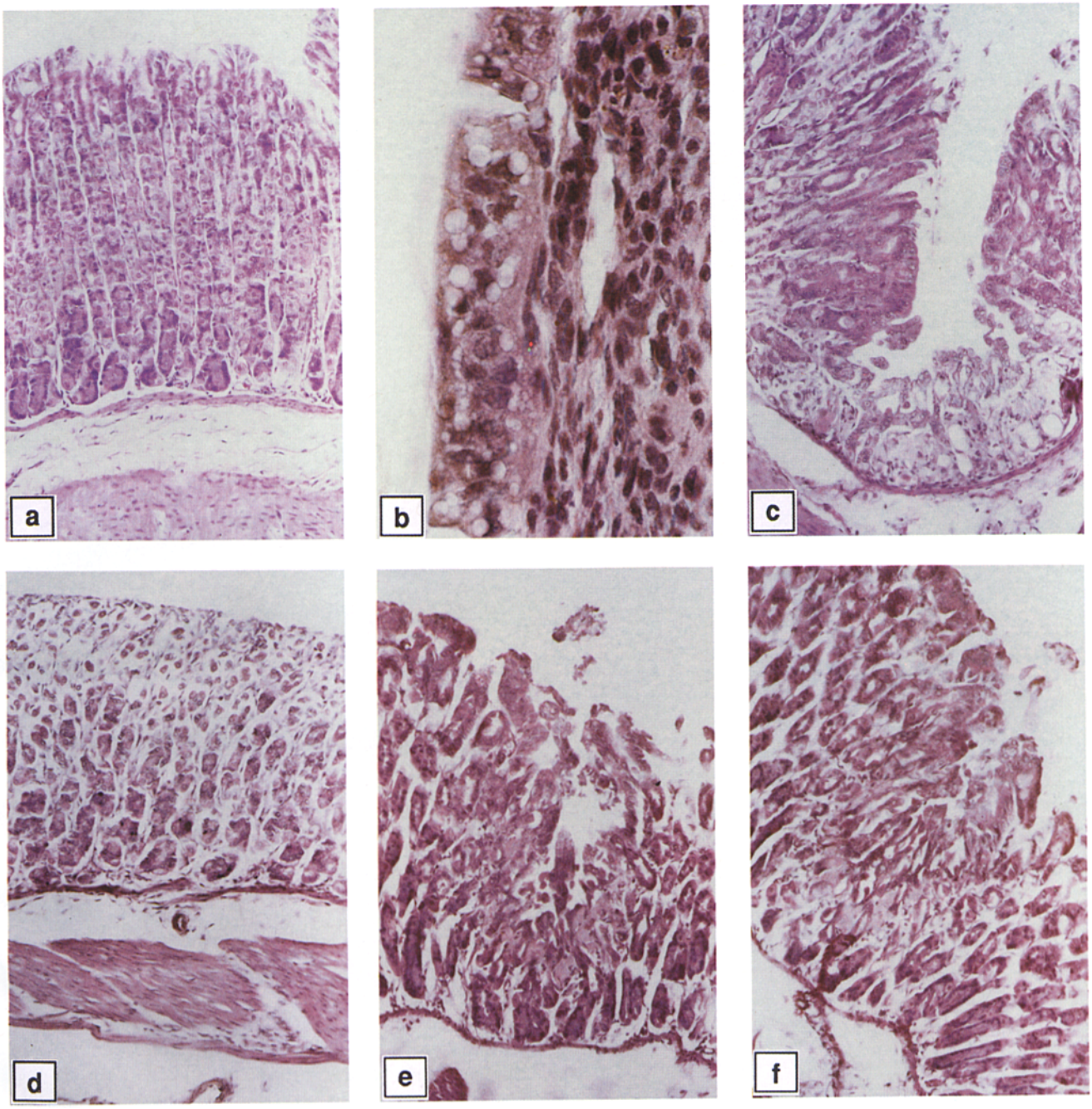

Figure 1. Cell vacuolation and damage to the gastric mucosa induced by *H. pylori* cytotoxin. (*a*) Gastric mucosa of a control mouse treated with saline (200×). (*b* and *c*) Gastric mucosa of a mouse treated with *H. pylori* sonicated (1,000× and 200×). (*d*, *e* and *f*) Gastric mucosa of a mouse treated with purified cytotoxin (200×).

Characterization of the Cytotoxin Gene Products. Different regions of the gene were expressed in *E. coli* as fusion proteins and used to obtain rabbit antisera (fragments A–E in Fig. 3 *a*). Antisera against peptides A, B, and C (Fig. 3 *a*) recognized a 94-kD polypeptide in both cell extracts and culture supernatant (Fig. 3 *b*, lanes *1–3* and *6–8*) of *H. pylori*. Antisera against the COOH-terminal region (peptides D and E in Fig. 3 *a*), failed to recognize this protein, however in the cell extracts they recognized a polypeptide of ~33 kD (Fig. 3 *b*, lanes *9* and *10*). This fragment was barely detectable in the supernatant fraction (Fig. 3 *b*, lanes *4* and *5*) suggesting that the 94-kD active toxin is released from the cell after cleavage of the COOH-terminal fragment of the precursor.

In the culture supernatant, the 94-kD polypeptide was further processed to produce a 37-kD NH_2-terminal fragment

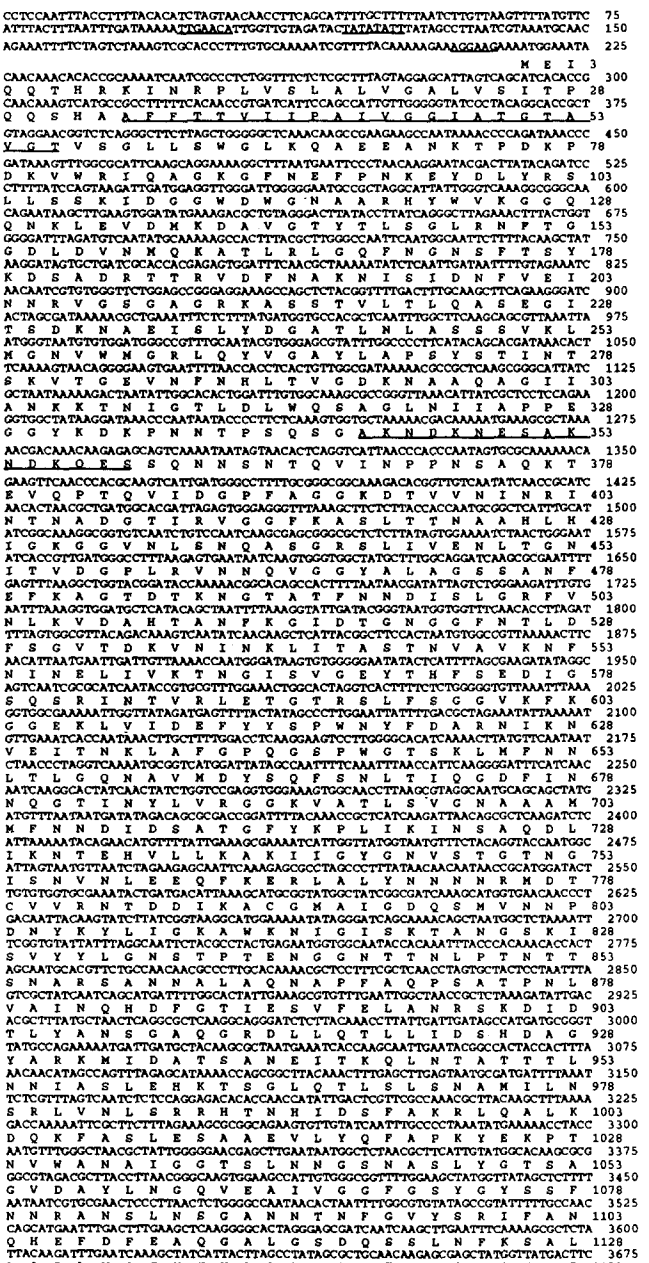

Figure 2. Nucleotide sequence and deduced amino acid sequence of the cytotoxin gene. Putative promoter elements, ribosome binding site, and transcription terminator are underlined. The 23 NH_2-terminal amino acids previously determined and the repeated hydrophilic motif are also underlined.

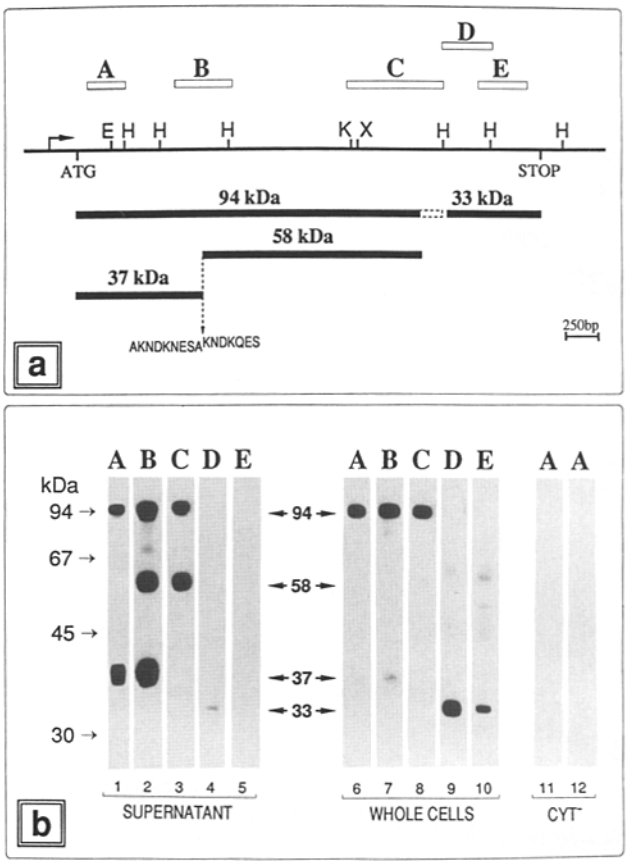

Figure 3. Immunoblot analysis of cell extracts and culture supernatants. (*a*) Schematic map of the fusion proteins used for generating the antisera and the polypeptides detected. (*b*) Lanes *1–5*, Immunoblots of cell extracts or lanes *6–10* of culture supernatants from *H. pylori* strain CCUG 17874 probed with antisera against region A (lanes *1* and *6*), region B (lanes *2* and *7*), region C (lanes *3* and *8*), region D (lanes *4* and *9*), or region E (lanes *5* and *10*). Lane *11*, cell extracts and lane *12* supernatant from a cytotoxin negative strain (G21) probed with antisera against region A.

and a 58-kD COOH-terminal fragment (Fig. 3 *b*, lanes *1–3*). These two polypeptides copurified on cationic exchange chromatography with the 95-kD molecule suggesting that the fragments remain associated after cleavage. NH_2-terminal sequencing of the gel-purified 58-kD fragment revealed that this processing occurs after the alanine at position 352. This residue is contained in a very hydrophilic region that includes a short almost perfect repeat of the sequence AKNDKXES. Cleavage occurs between the alanine and the lysine of the second repeat. Secondary structure predictions indicate that this region forms a flexible exposed loop in the protein.

Noncytotoxic Strains Contain a Silent Cytotoxin Gene. The antisera failed to detect the cytotoxin in either the cell extracts or culture supernatant of cytotoxin negative strains (Fig. 3 *b*, lanes *11* and *12*). The structure of the cytotoxin gene in three cytotoxin positive and three cytotoxin negative strains was analyzed by PCR using a panel of seven pairs of oligonucleotide primers spanning the complete gene. All pairs of primers used resulted in DNA fragments of the same length as measured by agarose gel electrophoresis, indicating no major rearrangement of the gene (data not shown). This is in contrast to the *cagA* gene which is completely absent in cytotoxin negative strains. We conclude that the cytotoxin gene is present also in noncytotoxic strains but it is not expressed. The reason

for the lack of expression of the gene and its relationship with the *cagA* gene will require further study.

Discussion

We have described a model that reproduces in mice prominent aspects of the pathology associated with *H. pylori* infection in humans. Gastric lesions were found after administration of sonic extracts and the purified cytotoxin from *H. pylori*. The lesions observed after administration of sonic extract reproduced several aspects of the histological lesions observed in biopsies from patients suffering from gastric ulcer disease, including epithelial vacuolation, mucosal erosion, necrosis, and ulceration. Sonicate-induced lesions lacked the neutrophil infiltration of the epithelium that is characteristic of *H. pylori*–associated gastritis in humans, suggesting that live bacteria may cause additional inflammation that the extracts fail to induce. It cannot be excluded however that there may be species differences in the inflammatory response.

The lesions induced by the purified cytotoxin were less severe than those obtained with the sonic extract, however they clearly showed extensive tissue damage and mucosal erosion. In particular, little if any inflammatory cell infiltration of the lamina propria was observed. A positive relationship between erosion and intraepithelial granulocytes has been observed in *H. pylori*–induced gastritis (21), however, the topography of the granulocytes did not necessarily overlap that of the epithelial erosions. It is likely, therefore, that the toxin induced erosion is due to direct cytotoxicity rather than an indirect inflammatory mechanism.

The sonicate induced larger, and more abundant vacuoles than the purified cytotoxin. In vitro, the presence of ammonia increases the size and number of toxin induced vacuoles (22). In vivo, the presence of the powerful *H. pylori* urease provides the ammonia that may account for the enlarged vacuoles. At this stage, we cannot exclude the possibility that additional molecules present in the sonicate may contribute to worsening of the lesions initiated by the cytotoxin. In particular, bacterial lipopolysaccharide may contribute to the increased inflammatory cell infiltration of the lamina propria. However the observation that extracts from noncytotoxic strains are unable to induce epithelial lesions in our model clearly indicates that the cytotoxin is necessary to induce the gastric damage. Since the toxin alone is able to induce in mice the mucosal erosion observed in human gastritis, we believe we have fulfilled Koch's postulates in a molecular fashion for this particular aspect of the disease.

The model we have described has several advantages over the existing models of *H. pylori* infection because it overcomes the inability of *H. pylori* to colonize small laboratory animals and the necessity to use surrogate Helicobacter species such as *H. felis*. In addition, pathology is induced in a period of time that is very short compared to the long lasting experiments involving gnotobiotic piglets and monkeys.

The availability of this mouse model of disease combined with the knowledge of the structure of the cytotoxin gene and protein represent the basic tools for understanding the molecular mechanisms of *H. pylori*–induced disease leading the way to the development of therapeutics and vaccines to combat such an important human pathogen.

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